SESSION 3

TRANSBOUNDARY, EMERGING AND ZOONOTIC DISEASES

The Importance of Emerging and Re-emerging Zoonotic Diseases: Recognition, Monitoring and Control

S. Mas-Coma[1]

ABSTRACT

Whilst communicable diseases mainly affect the developing world, new and emerging diseases have re-awakend the developed countries to the importance of these infections. Concern has been raised that climate change and other man-made changes to the environment could provide conditions for diseases to increase their range and affect countries where they have not normally been a problem. The zoonoses, infections naturally transmitted between vertebrate animals and humans, present considerable difficulties because eradication is almost impossible and control is problematic. However, control of many zoonoses can be affordable and reduction in human morbidity needs to become a priority task in many countries and regions. Many global, regional or locally occurring emerging or re-emerging infectious diseases are caused by zoonoses, including both vector-borne and non-vector borne diseases. Molecular biology has been instrumental in providing an understanding of zoonotic infections in relation to their transmission, epidemiology, clinical and pathological effects, treatment, development of vaccines and diagnosis and control. Examples of zoonoses in which molecular tools have helped decisively are mentioned in this review and examples are given on how molecular markers may help in the assessment and control of zoonotic diseases as illustrated in the insect vector-borne protozoan zoonosis, Chagas' disease and a snail-borne helminthic zoonosis, fascioliasis. Finally, emphasis is placed on the need to increase studies on animal reservoirs, to improve teaching and understanding of 'old-fashioned' disciplines such as medical malacology and entomology, as well as training and technology transfer and to actively pursue field work. Only by investing in these disciplines will we be in a position to go to the field, perform surveys, and acquire the data that will enable us to determine the presence of emerging or re-emerging infectious diseases.

Key words: *Emerging and re-emerging zoonoses, molecular biology tools, Chagas' disease, fascioliasis, animal reservoirs.*

INTRODUCTION

A communicable disease is one that is transmitted from one individual or inanimate source to another either directly, through a vector, or by some other means. Communicable diseases cover a wider range than the person-to-person transmission of infectious diseases: they include vector-borne parasitic diseases, zoonoses and all other transmissible diseases. Recently, communicable diseases have caught the attention of the world with the appearance of: avian flu caused by the H5N1 virus, severe acute respiratory syndrome (SARS), bovine spongiform encephalopathy (BSE or mad cow disease) and new variant Creutzfeld-Jacob disease (CJD), the relentless increase in human immunodeficency virus (HIV) infection, the use of anthrax and other microorganisms as biological weapons and more recently the new 'swine' flu A/H1N1. Communicable diseases have always been with us and although they are not considered a serious problem in developed countries, they are the main cause of death and infirmity in the developing world.

The impact of a communicable disease depends on the agent, its mode of transmission, the host and the environment as taken together they will determine the outcome of infection. The range of communicable diseases occurring throughout the world is considerable. Numerous types of agents are involved: Prokaryotes (microorganisms) including arboviruses and other viruses, bacteria, rickettsiae and spirochaetes; Eukaryotes (parasites) comprising Protozoa (with Sarcomastigophora, Apicomplexa, Ciliophora and Microsporidia), Helminths (with Trematoda, Cestoda, Nematoda and Acanthocephala), and Ectoparasites (with Arachnida and Insecta) and also Fungi. Others to be added are prions and toxins.

EMERGING AND RE-EMERGING ZOONOSES

Whilst communicable diseases mainly affect the developing world, new and emerging diseases have re-awakend developed countries to the importance of these infections (Morens et al., 2004). Although the major impact of these diseases arise within a developing country, the problem assumes international importance as more people travel to affected countries and incidents occur where exotic diseases are imported to the developed world. Concern has been raised that climate change due to global warming could provide conditions for diseases to increase their range and affect countries where they have not normally been a problem (Harvell et al., 2002). Similarly, global change factors, including increasing man-made modifications of the environment (Patz et al., 2000) and import/export of mainly domestic animals (farm animals, pets) and also exotic, sylvatic animal species, is also playing a role in the spread of infectious diseases (Chomel et al., 2007).

The key to the control of communicable diseases is the method of transmission. Communicable diseases fall into a number of transmission patterns: (i) direct transmission: without intermediate hosts (e.g. human to human, animal to animal, animal to human); (ii) human

1 Departamento de Parasitologia, Facultad de Farmacia, Universidad de Valencia, Burjassot, Valencia, Spain

* Corresponding author: S.Mas.Coma@uv.es

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reservoir with intermediate invertebrate host: the causal agent must undergo developmental stages in an intermediate host (e.g. snails in schistosomiasis); (iii) animal as intermediate host or reservoir: vertebrates play the role of intermediate host (e.g. taeniasis) or that can be reservoirs (e.g. Chagas' disease), and (iv) vector-borne transmission: an arthropod carries the infection from one host to another (e.g. *Anopheles* mosquitoes in malaria. Although often called vectors, snails are only intermediate hosts and not true vectors because they do not carry the infection from one host to another.

Communicable diseases fall into two main groups based on their transmission cycle, i.e. diseases in which only humans are involved and diseases in which there is an animal reservoir or intermediate host. The latter are the zoonoses, infections that are naturally transmitted between vertebrate animals and humans. According to the focus of the disease (intimacy of the animal to the human being), zoonoses can be grouped into the following types: (i) domestic: animals that live in close proximity to man (e.g. pets and farm animals); (ii) synanthropic: animals that live in close association with man, but are not invited (e.g. rats); and (iii) exoanthropic: animals that are not in close association with man and are not invited (e.g. monkeys). In a zoonotic disease, the animal reservoir is of prime importance to the success of any control measures. The most important difference between human diseases of zoonotic origin and those in which animals do not play a role as reservoirs is that in zoonoses, opposite to the latter, eradication becomes almost impossible and elimination becomes a task always believed to be far from affordable. Therefore, the greatest efforts by international agencies and national/international funding institutions are nowadays concentrated on human diseases of non-zoonotic nature/source, that is, in which there is no animal reservoir. The so-called big three, namely malaria, HIV/AIDS and tuberculosis, and the so-called neglected diseases including schistosomiases, filariases, onchocercosis, ascariasis, trichuriasis and ancylostomiasis-necatoriasis are at present priorities for the World Health Organization (WHO).

Additionally, there are other neglected parasitic diseases which need to be added to this priority list. These are diseases for which it is very difficult to get funds for research, despite being of high human impact globally, regionally or locally. Most of them are zoonoses which are emerging or re-emerging at present, including both vector-borne and non-vector borne diseases. They include (i) intestinal protozoal diseases such as giardiasis, cryptosporidiasis and amoebiasis, (ii) vector-borne protozoal diseases including leishmaniases, sleeping sickness and Chagas' disease, (iii) food-borne trematodiases such as fasciolosis, fasciolopsiasis, clonorchiasis, opisthorchiasis, paragonimiasis and gastrodiscoidiasis, (iv) cestodiases like taeniasis/cysticercosis, hidatidosis and alveococcosis, and (v) nematodiases as trichinelliases (or trichinosis) and strongyloidiasis.

For most of these zoonoses, the crucial facts are already known: (i) the characteristics of the disease including the transmission cycle of the causal agent are known; (ii) there are tools for the diagnosis of the disease in both humans and animal reservoirs, as well as in the intermediate host or vector in vector-borne diseases, and (iii) effective drugs for both animal and human use are available. The control of many of these zoonoses appears, therefore, affordable and a reduction of human morbidity may become a realistic goal for several of them in many countries and regions.

USE OF MOLECULAR BIOLOGICAL TECHNIQUES FOR UNDERSTANDING ZOONOTIC DISEASES

Molecular biology is a very broad field which has quickly evolved in recent years. It provides molecular tools for the genetic characterisation of living organisms and, through gene expression, the baseline for phenotypical analyses. There are many kinds of molecular approaches with different degrees of resolution, including methods and techniques for the genetic characterisation of: individual specimens, strains, populations, species and supraspecific taxa. Bioinformatics is a modern computer science which has evolved in parallel to molecular biology with the main objective of furnishing high capacities for the mathematical analysis of genetic data (mainly nucleotide and amino acid sequences of DNA) for both molecular phylogenetics and population studies.

Molecular marker combinations, including from high resolution DNA sequencing such as single nucleotide polymorphisms or SNPs (Mas-Coma and Bargues, 2008) up to less detailed techniques, such as banding analytical methods like random amplified polymorphic DNA or RAPD (Pacheco et al., 2003), restriction fragment length polymorphism or RFLP (Marcilla et al., 2002) or microsatellite markers (Hurtrez-Bousses et al., 2004) are very useful tools for studying zoonoses. For instance, in epidemiology they enable distinguishing different strains of the causal agent and their relationships with higher/lower prevalences and intensities in humans and animals, animal species which constitute the reservoirs and infection sources for humans, intermediate hosts or vector species which constitute the transmission sources for humans, climatic factors and environmental characteristics, geographical distribution and spreading capacities. In clinical studies and pathology, they enable distinguishing between different strains of the causal agent and their pathogenicity and immunogenicity. In diagnosis, they are useful for the highly sensitive and specific diagnosis of the causal agent in humans, reservoir animals and intermediate hosts and vectors. In treatment, they are used for the characterisation of resistant and susceptible strains. In control and surveillance, they furnish tools for the development of vaccines and the follow up of postreatment re-infections. The application of molecular tools in studies of avian influenza caused by the H5N1 virus is an excellent example of the application of this technology (WHO Global Influenza Program Surveillance Network, 2005).

Examples of zoonoses in which molecular tools have decisively helped in clarifying disease epidemiology and transmission are numerous. In cryptosporidiasis, molecular tools have proved that there is a higher number of different human-infecting species and specific reservoir hosts than initially believed (Xiao et al., 2000). In hidatidosis, different *Echinococcus granulosus* strains (genotypes) with different host ranges and geographical distributions are at present differentiated: sheep-dog, horse-dog, cattle-dog, camel dog, pig-dog, cervid strains (Le et al., 2002). Trichinosis was a disease in which only one species, *Trichinella spiralis*, was believed to be the causal agent but now it is known that there are in fact different *Trichinella* species with different sylvatic cycles and geographical distributions (Murrell et al., 2000).

Molecular tools are furnishing very useful information on the insect-borne protozoan zoonosis Chagas' disease and the snail-borne helminthic zoonosis, fasciolosis. These two diseases are reviewed in more detail to illustrate how molecular markers may help in the assessment and control of zoonotic diseases.

CHAGAS' DISEASE, AN EXAMPLE OF AN INSECT-BORNE PROTOZOAL ZOONOSIS

American trypanosomiasis or Chagas' disease caused by the haemoflagellate protozoan species *Trypanosoma cruzi* is widespread in Latin America from Mexico to Chile and southern Argentina. Although present estimates of 10 to 12 million people infected represent between six and eight million cases fewer than those reported in the 1980s, it is still the most serious parasitic disease of the Americas for its social and economic impact. Although it can also be transmitted by blood

transfusion and transplacentarily, human contamination usually occurs by vectorial transmission in poor rural or periurban areas of Central and South America (Schmunis, 2004). Moreover, the disease is recently emerging in western European countries such as Spain and France, but also Portugal and Italy, as a consequence of the present high immigration from Latin American countries- Although vector-borne disease transmission cannot take place in Europe because of the absence of triatomine vectors (Bargues et al., 2000), blood and vertical transmission are issues of concern (Schmunis, 2007).

Chagas' disease vectors are haematophagous reduviid (Hemiptera: Heteroptera) insects belonging to the subfamily Triatominae. The 138 species currently recognised within Triatominae are grouped into 17 genera forming five tribes, with all species appearing capable of transmitting *T. cruzi*. Among the triatomines, most of the species (76) are included in the genus *Triatoma*, within different complexes and subcomplexes in a classification which is progressively updated according to new genetic and morphometric data (Dujardin and Schofield, 2004).

In Chagas' disease, recent molecular results have shown that the causal agent *Trypanosoma cruzi* is very heterogeneous throughout the endemic countries of Latin America, including two main phylogenetic groups, I and II which diverged very long ago and in turn also appear to comprise different lineages (Briones et al., 1999). This, together with molecular studies showing that triatomine vectors are also more complex than previously believed, is giving rise to a completely new frame which indicates that known transmission patterns, clinico-pathological pictures, diagnostic kits and traditional control strategies will be in need to be reassessed.

The *Trypanosoma cruzi* Complex and the Heterogeneity of the Disease

Heterogeneity of *T. cruzi* does not only concern a different geographic distribution of the clinical presentation of the disease in the chronic stage and of the transplacentary transmission from mother to foetus, but also the different response to treatment and the different behaviour of the trypanosome in mice and triatomine bugs (Miles et al., 2004).

DNA-based techniques including RFLP, analysis of DNA of the kinetoplast (kDNA), RAPD, zymodeme analysis, comparison of polymorphisms between rDNA and miniexon gene, and analysis of the two internal transcribed spacers of the rDNA (ITS- and ITS–2), have shown that there are two main phylogenetic groups within *T. cruzi*, which by consensus have been designed *T. cruzi* I y *T. cruzi* II (WHO, 2002). Additionally, 5 subgroups have been distinguished among the second group and named *T. cruzi* II a-e (Tibayrenc *et al*., 1993; Fernandes *et al*., 1998; Oliveira *et al*., 1998). *T. cruzi* I has been linked to the sylvatic life cycle of the parasite and to strains showing low morbidity in humans, whereas *T. cruzi* II has been related to the domestic life cycle of the parasite and the chronic form of the disease in countries of the Southern Cone (Fernandes *et al*., 1998 and 1999). The subdivision of *T. cruzi* II into 5 subgroups was recognised from analysis of fragments of the 18S and 24S α genes of the rDNA (**Figure 1**) (Brisse et al., 2001).

T. cruzi I, initially considered uniform, is also progressively proving to be complex. When analysing *T. cruzi* I specimens from humans, domestic and sylvatic animals and also triatomine vectors, different lineages were found by intergenic miniexon gene sequencing in northern South America (Herrera et al., 2007) and also Mexico

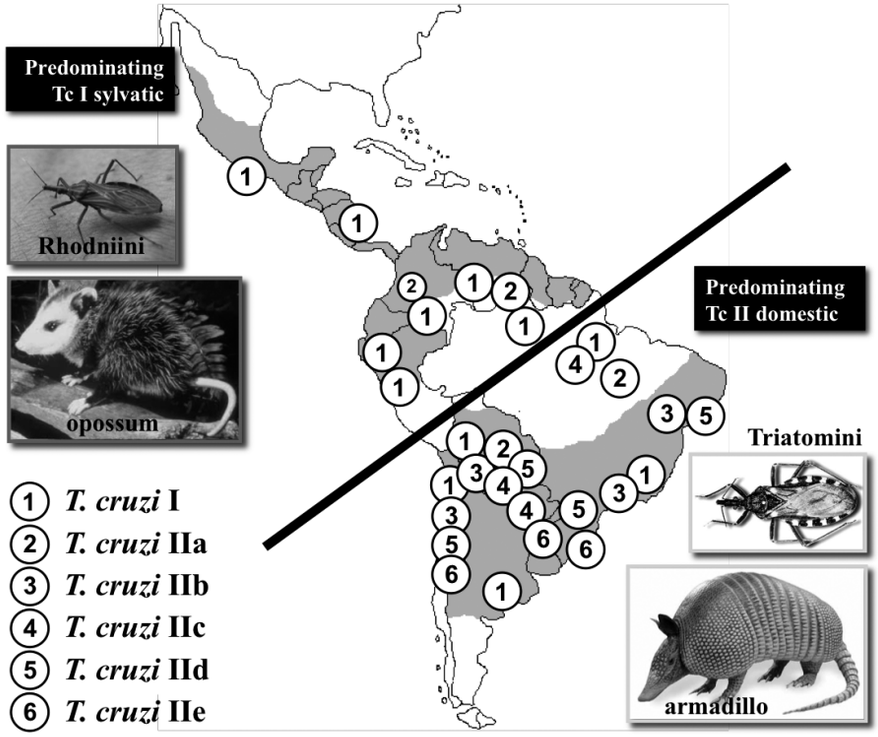

Figure 1. Geographical distribution and relationships with pressumably original definitive mammal hosts and triatomine vector groups of the causal agents of Chagas' disease, *Trypanosoma cruzi* I and *T. cruzi* II. Grey shading includes areas with Chagas' disease endemics or human reports.

(O'Connor et al., 2007). These lineages are also showing apparent links to the domestic, peridomestic and sylvatic life cycles of the parasite. These results indicate that *T. cruzi* I may also be as heterogeneous as *T. cruzi* II. All suggest that subgroups will need to be established within *T. cruzi* I, once more information from other molecular markers obtained in different geographic areas becomes available. So, there is still a long way to go until a sufficiently detailed mapping of the distribution of both *T. cruzi* I y *T. cruzi* II, as well as relevant information about relationships of the groups and subgroups with the differences in biology, transmission, epidemiology, clinical picture and pathology known to exist within Chagas' disease are obtained throughout their wide distribution in the Americas and which until now could never be satisfactorily explained.

Problems of Systematics in Triatomine Vectors, Disease Transmission, Epidemiology and Control

The geographical distribution of Chagas' disease, its local transmission patterns and main epidemiological characteristics depend on the vectors. The absence of effective drugs and an available vaccine mean that vectors become the main target for control measures. This is why great multidisciplinary efforts have always gone into understanding and cataloguing the triatomines, both vector species and those never found to be infected or involved in transmission. Vector systematics always plays a key role in vector-borne diseases. The cataloguing of vector characteristics related to disease transmission, human contamination, and epidemiology in general within different systematic units greatly facilitates the tasks.

The capacity of a triatomine species to become close to humans in an intradomiciliation process and feed on human blood is the key to becoming a good vector of the disease (Schofield et al., 1999; Dujardin and Schofield, 2004). A distinction is made between (i) intradomicilary vectors having colonised human dwellings (domestic vectors), (ii) sylvatic species in the process of adaptation to human dwellings (candidate vectors), and (iii) sylvatic species that remain closely associated with wild mammals (potential vectors) (Dujardin et al., 2002; Dujardin and Schofield, 2004). Thus, plasticity, adaptability and movement capacity of triatomines within the triangle constituted by the intradomicile, peridomicile and sylvatic habitats is crucial in the transmission, epidemiology and control of the disease (Schofield et al., 1999; Dujardin and Schofield, 2004).

Sequencing of nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) is the best method for providing information concerning triatomine systematics and vector characteristics up to the present. Both rDNA and mtDNA include neutral markers whose usefulness has already been emphasised in understanding Chagas' disease vectors (Monteiro et al., 2001; Bargues et al., 2002). Examples of aspects of Triatominae bionomy and of their relationships to Chagas' disease transmission, epidemiology and control that can be addressed from DNA sequence data are: (i) systematics and taxonomy; (ii) origin; (iii) evolutionary traits; (iv) evolutionary rates; (v) biogeography; (vi) specimen classification; (vii) population delimitation; (viii) hybrid characterisation; (ix) population changes; and (x) disease transmission (Bargues et al., 2002). Studies on the major vector species such as *Triatoma infestans* (Bargues et al., 2006), *T. dimidiata* (Bargues et al., 2008) and *Rhodnius prolixus* (Monteiro et al., 2000), have shown the valuable information that molecular techniques can furnish.

The broad usefulness of nuclear rDNA and mtDNA sequences explains why the number of studies using these markers published has increased so markedly in recent years. A review on selected, updated knowledge about nuclear rDNA and mtDNA in insects, concentrating on aspects useful for research on triatomines has recently been published (Mas-Coma and Bargues, 2009). This study analyses the efficiency, importance of their different characteristics, and the limitations and problems of each type of marker in the light of the results obtained in studies on populations, hybrids, subspecies and species of Triatominae, including several crucial genetic aspects newly or very recently detected. Emphasis is given to taxonomic units and biological entities presenting well-known problematics, as well as to molecular situations which can give rise to erroneus conclusions. The purpose of this review is to offer a baseline for future research on triatomines and their relationships to transmission, epidemiology and control measures of Chagas' disease, thereby facilitating future work on triatomines. This review highlights (i) present gaps, (ii) choice of the most appropriate markers, and (iii) marker aspects which must

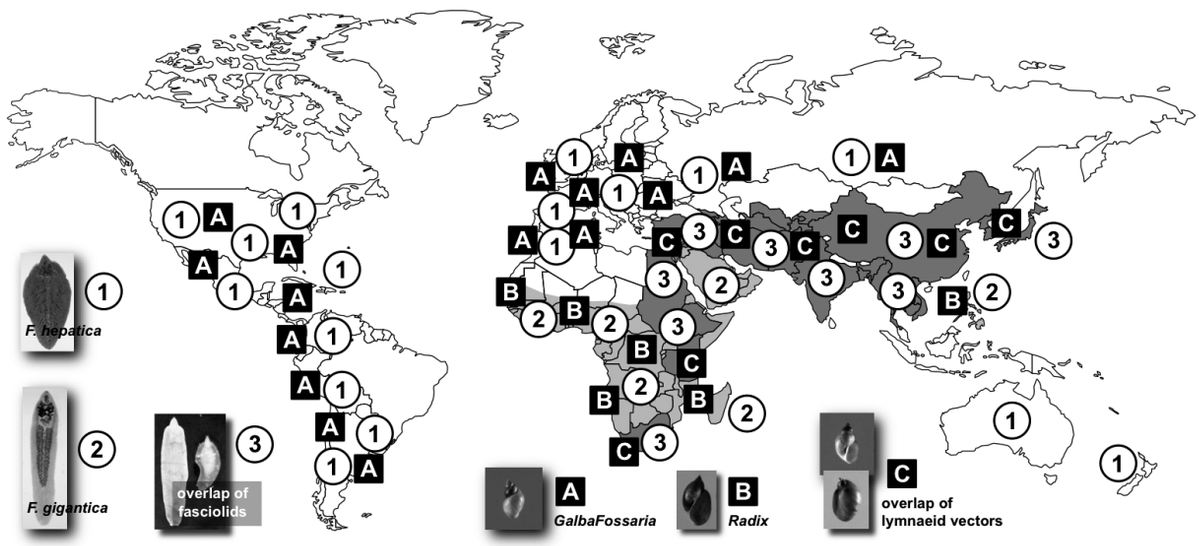

Figure 2. Global geographical distributions of fascioliasis.

be taken into account in sequence studies and phylogenetic analyses to obtain appropriate results and correct interpretations (Mas-Coma and Bargues, 2009).

HUMAN AND ANIMAL FASCIOLIASIS, AN EXAMPLE OF A SNAIL-BORNE HELMINTHIASIS

Molecular results are also giving rise to a revolution in the traditional knowledge on fascioliasis. Fascioliasis is an important disease caused by two digenetic trematode species of the genus *Fasciola*: *F. hepatica* distributed in the five continents and *F. gigantica* mainly in Africa and Asia, and transmitted by different freshwater snail species of the family Lymnaeidae (**Figure 2**) (Mas-Coma, 2004; Mas-Coma et al., 2005). The impact of this disease is markedly higher than that of Chagas' disease due to (i) its worldwide distribution, (ii) the present emerging situation almost everywhere, (iii) its great pathogenicity and adverse impacts on development, and (iv) the large number of around 17 million people affected, mainly children and females. All of this indicates that the large intraspecific variability of liver flukes and their lymnaeid snail vectors is important in determining the capacity of this disease to spread and emerge in very different areas and environments despite the different human behaviours. All in all, molecular tools appear to be in the forefront in ascertaining transmission patterns, human and animal infection sources, and epidemiological situations, as well as for establishing the appropriate global strategies and local measures of control.

Fasciola hepatica is present in the five continents, transmitted by lymnaeid vectors of the Galba/Fossaria group excepting in Oceania where it is transmitted by authochthonous as well as introduced lymnaeid vector species. *Fasciola gigantica* is transmitted by lymnaeid species of the Radix group and is distributed in sub-Saharan Africa, the Nile bassin, the Near East and overall southwards from the Himalayas and also partly northward from this mountainous chain, up to the Far East and Pacific islands as the Philippines and Hawaii. There is overlap of both species and respective lymnaeid vectors in eastern Africa and large parts of Asia. The molecular difference between both fasciolid species is only 10 mutations throughout the intergenic ITS–1–5.8S - ITS–2 region of the nuclear rDNA operon. Countries in white = presence of only *F. hepatica*; countries in grey = presence of only *F. gigantica*; countries in blackish = overlap of the two fasciolid species.

The Heterogeneity of Human Fascioliasis

In the last two decades, field studies have shown that human fascioliasis evolves very differently depending on the geographical area. Thus, epidemiological scenarios range from hyperendemic, mesoendemic and hypoendemic situations, up to epidemics in human endemic areas and in animal endemic areas, as well as areas with authochthonous, isolated, non-constant cases and others with only imported cases (Mas-Coma et al., 1999).

Moreover, different transmission patterns may be distinguished within the different human endemic areas: a very high altitude pattern in Andean countries, a Caribbean insular pattern, an Afro-Mediterranean lowland pattern in Egypt, and a pattern related to areas surrounding the Caspian Sea (Mas-Coma, 2005), to which another recently detected in Southeast Asia may be added. The relationships between the lymnaeid vector species and a specific transmission pattern should be emphasised. Lymnaeids show markedly different ecological and ethological characteristics depending on the species, with different factors being crucial in determining the characteristics of the disease, such as type of water collection, habitats, population dynamics, temperature thresholds, seasonality, or susceptibility regarding liver fluke infection. This indicates that, as in other vector-borne parasitic diseases, the lymnaeids may constitute excellent markers of disease characteristics useful for differentiating between different human fascioliasis situations and patterns. Consequently, an accurate classification and adequate genetic characterisation of the lymnaeid vectors is of the highest importance (Bargues and Mas-Coma, 2005).

The aforementioned heterogeneity of the disease in transmission and epidemiological characteristics is of such a level, that establishing convenient, simplified and uniform control programmes for the different endemic areas does not appear feasible. For many areas or countries, specific control measures should be recommended which may include several measures peculiar to that given place. Pragmatism demands the convenience of looking for markers which could easily and quickly distinguish each type of transmission pattern and epidemiological situation (Mas-Coma et al., 2009a). Genetic markers appear to be the frontline targets, since all the above-mentioned characteristics may be related to different combinations of species and strains of both liver flukes and lymnaeid vectors. Climatic-physiographic markers appear in the second line, because they need a previous characterisation of flukes and snails, both in the field and experimentally in the laboratory, to enable the application of accurate methods like mathematical modelling and remote sensing and GIS (Mas-Coma et al., 2008, 2009b). Summing up, molecular markers are key for an adequate characterisation of both parasites and vectors.

Molecular Markers to Assess Species and Strains of the Causal Agents of Fascioliasis

Among the different techniques used for the genetic characterisation of fasciolids, sequencing of nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) appears to be the best technique (Mas-Coma et al., 2009a). Among the nuclear rDNA operon, the 5.8S gene and the 18S gene are not useful markers due to their slow evolutionary rates (Mas-Coma and Bargues, 2009). The 28S rDNA is also highly conserved in *F. hepatica* and *F. gigantica*, with only few interspecific nucleotide differences. In spite of this, a 618-bp fragment was successfully selected for a PCR-RFLP assay using the restriction enzymes *Ava*II and *Dra*II to differentiate both fasciolid species (Marcilla et al., 2002). More recently, a nucleotide position among a 510-bp-long fragment of the 28S gene allowed differentiation between anthelmintic-resistant and susceptible fluke specimens in Spain (Vara-del-Rio et al., 2007).

The intergenic transcribed spacers ITS–1 and ITS–2 are the best markers for the differentiation of species (Mas-Coma and Bargues, 2009) and have also shown their usefulness for liver flukes. Both ITSs appear to be intraspecifically very conserved and with only five nucleotide mutations differentiating the two fasciolid species (Mas-Coma et al., 2009a).

With regard to mitochondrial gene markers, only partial sequences of two genes were initially used, cytochrome c oxidase subunit I (*cox*1) and the NADH dehydrogenase subunit I (*nad*1) (Hashimoto et al, 1997; Itagaki et al., 1998). Surprisingly, despite the availability of the whole mitochondrial genome sequence of *F. hepatica* for several years (Le et al., 2001), only short sequences of *nad*1 and *cox*1 have been used until recently, with the additional problem that the different fragments and sometimes their different lengths make comparative analyses difficult. However, recent work including complete *cox*1 and *nad*1 sequences and analysis of specimens from numerous countries of all continents offers opportunities to set new baselines for future studies involving (i) a global framework on the evolution of the disease, (ii) molecular characterisation of 'pure' *F. hepatica* and 'pure' *F. gigantica* and analysis of their respective intraspecific variabilities and also a detailed assessment of their interspecific differ-

ences; (iii) an exhaustive review of all sequences available so far, (iv) standardisation of methods and techniques, and (v) standardisation of DNA sequence nomenclature (Mas-Coma et al., 2009a).

The combined use of markers from both nuclear rDNA and mtDNA has proven to be very useful in fascioliasis, because it allows the detection of hybrid forms. The two fasciolid species are characterised by presenting introgression at the level of the mitochondrial genome. This enables an understandng to be gained of intermediate forms between the two fasciolid species known to affect animals and recently also detected in humans (Periago et al., 2007; Le et al., 2008; Mas-Coma et al., 2009a), as well as rare phenomena described in Asia, such as abnormal ploidy and aspermic partenogenesis (Terasaki et al., 2000).

Molecular Tools to Characterise Lymnaeid Snail Vectors

The role of lymnaeid vectors in fascioliasis transmission, epidemiology and control demonstrates the importance of getting new tools which could help in specimen classification, genetic characterisation of natural populations and laboratory strains, and in arranging the systematics and taxonomy of Lymnaeidae. The failure of all malacological and non-malacological tools used for these purposes up to the present suggests the convenience and usefulness of such tools to analyse whether DNA sequences and phylogenetic methods could be useful. The first attempt to develop a research collaboration of parasitologists, molecular biologists and malacologists was successful (Bargues et al., 2001).

A worldwide lymnaeid molecular characterisation initiative began progressively to make steps forward, including large, transboundary studies (Bargues and Mas-Coma, 2005; Mas-Coma et al., 2009b). The great spreading capacity of lymnaeids is evidence that sometimes not even a continental scale is sufficient, and intercontinental sequence comparisons are needed to correctly classify specimens. The intercontinental spreading of lymnaeids and its role in fascioliasis dissemination is well known (Mas-Coma et al., 2003, 2005; Pointier et al., 2007).

Among the different DNA markers used so far in lymnaeids, the 18S rRNA gene appears to be too conserved and its few variable positions may only be useful at generic and suprageneric taxon levels (Bargues and Mas-Coma, 1997, 2005). The ITS–2 and secondarily ITS–1 are the most appropriate for: (i) classification of lymnaeid specimens, (ii) characterisation of lymnaeid intraspecific genetic interpopulational variablity to furnish the genetic base on which to understand fasciolid-lymnaeid specificity, different susceptibilities or compatibilities of geographical strains or even resistances, (iii) establishment of valid species and their geographical distributions, and (iv) assessment of species interrelationships to arrange a natural systematic-taxonomic classification which would allow an analysis of coevolution with fasciolids (Bargues and Mas-Coma, 2005). One mutation at the level of the ITS–1 and another at ITS–2 have proven useful to distinguish between resistant and susceptible populations of *Pseudosuccinea columella* in Cuba (Gutierrez et al., 2003), although nothing evidently suggests that these mutations are linked to the resistance/susceptibility duality.

Within mtDNA, only fragments of the 16S and *cox*1 have been sequenced in lymnaeids (Remigio and Blair, 1997a; Remigio, 2002; Bargues et al., 2007). Recent knowledge indicates that mtDNA markers, including both mitochondrial genes and the ribosomal 12S and 16S genes, should be used with great caution when dealing with species belonging to different genera and even those well separated within the same genus (Mas-Coma and Bargues, 2009). Consequently, the use of mtDNA markers for this initiative are restricted to (i) sequence comparisons and phylogenetic analyses of only close species within the same genus, (ii) studies of intraspecific variability of species by sequence comparisons of individuals and populations, (iii) genetic characterisation of laboratory strains, (iv) studies on the spread of populations of a species, and (v) studies on genetic exchange between different neighbouring populations.

Very recently, the standardisation proposed for lymnaeids is expected to furnish the baseline needed to clarify the present chaotic systematics of this molluscan family and thus raise the possibility of moving forward in fascioliasis assessment. It should be borne in mind that fasciolid flukes show a strict specificity regarding lymnaeid species and sometimes apparently also geographic strains within a given lymnaeid species. Hence, molecular markers become crucial for assessing fascioliasis transmission both in the field and also experimentally in the laboratory, as well as in assessing fasciolosis transmission and establishing adequate control measures (Bargues and Mas-Coma, 2005; Mas-Coma et al., 2009a).

FUTURE EFFORTS IN VECTOR-BORNE ZOONOSES

The complexity of zoonotic infectious diseases poses a number of problems which must be solved. Although overall knowledge of the epidemiology and transmission of these diseases is available, specific knowledge relating to the local epidemiology and transmission characteristics is still lacking in many cases. Multisdisciplinary approaches and cross professional team networks are needed for both research and training. Efforts will be needed to convince different responsible ministries and health responsibles to work together and any political-strategic difficulties must be solved. Funding agencies need to be convinced about the need for increasing efforts at the animal level since studies on geographical distribution and epidemiology of zoonoses using modern tools are crucial to establish the appropriate local control measures.

The need for 'old-fashioned' disciplines such as medical malacology and entomology needs to be emphasised. Field work should again be encouraged. Today, one of the greatest problems is that in many developing countries little is known about disease epidemiology and therefore effective control methods cannot be applied.

Moreover, control of all kind of infectious diseases needs sustainability. Sustainability requires specifically trained scientists in endemic countries and areas. Consequently, training and technology transfer should be high on the agendas of research projects on zoonotic diseases.

Interestingly, results from field surveys usually suggest that many diseases are emerging or re-emerging. This could be related to the higher performance of today's diagnostic methods compared with older ones; however, one conclusion is evident: all these diseases are still there and continue to be as prevalent as ever!

CONCLUSIONS

Communicable zoonotic infectious diseases are very problematic due to (i) the complexity of the different organisms involved, (i) the difficulties posed by the numerous and changing biotic and abiotic factors influencing their epidemiology and transmission, and (iii) the huge challenges they pose for control. However, our capacities to tackle them in a multidisciplinarily manner have never been so strong. The moment to take decisive steps against these diseases by taking advantage of all these techniques simultaneously has arrived.

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An Efficient Stakeholder Driven Approach to Disease Control

D. O'Brien[1]*

ABSTRACT

The objective of this paper is to describe the work of the European Technology Platform for Global Animal Health (ETPGAH). The ETPGAH is a stakeholder driven initiative aimed at agreeing on the most important diseases, the most important gaps in our ability to control those diseases with the intention of focusing animal health research on filling those gaps. The stakeholders identified the need to prioritise animal diseases, carry out gap analysis, explore our fundamental research capacity, address enabling factors, review regulatory issues and take a global view as diseases do not respect borders. An Action Plan addressing all of these issues was published in 2007. The Action Plan identified 30 activities that need to be pursued. For each activity, the objectives, deliverables and tasks are stated. The ETPGAH must now oversee the delivery of the Action Plan and much progress has already been made. Mirror Groups have been established in a number of countries to communicate the Action Plan and to link its priorities into national research programmes. An ERA-Net has been created as recommended in the Action Plan and is launching research projects that will deliver additional actions contained in the Action Plan. The DISCONTOOLS project is aimed at developing a more sophisticated methodology for gap analysis and disease prioritisation. In conclusion, the ETPGAH has been successful in getting stakeholders to agree on priorities and is now actively delivering the Action Plan.

INTRODUCTION

The European Technology Platform for Global Animal Health (ETPGAH, http://www.ifaheurope.org/CommonTP.aspx?SubMenuId=47&MenuId=17) was established in December 2004 with the objective of identifying the most critical issues that need to be addressed in order to control diseases in animals. The ETPGAH was funded by the European Commission and as required by the Commission, was led by industry. European stakeholders and International organizations participated in the work of the Platform and developed a Vision, Strategic Research Agenda (SRA) and an Action Plan (http://www.ifaheurope.org/upl/4/default/doc/ETPGAH_ActionPlanAug07.pdf).

RESULTS AND DISCUSSION

The Vision developed is as follows 'To facilitate and accelerate the development and distribution of the most effective tools for controlling animal diseases of major importance to Europe and the rest of the world, thereby improving human and animal health, food safety and quality, animal welfare, and market access, contributing to achieving the Millennium Development Goals'.

The Vision foresees the speedier development of tools for disease control by focusing our research effort on the most important gaps in the most important diseases. These may be emerging, established or zoonotic diseases. By delivering better disease control, animal health and welfare is protected, human health benefits in terms of zoonotic disease control but human health also benefits from food security, safety and quality. Indeed, poverty and famine may be averted. The stakeholders to the ETPGAH recognise that diseases do not respect borders and take a global perspective in the knowledge that a global reduction in disease is to the benefit of everybody.

In developing the SRA, the stakeholders identified six major themes:

- prioritisation of animal diseases
- gap analysis
- fundamental research
- enabling factors
- regulatory issues
- global perspective

It was recognised that we need to prioritise our effort and focus on finding new disease control tools by collaborative research on a limited number of critical targets. By this mechanism, we can attempt to make greatest progress in the least amount of time. Critical to this concept is the need to identify and prioritise the most important gaps in our ability to control the critical diseases.

Having identified our targets, it is then vital that we have the fundamental research capacity — infrastructure and people — to carry out the necessary research. Establishing our research capability necessitates the creation of a database with the relevant information with gaps then being addressed. Along with filling gaps, efficiency can also be improved by avoiding unnecessary capacity development.

Enabling factors such as quality assurance, intellectual property rights and facilitation of technology transfer are critical components in moving from basic research to the development of a tool that can be used to fight a disease. Financial support at critical points in the development chain is also vital. Too often, projects are dropped because intellectual property rights have not been secured and nobody is willing to invest perhaps €100 million in taking the project from the laboratory bench through development and into the market.

The correct regulatory environment is vital to stimulate innovation. A balance needs to be reached between protecting human and animal health from the risks associated with a product versus the wish to eliminate all hazards. In addition, regulation needs to be focused on the needs of the veterinary sector which may be quite different to those of the human sector.

1 European Technology Platform for Global Animal Health, Brussels, Belgium

* Corresponding author: d.obrien@ifahsec.org

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From a global perspective, it is in the interests of everybody to reduce the global burden of disease. It may be much more beneficial to tackle disease at its source. This approach facilitates cooperation across the globe including capacity-building.

Having explored the broad areas that need attention in the SRA, the Action Plan was then developed and published in July, 2007 identifying the actions — research or information gathering — that needs to be carried out in order to deliver the SRA. The Action Plan follows the themes identified in the SRA with 30 activities outlined. For each activity, the objectives, deliverables and tasks are stated.

The purpose of the ETPGAH is to now oversee the delivery of the Action Plan. Each activity needs to be progressed and funding is an important factor. Funding from the European Commission is important as it stimulates collaborative research. However, nation states are the main source of research funding with more than 90% of funding coming from this source. As such, the ETPGAH has stimulated the creation of 'Mirror Groups' and seven have been formed to date (http://www.ifaheurope.org/CommonTP.aspx?SubMenuId=50&MenuId=17). The purpose of a Mirror Group is to communicate the content of the Action Plan to a national level and also to encourage the use of national funding to deliver some of the activities from the Action Plan best suited to that country. The Mirror Groups meet occasionally and now wish to develop common calls for research that may be funded by DG Research, individual Member States or a number of Member States.

In the overall context of the ETPGAH initiative, progress has been encouraging to date with many activities being funded by the European Commission.

A very important development has been the creation of the Emerging and Major Infectious Diseases of Livestock European Research Area Network (EMIDA ERA-Net). The EMIDA ERA-Net involves 19 countries with annual animal health research budgets of € 270 million. The EMIDA ERA-Net is progressing many of the information gathering exercises such as those related to infrastructure and human capacities. In addition, it is building a database of research carried out since 2005 along with information on ongoing research facilitating those who wish to make contact with and collaborate with scientists active in a given research area. The EMIDA ERA-Net has recently issued calls for research that will see projects being run across a number of countries thus contributing greatly to collaborative research across the countries involved in the initiative.

The DISCONTOOLS project commenced in March, 2008 and will run for four years. It is focused on developing a sophisticated prioritisation model based on a database of information in relation to approximately 45 diseases highlighted in the ETPGAH Action Plan. The database will be created by expert groups who will also be asked to propose prioritised gaps in relation to each disease. This information will then be scored via the prioritisation model and the output should be agreed priorities that should be the subject of focused research in order to lead to technological breakthroughs giving us the tools to control these disease more effectively. The technology will be web based and will be publicly available for comment and input thus leading to a database and prioritisation mechanism that will be continuously updated over time.

CONCLUSIONS

The ETPGAH has stimulated stakeholders to agree on a common Vision, Strategic Research Agenda and Action Plan. With the stimulus of DG Research in funding relevant research projects, the creation of the EMIDA ETRA-Net and the work of DISCONTOOLS, it can be concluded that much progress is being made in focusing our research effort on the priority gaps. In turn, this focus will lead to significant breakthroughs in technology at an earlier date than otherwise would have been possible leading to our earlier ability to control the diseases with which we currently struggle.

From the viewpoint of society, the ETPGAH initiative will help to protect animal health and welfare, as well as society from zoonoses. Most importantly, the aim of reducing the global disease burden should help to alleviate poverty and malnutrition in many parts of the world where endemic and epizootic diseases currently wreak havoc.

Development, Validation and Implementation of Animal Health Information Systems in an Environment without Uniquely Identified Animals in Transitional Countries

I. Naletoski[1]*, T. Kirandziski[2] & D. Mitrov[1]

ABSTRACT

Two animal health information systems were developed in Macedonia, the National Epidemiological Information System (NEIS) and the Laboratory Information System (LABIS). Both systems were aimed at collecting/interpreting animal disease data in a country where animals are not uniquely identified. The development of NEIS was based on the existing legislation of compulsory notification of infectious diseases. Field records are collected via the designated veterinary practices and entered into the NEIS via the veterinary inspectors who are employees of the Ministry of Agriculture, Forestry and Water Economy. Sources of data for NEIS are obligatory disease control programmes (Annual Order), endemic diseases, outbreaks, slaughterhouses and laboratory results of annual surveys. LABIS is a separate database for managing laboratory results. It collects data from samples submitted by Designated Veterinary Practices (DVPs). The samples can then be analysed in different laboratories using different methods and given a 'final status' by an authorised person. The final status is linked to the previously performed tests and entered into the NEIS. Using this concept, the Veterinary Department can trace back the background for each individual sample by reviewing the analyses performed on it. Both systems are designed as a referential integrity databases, where the field result is linked to the animal, owner, village (n = 1 803), epidemiological unit (n = 123) and epidemiological area (n = 30) in the country. NEIS can also present the same data in geographical maps, showing the infected village as the smallest unit of observation. Both systems have also different levels of authorisation access, allowing precise tracing of entered data.

Key words: *information systems, legislation, annual order, epidemiology, data entry, ISO 17205.*

INTRODUCTION

Veterinary activities for the control of animal diseases in Macedonia are based on the Law for Veterinary Health (Official Bulletin of R.M., 2007a), the Programme for control and eradication of especially dangerous diseases in animals (Official Bulletin of R.M., 2007b) and special programmes designed for specific diseases which are consistent with EU legislation. Under this legislation the country is divided into 30 Epidemiological Areas (EAs) and 123 Epidemiological Units (EUs). Each village belongs to a defined EU, which further belongs to a defined EA.

The Head Veterinary Office (HVO) comes under the Ministry of Agriculture, Forestry and Water Economy (MAFWE) and is divided into 5 sectors: Animal Health (AHS), Public Health, Border Inspection and Veterinary Legislation. It is lead by the Chief Veterinary Officer and the sectors are lead by the Heads of the sectors. The AHS is further divided into four departments: the Animal Health, Animal Welfare, Identification and Registration of Animals (I & R) and Veterinary Inspection in Animal Health (VIAHD). The veterinary inspectors (VIs) are employees of the VIAHD and are responsible for the animal health issues at the level of one or more EU.

The Designated Veterinary Practices (DVPs) are private enterprises, registered by HVO and licensed by the Veterinary Chamber. They perform field veterinary activities of Government interest. The designation is based on a public tender and is valid for five years. The DVPs are also responsible for one or more EU and can perform veterinary activities of government interest, only upon authorisation of the appropriate VI.

The Veterinary Institute is part of the Faculty of Veterinary Medicine in Skopje (FVMS) and is designated for official testing related to the animal health/public health issues. This testing is performed in the laboratories of the FVMS and are accredited under ISO 17025. Some of the testing methods (mainly upon request of the HVO) are accredited under ISO 17025.

In practice, the HVO publishes an 'Annual Order' (AO) based on the Programme for control and eradication of especially dangerous diseases in animals (Official Bulletin of R.M, 2007b). The AO defines all activities (examinations, vaccinations, checkups in the slaughterhouses, samplings, testings etc) which should be performed in a defined period of the year. Upon this order, the local VIs issue written orders to the appropriate DVPs. Activities are reported back to the local VIs, which forward the information to the HVO.

In case of laboratory testing, the samples are taken by DVPs, brought to the laboratories of FVMS and tested in the appropriate laboratory using predefined method/methods. The results obtained are sent back to the appropriate DVP, VI and at the HVO.

Until 2002, the results of all these activities were recorded on paper forms, cumulating progressively when collected at the level of

1 Faculty of Veterinary Medicine Skopje, Macedonia, Skopje, F.Y.R.Macedonia;
2 Ministry of Agriculture, Forestry and Water, Economy, Veterinary Department, Animal Health Unit, Skopje, F. Y. R. Macedonia

* Corresponding author: naletoski@fvm.ukim.edu.mk

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HVO and causing continuous problems when certain data analysis were needed. Additional problems have arisen because of the lack of permanent identification, control of movement and definition of the target group for examination (flock, backyard animals, mixing between flocks and houses etc.). During 2004, a Law for Identification and registration of Animals (Official Bulletin of R.M, 2004) was issued which is supposed to cover all domestic animals with permanent identification numbers, with strengthened, computerised management of farming, animal health and public health control. Up to now, only cattle have been permanently tagged; sheep and goats, as well as pigs are in the process of being tagged.

To solve these problems, we have developed the NEIS, a computerised system for animal disease data collection, which imitates the original flow of information. The system is based on collection and recording of data by the local VIs, which is later transferred to the HVO, enabling immediate follow up of the planned activities.

As the majority of the results originate from the laboratory, there have been problems with entering the huge amount of results into the NEIS. A solution was the development of LABIS (MAFWE, 2004), a system which can store different laboratory analyses performed on a single sample, deliver the final status (result) and transfer it into the NEIS.

Using this concept, we have succeeded in improving the collection of epidemiological data, enabling their urgent analyses and undertaking appropriate measures in the field. As both systems are created for environments lacking strict animal breeding and disease control, they are suitable for application in developing and transitional countries.

MATERIALS AND METHODS

National Epidemiological Information System (NEIS)

As a starting point, the main components of NEIS were defined: the nomenclatures, the planning of the AO, the structure of the national flock and the main sources of animal disease information. The nomenclatures were practically lists of commonly used attributes necessary for data entry and analysis. In this way, lists were made of villages, EUs, EAs, animal species, diseases etc. Afterwards, the necessary referential integrated links between these lists were established e.g. the link between an individual village and appropriate EU, as well as the link between the EU and the appropriate EA (**Figure 1**). Similar links have been established between the animals and the diseases, owners and the appropriate villages and users of the software and their user authorisations.

Using such linked nomenclatures, we were able to facilitate compact data entry for the activities performed and the existing predefined attributes.

The planning of the Annual Order (**Figure 2**) is usually made during the last quarter of the year for the upcoming year (e.g. between October and December 2008 for the year 2009). Creating the computer recorded plan could enable the HVO to follow up the performed activities in real time at the level of EU/village/owner on the one hand, and the VP/DVI on the other. Consequently, deviations from the planned activities can be immediately seen and corrective measures applied.

The analysis of the structure of the national flock is of great importance from many organisational reasons. Firstly, the distribution of animals inside the units of observation (EAs, EUs and villages) is important for organising human resources for the planned activities and for implementing consequent measures (easy to vaccinate or control a flock of 10 000 sheep, difficult to do the same in 2 000 flocks, each of 5 sheep!).

The structure of the national flock in the country is as follows:

The total area of the F.Y.R. of Macedonia is 25 713 km^2; divided into 30 EAs, within which 123 EUs are defined. There are 1 803 administrative villages, each of which belongs to a defined EU.

Approximately 87% of the cattle population is bred in farms (holdings) smaller than 30 animals. Sheep and goats are mainly kept in flocks of 50–100 animals/flock. Pigs and poultry are bred mainly in smaller (backyard) farms. The rest of the animals (approx. 13% of cattle, 20–30% of sheep and goats, 27% of pigs and 50% of poultry) are bred in professional farming facilities. This structure of the national flock results in the wide dissemination of large number of small flocks (holdings). Having these data, the HVO can easily estimate the number of official veterinarians, designated practices and intensity (timeframes) of activities for fulfillment of the AO.

The main sources of disease information were investigated because there was a need to match real sources of disease data and

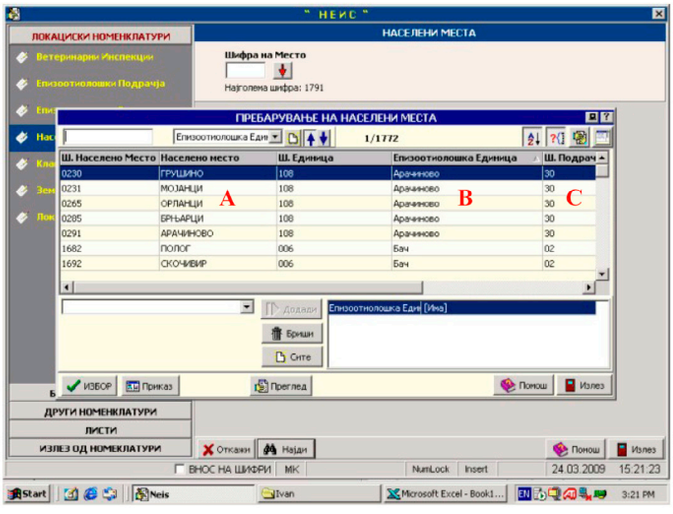

Figure 1. Nomenclatures: linking villages (column A) to appropriate EU (column B) and EA (column C).

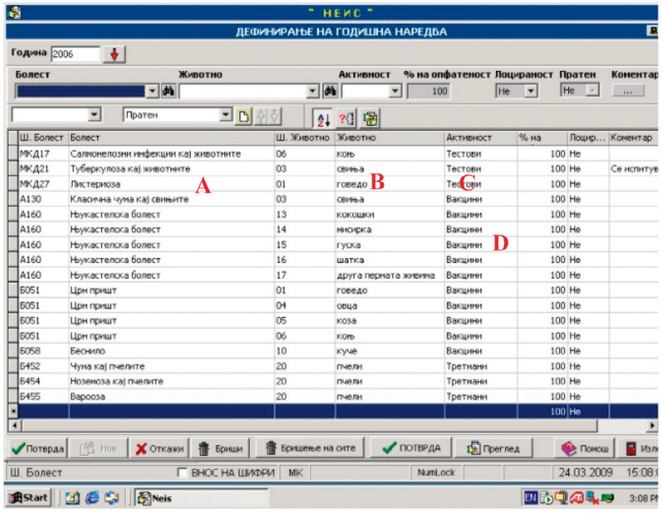

Figure 2. Planning of the AO by disease (column A), animal species (column B), type of activity (column C) and coverage (column D).

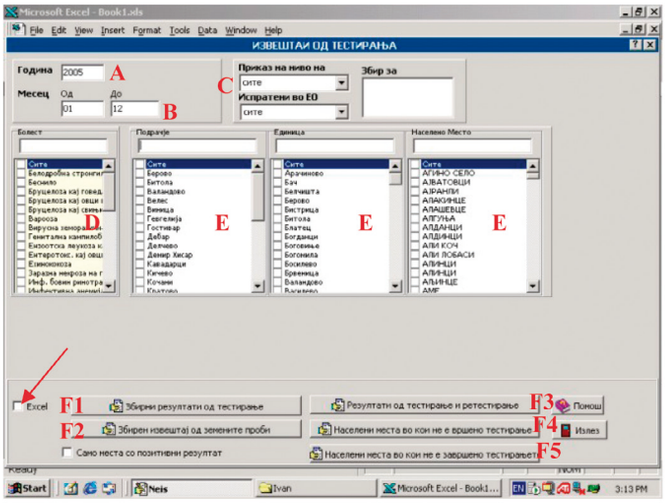

Figure 3. Example of a request for a report on performed testing (explanation in the text)

Figure 4. Example of a report for performed testings, after data filtering (explanation in the text)

entry tables/forms of the NEIS. These sources of information were divided into five classes, and for each of them a separate entry port (screen) was created. The classes of disease information are shown in **Table 1**.

Each of the five entry screens for the appropriate class of information is in compliance with the existing forms of DVPs and VIs, which enables a user-friendly entry by appropriate VIs. For each entered diagnosis, the source is also entered (clinical, post mortem, laboratory), to enable the HVO to establish its relevance. Once the data are entered into the local NEIS database, they are automatically transferred into the central database at HVO on a daily basis. However, if an outbreak occurs, the local VI can transfer the recorded data to the HVO immediately by pressing a single button. This tool is enabling the HVO to have an immediate overview of the situation in the field. Analysing entered data is enabled via the predefined reports. However, the system allows user-defined reports by selecting data the users want to see at any given moment. Additionally, the reports are generated using 'menu forms' where significant filtering of data is enabled, the aim being to select only critical information required during each request. An example of a typical report menu is shown in **Figure 3**. The report contains a selection of the parameters of interest, such as: selection of year (A), months from/to (B), selection of all testing performed or a defined testing (C), selection of disease (D), village within appropriate EU and EA or selection of certain EUs, EAs or villages (E).

Letters F1-F5 generate reports of filtered data in different ways e.g. cumulative results of all testing (F1): cumulative results of all sampled animals, showing pending results (F2): results of testing and retesting on same animals (F3); only villages where testing has not been performed (F4); and villages where testing is still not finished (F5). The menu offers a button (red arrow) which exports the data into Microsoft Excel, for eventual further analysis by the user. Such a pattern of filtering is used for all the reports on different queries. A typical report after data filtering is shown in **Figure 4**.

The filtering form has been adjusted to show summarised data for each EU and all diseases tested for the period between January 2004 and December 2004 (M). Simultaneously, the filter has been set up to show the name of the EA (A), disease tested (in the example: bovine brucellosis (B1), ovine/caprine brucellosis (B2) and bovine tuberculosis (B3), animals species (column C), number of villages covered during testing (column D), number of villages with positive animals (column

Table 1. Sources of entry data for the NEIS.

Type of information collected in the NEIS	Responsibility/method of collection
Obligatory disease control programmes (vaccinations, anthelmintic treatments and field diagnostics /TBC/ etc.)	VI/manually, in the local NEIS database
Endemic diseases (anthrax, clostridia etc.)	VI/manually, after laboratory confirmation, in the local NEIS database
Outbreaks (once an outbreak occurs /FMD/ the veterinarian must not test every single animal in the lab., but count the number of animals showing clinical signs in the village	VI/manually, in the local NEIS database
Slaughterhouses (findings during slaughtering e.g. *Echinococcus, Trichinella* etc.)	VI/manually, in the local NEIS database
Laboratory results of annual surveys	LABIS at FVMS/Automatically

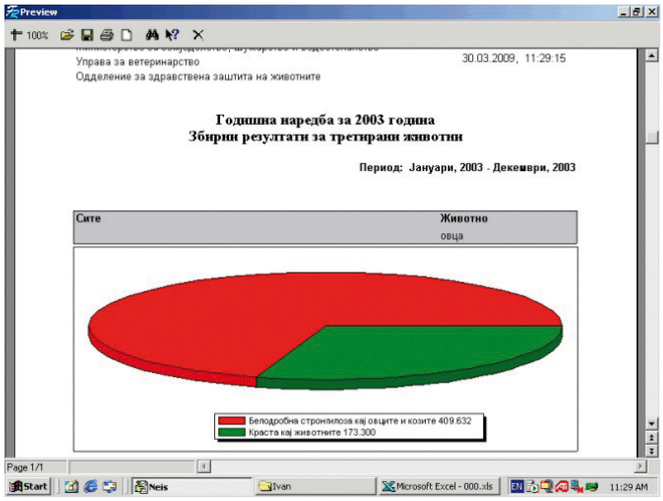

Figure 5. Example of a graph in a pie format (explanation in the text).

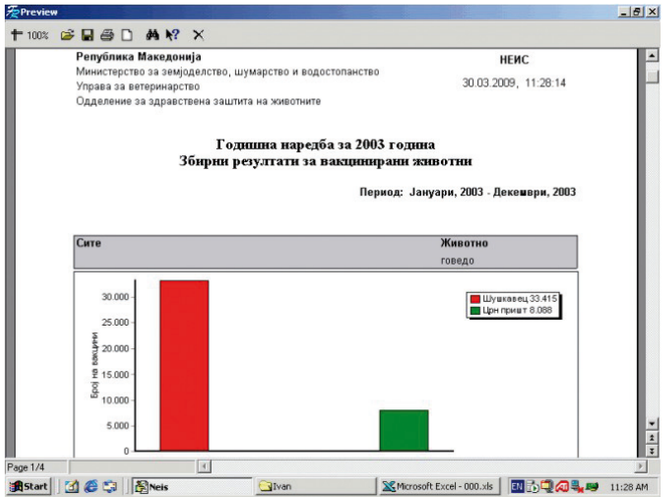

Figure 6. Example of geographical maps, upon different filtering criteria (explanation in the text).

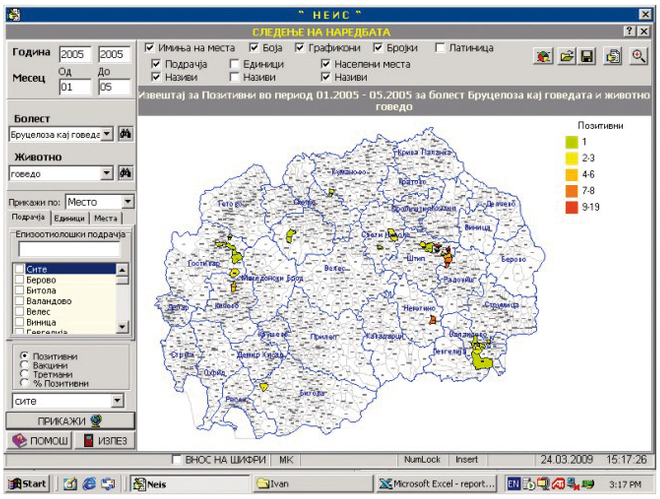

Figure 7. Example of a report as a geographical map, with appropriate filters over and left from the map (explanation in the text).

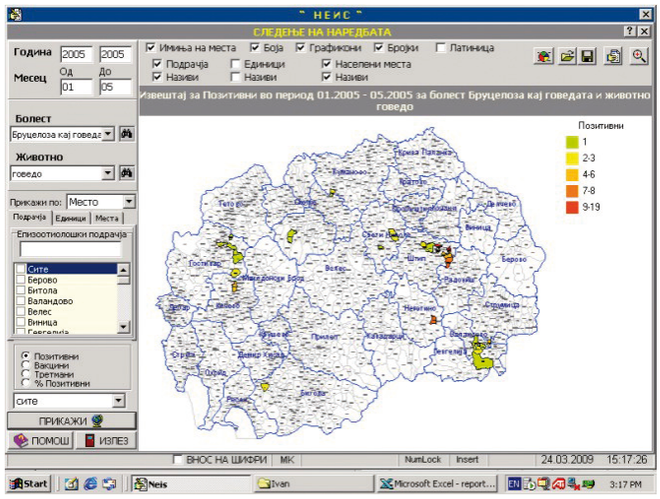

Figure 8. Entry form for the submission letter (description in the text).

E), percentage of villages with positive animals (column F), total number of tested animals (column G), total number of positive animals (column H), percentage MAFWE, 2002 of positive animals (column I), number of tests performed to obtain the final results (column J), number of slaughtered animal (column K) and number of destroyed animals (column L). Similar reports can be generated if the menu form is set up to filter the data to a lower level of observation (EU or village). An additional possibility for the user is to select columns he/she wants to see after the report is generated. This offers an opportunity to additionally avoid unnecessary data. This pattern of report is similar for all other reports generated by NEIS.

An additional potential of NEIS is to transfer the tabular data into graphs and maps. Two formats of graphs can be generated, the pies (**Figure 5**) and columns (**Figure 6**). The maps (**Figure 7**) are generated using mapping software. The maps show the data by linking the geographical borders of the administrative units (EAs, EUs and villages) to the tabular data from the reports. Consequently, the maps can show cumulative data based on a village, EUs or EAs. The same filtering principles are available for the maps as for the classical reports. The difference is only that the maps show coloured surfaces of the required geographical unit, which indicate the number of the required parameter or (upon request) a percentage of it.

Figure 5 is a pie chart where the occurrence of strongilosis (red colour) is compared to scabies (green color), for all the country. The coloured labels indicate the required parameter and the absolute number of variables for each parameter. **Figure 6** (just an example), shows a column graph, where the occurrence of blackleg (red colour) is compared with the occurrence of anthrax (green colour), cumulative at the level of the whole country. The coloured labels (lower part of the graph) indicate the required parameter and the absolute number of variables for each parameter.

Similar graphical presentations can be generated for the level of EA, EU or village.

Laboratory Information System (LABIS)

LABIS is designed to work in conjunction with the NEIS. For that reason, the nomenclatures are identical and picked up from one

Table 2. Steps for entering data in the LABIS.

Step	Activity	Comment
1.	Reception of samples	Samples are received, data of the main letter entered into LABIS and 'Certificate of Reception' issued to submitters.
2.	Entering the main submission letter with the corresponding forms with details	Required component for further processing of the samples in the laboratory.
3.	Electronic sending of the samples through the laboratory/ies.	Records on by whom, where and when the samples are processed.
4.	Definition of tests which are required.	Activity performed by request (HVO) or by decision of the head of the laboratory (outbreak/mortality?)
5.	Creating results from different tests.	If necessary, the head of the laboratory can send the sample to another laboratory for additional testing.
6.	Creating of the final result, based on one or more analyses for a certain disease.	The system will not allow generating the final result unless all requested testing and diseases are finished!
7.	Reports on tests performed, final results issued and epidemiological information based on the laboratory result.	These records are important as information support for the laboratory and communication with the HVO.

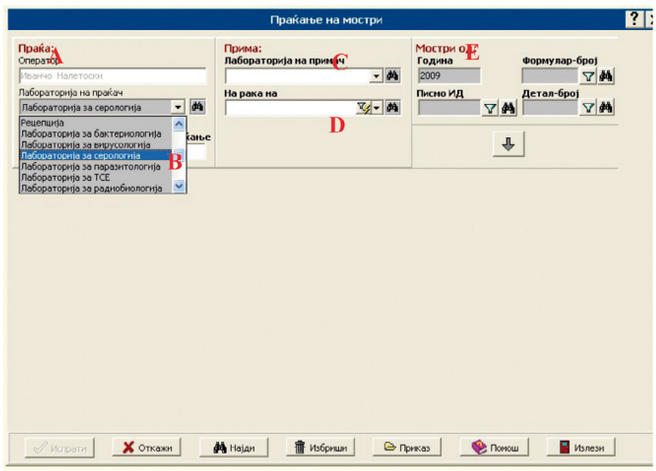

Figure 9. Entry form for sending of the samples to the laboratory.

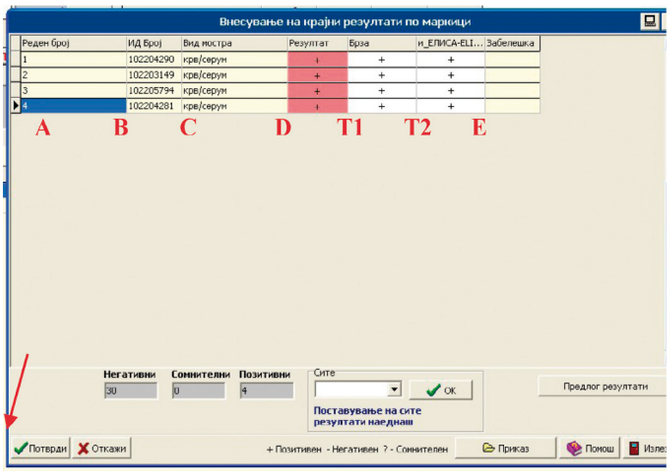

Figure 10. Entry form for sending of the samples to the laboratory.

source. This enables a synchronised transfer of data between the two systems.

The data flow in LABIS is designed to follow several steps which are in accordance with the former procedures involving manual sample submission and processing through the laboratory/ies. Briefly, the samples are submitted with the submission letter (obligatory document for submission) at the reception desk of FVMS. The submission letter is comprised from a main letter and the corresponding forms with details for the samples. The main letter contains general data about the village, owner, farm, the DVP and the aim for the sampling. The corresponding forms with details contain details about the sampled animals. The steps required for data entering into the LABIS are shown in **Table 2**.

The same pattern is used also in LABIS (**Figure 8**), with a remark that all forms with details are obligatory for entering in the database. Using this way of data entry, systematic data are stored in the database and the manual miss-filling of the forms omitted.

After entering the main letter (**Figure 8**, area A), the system generates a unique ID of the submission letter and a Certificate for Reception, which is given to the submitter.

The detailed forms (**Figure 8**, area B) can be entered into the system immediately or if needed, later, but in any case before sending the samples to the laboratory.

A special command button is available to enter the sample ID numbers in the system (**Figure 8**, area C). Entering of sample IDs into the system is not obligatory. They all can be added into the system, or upon request of the submitter (HVO), only positive samples will be added.

Upon entering the basic data into the system, the staff of the reception desk enter the name of the targeted laboratory/ies for testing (**Figure 9**). This process records who has sent the samples (A), when (date and time), (B), which is the recipient laboratory (C), which person should receive the samples (D) and the details on the submission letter to which the samples belong (E). This entry is important to follow up the activities on the samples through the responsible persons for them.

Once the responsible person in the first recipient laboratory opens the form with sent samples to his lab., the system will show him/her what has been sent and what kind of tests are required. If needed, the responsible person from the first recipient laboratory can resend

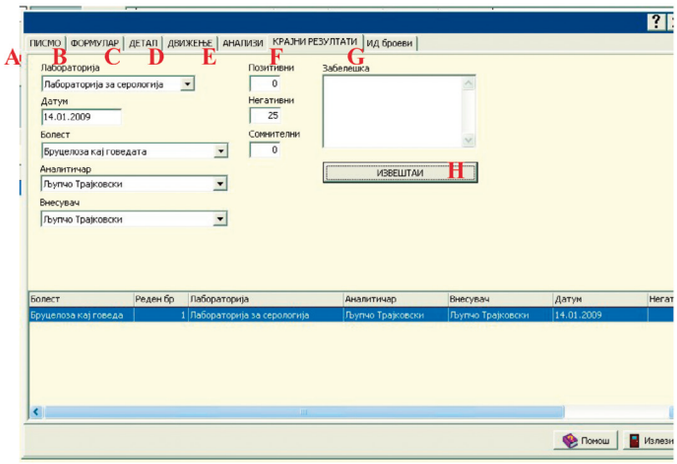

Figure 11. Form showing all details performed on the samples from a single submission letter.

the samples to an additional laboratory for which the same data from **Figure 9** are entered.

Different laboratories can test the samples using one or more tests (methods), (**Figure 10**). The results of all these tests are recorded for each sample individually (column B), showing the type of sample (column C), proposed final result (column D), and the performed tests to achieve the result (T1-T2). Separate columns with the computer generated unique ID of the sample is shown as is a column for free text for each sample (column A and remarks, column E respectively).

The proposed result (column D) is generated by the computer, based on the majority of the test results. The diagnostician is allowed to deliver his/her own final result based on knowledge related to the diagnostic performances of the tests performed. However, the system keeps all data, according to which the final status of the sample has been delivered, together with the name of the person who has generated it. Once the final result for a certain disease is confirmed by pressing the confirmation button (red arrow), it is stored in the database and cannot be edited anymore.

The same principle is used to confirm the diagnosis if multiple diseases are required for testing. The final result can be issued only after the status for all requested diseases has been confirmed.

After issuing (closure of the submission letter) of the final result, the authorised person can have a detailed look it using a special form (**Figure 11**). This form offers basic details related to the required submission letter (A), its forms and details (B and C), movement of the sample/es.

These reports are linked only to the laboratory results obtained in the designated labs. They are aimed to support the organisation of the laboratory, to gain epidemiological information from laboratory results and to support the HVO in the process of planning.

Since 2004, a Law for I & R of Animals has been issued (Official Bulletin of R.M., 2004). This law defines precisely the registers of owners, holdings, animals tagged with unique IDs and their relationships (actions to be taken when changing the owner, holding movement etc). As some of the species are identified at an individual level (cattle, sheep, goats) and some on a flock level (feeding lambs, farming pigs), the transfer of data from LABIS to I & R may cause difficulties. Additionally, some species are not subject to I & R, but will be of epidemiological interest (migratory birds, wild boars, wild carnivores). For this reason, a intermediate module is planned, aimed on enabling communication between LABIS and I & R.

RESULTS AND DISCUSSION

I & Rs are based on a strict link between the animal ID and the computer database. This requires almost perfect performance of the veterinary service, farmers and supporting staff (database users and managers). However, from a technical standpoint, while a good information system can be easily created, the difficult task is to build up a continuously supportive veterinary service. Such support should cover registration of any activity related to the animals (newborns, slaughtered/destroyed, animal movements etc.), as well as registration of epidemiological events, all of which are quite dynamic. Additionally, veterinary services should keep a history of all these events, which should be available for reviewing at any time.

If such a system is not working continuously and properly, early discrepancies will occur (experiences from Macedonia) in terms of missing young animals (unregistered calves), missing previously existing animals during sampling (unreported slaughtered/sold animals), or sampling animals that exist in the national flock occurring in totally different places (unregistered animal movement-trade).

Having this in mind, the concept of NEIS and LABIS was based on continuous collection of field information (NEIS) or from the annual obligatory testing campaigns (LABIS), simulating a 'snapshot' of the epidemiological situation.

Several major advantages have resulted from the two databases. Firstly, the traditional paperwork was largely replaced by computerised data collection, although some phases are still based on paper recording. Additionally, manipulation and interpretation of epidemiological data were significantly improved as all the data are permanently stored in the database. Finally, prompt reporting of data was enabled, either as tables, graphs or maps.

Using this concept, we have succeeded to link the animal to the owner, village, EP and EA which has resulted in the possibility of linking the epidemiological event related to the animal with its geographical origin, defined by the date of the event. Also, since these events were linked to the DVPs, the same events could be reviewed through them. When showing these data in a cumulative manner (quarterly, annually), we could generate an ongoing 'snapshot' of the epidemiological situation, although the I & R of animals is still not implemented. Using these 'snapshots' the HVO can have precise idea on the epidemiological situation in the country, focus activities (human and financial resources) towards the most actual problems and monitor the results of control/eradication campaigns. Reporting and monitoring of outbreaks by prompt transmission of field data has also improved the capacity of the HVO to monitor up-to-date activities.

In case of LABIS, we have succeeded to systemise multiple events connected with the sample from the moment of submission through to issuing the final result. These events include testing of one or more samples (blood, tissues, liquids) from one or more animals against multiple diseases using multiple diagnostic methods. As described above, all the events related to a single disease are interpreted via the 'final result', which can be transferred to NEIS. This concept of data recording enabled us to link the final result to its history (performed tests) and offer evidence on the relevance of the established diagnosis. Additionally, it has improved many pre-existing organisational problems such as sample flow through the laboratory and communication between the designated laboratory/ies and the HVO.

In terms of sample flow through the laboratory, where the samples are analyzed, using which methods, which technicians are producing more results and which protocols have been used for

individual samples as well as which submission letters (samples) are pending can be easily monitored. This information is directly improving the organisational capacities of laboratory management, such as engagement of people, planning of purchasing and supporting ISO 17025 requirements by systematic data recording.

In terms of the communication between the designated laboratory and the HVO, the system is promptly reporting both the final results obtained to the HVO and the history (performed analyses). After transfer of the data to NEIS, the laboratory results can also be presented in tabular, graphical and/or mapping formats.

Based on current experiences, both systems have some problems to be solved in the field. In the case of NEIS, during the start-up phase there were many older VIs who were not familiar with using computers at all, although the entering forms were practically identical to the old, paper forms. This caused problems in entering of data into the system and was generally overcome by employments of younger (computer friendly) VIs. An additional and remaining actual problem is the lack of sufficient numbers of VIs in the field which results in an accumulation of duties to the existing once.

In case of NEIS, there are two major problems. The first problem is recording of sample IDs in the system during large scale surveys. The recording is still manual, and the temporary solution agreed with the HVO is to record only the IDs of positive samples, leaving negatives only with the number of tested samples linked to the submission letter.

The second problem is recording of results according to the ISO 17025:2005 standard (page 20, chapter 5.10.1), which requires the laboratory to keep the records even when the customer agrees to simplified reporting. This problem is temporarily solved by keeping the hard copies of submission letters and linking them to the recorded results from the database.

Development of the I & R has created a demand to synchronise the work of NEIS/LABIS according to the I & R. The I & R can record data using individual animal numbers or flock (holding) number. Both numbers are coded according to a certain algorithm which requires recognition by LABIS. However, animals having flock (holding) numbers can be either retagged using temporary individual numbers, or the flock can be given a 'flock' result. An additional problem is the animals which are not covered by I & R (wild/ free-living animals etc.) which will have to be tagged using uncoded numbers. Having these two types of numbers, LABIS should be reprogrammed to recognise and distinguish them.

As a solution to these problems, we are planning to build a 'between module' which will represent a communication between LABIS and I & R. Additionally, we plan to redefine the preparation of the submission letter to be filled at the DVPs, the idea being that after sample collection, the DVPs will have to submit the submission letter directly in a local LABIS database. These data will be transferred to the LABIS via the 'between module'. The veterinarians will print their own 'Certificate of Reception' and will bring the formatted racks directly to the reception desk at the FVMS. The employees of the FVMS will find the filled submission letter in the computer according to the unique ID of the Certificate of Reception and recheck the submitted samples using a barcode reader. Each record from the barcode reader should find its match in the database belonging to the defined unique letter ID. Correct matching will be proof that the samples are correctly submitted. This action will also solve the problems occurring during large scale surveys and fulfill the requirements of ISO 17025.

Finally, it should be mentioned that after development of the two softwares, a period of 1–2 years is required to find gaps and 'fine tune' the systems with user-defined queries and reports, which should be a subject of guarantee for the programmers.

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Novel and Rapid Technologies for the Early Diagnosis and Molecular Epidemiology of Viral Diseases

S. Belák[1], N. LeBlanc[1], A. Diallo[2]*, P. Thorén[3] & G. Viljoen[2]*

ABSTRACT

Early and rapid identification of disease-causing pathogens, particularly those responsible for serious epidemic diseases, is a key element in the prevention of outbreaks and protection of susceptible populations. The detection of pathogen-specific nucleic acids has proven to be an invaluable tool in the diagnostic field. The advancement of technology involving the integration of amplification and signal detection systems has increased diagnostic capability, enabled development of robust, standard quantification techniques, as well as sequence analysis, by exposing products of the polymerase chain reaction (PCR) to a thermal gradient, i.e. melting and hybridisation curves. The most recent molecular technologies provide precision or broad detection facility, which is crucial to finding low level viraemia, distinct subtypes of interest, or mutants. In order for the development of new technologies and assays to proceed, a detailed knowledge of the diagnostic requirements is needed to create fit-for-purpose tools for the detection and discrimination of present and emerging diseases. The development of fit-for-purpose tools only makes sense if they can be transferred to, and applied in, appropriate laboratories and the field to provide reliable results using the most efficient methods for recognition of pathogens thereby allowing effective control measures to be employed as soon as possible. The prime objective in using these diagnostic tools should be to detect the pathogen at the earliest stage of the disease to prevent its spread.

INTRODUCTION

Currently, there are a number of factors that create an increased threat from emerging and re-emerging animal diseases (including 'unknown diseases'), and those that affect humans and seriously impact on a secure and safe food supply. Those factors include increased and more concentrated human and domestic animal populations (urbanisation and intensive husbandry practices), increased human and animal movement (both in frequency and geographically), including the globalised trade of live animals and animal products, bedding and feeds. Perhaps the biggest concern for food security is the outbreak of a pandemic event. Despite considerable efforts -— such as programmes for disease control, diagnostics, vaccination (conventional and marker) (Lubroth et al., 2007), regulation of animal transfer or movements and even stamping-out policies — animal diseases still continue to have a high bearing on animal health and human welfare.

Highly pathogenic H5N1 avian influenza (HPAI), and the pandemic H1N1 influenza ('swine flu') viruses are the most recent pathogens to present a global zoonotic hazard in terms of human health, with pandemic potential. These two pathogens each represent one half of a nightmare scenario. With a mortality rate, which may be greater than 50%, highly pathogenic H5N1 is truly a killer virus, while the 'pandemic H1N1', as the name and status given by the World Health Organization (WHO) indicates, possesses features of transmissibility that allowed it to spread around the world rapidly, with much lower mortality rate, as recorded so far. In addition to influenza, other diseases such as those caused by Hantaviruses, Japanese encephalitis virus, Human immunodeficiency virus (HIV), Dengue viruses, Menangle virus, Australian bat lyssavirus, Ebola virus, severe acute respiratory syndrome (SARS) coronavirus, Nipah virus and Hendra virus, also raise real concerns for human health, often in terms of their potential for global spread.

The globalisation of many aspects of animal production, from 'farm to fork', and the opening of borders between countries such as the European trading block, have created new risks and challenges for maintaining safe and stable food supplies. Even in the developed world, major disease epidemics have caused immense losses in the animal production sector. Foot-and-mouth disease (FMD), bluetongue (BT), and classical swine fever (CSF) have caused major problems throughout the EU. Rift Valley fever (RVF) moving into the Arabian Peninsula is another example of the significant economic and social consequences that result from the spread of highly contagious transboundary animal diseases (TADs).

Climate change is another factor with a global impact that is likely to affect the incidence, appearance and proliferation of viral pathogens, particularly arboviruses, whose lifecycle involves arthropods as vectors. Some examples that appear to support this predicted trend is the spread of BT virus and African horse sickness (AHS) virus into more northern regions, particularly in Europe (Schwartz-Cornil et al.,

1 Joint Research and Development Division, Department of Virology, Swedish University of Agricultural Sciences (SLU) and National Veterinary Institute (SVA), The Collaborating Center for the Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine of the OIE (World Organisation for Animal Health), Uppsala, Sweden

2 Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, International Atomic Energy Agency, The Collaborating Centre for ELISA and Molecular Techniques in Animal Disease Diagnosis of the OIE, Vienna, Austria

3 Section for Molecular Diagnostics, Department of Virology, Immunobiology and Parasitology, National Veterinary Institute, (SVA), The Collaborating Center for the Biotechnology-based Diagnosis of Infectious Diseases in Veterinary Medicine of the OIE (World Organisation for Animal Health), Uppsala, Sweden

* Corresponding authors: G.J.Viljoen@iaea.org and Adama.Diallo@iaea.org

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2008). Another vector-borne viral disease, African swine fever (ASF) has also spread outside of Africa to various regions of the world, including the recent spread into the Caucasus regions and beyond.

In addition to TADs, endemic viruses also have very high socio-economic impact. Pathogens such as bovine herpesviruses (BHV 1–5), bovine viral diarrhoea virus (BVDV), bovine respiratory syncitial virus (BRSV), porcine reproductive and respiratory syndrome virus (PRRSV), swine vesicular disease virus (SVDV), porcine circovirus type 2 (PCV2), among others, are found in cattle and swine populations around the world and activities designed to eradicate them require considerable inputs of resources. In fact, while the terms TAD and endemic are useful for classifying diseases they are not mutually exclusive. For example, CSF can be termed both as a TAD and as an endemic disease; it has been eradicated from domestic pigs in the EU, but wild boar populations are still infected in several countries of Europe. Therefore, this virus now poses a permanent danger to domestic herds with the likelihood of re-infecting them with a serious TAD. Another example is the arterivirus PRRSV; which was first detected and described in the Netherlands in 1991 and today is found around the globe.

The above examples clearly indicate that there is a need for reliable detection of targeted pathogens in the earliest phase possible of infection or in low copy contamination of various animal products or fomites. Although there are now well established techniques in molecular biology, nucleic acid amplification platforms such as PCR are relatively new compared with other diagnostic techniques. However, such amplification methods have proven to be unmatched in providing rapid and sensitive diagnostic tests, therefore the focus of this review is on thermal amplification platforms, their associated technology and the various detection chemistries employed. This review is intended to provide information on the current state-of-the-art and looks to future technological and molecular developments that can improve diagnostic capabilities for both human and animal populations.

One issue that is crucial in reaching the goal of producing effective diagnostic tools is the harmonisation and validation of new assays and technologies. In the environment of continual assay development and technological advancement (both simple and sophisticated), new diagnostic tools can only be widely adopted if they have been definitively shown to be effective and fit for their intended purpose. Furthermore, for early warning against many important livestock diseases, inexpensive and robust methods and devices are needed to cover the entire analytical process from sample extraction to reporting results to appropriate authorities. This review looks primarily at the field of biotechnology-based molecular diagnostic approaches.

TECHNOLOGY REVIEW

Application of Molecular Biology for the Diagnosis of Viral Diseases

Since the advent of nucleic acid amplification with the invention of the PCR in the mid-1980's, the development of diagnostic tools using molecular biology has exploded. This has been accompanied by a concomitant, parallel development in hardware technology, both simple and sophisticated. The most important driver of these advancements is the increased demand for secure and safe food, which includes the need to detect and control plant and animal pests and diseases. This technology review concentrates on the molecular diagnostic and characterisation technologies that are showing promise for providing diagnostic tools that deliver early and rapid detection of pathogens that threaten both human and animal health, as well as the food supply chain, under a range of circumstances from penside diagnosis in the field, through various levels of sophistication to well equipped diagnostic laboratories with skilled human resources.

PCR Platforms

Since the late 1980s, starting with gel-based PCR assays, a large variety of hardware technology and molecular chemistries have been applied to the diagnosis of pathogens including TADs and zoonoses in food, feed and fomites (Viljoen et al., 2005a). In gel-based systems, sensitivity and specificity may be increased by performing a nested PCR. This approach involves amplifying a region of the genome and then an inner region within the original amplified PCR product for another round of PCR. Real-time PCR has revolutionised molecular diagnostics, offering platforms that can readily be used in routine diagnostic laboratories as well as in research (Belák and Thorén, 2001; Belák et al., 2009). The list of real-time PCR platforms and their respective chemistries is extensive. Some examples include TaqMan[1] (the most widely used chemistry), molecular beacons (MB), primer-probe energy transfer (PriProET), linear-after-the-exponential PCR (LATE PCR), Scorpion primers, and SYBR Green. All these chemistries have been used to create highly sensitive and specific assays for the detection of pathogens (Belák, 2007). Some real-time PCR platforms are able to detect fewer than ten genome copies of the targeted viruses. Along with this very high analytical sensitivity, the majority of these platforms are able to amplify and detect exclusively the selected target nucleic acids. The chemistries all detect nucleic acids but the manner in which they do so differs at the molecular level. Some chemistries function by hybridising to a unique string of nucleic acids in the target genome, e.g. TaqMan and MB. The TaqMan assays rely on enzymatic degradation of a probe while in MBs there is a conformational change. SYBR Green chemistry functions as an intercalating dye which binds to any double-stranded DNA (Viljoen et al., 2005a). Sometimes co-development of assays makes sense in order to produce the best possible detection method (Gyarmati et al., 2007).

Real-time PCR provides sensitivity and specificity close to or equal to traditional nested PCR. Its value in the field of diagnostics comes from a number of important, well-proven advantages. For routine diagnostics, such as monitoring programmes, the main advantage is minimising the contamination risk posed by post-amplification products by measuring the amplified products in the reaction vessel, thereby reducing exposure to other samples or any other part of the outside environment. There are other significant advantages as well. In addition to providing a positive or negative result, by using a standard curve of known copy number the tests can give a quantitative estimation of the target nucleic acid. Pathogen load is often of interest but for certain pathogens it is crucial to the onset of disease as is the case in the diagnosis of PMWS in swine, where the viral load of porcine circovirus type 2 (PCV2) has to be determined (Segalés et al., 2005). As there is no requirement for gel preparation and staining, manual efforts are reduced. Automation of most of the process (extraction, preparation, amplification) is possible and with the use of a microtitre plate format, high throughput can be achieved.

A number of probes can be labelled with several different detection molecules, (i.e. fluorophores), so that multiplex PCR systems can be created. Multiplex PCR platforms use multiple primers to allow amplification of several different templates within a single reaction. This approach can be used to analyse a single nasal swab for a respiratory disease or a rectal swab collected from an animal suffering enteritis/diarrhoea syndrome. By performing multiplex PCR,

1 TaqMan is a registered trademark of Roche Molecular Sytems, Inc.

the detection specificity is broadened to detect all possible pathogens present that can be considered as agents for this particular disease syndrome. This is useful for detecting multiple targets as well as providing various internal controls that verify the assay is functioning properly. Compared with single-target PCR platforms, construction of multiplex platforms is more complex as the number of primer pairs increases (Viljoen et al., 2005a). Potential competition between the oligonucleotides can also be a hindrance to the development process, but with expertise and the use of bioinformatics and design software; sensitive and specific multiplex PCRs using dozens of primer pairs have been developed (Viljoen et al., 2005a). This level of multiplexing may not often be useful in real-time PCR platforms, but does allow microarray platforms to become a powerful tool at the diagnostic front-line (see below). That is not to say that real-time PCR platforms cannot effectively use multiple primers. For instance, LATE-PCR employs MBs read at different temperatures, allowing for double-digit primer pairs to be used effectively. Another multiplex technology, that uses an advanced gel-based system for detection has been put forward by Seegene Inc (Rockville, MD and Seoul, Korea). It has 16 capture elution (CE) marked products most of which include viral detection. One such assay is the Seeplex® RV12 affinity capture elution (ACE) detection assay designed to detect 12 major respiratory viruses (11 RNA and one DNA virus). For the veterinary field, the Seeplex® Porcine Diarr-V detection kit and Seeplex® Porcine Diarr-B detection kit are designed to detect five viruses and bacteria causing swine gastrointestinal diseases, including porcine epidemic diarrhoea. The general system used for these assays combines a novel chemistry using bipartite primers connected with a poly-inosine linker and conventional gel-based detection using amplicon size (Jun et al., 2008; Kim et al., 2008). Finally, although real-time systems have a higher up-front cost, when large sample numbers are to be tested, it provides lower costs per detected agent (Belák and Thorén, 2001).

The specification of the real-time PCR platform with regards to both hardware and chemistry is important, considering that the various equipment and chemistries have different strengths and weaknesses. For hardware, cost, reliability, maintenance requirements, flexibility, format (e.g. 96-well plate), speed, as well as other technical specifications like laser channels and filters, all have to be considered in deciding which platform is most suitable. For different chemistries, detection range, sensitivity, specificity, sensitivity to mismatch etc., can all affect the platform's results. It should be noted that at times, unexplained variation can occur within and between laboratories using the same platform (Belák and Thorén, 2001). It is crucial to be exact when describing a real-time PCR platform, chemistry, and assay in order to produce sound longitudinal and/or comparative data for routine or research purposes.

In reality, most molecular diagnostic laboratories generally employ a number of assays and it is not practical if all assays used are of different design or utilise different real-time PCR machines. Therefore, often it is not feasible for these laboratories to set up the recommended assays exactly as prescribed or to choose what may be the absolute best assay. As an example, the routine molecular diagnostic laboratory at the National Veterinary Institute (SVA) in Sweden is relatively small, running less than 20 000 assays/year (the Animal Health Service, GD, in the Netherlands runs at least ten times as many); however, SVA still has dozens of assays in use. Since the pathogen threat list is essentially decoupled from throughput, smaller laboratories must still be capable of testing for many pathogens. For the purposes of monitoring and early warning, it is often not wise to set up assays *ad-hoc*, based on perceived risks. Generally, diagnostics must be in place for all pathogens that are likely to present a risk. Recently at SVA, the molecular diagnostic laboratory has adapted virtually all of its assays to identical reaction conditions. This allows several different assays to be run simultaneously, thus creating a very efficient and economical work scenario. This is possible due to the combination of the robust TaqMan chemistry and a new molecular diagnostic kit that enables all PCR assays to be run using one thermal cycling routine.

Another strategy employed by smaller laboratories is to split up responsibilities for less common tests among laboratories. This can compromise the strategy of providing the earliest diagnosis possible but may be the most viable option when considering the economics of maintaining reagents for assays that may only be needed a few times in a year within a large region. Clearly therefore, in the real world, the ideal will usually not be possible. Adaptation to different nucleic acid extraction methods, real-time PCR machines and kits will always be the case. This illustrates the importance of reference laboratories for aiding proficiency testing, coordinating ring trials, and maintaining reference sample banks. Laboratories will often not be using the exact same testing procedures but it is essential that the various modifications still give comparable assay characteristics.

Automated Sample Preparation

Effective molecular diagnostic assays are dependent on the efficient processing of samples to extract nucleic acids (Viljoen et al., 2005a). This process can be difficult and varies depending upon the nature of the sample. Many sample matrices contain elements that can inhibit PCR to varying degrees and this in turn affects the effectiveness of the assay. When there are many samples, the extraction of nucleic acids can delay the throughput of samples and the analysis process. The use of nucleic acid extraction robots is common in both routine and research laboratories. Molecular diagnostics has evolved in a similar way to ELISA-based diagnostics, using automated systems for most of the procedures from sample processing to analysis. This allows laboratories to provide rapid, robust, low-cost diagnostics with improved reliability and less chance of contamination. Extraction robots generally function on two basic premises, using either filter- or magnetic bead-based extraction protocols. Examples of the latter include the Magnatrix 8000[2] and the more recent Nordiag Bullet[3] and the QIASymphony SP[4]. Larger robotic platforms can typically purify nucleic acids from 96 samples within 3–4 h (Belák and Thorén, 2001; Belák, 2007). Pipetting robots can prepare the PCR mixture and add the sample; other automated components can feed assays into the analytic machines.

Alternative Platforms to Real-Time PCR

Microarray technologies allow many specific sequences to be analysed simultaneously. They exist in several formats and they all allow many nucleic acid sequences to be identified through the use of complementary probes. The first microarray-based kit approved by the Food and Drug Administration (FDA) of the USA for use in the EU was the AmpliChip Cytochrome P450 Genotyping Test (Roche Molecular Systems Inc. Pleasanton, Calif.) in 2008. The assay uses a solid phase, planar microarray format and was designed to identify dozens of variants of the genes cytochrome P450 2D6 and cytochrome P450 2C19 in patients to help determine their proficiency in drug metabolism. Microarray-based diagnostics has now expanded into the field of viral diagnostics, aided by new array technology which is more suitable for use in diagnostic laboratories. The traditional microarray

2 NorDiag AB, Sweden

3 NorDiag AB, Sweden

4 QIAGEN Inc., Gaithersburg, MD, USA

Figure 1. Clondiag Chip technolgies ArrayTube (AT) platform. From left to right: array tube (with probe array chip on the bottom of the tube), the ATR 03 reader (connected to a PC or laptop to run image acquisition and analysis), magnification of probe array chip (detection reaction consists of horseradish peroxidase and HRP-substrate tetramethylbenzidine which reacts to form a blue precipitate.

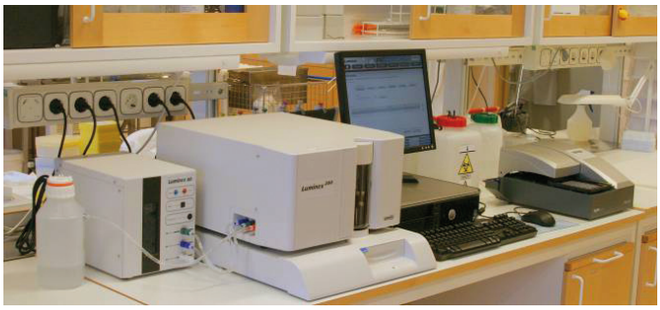

Figure 2. Luminex 200 system including from left to right: waste bottle, the Luminex SD sheath fluid delivery system the Luminex 200 instrument (above), the Luminex XY platehandling platform (below), Luminex xPONENT 3.0 software and a PC.
The microsphere-based detection system allows for 100 different analytes to de detected in each sample (100-plex). Also pictured (far right), is the Tecan Hydroflex wash station with buffer and waste bottles. This instrument performs automated microplate washing and vacuum filtration for use with both bead types (magnetic and polystyrene) used in the Luminex system.

format uses spotted glass slides and is not particularly user friendly. The company, CLONDIAG chip technologies GmbH (Jena, Germany) has produced the ArrayTube and ArrayStrips systems which use a planar array printed on the bottom of custom sample tubes or strips and read in their specialised workstations (**Figure 1**). The UK's Veterinary Laboratories Agency has developed a test based on the microarray technology or analysing antimicrobial resistance or virulence in a range of different bacteria (www.identibac.com). Examples of this technology applied to viral diagnostics include the detection and subtyping of avian influenza (Gall et al., 2008) and the detection of herpesvirus and adenovirus co-infections (Müller et al., 2009).

Another multiplex system, which has entered the field of viral diagnostics uses nucleic acid probes which are covalently linked to microspheres instead of attached to a solid surface, thus the microarray is in liquid suspension. This technology was brought to the market by the Luminex Corporation (Austin, Texas, **Figure 2**). In this system, nucleic acids are coupled to coloured microspheres creating up to a 100-plex assay on the Luminex® 200™. A 500-plex instrument is currently in the final stages of commercial development. The technology is an open platform with 'naked beads' available to be coupled to custom oligonucleotides (Dunbar, 2006). This technology can also be used to detect proteins (de Jager and Rijkers, 2006). The xTAG Respiratory Viral Panel (RVP) (Mahony et al., 2007) received 510(k) clearance from the U.S FDA in 2008 and is also CE marked for use in EU. This test was FDA cleared for 12 viruses and subtypes and CE marked for 19. The xTAG RVP is the first multiplexed nucleic acid test for respiratory viruses cleared for *in vitro* diagnostic use by FDA, the first test the FDA cleared to detect human metapneumovirus, to subtype influenza A, and to detect adenovirus.

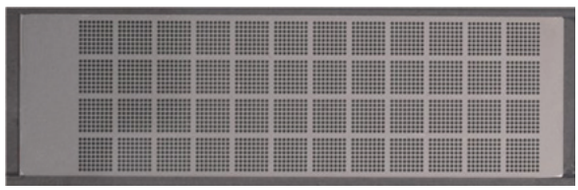

Figure 3. BioTrove OpenArray platform plate. A plate consists of 48 sub-arrays each containing 8 × 8 through-holes (wells) for a total of 3 072 reaction chambers per plate. Three plates can be read in the NT instrument (not shown) for a total of 9 216 simultaneous real-time qPCR results.

Besides probe-based methods, there are other technologies being employed in viral diagnostics including surface plasmon resonance (SPR) and mass spectrometry. The IBIS T5000 Universal Biosensor system uses mass spectrometry on PCR products to derive base compositions (Ecker et al., 2008; Blyn et al., 2008). Microfluidic platforms such as droplet-based PCR are a recent technology for high-throughput parallel PCR analysis, which allows for millions of discrete picoL reactions (Williams et al., 2006). The BioTrove OpenArray system[5] is a low volume, multi-well, PCR based system using nano-volume reaction mixes. Using a standard PCR cycling protocol, it produces 3 072 simultaneous PCRs in 33 nL reaction volumes in one plate (approx. dimensions of conventional microarray slide). The plate composition consists of 48 sub-arrays each with 64 through-holes or wells (**Figure 3**). The OpenArray plate can be composed in two ways: the wells can be spotted with primers or they can be empty with primers being added as part of the reaction mix. The cycling instrument (NT cycler) can run up to three plates simultaneously (9 216 individual PCR assays) and monitors the reaction progress in real-time, using either SYBR Green or TaqMan chemistry.

Alternative Chemistries to Polymerase Chain Reaction (PCR)

Methods such as Invader[6], nucleic acid sequence based amplification (NASBA) or loop-mediated isothermal amplification (LAMP) technologies, which use isothermal conditions in the amplification process instead of varying temperature amplification methods (as in PCR) are becoming more accepted (Hjertner et al., 2005; Blomström et al., 2008). For example, the LAMP method is simple and rapid, being performed in less than one hour.

A one-step reverse transcriptase LAMP (RT-LAMP) assay was developed recently to improve the detection of swine vesicular disease virus (SVDV). In this method, a set of six specially designed primers targeted eight distinct sequences of a target gene. The assay detected all 28 isolates tested in the developmental phase. Clinical

5 Life Technologies Corporation, Carlsbad, California

6 Invader is a registered trademark of Third Wave Technologies, Inc

Figure 4. BioSeeq Portable Veterinary Diagnostic System. Pictured left: portable PCR instrument with five independent thermocyclers (running assay in far left thermocycler) with built-in communications including GPS, WiFi and Bluetooth technology. Picture right: disposable automated sample preparation unit (SPR) processes raw sample into PCR mixture allowing test to be conducted at pen-side.

samples from nasal swabs, serum and faeces were used to evaluate the performance of the RT-LAMP compared with real-time PCR assays. The results from nasal swabs and serum were not significantly different from the TaqMan assay but with faecal samples the RT-LAMP assay performed significantly better than real-time PCR (Blomström et al., 2008).

A novel system for nucleic acid detection using Zinc finger proteins has been developed (Osawa et al., 2008). Zinc finger proteins are DNA-binding proteins that can bind to double stranded (ds) DNA with high affinity and specificity. They directly detect PCR products and check for specific PCR amplification. This has been used to detect simultaneously three pathogens: *Legionella pneumophila, Salmonella spp*. and Influenza A virus (Osawa et al., 2008).

Technology in the Field

Portable PCR technology is now coming to the marketplace. Companies are producing battery-powered machines that are simple to operate, work under field conditions, can be disinfected and have simple sample preparation methods. They are designed to bring laboratory facilities and the ability to make diagnosis closer to field cases and disease outbreaks (Viljoen et al., 2005b). For example, several companies (Smiths Detection[7], DxNA[8], Qiagen[9]) have produced portable devices and technology platforms specifically for field veterinarians. The system consists of a briefcase-sized PCR instrument, a disposable sample preparation unit and LATE PCR chemistry. It provides on-site identification under a wide range of weather conditions and the operator requires no technical training in PCR methodologies (**Figure 4**). Another company that has entered the high-tech side of portable diagnostics is UK-based Enigmadiagnostics (Salisbury, UK) whose Enigma FL instrument has been tested successfully by the UK Veterinary Laboratory Agency.

Some isothermal methods, such as LAMP assays have several features that support their use for penside diagnosis as they can be performed in modestly equipped field laboratories. Isothermal amplification requires a simple thermal block, can be obtained within 30–60 min, is highly specific and sensitive and the result can be assessed either by gel electrophoresis or directly, visually through the addition of SYBR Green. There are also several companies that produce low tech portable PCR lateral flow devices, which are designed to detect amplified nucleic acids using simple and cheap dipsticks, similar to pregnancy tests. These companies generally do not provide the entire system but there are firms which sell the devices e.g. BESt cassettes, Biohelix Corp (Beverly, MA, USA); production equipment, BioDot Ltd. (Chichester, UK) and even aid in the construction of portable systems such as Diagnostic Consulting Network Inc. (Carlsbad, CA, USA).

Multiplex methods are also being developed for use in field laboratories. A novel assay has been developed for the detection and discrimination of pestiviruses, i.e. BVDV types 1 and 2, CSFV and BDV using magnetic bead detection of PCR products in microarrays. In this method the PCR products are hybridised onto an array, followed by visualisation with streptavidin-coated magnetic beads for visual inspection or microscope examination. This assay was evaluated using a panel of pestiviruses comprising members of all four accepted species and performed as well in the ring trial as real-time PCR. This magnetic bead-based assay offers a novel technology for multiplex molecular diagnostics in virology (LeBlanc et al., 2009).

Molecular Methods for Monitoring Disease Epidemiology

Specific DNA products yielded by different chemistries can be analysed, sequenced and characterised. This means that exact information can be derived and stored in databases to enable comparison among samples. Large international databases such as GenBank, are powerful tools that aid in the phylogenetic identification, classification, and tracing of pathogen evolution and spread. For example, it is now hypothesised that EU and US genotypes of the porcine respiratory and reproductive syndrome virus (PRRSV) evolved from a common ancestor that was suspected to have originated from Eastern Europe (Stadejek et al., 2002; Ståhl et al., 2005 and 2007). In terms of early warning, molecular epidemiology can be combined with simple detection methods to better monitor potential health risks. For instance, influenza is a virus where in-depth sequence data could be very useful. If swine populations were better monitored with detailed analysis performed at routine intervals, then perhaps the current Influenza A H1N1 ('swine flu') pandemic could have been avoided.

Standardization and Validation according to OIE Standards

The increasing tendency to place molecular diagnostic assays at the forefront of routine analysis, combined with the tendency towards 'in-house' assay development and modification, have made it imperative to ensure international standardization and validation of developed assays. It is crucial to the success of these technologies that validated techniques exist to ensure they are fit-for-purpose and that results can be compared among laboratories. This approach provides the best prospect for the successful control of TADs and endemic diseases on a broad scale, while following the 'One world, one health' principle. Authorities, both national and international, require proof that assays used in laboratories are as reliable as possible (Belák and Thorén, 2004). International agencies such as the OIE and FAO, national diagnostic laboratories and research institutions as well as commercial companies have their own requirements and it is important that all of them work together on the international standardisation of protocols. The OIE has the lead role in this activity by publishing standards for the validation of diagnostic assays

7 Smiths Detection, Watford, UK

8 DxNA LLC. Saint George, UT, USA

9 QIAGEN Inc, Valencia, CA, USA

(http://www.oie.int/vcda/eng/en_background_VCDA.htm?e1d9) and publishing these in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 (http://www.oie.int/eng/normes/manual/A_summry.htm).

Whole-genome Sequencing

The design of suitable molecular assays will rely increasingly on available bioinformatics since sequence data are required for suitable probe and primer design. Whole-genome shotgun sequencing together with powerful computational algorithms to facilitate sequence data assembly, gene prediction and functional annotation have played important roles in this process. Primers designed to target conserved sequences of bacteria e.g. rRNA have allowed for the development of broad spectrum PCR for detecting bacteria (Picard and Bergeron, 2002). The variable internal rRNA gene regions can be sequenced and the data obtained compared with sequences from databases to make potential identifications at the species level, for contributing to epidemiological studies or for strain typing purposes, so as to determine traits such as antimicrobial resistance and virulence. Sequence data from 140 viruses have been used to design long oligonucleotide DNA microarrays with the potential of simultaneously detecting hundreds of viruses (Wang et al., 2002).

Bioinformatics, Comparative Analysis of Sequence Data

Many challenges also exist regarding the storage, analysis, management and integration of generated data. In the case of microarrays, pattern recognition tools are becoming increasingly important not only for direct pathogen detection, but also for analysing expression profiles from infected cells (Stenger et al., 2002). Methods of improving internet access to vast amounts of biomedical literature, using improved search engines and data mining programmes will also make important contributions to bioinformatics.

Internet and wireless communications technologies are also being harnessed to facilitate diagnostic systems (Cranfield Centre for Analytical Science Institute of BioScience and Technology, Cranfield University). Data that have been collected remotely can now be readily transferred to a central application for processing. Broadband internet, mobile phones and wireless communications technology among others, will allow the development of fully integrated, distributed applications across the internet.

Detection of Emerging, Re-emerging or 'Unknown' New Viruses

Various methodologies including random amplification followed by partial or full-genome sequencing, have been developed and are under development with the intention of discovering emerging and/or new viruses in humans and animals (Ambrose and Clewley, 2006; Delwart, 2007). The increasing availability and use of new sequencing technologies such as the 454 (Roche), SOLID (Applied Biosystems) and Solexa (Illumina) are accelerating this approach, which is becoming a frequently used tool to study different metagenomic issues (Shendure and Ji, 2008; Bosch and Grody, 2008).

In the development of 'discovery' technologies to identify pathogen genomes that no-one has identified before the group of Ian Lipkin, has played a pioneer role at the Columbia University's Mailman School of Public Health. This group has been working on technologies focusing on viral discovery since the late 1980s, being among the first researchers to identify microbes using only molecular tools. The team has identified close to 200 new viruses so far (see details and future ideas in recent summaries and discussions [Lipkin, 2009; Westley, 2009]). Other groups are following suite - Blomstrom et al. (2009) recently reported the detection of a novel porcine bocalike virus in the background of porcine circovirus type 2 viruses that induced a postweaning multisystemic wasting syndrome.

Biosensors

Biosensors consist of a biological recognition element in intimate contact with a transducer. The latter may include amperometric and potentiometric electrodes, field-effect transistors, magnetoresistive sensors, piezoelectric crystals, optical and optoelectronic devices, as well as miniature cantilevers. Developments in DNA-based biosensors are one of the fastest growing areas in nucleic acid analysis. Nucleic acid probes are used as the biological recognition element although ion channels have also been proposed for detecting base pair composition of DNA, with changes in electron current monitored according to the degree of pore blockage by different bases as single-stranded (ss) DNA is pulled through (Meller et al., 2000; Kristensen et al., 2001; Scheller et al., 2001). Electrochemical methods include the voltametric detection of redox intercalators and the mediated oxidation of guanine within the DNA. One format utilises signalling probes labelled with the redox agent ferrocene which bind to the target DNA previously immobilised on a capture probe embedded in a self assembling monolayer coating a gold electrode (Umek et al., 2001). Alternatively, the hybridisation event can be amplified using an enzyme label followed by monitoring of impedance. The conductive properties of a DNA duplex for electrons or holes can allow for monitoring using a redox probe at one end and the electrode on the other. Charge transfer is influenced by distortions with mismatches during hybridisation. In the future, ultraminiaturised electrochemical DNA sensors should allow for online monitoring and analysis. Electrochemical methods in combination with microfabrication techniques are likely to play important roles in providing highly sensitive assays.

The Bead ARray Counter (BARC) uses DNA hybridisation, magnetic microbeads, and giant magnetoresistive (GMR) sensors to detect and identify biological warfare agents. The current prototype is a table-top instrument consisting of a micro-fabricated chip (solid substrate) with an array of GMR sensors, a chip carrier board with electronics for lock-in detection, a fluidics cell and cartridge, and an electromagnet. DNA probes are patterned onto the solid substrate chip directly above the GMR sensors, and sample analyte containing complementary DNA hybridises with the probes on the surface. Labelled, micron-sized magnetic beads that specifically bind to the sample DNA are then injected. A magnetic field is applied, removing any beads that are not bound to the surface. The beads remaining on the surface are detected by the GMR sensors, and the intensity and location of the signal indicate the concentration and identity of pathogens present in the sample. The current BARC chip contains a 64-element sensor array. With recent advances in magnetoresistive technology, however, chips with millions of these GMR sensors will soon be commercially available, allowing simultaneous detection of thousands of analytes (Edelstein et al., 2000).

Piezoelectrical sensors are quartz crystal acoustic sensors that detect changes in mass on the crystal surface and would thus detect DNA-DNA hybridisations. The thickness shear mode type acoustic model is now being used especially for biomedical analysis (Pavey, 2002). Surface plasmon resonance (SPR) allows for real-time monitoring of binding between nucleic acid target and probe on the surface of a gold-coated prism. SPR monitors accumulating changes in surface mass following DNA-DNA hybridisation and can be used to confirm the specificity of a PCR reaction (Bier et al., 1997; Caruso et al., 1997). Single-stranded DNA obtained following asymmetric

PCR has been used to hybridise with a biotinylated probe attached to the sensor surface (Bianchi et al., 1997). Cantilevers coated with receptor layers act as force transducers and are being used in microfabricated biosensor devices (Oak Ridge National Laboratory [http://www.ornl.gov]; Graviton Inc. [http://www.graviton.co.jp]; Protiveris Inc. [http://www.protiveris.com]; Cantion A/S [http://www.cantion.com IBM, http://www.ibm.com]). Changes in surface stress, temperature and magnetisation can be monitored following receptor-target binding, such as DNA hybridisation. Monitoring is done using optical lever, interferometry or beam-bounce techniques. In the case of interdigitated cantilevers, a diffraction pattern is monitored in accordance with cantilever deflection. Capacitor plates or peizoelectrical cantilevers can be used to monitor changes in capacitance or conductivity in response to surface stresses (Bianchi et al., 1997). Silicon nanowires (Nanosys, CA, http://www.nanosysinc.com) and carbon nanotubes (Molecular Nanosystems, CA, http://www.monano.com) have been described which can monitor changes in conductance during binding of biological molecules to the surface (Alivasatos, 2001). DNA labelled with gold nanoparticles can be used as probes on chips that can then be monitored electrically. Carbon nanotubes, molecular transistors and switches including allosteric and ribozymal nucleic acids, have exciting contributions to make as components of biosensors (Soukup and Breaker, 1999).

Microfabrication, Microfluidics and Integrated Systems

The integration of sample processing, amplification and detection systems is an important goal in achieving suitable point-of-care with on-site devices. Several partly integrated systems have been developed for the detection of biowarfare agents. The Lawrence Livermore National Laboratory and University of California, Davis (LLNL-UC Davis) consortium have developed a self-contained system that continuously monitors air samples and automatically reports the presence of specific biological agents. The LLNL Autonomous Pathogen Detection System (https://ldrd.llnl.gov)is an integrated aerosol collector, sample preparation and detection module that detects and identifies pathogens and/or toxins by a combination of an immunoassay and PCR. Nanofabrication and molecular electronics are being used by Nanogen (http://www.epochbio.com) to develop various sample-to-answer devices.

Micro-fabricated devices are being used to perform PCRs for faster cycling using small chambers and integrated heaters. A variation of this approach uses the well of a microchip as the PCR chamber, with the entire chip undergoing thermal cycling. Electrophoretic separation at the level of the wells of the microchips can be achieved by transferring samples between a PCR chamber and a well. The PCR has also been integrated with detection using fluorogenic DNA probes. Most integrated systems combine the processing and detection phases using a microfluidic platform. Microfluidic systems consist of microchannels and tiny volume reservoirs and utilise electrokinetic or pneumatic mechanisms to transport fluids. The flow rates are in the nL/sec range through flow channels with cross-sectional dimensions in the tens of μ meters. Advantages include improved speed of analysis, reproducibility, reduced reagent consumption and the ability to perform multiple operations in an integrated fashion. Further development of this technology is expected to yield higher levels of functionality of sample throughput on a single microfluidic analysis chip.

Integrated systems using microfluidics are usually termed 'lab-on-a-chip' technologies. Although still in the developmental phase, they are likely to make dramatic impacts on molecular diagnostics in the future, especially as point-of-care devices with important contributions from nanotechnology.

Cepheid (Sunnyvale, CA, USA) has developed a device that consists of disposable cartridge-containing reagents and chambers for bacterial cell lysis and test sample preparation for fluorescent-based nucleic acid detection in a device called GeneXpertTM. Infection Diagnostic developed a rapid sample preparation method for both Gram positive and negative bacteria in different sample specimens. Lin et al. (2003) constructed a micro-fabricated device for separating and extracting ds DNA fragments using an array of microelectrodes and a cross-linked polyacrylamide gel matrix that is amenable to integration with reaction chambers into a single device for portable genetic-based analysis.

Microfluidic-based laboratory card devices have resulted in credit card-sized designs suited for processing whole blood for haematological applications, and involves flows of sample reagents and control solutions in microchannels using capillary flow, hydrostatic pressure and fluid adsorption (Micronics Inc. WA, http://www.micronicsinc.com) (Bousse et al., 2000; Cronin and Mansfield, 2001). Optical microchips have also been devised whereby a microarray of optical scanning elements is integrated with microfluidic circuits (Ruano et al., 2003). These allow for fluorescence detection and have the potential to provide other optical biosensor platforms such as SPR, evanescent field technology and interferometry.

DISCUSSION AND CONCLUSIONS

Given the socio-economic impact of infectious diseases in human and animal health today, there is clearly a demand for highly sensitive and robust diagnostic techniques which facilitate the early detection of the dangerous pathogens and assist in the immediate implementation of disease control measures. Globalisation and extensive international trade have created a scenario where infectious agents have the potential for global spread within hours or days, causing serious epidemics. It is extremely important to apply techniques, which can detect pathogens rapidly and effectively. Advances in real-time PCR methods, such as PriProET and LATE PCR are providing new opportunities to provide the best validated, fit-for-purpose assays. Portable PCR machines and isothermal amplification techniques such as NASBA and LAMP, and LFDs allow the application of PCR for 'point-of-care' or 'on-site' detection of pathogens. 'On-site' tests provide powerful early warning tools to the authorities responsible for prevention and control of infectious diseases; however, in general and especially in the case of important pathogens such as TADs, the results from on-site tests have to be confirmed in the laboratory. Microarrays allow a more complex investigation of pathogens. By combining a wide range of highly specific target sequences, a detailed investigation of the pathogen responsible for the disease outbreak is possible. Microarrays can simultaneously detect several agents or even a high number of pathogens, co-infections, subtypes and pathotypes, providing information rapidly so that appropriate actions can be taken based on a detailed understanding of the disease status in affected population(s). Microarrays, especially liquid-phase systems, are also amenable to automation and the use of high-throughput platforms.

Other detection methods, which are still at the more developmental stages include surface plasmon resonance and matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (Sampath et al., 2007; Jores et al., 2009). Advances in biosensors, the development of integrated systems such as lab-on-a-chip devices and enhanced communications systems are all promising. No doubt, some of these newer technologies will provide valuable early warning tools in the future.

In addition to the control of disease outbreaks, the need for safe tracing, identification and testing of various pathogens in food and feed products is also increasing. Furthermore, for zoonotic diseases, transmission of pathogens between various hosts in animal and human populations necessitates extensive monitoring programmes. The appearance of 'unknown' or 'completely new' pathogens creates new epidemiological situations that rapidly cover large regions of the globe (e.g. causative agents of SARS, H5N1, H1N1 'swine flu', pandemic simian immunodeficiency virus [SIV]). In this area, the most advanced technologies available will be required to provide the most complete analysis possible (e.g. full-genome sequencing).

With the continual risks from transboundary and endemic diseases which are compromising food safety, and also the risk posed by zoonoses, the 'One World, One Health' approach is imperative; particularly today, in the current environment of intense globalisation, international trade, global tourism, and climatic changes as well as financial, energy and political instability. Diagnostic research laboratories will be expected to improve detection capabilities by developing novel biotechnology-based diagnostic techniques, some of which have been outlined in this article. Finally, although not a subject of this article, the need to complement new techniques with the maintenance of classical ones is vital. The devices and technologies described in this article enable ever more efficient and speedy diagnosis without the user needing to process live pathogens in the laboratory, but it is in danger of creating a generation of scientists whose knowledge of the actual organism is seriously deficient. Classical methods in virology and bacteriology, with special regards to virus isolation in cell culture, and *in vitro* and *in vivo* characterisation are in jeopardy of being lost as laboratories replace them with modern technology. This is very short-sighted considering the likely impact on science if laboratories lose the ability to isolate and cultivate different strains of pathogenic micro-organisms, particularly those causing newly emerging diseases, where it is necessary to understand their pathogenesis, develop diagnostics, perform repeatable experiments, produce vaccines as well as study many other aspects of infection biology.

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Molecular Detection Technologies for Arboviruses

W. Wilson[1]*, J. Jiang[2] , H. Weingartl[2] , L. Rodriguez[3], C. Murrieta[1], B. Drolet[1] & M. Miller[1]

ABSTRACT

Arthropod-borne animal viruses (arboviruses) cause significant livestock and economic losses to world agriculture. This paper discusses the current and potential impact of these viruses, as well as the current and developing molecular diagnostic tools for those emerging and re-emerging insect transmitted viruses affecting livestock and wildlife. The emphasis is on viruses for which there have been significant recent outbreaks in livestock including: bluetongue virus (BTV), epizootic hemorrhagic disease virus (EHDV), vesicular stomatitis virus (VSV), and Rift Valley fever virus (RVFV). The current readiness for rapid detection of arboviruses is fairly high, but there is a need for global harmonisation and continued evaluation due to the genetic variation of these unique pathogens. The tool chest for molecular detection contains a range of assays from low technology to high-throughput sophisticated devices.

Key words: *Arbovirus, bluetongue, Rift Valley fever, detection.*

INTRODUCTION

Arthropod-borne animal viruses (arboviruses) are of increasing concern to both veterinary and public health due to the recent expansion of these viruses into new areas. The introduction of West Nile virus into the USA initiated this growing concern (Lanciotti et al., 1999). The danger of the spread of arboviruses into new geographic locations was also unfortunately realised by the introduction of bluetongue virus (BTV) serotype 8 into northern Europe (Thiry et al., 2006; Toussaint et al., 2006). This has resulted in tremendous efforts to understand the epidemiology of the disease in Europe as well as detect and control the spread of the virus. Biting midges of the genus *Culicoides* transmit BTV and the related epizootic haemorragic disease virus (EHDV). These viruses cause sub-acute to lethal disease in cattle, sheep, goats and/or wild ungulates, the resulting worldwide losses attributable to BTV alone being estimated at $3 billion annually. There was a fairly good understanding of the epidemiology of BTV until the recent introduction of BTV into Europe. Of particular concern is the economic and unique disease impact that BTV–8 has had in Europe and the fact that there have been multiple isolations of exotic BTV serotypes in the USA over the past 3 years. In Europe, killed BTV–8 vaccines are being used to control and potentially eradicate the disease (Mintiens et al., 2008). In the USA, there is only one commercial vaccine available, and it is specific to BTV type 10. There is limited or no cross-protection between serotypes, thus complicating the control of the disease. The related orbivirus, EHDV, is of considerable interest to the captive cervid industry, and EHDV serotype 7 has been associated with clinical disease in Israeli cattle (Yadin et al., 2008) A number of assays are available for detection of viral RNA using reverse transcriptase-polymerase chain reaction (RT-PCR) genome amplification for BTV and EHDV (Wilson 1994; Shad et al., 1997; Aradaib et al., 1998). Additionally, real-time RT-PCR (rRT-PCR) assays are available to detect all BTV serotypes (Shaw et al., 2007; Toussaint et al., 2007) and all EHDV serotypes (Wilson et al., 2009b). This report summarises a multiplex assay developed to detect BTV and EHDV and distinguish between the two viruses in a single closed tube (Wilson et al., 2009c).

There are periodic outbreaks of vescicular stomatitis virus (VSV) in the USA presumably introduced by insect vectors from enzootic regions of Mexico (Rodriguez et al., 2000). *Culicoides*, black flies, and sand flies transmit VSV to cattle and horses (Drolet et al., 2005). Insects are believed to play an essential role in transmitting the virus to domestic livestock. Once initial infection has occurred, direct contact transmission is believed to be another route of infection. Humans associated with infected livestock can become infected resulting in mild to severe febrile illness. The clinical severity of VSV, and its similarity to clinical foot-and-mouth disease (FMD), results in quarantines, sale barn closures, and restrictions on the movement of livestock and animal products. As with BTV, there are a number of standard diagnostic tools available for detection of VSV, including a recently developed multiplex rRT-PCR for detecting and distinguishing VSV Indiana from VSV New Jersey (Rodriguez et al., 2009; Wilson et al., 2009a).

Recent outbreaks of Rift Valley fever (RVF) in East Africa have raised worldwide concerns of the potential spread and disease impact of this virus, which can cause high mortality in young small and large ruminants and abortions in older animals. This is a zoonotic virus resulting in mild to lethal disease in humans. Retinal degeneration has been reported in up to 10% of infected humans. Few veterinary diagnosticians are immunised with the expensive investigational vaccine to allow them to work safely with this virus. Therefore a rapid, sensitive, and specific diagnostic tool such as a molecular amplification technology that quickly inactivates the virus during sample processing is ideal. In addition, diagnostic assays are being developed to differentiate infected from vaccinated animals (DIVA). A multiplex

1 Arthropod-Borne Animal Diseases Research Laboratory , Agricultural Research Service, U.S. Department of Agriculture, Laramie, WY, USA
2 National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, Manitoba, Canada
3 Foreign Animal Disease Research Unit, Agriculture Research Service, U.S. Department of Agriculture, Plum island Animal Disease Center, Greenport, NY, USA
* Corresponding author: william.wilson@ars.usda.gov

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real-time RT-PCR for all three virus segments is in development to provide a robust and DIVA compatible assay for RVF.

MATERIALS AND METHODS

The general approach to real-time PCR (rRT-PCR) design was to select specific gene target(s) based on what is known about the molecular biology of the arbovirus. If insufficient sequence information was available, then sequencing of appropriate diverse virus populations was done. The sequence information was submitted to various bioinformatic analysis tools to select potential real-time RT-PCR signatures (primer and probe combinations). General descriptions of the assay compents are provided below, but specifics can be found in the reference cited for each virus.

RNA Extractions

Three RNA extraction systems were applied to the various arboviruses. The first systems used phenol-based extractions such as Trizol (Invitrogen Corporation, Carlsbad, CA), TriReagent (Applied Biosystems/Ambion, Austin, TX) and TriPure (Roche Applied Diagnostics, Indianapolis, IND). The second method used was a spin column system (Qiagen Inc. CA). The final system used was magnetic bead-based (Applied Biosystems/Ambion, Austin).

PCR Procedures

Amplification was first confirmed using standard SYBR green RT-PCR reagents (Applied Biosystems/Ambion, Austin). Once amplification was confirmed then dual-labeled probes were obtained (Biosearch Technologies, Inc., Novato, CA; Integrated DNA Technologies, Inc., Coralville, IA). Standard one-step real-time RT-PCR reagents were used for the TaqMan® assays (Applied Biosystems/Ambion, Austin, TX).

RESULTS AND DISCUSSION

Bluetongue virus continues to be an important arbovirus of livestock and the recent introduction of BTV–8 demonstrates the need for continued diagnostic advances. The sequence analysis performed to develop the BTV/EHDV multiplex real-time RT-PCR demonstrated a greater sequence variability in the target genes than previous studies predicted (Wilson, 1994). The target genes were selected based on previous phylogeny studies (Wilson, 1994), but it was found that the sequence information at the time was insufficient to design universal primer sets for BTV (Wilson et al., 2004). Additional sequence data from the prototype strains was generated and a bioinformatics approach was used to design a more robust rRT-PCR assay that rapidly detects and distinguishes between BTV and EHDV strains. All of the RNA extraction methods commercially available worked with the assay, but the magnetic bead extraction afforded the best sensitivity. This was estimated using spiked blood and found to be 10 $CCID_{50/ml}$ for BTV and 1 $CCID_{50/ml}$ for EHDV (Wilson et al., 2009c).

Vesicular stomatitis virus is important not only because of the severe, but usually non-lethal disease it can cause, but because the clinical signs in cattle and swine resemble that of FMD. As with BTV/EHDV, a bioinformatic approach based on phylogenetic studies was used to develop a robust rRT-PCR design (Rodriguez et al., 2009). Since there are multiple serotypes of VSV; the assay was designed to detect and distinguish between VSV New Jersey and VSV Indiana, the most prevalent serotypes. The VSV multiplex assay performed very well using both phenolic and column-based RNA extraction methods in the laboratory. Field evaluation was performed using the column-based RNA extraction method to be consistent with the FMD assay developed previously (King et al., 2006). The field evaluation was performed in endemic countries and a distinct genetic VSV population in Costa Rica was found not to be detectable by the assay. Additional sequence analysis was performed and a new primer/probe design developed that resulted in near 100% sensitivity and specificity. This assay has also been successfully applied to experimental VSV infection studies of insects (Mead et al., 2009).

The 2006–2007 outbreak of RVF in Kenya, Tanzania and Somalia has resulted in increased research efforts for early detection and control of this deadly and zoonotic virus that is associated with high abortion rates in livestock. Three RT-PCR assays have been developed for RVF (Garcia et al., 2001; Drosten et al., 2002; Bird et al., 2007). These assays all target different segments of the tripartite genome. Two of the assays were shown to be effective with clinical samples (Bird et al., 2007; Njenga et al., 2009). We have applied these assays to samples generated in experimental virulent and vaccine RVF strain infections of sheep, cattle and mosquitoes. The three available commercial extraction methods worked well with these assays. The choice of extraction method is primarily based on throughput and approved institutional safety protocols. The three rRT-PCR protocols were combined to generate a robust single tube multiplex RT-PCR assay which detects all of the three genome segments using different reporter dyes for each segment for confirmation purposes. During optimisation, the L-gene primer set (Bird et al., 2007) had low sensitivity and the S-gene primer set (Garcia et al., 2001) was consistently less sensitive in our single step format as compared with the original protocols. The newly designed L and S primer sets worked well as individual primer/probe sets and their incorporation into the multiplex rRT-PCR is currently in progress. There are two real-time reverse transcription-loop-mediated isothermal amplification assays (LAMP) available that provide alternative amplification detection tools for RVF viral RNA (Peyrefitte et al., 2008; Le Roux et al., 2009).

CONCLUSIONS

Standard RT-PCR viral gene amplification assays are being discarded by many diagnostic laboratories because they are prone to cross-contamination problems. The real-time RT-PCR assays such as those described here for the arboviruses, can be performed in a closed tube environment. This decreases the cross-contamination that occurs more frequently with gel-based assays and the instrumentation required is becoming more comon even in developing countries. These assay are specifically designed to be robust and flexible. On routine analysis, small numbers of samples can be run, but in outbreak situations the real-time RT-PCR assays can be automated to allow high throughput. In addition, the assay can be rapidily modified to detect new genetic variants. Additonal modifications including multiplexing for differential pathogen disease targets, and inclusion of an internal or positive RNA control such as in the multiplex vesicular disease panel (Lenhoff et al., 2008), could provide future improvements. New technologies such as linear-after-the-exponential (LATE)-PCR (Sanchez et al., 2004), LAMP (Peyrefitte et al., 2008; Le Roux et al., 2009) and surface enhanced raman spectroscopy (SERS, (Harpster et al., 2009) are taking advantage of the existing amplification detection knowledge base and providing both low and high technology solutions for virus genome detection. The development of validated diagnostics tools is needed for effective control strategies and the formulation of reasonable animal regulatory statutes to reduce the economic impact of these arboviruses.

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A Recombinant Nucleocapsid-based Indirect ELISA for Serodiagnosis of Rift Valley Fever in African Wildlife

J.T. Paweska[1]*, P.J. van Vuren[1], A. Kemp[1], R. Swanepoel[1], Peter Buss[2], R.G. Bengis[2], F. Gakuya[3], R.F. Breiman[4] & M.K. Njenga[4]

ABSTRACT

An indirect ELISA (I-ELISA) based on the recombinant nucleocapsid protein (rNp) of Rift Valley fever virus (RVFV) was evaluated for the detection of specific serum IgG antibody in African wildlife. Data sets derived from field-collected sera (n = 918) in Africa (antelopes = 570, black rhinoceros = 43, common zebra = 24, elephant = 73, giraffe = 81, grevy zebra = 78, warthog = 49) were categorised according to the results of a virus neutralisation test (VNT). At cut-offs optimised by the two-graph receiver operating characteristics analysis, the diagnostic sensitivity of the I-ELISA was 100% and diagnostic specificity ranged from 99.8% to 100% while estimates for the Youden's index (J) and efficiency (Ef) ranged from 0.99 to 1 and from 99.7% to 100%, respectively. The rNp-based I-ELISA is highly accurate, safe, and offers a single assay format for rapid detection of IgG antibody to RVFV in sera of different wildlife species.

Key words: *Rift Valley fever virus, recombinant nucleocapsid protein, IgG antibody, indirect ELISA, African wildlife.*

INTRODUCTION

The recent occurrence of the first confirmed outbreaks of Rift Valley fever (RVF) outside Africa (Jupp et al., 2002), together with the ability of RVF virus to replicate in a wide range of mosquito vectors (Turrel et al., 2008) and the effects of global warming which facilitate spread of arthropod-borne viruses (Purse et al., 2005) into non-endemic regions of the world are of medical and veterinary concern. Antibodies to RVF virus have been found in many wildlife species (Davies, 1975; Anderson and Rowe, 1998; Fischer-Tenhagen et al., 2000; Paweska et al., 2005; Evans et al., 2008; Paweska et al., 2008) but their importance in the epidemiology of the disease during the inter-epidemic and epidemic periods has yet to be elucidated.

Various forms of enzyme-linked immunoassays (ELISA) for serodiagnosis of RVF in different host vertebrates have recently been validated. Whilst these assays were shown to be highly sensitive and specific, they are based on β-propiolactone inactivated and/or gamma-irradiated, sucrose-acetone-extracted whole antigens (Paweska et al., 2003a; Paweska et al., 2003b; Paweska et al., 1995). The production of such antigens requires high bio-containment facilities to limit the risk of exposure of laboratory personnel while culturing the virus prior to inactivation. Other disadvantages include high production costs and the risk of incomplete inactivation. An indirect ELISA (I-ELISA) based on recombinant nucleocapsid protein (rNp) of RVF virus was reported to have high analytical accuracy for the detection of IgG antibody in experimentally infected and vaccinated sheep (Jansen van Vuren et al., 2007; Fafetine et al., 2007). The test was also shown to have high diagnostic performance characteristics in testing African buffalo sera (Evans et al., 2008; Paweska et al., 2008).

This paper describes evaluation of the rNp I-ELISA as a single test format for rapid detection of IgG antibody to RVF virus in different wildlife species.

Table 1. Number of field-collected wildlife sera tested in the VNT.

Species	Total Tested	VNT-[a]	VNT+[b]
Black rhinoceros	43	29	14
Common zebra	24	24	0
Elephant	73	69	4
Giraffe	81	81	0
Grevy zebra	78	77	1
Warthog	49	47	2
Eland	66	63	3
Gerenuk	6	1	5
Hartebeest	10	10	0
Impala	339	330	9
Kudu	73	66	7
Waterbuck	42	40	2
Thomson gazelle	8	1	7
Grand Total	918	864	54

[a] = number of sera tested negative in VNT; [b] = number of sera tested positive in VNT.

1 Special Pathogens Unit, National Institute for Communicable Diseases, Sandringham, South Africa
2 Kruger National Park, Skukuza, South Africa
3 Kenyan Wildlife Service, Nairobi, Kenya
4 GDD/IEIP-Kenya, Centers for Disease Control, Nairobi, Kenya

* Corresponding author: januszp@nicd.ac.za

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MATERIALS AND METHODS

Serum Specimens

A total of 918 wildlife sera collected between 1978 and 2008 in Kenya, South Africa and Zimbabwe were used. Sera which tested negative in the virus neutralisation test (VNT) were regarded as reference panel from non-infected animals, and sera which tested positive as reference panel from animals infected with RVF virus (**Table 1**).

Virus Neutralisation Test

Duplicates of serial two-fold dilutions of sera inactivated at 56°C for 30 min were tested as previously described (Paweska et al., 2003b). Titres were expressed as the reciprocal of the serum dilution that inhibited ≥ 75% of viral cytopathic effect. A serum sample was considered positive when it had a titre of ≥ $\log_{10}$ 1.0, equivalent to a serum dilution ≥ 1:10.

ELISA Antigen and Procedure

The assay procedure was carried out as previously described (Paweska et al., 2008), and ELISA results were expressed as a percentage of the high-positive control serum (PP) (Paweska et al., 2003b). The assay runs were accepted within the upper and lower control limits for the internal controls as previously statistically determined (Paweska et al., 2008).

Selection of Cut-off Values and Determination of ELISA Diagnostic Accuracy

Cut-off values at 95% accuracy level were optimised using the misclassification cost term option of the two-graph receiver operating characteristics (TG-ROC) analysis (Greiner, 1996). In addition, cut-off values were determined by mean + 2 standard deviations (SD). and by mean + 3SDs derived from PP values in uninfected animals. Estimates of diagnostic sensitivity and specificity and other measures of combined diagnostic accuracy were calculated as previously described (Paweska et al., 2008).

RESULTS

Antibody Dilution Curves

Dose response curves using different dilutions of sera known to be positive or negative in the VNT had the expected analytical slope and the I-ELISA clearly differentiated between different levels of specific IgG antibody against RVFV in African wildlife (**Figure 1**).

Cut-off Values and Diagnostic Accuracy

Threshold values for IgG I-ELISA were derived from data sets dichotomised according to the results of the VNT (**Table 1**). The effect of differently determined cut-off values on distinguishing between sera which tested negative or positive in this assay, and consequently on estimates of sensitivity, specificity, and other estimates of diagnostic accuracy is given in **Table 2**. Optimisation of cut-off values using the misclassification cost term option of the TG-ROC analysis was based on the non-parametric programme option (Greiner, 1996) due to departure from a normal distribution of data sets analysed. Graphical presentation of the TG-ROC analysis is shown in **Figure 2**.

At a cut-off value of 20.4 PP the overall misclassification costs become minimal under the assumption of a 50% disease prevalence and equal costs of false-positive and false-negative test results. The two curves represent MCT values based on non-parametric (smooth line) or parametric (dashed line) estimates of sensitivity and specificity derived from data sets in field-collected sera. Optimisation of cut-off values was based on the non-parametric program option due to departure from a normal distribution of data sets analysed.

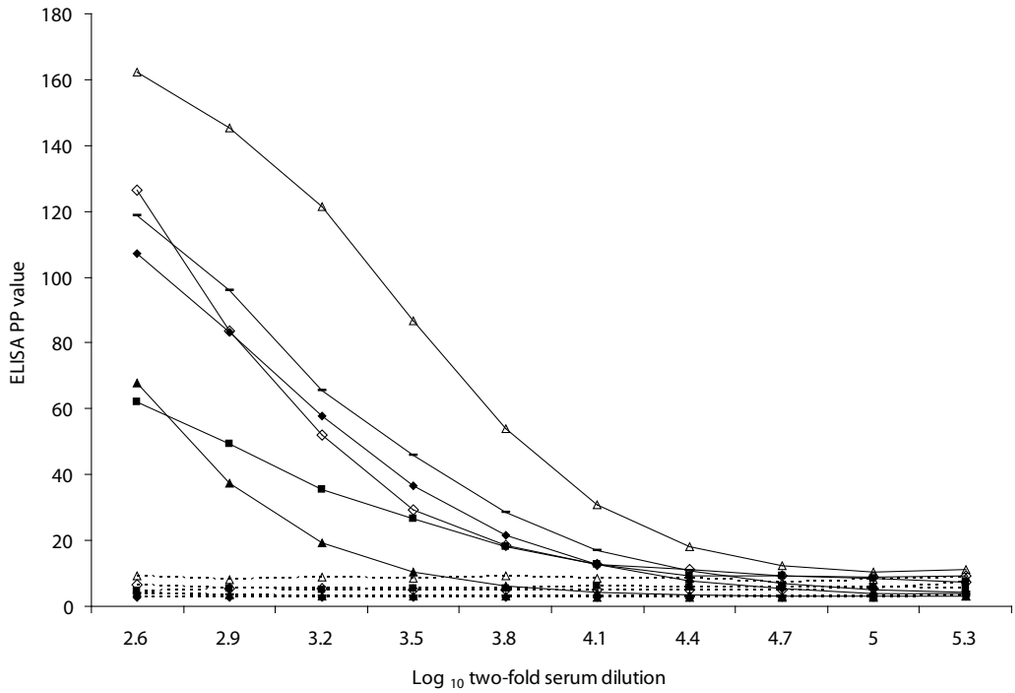

Figure 1. Dose response curves of wildlife sera in IgG I-ELISA tested positive (□) or negative (---) in a VNT: black rhinoceros (♦), eland (▲), gerenuk (■), kudu (◊), impala (Δ), Thomson gazelle (—). VNT titres in positive sera ranging from $\log_{10}10^{1.9}$ (■) to $\log_{10}10^{3.1}$ (Δ).

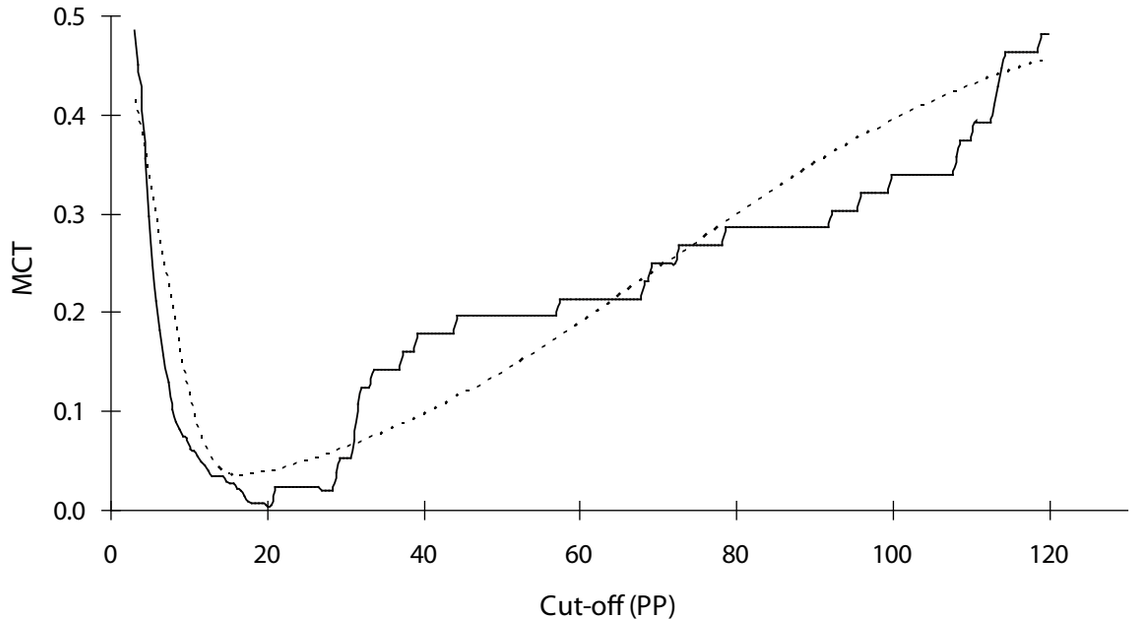

Figure 2. Optimisation of cut-off value for Rift Valley fever nucleocapsid-based I-ELISA in African antelopes using the misclassification cost term (MCT) option of the two-graph receiver operating characteristic analysis.

Table 2. Diagnostic accuracy of Rift Valley fever recombinant nucleocapsid–based I–ELISA in African wildlife.

Species	Cut–off[a]	D–Sn[b]	D–Sp[c]	Y[d]	Ef[e]	PPV[f]	NPV[g]
Black rhinoceros	33.6[h]	100	100	1	100	100	00
	27.5[i]	100	91.3	0.91	93.7	81.5	00
	35.5[j]	100	100	1	100	100	00
Common zebra	–	–	–	–	–	–	–
	13.9	–	100	–	–	–	–
	17.9	–	100	–	–	–	–
Elephant	28	100	100	1	100	100	100
	10.6	100	95.8	0.96	99.7	99.7	100
	13.6	100	97.2	0.97	99.8	99.8	100
Giraffe	–	–	–	–	–	–	–
	11.7	–	100	–	–	–	–
	14.3	–	100	–	–	–	–
Grevy zebra	–	–	100	–	–	–	–
	17.3	–	100	–	–	–	–
	22.5	–	100	–	–	–	–
Warthog	27.7	100	100	1	100	100	100
	13.5	100	95.9	0.96	96	49.7	100
	17.5	100	97.9	0.98	98	66.4	100
Antelopes[l]	20.4	100	99.8	0.99	99.7	95.6	100
	8.4	100	88.1	0.88	88.7	32.4	100
	14.4	100	97.0	0.97	97.1	67.9	100

Animals were categorised according to the results of the VNT.

[a] — cut–off value expressed as a percentage positivity (PP) of an internal high–positive serum control; [b] — diagnostic sensitivity (%); [c] —diagnostic specificity (%); [d] —Youden's index; [e] — efficiency (%); [f] — positive predictive value (%); [g] — negative predictive value (%); [h] — cut–off value optimised by TG–ROC analysis; [i] — cut–off value based on mean + 2 SD of ELISA PP values in VNT–negative population; [j] — cut–off value based on mean + 3 SD of ELISA PP values in VNT–negative population; [k] — not determined due to unavailability or very limited number of VNT–positive sera; [l] — eland, gerenuk, hartebeest, impala, kudu, Thomson gazelle, waterbuck.

DISCUSSION

Traditional methods for detecting antibodies to RVF virus include haemagglutination-inhibition, complement fixation, indirect immunofluorescence, and virus neutralisation assays. The last of these is regarded as a gold standard but is laborious, expensive and dependent on the availability of live virus and tissue cultures. Therefore, it is only used in specialised reference laboratories housing high biocontainment facilities. Laboratory safety and other advantages of the rNp-based I-ELISA compared with the VNT have been discussed recently (Paweska et al., 2008).

Antigenic cross-reactivity studies in animals (Swanepoel et al., 1986) and recent results in the I-ELISA failed to provide any evidence that other African phleboviruses could hamper reliable serodiagnosis of RVF (Paweska et al., 2007). However, to account for possible cross-reactivity with unknown phleboviruses, sera in this study were tested at relatively high dilution. The I-ELISA had high estimates of diagnostic sensitivity when cut-offs determined by traditional statistical approach were used but the diagnostic specificity and combined measures of assay accuracy were lower compared with those which were based on the cut-offs derived from the TG-ROC analysis. A cut-off determined as two or three SDs above the mean in uninfected individuals which is still commonly used for interpretation of serodiagnostic assays, assumes a normal distribution of test values in targeted populations, and therefore provides only an estimate of diagnostic specificity but not sensitivity (Jacobson, 1998). Deviations from normality are often observed in serological data and should be addressed in the selection of threshold values (Vizard et al., 1990). The prevalence assumed in the study sample may not be representative of the prevalence in the target populations and this should be borne in mind in applying the estimates of diagnostic accuracies reported for the rNp-based I-ELISA in the present work.

This study confirm previous findings (Paweska et al., 2008) that the rNp-based I-ELISA accurately identifies sera with different concentrations of specific IgG antibodies to RVF virus, and compared with the VNT it has very high diagnostic performance in various wildlife animal species. As a single and safe test format, it provides a useful tool for seroepidemiological studies of RVF virus infections in African wildlife species. Such investigations might help to elucidate their specific role in the epidemiology of the disease during the inter-epidemic and epidemic periods, and including enigmatic mechanisms of the virus cryptic maintenance within the host-vector natural cycle.

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Variability of the IFN-β Promoter Repressing Activity of NSs Proteins Derived from Field Isolates of Rift Valley Fever Virus

M.M. Lo[1], M. Spiegel[2]*, G. Hunsmann[2], M. Weidmann[2], F. Hufert[2], Y. Thiongane[1] & A.A. Sall[3]

ABSTRACT

Variability of viral and host genetic factors may be involved in the pathogenesis of Rift Valley fever virus (RVFV) infections and may explain the wide range of clinical outcomes in susceptible vertebrate hosts. The differences in virulence among RVFV isolates may be due to the interference with either the innate and/or adaptive immunity. In this work, the interferon-β antagonistic function of NSs of RVFV isolates from different sources (animals, humans, and insects) was assessed after cloning and sequencing the non-structural S segment gene (NSs). The NSs clones were monitored for their immune modulatory effects by analysing their ability to suppress the activation of the IFN-b promoter using a reporter assay system. Additionally, expression of NSs in Vero E6 cells was monitored by immunofluorescence staining. Two RVFV NSs proteins (derived from isolates R7 and R18) failed to inhibit IFN-β promoter activation whereas the remaining 24 showed efficient suppression of IFN-β promoter activity. Additionally R7-NSs and R18-Nss were unable to form nuclear filaments which are a typical feature of wild-type RVFV-NSs. Sequencing of R18-NSs revealed a large internal in-frame deletion identical to the mutation described for the naturally occurring RVFV mutant clone 13, which leads to a non-functional NSs-protein. Indeed, R18 was later identified as a RVFV clone 13 isolate. In contrast, R7-NSs contains a point mutation in the NSs gene, which results in the replacement of a leucine by proline. Interestingly, this unique point mutation has effects comparable to the large in-frame deletion of clone 13 NSs.

Key words: *interferon-β, Rift Valley fever virus, NSs protein.*

INTRODUCTION

A Rift Valley fever (RVF) outbreak leading to heavy mortality in newly-born lambs on a farm in Kenya was first described in 1931 (Daubney et al., 1931). RVF virus (RVFV) mainly causes disease in domestic ruminants inflicting high rates of abortion and mortality. Recurrent enzootic and epizootic outbreaks have been documented in eastern, southern and western Africa, Madagascar and Egypt. In 2000 RVFV even spread to the Arabian Peninsula, thereby emerging outside of Africa for the first time. The zoonotic disease can also cause epidemics in humans as recorded in Egypt in 1977, in Saudi Arabia and Yemen in 2000, and in Kenya and Tanzania in 2007 (Imam and Darwish, 1977; Jouan et al., 1990; Thiongane et al., 1996; Abd el-Rahim et al., 1999; Madani et al., 2003) RVFV survives dry periods in vertically infected eggs of different mosquito species and disease outbreaks are often linked to preceding heavy rainfalls.

RVF outbreaks usually begin in livestock with elevated abortion counts. In humans the symptoms range from mild fever to encephalitis, retinitis and fatal hepatitis with haemorrhages. The more severe forms occur in less than 1% of patients of which up to 50% may die (Woods et al., 2002). Although RVFV is mainly transmitted by mosquitoes (Hoogstraal et al., 1979), transmission to humans can also occur by contact with infected tissues e.g. from abortions from livestock (Hoch et al., 1985; Logan et al., 1992) or by aerosols from slaughtered animals (Chambers & Swanepoel, 1980) and consequently livestock workers and wildlife rangers in Nigeria (LaBeaud et al., 2007; LaBeaud et al., 2008) but also nomadic tribes in Kenya (Olaleye et al., 1996) show a very high seroprevalence towards RVFV.

RVFV is a member of the genus *Phlebovirus* in the *Bunyaviridae* family. The segmented negative single strand RNA genome of the virus codes for the polymerase (L-segment), the glycoproteins G1 and G2 and two non-structural proteins NSm14 and NSm78 (M-segment) and for the nucleocapsid (S-segment). The S-segment also codes for a non-structural protein (NSs) and a nucleoprotein (N). The NSs of RVFV is a 31-kDa protein composed of 265 amino acids which is phosphorylated by casein kinase II at two serine residues located in the C-terminus. It accumulates in the nuclei of infected cells, where it forms filamentous structures. A carboxy-terminal domain mediates oligomerisation and is responsible for filament formation (Yadani et al., 1999). Non-U.S. Gov't</keyword><keyword>Viral Nonstructural Proteins/*chemistry/physiology</keyword></keywords><dates><year>1999</year></dates><accession-num>10233964</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/htbin-post/Entrez/query?db=m&form=6&dopt=r&uid=10233964</url><url>http://www.ncbi.nlm.nih.gov/pmc/articles/PMC112546/pdf/jv005018.pdf</url></related-urls></urls></record></Cite></EndNote>. The nuclear localisation of NSs is intriguing because all steps of the viral life cycle are known to occur in the cytoplasm (Billecocq et al., 2004).

The naturally attenuated RVFV strain clone 13 originally isolated from a non-fatal human case in Bangui, Central African Republic

1 ISRA/LNERV, Dakar/Hann, Senegal
2 Institute of Virology, University of Goettingen, Goettingen, Germany
3 Institut Pasteur, Dakar, Senegal

* Corresponding author: martin.spiegel@medizin.uni-goettingen.de

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(Muller et al., 1995) carries a large in-frame deletion in the NSs gene. It is not virulent *in vivo* (in mice and hamsters) but grows as well as wild-type RVFV in cell cultures (Muller et al., 1995).

Various cells in the body are capable of sensing infectious viruses and initiating reactions collectively known as antiviral innate responses. These responses include the production of antiviral cytokines such as type I interferon (IFN) and subsequent synthesis of antiviral factors, which are responsible for impairing viral replication and promote adaptive immune responses (Samuel, 2001). IFN-α/β is induced by pathogen-associated molecular patterns like double-stranded RNA (dsRNA) or single-stranded RNA with a non-modified triphosphate residue at the 5' end. It activates the Jak/Stat signaling pathway which leads to the expression of antiviral active factors. To overcome these antiviral responses, viruses have evolved various strategies to inhibit IFN production, IFN signaling, or IFN action. The efficiency by which a virus antagonises the IFN system is critical for its pathogenicity and its ability to infect and spread in a host (Billecocq et al., 2004).

A comparative analysis in mice showed that the RVFV clone 13 (carrying a mutant NSs gene) though avirulent in wild type mice can kill IFN-α/β receptor knockout mice, which cannot mount an antiviral response. In contrast to RVFV clone 13, virulent RVFV strains (carrying an intact NSs gene) like the strain ZH548 do not induce an IFN-response in wild type mice. This observation led to the conclusion that NSs is an IFN antagonist (Bouloy et al., 2001; Billecocq et al., 2004). Further analysis revealed that NSs is a potent repressor of the IFN-b gene expression. In addition, clone 13 proved to be an excellent inducer of early IFN-α/β production *in vivo*. In contrast, the virulent strain ZH548 failed to induce detectable amounts of IFN-α/β and replicated extensively in both IFN-competent and IFN-defective mice.

NSs is located exclusively in the nucleus of RVFV-infected cells which is rather surprising since this virus, like all the members of the family *Bunyaviridae*, utilises only the cytoplasm as its site for replication. Intranuclear inclusions were first detected in the hepatocytes of RVFV-infected animals (Daubney et al., 1931). Later, Swanepoel and his group (Swanepoel and Blackburn, 1977) detected nuclear filaments in cells infected with various virulent RVFV strains and showed that the nuclear filament is composed of bundles of 50-nm-thick fibrils, which occupy half the length of the nucleus and are confined exclusively to the nuclei but not associated with nucleoli.

The immune compromising effect of wild type RVFV strains through NSs-mediated interference with the innate immune system is an important virulence factor. Variations in the NSs sequence may influence the NSs function and might therefore contribute to the wide range of clinical outcomes of RVFV infections in man and cattle. In this work the ability of NSs of wild type RVFV isolates to block type I IFN induction (i.e. activation of the innate immune system) was assessed after cloning and sequencing the NSs gene. Additionally NSs expression, nuclear localisation and filament formation was monitored by immunofluorescence analysis.

MATERIAL AND METHODS

cDNA Preparation

Total RNA was prepared from virus culture supernatants of strains previously described (Weidmann et al., 2008). The strains were blinded and coded R1-R33. After RNA extraction, the complementary DNA (cDNA) was prepared for each strain. The first strand cDNA synthesis was performed using Superscript III (Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions. The cDNAs obtained were stored at –80 °C until used.

The cDNAs were used as templates in PCRs using Pfx DNA polymerase (Invitrogen) with RVFV-NSs specific forward primer which adds a 5' BglII restriction site (5'GACAGAAGATCTATGGATTACTTTCCTGTGATATCTG3') and reverse primer (5'GTCGACTCACTTGTCATCGTCGTCCTTGTAGTCATCAACCTCAACAAATCCATC3') which adds an immunogenic FLAG tag as well as a 3' SalI restriction site. The addition of the FLAG tag at the C-terminus of RVFV-NSs neither affects the IFN-antagonistic function of NSs nor the ability to form nuclear filaments (Billecocq et al., 2004). A touch down program for PCR was used: after the denaturation step at 95 °C for 120 s, ten cycles were performed with denaturation at 95 °C for 30 s, an annealing step for 30 s with an initial temperature of 57 °C which was decreased by 0.5 °C for each cycle and an extension step at 68 °C for 60 s. Then, 25 cycles were performed with a constant annealing temperature of 52 °C while the other parameters remained unchanged. After the final extension step at 68 °C for 300 s and subsequent cooling, the amplification products were separated by electrophoresis in a 0.8% TAE agarose gel and the PCR products were recovered using peqGOLD Gel Extraction kit (PEQLAB Biotechnologie GmbH, Nürnberg, Germany).

Cloning Experiments

TA Cloning of Purified PCR Products

TA cloning of the purified PCR products into the vector pCRII was performed according to the manufacturer's instructions (Invitrogen). Positive clones were pre-screened by blue/white staining. White colonies were picked and amplified in LB-Amp selection medium. Subsequently, plasmid DNA was isolated using PeqGold Plasmid Miniprep Kit (PEQLAB Biotechnologie GmbH). A restriction digest using EcoRI enzyme was done to identify plasmids carrying the NSs cDNA and a 1% agarose gel electrophoresis was run to visualise and differentiate the digestion products. Finally, positive plasmids were sequenced using the primers M13 forward (5'GTAAAACGACGGCCAG3') and M13 reverse (5'CAGGAAACAGCTATGAC3') to determine orientation and sequence integrity of the cloned NSs cDNAs as well as the presence of the C-terminal added FLAG tag (Seqlab Laboratories, Göttingen, Germany).

Subcloning of NSs cDNAs into the Eukaryotic Expression Vector pI.18

The 26 different pCRII-RVFV-NSs plasmids obtained were digested with BglII and SalI (Fermentas, St. Leon-Rot, Germany) to cut out the NSs cDNA fragments. Following agarose gel electrophoresis and gel purification using Zymoclean gel DNA recovery kit (HISS Diagnostics, Freiburg, Germany), the NSs cDNAs were subcloned into BglII/SalI-digested eukaryotic expression vector pI.18. Ligation products were transformed into Z-competent E. coli XL1blue and transformed bacteria were grown on LB-amp agar plates. Subsequently clones were picked and amplified in LB-Amp medium followed by plasmid DNA isolation. Isolated plasmids were screened for NSs cDNA insertion by BglII/SalI digest. Positive clones were amplified in 50 ml LB-Amp medium and plasmid DNA was isolated using the Nucleobond 100 kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany).

Monitoring the Repression of IFN-β Promoter Activation by RVFV-NSs Proteins

The inhibitory effect of RVFV-NSs on VSV-RNA-mediated IFN-β promoter activation was tested in a luciferase reporter assay. With this aim $1x10^5$ Vero E6 cells were cotransfected with p125-luc (firefly luciferase cDNA expression driven by the human IFN-β promoter),

pRL-SV40 (renilla luciferase cDNA expression driven by the $SV40_{early}$ promoter, Promega, Mannheim, Germany) and pI.18-RVFV-NSs (NSs cDNA expression driven by the hCMV-IE promoter linked to the hCMV intron A; plasmid pI.18 was kindly provided by J. Robertson, Hertfordshire, UK) using FuGene HD (Roche Biochemica, Mannheim, Germany) according to the manufacturer's instructions. After 24 h the transfected cells were either stimulated with RNA from VSV-infected Vero E6 cells or with RNA from uninfected Vero E6 cells as a control. RNA from VSV-infected cells contains high amounts of viral RNA with an unmodified triphosphate 5' end which is a potent inducer of IFN-β expression (Pichlmair et al., 2006). For this purpose, cells were transfected with 1 µg RNA using FuGene HD. Cells were lysed 16 h post stimulation and a dual-luciferase assay performed according to the manufacturer's instructions (Promega, Mannheim, Germany) using a Fluostar Optima reader (BMG Labtech, Offenburg, Germany).

Monitoring the Formation of Filaments in the Nucleus of Vero E6 Cells Transfected with RVFV-NSs Expression Plasmids using an Indirect Immunofluoresence Assay

Vero E6 cells were cultured on coverslips and transfected with RVFV-NSs Flag expression plasmids using FuGene HD (Roche) as transfection reagent. At 24 h post transfection, the cells were fixed with 3% paraformaldehyde and permeabilised with 0.5% TritonX100 (Sigma, Deisenhofen, Germany). The permeabilised cells were then incubated with a monoclonal mouse anti-FLAG antibody (Sigma) as primary antibody (diluted 1:200 in PBS containing 1% FCS), followed by incubation with an Alexa Fluor 555 labelled anti-mouse IgG (Invitrogen) as secondary antibody. Then coverslips were mounted in Fluosave mounting medium (Calbiochem, Bad Soden, Germany). After solidification of the mounting medium, the slides were examined by fluorescence microscopy using a Nikon TE2000-S inverted microscope.

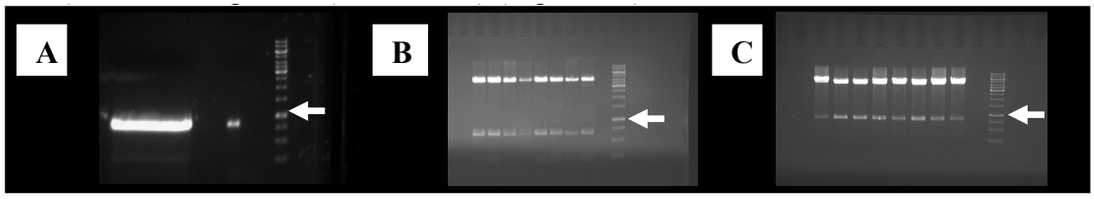

Figure 1. (A) PCR product from cDNA of RVFV-NSs R17 in 0.8% agarose gel and 0.05% ethidium bromide, (B) Eight clones of the pCRII-RVFV-NSs R17 construct after restriction digest using EcoRI in 1% agarose gel and 0.05% ethidium bromide. The 200 bp fragment of the 5'NSs fragment is hardly visible, (C) Eight clones of the pI.18RVFV-NSs R17 construct after restriction digest using SalI and BglII in 1% agarose gel and 0.05% ethidium bromide. The arrows indicate the 1 000 bp band of the DNA marker.

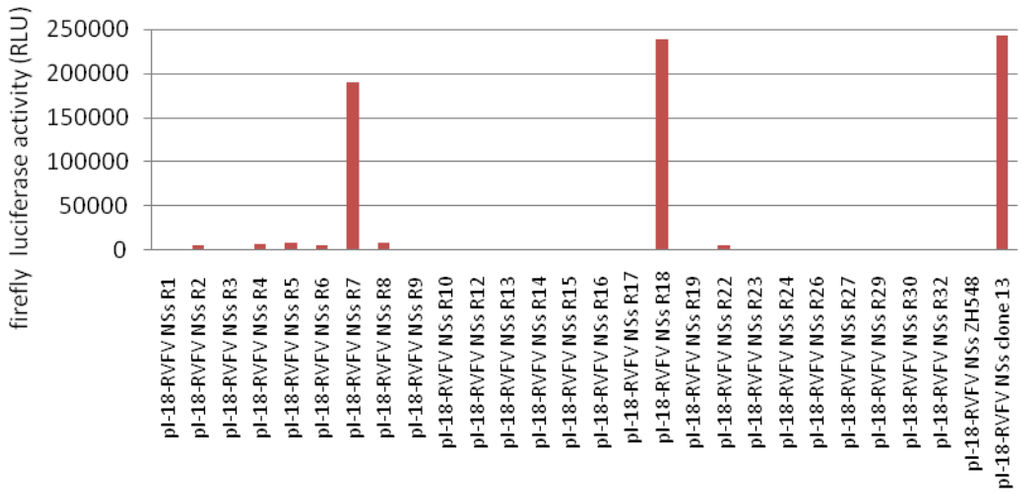

Figure 2. Effect of NSs on IFN-β promoter activity after stimulation with RNA from VSV-infected cells for the 26 RVFV-NSs clones, RVFV-NSs ZH548, and RVFV-NSs clone13 in a luciferase reporter assay. Relative light units (RLU) represent the readout for firefly luciferase activity which indicates the activity of the IFN-β promoter. The diagram represents the mean values of five independent experiments for each NSs clone.

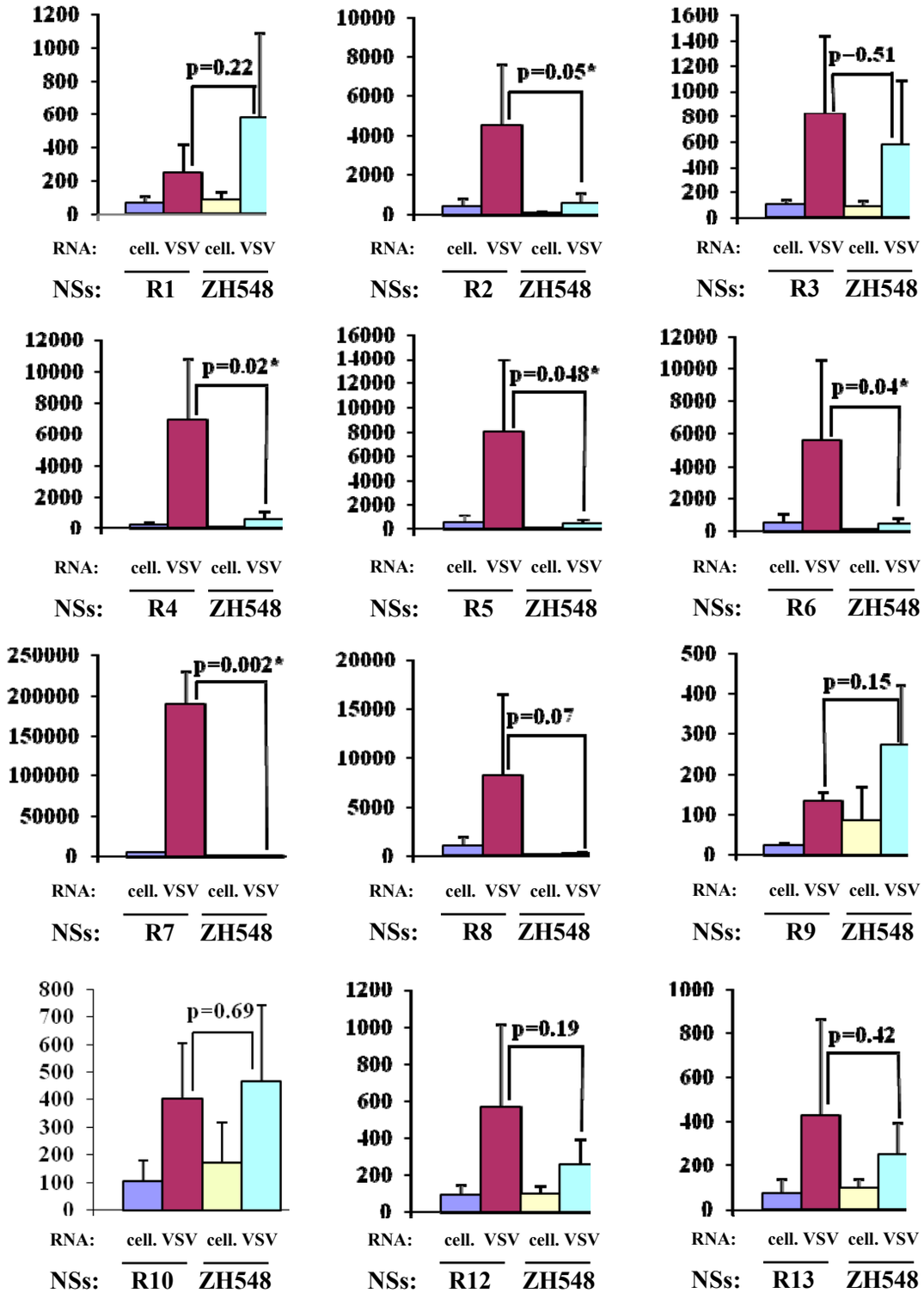

Figure 3. IFN-β promoter activity measured by firefly luciferase activity after stimulation with RNA of VSV-infected Vero cells (VSV) or with RNA of uninfected cells (cell.) for RVFV-NSs R1, 2, 3, 4, 5, 6,7, 8, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24, 26, 27, 29, 30, 32 in comparison to wt RVFV-NSs ZH548. The diagrams represent the mean values of five independent experiments for each NSs clone; p values were calculated by Student's t-testtest. Significant differences (p<=0.05) are marked with an asterisk.

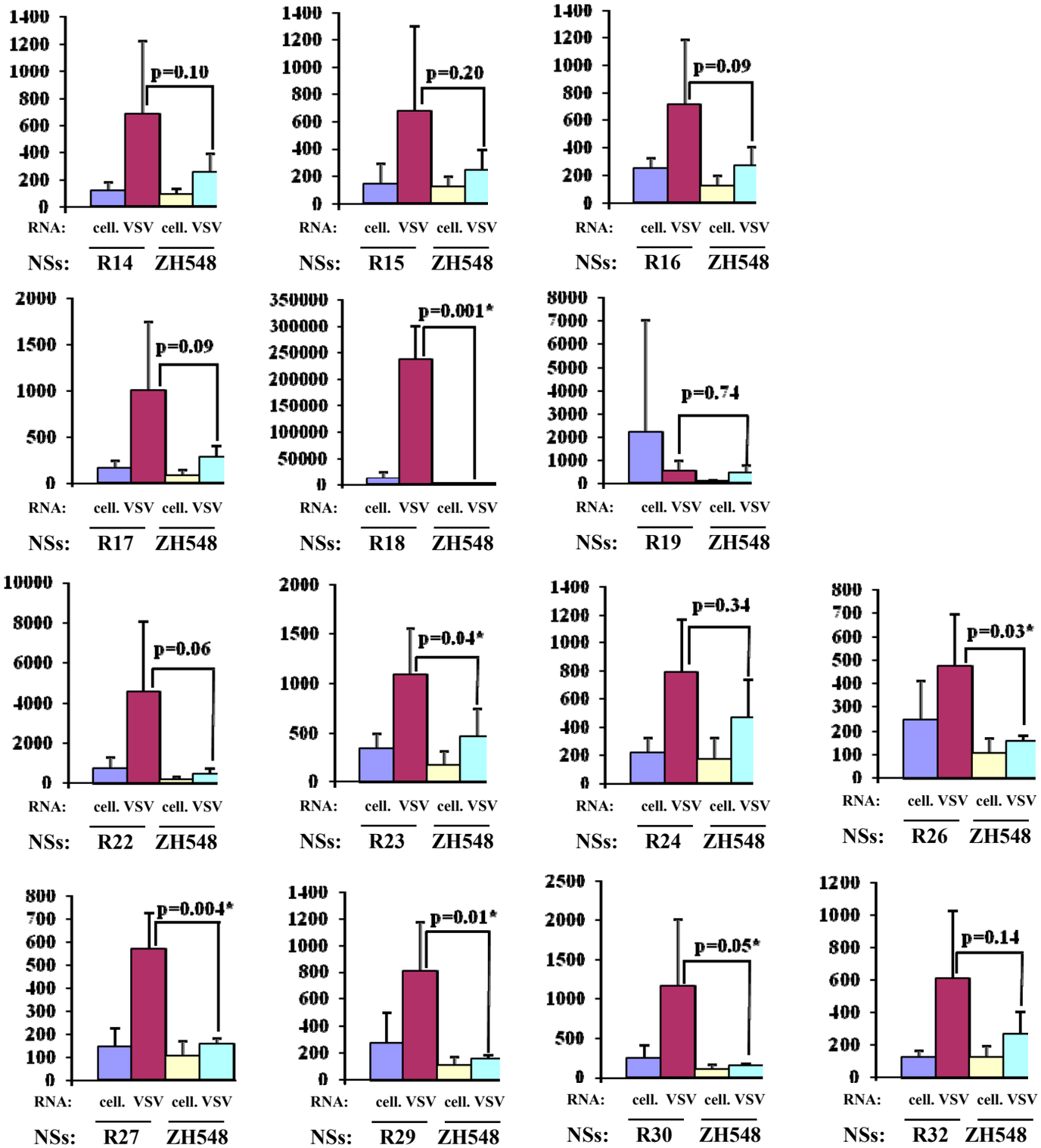

Figure 3. (continued).

RESULTS

Cloning of the NSs of RVFV Isolates into the Eukaryotic Expression Vector pI.18

First, NSs cDNAs were obtained by reverse transcription of viral RNA followed by PCR amplification and cloning into the TA-vector pCRII. Subsequently, the cDNAs were subcloned into the eukaryotic expression vector pI.18.

An example of the successful cloning procedure is shown for RVFV-NSsR17 in **Figure 1**. The expected band size for RVFV-NSs after PCR amplification is around 800 base pairs which indeed was observed for R17-NSs (**Figure 1A**). Ligation of the NSs cDNA into pCRII was monitored by EcoRI digest which results in a 4 000 bp band (vector backbone), a 600 bp band (3' fragment of NSs cDNA) and 200 bp band (5' fragment of NSs cDNA) (**Figure 1B**). After subcloning the NSs cDNA into the eukaryotic expression vector pI.18, successful ligation was confirmed by BglII/SalI digest which results in a 4 300 bp band (vector backbone) and a 800 bp band (NSs cDNA) (**Figure 1C**).

Inhibition of IFN-β Promoter Activation by RVFV-NSs

From the 33 RVFV isolates available, 26 RVFV NSs cDNAs were successfully cloned and ligated in pI.18. For the remaining isolates we were not able to amplify the NSs cDNA most probably due to minor quality of the RNA used as template for reverse transcription. To test whether expression of the NSs cDNAs inhibits the IFN-β promoter activation, Vero E6 cells were cotransfected with expression plasmids for (i) NSs of wild type virus ZH548 as a positive control, (ii) NSs of the attenuated RVFV clone 13 as a negative control, and (iii) NSs of the 26 RVFV isolates together with the reporter plasmids p125-luc and pRL-SV40. After stimulation with RNA of VSV-infected Vero E6 cells or uninfected cells IFN-β promoter activities were determined by luciferase assay.

The results of the luciferase assays (**Figure 2**) show that two RVFV-NSs clones (R7 and R18) failed to suppress IFN-β promoter activation after stimulation with RNA of VSV-infected cells as indicated by the high firefly luciferase activities when compared to RVFV ZH548- NSs (reference virulent wild-type strain). In contrast, the other RVFV-NSs clones showed efficient suppression of IFN-β promoter activity since only low firefly luciferase activities were detected. While RVFV-NSs R7 may have some residual activity, RVFV-NSs R18 has no inhibitory effect at all which is very similar to the NSs from the avirulent RVFV strain clone 13. Furthermore, the NSs clones with inhibitory effect on IFN-β promoter activation as well as RVFV ZH548-NSs were able to decrease the activity of the constitutively active SV40 promoter as indicated by low renilla luciferase acitivities when compared with RVFV-NSs clone 13 (data not shown). This is in line with previous observations that RVFV-NSs affects RNA polymerase II mediated transcription (Billecocq et al., 2004; Le May et al., 2004).

Comparison of the Activity of the RVFV-NSs Clones with the Reference Wild-type RVFV ZH548-NSs

The results show significant differences ($P <= 0.05$) in the ability to inhibit IFN-β promoter activation between the wild type RVFV ZH548-NSs and R2, R4, R5, R6, R7, R18, R23, R26, R27, R29, R30 RVFV-NSs (**Figure 3**). Although significant, the observed differences are small with the marked exception of R7- and R18-NSs. There is no significant difference in blocking IFN-β promoter activation between the ZH548-NSs and the remaining RVFV-NSs clones.

Comparison of Filament Formation in the Nucleus of Vero E6 Cells Transfected with 26 RVFV-NSs Expression Plasmids

The immunofluorescence staining allowed detection of filamentous structures in the nucleus of transiently transfected Vero E6 cells for most of the RVFV-NSs isolates as well as for the wild-type strain ZH548-Nss (**Figure 4z**), however for the NSs of three RVFV isolates (R7, R10, and R18) nuclear filament formation was not observed.

Interestingly, R7-NSs and R18-NSs which failed to inhibit IFN-β promoter activation weren't detectable in immunofluorescence tests but had an intact FLAG-tag (**Figures 4g** and **4p**). For unknown reasons we were unable to detect filament formation for NSs-R10 (**Figure 4j**) which showed efficient inhibition of IFN-β promoter activation as well as an intact FLAG-tag. However, we cannot rule out the possibility, that the FLAG-tag was destroyed during subcloning of the NSs-cDNA into pI.18 and resequencing is required to check this hypothesis.

A Point Mutation in the NSs Sequence of RVFV-NSs R7 Affects NSs Function

All cloned NSs genes were sequenced and compared to the NSs sequence of the reference strain RVFV ZH548. We found nucleotide exchanges in 71 positions of the NSs ORF which has a length of 798 nt, however the vast majority of these mutations are silent. In the NSs sequence of RVFV isolates R1, R2, R3, R4, R5, R6, R7, R8 and R32 a G to A exchange at position 71 of the NSs ORF was detected which leads to the replacement of arginine by lysine on amino acid level. With the exception of R7-NSs these NSs clones were efficient inhibitors of IFN-β promoter activation (**Figures 2** and **3**) and formed nuclear filaments (**Figure 4**) indicating that this mutation has no effect on NSs function and stability. Frequent mutations were observed at positions 724–726. While the RVFV ZH548-NSs sequence contains the triplet ATT at these positions which corresponds to isoleucine, NSs clones R1 and R3 contain GTC (corresponding to valine), NSs of RVFV isolates R4, R5, R6, R7, R8, R22, R23, R24, R26, R27, R28, R29, R30 and R32 contain GTT (also corresponding to valine) and the NSs of the isolates R12, R13 and R14 contain ATC (which is a silent mutation). The remaining NSs sequences of isolates R9, R10, R15, R16 and R17 carry no mutations at these positions. Similar to the replacement of arginine by lysine in the N-terminal part of NSs, the isoleucin to valine exchange in the C-terminal part of NSs had no consequences with respect to suppression of IFN-β promoter activation (Fig 2 and 3) and nuclear filament formation (**Figure 4**). R7-NSs contains an additional mutation at position 344 (T to C) which leads to the replacement of leucine by proline. This mutation seems to have a strong effect on the NSs function since NSs R7 no longer inhibits activation of the IFN-β promoter (**Figures 2** and **3**). Additionally, this mutation might be detrimental for the stability of R7-NSs since no nuclear filament formation was observed (**Figure 4**) although the C-terminal domain (amino acids 249 to 265 of the NSs protein) which is required for oligomerisation and filament formation (Yadani et al., 1999) is present in R7-NSs. In contrast, R8-NSs which is — apart from the leucin to proline exchange — otherwise identical to R7-NSs (on nucleotide as well as amino acid level) is an efficient inhibitor of IFN-β promoter activation (**Figures 2** and **3**) and forms nuclear filaments (**Figure 4j**).

Sequencing of RVFV R18-NSs revealed a large in-frame deletion of 549 nucleotides starting at nucleotide position 46 of the NSs ORF which is identical to the deletion observed in RVFV clone 13-NSs. Interestingly, the point mutation observed in R7-NSs and the in-frame deletion observed in R18-NSs (which includes position 344

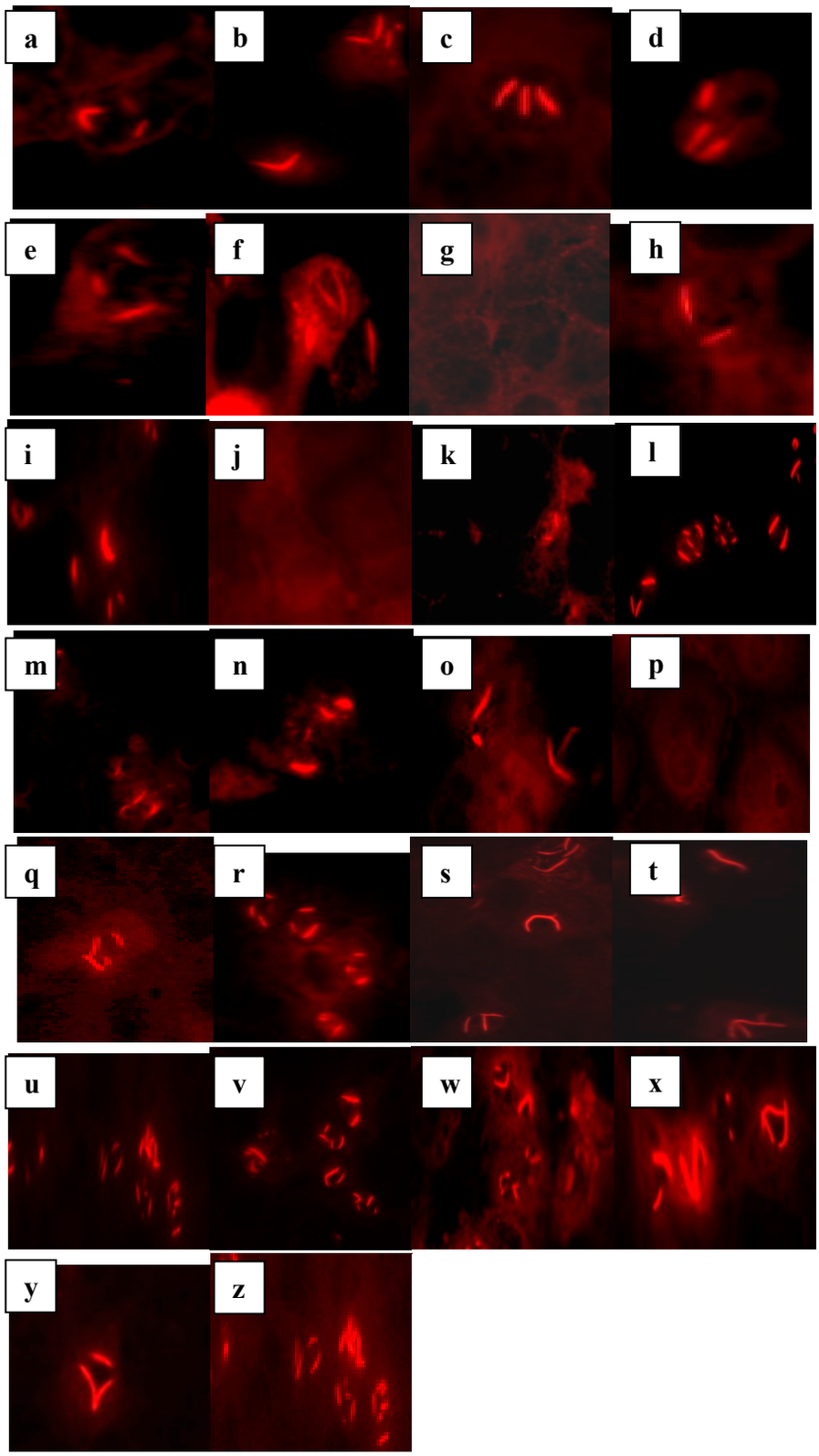

Figure 4. Filamentous structure formation of RVFV-NSs in transfected cells. (a) R1, (b) R2, (c) R3, (d) R4, (e) R5, (f) R6, (g) R7, (h) R8, (i) R9, (j) R10, (k) R12, (l) R14, (m) R15, (n) R16, (o) R17, (p) R18, (q) R19, (r) R22, (s) R23, (t) R24, (u) R6, (v) R27, (w) R29, (x) R30, (y) R32, (z) ZH548. No filament formation was observed for R7 (g), R10 (j) and R18 (p).

of the R7-NSs mutation) have similar effects: Both NSs clones fail to inhibit IFN-β promoter activation and do not form nuclear filaments.

DISCUSSION AND CONCLUSIONS

Significant differences in IFN-β promoter activation in the presence of NSs from RVFV isolates R2, R4, R5, R6, R7, R18, R23, R26, R27, R29, R30 were observed in this study when compared with the wild type RVFV ZH548-NSs. However, with the exception of R7- and R18-NSs all these NSs clones were still efficient inhibitors of IFN-β promoter activation. R7-NSs and R18-NSs were unable to suppress IFN-β promoter activation and did not form nuclear filaments. A follow up of viral RNA samples revealed that strain R18 is indeed a RVFV clone 13 isolate. The follow up has not yet revealed the identity of the strain from which R7-NSs was amplified. Consequently the results for R18-NSs simply confirm the loss of function of clone 13-NSs (Vialat et al., 2000) whereas R7-NSs truly shows a significant inability to suppress IFN-β promoter activation induced by RNA from VSV-infected cells.

Our reporter assay results are consistent with previous findings that RVFV clone 13 (lacking a functional NSs), but not wild type virus induces IFN-β gene expression (Billecocq et al., 2004). Previous work also demonstrated that recombinant NSs of wild-type strain ZH548 forms filamentous structures in the nuclei of transiently transfected cells; these structures were identical to those observed in ZH548-infected cells (Billecocq et al., 2004). In contrast, the truncated NSs of RVFV clone 13 was barely detectable and located mainly in the cytoplasm (Billecocq et al., 2004). It was reported that — except for RVFV clone 13 — the NSs proteins of all the RVFV strains analyzed so far form filamentous structures in the nuclei of infected cells (Muller et al., 1995). Interestingly, we showed here that R7-NSs, which failed to inhibit IFN-β promoter activation, wasn't detectable in IFA tests although sequencing results revealed an intact C-terminal domain required for oligomerisation and filament formation as well as an intact FLAG-tag. Therefore, the observed absence of filament formation as well as the loss of function can be attributed to the point mutation found in the R7-NSs sequence which results in a replacement of leucine by proline. This mutation probably leads to an unstable conformation which might result in proteasomal degradation as it is the case for RVFV clone 13 NSs (Vialat et al., 2000). RVFV-NSs is normally quite stable, and the situation observed for RVFV clone 13-NSs is exceptional. Obviously, the unique point mutation leading to a leucine to proline exchange at amino acid position 115 in R7-Nss has effects comparable to the large in-frame deletion of clone 13 NSs and is critical for NSs function and stability. Further experiments have to be done to test whether strain R7 is less virulent than the reference strain ZH548 *in vivo* or even non-pathogenic like RVFV clone 13.

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Phylogenetic Analysis of the Capripoxvirus RPO30 Gene and its Use in a PCR Test for Differentiating Sheep Poxvirus from Goat Poxvirus

C.E. Lamien[1]*, M. Lelenta[1], R. Silber[2], C. Le Goff[3], D. Wallace[4], V. Gulyaz[5], E. Tuppurainen[6], A.G. Luckins[1], E. Albina[3] & A. Diallo[1]

ABSTRACT

The Genus Capripoxvirus (CaPV) of the Poxviridae family comprises sheep poxvirus (SPPV), goat poxvirus (GTPV) and lumpy skin disease virus (LSDV) which are responsible for economically important diseases affecting sheep, goats and cattle respectively. To date, there have been no molecular criteria upon which to base strain designation. The complexity of CaPVs host specificity shows the need to develop more reliable tools for CaPVs identification than the current method which is based on the host origin. Previous reports, based on partial or full genome sequencing indicated that CaP viruses are genetically distinct from each other and can be grouped as three different species: SPPV, GTPV and LSDV.

In contributing to the creation of more stringent data for genotyping CaPVs, we have analysed the RPO30 gene of several isolates. The phylogenetic reconstructions have shown that the viruses can be segregated into three different lineages according to their host origins: the SPPV, the GTPV and the LSDV lineages. In addition, a 21-nucleotides deletion found in all individuals within only the SPPV group was exploited to design a classical PCR method to differentiate SPPV from GTPV. This test allows the rapid differential diagnosis of diseases caused by either SPPV or GTPV strains.

Key words: *Capripoxvirus, RPO30 gene, lineages, sequencing, differential diagnosis, polymerase chain reaction.*

INTRODUCTION

The genus *Capripoxvirus* (CaPV) of the *Poxviridae* family comprises sheep poxvirus (SPPV), goat poxvirus (GTPV) and lumpy skin disease virus (LSDV) which are responsible for economically important diseases affecting sheep, goats and cattle respectively. The criteria upon which CaPVs are named remain the host origin. This tends to suggest that they are strictly host specific. However there are several reports indicating the involvement of both sheep and goats in different outbreaks and therefore, this way of naming CaPV cannot be fully reliable (Diallo and Viljoen, 2007; Babiuk et al., 2008). In the same way, the fact that CaPV infections cannot be distinguished clinically or serologically, emphasises the need to establish more reliable tools such as those based on molecular methods for strain identification. Previous reports based on partial or full genome sequences, have shown that CaPVs are genetically heterogeneous and can be grouped as three different species SPPV, GTPV and LSDV (Tulman et al., 2002; Hosamani et al., 2004; Le Goff et al., 2009).

Here we describe the suitability of the CaP viruses' RPO 30 gene, the orthologue of the *Vaccinia* virus E4L gene encoding the 30 kD RNA polymerase subunit for virus-animal origin discrimination, and the design of a polymerase chain reaction (PCR) method to differentiate SPPV from GTPV based on the presence of a 21 nucleotide deletion found exclusively in the SPPV sequences.

MATERIALS AND METHODS

Genomic DNA from CAP viruses (**Table 1**) was extracted with AllPrep DNA/RNA Mini Kit (Qiagen) and a region containing the full RPO30 gene was amplified, cloned into pGEM-T plasmid (Promega) and sequenced. The sequencing data were analysed with Vector NTI.10 and the BioEdit software was used to perform the multiple alignments of the nucleotides and amino acid (aa) sequences. The Mega 4 software was used to generate the phylogenetic tree.

The PCR primers SpGpRNAPol F (tctatgtcttgatatgtggtggtag) and SpGpRNAPol R (agtgattaggtggtgtattattttcc) were designed (with Allele ID 6 software) on both sides of the region containing a deletion in SPPV sequences so that the PCR products from SPPV would be shorter in comparison with GTPV and LSDV.

RESULTS AND DISCUSSION

Phylogenetic and Sequence Analyses

The phylogenetic analyses using the Neighbor-Joining method showed three lineages: the LSDV lineage, the GTPV lineage and the SPPV lineage (**Figure 1**). A few discrepancies were found: isolate

1 Animal Production and Health Laboratory, FAO/IAEA Agriculture & Biotechnology Laboratory, IAEA, Vienna, Austria
2 Institute for Veterinary Disease Control, Austrian Agency for Health and Food Security, Moedling, Austria
3 CIRAD, UMR Contrôle des Maladies, Montpellier, France
4 Biotechnology Division, ARC-Onderstepoort Veterinary Institute, Onderstepoort, South Africa;
5 Pendik Veterinary Control and Research Institute, Pendik/Istanbul, Turkey
6 Institute for Animal Health, Pirbright Laboratory, Woking, Surrey, UK

* Corresponding author: c.lamien@iaea.org

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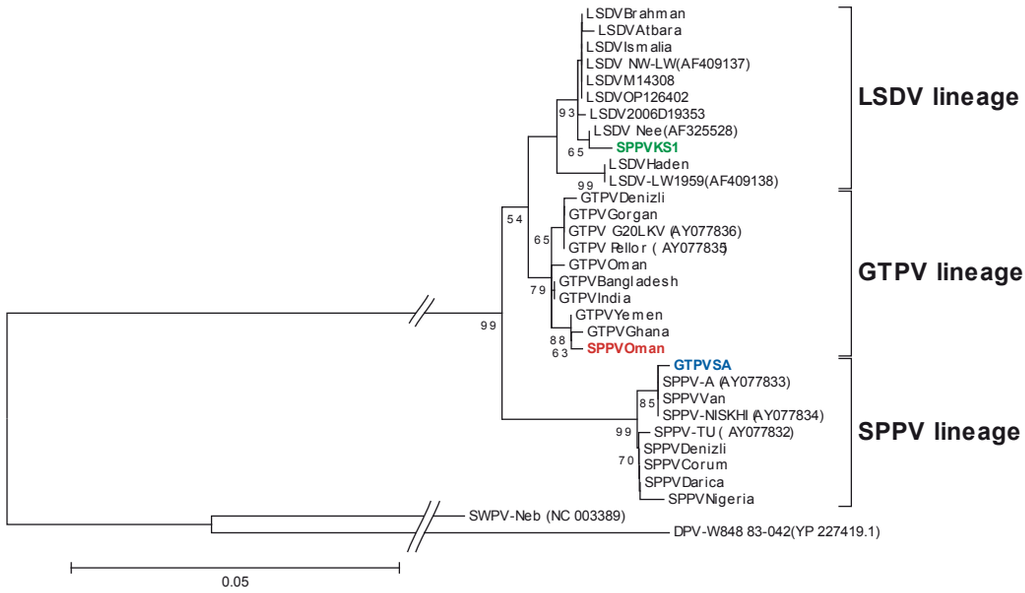

Figure 1. Phylogenetic tree derived from the nucleotide sequences of 30 CaPVs RPO30 genes by the Neighbor-Joining method Distance analysis based on Kimura 2-parameter distance. Deer poxvirus (DPV) and swine poxvirus (SWPV) retrieved from the gene bank were used as out-groups. The numbers at the nodes are the bootstrap confidence levels (in percentages) obtained for 1 000 replicates. Only bootstrap > 50 are shown.

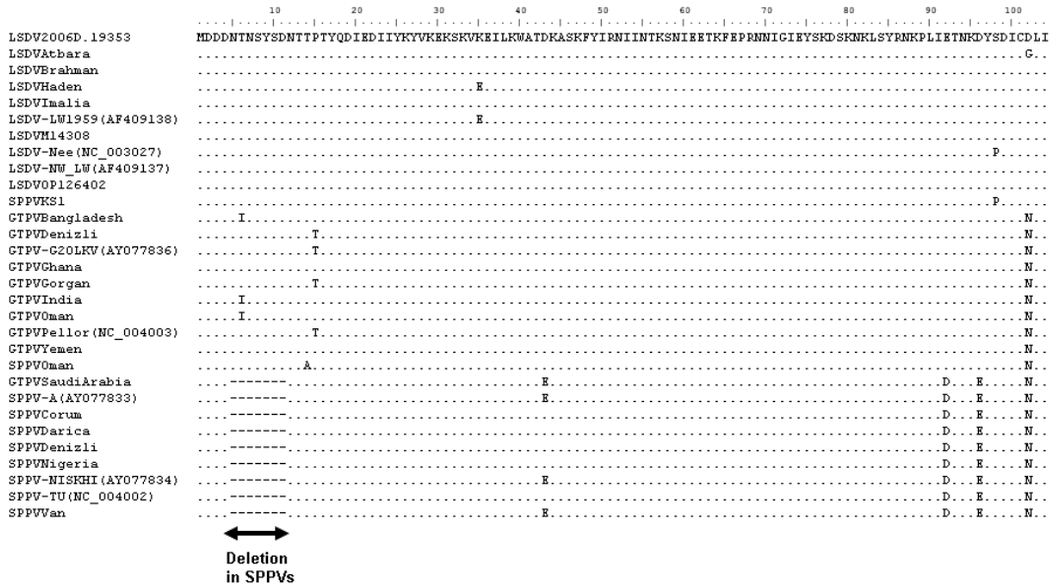

Figure 2. Alignment of the amino acid sequences of 30 CaPVs RPO30 genes (Partial representation) showing a 21 nucleotide deletion in SPPV sequences.

SPPV Oman was found to be located in the GTPV lineage, while isolate SPPV KS1 was in the LSDV group and GTPV Saudi Arabia was present in the SPPV group. Interestingly, these results are fully in agreement with our previous study using the CaPVs' GPCR gene (Le Goff et al., 2009). In addition, the SPPV KS1 and SPPV Oman have been shown to be related to LSDV and to GTPV respectively (Black et al., 1986). The alignment of the aa sequences showed seven aa deletion (corresponding to a 21-nucleotide deletion) in all and only the members of the SPPV lineage at positions 5 to 11 (**Figure 2**).

PCR for Differentiating SPPV from GTPV

Because a 21 bp deletion was found in all individuals belonging to the SPPV lineage (**Figure 2**), we have used it as a marker for differentiating SPPV from GTPV in a single PCR step. Primers were designed for both side of this region so that the PCR products from GTPV/LSDV would differ in length from those produced with SPPV. After the migration of the PCR products on a 3% high resolution agarose gel, all individuals in the SPPV group were found to produce a shorter amplicon (151 bp) compared with GTPV/LSDV (172 bp) as

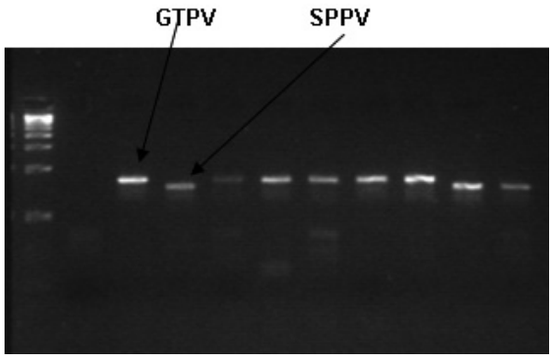

Figure 3. Classical PCR for differentiating GTPV from SPPV
SPPV and GTPV PCR products have different lengths due to a deletion in the target region within SPPV RPO30 gene.

shown in **Figure 3**. This system can therefore be used to differentiate GTPV from SPPV, assuming that the samples are coming from domestic small ruminants since with the exception of KS1, no strain of LSDV has been reported in small ruminants (Diallo and Viljoen, 2007; Babiuk et al., 2008). Results of the genotyping using this PCR method are similar to those obtained by sequencing the full RPO30 gene and also in agreement with our previous phylogenetic studies using the CaPVs' GPCR gene (Le Goff et al., 2009).

CONCLUSIONS

This study shows that the animal species origin of CaPVs can be determined by sequencing their RPO30 gene. Furthermore, using the 21 nucleotides deletion found only in all members of the SPPV lineage, a simple and quick PCR method was developed to differentiate CaPVs in small ruminants without the need for sequencing. Also, since it does not require the use of any genotype-specific primers or any multiplexing, this method is suitable for use in large scale screening and genotyping during disease outbreaks.

Table 1. List of Capripoxviruses used in this study.

	Strain name	Species of origin	Source/Accession number
1	LSDV Brahman	Cattle	OVI/South Africa
2	LSDV Atbara	Cattle	OVI/South Africa
3	LSDV Ismalia	Cattle	AGES/Austria
4	LSDV NW-LW	Cattle	AF409137
5	LSDV M14308	Cattle	OVI/South Africa
6	LSDV OP126402	Springbok	OVI/South Africa
7	LSDV 2006D19359	Cattle	OVI/South Africa
8	LSDV Nee	Cattle	AF325528
9	LSDV Haden	Cattle	OVI/South Africa
10	LSDV LW1959	Cattle	AF409138
11	GTPV Denizli	Goat	VCRI-Pendik/Turkey
12	GTPV Gorgan	Goat	Pirbright/UK
13	GTPV G20LKV	Goat	AY077836
14	GTPV Pellor	Goat	AY077835
15	GTPV Oman	Goat	Pirbright/UK
16	GTPV Bangladesh	Goat	Pirbright/UK
17	GTPV India	Goat	Pirbright/UK
18	GTPV Yemen	Goat	Pirbright/UK
19	GTPV Ghana	Goat	Pirbright/UK
20	GTPV Saudi Arabia	Goat	Pirbright/UK
21	SPPV A	Sheep	AY077833
22	SPPV Van	Sheep	VCRI-Pendik/Turkey
23	SPPV Niskhi	Sheep	AY077834
24	SPPV TU	Sheep	AY077832
25	SPPV Denizli	Sheep	VCRI-Pendik/Turkey
26	SPPV Çorum	Sheep	VCRI-Pendik/Turkey
27	SPPV Darica	Sheep	VCRI-Pendik/Turkey
28	SPPV Nigeria	Sheep	Pirbright/UK
29	SPPV KS1	Sheep	AGES/Austria
30	SPPV OMAN	Sheep	Pirbright/UK

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Rift Valley Fever, Disease Ecology and Early Warning

S. de La Rocque[1,2]* & P. Formenty[3]

ABSTRACT

Rift Valley fever (RVF) once again dramatically affected the Horn of Africa (Kenya, Somalia, and Tanzania) in 2006–2007. This outbreak was linked to unusual rainfall associated with climatic events (El Niño) which affected the populations of the mosquitoes acting as vectors and reservoirs of the disease. The disease also reappeared in Sudan in the autumn of 2007, following excessive rainfall driven by a post-El Niño and unusually warm sea temperatures in the Indian Ocean. In 2008, the disease affected southern African countries (Swaziland, South Africa) and islands in the Indian Ocean (Comoros, Mayotte Island, Madagascar). Based on near real-time climatic data, forecasting models and early warning systems were available at the continental level and proved to be efficient in raising the alert before the onset of the epidemic, at least for the coastal countries of eastern Africa. These recent events provided an opportunity to review the natural history of RVF, especially in some places where its ecology was poorly documented. FAO and WHO officers used outcomes from the different models and then identified gaps or needs that could be filled in order to improve the use of these predictions. A brainstorming meeting was organised in Rome in September 2008 to discuss adjustments and complementarities to the existing models, as forecasting and early warning systems are the keys that may provide a time window for preventive measures before amplification of the virus is out of control.

Key words: *Rift Valley fever, zoonotic disease, arthropod vectors, ecology, areas of risk, early warning systems.*

INTRODUCTION

Rift Valley fever (RVF) is a per acute or acute viral disease of domestic and wild ruminants, caused by a mosquito-borne virus and characterised by necrotic hepatitis and a hemorrhagic state, but infections are frequently unapparent or mild. The disease is considered more severe in sheep, cattle and goats, producing high mortality rates in new-born animals and abortion in pregnant animals. However, there are within- and between-species variations in the manifestation of disease, depending on several factors including herd immunity levels (FAO, 2003).

RVF is a zoonotic disease and the vast majority of human infections result from direct or indirect contact with the blood or organs of infected animals while human infections have also resulted from the bites of infected mosquitoes. Infection in humans is usually associated with mild to moderately severe influenza-like illness. While most human cases are relatively mild, a small percentage of patients develop a much more severe form of the disease. This usually appears as one or more of three distinct syndromes: ocular disease (0.5%–2% of patients), meningo-encephalitis (less than 1%) or hemorrhagic fever (less than 1%). The case-fatality ratio for patients developing the hemorrhagic form of the disease is high at approximately 50% (WHO, 2007).

RVF was first identified by Daubney in 1931 after heavy seasonal rains in the Rift Valley in Kenya between lakes Naivasha and Elementaita (Daubney et al., 1931). It has been reported in most sub-Saharan countries, as well as in Mauritania, Egypt and the Indian Ocean islands (Comores, Madagascar) and has also spread in the Middle East (Saudi Arabia, Yemen).

The virus may be transmitted by a very large number of arthropods. Thirty-eight species of mosquito have been found infected in nature, of which at least 35 have proved their vector competence in controlled conditions (European Food Safety Agency, 2005). For mosquitoes only, six genera are represented in the first list: *Mansonia*, *Anopheles*, *Coquillettidia*, *Eretmapodites*, *Culex, Aedes* inc. *Ochlerotatus*. Some species of the latter two are considered to be the main vectors (McIntosh et al., 1980; Meegan et al., 1988). In addition, mechanical transmission has been demonstrated with other haematophagous insects, including *Stomoxes*, phlebotomies or *Culicoides* midges. The wide diversity of arthropods from which the virus has been isolated emphasises the difficulty of understanding the epidemic/endemic cycle of the virus.

ECOLOGY OF RVF

Endemic versus Epidemic

RVF endemic cycles occur in temperate, tropical and sub-tropical zones of Africa. The virus is capable of inhabiting a variety of different bioclimatic conditions, including: wet and tropical areas, e.g. Ivory Coast and Congo; hot and arid areas, e.g. Yemen or Chad; and irrigated regions, e.g. the Senegal River valley and the Nile Delta (Chevalier et al., 2004). Most RVF viral activity is cryptic, at a low level, and not associated with detectable disease in humans and animals. Many African countries have found significant sero-prevalence in sheep, goats and cattle for the RVF virus throughout various agro-climatic zones in their country, without clinical signs being reported in humans or in animals (Davies, 2006). Most countries are not really

1 EMPRES/Animal Production & Health Division, Food and Agriculture Organization of the United Nations, Rome, Italy
2 Centre International de Recherche Agronomique pour le Développement, Campus International de Baillarguet, France
3 Global Alert and Response Department, World Health Organization, Geneva, Switzerland

* Corresponding author: Stephane.DeLaRocque@fao.org

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aware of the circulation of the virus because of a lack of systematic surveillance activities.

At irregular intervals of about 5–12 y, large epidemics of RVF have occurred in southern and eastern Africa and these epidemics have been associated with above average rainfall, huge activity of the vectors and the presence of susceptible livestock. The 1997–1998 epidemic in the Horn of Africa, which is considered as one of the most devastating RVF epidemics in East Africa, was associated with torrential rains (60–100 times the seasonal average) that resulted in the worst flooding in the Horn of Africa since 1961 (WHO, 1998). Flood plain zones are particularly affected, as are any areas where the water table has been raised by irrigation or other water conservation practices and serves as breeding habitats for mosquito vectors of RVF. Because climatic events tend to occur over large areas, there is a tendency for outbreaks to occur simultaneously in adjacent territories.

Throughout its wide ecological range, the pattern of RVF activity is much influenced by the ecological characteristics of the particular biotope under consideration. The agro-climatic zones of Africa can give a good guide as to the expectations from RVF virus activity (Davies, 1998) and can be schematically summarised as follows:

- enzootic activities (+/-cryptic) in high rainfall forests and forest edges;
- periodic increased virus activity to epizootic proportions in bushed and wooded grasslands;
- rare but explosive epizootic RVF activities in dry grassland and semi-arid zones associated with flood plains;
- very rare but long lasting RVF outbreaks in animals and humans following introduction of viraemic animals in irrigation scheme areas (**Table 1**).

Persistence and Spread of RVF Virus

Current evidence suggests that the RVF virus in sub-Saharan Africa is maintained in inter-epidemic periods, primarily by transovarial transmission in *Aedes* mosquitoes (Linthicum et al., 1985). *Aedes* eggs can resist desiccation for long periods and hatch in water inundation, such as occurs following prolonged rainfall or flooding. *Aedes* mosquitoes are thought to be the reservoir of the RVFV between those epidemics triggered by above-average rainfall that leads to a rapid increase in vector numbers. Once infection has been amplified in livestock, secondary epidemic vectors such as *Culex* or, *Anopheles* mosquitoes that breed in semi-permanent pools of water and get infected by biting infected vertebrates can become involved in transmission and some, like *Culex*, serve as excellent secondary vectors if immature mosquito habitats remain flooded for long enough (Linthicum et al., 1985).

It is not always clear if the RVFV is maintained between epidemics or is re-introduced. In some sub-Saharan countries, it has been speculated that limited *Aedes* populations may still play a role in maintaining a low level of viral transmission to livestock every year (Chevalier et al., 2005). Recent studies conducted in the Ferlo region of Senegal demonstrated that several generations of *Aedes vexans* can emerge during the same rainy season (Mondet et al., 2005), depending on the succession of rains and dry periods and consecutive changes in the water levels of temporary ponds. This mechanism could be the way the disease persists at low incidence in livestock.

The persistence of the activity of the virus is also modulated by the immunity of the affected host populations. Classical epidemic curves show a few weeks/months decrease in incidence after the peak. Depending also on the natural resistance of the animals, and subsequent level and duration of viraemia, the outbreak picture can significantly differ. In the irrigated highly cultivated area of Gazeera in Sudan, the abundance of so-called secondary vectors and a significant proportion of naive exotic dairy herds were favourable for an outbreak in 2007 that lasted for several months.

THE 2006–2009 OUTBREAK IN EAST AND SOUTHERN AFRICA

An updated map of the distribution of RVF outbreaks is given in **Figure 1**.

Kenya

By mid-December 2006, the disease emerged in its epidemic form in the North-Eastern Province of Kenya, where the 1997–98 outbreak was also centred (WHO, 1998). By the middle of March 2007, 684 human cases were reported by the Ministry of Health, including 155 deaths. The disease started in the Garissa District and half of the cases were reported in the North-Eastern Province (also in the Ijara and Wajir Districts). It then appeared in one district of the Rift Valley Province (Kajiado); five districts of the Coastal province (Kilifi, Tana River, Malindi, Isiolo, Taita Taveta); two districts

Table 1. RVF related characteristics of different ecological systems potentially suitable for RVF occurrence.

Ecological Zone	II	III	IV	V	VI
Vegetation	Forest & derived grasslands	Bushed & wooded grasslands	Dry grassland	Semi-arid lands	Irrigation scheme
Virus activity in inter-epizootic period	Yes	Possible every 3–5 y	Unlikely, unless floods	No-except in riverine flood zone	No-except if viraemic animals are imported
Mosquitoes population	Constant and diverse	Seasonal	Explosive	Explosive	Constant: *Culex, Anopheles*
Herd Immunity	Stable	Stable	Low	Very Low	Very Low
Epizootic virus activity	Yes	Yes	Usually	Not usually	Not usually
Duration of Epizootics	Peak 3–6 months; Cases 1–3 y	Peak 3–6 months Some foci one y	3–6 months	3–4 months	6–10 months
Periodicity of Epidemics	3–15 y	5–15 y	5–25 y	25–35 y	10–25 y

Adapted from (Davies, 1998). The ecological zones have been defined by Pratt et al. (1966).

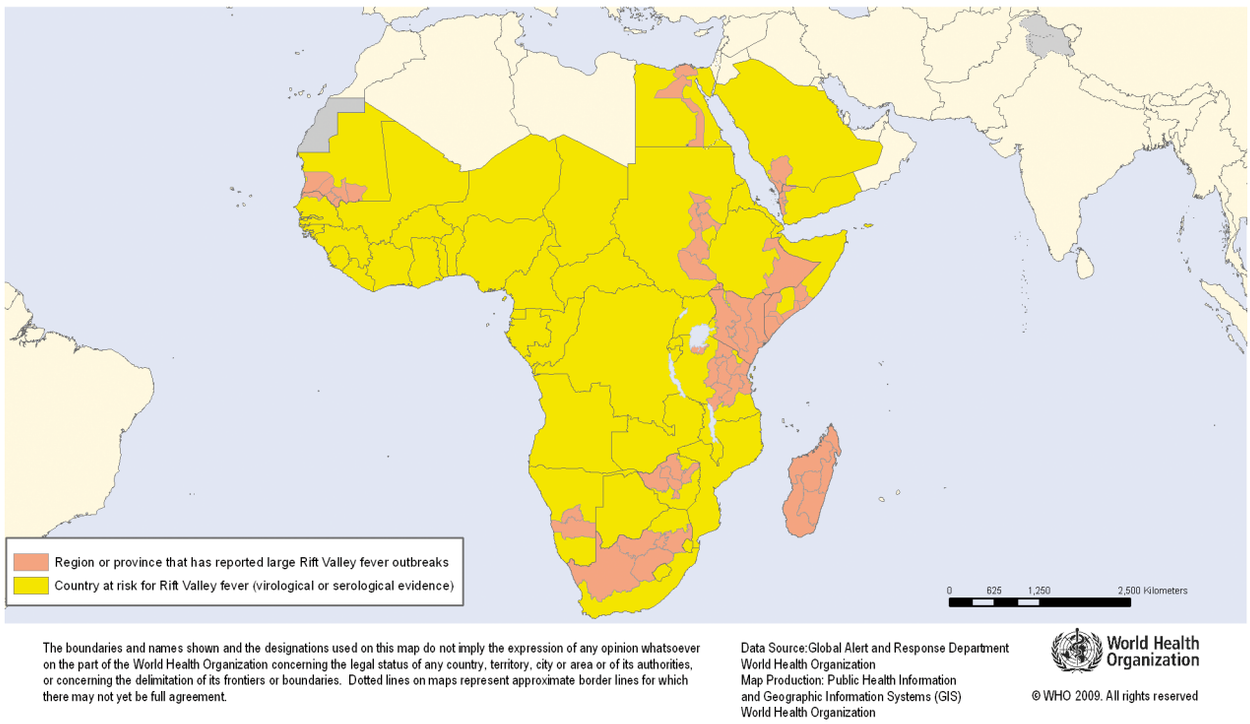

Figure 1. Location of large Rift Valley fever outbreaks and infected areas (the map includes historical and recent reports of RVF in human and/or animals).

of the Central province (Kirinyanga, Marangua), two districts of the Eastern Province (Banngo, Nakuru); and one district in the Nairobi area (WHO, weekly epidemiological record, 18/05/2007). Bans on livestock slaughtering and vaccination of livestock with live attenuated vaccine were put in place.

Somalia

In mid-December 2006, suspected cases were also reported to WHO from the Lower Juba Region. Investigations were limited by the civil unrest, but a final total of 114 human cases were reported, including 51 deaths. The geographical distribution of cases was mainly in the Lower Juba Region, followed by the Gedo Region, the Hiran Region, the Middle Juba Region, the Middle Shabelle Region and the lower Shabelle Region (WHO, weekly epidemiological record, 18/05/2007).

Tanzania

In mid-January 2007, RVF was suspected in Tanzania in both animals and humans in the Arusha Region in the north of the country. The disease was then reported in the central part of the country, including the regions of Dodoma, Iringa, Manyara, Morogoro, Mwanza, Pwani, Singida and Tanga. At the beginning of May, there were 290 reported human cases, including 117 deaths. Clinical suspicions in livestock also concerned southern provinces bordering Mozambique, Malawi and Zambia. It should be noted that the previous epidemic in 1997–98 also started in the northern provinces, but did not affect the central region of the country. However, field evidence of high prevalence of antibodies in livestock in 2007 in the south-west of the province suggests that the disease is probably widely endemic in Tanzania and not a recent introduction from Kenya, as was initially believed (B. Swanepoel, pers. comm.).

Sudan

WHO reported an RVF outbreak in humans in Sudan at the beginning of November 2007. A total of 738 human cases, including 230 deaths, were officially reported in White Nile, Sinnar and Gesira States. Some serological and virological analysis conducted on animal samples revealed the presence of specific antibodies and RVF virus, confirming, if it were necessary, the circulation of the virus in livestock.

Neighbouring Countries

Animal cases were reported from Burundi and Swaziland in June 2008 while, surprisingly, no cases were reported from Ethiopia, Zambia, or Mozambique. During the first few months of 2008, small focal outbreaks of RVF affecting mostly captive buffalo, cattle and small ruminants were also reported in South Africa, as well as about 20 human cases.

Comoros and Mayotte Islands

In July 2007, a 12 year-old child originating from Moroni, Comoros, was hospitalised in Mayotte and found positive for RVF. Clinical suspicions in small ruminants were also reported in Comores (unpublished data). Eight human cases were then reported on Mayotte between September 2007 and May 2008. A serological survey in cattle in 2008 confirmed the active transmission of the disease on Mayotte (23% of herds affected). The origins of the disease in these islands have never

been clearly established, but seem to involve illegal trade of livestock from Tanzania (Cêtre-Sossah et al., 2009).

Madagascar

Central Madagascar experienced events of cattle mortality in December 2007. By the end of January 2008, human cases were reported in Tolagnaro city in the south and almost simultaneously in the highland Anjozorobe District, 80 km north of Antananarivo and on the eastern coast of Madagascar. The outbreak lasted until June 2008, and a total of 476 suspected human cases were reported from 15 districts, with 19 deaths from the disease. The disease was then again detected in cattle in October 2008 in the Fianarantosoa Districts, central highlands, and 236 cases were suspected from four contiguous districts, where seven people died. The real incidence of RVF in Madagascar was difficult to establish over the course of the outbreak because of poor reporting channels and the limited capacities for active surveillance in livestock. Recently, a serological investigation carried out among cattle sampled after the 2008 RVF outbreak confirmed the wide distribution of recent antibodies on the island with high prevalence in Toliara, the traditional livestock breeding areas of Madagascar (E. Jeanmaire, in preparation). A national cross-sectional serological survey among people working in slaughterhouses confirmed that the virus had circulated in at least in 92 of the 111 districts of the country (Andriamandimby et al., in preparation).

AREAS AT RISK

Persistence and Emergence

Certain types of environment may be favourable to the persistence of the virus and this could explain why the disease re-emerges from the same sites. Studies in Kenya have shown that flooding of grasslands may be important in the generation of RVF epidemics (Davies, 1975). In this country, the typical dambo habitats are shallow depressions often located near the head of a drainage system, associated with tall grasses, sometimes bushes or wooded savannah. They form temporary ground pools, varying from tens of metres to several km in length and remain dry for prolonged periods (y). The previously cited hypotheses regarding the possible reservoir role species from the *Aedes* genus (i.e. *Ae. cumminsii*, *Ae. circumluteolus, Ae. mcintoshi*) have derived from studies of mosquito ecology within these ecotypes that are abundant in the Garissa area (Linthicum et al., 1984; Linthicum et al., 1985).

In Sudan, the Kosti region 200 km south of Khartoum in the White Nile Province consists partly of low lying areas with many swamps and small tributaries of the White Nile. A biotype particular to the Sudan is the toich, seasonally flooded grassland in the catchment areas of the White Nile and its tributaries, which form a large part of the southeast of the country. After 6–12 weeks of flooding, these areas are extensively grazed by cattle. The disease is endemic in most parts of the Sudan (ecological zones II, II and IV) and the last major epidemic in 1973 also started in the Kosti agricultural district, and then extended as a devastating epizootic to the Upper and Blue Nile provinces (Eisa et al., 1977). Additionally, in June 1976, clinical signs and serological evidence of RVF in cattle and humans were reported in a dairy farm in Khartoum North, following the introduction of new cattle from the White Nile province (Eisa et al., 1980).

In Madagascar, the epidemiology of RVF is still very poorly understood. The recent outbreak occurred during a low rainfall period. In the past, RVF had already affected Madagascar in 1990–91 (Morvan et al., 1991), and it was established that the virus circulating in 2007–2008 is similar to the one isolated in the Horn of Africa in 2006–2007 (Andriamandimby, in prep). Therefore, while the origin of the disease in Madagascar has not been clearly established, it may involve illegal trade of livestock from the Horn of Africa (Kenya, Tanzania or Comoros). No cases were reported during the long inter-epizootic period, but anecdotic serological investigations revealed contacts of humans and animals with the virus in Toliara and the south-western provinces (Zeller, 1998), feeding the hypothesis of a low-level circulation of the virus and/or the possible existence of endemic areas. This area contrasts with the rest of the island in terms of ecotype, with agro-ecoclimatic patterns similar to those of the endemic areas of Kenya (Le Houérou et al., 1989). In addition, investigations conducted during the course of the 2008 outbreaks revealed that herds in the area around Toliara experienced a high rate of unreported abortions the year before and a significant number of sero-positives confirmed that these animals suffered from RVF several months before any report of cases (FAO, unpublished data).

Primary versus Secondary Foci

From endemic areas, the disease may spread with livestock movements and the introduction of viraemic animals in suitable areas (e.g. irrigation schemes). At the primary focus site the virus is maintained in the vectors and/or in the hosts while in secondary foci the virus is imported and spread between naïve ruminants thanks to local mosquitoes that are competent vectors for RVF (*Culex, Anopheles*…). During the 2007–08 outbreak in Sudan, the disease reached the irrigated area of Gazeera through sheep, camels and livestock on their way to the capital and the northern coast, from where hundreds of thousands of sheep are exported to Mecca for the Hajj. The presence of high densities of vectors and the abundance of naïve ruminant populations including highly susceptible exotic dairy cattle, acted as highly favourable factors for the amplification of the virus, with high impact on humans. The same applied for the Ifakarra rice valley in Tanzania, 2007, and in Madagascar in 2007–08. In Madagascar the cases were initially detected in the highlands, but the infection may have been imported from the livestock breeding areas in the south and north-west when the animals were transported to meat markets around the capital or to be used as oxen in the rice fields, where the mosquitoes are abundant. **Figure 2** schematically illustrates these different epidemiological processes.

The differentiation between primary and secondary foci is made more complex by the usual absence of clear chronology in cases reporting and the poor quality of reports, especially on the veterinary side. In addition, no systematic system of traceability or identification exists in most of these countries, and the history of an event is often difficult to document in detail. Other economical, sociological or cultural factors may also make the investigation even more complex.

DISEASE FORECAST AND EARLY WARNING

Land tenure, vegetation activity and some bio-climatic parameters can be captured by high level resolution satellite observations Associated with other data, they have been tentatively used to determine weather events or areas at risk and are of particular interest for RVF forecasting

Map of Suitability for Presence of RVF

Data derived from the AVHRR-NOAA satellites, including land surface temperature and NDVI data are processed and associated with a Digital Elevation Model (DEM), then confronted with a set of disease presence and absence points. Non-linear discriminant analysis captures the characteristics of sites of disease presence and absence, and these are used to define the status within a multivariate space of

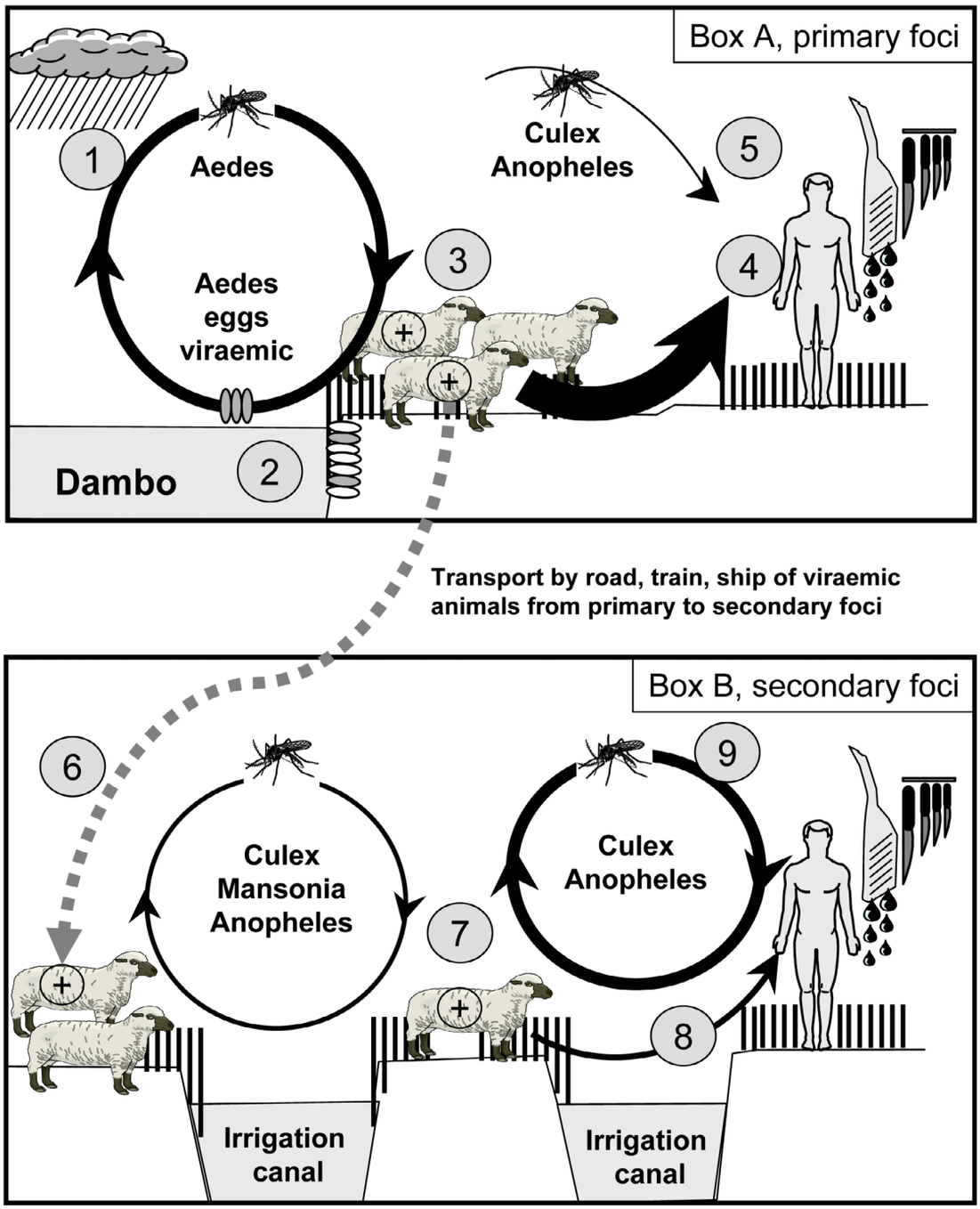

Figure 2. Schematic transmission of Rift Valley fever (Credits: P. Formenty, WHO).

Box A, primary foci — (1) Following heavy rainfall, dambos with *Aedes* breeding sites are flooded for at least a 10–15 days period of time. (2) A portion of the infected *Aedes* eggs leave dormancy and after hatching adults emerge infected with RVF virus. (3) *Aedes* females mosquitoes are aggressive mammophilic blood feeders and bite surrounding domestic ruminants (cattle, camels, sheep, goats) that serve as amplification host for the virus. RVF animal outbreaks are characterised by massive abortion of females and death of young ruminants (4). The large majority of human infections result from direct or indirect contact with the blood or organs of infected animals during slaughtering or butchering, assisting with animal births, delivering veterinary care or from the manipulation of fresh carcasses or foetuses. (5) Later in the course of the outbreak, secondary vectors such as *Culex* breeding in stagnated waters also contribute in the transmission of RVF virus to humans after acquiring the virus during a previous blood meal on an infected animal. The + within the circles indicates RVF infected sheep.

Box B, secondary foci — (6) Domestic animals infected with RVF at the primary site are imported to irrigation areas by route, train or ship. (7) Irrigation schemes are persistent habitats for *Culex, Mansonia* and *Anopheles* mosquitoes, whose abundant populations increase the circulation of the virus between amplifying hosts (cattle, camels, sheep, goats). (8) As in primary foci, human infections may result from direct or indirect contact with the blood or organs of infected animals. (9) However, most of the human infections result from bites from infected mosquitoes having acquired the virus during a previous blood meal on an infected animal.

any point within the risk-mapped area. On this basis, the probability with which the point belongs to the category of disease presence or absence is calculated and this probability is entered into the final risk map. This approach has been successfully used to draw maps of suitability for presence with various diseases or vectors, including Rift Valley fever (Rogers, 2006). However, the dataset used for the latter exercise is derived from a published literature search covering the period from 1968 to 2004 and therefore is far from exhaustive.

Monitoring Rainfall Anomalies

RVF outbreaks in East Africa have been associated with warm phases of the El Niño/Southern Oscillation (ENSO) that are usually a result of very heavy rainfall after a few weeks of drought. It has proved possible to predict periods of RVF epizootic activity in East Africa using data acquired by remote sensing, allowing national and regional monitoring of the rainfall and climate patterns (Linthicum et al., 1990). In operation since 1999, the monitoring and prediction system developed by NASA's Goddard Space Flight Center relies on interpretation of rainfall and analysis of NDVI anomalies to map areas showing conditions that would support RVF vector emergence, production and propagation (Linthicum et al., 1999). This system has a pre-epizootic predictive period of two to five months before virus activity occurs and has enabled the successful development of forecasting models and early warning systems for RVF. It processes in a Potential Epizootic Areas Mask (PEAM), based on RVF literature survey and climate variable (rainfall and NDVI) thresholds to derive what is referred to as potential epizootic area. These maps are made public on a monthly basis (http://www.geis.fhp.osd.mil/GEIS/SurveillanceActivities/RVFWeb/indexRVF.asp) but warnings are sent to key partners, including WHO and FAO in real time.

Early Warning in Real Life

In early autumn 2006, these models confirmed a sharp increase in the probability of observing heavy rainfall and floods in high-risk areas of Kenya, Somalia, Tanzania, Sudan and Ethiopia (Anyamba et al., 2009). The first warnings of a potentially serious outbreak were sent by the Nasa/Goddard Space Flight Center in mid-September. FAO and WHO forwarded this warning to their representatives in the at-risk areas and a consolidated warning was made public in November (FAO, 2006).

FAO and WHO, in collaboration with other national and international organisations (CDC, Pasteur Institute, OIE, UNICEF etc.) and national authorities in the countries were very involved in the emergency response. National action plans were reviewed, disease control activities were implemented and a significant amount of resources allocated to increase disease detection and surveillance. As a result, the existing models can now benefit from recent and unique data on both humans and animals, allowing adjustments and complementarities. At the same time, FAO and WHO officers had the opportunity to widely use outcomes from the different models to identify gaps or needs that could be filled in order to improve the use of these predictions in an integrated disease prevention and control perspective. In September 2008, experts on RVF modelling and forecasting were invited by FAO and WHO to a two-day workshop to share experience, identify gaps and explore potential improvement in the models, in order to further adapt outcomes to fit with the needs of the disease control strategies. The objective of this workshop was to review the natural history of RVF, review the forecasting models and risk distribution maps available and being developed, and propose a roadmap to improve these tools. The final goal was to define a roadmap for the development of tools for forecasting and real-time RVF outbreak management.

The conclusions of this meeting are available at: ftp://ftp.fao.org/docrep/fao/012/ak144e/ak144e00.pdf. Improvements and adjustments were proposed as follows:

- the model on suitability for presence could be improved significantly by completing the dataset and by distinguishing the primary foci (i.e. the area where the disease emerged) from secondary foci (e.g. where the disease was later introduced by animal movement). An updated dataset including human and animal outbreak data, and distinguishing primary from secondary foci, is currently being prepared by WHO and FAO;
- a MODIS-based mapping algorithm could be applied to capture the flood dynamics. With improved flooding maps (where, when and duration), statistical spatial analysis could be developed (using historical data on RVF outbreaks) and applied in order to better identify risk areas and risk conditions for the emergence of RVF;
- the explicit incorporation of rainfall into the climate-based risk mapping component, would enable improvement of the risk mapping through a ranking of risk based on accumulated rainfall;
- the map of suitability for presence could help in improving the Potential Epidemic Area Mask as some of the RVF outbreaks along coastal Kenya in 2006–2007, in South Africa in 2008, or in central Madagascar in 2008–2009 were outside the currently used PEAM;
- the use of existing climate forecasting models should be further explored.

In conclusion, RVF outbreak warning messages were sent in 2006, but the implementation of preventive measures and key control activities was difficult in this context, for various reasons, including a decrease in awareness and resources dedicated to RVF, lack of clear regional strategies and common contingency plans at the country and regional levels. FAO and WHO are currently preparing guidelines and options to improve the level of preparedness and the capacities of the countries for early response. Part of this effort is dedicated to early warning, as it is a key point that may provide a time window for preventive measures, before the amplification of the virus is out of control.

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Epidemiological Survey of Bovine Pleuropneumonia in Mali

M. Niang[1]*, A. Sery[1], O. Cissé[1], M. Diallo[1], S. Sidibé[1], M. Doucouré[1], M. Koné[1], L. Doumbia[2], F. Roger[3] & F. Thiaucourt[3]

ABSTRACT

Now that rinderpest has been eradicated, contagious bovine pleuropneumonia (CBPP) remains the most important infectious disease of cattle in many tropical African countries including Mali. It is considered as a priority disease by the World Organization for Animal Health, the African Union /Inter-African Bureau for Animal Resources, the Pan-African Programme for the Control of Epizootics (PACE) and the FAO-Emergency Prevention System for Transboundary Animal Diseases (EMPRES). All these institutions and programmes have recognised that the lack of sufficient epidemiological data on this disease is an obstacle to implementing efficient plans for its control. Therefore, research must be conducted in order to generate the accurate data needed to prepare a technically appropriate and well coordinated control programme. The present epidemiological study was conducted in Mali with the aim of evaluating the seroprevalence of the disease in cattle and its geographical distribution. Serum samples collected from 7 628 cattle in different parts of the country were tested for the presence of specific antibody against CBPP by a c-ELISA. In parallel to this serological study, data on CBPP outbreaks and abattoir lung seizures over a 10-year period (1997–2006) were collected. Preliminary results indicate a national seroprevalence rate of 16.28% (1 242/7 628) and a herd seroprevalence rate of 85.18% (161/189). In general, both rates correlated and were relatively higher in the central and southern regions (17.77–28.33%; 89.29–100% respectively) than in the northern and western regions (4.63–11.89%; 60–83.33% respectively). The total number of outbreaks reported from the field was 121, while the number of suspected lung lesions detected in abattoirs was 12 470. The distribution of these figures varied between the country's regions but did not correlate with the observed seroprevalence rates.

Key words: *Contagious bovine pleuropneumonia, epidemiology, seroprevalence, Mali.*

INTRODUCTION

Cattle husbandry plays an important role in the economy of many tropical African countries including Mali. It is important for the rural populations as it contributes to their well-being through the production of milk, meat, hides, animals and draught power. In many of these countries cattle are considered as a primary source of wealth. Cattle can therefore be used for household consumption or to generate cash income. It is also important for the well-being of urban populations since demand for milk and milk products are increasing steadily. At the national level, cattle husbandry plays an important economic role through exports to regional markets. Therefore, improving cattle production in these countries is a major objective of the governments, veterinary services, international organizations and institutions involved in development. To achieve this objective, the control of animal diseases is a necessary requirement.

Among a panel of cattle tropical transboundary diseases present in African countries including Mali, our main interest and expertise concern contagious bovine pleuropneumonia (CBPP). The disease is caused by *Mycoplasma mycoides* subsp. *mycoides SC* (*MmmCS*) and characterised by a severe fibrinous exudative pleuropneumonia. With the successful achievement of the rinderpest eradication programme, it is now considered as a priority disease by the World Organization for Animal Health (OIE), the African Union /Inter-African Bureau for Animal Resources (UA/IBAR), the Pan-African Program for the Control of Epizootics (PACE) and the FAO-Emergency Prevention System for Transboundary Animal Diseases (EMPRES). In addition, the veterinary services in Africa all agree that the incidence of CBPP is increasing. Paradoxically, despite the recognition of the importance of CBPP, no significant studies have been conducted to evaluate its prevalence and economic impact. This is a major limiting factor for designing cost-effective control strategies.

The objective of this study was to evaluate the seroprevalence of CBPP among cattle in Mali and its geographical distribution.

MATERIALS AND METHODS

Sampling Methods

The cattle population in Mali, which is the subject of this investigation, is estimated to be approximately 8 385 703 heads (Direction Nationale des Services Vétérinaires, 2008) and is distributed as described in **Table 1**. Each region is subdivided into prefectures ('circles'), making a total of 49; each prefecture or 'circle' is divided into communes, making a total of 703 communes; and each commune consists of a number of villages, giving a total of 12 044 villages. The capital city of Bamako, called the District of Bamako comprises 6 urban communes (www.dgemali.net).

According to this distribution of cattle and administrative structure in Mali, seven out of the eight regions (excluding the region of

1 Laboratoire Central Vétérinaire (LCV), Bamako-Mali
2 Direction Nationale des Services Vétérinaires (DNSV), Bamako-Mali
3 CIRAD-UMR15, Montpellier, France

* Corresponding author: mniangm@yahoo.fr / mamadouniang@hotmail.com

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Table 1. Sampling frame according to the administrative structure and distribution of cattle in Mali.

Region	Estimated cattle population*	Number of prefectures	Number of communes	Estimated total number of sampling units**	Number of units sampled	Number of serum samples collected
Kayes	893 077	7	129	1 623	28	1127
Koulikoro	1 203 348	7	108	2 032	28	1112
Sikasso	1 336 681	7	147	2 324	28	1120
Ségou	945 908	7	118	2 280	28	1160
Mopti	2 347 997	8	108	2 107	33	1346
Tombouctou	848 633	5	52	1 035	20	800
Gao	722 848	4	24	414	18	723
Kidal	59 538	4	11	150	ND	ND
Bamako	27 673	6	6	79	6	240
Total	8 385 703	55	703	12 044	189	7628

* DNSV Annual Report, 2008; ** village or herd; ND = not done.

Kidal, with the lowest number of cattle) were studied; the District of Bamako was also included in this study. Consequently, all the prefectures (45) representing the seven regions, and the six communes of the District of Bamako were involved.

The sampling unit was the herd. Animals in the same village or communal corral which share common grazing fields or water bores were considered as a single herd. This means that the sample selection was based on villages (in the regions) and on corrals (in the District of Bamako).

In collaboration with the veterinary services, a total of 189 villages and corrals were purposively selected from the 45 prefectures and the six communes of Bamako District, based on their accessibility and the willingness of cattle owners to cooperate. From each herd at least 40 serum samples were collected taking ten animals from each of the following four age groups: 0–1 y, 1–2 y, 2–3 y and more than three years. A total of 7 767 serum samples were collected (**Table 1**).

Data Collection

A detailed questionnaire of the herd history (type of husbandry, disease status and vaccination history) and relevant data on the animals (age and sex) being sampled was completed at the sampling sites. In parallel, data on the number of outbreaks declared and the suspected lung lesions detected in abattoirs over a ten-year period (1997–2006) were also collected respectively from local veterinary services and from abattoir files.

Serum Collection and Serological Testing

Since vaccination against CBPP is practised in Mali, the date of the last registered vaccination against the disease exceeded three months at the time of sampling. Blood samples were collected by jugular vein puncture using sterile disposable needles and syringes. Samples were allowed to clot at room temperature and then centrifuged to harvest the serum that was aliquoted and stored at –20 ^{o}C until tested for antibodies (Ab) against *MmmSC*.

The *Mmm*SC-specific antibody (Ab) response was assessed in serum samples using a competitive enzyme linked-immunosorbent test (cELISA) (Institut Pourquier, Montpellier, France), according to the protocol provided with the kit. This test is recommended by the OIE as the standard serological method for CBPP diagnosis (Niang et al., 2006). Moreover, as the antibodies induced by T1/44 vaccines do not persist at a detectable level for more than three months after vaccination, this c-ELISA kit according to its manufacturer can be used for the detection of natural infections, even in areas where vaccination is used. For this reason, assurance was made that in all sites visited, the date of the last registered vaccination against the disease exceeded three months at the time of sampling.

Briefly, 90-well pre-coated microplates with *Mmm*SC antigens were used for the assay. Sera were diluted one in ten and incubated with the monoclonal antibody. The reaction was then revealed by a peroxidase anti-mouse conjugate and a tetramethyl benzidine (TMB) substrate. The reaction was stopped by addition of sulphuric acid solution and optical densities were read at 450 nm with a Titretek Multiscan Plus MKII microplate reader (Flow Laboratories, Finland) and recorded by the ELISA Data interchange (EDI) version 2.2 software connected to the reader.

Data Analysis

National and regional/District prevalences were estimated by calculating the proportion of sera giving a positive result over the total number of tested sera. The herd prevalence was estimated by calculating the proportion of herds with at least one positive animal over the total number of herds visited.

RESULTS

Results on the disease seroprevalence are presented in **Table 2**. Overall, the national prevalence was 16.28% (1 242/7 628), while the herd prevalence was 85.18% (161/189). The prevalence per region ranged from 4.63% in Tombouctou to 26.52% in Mopti, while herd prevalence ranged from 60% in Tombouctou to 96.97% in Mopti. In the Bamako District the prevalence was 28.33%, while the herd prevalence was 100%.

The prevalence of *MmmSC* reactors by age is given in **Table 3**. Analysis of the results reveals that animals aged between 2 and 3 y and above had higher prevalence rates than those of the youngest age groups.

A total of 121 outbreaks of the disease occurred during the period 1997–2006 (**Table 4**). Except in the region of Kidal and the District of Bamako where no cases were reported, all the other regions had reported outbreaks with the highest number being in the regions of Segou (30), Koulkoro (26), Kayes (21) and Sikasso (20)

Table 2. Individual and herd prevalence rates of CBPP antibodies in the different regions of Mali.

Regions	Sampled herds	Sampled sera	Positive sera	Individual antibody prevalence (%)	Positive herds	Herd antibody prevalence (%)
Kayes	28	1 127	125	11.09	21	75.00
Koulikoro	28	1 112	125	11.24	23	82.14
Sikasso	28	1 120	199	17.77	26	92.86
Ségou	28	1 160	245	21.12	25	89.29
Mopti	33	1 346	357	26.52	32	96.97
Tombouctou	20	800	37	4.63	12	60.00
Gao	18	723	86	11.89	15	83.33
Kidal	ND	ND	ND	ND	ND	ND
Bamako	6	240	68	28.33	6	100.00
Total	189	7 628	1242	16. 28	161	85.18

ND — not done.

Table 3. Prevalence of CBPP antibodies according to age groups in different regions.

Regions	Age group (y)											
	0–1 y			1–2 y			2–3 y			> 3 y		
	Sera	Pos.	%	Sera	Pos.	%	Sera	Pos.	%	Sera	Pos.	%
Kayes	280	21	7.50	280	30	10.71	280	41	14.64	287	33	11.50
Koulikoro	280	15	5.36	273	32	11.72	279	40	14.34	280	38	13.57
Sikasso	280	27	9.64	280	59	21.07	280	51	18.21	280	62	22.14
Segou	283	49	17.31	296	62	20.95	294	58	19.73	287	76	26.48
Mopti	355	69	19.44	343	93	27.11	328	103	31.40	320	92	28.75
Tombouctou	200	2	1.00	200	10	5.00	200	11	5.50	200	14	7.00
Gao	190	29	15.26	181	21	11.60	180	19	10.56	172	17	9.88
Kidal	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
D. Bamako	60	11	18.33	60	18	30.00	60	20	33.33	60	19	31.67
Total	1928	223	11.56	1913	325	16.98	1901	343	18.04	1886	351	18.61

ND = not done.

Table 4. Summary of annually reported CBPP outbreaks in the different regions of Mali (1997–2006).

Year	Number of reported outbreaks per Region									
	Kayes	Kkro	Sikasso	Segou	Mopti	Tmbtou	Gao	Kidal	Bamako	Total
1997	6	3	3	3	0	0	0	0	0	15
1998	1	2	0	5	1	0	0	0	0	9
1999	2	3	0	5	2	0	0	0	0	12
2000	4	1	2	3	1	1	0	0	0	12
2001	1	7	3	2	0	1	1	0	0	15
2002	1	0	3	1	0	0	0	0	0	5
2003	1	5	4	3	1	0	0	0	0	14
2004	2	0	2	2	0	0	1	0	0	7
2005	2	3	1	4	1	3	8	0	0	22
2006	1	2	2	2	0	2	1	0	0	10
Total	21	26	20	30	6	7	11	0	0	121

Table 5. Summary of the annually reported CBPP lung seizures in the different regions of Mali (1997–2006).

Year	Number of reported lung seizures per region									
	Kayes	Kkro	Sikasso	Segou	Mopti	Tmbtou	Gao	Kidal	Bamako	Total
1997	967	104	0	13	0	0	0	0	0	1084
1998	1 074	132	0	67	0	0	0	0	0	1 273
1999	1 239	140	0	30	0	0	0	0	0	1 409
2000	925	135	0	59	0	0	0	0	0	1 119
2001	1 266	137	4	46	0	0	0	0	0	1 453
2002	1 142	136	0	49	0	0	0	0	0	1 327
2003	970	135	14	20	0	0	0	0	0	1 139
2004	1 039	135	0	17	0	0	0	0	0	1 191
2005	1 029	145	41	42	0	0	0	0	0	1 257
2006	1 049	122	7	36	5	0	0	0	0	1 219
Total	10 700	1 321	66	379	5	0	0	0	0	12 470

and the lowest number registered being in the regions of Mopti (6), Tombouctou (7) and Gao (11).

A total of 12 470 lungs were seized due to CBPP lesions during the 1997–2006 period (**Table 5**). Most seizures occurred in the regions of Kayes (10 700 cases), Koulikoro (1 321 cases) and Segou (379 cases). Conversely, the regions of Kidal and the District of Bamako did not report any cases.

DISCUSSION

This first nation-wide serological survey has provided data for a better assessment of the prevalence of CBPP in Mali. Serum samples collected from 7 628 cattle in different parts of the country were tested for specific antibodies against CBPP by c-ELISA. The test is recognised by the OIE as reference test for the serodiagnosis of CBPP (Niang et al., 2006), but like any serological test, it has its limitations since its does not differentiate between antibodies from vaccinated and non-vaccinated animals. Nevertheless, according to the manufacturer, this kit can detect antibodies after infection even in areas where vaccination is practised because it is accepted that the antibodies induced by T1/44 vaccines do not persist for more than three months at a level detectable by this test in animals vaccinated against CBPP. Therefore, the positive results obtained in this study likely reflect the presence of specific antibodies post-infection since at all sites visited, the last recorded vaccination against CBPP was more than three months before the time of sampling.

The survey showed the prevalence of the disease nation-wide, with higher individual and herd prevalence rates observed in the central and southern regions which include Mopti, Segou, Sikasso and the District of Bamako. This strengthens the view that these regions are endemic areas. This might be due to the fact that these regions happen to include many common dry pastures used by transhumant herds not only from neighbouring regions within Mali but also from neighbouring countries. Therefore the close contacts among healthy and carrier animals within these dry season pastures probably enhanced the transmission of the infection.

Highly noticeable was the high individual and herd seroprevalence rates observed in the capital District of Bamako (28.33% and 100%) where the communal corrals are supposedly well managed and the owners utilise the well-organised State and private veterinary services extensively to assist production. This is probably due to the presence in Bamako of many cattle markets where animals of unknown status converge. These cattle are purchased by cattle owners and then newly introduced into the communal corrals. The status of the region of Koulikoro (11.24% and 82.14%) which is located between the central and the northern part of Mali is somewhat confusing but may be explained by the presence of common pastures in its northern part which are used by transhumant herds coming from Mauritania.

In contrast to the above regions, prevalence rates were relatively lower in the northern and western regions which include Toumbouctou, Gao and Kayes. Although these regions have some common pastures used by transhumants, the concentration of animals coming from neighbouring countries is not as high as that of the southern and central regions. Also, in contrast to the southern and central regions, the climate in these regions is harsh and might not be favourable for the long-term survival of the *Mycoplasma* organisms.

The results of the survey also revealed that older animals had the highest prevalence rates, suggesting that adult cows are more susceptible than young animals which tend to develop joint lesions instead of pulmonary disease (Masiga et al., 1996).

The only statistics available on the epidemiology of CBPP in Mali are those provided by the DNSV which unfortunately include only the number of outbreaks from which morbidity and mortality rates are deduced. These data are clearly very incomplete and do not address the prevalence of the disease, its exact distribution per region and area and per age groups taking into account the structure of the herd.

Similarly few data exist at both regional and continental level. In the West African sub-region, only in Guinea has a limited serological survey been used to evaluate the prevalence of CBPP. This gave a prevalence rate of 6.3% (Diallo et al., 1999). A similar survey conducted in Eritrea showed a seroprevalence rate of 1.43% (T. Tekleghiorghis and U. Ghebremicael, 2004).

Analysing the number of outbreaks declared from the field and the suspected lung lesions detected in abattoirs, it appears that their distribution varied among regions but did not correlate with the observed seroprevalence rates. Indeed, highly noticeable was their low number nationwide, specifically in the region of Mopti which is considered to be a highly endemic area as shown by the results of the present serological survey. This may be due to

inadequate reporting of CBPP cases or to the refusal of herdsmen to report outbreaks because of the fear of quarantine or other sanctions. Also, lung sequestra indicating the chronic stage of CBPP may be mistaken by inexperienced meat inspectors for other lung lesions such as abscesses, tubercular nodules, *Echinococcus* cysts and farcy (nocardiosis) lung lesions.

CONCLUSIONS

The main objective of this study was to evaluate the seroprevalence of CBPP among cattle in Mali and its geographical distribution. Preliminary results indicate the prevalence of the disease nationwide, with higher individual and herd prevalence rates occurring in the central and southern regions of the country than in its northern and western regions. The total number of outbreaks declared from the field and the suspected lung lesions detected in abattoirs varied between regions but did not correlate with the recorded seroprevalence rates.

It is hoped that this study will pave the way for the other West African countries to undertake similar studies for the establishment of a successful sub-regional coordinated CBPP control programme.

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Surveillance of Antibodies to Bluetongue Virus in Livestock in Mongolia using C-ELISA: Preliminary Results

C.H. Tungalag[1]*, D. Orgil[2], Ts. Altansukh[1], A. Bayanzul[1], B. Chemedtseren[1], S. Ganzorig[1] & J. Dabaadorj[1]

ABSTRACT

A competitive enzyme-linked immunosorbent assay (C-ELISA) was used to conduct surveillance of bluetongue virus antibodies (BTV) in sheep, goats and cattle in Mongolia. The highest prevalence was recorded in goats (86%) followed by sheep (51%) and cattle (9%). The results are the first confirmation of the presence of such antibodies in Mongolian livestock. Studies are now underway to conduct more detailed investigations concerning bluetongue, including to determine the virus serotypes that are and have been circulating in the country.

Key words: *bluetongue, serosurveillance, C-ELISA, prevalence.*

INTRODUCTION

Bluetongue disease is a non-contagious, arthropod-borne viral disease of ruminants, mainly sheep and less frequently of cattle, goats, deer and antelope. The pathogenic virus belongs to the genus *Orbivirus* and is a member of the Reoviridae family. There are 25 serotypes. A midge, *Culicoides imicola* and other culicoid species are responsible for its transmission.

The disease can cause serious losses to small ruminant production as well as constituting yet another constraint to trade. Not suspected to have occurred in Mongolia, the disease was, until recently, limited in its geographical distribution to areas south of latitude 40°. However, it appears to be moving north as a consequence of climate change resulting from global warming and it is now established in Inner Mongolia of the People's Republic of China and in the north-east of that country. Also, an alarming spread north has occurred from the Mediterranean basin but, as yet, there is little information available about the situation from the eastern side of the Eurasian continental mass although there are anecdotal reports of bluetongue occurring in northern China (Manchuria) and Inner Mongolia close to the Mongolian border which are a cause for concern that a similar situation is developing there.

Our aim therefore was to establish the status of bluetongue in Mongolia as an early warning of possible future disease problems so that the country can prepare to protect its 24 million small ruminants which generate a significant proportion of income for livestock keepers.

MATERIALS AND METHODS

Blood Sampling

The serosurvey was planned between August and September 2007 and data collected over the following eight weeks followed by laboratory testing between November 2007 and August 2008. Results were analysed between November 2008 and April 2009. Blood samples were collected based on epidemiological statistical calculations and random selection (Putt et al., 1993) Administratively, Mongolia divided into 22 aimags (districts) which are subdivided into 332 soums (municipalities) comprising 1 386 bags. Eighty soums were chosen randomly from all aimags and two cities, the number of soums selected being proportional to the number of livestock. For each soum, six herds were selected at random, each defined on the basis that they had different grazing pasture, and from each selected herd, ten cattle, five sheep, and five goats were chosen at random.

A two-stage sampling method was chosen, the primary sampling unit being the soum and the secondary unit, the animal. Sample size was calculated to detect a prevalence of 5% at soum level with a 95% level of confidence. Overall, the survey involved 4 800 cattle, 2 400 sheep and 2 400 goats. Information was collected on the age, status, geographical location, name of owner, bag, soum and aimag/administration unit.

Testing of Sera and Calculation of Prevalences

Screening of sera for bluetongue was through a competitive (ELISA) using a horseradish peroxidase labelled bluetongue virus-specific monoclonal antibody for detecting BTV antibodies (test kit 5010.20, VMRD Inc., Pullman,USA). The kit has a demonstrated sensitivity of 100% and a specificity of 99%. Testing was conducted at the Department of Infectious Diseases and Microbiology, Mongolian State University of Agriculture.

1 Department of Infectious Diseases and Microbiology, The Mongolian State University of Agriculture, Zaisan, Ulaanbaatar, Mongolia
2 Ministry of Food, Agriculture and Light Industry of Mongolia, Ulaanbaatar, Mongolia

* Corresponding author: hudererdene@magicnet.mn

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Table 1. Serological prevalence of bluetongue virus in livestock in Mongolia.

Species	Neg	Pos	Total	AP[1]	TP[2]
Cattle	4 277	427	4 704	9.1%	8.2%
Sheep	1 165	1 217	2 382	51.1%	50.6%
Goats	334	2 041	2 375	85.9%	85.8%
Total	5 776	3 685	9 461	38.9%	38.3%

[1] Apparent prevalence
[2] True prevalence

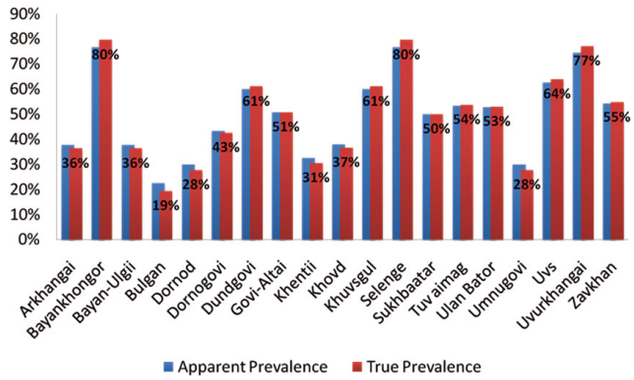

Figure 1. Apparent and true prevalence of bluetongue in sheep by aimags.

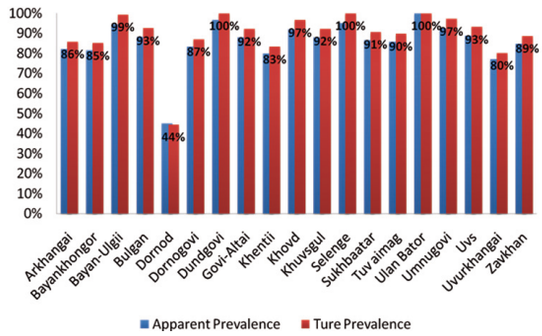

Figure 2. Apparent and true prevalence of bluetongue in goats by aimags.

RESULTS AND DISCUSSION

Results of the serological testing are summarised for each aimag and livestock species in **Table 1** and **Figures 1–2**. They show a wide variation in prevalence among the different aimags and that while bluetongue virus antibody was detected in all aimags and cities, highest prevalence was recorded in goats (86%) followed by sheep (51%) and cattle (9%).

However, because of BTV's wide pathogenic variability and the fact that cross reactions may occur between other orb viruses, a positive ELISA result with the bluetongue group test does not mean that clinical signs were caused by BTV itself.

CONCLUSIONS

The results presented here record the first confirmation of BTV antibodies in sheep, goats and cattle in Mongolia. Future studies should be undertaken to investigate bluetongue in more detail, and in particular to determine the BTV serotypes that are and have been circulating in Mongolia.

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SESSION 4

ONE HEALTH

One Health: Is there a Need for a Global Research Agenda?

E. Schelling[1]* & J. Zinsstag

ABSTRACT

Zoonotic infections receive internationally more and more attention as neglected diseases impoverished communities. Professional organisations and government institutes have created joint public and animal health working groups and numerous surveillance and research and programmes under the umbrella of 'One Health'. Still, there remains a divide between human, animal and ecosystem health specialists. Because most zoonoses go unrecorded, a rethinking of research and control efforts and their economic consequences is needed. Innovative research approaches promise to better capture the impacts of zoonoses from a societal perspective through more comprehensive frameworks that consider benefits and costs of zoonoses control in different sectors, notably the public health, livestock and private sectors. Such cross-sectoral studies promise to foster communication and exchange of information between sectors. Building on established national and regional technical support agencies for avian influenza will be important, despite the challenges to capture the momentum of partnerships, to establish permanent dialogue between sectors and to create sustainable national and regional bodies. Regional and international research groups applying a 'One Health' approach are well placed to generate the data required on levels of under-reporting, disability adjusted life years (DALY) estimates of zoonoses and the epidemiological and financial information for analyses of costs and benefits and cost-effectiveness and thereby identify the appropriate option for zoonoses prevention and control in particular settings from a range of possible interventions.

Key words: *One Health, neglected zoonoses, DALY, cost-benefit, cost-effectiveness, control.*

BACKGROUND

Most zoonoses occur more frequently among the poor and the poor are more vulnerable to the dual burden of zoonotic infections in people and livestock (WHO, 2006). Poor communities are also characterised by co-infection of several zoonoses and co-morbidity with other severe diseases. Risk factors of cystic echninococcosis in people, for example, are low socio-economic conditions including education levels, poor diagnostic facilities, the lack of dog population control measures and the absence of anthelmintic treatment (Macpherson, 2005). Zoonoses now belong to the category of neglected (tropical) diseases (WHO, 2007). Many countries lack information on the distribution of zoonotic diseases.

Control of zoonoses and improved food safety are public goods and therefore the public sector is seen as an appropriate delivery channel (Ahuja, 2004) although the State may sub-contract tasks such as vaccination and meat inspection to the private sector. However, services in developing countries continue to be poor, particularly in sub-Saharan Africa. As a consequence of the acute human resources crisis affecting the health sector (Wyss et al., 2003) and the structural adjustment programmes followed by the privatisation of health and veterinary services, large areas are not covered by sufficient qualified public and animal health professionals to assure surveillance and reporting of new outbreaks.

Communication between physicians and veterinarians is nearly absent or very weak. Veterinarians and physicians perceive the risks of zoonoses differently in the same context. For example, veterinarians rather than physicians speak with people living with HIV about the zoonotic risks of pet keeping (Grant and Olsen, 1999; Kahn, 2006). One obstacle to improved communication between public health specialists and veterinary professionals — the basis for cooperation between the health and veterinary sectors — has also been the lack of a common measure for the importance of zoonoses. On the human health side, the disability-adjusted life year (DALY) has been developed as a standardised measure of disease burden in people combining the years of life lost (YLL) due to premature death and the years of life lived with a disability (YLD). Since their introduction in the mid 1990's, DALYs have been used to prioritise disease control in the health sector by comparing interventions on the basis of effectiveness to reduce disease burden. Yet, disease burden of zoonoses in terms of DALYs, and particularly global disease burdens, have so far been determined for very few zoonoses e.g. for echinococcosis (Budke, 2006). Huge gaps in reporting of disease outbreaks exist for individual countries (Cowen et al., 2006). Widespread underreporting of zoonotic diseases may explain why they do not score high from a total disease burden perspective and why they often do not figure high on international public health agendas.

ONE HEALTH INITIATIVES

In the past decade, the 'One Medicine' concept by Calvin Schwabe (Schwabe, 1984) has received new attention. By extension of the concept into aspects of health systems it evolved towards 'One Health' (Zinsstag et al., 2005). 'One Health' was adopted by the World Conservation Union in the Manhattan declaration considering that the survival of wildlife requires healthy animals and healthy people. The American Medical Association (AMA) and the American Veterinary Medical Association (AVMA) have created the 'One World One Health' initiative (Enserink, 2007; King et al., 2008). The Consultative

1 Swiss Tropical and Public Health Institute, Basel, Switzerland

* Corresponding author: esther.schelling@unibas.ch

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Group of International Agricultural Research (CGIAR, the alliance of international agricultural research centres) has set up a platform on agriculture and health. The World Health Organization (WHO), the Food and Agriculture Organization (FAO) and the World Organisation for Animal Health (OIE) have cooperated through a global early warning system for animal diseases transmissible to humans (GLEWS) since 2006. The WHO Study Group on Future Trends in Veterinary Public Health, emphasising the need to expand the links between human and animal medicine, recognises that developing countries, especially in Africa, may lack the necessary technology, infrastructure and resources, leading to poor or non-existent surveillance and even inappropriate setting of priorities (WHO, 2002).

Other integrative concepts go beyond the direct animal-human interconnectedness and explore the relationships between various ecosystem components to define and value the priority determinants of health and human well-being (Forget and Lebel, 2001; Waltner-Toews, 2009). The drivers of emergence and re-emergence of diseases are related to disturbances of social and ecological equilibriums. Environmental changes can either not or hardly be reversed, for example pollution with heavy metals (Forget and Lebel, 2001). Ecohealth approaches have been conceptualised and promoted for many years by the International Development Research Centre (IDRC). Ecohealth can be defined as systemic, participatory approaches to understanding and promoting health and well being in the context of social and ecological interactions (Lebel, 2004). Patz et al. (2004) have summarised results that have been achieved with ecohealth approaches. An example is the recognition of fish becoming a human health risk in the Amazonas because with deforestation and soil erosion increased mercury loads were washed into the water.

Shared surveillance databases for disease events in animals and people are being developed such as the GLEWS by WHO, FAO and OIE, the Program for Monitoring Emerging Diseases (ProMED)-mail electronic early warning system (Cowen et al., 2006); other databases are designed to monitor antimicrobials and antimicrobial resistance or environmental toxicology (Zinsstag et al., 2009). In many countries working groups and task forces with members from both the health and livestock sectors were established for preparedness and control of avian influenza. Some countries, for example Ethiopia, started including other zoonoses in their mandate, which makes them permanent partners in a countries' disease control strategy (WHO, 2009). These countries have likely perceived the benefits and added value of coordinated work across sectors, and it is now important to sustain these efforts and the finances invested (US$ 2.7 billion until 2009) in preparedness and control of avian influenza.

The challenge then is to create functional linkages and better cooperation between the human and animal health sectors and aligning these with the needs and potentials of communities and policy makers, while acknowledging that a 'One Health' agenda should embrace ecological thinking. Coordinated working across and between sectors (i.e. 'intersectoral cooperation' for the achievement of a common goal is described below, involving primarily the public health and veterinary sectors, the private sector, and others for example the financial sector.

TAKING A MULTSECTORAL PERSPECTIVE

Communities play an essential role at the first level of any surveillance system (Jost et al., 2007) and are often excellent observers and know the priority diseases of humans and animals in their context. Thanks to livestock holders' reports on perceived poor anthrax vaccine quality, contamination problems in local vaccine production were detected in Chad (Schelling et al., 2008). Interdisciplinary working groups ideally include anthropological and social scientists along with physicians and veterinarians. Ownership by the communities for such endeavours is possible with their participation in knowledge generation as equal partners together with local authorities and scientists. Where an interdisciplinary team involves communities, authorities, lay people and local professionals with knowledge to a given question, then priorities, acceptable institutional and legal arrangements can be identified. Such a process with regular consultation of key partners from academia and society is a transdisciplinary process.

Joint field research studies by mixed public health-veterinary-livestock production-wildlife-plant health teams can serve as the nucleus for enhanced sharing of information. In former Soviet Union countries such as Kyrgyzstan, brucellosis was rather well controlled by veterinarians. However, after the break-up, the veterinary services lacked the needed organisation and human and financial resources. The health sector was then confronted with a sharp increase in human cases and criticised the veterinarians' inability to control the disease. In return, the veterinarians stated that the health sector exaggerated the seriousness of the problem. A representative sampling of people and their livestock then showed the current disease situation. In the aftermath of this survey, the health and veterinary sectors exchanged information more regularly (Zinsstag et al., 2009). Simultaneous surveys in people and their animals allow the recognition of the most important animal species involved in transmission to people (Schelling et al., 2003). In addition, joint studies in the human, animal and wildlife populations can provide the needed evidence for authorities to start joint preparedness and surveillance planning (Kuehn, 2006). However, in reality public health and veterinary governmental authorities often only start cooperating when faced with an outbreak of a disease.

Attempts to assess burdens of diseases in terms of DALYs for neglected zoonoses are hampered by missing knowledge from underreporting, economics and also epidemiology of the diseases. Several research groups work on new diagnostic tools for zoonoses because improved tools are essential for correct diagnosis, to estimate underreporting of zoonoses and to monitor control activities. Efforts go towards developing tests that provide an immediate result, which professionals need to make an appropriate decision, for example on treatment of a patient. These tests are called point of care/bed-side tests for people and pen-side tests for livestock. For example for brucellosis, bed-side and pen-side tests would allow brucellosis to be differentiated from other illnesses causing fever and thus to treat a patient with the recommended antibiotics, as well as to monitor control activities in livestock. Surveys are often needed to obtain an overview on the situation of a zoonosis in a country. Where there is no sampling frame with lists of sampling units, a multi-stage cluster sampling approach can lead to a representative sample. Costs are high for any survey. Therefore, alternative approaches for obtaining accurate estimates are explored such as ecological niche factor analysis and risk mapping.

A good example of an innovative approach is described by Cleaveland et al. (2002). To estimate human rabies deaths (where active detection of human often is too costly because the incidence is very low and passive surveillance of suspect dogs is insufficient [Kitala et al., 2000]), they have surveyed animal bites that are rather frequent. With a probability decision tree using information on bite location and follow-up of bites from suspected rabid animals, they could estimate human deaths. After validation of the approach in field studies, they could show that in rural Tanzania the true incidence of human rabies is ten to 100 times higher than reported.

Costs of uncontrolled zoonoses can be demonstrated in monetary terms for the livestock sector (reduced productivity and market losses), the public health sector (diagnosis, treatment, hospitalisation costs) and the private sector (patient or animal owner out-of-pocket

expenditures, opportunity costs). Where benefits from control within different sectors have been estimated, the cost could be distributed proportionally to the sectors according to their monetary benefit from control. When this comprehensive cross-sectoral analysis was applied, control interventions emerge as highly cost-effective for the public health sector i.e. US$ 25 or less per DALY averted (Roth et al., 2003; Budke et al., 2005; Knobel et al., 2005). Control of cysticercosis is one example of a neglected zoonosis that will likely benefit from a framework that assesses the impact of zoonoses across different sectors (Praet et al., 2009).

Research should also be directed towards demonstrating the additional cost-effectiveness of integrated control of multiple zoonoses compared with efforts focused on individual diseases. This would foster integrated services and control within the human health sector. For example, integrating vaccination of children together with distributing vitamin A, selling of insecticide-treated mosquito nets, de-worming and malaria treatments are encouraged by WHO and UNICEF because serving people with multiple interventions is more equitable and sustainable. Although prior evaluation of the cost-effectiveness of combined approaches provides the needed data for prioritising interventions (Grabowsky et al., 2005), there are so far no good examples of implementing a zoonoses control programme with funding from different sectors.

To achieve such cost-sharing between sectors, decision makers from relevant ministries such as the Ministry of Finances should clearly be involved early in the process of outlining financial flows.

However, health services for humans and animals are provided separately. Joint health service provision and information dissemination should nevertheless be considered since sharing of infrastructure, cold chain and personnel may widen the radius of operation of health and veterinary services in rural zones. McCorkle (1996) argues that an intersectoral approach combined with traditional, non-Western patterns for the joint delivery of basic healthcare services to both humans and animals would be more appropriate and feasible than imposing Western medical approaches alone.

CONCLUSIONS

Zoonoses are among the most important animal and public health problems that affect the well-being of societies worldwide, yet they are too often forgotten or neglected. Since most zoonoses go unrecorded, a rethinking of research and control efforts and the economic consequences is required. Despite the challenges to capture the momentum of partnerships between sectors, building on established national and regional technical support agencies for avian influenza will be important to establish permanent dialogue between sectors and to create sustainable national and regional bodies applying the 'One Heatlh' concept. A more widely, applied 'One Health' research agenda fosters South-South and South-North collaborations, which, in turn, accelerates exchange on lessons learned in different parts of the world. International bodies like the OIE , FAO and the WHO have joined forces to establish global standards for zoonosis surveillance and control, which is very encouraging.

New collaborations, alliances and institutional arrangements can support countries to identify where they can add value to health programmes by strengthened cooperation between different sectors. Public services will certainly continue to play an important role in zoonosis control and service delivery, but conventional strategies are increasingly complemented and, in some cases, replaced by contracting with private operators and by public - private initiatives (also between resource-poor and industrialised countries).

Regional and international research groups with a 'One Health' approach such as the Foodborne Disease Burden Epidemiology Reference Group (FERG), the Cysticercosis Working Group in Eastern and Southern Africa, and the EU-funded research consortium on Integrated Control of Neglected Zoonoses in Africa (ICONZ) are well placed to generate the needed data on levels of underreporting, DALY estimates of zoonoses and the epidemiological and financial information needed for analyses of costs and benefits and cost-effectiveness and thereby identify the most appropriate option for zoonoses prevention and control in particular settings.

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Integrating Results of Laboratory Surveillance of Human Illness and Monitoring of Animals and Foods

D.M.A. Lo Fo Wong[1]*

ABSTRACT

Cost-efficient monitoring of food contamination and surveillance of foodborne diseases requires a coordinated multidisciplinary approach with the participation of stakeholders from all sectors of the 'farm-to-consumption' continuum including the public health sector. To facilitate communication and coordination, establishment of a coordinating body with the participation of relevant stakeholders is recommended. Furthermore, relevant surveillance data from all stages in the food production chain and from the surveillance of human disease should be continuously collected and analysed to evaluate trends and sources of foodborne disease. The establishment of a dedicated multidisciplinary surveillance unit involving epidemiological and microbiological expertise from all sectors can facilitate this type of coherent data analysis and feed back. Systems such as these can be operated at the national, regional and global levels.

Key words: *food safety, foodborne diseases, surveillance, communication, coordination, multidisciplinary.*

INTRODUCTION

Food safety is the assurance that food will not cause harm to the consumer when it is prepared and/or eaten. The provision of this assurance covers an incredibly complex area of roles and responsibilities. It crosses multiple sectors of government, including Ministries of Health, Agriculture and Trade, and requires the involvement of multiple professional disciplines and a broad array of stakeholders. An effective food safety system, national and international, requires the sharing of information and expertise in order to face the global nature of modern food safety issues.

The contamination of food is a worldwide public health concern and a leading cause of trade issues internationally. Food contamination by chemical hazards may occur through environmental pollution of the air, water and soil, e.g. with toxic metals, polychlorinated biphenols (PCBs) and dioxins, or through the intentional use of various chemicals, such as pesticides, animal drugs and other agrochemicals. However, this paper will not address specific issues related to trade or chemical food contamination, but focus mainly on the control of foodborne pathogens and disease throughout the food production chain.

1 Food Safety and Zoonoses, World Health Organization, Switzerland

* Corresponding author: lofowongd@who.int

Microbiological food contamination and subsequent transmission can occur at any point of the food production chain, from livestock feed, via the on-farm production site, at the slaughterhouse or packing plant, in manufacturing, processing and retailing of food, through catering and home preparation. Each link in the chain is responsible for the quality and safety of its products. Since there are numerous possible routes for transmission of contamination throughout production, isolated actions (e.g. decontamination of animal feed) will in most cases not ensure lasting consumer protection. Therefore, control measures against the introduction and transmission of foodborne pathogens should be considered at all levels of production.

CONTROL OF FOODBORNE DISEASES

In order to control foodborne disease, it is important to have knowledge of the potential source(s) of food contamination hazards and the most important route(s) of transmission. Once some basic knowledge of the origin and modes of spread of contamination is present, efficient control strategies can be devised and the burden on human and animal health decreased.

Professionals and technical staff working in veterinary, food and public health disciplines have a number of tools available that rely on each other. There are diagnostic tools that can help detect and distinguish foodborne pathogens, chemicals, toxins or other types of residues. There are epidemiological methods to analyse these data and expose association between factors and disease. There are mathematical modelling tools to help understand the complexity of food safety issues and how factors inter-relate. All these technical tools together provide the basis for informed decision support to risk managers and decision makers.

When results obtained from these tools are combined intelligently, food safety and public health systems can be transformed from a reactive to a proactive approach. This means that when signals of contamination early in the chain are communicated timely and accurately, the food safety system can expand from outbreak investigation and trace-back to include predicting forward, and from control to prevention of food safety events. Regular and open communication between all major stakeholders in food safety is imperative for the successful integration and coordination of all food safety efforts.

MAJOR STAKEHOLDERS IN FOOD SAFETY

The food industry is responsible for the quality and the safety of its products and is therefore a major stakeholder in food safety. Production may be monitored through, for example, certification programmes, process control schemes or HACCP (Hazard Analysis

N.E. Odongo, M. Garcia & G.J. Viljoen (eds), Sustainable Improvement of Animal Production and Health. Food and Agriculture Organization of the United Nations, Rome, 2010: **349–352**

Critical Control Points) based control programmes. These control activities generate data that can constitute an important contribution to national surveillance programmes. During outbreak investigations, additional sampling may be required to trace-back human illness to the point of contamination in the food-production chain. Close cooperation between the private and public sectors is therefore imperative.

In general, the main stakeholders in food safety representing the government are the Ministries of Health and the Ministries of Agriculture/Food, but may also include Ministries of the Environment, Trade/Commerce/Industry, Consumers and others. Under them, are agencies that are responsible for the legislative, technical and practical implementation of food safety programmes, and each agency often has a dedicated reference laboratory associated with it. The access to surveillance data often goes through these laboratories. These governmental organisation structures often run independent of each other. In order to get a comprehensive view of the national food safety status, the Ministries and their respective agencies and reference laboratories should work closely together.

Finally, other stakeholders of food safety are the non-governmental organisations. They may represent consumer groups, food industry workers or environmentalists. Although these organisations seldom are directly involved in the generation of data, they can influence the launching of food safety initiatives and serve as a driving force behind initiation of surveillance efforts.

The main challenge is to develop structures that ensure the systematic collection, collation, analysis and interpretation of surveillance data and communication to all public and private stakeholders involved (**Figure 1**). Such a structure relies on the acknowledgement of the major stakeholders that they all play an important role in food safety and that they all gain from this collaboration. For this purpose, one or more coordinating bodies or steering committees with representatives of all stakeholders may be formed. The integration of all surveillance data from farm-to-consumption in a coherent analysis and subsequent interpretation may be the task of a specialized multidisciplinary research unit, which reports to the relevant coordinating bodies or steering committees. The evaluation by these committees can then lead to coordinated efforts of prevention and control.

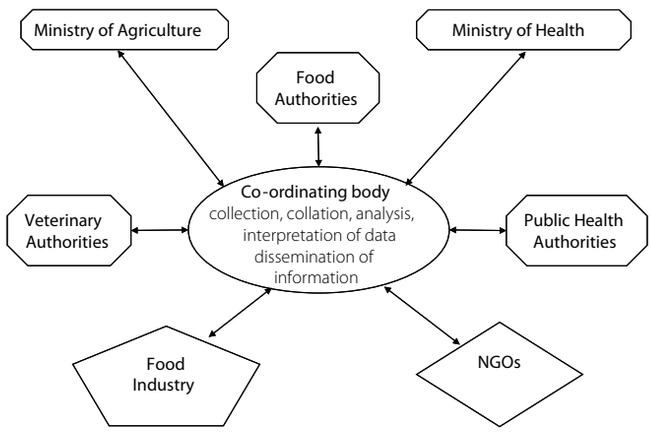

Figure 1. Schematic presentation of the collection, collation, analysis and interpretation of surveillance data and the subsequent dissemination of information to major stakeholders in food safety.

INTEGRATION OF FOODBORNE PATHOGENS AND DISEASE SURVEILLANCE

Integration of surveillance activities at the national level facilitates optimisation and cost efficiency in the generation and utilisation of surveillance data. The challenge is to optimise the sensitivity of the surveillance system while minimising the costs. For example:

- Integration of surveillance components within and between links of a production chain, e.g. to investigate possible associations between the levels of food-borne pathogens in food animals and in food products at retail;
- Integration of different surveillance programmes of the same production animal, e.g. using the same serum samples for the detection of antibodies against both *Salmonella* and porcine reproductive and respiratory syndrome (PRRS);
- Integration of different surveillance programmes for different production animals, e.g. to estimate the relative contribution of the main reservoirs to the total number of human cases of foodborne illness;
- Integration of national surveillance programmes to rapidly recognise and report international outbreaks, a so-called network of networks, e.g. PulseNet International, OzFoodNet, and the Global Foodborne Infections Network (GFN), formerly known as WHO Global Salm-Surv;
- Integration of surveillance programmes run by international organisations, such as the Global Early Warning System for Major Animal Diseases, including Zoonoses (GLEWS), a joint system that builds on alert mechanisms of the World Health Organization (WHO), the Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) and the International Food Safety Authorities Network (INFOSAN) of FAO and WHO.

The integration of foodborne pathogens and disease surveillance activities can be achieved through: i) communication, ii) collaboration, iii) coordination and iv) central collection, collation, analysis and interpretation of data. Communication between major stakeholders can be maintained during regular meetings and direct, informal contact between veterinary and public health workers in key positions. Collaboration consists mainly of the routine exchange of data and participation in outbreak investigation and response. Control activities and the sharing of information need to be coordinated, within and between programmes. Managing a central database containing all surveillance data or linking databases which contain complementary data allows for coherent analyses of the relation between foodborne pathogen reservoirs and disease in time and space. These four components ensure the optimal use of data that are already being generated.

SALMONELLA SURVEILLANCE IN DENMARK: AN EXAMPLE OF AN INTEGRATED APPROACH

In Denmark, the successful implementation of a number of surveillance and control programmes can be accredited to the close cooperation between the public sector and private industry (Wegener et al., 2003). The authorities have delegated the responsibility for technical coordination of the programmes to committees with representatives from the industry, government bodies and science. In the planning and implementation of programmes, there has been a close involvement of microbiologists and epidemiologists. In addition, there is a very close collaboration between medical and veterinary epidemiologists and microbiologists in assessing the effect of the programmes on the incidence of human infection.

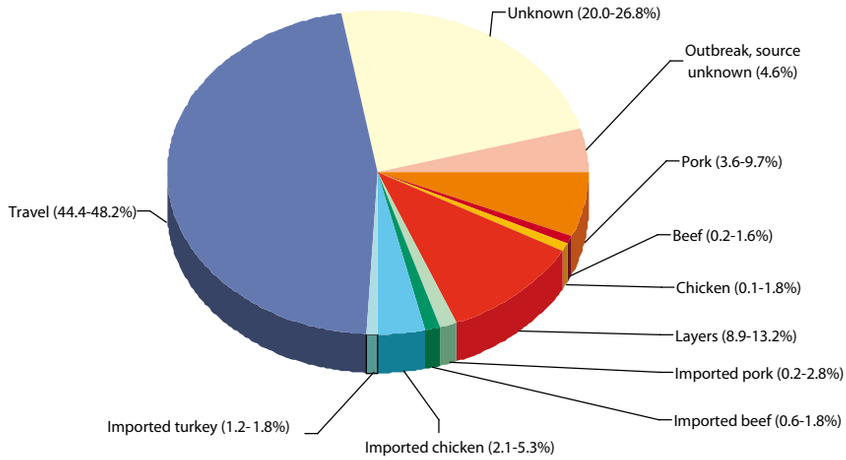

Figure 2. Estimated sources of 1 647 registered cases of human salmonellosis in Denmark, 2007. Source Danish Zoonosis Centre.

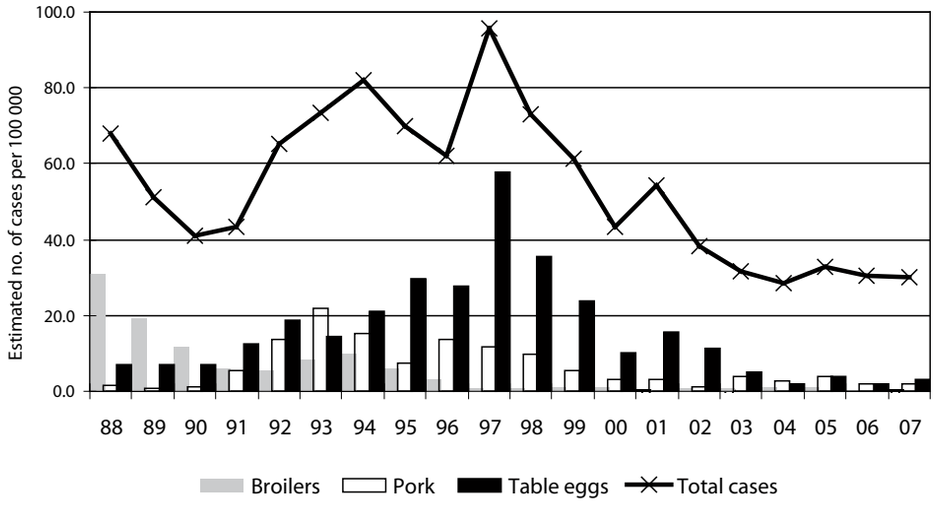

Figure 3. Trends and sources of human salmonellosis in Denmark, 1988 to 2007. Source: Danish Zoonosis Centre.

To initiate and generate the basis for targeted action, The Danish Zoonosis Centre was established in January 1994. The Zoonosis Centre is an epidemiological surveillance and research unit hosted by the National Food Institute, Technical University of Denmark. As the national coordinating body for food safety and zoonosis, the Zoonosis Centre collects all data from all national surveillance and control programmes on zoonoses and conducts an ongoing analysis of the national zoonosis situation from farm-to-consumption, including the identification of outbreaks, the assessment of sources of human foodborne disease as well as basic epidemiological research. An overview of the data is published and commented on in hard copy (Anonymous, 2009) and on the internet (http://www.food.dtu.dk/Default.aspx?ID=9606). The report includes an annual account of major sources of foodborne salmonellosis based on surveillance (**Figure 2**), as well as an overview of the trends in the estimated attribution of these sources to human infection since 1988 (**Figure 3**). The principle of the attribution method is to compare the number of human cases caused by different *Salmonella* subtypes with the distribution of the same subtypes in the various animal and food sources (Hald et al., 2004).

Figure 3 shows that Denmark experienced three major waves of human salmonellosis incidence between 1988 and 2007 that were mainly associated with broilers, pork and eggs, respectively. Following the introduction of a control programme in broiler production in 1988, the broiler-associated salmonellosis incidence (cases/100 000) has been reduced from 30.8 in 1988 to 0.2 in 2007; following the implementation of a control programme in pig herds in 1993, the pork-associated salmonellosis incidence has been reduced from 22.0 in 1993 to 2.0 in 2007; and the egg-associated salmonellosis incidence has been reduced after implementation of a control programme in laying hen production in 1997, from 57.7 in 1997 to 3.3 in 2007. By combining data from animal, food and human sources in a comprehensive analysis, the attribution method has proven to be a useful tool to support the Danish risk managers in their decision to

implement new intervention strategies and evaluate their impact on public health (Anonymous, 2009).

The success of the Danish control programme which has reduced the reported human salmonellosis incidence from 95.6 in 1997 to 31.5 in 2003 has been an inspiration for many countries. However, the incidence has remained at approximately 30/100 000 since then. The risk management options to further reduce the human incidence are less clear. Additional information may be needed to identify sources that are currently not being monitored, including abroad, and implement new prevention measures to decrease the human incidence further.

Notwithstanding this, the recognition of the central role, expertise and leadership displayed by the Danish Zoonosis Centre, has led to its nomination as the Danish reference laboratory for zoonosis epidemiology in EU (NRL–5), WHO Collaborating Centre for Antimicrobial Resistance in Foodborne Pathogens and Zoonosis Collaboration Centre (ZCC) of the European Food Safety Authority (EFSA) with the main task of preparing the Community Summary Report on trends and sources of zoonoses, zoonotic agents and antimicrobial resistance in the European Union (e.g. EFSA, 2009).

An increasing number of other countries have in recent years established similar or related structures to integrate surveillance efforts and facilitate communication and coordination. These countries include Sweden, Norway, Finland, UK, Ireland, Canada and many more. There is also a growing interest of countries to adapt existing or develop new methods to attribute human foodborne illness to specific animal and food sources (Batz et al., 2005).

GLOBAL CAPACITY BUILDING FOR INTEGRATED SURVEILLANCE AND CONTROL

There are various national and regional initiatives that aim to build and improve technical capacity of laboratory diagnostics or foodborne outbreak investigation. However, few initiatives focus specifically on the integration of efforts between sectors and disciplines that are involved in food safety and zoonoses.

The WHO Global Foodborne Infections Network (GFN) (formerly known as WHO Global Salm-Surv) is a capacity-building network to detect, control and prevent foodborne and other enteric infections from farm to table. GFN promotes integrated, laboratory-based surveillance and intersectoral collaboration among human health, veterinary and food-related disciplines (www.who.int/gfn). Created in 2000, the programme now has over 1 500 members from more than 700 institutions in 177 countries. GFN has six main programme components: International Training Courses, Global Salmonella Country Databank, the External Quality Assurance System, the Electronic Discussion Group, Focused Regional and National Projects, and Reference Testing Services.

GFN conducts training courses for microbiologists and epidemiologists from veterinary, food and human health disciplines at 17 training sites around the world. The training modules include benchtop training for microbiologists for laboratory testing of a variety of foodborne pathogens, lectures and case studies for epidemiologists as well as joint activities for all course participants such as integrated surveillance, risk assessment and attribution methodology. To date the programme has held over 65 international training courses in Chinese, English, French, Portuguese, Spanish, and Russian for approximately 1 300 microbiologists and epidemiologists from over 120 countries. Also, more than 80 countries have provided data to the Country Databank on over 1.5 million human isolates and close to 400 000 isolates from non-human sources to help provide a global overview of the epidemiology of *Salmonella*. The External Quality Assurance System of GFN is one of the world's largest annual proficiency tests with more than 150 laboratories participating worldwide (Hendriksen et al., 2009). Though originally focusing on *Salmonella* diagnostics and epidemiology, the training programme has evolved into a capacity-building platform that accommodates a variety of foodborne and other enteric pathogens and diseases of importance in the various regions.

CONCLUDING REMARKS

The detection of changes in patterns of foodborne diseases and variations in the contamination in the food production process are an absolute necessity for the monitoring and continuous improvement of food quality and safety. These programmes need to be sensitive, sensible and cost efficient. Food contamination monitoring and foodborne disease surveillance at national level provide a timely and comprehensive overview of the veterinary and public health status of a nation. The integration of foodborne disease surveillance has the goal to gather all national surveillance activities in a common public service that carries out many functions using similar structures, processes and personnel. The infrastructure of an established surveillance programme in one area may serve as a framework for strengthening other surveillance activities. Though some foodborne diseases may have specific information needs requiring specialised systems, there may be the potential for synergy and the sharing of common resources.

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Molecular Characterisation of Human and Animal Fascioliasis in the Americas

M.D. Bargues & S. Mas-Coma[1]*

ABSTRACT

In Latin America, fascioliasis is an important human and animal health problem in many countries. Molecular studies were performed to determine the genetic characteristics of both liver flukes and lymnaeid vectors by combined haplotyping. Molecular markers obtained were the complete sequences of the nuclear ribosomal DNA ITS–1 and ITS–2 and the mitochondrial DNA genes *cox*1 and *nad*1 and the respective amino acid sequences of the proteins COX1 and NAD1. Fasciolid flukes from Latin America showed a surprising homogeneity both at nuclear rDNA and at mtDNA levels. Differences detected when comparing *cox*1 and *nad*1 were so few that bootstrap values obtained in phylogenetic analyses by using one or the other gene independently proved to be insufficient. Significant values in mtDNA were only obtained by combining both genes within the same analyses and when comparing different countries. These results contrast with those obtained through sequencing studies of lymnaeid snails, in which several different species showing vectorial capacity appeared. Almost all vector species proved to belong to the problematic *Galba/Fossaria* group of small-sized lymnaeids, and, with few exceptions, endemic areas had more than one vector species involved in disease transmission. Thus, owing to the intraspecific genetic homogeneity of the fasciolids, the differences in transmission patterns and epidemiological situations may be related to differences in the lymnaeid vector species present in endemic areas. Results obtained open new doors for future molecular research to establish appropriate control measures for endemic areas in different Latin American countries.

Key words: *fascioliasis, lymnaeid snails, genetic analysis, haplotyping, molecular markers.*

INTRODUCTION

Fascioliasis is a zoonotic disease of domestic ruminants caused by liver fluke parasites and transmitted by freshwater lymnaeid snail vectors. This disease is of well-known veterinary importance because of its great pathogenicity and impact in livestock, especially sheep, goats and cattle, but also pigs, buffaloes and donkeys, as well as horses, camelids and other domestic herbivores. Moreover, this disease is today emerging in humans in Europe, Africa, Asia and the Americas, with 51 countries recording human infection. This parasitic disease is caused by the liver fluke species *Fasciola hepatica* and *F. gigantica*, whose geographical distribution differs. *Fasciola hepatica* is present in Europe, Africa, Asia, the Americas and Oceania, whereas *F. gigantica* is only found in Africa and Asia (Mas-Coma and Bargues, 1997; Mas-Coma, 2004). Although being long recognised for its great veterinary importance, fascioliasis by both *F. hepatica* and *F. gigantica* has only recently been shown to be a widespread human health problem (Mas-Coma et al., 1999a,b; Mas-Coma et al., 2005) with severe symptomatology and pathology in both acute and chronic phases (Chen and Mott, 1990; Mas-Coma et al., 1999b, 2000; Valero et al., 2003). Recent estimates suggest that between 2.4 million (Rim et al., 1994) and 17 million people (Hopkins, 1992) are infected and the figure may be even higher considering the lack of data from many countries, mainly of Asia and Africa (Mas-Coma, 2004).

This old disease has a great powers of geographical expansion due to the large colonisation capacities of its fasciolid causal agents and freshwater lymnaeid snail vector species (Mas-Coma et al., 2001), and is at present emerging or re-emerging in many countries, showing both increases in prevalence and geographical expansion (Mas-Coma, 2004). Throughout its wide geographical distribution range, human fascioliasis shows very unique epidemiological characteristics and human endemic areas that range from hypo- to hyperendemic (Mas-Coma et al., 1999a, b). A recent global analysis of these characteristics concluded that fascioliasis was the vector-borne parasitic disease with the widest latitudinal, longitudinal and altitudinal distribution known (Mas-Coma et al., 2003; Mas-Coma, 2004).

In Latin America, large hot spots of disease have been detected in high altitude areas of Argentina, Chile, Bolivia, Peru, Ecuador and Venezuela, with very high prevalences in livestock and humans in endemic areas where transmission and epidemiology follow whether altiplanic-permanent or valley-seasonal patterns related to vectors of the *Galba/Fossaria* group. Other hot spots include Caribbean Islands like Cuba and Central American countries like Mexico where transmission and epidemiology are determined by lymnaeids such as *Lymnaea cubensis* and *Pseudosuccinea columella*, and where animals have very high prevalences and intensities of infection and a hypoendemic situation with periodic epidemics exists in humans (Mas-Coma, 2005; Mas-Coma et al., 2005).

Molecular studies have been performed during several years to determine the genetic characteristics of liver flukes and to classify lymnaeid snail vectors in the New World. Like other vector-borne infectious diseases, the vectors are crucial for establishing the patterns of transmission and epidemiological features of the disease (Bargues and Mas-Coma, 2005). Lymnaeids are freshwater snails which pose great difficulties in classifying specimens, because of their anatomical uniformity and the intraspecific variability of the shell morphology: sometimes even expert malacologists cannot classify

1 Departamento de Parasitologia, Facultad de Farmacia, Universidad de Valencia, Valencia, Spain

* Corresponding author: S.Mas.Coma@uv.es

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specimens correctly (Bargues et al., 2001). In the case of controversial groups, such as *Galba/Fossaria* and *Radix* which include the main vector species of *F. hepatica* and *F. gigantica* respectively, accurate classification of specimens is only possible by DNA marker sequencing (Bargues and Mas-Coma, 2005).

MATERIALS AND METHODS

The specific objectives of the studies reported was to genetically characterise both liver flukes and lymnaeid vectors by combined haplotyping. A total of 141 adult flukes, obtained from animal livers in slaughterhouses of 37 different localities from the endemic countries of Argentina, Bolivia, Chile, Ecuador, Mexico, Peru, Uruguay and Venezuela, were sequenced. Lymnaeid vectors were collected during field activities in human endemic areas of the same countries. For liver flukes, the molecular markers obtained were complete sequences of the nuclear ribosomal DNA (rDNA) internal transcribed spacers ITS–1 and ITS–2, as well as the complete sequences of the mitochondrial DNA (mtDNA) genes of the cytochrome c oxidase subunit I (*cox*1) and NADH dehydrogenase subunit I (*nad*1) and the respective amino acid sequences of the proteins COX1 and NAD1 (Mas-Coma et al., 2009a). Primers used for ITSs were the same as previously used (Mas-Coma et al., 2001 and 2009a); mitochondrial markers were designed on sequences of the complete mitochondrial DNA genome of *F. hepatica* from Australia (Le et al., 2001). For lymnaeid snails, the molecular markers used included the complete sequences rDNA ITS–1 and ITS–2 and mtDNA *cox*1, plus the small subunit or 18S rRNA gene (Bargues et al., 2007).

Sequences were aligned using CLUSTAL-W, homologies assessed using BLAST, genetic distances were measured using PAUP, and pairwise alignments were made with MEGA. Genetic variation was evaluated using DnaSP and a hierarchical analysis of molecular variance (AMOVA) performed using Arlequin. Phylogenies were inferred by maximum-likelihood (ML) using PAUP and PHYML. The evolutionary model was determined using the hierarchical Likelihood Ratio Test (hLRTs) and the Akaike Information Criterion implemented in Modeltest. A median-joining network analysis was performed using Network. Distance-based phylogeny was obtained using the neighbour-joining algorithm with the ML pair-wise distances. Statistical support was evaluated with 1 000 bootstrap replicates. A Bayesian phylogeny was applied to obtain posterior probabilities with MrBayes (Bargues et al., 2007 and 2008).

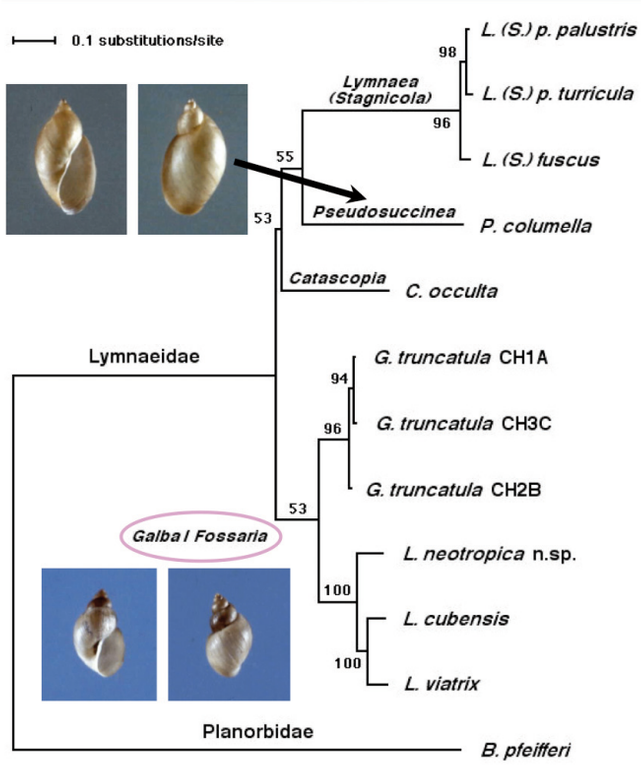

Figure 1. Phylogenetic tree of lymnaeid vector species from Latin America and Europe, based on combined sequences of rDNA ITS–1 and ITS–2, derived from the maximum likelihood (ML HKY85+G) model. Numbers represent the percentage of 1000 puzzling replicates. Scale bar indicates the number of substitutions per sequence position. Modified from Bargues et al. (2007).

RESULTS

DNA Sequencing and Phylogenetic Analyses of Fasciolids

In fasciolids from Latin America, the complete sequence lengths and nucleotide compositions of rDNA ITS–1, ITS–2, and mtDNA *cox*1 and *nad*1 were 432 nucleotides and 51.85% GC content, 364 nucleotides and 48.35% GC content, 1533 nucleotides and 37.24% average GC content, and 903 nucleotides and 34.83% average GC content, respectively. The proteins of the two mtDNA genes were 510 aa long, with start/stop codons of ATG/TAG in all individuals analysed; in the case of COX1, they were 300 aa long, with start/stop codons of GTG/TAG in all individuals analysed, in NAD1.

Fasciolid flukes showed a surprising homogeneity both at nuclear rDNA and at mtDNA levels. No one nucleotide difference appeared in the ITS–1 sequences of the different South American, Central American and Caribbean countries, whereas only one mutation appeared in the ITS–2 sequence in 41.6% of the populations. Similarly, differences detected when comparing *cox*1 and *nad*1 sequences were so few that bootstrap values obtained in phylogenetic analyses by using one or the other gene independently proved to be insufficient. Significant values in mtDNA were only obtained by combining both genes within the same analyses and when comparing different countries.

Interestingly also, when comparing the sequences of both ITS–1 and ITS–2 of all fasciolids of the Americas with corresponding sequences of 'pure' *F. gigantica* from sub-Saharan African countries, the same differences appeared in the polymorphic sites 24, 114, 208, 286 and 306 in ITS–1, and in positions 234, 273, 279, 330 and 337 in ITS–2.

DNA Sequencing and Phylogenetic Analyses of Lymnaeid Vectors

Results obtained in lymnaeid snails contrasted with those obtained in the sequencing studies of fasciolid flukes in that very different sequences were found. These sequences corresponded to several different vector species having the capacity to transmit fascioliasis. Almost all vector species proved to belong to the *Galba/Fossaria* group of small-sized lymnaeids, and, with a few exceptions, endemic areas had more than one vector species involved in disease transmission. The main species found within this problematic group were *Lymnaea viatrix* (= *L. viatrix* var. A ventricosa), *L. cubensis*, *L. neotropica* (= *L. viatrix* var. B elongata) and *Galba truncatula*. The only species

not belonging to the *Galba/Fossaria* group is the widely dispersed, large-sized *Pseudosuccinea columella*.

The phylogenetic analysis with maximum likelihood (HKY85 + G) showed a tree (**Figure 1**) in which the close relationship appears between the American *L. viatrix*, *L. cubensis*, *L. neotropica* and the species *G. truncatula* of European origin. Additionally, *P. columella* , a well-known vector species, appears within the clade of secondary vector species as the stagnicoline lymnaeids, separating the Palaearctic *Catascopia occulta* from the European *L.* (*Stagnicola*) *palustris*, *L.* (*S.*) *turricula* and *L.* (*S.*) *fuscus*.

DISCUSSION AND CONCLUSIONS

Studies on Liver Flukes

The uniformity of American fasciolids with respect to rDNA ITS–1 and ITS–2 and their very low intraspecific variability in nucleotide differences in the two mtDNA genes *cox*1 and *nad*1 are worth emphasising (i) due to its contrast with the high diversity shown by the liver fluke in the Old World linked to its geographical origin and spreading peculiarities in the pre- and posdomestication periods, as verified by the same DNA markers (Mas-Coma et al., 2009a), and also detected in Ireland, The Netherlands and Greece with the mtDNA PCR-RFLP technique (Walker et al., 2007), and (ii) because of the problem they pose for future analyses if only fragments of the two aforementioned mtDNA genes are used (e.g., Hashimoto et al., 1997; Itagaki et al., 1998) (**Figure 2**). Results obtained with liver flukes in American countries using the complete sequences of the *cox*1 and *nad*1 genes show that mutations in the Americas are so few that phylogenetic analyses are not able to furnish significant results when using each mtDNA gene independently. Significant results were only obtained when combining both complete gene sequences and only when comparing flukes from different countries. This means that (i) the uncomplete sequences of the short fragments of these two mtDNA genes that have been always used until now (**Figure 2**) are useless in the Americas, and that (ii) even when combined, the complete sequences of these two mtDNA genes are also useless for analysing disease transmission within a specified endemic area. This suggests that DNA markers evolving much faster than *cox*1 and *nad*1 or other molecular methods involving banding of larger sequences (e.g. RAPDs and microsatellites) will be needed to analyse disease transmission.

A positive conclusion can, however, be deduced from the homogeneity of the liver flukes in the Americas, namely the simplification of both field epidemiological studies and disease control activities (Mas-Coma et al., 2009a). This picture appears to be different from the one known in the Old World, where different epidemiological scenarios and transmission patterns are known (Mas-Coma et al., 1999a, b; Mas-Coma, 2005). In the Americas, nevertheless, the possibility that climate change has different effects on fascioliasis transmission and epidemiology in different areas such as Andean countries and Caribbean Islands cannot be overlooked (Mas-Coma et al., 2008 and 2009b).

Among the several conclusions which can be reached from this analysis of fascioliasis in Latin American endemic countries, the following shall be emphasised because of their applied interest. The homogeneity of liver flukes in the Americas suggests that domestic livestock trade between different countries is important in disease spread. For the same reason, no important clinical and pathogenic differences are to be expected in different endemic areas, except those possibly linked to breed or ethnic group-related differences in susceptibility. Likewise, the susceptibility of flukes to treatments and their capacity to give rise to resistance may be expected to be uniform throughout. The later constitutes a real problem due to the high risk of rapid spread of resistance to triclabendazole if it appears one day in the Americas, the drug most used in animals (Fasinex®) (Fairweather and Boray, 1999; Fairweather, 2005) and the drug of choice of human treatment at present (Egaten®) (Savioli et al., 1999; Mas-Coma et al., 1999b and 2005).

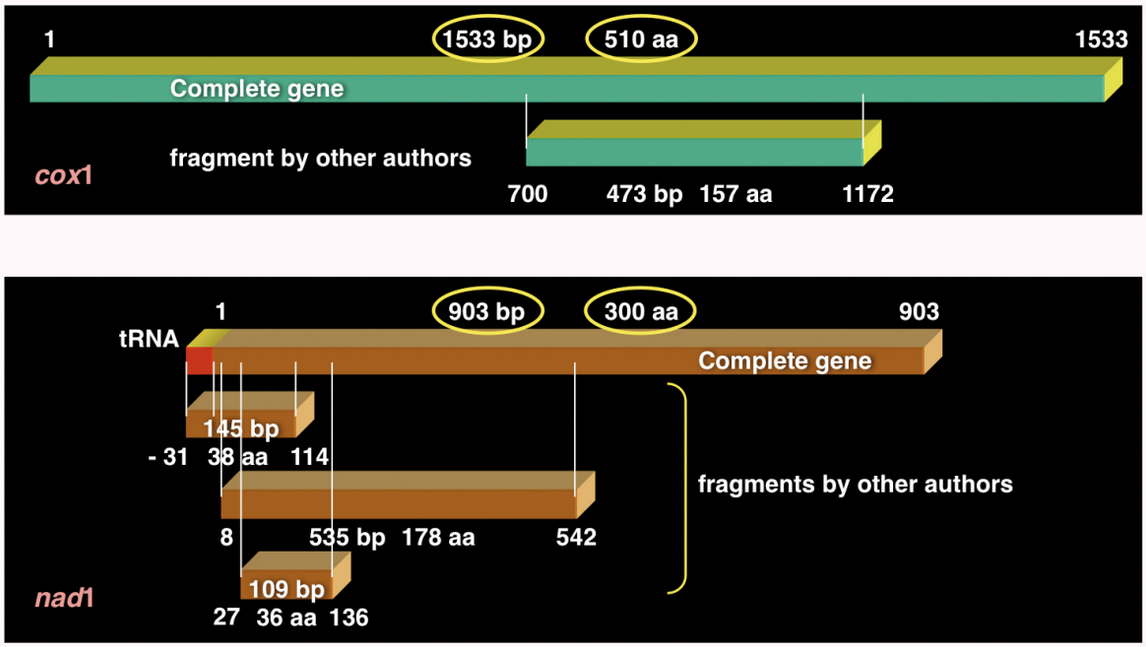

Figure 2. Comparison of the complete sequences of the mtDNA genes *cox*1 and *nad*1 with uncomplete sequences of fragments having been used until now, illustrating nucleotide sequence regions and respective information lost when only fragments are considered.

Studies on Lymnaeid Vectors

The first conclusion from the results of the sequencing studies on lymnaeid snails is that compared with Europe, lymnaeid vector species in the Americas are much more complex genetically. In Europe, there is only one main vector species of human and animal fascioliasis, *G. truncatula*, and with stagnicolines as the only secondary lymnaeids able to transmit the disease under specific circumstances (Bargues et al., 2001, 2003 and 2006a). Contrarily, Latin America lymnaeid vectors linked to endemic areas of zoonotic transmission of fascioliasis appear to involve different species of the *Galba/Fossaria* group, including both autochthonous American species and introduced European ones (Bargues et al., 2006b and 2007). The lymnaeids of this problematic group pose considerable classification problems, because the different species look very much alike in terms of both shell morphology and inner anatomy. Moreover, they appear to show similar ecological characteristics which increases the dificulties in their differentiation. Together, these will represent huge challenges for health workers charged with assessing the characteristics of transmission and epidemiology of the disease in different endemic areas and designing appropriate control measures.

Owing to the intraspecific genetic homogeneity of the causal fasciolids, the differences in transmission patterns and epidemiological situations may be related to differences in the lymnaeid vector species present in endemic areas. Indeed, results described here fully agree with previous assumptions about transmission patterns in human endemic areas and which, in the Americas, can be distinguished as follows according to analyses from field studies (Mas-Coma, 2005):

- a very high altitude pattern related to only *F. hepatica* transmitted by imported *Galba truncatula* in Andean countries following transmission throughout the year; within this category, two subpatterns may be distinguished according to physiographic and seasonal characteristics:
  - — the altiplanic pattern, with transmission throughout the whole year (e.g. in the northern Bolivian Altiplano and the Puno Altiplano);
  - — the valley pattern, with seasonality and with prevalences and intensities related to altitude (e.g. in the Peruvian valleys of Cajamarca and Mantaro);
- a Caribbean insular pattern, with reduced but repeated outbreaks in human hypoendemic areas and lymnaeid species other than the main vector species being involved in transmission (e.g. the Pinar del Rio Province in Cuba).

Finally, in Latin America, knowledge of lymnaeid vectors is required for control initiatives and the results presented here open new doors for future, crucial molecular research as a means to establish the appropriate control measures for endemic areas of the different Latin American countries.

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Climatic Characteristics of Areas with Lymnaeid Snails in Fascioliasis Endemic Areas of Mendoza Province, Argentina

P.F. Cuervo[1]*, R.L. Mera y Sierra[1,2], E. Deis[1] & L. Sidoti[1]

ABSTRACT

Fascioliasis is a zoonotic trematodiasis which is both emerging and spreading all over the world, with important human endemic areas in South America. Its prevalence in Argentina, and particularly in Mendoza Province, appear to be high. This study was designed to characterise the main climatic conditions of sites in endemic areas of fascioliasis where freshwater snails of the Lymnaeidae family (the intermediate vectors of Fasciola hepatica) are present This was done by analysing the sites by digital climatic analysis using DIVA-GIS 5.2 software, coupled with information gained through earlier research. Temperature showed a small dispersion among sites, possibly indicating that temperature may have a greater influence on the distribution of lymnaeids than precipitation. Also there was convergence in the dispersion graphic between the values for 'minimum temperature of the coldest month' and 'precipitation of the driest month', showing that these aspects could be considered as limitations to the snails' survival. It is concluded that lymnaeid snails have great adaptability and survival capacities, enabling them to colonise and survive in extreme and diverse environments such as the high altitudes of the Andes and the arid plains of central Mendoza Province. The impact of global climate change should not be overlooked as a factor enhancing vector spread.

Key words: *fascioliasis, lymnaeid snails, climatic analysis, adaptability and survival.*

INTRODUCTION

Fascioliasis is a zoonotic trematodiasis caused by the liver flukes *Fasciola hepatica* and *F. gigantica*, which are transmitted by freshwater snails of the family *Lymnaeidae*. Definitive hosts of fasciolid flukes are mainly herbivorous ruminants, especially livestock species such as sheep, cattle and goats. In the New World fascioliasis is only caused by *F. hepatica* (Mas-Coma, these proceedings). Its high pathogenicity causes substantial economic losses in animal production throughout all countries of South America (Torgerson and Claxton, 1999). From a public health standpoint, the disease has become increasingly important over the last two decades, affecting an estimated 17 million people worldwide. In fact, human infections have been noted in most South American countries (Mas-Coma et al., 1999a and 2005), with important human endemic areas being in Andean areas of countries such as Chile, Bolivia and Peru (Mas-Coma et al., 1999b; Apt et al., 1993; Esteban et al., 2002).

As in all other vector-borne diseases, the transmitting freshwater snails (family *Lymnaeidae*) are crucial in determining epidemiological and transmission patterns of the disease, with snail host population dynamics being related to disease intensities, distribution and transmission. Lymnaeid snail population dynamics, like those of other snails are profoundly affected by climatic factors such as air temperature, rainfall and/or potential evapotranspiration.

Rainfall is known to be crucial in fascioliasis transmission, as the appearance of temporary fresh water bodies or the widening of permanent ones offer lymnaeid snails the necessary habitats for their fast evolving populations (Mas-Coma et al., 2009). Thus rainfall is crucial in regions such as the Argentinian latitudes, where the weather characteristics follow a marked seasonality. Snail population dynamics are also highly dependent on temperature. Climate change is therefore likely to affect the distribution and survival of intermediate hosts like lymnaeid snails as well as influence the rates of reproduction and maturation of the parasites carried by them (Mas-Coma et al., 2008).

The prevalence of fascioliasis in the Mendoza Province of Argentina appears to be very high. Most positive animals (76%) from a total of 258 infected cattle came from Andean valleys in the localities of Las Heras, Malargüe, San Carlos, Tunuyán and Tupungato (Mera et al., 2005, 2006; Gonzalez et al., 2006).

Regarding the lymnaeid snails present in the Province, the species of most concern is *Galba truncatula* (the most effective vector known to date); it has recently been characterised by DNA sequence comparisons in affected areas (Bargues et al., 2006 and 2007). Considering the above, the aim of this study was to characterise the climate of sites where snails of the Gastropoda: *Lymnaeidae* family are present in endemic areas of fascioliasis; as well as to identify some probable limiting climatic factors. The information generated thereby could be important for assessing risk areas and control measures, as well as for predicting the future impact of the disease in the event of climate change.

1 Cátedra de Parasitología y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias, Universidad J.A. Maza, San José, Guaymallén, Mendoza, Argentina
2 Área de Infectología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina

* Corresponding author: pablofcuervo@hotmail.com

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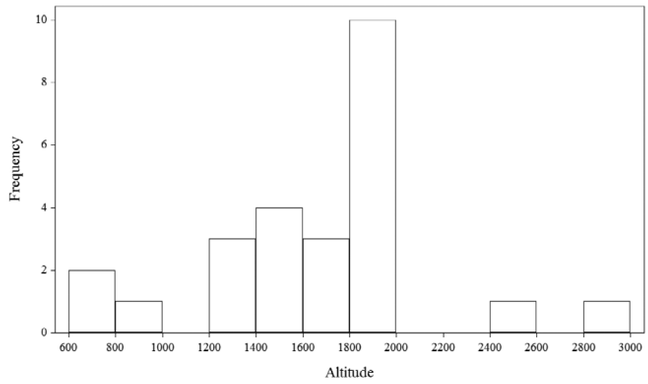

Figure 1. Altitudinal groups.

MATERIALS AND METHODS

A digital climatic analysis of fascioliasis endemic areas with confirmed presence of lymnaeid snails was performed by using DIVA-GIS 5.2 software.

The study performed took into account 25 sites where lymnaeids had been sampled during the last five years by continuous field studies covering the most important river basins of the Province, targeting particularly those areas described in previous research as being endemic for livestock fascioliasis (Mera y Sierra et al., 2005 and 2006; Gonzalez et al., 2006). Coordinates were registered with a standard GPS (Garmin Vista Cx®).

The altitudes of the sampling sites were represented in a histogram (Statistix 7.0), in order to obtain a sampling distribution by altitudinal groups. By using the DIVA-GIS 5.2 software and WorldClim climate data (WorldClim 1.4, 2.5 min resolution climatic layers, Hijmans et al., 2005), digital climatic information was obtained for every site. WorldClim provides monthly maximum and minimum temperatures and monthly precipitation, as well as 19 derived bioclimatic variables. This information was analysed by altitudinal groups with descriptive statistics (Statistix 7.0). A combined dispersion graphic (provided by DIVA-GIS 5.2 — Outliers Graphic, on Ecological Niche Modeling) was developed for the 19 bioclimatic variables, representing every site.

RESULTS

A histogram enabled separating the samplings into three altitudinal groups for the purposes of analysing the information. Group 1: 600 –1000 m above sea level (m.a.s.l.), with three sites (3/25); Group 2: 1200–2000 m.a.s.l., with 20 sites (20/25); Group 3: 2400–3000 m.a.s.l., with two sites (2/25) (**Figure 1**).

The main climatic characteristics of each group were as follows:

- Group 1 (**Table 1** and **Figure 2**): annual mean temperature (15.6°C), maximun temperature in the warmest month (31.5°C); minimun temperature in the coldest month (0.03°C), mean temperature of wettest and driest quarters (22.1°C and 8.2°C), precipitation in wettest and driest quarters (105.3 and 20 mm), and precipitation in warmest and coldest quarters (102.7 and 20 mm).
- Group 2: annual mean temperature (7.41 °C), maximun temperature in the warmest month (22.46°C); minimun temperature in the coldest month (–5.36°C), mean temperature of wettest and driest quarters (3.54 and 11.03°C), precipitation in wettest and driest quarters (153.90 and 51.95 mm), and precipitation in warmest and coldest quarters (58.65 and 144.85 mm) (**Table 1** and **Figure 2**).

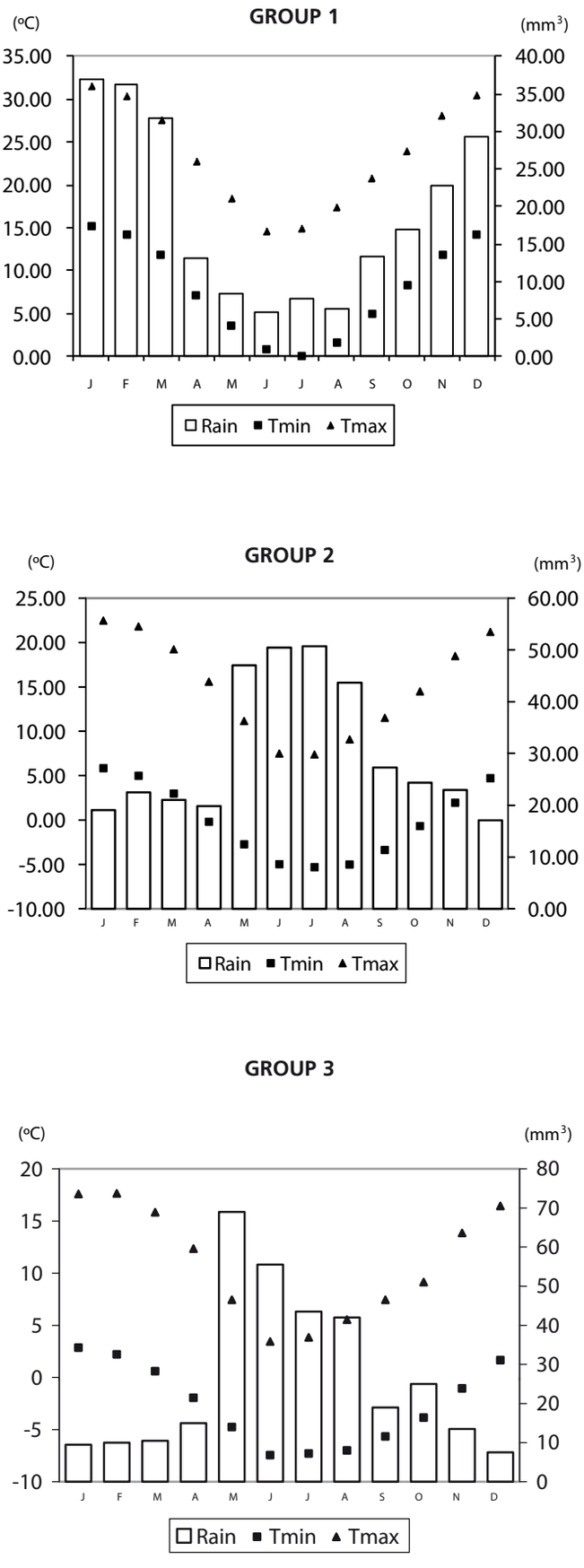

Figure 2. Mean temperatures and monthly precipitation.

Table 1. Climate conditions during the period of the study.

Bioclimatic Variable	Group 1		Group 2		Group 3	
	Mean	s.d.	Mean	s.d.	Mean	s.d.
Annual mean temperature	15.60	0.721	7.41	4.417	4.15	0.78
Mean monthly temperature range	15.60	0.265	15.13	0.615	13.50	0.57
Isothermality (*100)	49.50	0.755	54.45	1.504	53.80	0.57
Temperature seasonality (STD *100)	589.30	16.076	489.27	36.016	461.25	11.38
Max temperature of warmest month	31.53	0.709	22.46	5.010	17.70	1.13
Min temperature of coldest month	0.03	0.416	–5.36	3.810	–7.45	0.35
Temperature annual range	31.50	0.436	27.82	1.635	25.15	0.78
Mean temperature of wettest quarter	22.07	1.361	3.54	6.529	–0.80	0.57
Mean temperature of driest quarter	8.20	0.529	11.03	4.575	9.70	0.99
Mean temperature of warmest quarter	22.63	0.874	13.49	4.803	9.70	0.99
Mean temperature of coldest quarter	8.20	0.529	1.44	4.100	–1.50	0.57
Annual precipitation	228.67	20.984	365.95	122.392	320.00	26.87
Precipitation of wettest month	37.33	0.577	57.40	26.428	69.00	18.38
Precipitation of driest month	5.67	1.528	15.05	4.097	7.50	4.95
Precipitation seasonality	63.10	9.968	45.82	18.333	76.65	27.08
Precipitation of wettest quarter	105.33	4.041	153.90	72.424	168.00	45.25
Precipitation of driest quarter	20.00	5.196	51.95	13.256	27.00	16.97
Precipitation of warmest quarter	102.67	4.509	58.65	10.956	27.00	16.97
Precipitation of coldest quarter	20.00	5.196	144.85	76.024	141.00	36.77

- Group 3 (**Table 1** and **Figure 2**): annual mean temperature (4.2 °C), maximun temperature in the warmest month (17.7 °C); minimun temperature in the coldest month (–7.5 °C), mean temperature of wettest and driest quarters (–0.80 °C and 9.70 °C), precipitation in wettest and driest quarters (168 and 27 mm), and precipitation in warmest and coldest quarters (27 and 141 mm).

The combined dispersion graphic showed a small dispersion between temperature-related variables (1–11), but the greatest divergence between precipitation-related ones (12–19). However, convergence between values is specially noticeable for variables 6 (minimun temperature of coldest month) and 14 (precipitation of driest month) (**Figure 5**).

DISCUSSION AND CONCLUSIONS

The small dispersion seen on temperature variables may indicate that at Andean sites temperature may have a more important influence on lymnaeid distribution than precipitation. Also, the convergence noted between minimun temperatures of the coldest month and levels of precipitation in the driest month, suggests that these aspects could be considered as limitations to the survival of snails. In this regard, the minimun temperature of the coldest month may reflect the lowest temperature at which these snails are capable of surviving (with an extreme minimum temperature of –7.7 °C, at 2 971 m.a.s.l.), which is well below water freezing point (the 'ice point'). Also, precipitation during the driest month (5.67 mm^3, in Group 1 sites) reflects the extreme conditions of some of the sites, with great risks of drought. In both situations it is known that these snails may survive by burrowing themselves into soil, while waiting for better external conditions.

It was also noted that at Group 1 sites at lower heights the driest quarter is also the coldest, as rains mainly occur in summer, while in Groups 2 and 3 the situation is quite different, as the main precipitations occur during the coldest quarter in the form of snow. In these places during summer, snails are present on the streams fed by snowmelt.

In light of these results, it is apparent that lymnaeid snails have great adaptibility and survival capacities, enabling them to colonise and survive in extreme and diverse environments such as the high altitudes of the Andes (with a mean minimun temperature in the coldest month of –7.45 °C, in Group 3) or on the arid plains of central Mendoza Province (with precipitation in the driest month of just 5.67 mm^3, in Group 1). Clearly, the impact of global climate change should not be overlooked, as changing climatic conditions may enhance the spread of lymnaeid vectors and the colonisation of new sites, thereby increasing the risk of fascioliasis.

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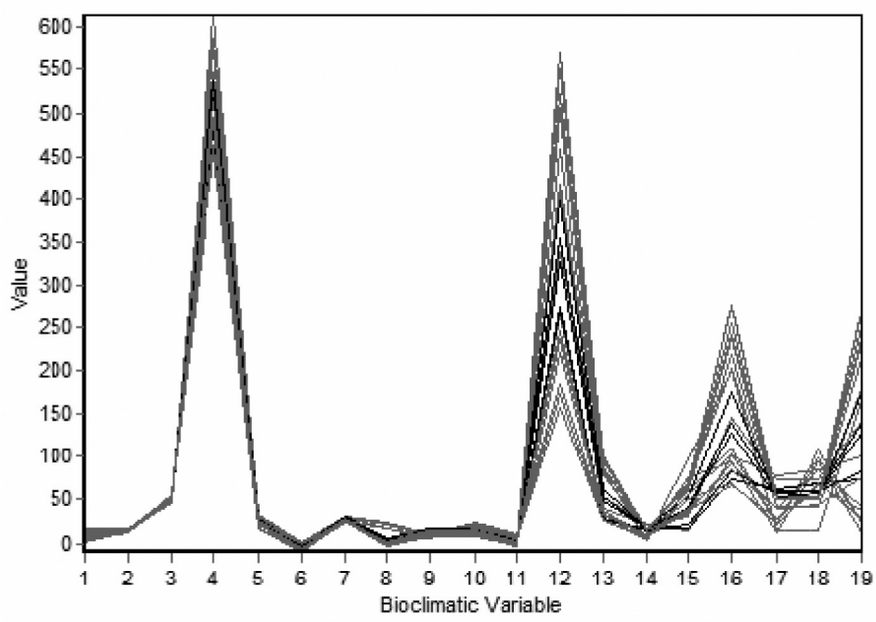

Figure 5. Dispersion graphic showing the 19 bioclimatic variables.

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Prevalence of Rift Valley Fever IgG Antibody in Various Occupational Groups before the 2007 Outbreak in Tanzania

E.S. Swai[1]* & L. Schoonman[2]

ABSTRACT

Rift Valley fever (RVF), caused by RVF virus is a mosquito-borne viral disease that is a significant global threat to humans and livestock. In Tanzania, RVF virus infection in human is not well studied. In this study we aimed to determine the seroprevalence of RVF, assess its zoonotic importance and identify factors associated with seroprevalence. A cross-sectional serological survey of 199 apparently healthy persons from various occupations was carried out in Tanga, Tanzania in November 2004 to determine exposure to RVF virus. Sera were tested for the presence of antibodies to RVF virus by an inhibition enzyme linked immunosorbent assay (ELISA) for detecting immunoglobulin G (IgG). All reactive sera were further tested using an immunoglobulin M (IgM) capture ELISA test for detection of specific RVF virus to determine if there were any instances of recent infection. Eight samples (4%) tested positive for IgG but none of them tested positive for IgM antibodies. Among the occupational groups examined, the seroprevalence was 7.3, 1.5 and 9.5% respectively among 'abattoir workers', 'livestock keepers' and 'other' categories. Seropositivity was higher in males (5.3%) than females (1.5%), with no significant differences among the age groups and sexes. The results indicate that a small proportion of people in Tanga municipality were exposed to RVF virus infection prior to the 2007 disease outbreak in Tanzania. Public health actions for RVF control will need to target not only the occupational groups at risk of infection with severe forms of this disease, but also the general population at large.

Key words: *Epidemiology, sero-prevalence, zoonoses, vector-borne, Tanzania.*

INTRODUCTION

Rift Valley fever (RVF), caused by RVF virus is a mosquito-borne viral disease that is a significant global threat to humans and livestock. Transmission in humans is via direct contact through infected animal products or contaminated foods or aborted foetuses and from the bites of infected mosquitoes, most commonly the *Aedes* species (Corso et al., 2007). Humans infected with RVF virus typically develop a mild self-limited febrile illness, but retinal degeneration, severe encephalitis, fatal hepatitis and haemorrhagic fever may also occur (Swanepoel and Coetzer, 2004). Although few studies have been conducted to asses the economic impact attributable to human RVF, it is thought to be substantial (Meegan, 1981; Davies and Martin, 2003; Davies 2006).

Previous RVF outbreaks in Tanzania, were confined mainly to livestock and mostly affecting northern parts of the country and recorded in 1956, 1978/79 and 1997/98 (Kondela et al., 1985; Woods et al., 2002). The recent re-emergence (in early 2007) of the disease among humans and livestock covering nine different geographical regions, and the fact that RVF virus replicates in a wide range of competent mosquito vectors (Turell et al., 2008) have raised concern that the virus might spread further into non-endemic regions of Tanzania (e.g. Tanga, Coast, Mtwara, Ruvuma, Rukwa, Kigoma and Shinyanga). These threats emphasise the need to have capable surveillance tools and a sound disease control strategy in place. Unpublished, hospital-based reports from the recent outbreak in Tanzania indicate that RVF claimed 144 lives with a corresponding case fatality rate of 46.6% (WHO, 2007).

RVF is known to be endemic in most sub-Saharan countries and in some regions in Tanzania (FAO/UNDP/OAU/IBAR, 2001; FAO, 2002). Between epidemic waves, RVF virus circulates at very low prevalence and without noticeable clinical manifestations in both humans and animals (Davies and Martin, 2003). Much less is known of the prevalence in man and of the effect on human health in this region of the world. This information is important when designing appropriate strategies that would help reduce its prevalence and effects. This inadequacy of data, and the availability of a serum bank comprising samples collected in 2004 prompted the initiation of this study with the aim of establishing past exposure to RVF virus before the 2007 outbreak. The objective of the present study was to estimate the RVF antibody prevalence in various, apparently healthy, occupational risk groups of inhabitants in the Tanga Municipality of Tanzania. The overall purpose was to collect baseline data to enhance understanding of the epidemiology of the disease.

MATERIALS AND METHODS

Study Area

This cross-sectional field study was conducted in Tanga Municipality, Tanga Region, one of eight regional districts in the country. The area is located between latitude 4° 21′ and 6° 14′ S, and longitude 36° 11′ and 38° 26′ E, in northeast Tanzania. It has a human population of around 242 640 growing at 2.9% per annum (URT, 2002). Most rural inhabitants are subsistence farmers, whilst Tanga town, the regional centre, provides many jobs in industry and trade. Tanga Municipality has a hot and humid tropical climate with two rainy

[1] Veterinary Investigation Centre (VIC), Arusha, Tanzania
[2] Tanga Dairy Trust (TADAT), Tanga, Tanzania

* Corresponding author: ESswai@gmail.com

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seasons: an intense one observed during the months of March, April and May, and a mild one occurring in November and December. The mean annual rainfall varies from 500 to 1 400 mm/y. The relative humidity of the day ranges from 60–90% for most of the year. Monthly mean temperatures range typically from 15 °C between June and August and 35 °C between December and March, and the area receives between 2 300 to 3 100 h of sunshine /y. Subjects for the study were selected according to the willingness of individuals to be included in the study frame for testing.

Data Collection

A pre-tested individual questionnaire comprising closed ended questions was used to obtain demographic (age, gender, location) and occupational data (categorised as 'abattoir workers'; 'livestock keepers'; 'non-livestock keepers'; 'veterinary/livestock farmers' and 'other'). The 'other' category comprised people from the general community outside the traditional occupational risk groups i.e. business people, housewives, students, soldiers etc. Other information collected included contact with livestock at home, type of activities in which the individual was engaged, as well as awareness regarding zoonoses including RVF.

Ethical Consideration

During survey visits, interviewers introduced themselves and explained the objectives and all procedures to all potential interviewees participating in the study. A written consent form was obtained from adults or guardians of those individuals aged less than 18 y prior to inclusion. Ethical review and approval were granted by the Human Ethics Committee of the National Institute for Medical Research (NIMR), Dar-es-Salaam, Tanzania. Research clearance was obtained from the Tanzania Commission of Science and Technology.

Laboratory Analysis of Sera

During 2004, a total of 199 human (aged from 14 to 84 y.) serum samples were collected from urban and peri-urban areas surrounding Tanga town. The radius was about 30 km of the town centre. Serum samples from each individual were then stored at -20 ^{0}C until further use. Serodiagnosis of RVF was performed using a Rift Valley fever Inhibition ELISA kit (batch no. 2007/10) obtained from the National Institute for Communicable Disease, Sandringham, South Africa. Results or cut-off values were expressed as percent inhibition (PI) values using the equation: [(100 – (mean net optical density (OD) of test sample/mean net OD of negative control) × 100]. For ease of interpretation, and comparison with other studies, test sera were classified as seropositive if the PI was ≥ 38.6%. RVF IgG positive (past exposure to virus/infection before or during November 2004) samples were tested using a capture ELISA to evaluate the level of anti-RVF IgM antibodies (which reflect recent infection at least one month prior to November 2004) using test kit batch no. 2007/06. Results were expressed as percent positivity (PP) values of optical densities (Paweska et al., 2005), relative to those of a strong positive control serum. Threshold PP values ≥ 7.1% were considered to be positive and values less than this as negative.

Statistical Analysis

Questionnaire and laboratory data were handled and analysed using Epi-info (version 6.04, CDC, Atlanta, USA). The differences in RVF antibody prevalences were compared across the investigated variables using the Mantel-Haenszel chi-square. A value of $P < 0.05$ was considered significant. Biostatistical analysis was performed using Epi-info (CDC, 1996).

Table 1. The proportion of individuals in each category of each variable investigated during the study (n = 199)

Variable	Category	No examined	%	No positive	Prevalence (%)
Occupation group					
	Abattoir	41	20.6	3	7.32
	Livestock farmer	67	33.6	1	1.50
	Non-livestock keepers	38	19.1	0	0
	Veterinary/meat inspectors	11	5.52	0	0
	Other	42	21.1	4	9.5
Sex					
	Female	67	33.7	1	1.50
	Male	132	66.3	7	5.30
Age (y)					
	≤ 20	18	9.04	0	0
	20-30	48	24.12	3	6.25
	30-40	67	33.6	4	5.97
	40-50	41	20.6	0	0
	50-60	14	7.03	0	0
	≥ 60	11	5.52	1	9.09
Total		199	100	8	4.02

RESULTS

Descriptive Statistics

Most of the sampled subjects (n = 156; 78%) were between 20 and 50 y of age, and two-thirds were male and one-third female. The abattoir group consisted of only males. All of the non-livestock farmers group and almost all persons of the 'other' group did not have cattle at home. Non-livestock farmers were those involved in other agricultural activities like growing crops. Some of the abattoir workers (n = 9; 23%) and most of the veterinary staff (n = 1 0; 90%) reported keeping livestock at home and being involved activities related to cattle keeping (**Table 1**).

Presence of Antibodies to RVF Virus Infection

The overall prevalence of anti RVF virus immunoglobulin (IgG) antibody was 4.02% and none had IgM antibodies. The sero-prevalence of anti-RVF virus immunoglobulin (IgG) antibody was higher in males than in females but the difference was not statistically significant (P = 0.34). Seroprevalence increased markedly in males aged between 20 and 40 y (data not shown), while antibodies were not detected in young people under 20 y. Among the occupational groups examined, the seroprevalence was 7.3%, 1.5% and 9.5% in the 'abattoir workers', 'livestock keepers' and 'other' categories, respectively (**Table 1**) although no statistical difference was found between any of these occupational groups (P = 0.086). None of the study participants considered RVF to be a zoonotic disease.

DISCUSSION

There is a paucity of virological and epidemiological information concerning the seroprevalence of RVF infections in the general population and various occupational groups in Tanzania. Overall, 60% of the sera investigated were from people who had contact with cattle, either through cattle keeping at home, or through occupations like working in abattoirs and as veterinary staff, and 40% were from people who had no close contact with cattle, like crop farmers and others. This last group might still have contact with cattle products, like raw meat and milk when preparing or consuming food. Interestingly, the detected seropositive status of the general population before the 2007 outbreaks support the endemicity of this disease in Tanzania (Kondela et al., 1985; FAO/UNDP/OAU/IBAR, 2001; Woods et al., 2002).

The low reactor rate for females in this study could probably be due to the small number of females (n = 67) studied compared with males (n = 132). Recent RVF outbreaks in Tanzania showed that the ratio of male to female mortalities was 1.6:1 and more patients aged 16–60 yrs died than in any other age group (MoHSS, 2007). The higher proportionate infection rate in males than females agrees with the findings of other workers (Nabeth et al., 2001). Traditionally, the majority of abattoir and cattle keeping activities in Tanzania are carried out by men. This may, in part, explain the high prevalence rate detected in males and in abattoir workers.

The overall prevalence of infection in the present study was lower than that reported from other parts of Africa such as a prevalence of 14.8% (Olaleye et al., 1996) in Nigeria, 24.4% (Nabeth et al., 2001) in Mauritania, and 22.3% in Senegal (Wilson et al., 1994). On the other hand, the prevalence in Tanga was higher than found from studies in pregnant women in Mozambique (Niklasson et al., 1987), where a prevalence of 2% was reported. This variability could be attributed to differences in sampling techniques, investigator technical know-how, climate-agro-ecological factors, as well as the diagnostic methods used. The IgG-sandwich ELISA and the IgM-capture ELISA used here, when compared with the virus neutralisation test (sensitivity 96.2% and specificity 95 2%), have sensitivities of 100% and 96.47% respectively and specificities of 99.95% and 99.44% respectively on field sera (Paweska et al., 2005).

Tanga Municipality is characterised by hot, humid and wet climate variables. This is thought to allow a build-up of high densities of mosquitoes of veterinary and medical importance (James, 1979) and may therefore increase the abundance of mosquito species that are potential vectors of RVF virus i.e. *Aedes* and *Culex* (Turell et al., 2007 and 2008). Laboratory-based vector competence and field isolation studies carried out with selected African mosquitoes suggest *Culex zombaensis*, *Culex poicilipes*, *Culex pipiens* and *Aedes caspius* to be potential vectors for RVF virus transmission (Meegan et al., 1980; Diallo et al., 2005; Turell et al., 2007 and 2008). Although *Aedes* and *Culex* species are known to be prevalent in Tanga, their role in transmission of RVF warrants further investigation (Mboera et al., 2000; Magesa et al., 2006).

A number of studies, (Wilson et al., 1994; Elfadil et al., 2006; Turell et al., 2008), have shown that the presence of, and exposure to bites from a wide range of mosquito vectors e.g. *Aedes* spp. was associated with an increased likelihood of the presence of antibodies to RVF virus in the human population. This would be consistent with RVF virus being transmitted by mosquito vectors in addition to any contact that individuals might have with infected animal parts (tissue, blood, urine, uterine fluid etc) during slaughtering (Chevalier et al., 2004; Gerdes, 2004) when assisting with the delivery of newborn animals, or iatrogenically by the use of contaminated needles during mass vaccination (FAO, 2002).

The finding that none of the participants was aware of RVF as a zoonotic disease is striking, and this is a reflection of the poor knowledge of zoonoses by livestock keepers, veterinary field staff and staff in the heath facilities coupled perhaps with the inter-epizootic nature of disease occurrence. The limited knowledge level recorded in this study may also be due to the general lack of data on RVF and inadequate communication between veterinary and human healthcare professionals. This shows also that emergency preparedness for RVF epidemics is low. Furthermore, low awareness is likely to expose people to increased risk of contracting RVF since they might not take proper precautions i.e. use protective clothing when dealing with abortion cases and during on-farm activities like slaughtering cattle. For instance, during the current study period most abattoir staff had no protective clothing. Also, the consumption of raw milk, raw blood and raw or undercooked meat is still common practice in some communities in Tanzania (Shirima et al., 2003).

CONCLUSIONS

This study highlights the importance of RVF as a public health risk and occupational disease in the area under study and supports the need for effective surveillance and future prospective studies to assess specific risk factors. Such studies will be crucial for the design of prevention strategies, which are likely to include instituting vector (i.e. mosquito) control measures and avoiding infected animal parts (urine, blood, uterine fluid). The link between relevant professionals needs strengthening on a broader scale (i.e. through combined veterinary/medical student training and continuing education) to embody the principle of collaborative approaches for epidemiological studies and control of zoonotic diseases.

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SESSION 5

ACHIEVING FOOD SAFETY AND SECURITY IN THE 21ST CENTURY

Biosecurity in a Global Market Place

M. Jeggo[1]*

ABSTRACT

International travel and free trade are modern bywords and the international movement of people, animals and livestock products seen as essential for the global market place to function. Yet is this compatible with a national bio-secure environment? Governments around the world seek to manage the risks posed by infectious disease to livestock, man, the environment and related ecosystems whilst at the same time permitting free trade. Ample examples exist of these competing elements as illustrated by recent outbreaks of avian influenza, bluetongue, severe acute respiratory syndrome (SARS) and most recently in Australia, equine influenza. Whilst the recognition that some 70% of new infectious diseases in man come from animals, even those diseases that affect only animals such as foot and mouth disease, can have devastating effects on trade and economies. The word 'biosecurity' now encompasses most of these elements with processes being developed to identify, mitigate or eliminate these biosecurity risks, and ultimately to prevent adverse events. An added dimension to be considered recently is that of bio-terrorism. So is it time for a new global co-ordinated and collaborative approach to managing biosecurity that recognises the need to encourage not restrict, the global market place? Are there newer approaches that could encourage global trade in livestock and livestock products? One such strategy could be to consider the biosecurity risks of the commodity as opposed to the disease status of the country of origin as a more effective approach for the future.

Key words: *trade, infectious diseases, biosecurity, risk assessment, health status, commodity-based, international standards.*

THE GLOBAL MARKET PLACE FOR LIVESTOCK AND LIVESTOCK PRODUCTS

In 1999 it was recognised that a global livestock revolution was underway characterised by a doubling in demand for livestock products over the next 15 years (Delgado et al., 1999). Driven by urbanisation, an increase in available incomes and a move away from a cereal- to a livestock-based diet, it was foreseen that this demand-driven process would provide a major opportunity for those in the livestock sector. Whilst not a global trend, it was clear that in both Asia and Latin America this revolution had the potential to seriously revitalise rural communities and for many provide a way out of subsistence farming. Now ten years on, every indication continues to support this basic premise (Dijkman et al., 2008). However, more recently there has been a shift of production away from the temperate and dryer areas, to the warmer and more humid areas but with an associated increase in livestock disease risks for producers in such regions. In response to these and other pressures, there has been a move from local multi-purpose activities to a more market orientated integrated process-driven production approach. Significantly, this has increased pressure on communal resources such as grazing areas and water.

As the livestock revolution has unfolded, in some regions there has been a focus on large-scale industrial type production systems with a major concentration on monogastric species (pigs and poultry) (Naylor et al., 2005). There are, however, large regional differences e.g. Brazil has emerged as the major global poultry and pig producer with China and Japan as major importers whilst sadly, the situation in Africa has remained static. Critically these changes have been associated with significant threats to both the environment and human health. The overall trend has continued to be associated with significant shifts of production from developed to developing countries (**Figure 1**).

FOOD SECURITY

There has been a growing focus on global food security since the Millennium Development Goals were set, and whilst still targeting developing countries the global nature of this challenge has become increasingly clear. The matter has now become significantly more acute with the new awareness of the impact of climate change on primary food production and the vital role played by water availability in the process. For livestock, the negative impact of methane production and the associated carbon trading issues have somewhat tempered producer opportunities. A major factor leading to volatility

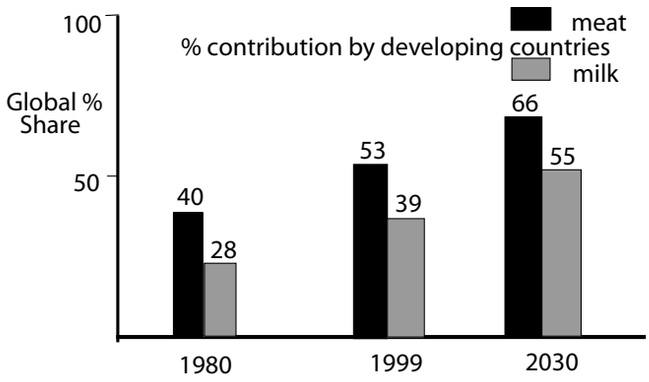

Figure 1. Shifting production of livestock products from developed to developing countrie.

1 Australian Animal Health Laboratory, Geelong, Victoria, Australia

* Corresponding author: Martyn.Jeggo@csiro.au

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in the financial viability of supply chains has been the availability and price of grain. Considered within the concept of the three 'Fs' (feed, fuel and food), the influence of grain has been significant in the past ten years. Effects such as the availability and price of oil, and the use of grain as an alternative fuel source have had profound effects (Steinfeld, 2003). Yet the financial viability of livestock as a key food source depends intrinsically on the price of grain. The longer term implications of these fluctuations on livestock production profitability remain unclear.

DO INFECTIOUS DISEASES MATTER?

In the late 1980's infectious disease both of man and animals were considered threats of the past. The range of effective vaccines and therapeutics had persuaded many that health risks of the future would be focused on nutritional diseases and those associated with longevity. This paradigm, however, changed somewhat dramatically with the advent of mad cow disease and then subsequently, to name but a few, SARS, avian influenza, foot and mouth disease and most profoundly, AIDS (Woolhouse et al., 2005). By the turn of the century it was clear that not only were new and emerging diseases having a profound impact e.g. SARS, henipavirus infections, but that many diseases thought to be under control were re-gaining importance e.g. tuberculosis, malaria.

An example of the speed of spread can be seen with the introduction of bluetongue into Europe. The disease had been confined to one or two islands in the Mediterranean area but this changed dramatically in 2002 with an introduction into Holland and the subsequent spread throughout Europe.

Importantly, the cost of disease relates not only to the effect on the individual animal and producer, but the knock-on effects to other industries and related areas. The 2001 foot and mouth disease outbreak in Europe, whilst serious enough for livestock producers, had a huge impact on the tourist industry in the UK, whilst for the global SARS pandemic, the biggest economic impact was on the airline industry (**Figure 2**).

Overall, the incidence of infectious disease continues to grow globally, with known infectious diseases gaining further ground e.g. rabies, tuberculosis, salmonella, Rift Valley fever; and with new diseases continuing to arise e.g. acquired immunodeficiency syndrome (AIDS), bovine spongiform encephalopathy (BSE), Hendra virus infections, Nipah virus infections, SARS, and Ebola Reston virus. Significantly 75% of new diseases affecting man now originate in animals (Jones et al., 2008). Gradually the new concept of 'one health' has emerged within the framework built around the 'biosecurity' approach. Biosecurity is essentially about the risks associated with infectious disease and the related causative pathogens and has now been extended by some to include all invasive species and their impact on animals, man and the environment (**Figure 3**).

THE CONCEPT OF RISK

Managing the threats from infectious disease requires an understanding of the risks involved and an underlying perception that there are no certainties (Wooldridge et al., 2006). The threats are unpredictable and the associated risks need to be managed within this framework. Risk is determined through an analysis of likelihood and consequence with a crucial underpinning i.e. understanding that zero — risk does not exist. In risk management it is necessary at the outset to undertake a hazard identification followed by a detailed risk assessment before considering risk mitigation strategies and importantly a process of risk communication. A useful approach is to consider the twin elements of likelihood and consequence within the framework of a 'Bow Tie' structure (**Figure 4**).

A careful review would indicate that the biggest biosecurity risk posed by invasive species comes from those infectious agents that can evolve and adapt both to their current hosts and through host switching. In this context, the virus is ideally suited and therefore

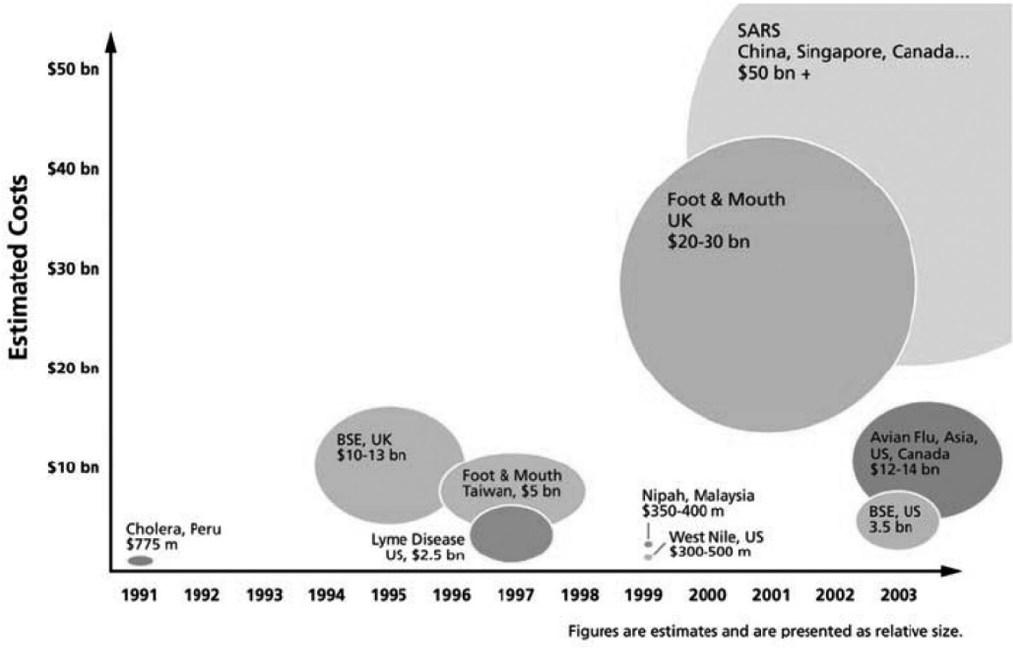

Figure 2. Economic impacts of selected emerging and re-emerging infectious diseases.

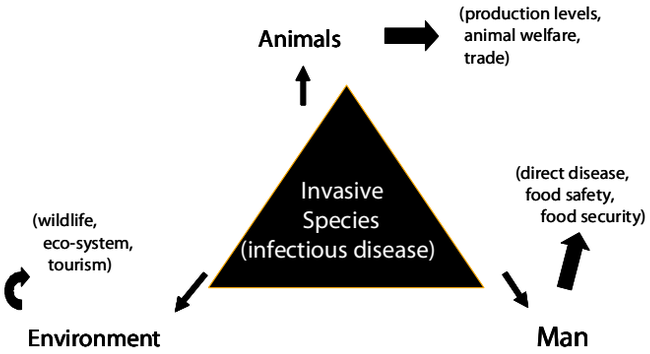

Figure 4. The 'Bow Tie' approach to risk assessment.

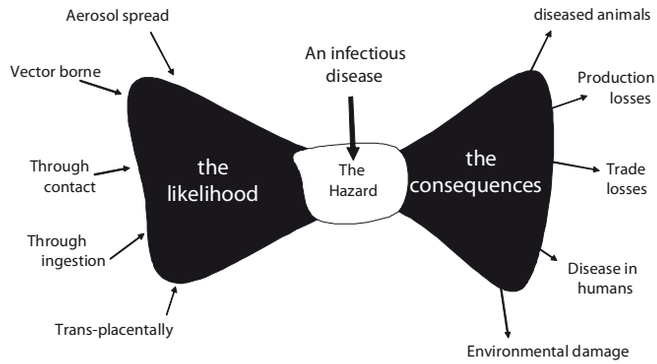

Figure 3. The current biosecurity framework.

not surprisingly seen as the biggest threat. However, other factors serve to enhance the ability of infectious agents to pose new threats including climate change, the global movement of people, animals and their products, the urbanisation and centralisation of people and livestock and the overall trend towards intensification of animal production systems.

World Organisation for Animal Health (OIE)

The OIE was established in 1924 to manage the risks of animal disease brought about by trade in livestock (e.g. rinderpest and foot and mouth disease). At this time it was recognised that the main risks were associated with the movement of live animals and therefore there was a clear focus on establishing the disease status of the country of origin of the animals being imported. This resulted in trade essentially taking place with countries of similar disease status or with those of a better status. Although increasingly this provided a 'global' framework, bilateral processes between countries proved to be the main way of operating for many years.

The OIE approach is not the same today (OIE, 2008) and the OIE's Terrestrial Animal Health Code (TAHC) now permits the use of zonation and compartalisation to assist trade. There is now a more sophisticated risk framework, which clarifies the processes for defining the disease status of a country, detailed guidelines on the laboratory tests to be used, and processes for assessing the quality of national veterinary services and their ability to correctly determine a national disease status. Although not directly a requirement, animal welfare issues are now being addressed by OIE and it works in close co-operation with the World Health Organization in addressing issues around zoonotic diseases. However, despite all these changes, for most developing countries, the fundamental basis for trade is still as it was in 1924 i.e. the health status of the 'national herd'.

Does the market really care that things have remained fundamentally the same over so many years? Certainly there remains real concern of the risks to the national 'herd' through the importation of infected stock or stock of unknown disease status. Many processes are in place to manage this risk today. Moreover, there was, and is today, considerable trade advantage for countries free of many of the OIE diseases. Additionally and increasingly Governments are concerned not only about the trading issues but the risks to man and the environment posed by such trade. Finally, issues surrounding the management of animal welfare in exporting countries are now having serious trade implications.

But viewed from some perspectives, this is not a 'level' playing field. Developing countries are placed at a serious disadvantage and unfortunately many of these countries which have agriculturally-based economies find themselves unable to trade effectively in their primary commodity. The starkest examples exist in Africa, where resources for establishing and maintaining veterinary services remain limited, international trade in livestock and livestock products is minimal and these countries remain within the 'poverty trap'. Is there an alternative?

Commodity Versus Product

In the past few years, the OIE has begun to accept the principle of commodity-based trading (CBT) and uses this as a guide for bilateral trade agreements (see www.oie.int). It is important to distinguish this from the extensive work undertaken by the FAO/WHO Codex Alimentarius Commission which deals with setting standards for products, albeit confined exclusively to issues of human food safety. There are regrettable overlaps and gaps in the standards established for commodities (OIE) and products (Codex Alimentarius). Harmonising these standards could do a great deal but would still require drastic modification of current certification and auditing procedures to have a real impact.

Building on this Paradigm

Can we therefore build on this alternative approach and trade in the livestock commodity or processed product and not in the live animal — and base the biosecurity risk assessments on the commodity or product rather than on the disease status of the country (Perry et. al. 2005)? For subsistence farmers in developing countries participation in the 'livestock revolution' is essential to create a pathway out of poverty.

Support for driving this change would need to focus on establishing processing plants and operations in developing countries and to undertake production in ways that address any biosecurity risks in the final exported product. The national disease status thereby becomes irrelevant and risk is assessed from the perspective of the commodity and not the animal. This approach would involve further investment in infrastructure to create the necessary post-farm gate processing capability but could have real advantages for the many developing countries currently excluded from the international livestock market place because of their current disease status. In creating this processing infrastructure, ways would need to be found to fully engage livestock producers in the process, through, for example, whole of chain co-operatives. In this way the benefits would feed all the way down the production chain and not just accrue post-farm gate.

Successes in this area have already been achieved, as for example in Kenya and Ethiopia (Perry et al., 2005) but international bodies such as the World Bank need to drive the process further to ensure the necessary investments are available to establish the needed infrastructures including processing plants. Of course, none of this precludes the need to continually improve the disease status of these countries, to improve their veterinary services and reduce the risks from disease on their livestock, people and ecosystems. Indeed the more these issues are addressed at the level of the farm the less will be the need to manage the risks at the processing end.

CONCLUSIONS

Global trade in livestock and livestock products continues to increase and is now clearly demand- rather than supply-driven. Ensuring a food secure environment will therefore in part only be achieved through meeting this demand. Biosecurity and particularly the risks from infectious diseases in live animals limit our ability to meet this demand at the global level. Investments in processing capacity at or near livestock production areas and importantly, prior to export from a country, could significantly reduce biosecurity risks. This has the potential to provide substantial benefits to those countries currently excluded from the opportunities provided by the livestock revolution because of their poor national disease status. Is this the way for livestock producers in developing countries to trade their way out of the poverty trap?

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Quality Comparison between Gamma-Irradiated and E-beam Irradiated Pork Patties

B.S. Song, W.G. Kim , J.G. Park, J.H. Kim, Y. Yoon, J.I. Choi , M.W. Byun & J.W. Lee[1]*

ABSTRACT

This study compared the effects of gamma and electron beam (e-beam) irradiation on the quality of pork patties. Pork patties (diameter: 100 mm, thickness: 10 mm) were vacuum-packaged and irradiated by gamma ray (^{60}Co with a 490 kCi source) and e-beam (2.5 MeV) at five, ten, 15, and 20 kGy at room temperature. During accelerated storage at 30 °C for 10 d, determination of total bacterial populations, hardness, and sensory evaluation was conducted at appropriate sampling intervals. The results of total bacterial populations showed that the gamma-irradiated (GR) samples had lower ($P < 0.05$) total bacterial counts than e-beam-irradiated (EB) samples during sotrage at 30 °C for 10 d, regardless of irradiation dose. The hardness and sensory properties such as colour, chewiness, taste, and overall acceptability of pork patties were decreased depending upon irradiation dose. GR samples had lower hardness and sensory scores than those of EB samples. In conclusion, gamma irradiation on pork patties should be useful in decreasing bacterial populations when compared with e-beam irradiation. However, further studies should be conducted to reduce the quality deterioration of GR pork patties.

Key words: *pork patties, gamma irradiation, E-beam irradiation, bacterial populations, hardness, sensory properties.*

INTRODUCTION

The meat processing industry has grown substantially in recent years, and the development of new processed meat products has increased because of the demand for ready-to-eat meat products and the excellent nutritional properties of the foods. However, slaughter, cutting, and processing procedures may increase the possibility of microbial contamination of foods. The studies by Taha (1999), and Woodburn and Raob (1997) showed that fresh meat and processed meat have been implicated in the transmission of foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella*, *Pseudomonas* spp., *Listeria monosytogenes*, *Staphylococcus aureus*, and *Bacillus cereus*.

One of the decontamination technologies for ensuring the microbiological safety of meat is radiation processing. In addition to spoilage bacteria, meat products may contain parasites and pathogenic bacteria, which can be inactivated by irradiation (Olson, 1998). Many researchers have also reported that gamma or electron beam (e-beam) irradiation in low doses (< 10 kGy) kills most microorganisms with no deterioration of food quality (Mohamed, 1999; Thayer et al., 1995; Youssef, 1994). Indeed, several reports have demonstrated the antimicrobial effects of radiation in meat products such as bacon, ham (Weirbicki and Heilgman, 1980), hamburgers (Dempster et al., 1985) and sausage (Kiss et al., 1990).

Meanwhile, Mitchell (1994) suggested that e-beam processing is regarded more favourably than gamma irradiation by consumers who may associate gamma processing with the nuclear industry. Thus, comparison was needed of the effects of gamma and e-beam on microbial, physicochemical and sensory properties of different foods. However, few studies have been conducted to compare the effects of gamma and e-beam irradiation on quality and reduction of bacterial populations in meat and meat products (Mitchell, 1994; Song et al., 2009; Park et al., 2010). Therefore, the objective of this study was to compare the effects of gamma irradiation and e-beam irradiation on the qualities of the pork patties as well as reduction of microbial population.

MATERIALS AND METHODS

Pork Patty Preparation and Packaging

Pork loins were purchased from three local grocery stores. Ground pork (53 g) was then mixed with various ingredients: (pork back fat: 15 g, ice water: 6 g, ginger: 1 g, onion: 8.5 g, egg white: 4.3 g, tomato ketchup: 1.6 g, isolated soy protein: 4.1 g, dried bread powder: 4.1 g, nutmeg powder: 0.05 g, NaCl: 0.65 g, flavour enhancing wine: 0.41 g, black pepper powder: 0.21 g, red colour reagent: 0.01 g, trisodium phosphate: 0.22 g, sugar: 0.85 g) as described by Lee et al. (2005), and 100 g of the meat batter was used to prepare patties (diameter: 100 mm, thickness: 10 mm) using a patty maker (Large Hamburger press, Tupperware, Inc., Orlando, FL, USA). The patties were then heated in a cooker (NUVUES–3 cooker, Menominee, MI, USA) up to 70 °C of internal temperature, removed from the cooker and cooled down at room temperature (25 °C). Each patty was placed in a retort pouch laminated with polyester, aluminum and polypropylene (MULTIVAC, Wolfertxchwenden, Germany), followed by vacuum-packaging. The internal temperature was monitored with a thermocouple (TES–1300 thermometer, TES, TAIWAN).

Irradiation and Storage Conditions

The vacuum-packaged samples were irradiated at 0 (control), 5, 10, 15, and 20 kGy of gamma rays, while the e-beam irradiated both sides of the patties at same dose as the gamma irradiation. Gamma irradiation was conducted using a ^{60}Co irradiator (point source AECL, IR–79, MDS Nordion International Co. Ltd., Ottawa, Ontario, Canada) in the Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute (Jeoung-Eup, Korea). The source strength was

1 Team for Radiation Food Science & Biotechnology Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, Jeongeup, Korea

* Corresponding author: sjwlee@kaeri.re.kr

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approximately 300 kCi with a dose rate of 10 kGy/h. Dosimetry was applied using 5 mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). E-beam irradiation was performed with an ELV–4 Electron-Beam-Accelerator (2.5 MeV) at the EB-Tech (EB-Tech Co., Daejeon, Korea). The beam currents were 2.5, 5, 7.6, and 10.5 mA for 5, 10, 15, and 20 kGy, respectively.

After irradiation, the patties were stored in an incubator (Mir 552, Sanyo Co., Tokyo, Japan) at 30°C for 10 d.

Total Aerobic Bacteria

Total aerobic bacterial populations in patties were determined on days 0, 2, 5, and 10. The 10 g portions of patties were placed aseptically in sterile nylon bag (10 × 15 cm; Sunkyung Co., Ltd., Seoul, Korea) containing 90 mL of 0.1% sterile peptone water (Difco Laboratores, Detroit, MI, USA) and blended for 2 min using a Lab-blender 400 stomacher (Seward medical, London, UK). The blended sample was used to test the growth of the total aerobic bacterial populations in a plate count agar (Difco Lab., St. Louis, USA). Plates were prepared in triplicate and incubated at 37°C for 48 h, and aerobic bacterial populations on a plate were determined as colony forming units (log CFU)/g.

Hardness and Sensory Evaluation

The hardness of the patties was also determined on day 0 using a penetrating test by a texture analyser system (TA-XT2i, Stable Micro System, England) equipped with a probe (1.0 cm thickness). Sensory evaluation of the patties was conducted by 21 panelists who were trained according to the method described by Civille and Szczesniak (1973). Colour, chewiness, taste, off-flavour, and overall acceptance of non-irradiated, gamma and e-beam irradiated samples were evaluated using a seven-point descriptive scale where 1 = extremely disliked or extremely weak to 7 = extremely liked or extremely strong. After irradiation, patties were removed from pouches and reheated in a cooker (NUVUES–3 cooker, Menominee, MI, USA) at 130 °C for 10 min for sensory evaluation.

Statistical Analysis

One-way analyses of variance (ANOVA) were used to determine the effect of a combined treatment of *Kimchi* on the growth of the microorganisms and the quality properties of four groups by a Statistical Package for Social Sciences (SPSS, 10.0). Duncan's multiple range test was used to compare the differences among the means at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Irradiation on Bacterial Growth

After irradiation (day zero), gamma irradiation decreased bacterial populations more than e-beam irradiation ($P < 0.05$), and GR samples at more than 5 kGy and EB samples at more than 10 kGy had levels

Table 1. Effect on growth of total aerobic bacteria of pork patties with vacuum packaging and gamma ray or electron beam irradiation during storage at 30°C (log CFU/g).

Days	Gamma ray (kGy)					Electron beam (kGy)				
	0	5	10	15	20	0	5	10	15	20
0	3.65	ND[2)]	ND	ND	ND	3.56	3.32	ND	ND	ND
2	–[1)]	6.89	5.76	3.32	ND	-	7.91	6.83	6.61	5.51
5	-	-	6.49	5.72	3.75	-	-	-	-	-
10	-	-	-	7.26	5.46	-	-	-	-	-

1 indicates no determination of cells because of spoilage.

2 not detectable.

Table 2. Evaluation of hardness and sensory qualities of gamma ray or electron beam irradiated pork patties after vacuum packaging.

	Dose (kGy)	Hardness (g)	Colour	Chewiness	Taste	Off-flavour	Overall acceptance
Gamma ray	0	431.76±45.35^{a}	6.8±0.8^{a}	6.7±0.7^{a}	6.8±0.6^{a}	1.2±0.1^{c}	6.8±0.8^{a}
	5	395.67±50.32^{a}	6.1±0.6^{a}	6.1±0.5^{a}	6.2±0.4^{a}	2.2±0.2^{b}	5.7±0.4^{a}
	10	385.06±27.59^{a}	5.6±0.5^{ab}	5.7±0.4^{ab}	5.6±0.6^{a}	2.7±0.3^{ab}	5.3±0.5^{ab}
	15	381.43±20.32^{a}	5.1±0.4^{b}	5.2±0.4^{b}	5.3±0.4^{ab}	3.1±0.3^{a}	4.6±0.2^{b}
	20	375.69±28.35^{a}	4.7±0.5^{b}	4.4±0.3^{b}	4.1±0.3^{b}	3.3±0.2^{a}	4.2±0.4^{b}
Electron beam	0	431.76±45.35^{a}	6.7±0.4^{a}	6.9±0.6^{a}	6.7±0.7^{a}	2.1±0.2^{b}	6.9±0.5^{a}
	5	424.38±36.22^{a}	5.9±0.6^{a}	5.8±0.6^{ab}	6.5±0.3^{a}	2.3±0.2^{ab}	5.6±0.4^{b}
	10	423.21±61.62^{a}	5.8±0.5^{ab}	5.5±0.3^{b}	5.8±0.5^{ab}	2.7±0.1^{a}	5.4±0.4^{bc}
	15	419.93±83.64^{a}	5.4±0.3^{b}	4.6±0.4^{c}	5.4±0.6^{b}	2.9±0.2^{a}	4.9±0.2^{c}
	20	407.34±69.88^{a}	5.1±0.4^{b}	4.3±0.3^{c}	4.3±0.4^{c}	2.7±0.2^{a}	4.4±0.4^{c}

$^{a-c}$ Means within the same column different letters differ significantly ($P < 0.05$).

below detection limit (2 log CFU/g) (**Table 1**). This was also found in studies by Chung et al. (2000), Song et al. (2009) and Park et al. (2010) indicating that gamma irradiation was more effective than e-beam irradiation for the destruction of *P. fluorescens* or total aerobic bacteria in refrigerated beef or beef patties. However, bacteria in the samples below the detection limit were recovered during accelerated storage at 30 °C.

Hardness and Sensory Evaluation

The results of the hardness and sensory evaluation of patties are shown in **Table 2**. The hardness of the GR and EB samples significantly decreased ($P < 0.05$) depending on the irradiation doses. The hardness of the GR patties was lower than that of the EB patties. Yook et al. (2001) studied the effect of gamma irradiation on morphological properties and post-mortem metabolism in bovine *M. sternomandibularis* with special references to ultrastructure, shear force, pH, and ATP breakdown. This observation suggests that the bonds between myosin and actin are disrupted by irradiation and is supported by the report of Lee et al. (2000), that myosin was denatured by gamma irradiation.

Sensory properties such as colour, chewiness, taste, and overall acceptability of the GR and EB samples were decreased depending on the irradiation doses (Luchsinger et al., 1996; Park et al., 2010). The GR samples had lower sensory scores than the EB samples. These adverse changes (off-flavour) may be caused by free radicals generated from irradiation (Smith et al., 1960). However, the generation of off-flavour in irradiated meat and meat products can be reduced by various methods such as modified atmosphere packaging, reducing the temperature (freezing) prior to irradiation and addition of antioxidants (Brewer, 2009).

CONCLUSIONS

Gamma irradiation is more effective for inactivating microorganisms in patties than e-beam irradiation. However, gamma irradiation decreased the hardness and sensory scores of patties to a greater extent than e-beam irradiation. Therefore, combination treatments such as modified atmosphere packaging, reducing the temperature (freezing) prior to irradiation and addition of antioxidants will be necessary for quality improvement of irradiated patties.

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Current Status, Surveillance and Control of Avian Influenza in Domestic and Wild Bird Populations in Bulgaria

G. Georgiev[1]*, G. Gujgulova[1], L. Polihronova[1], A. Teneva[2], I. Dimitrova[2], K. Ivanova[3] & E. Todorovska[3]

ABSTRACT

This report describes the history and current status of avian influenza (AI) infection and control in Bulgaria. The country has a unique geographic position in Europe with regard to wild bird populations and their migration routes which pass through its territory. In recent years, Bulgaria did not remain free from AI. The region with the highest rate of isolation of H5N1 virus strains were the Black Sea coast and wet territories connected with the Via Pontica migration pathway in the administrative districts of Dobrich, Varna and Bourgas. Low pathogenic (LP) AI strains isolated from ducks were subtypes H3, H4 and H6 from the areas of Plovdiv, Pazardjik, St. Zagora, Yambol, Sliven and Haskovo. Raising ducks for liver production is a popular practice in south and southeast Bulgaria. From an epidemiological standpoint, controlling circulation of AI viruses among duck flocks, especially before their gathering in larger farms for fattening is a mandatory requirement of official authorities. To prevent the spread of highly pathogenic (HP) AI, surveillance of domestic poultry as well as wild birds should be strengthened in countries at risk, especially along bird migration routes. Monitoring, sampling and analysis of the viral subtypes of AI found in wild birds needs to be carried out to fully understand their role in the propagation and spread of HPAI viruses.

Key words: *Avian influenza, Bulgaria, migratory routes, National Veterinary Service, wetlands, wild birds.*

INTRODUCTION

Avian influenza (AI) is a highly contagious viral disease affecting domestic poultry (chickens, turkeys, quails, guinea fowl, etc.), as well as pet birds and wild birds. It is a disease of varying severity but may be of great importance for animal health, with serious implications for the poultry industry and, in some cases, for human health. Influenza A viruses infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease in susceptible birds: low-pathogenic AI (LPAI) and highly pathogenic AI (HPAI) (Lamb and Krug, 2001; Fouchier et al., 2005). Highly pathogenic avian influenza (HPAI) virus spreads rapidly, and may cause serious disease and high mortality in affected birds (up to 100% within 48 h). The low pathogenic avian influenza (LPAI) causes mild disease that may be undetected as some species of birds show no symptoms. Subtyping of influenza A viruses is based on antigenic differences between the two surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA and 9 NA subtypes of influenza A viruses have been identified.

Wild birds play a role on the circulation of influenza A virus. They often carry LPAI viruses (Alexander, 2000; OIE, 2004; Mekushinov, 2006), and infected individuals can spread these over a wide area during their migration between breeding and wintering grounds. The mallard is one of the most abundant waterfowl in the world, and Munster et al. (2005) detected numerous influenza A virus subtypes, including the strains H5 and H7, which may sometimes be highly pathogenic in this species (Mekushinov, 2006).

Once domestic birds are infected, outbreaks caused by HPAI can be difficult to control and often have major economic impacts for poultry farmers in affected countries, since mortality rates are high and infected birds must be destroyed in order to prevent the spread of the disease. Indeed, since 1997 millions of domestic poultry died or had to be destroyed due to outbreaks of HPAI H5N1 in the countries of Southeast Asia.

AVIAN INFLUENZA IN BULGARIA

The Danube Delta forms the most extensive wetland in Europe after the Volga delta. This is one of Europe's most important sites for breeding, passage and wintering of water birds, particularly wintering; it regularly holds more than 20 000 water birds. In winter 2005–2006, the Danube Delta area faced several outbreaks of HPAI H5N1 in both domestic and wild birds. H5N1 avian influenza was first reported in Romania in October 2005. In February 2006, the OIE confirmed a further outbreak in poultry in the Jurilovca district of Tulcea County. Several outbreaks had previously been reported in this County, although this was the first in the Jurilovca district. Birds on the infected farm and neighbouring premises were culled and movement controls on people and poultry applied.

The poultry livestock industry in Bulgaria is still not affected by the AI subtype H5N1 virus. However, in 2006 a pathogenic strain was isolated from five sick and dead swans and in the course of surveillance for AI viruses (AIVs) low pathogenic strains of types H4, H5, H6, H7 and H10 were also isolated. These findings show that the country is threatened by potential AI infection connected with wild migratory water fowl.

1 National Diagnostic and Research Veterinary Institute, Sofia, Bulgaria
2 Universiry of Forestry, Sofia, Bulgaria
3 AgroBioInstitute, Sofia, Bulgaria

* Corresponding author: georgivet2@yahoo.com

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Bulgaria possesses a unique flora and fauna with some 394 wild bird species recorded in the country. Bulgaria has a special geographic position in Europe with regards to wild bird populations (Nankinov et al.1997; Kostadinova and Gramatikov, 2000) and is the second country in Europe after Spain in terms of the diversity of its bird fauna. Although the territory represents only 1% of the European land mass, it contains 76% of the European ornitho-fauna (Nankinov et al.1997). Many of them are migratory birds and some are included in the Red Book of endangered species. There are two major migratory routes that wild birds use to cross the territory of Bulgaria - the Via Pontica and Via Aristotle. These are used by migratory birds from Northern, Central and Eastern Europe and western Siberia.

Anatidae (ducks, geese and swans) are a group of water birds that are ecologically dependent on wetlands for at least some aspects of their annual cycle. These species use a wide range of wetlands, from the high arctic tundra, rivers and estuaries, freshwater or saline lakes, and ponds or swamps, to coastal lagoons and inter-tidal coastal areas such as mud-flats, bays and the open sea. They also utilise man-made wetlands such as rice fields and other agricultural areas. Many Anatidae populations migrate between wetlands in the northern breeding areas and southern non-breeding areas and in doing so, regularly cross the borders of two or more countries.

These migrations create possibilities for multiple contacts with other bird and animal species and enhanced circulation and spread of AIVs. It is well known that most of the influenza A subtype viruses circulate in their principal host, the wild duck. The main migratory species in Bulgaria belong to two orders, Anseriformes (ducks, gooses, swans, etc.) and Charadriformes (*Arenaria inteprea, Vanellus spinosus, Scolopax rusticola, Sterna fuscata, Larus crassirostria,* etc).

One of the widespread migrating species in the country is the mallard (Anas *platyrhynchos*), which has a breeding population of up to 6 000 birds and a wintering population of up to 148 600 birds (BirdLife International, 2004). The greatest numbers are observed in October, with numbers falling until the end of December, and increasing again in January (Munster et al., 2005; Olsen et al., 2006). This is because the autumn migration has two stages. The first one is the movement of mallards to the Mediterranean Sea and western Asia, mainly during October and November, while the second comprises mallards that come for wintering in Bulgaria at the end of December and beginning of January. During the spring migration, which takes place in February until mid- March, mallards go back to northeastern Russia and Siberia (Munster et al., 2005; Olsen et al., 2006). Wintering of birds from Central Europe in Bulgaria is considered an exception (Munster et al., 2005).

High and Low Pathogenic Strains Isolated

The prevalence of infection with AI has a seasonal character, being greatest in late autumn and winter in the Northern hemisphere, when the birds come back from the regions where they lay their eggs and care for the young, namely the Arctic zone where there is a very dense presence of different bird species and nests and oral/faecal contamination is massive. Clinical symptoms of the disease appear in some of the resting stops during the migration e.g. an H5N1 epizootic in Romania in 2005 and cases of infected swans and wild ducks in the period after January 25, 2006 in Bulgaria. The severe cold of –25 °C was thought to be the cause of dissemination in all directions and to neighbouring Romania of subtype H5N1by sick and infected swans.

The regions in Bulgaria within the last three years where isolates of highly pathogenic viruses of the H5N1 subtype and the low pathogenic viruses of H4, H6, H7 and H10 have been found have been clearly defined (**Table 1**). The region with the highest rate of isolation of H5N1 virus strains completely overlapped the Black Sea coast and wet territories connected with Via Pontica in the administrative districts of Dobrich, Varna and Bourgas. Only one H5N1 strain was isolated in 2006 in the Vidin region as a result of the migratory movement of swans infected with this viral type originating from the Danube river delta. The preserved territory of Poda covers a surface of 2 270 hectares. It is located on the south of Bourgas, close to and connected with the Black Sea. Two hundred and forty five bird species occur in the region, some of them being included in the Red Book of Bulgaria and some of them of European importance. Many migrating and wintering species are found. The territory is on the migration route Via Pontica.

The first case of HPAI in wild birds was found in a dead mute swan found on the river Danube near the town of Vidin. It was confirmed as H5N1. This case was likely the result of spread by H5N1-infected migrating swans and wild ducks from the Danube delta. Mute swans were also found dead around Lake Durankulak at the coast in Kraimorie, Burgas as a consequence of AI-H5.

The Bulgarian National Reference Laboratory on Avian Influenza and Newcastle disease in birds also holds several LPAI isolates:

- influenza A/H10N7/Mallard/ Montana/08., isolated from the fresh faeces of live mallards, with no clinical signs inhabiting the river Ogosta in the district of Montana;
- influenza A/H7N7/Mallard/ Han Krum/08, isolated from the internal organs of a shot mallard, inhabiting the river Kamchia, near Han Krum village in the district of Shumen.

Areas and Species at Risk

Based on AI cases in Bulgaria over the last three years, several regions can be identified: highly pathogenic viruses of the subtype H5N1 and low pathogenic subtypes H4, H6, H7 and H10.

The risk zones of AI penetration are connected with the wet areas and territories via the main migratory path of wild birds and the Black Sea coast (**Figure 1**). The national early warning and surveillance system was adapted to and covered these risk areas on the basis of periodic risk assessment.

Two well equipped laboratories in Sofia and Varna cover the needs for sample investigation and research activities connected with AI in the country. The national surveillance plan includes domestic and wild bird populations and domestic small and large scale poultry production in the country.

Based on risk assessment, we believe that future research should focus on the populations of several species of wild migratory ducks wintering at Shabla Lake (district of Dobrich), Varna-Beloslav Lake (district of Varna) and the wetlands of Poda connected to the Mandra Lake (district of Burgas). Surveillance will include marking of caught birds to monitor their AI status in case they are caught again. These three lakes have been selected based on the advice of the Bulgarian Society for the Protection of Birds (BSPB) Varna, and on the basis of their hydrologic features which make placing of ornithological nets for catching ducks possible. Other lakes do not allow access by boat or they freeze over in the winter.

Geographic coverage

Shabla Lake Complex

Area: 3 195 ha; altitude: 0–40 m.

Shabla lake (Dobruch district) one of the favorite places where wild birds stop when they migrate. The lake complex includes the lakes of Shabla, Ezerets and Shabla Tuzla, located over Sarmatian limestone in northeastern Bulgaria, 5 km northeast of the town of

Table 1. AIVs isolated in Bulgaria during 2005–2008.

Isolate	Date	District	Location	Sample	Bird species	Virus isolates
A/malard duck/05	11.11.2005	Bourgas	Poda dam	Faecal sample	Malard duck	H6N2
A/swan/Vidin/06	31.01.2006	Vidin	Danube river	Internal organs	Swan	H5N1
A/swan/Varna/06	06.02.2006	Varna	Tzonevo dam	Internal organs	Swan	H5N1
A/swam/Kraimorie/06	07.02.2006	Bourgas	Kraimorie	Internal organs	Swan	H5N1
A/swan/Dobrich/06	06.2.2006	Dobritch	Duranculak Lake	Internal organs	Swan	H5N1
A/swan/Bourgas/06	17.02.2006	Bourgas	Chengenez Skale village	Internal organs	Swan	H5N1
A/mule duck/Parvomay/06	25.04.2006	Plovdiv	Parvomay town	Cloacal swab	Mule duck	H6N5
A/mallard/Pazardjik/06	21.03.2006	Pazardjik	Kovatchevo village	Faecal samples	Malard duck	H4N6
A/mule duck/Rajevo konare//07	14.05.2007	Plovdiv	Rajevo konare village	Cloacal sample	Mule duck	H4N6
A/malard/Krepost/07	18.04.2007	Haskovo	Krepost village	Cloacal sample	Mule duck	H4N2
A/mule duck/Rajevo konare/07	22.11.2007	Plovdiv	Rajevo konare	Cloacal swab	Mule duck	H6N5
A/malard/Chan Krum/08	31.01.2008	Shoumen	Chan Krum village	Internal organs	Malard duck	H7N7
A/malard/Montana/07	31.01.2008	Montana	Ogosta river	Faecal sample	Malard duck	H10N7

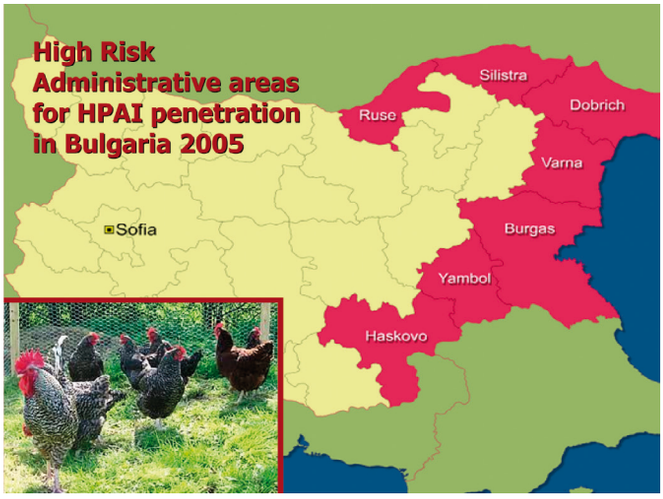

Figure 1. Areas in which there is a high risk of HPAI in Bulgaria.

Shabla. Shabla Lake unites two closely located coastal firth lakes — Shabla and Ezerets — connected through an artificial canal. On the east, the lake is separated from the sea by a 30–50 m sand strip. Shabla Tuzla is a semi-saline lagoon, located 1.5 km southeast of Shabla Lake and separated from the sea by high dunes. The territory of Shabla Lake complex supports 259 bird species and is of strategic importance for the globally threatened red-breasted goose in winter, as, together with Durankulak Lake, it holds almost the entire global population of this species. The lake is one of the sites with considerable concentrations of whooper swan and mallard.

Varna- Beloslav Lakes Complex

Area: 4 681.8 ha; altitude: 0–101 m.

The complex includes two lakes, Varna and Beloslav, connected by an artificial canal and located to the west of the city of Varna. Varna Lake is a coastal firth lake of natural origin, although Beloslav Lake was a closed freshwater firth until 1923. Due to the digging of artificial canals connecting Varna Lake with the Black Sea and another one between the two lakes, the water salinity increased. Since lakes do not freeze in winter, they are preferred as a wintering site by different ducks, cormorans and other waterfowls.

Mandra-Poda Complex, District of Burgas

Area: 5 988 ha; altitude: 0–101 m.

The complex includes Mandra Lake with its adjacent wetlands. Mandra Lake is located at the Black Sea coast and is the southernmost of the Burgas lakes. Its northeastern part touches on the city of Burgas. This former semi-saline lake has been turned into a freshwater reservoir. A lagoon covering the areas of Poda and Uzungeren has been preserved between the reservoir wall and the Black Sea (Roberts, 1978). The complex has international importance for the regular wintering of up to 69 000 waterfowl belonging to 82 species.

Bourgas lake system (Mandra Dam, area Poda and Vaya Lake

The areas with the highest number of isolated subtypes H5N1 virus of AI cover almost entirely a strip along the Black Sea coast and wet territories under Via Pontica in the areas of Dobrich, Varna and Bourgas.

Lake system and wet territories in Varna and Dobrich districts

Two findings were identified as important for AIV infections based on the epidemiological data. First, the availability of water reservoirs, some of which are warm waters being located near to electric power stations; and second, the availability of farms with ducks which are stocked with one-day chicks imported from France or produced from imported breeding eggs delivered by the same suppliers. Ducks are raised extensively in the first three months and afterwards are transferred to fattening farms with relatively good biosecurity standards.

Epidemiology and Surveillance

As shown in **Table 1**, isolated low pathogenic viruses from ducks were of subtypes H4 and H6 in the areas of Plovdiv, Pazardjik and Haskovo. Raising ducks for liver production on such farms is a popular practice in south and southeast Bulgaria (regions of Yambol, Sliven, Stara Zagora, Bourgas, Haskovo).

Table 2. Species and numbers of birds of interest for surveillance in the lakes.

Species	Size of wintering population on Shabla Lake	Size of wintering population on Varna-Beloslav Lake	Size of wintering population on Poda Lake
Ruddy Shelduck	1–27	0–8	1–6
Common Shelduck	1–20	1–43	8–641
Eurasian Wigeon	3–550	3–112	20–3 530
Gadwall	8–15	2–15	19–102
Common Teal	4–408	15–336	175–3 700
Mallard	80–62 210	97–4 004	268–11 883
Northern Pintail	3–19	1–8	3–57
Garganey	3–14	18–87 at the time of migration	Up to 1 112 at the time of migration
Northern Shoveler	6–42	2–35	30–1 109
Red-crested Pochard	4–32	1–28	1–54
Common Pochard	36–3 520	1 125 – 10 240	371–1 3170
Ferruginous Duck	Up to 88 at the time of migration	0–2	4–45
Tufted Duck	16–735	75–2408	486 – 12 800
Greater Scaup	2–26	-	5–100
Longtailed Duck	3–6	-	0–3
White –winged Scoter	-	-	2–12
White-headed Duck	-	3–5	24–202
Black Scoter	-	-	0–3

From an epidemiological standpoint, control over the circulation of AIVs among duck flocks, especially before their gathering in bigger farms for fattening, is mandatory but often overlooked by official authorities due to lack of sufficient resources. During the first three months of rearing the ducks are exposed to high risks of contact with other domestic and wild birds, including migrating waterfowl. If researched, the study will ensure monitoring of a very important factor for circulation and ecological migration of AIVs in the country. Understandably, it will help the local duck industry (a major export earner for the country) to improve its biosecurity standards.

In the past few years the surveillance programme of the Bulgarian National Veterinary Service has included both passive and active surveillance of wild birds. In fact, mainly dead birds were submitted to laboratories, and very rarely faecal samples from places the wild birds inhabit. Also, attempts had not been made either to catch live wild waterfowl, or to monitor the status of marked birds and assess their potential for carrying of AIVs during different life stages. For that reason active surveillance and using microsatellite markers for bird migration tracing will improve the early warning and preventative AI control system.

The currently ongoing outbreaks caused by HPAIV of the subtype H5N1 are of concern not only to the poultry industry but also to public health. This virus, which causes a high fatality rate among infected patients, may adapt to efficient human-to-human transmission and thus initiate a new human influenza pandemic (Alexander, 2000; BirdLife International, 2004; Wallenstein et al., 2007). Since 1996, when the ancestor virus was identified in domestic geese from China, outbreaks have spread and now encompass countries in Asia, the Middle East, Europe, and Africa. This spread of HPAIV among poultry flocks is traditionally thought to occur by transport of infected poultry, contaminated equipment, and persons associated with the poultry industry. HPAIV has occasionally been detected in wild birds near affected poultry flocks, but these birds have had limited or no role in virus dissemination. In the current outbreaks, however, wild birds are suspected of playing a major role as long distance virus vectors.

During the expansion of HPAI (H5N l) outbreaks from Asia to Europe, two events implicated wild birds, particularly water birds, as long distance virus vectors. First, virus outbreaks in 2005 spread rapidly westward from Russia and Kazakhstan in July and August to Turkey, Romania, and Ukraine in October. Wild water birds were suggested as vectors because the virus spread through areas that had no previous record of any virus presence and coincided with the fall migration of wild water birds between these areas. Second, at the beginning of 2006, HPAIV (H5N1) was detected in many wild water birds in western Europe, often in areas where no outbreaks had been detected among intensively surveyed poultry; this event overlapped with unusual water bird movements associated with cold weather in the Black Sea.

To prevent further spreading of H5N1, surveillance in domestic poultry as well as in wild birds should be strengthened in countries at immediate risk, especially along migrating bird routes. Resources should be focused on the reduction of close contacts between humans, domestic poultry and wildlife through better management practices and improved biosecurity in poultry production enterprises, especially those that are small and 'open-air' - where domestic poultry and waterfowl are allowed to mingle with wild birds.

Official competent authorities such as the Chief Veterinary Officer would also need to monitor 'wet' and wildlife markets, where wild and domesticated species are kept in close proximity and are at risk of exposure to a wide range of pathogens. Limiting contact with wild birds should therefore be part of any AI control strategy. The control

of AI infection in wild bird populations at this stage is not feasible from logistical, environmental and biodiversity points of view. Indiscriminate culling of wild migratory bird populations would be ineffective in preventing further spread of AI and their hunting would likely cause dispersion of the birds.

PRESENT AND FUTURE NEEDS

Monitoring, sampling and analysis of the viral subtypes of AI found in wild birds needs to be done in order to fully understand their role in the propagation and spread of HPAI viruses. Multidisciplinary research is required that brings in the competencies of veterinarians, wildlife specialists, ornithologists, virologists, molecular biologists and other specialities. Besides the current regional and country specific AI projects being implemented by FAO, Mongolia has been assisted through a regional technical co-operation to review emergency preparedness and surveillance activities for HPAI since the outbreak in wild birds was reported.

A Global Strategy for the prevention and control of HPAI has been prepared by FAO and OIE under the umbrella of the Global Framework for the Control of Transboundary Diseases (GF - TADs). This Global Strategy addresses country level activities as well as the indispensable regional and international coordination. Within the epidemiological context of the current HPAI outbreaks, there is an urgent need to strengthen the joint FAO/OIE/WHO Global Early Warning System (GLEWS) so as to improve the regional capacity for early detection and response to AI incursions. Immediate support to national Animal Health Services will be required in Eastern Europe for emergency preparedness, surveillance and early response activities Diagnostic capability in the region for avian influenza have been substantially enhanced by national efforts, with coordination and support provided by international organization. However, surveillance methods are still seriously inadequate to allow confident national and regional decisions to be made.

Specifically, surveillance efforts should be shifted from simple case-finding to identification of risk factors influencing maintenance of infection, and integrate surveillance procedures into control strategies. Also, for each country, the transmission pathways which are considered to have been responsible for infection dissemination in the current epidemic should be identified, and a surveillance strategy developed which will allow each of these pathways to be monitored, and changes in prevalence to be assessed. For example, market surveillance and interviews should be used to identify high-risk markets, bird types and bird sources, and control strategies built around this information. Further, for each country which no longer has active infection (or cases), surveillance measures that would minimise the time to detect a new incursion should be determined within the limits of available resources.

Other steps would include applying nuclear molecular epidemiological investigation methods more comprehensively, to clarify epidemiological processes which are influencing the evolution of the epidemic: assessing the level of human exposure under various circumstances, in order to evaluate the risks of emergence of a virus capable of human to human transmission, and use this information to help guide the allocation of resources to different elements of the control strategy. Further needs include developing rapid and standardised methods for the routine analysis of surveillance data which would identify important changes in the H5N1 situation, and enable notification of this information to the competent authorities; and harmonised collection and presentation of surveillance and control data across countries in the region, so that information can be interpreted in a compatible way across the region.

CONCLUSIONS

The region with the highest rate of isolation of H5N1 virus strains completely overlapped the Black Sea coast and wet territories connected with the Via Pontica migration pathway in the administrative districts of Dobrich, Varna and Bourgas. Isolated LPAI strains from ducks were of subtypes H3, H4 and H6 in the areas of Plovdiv, Pazardjik, St. Zagora, Yambol, Sliven and Haskovo.

Raising ducks for liver production is a popular practice in south and southeast Bulgaria. From an epidemiological standpoint, control over circulation of AIVs among duck flocks, especially before their gathering in larger farms for fattening is a mandatory requirement of official authorities. To prevent the spread of HPAI, surveillance of domestic poultry as well as wild birds should be strengthened in countries at risk, especially along bird migration routes. Monitoring, sampling and analysis of the viral subtypes of avian influenza found in wild birds needs to be done in order to fully understand their role in the propagation and spread of HPAI viruses.

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Vitamin D Metabolism in Experimental Animals: Kinetics of *Solanum glaucophyllum* Active Principle in Cows and Assessment of Calcium, Phosphorus and Vitamin D3 Requirements in Broilers

M. Dallorso[1*], J. Azcona[2], B. Iglesias[2], S. Gil[3] & N. Belmonte[1]

ABSTRACT

In 1990 our group began working on the development of a sensitive method to measure the active principle (1,25 dihydroxy-vitamin D_3-glycoside) of *Solanum glaucophyllum*, a plant which grows wild in Argentina and causes calcinosis in breeding cattle. A radioreceptor assay (RRA) was applied to measure the free vitamin D metabolite in the plasma of experimental cows that were fed the plant in order to study the kinetics of the active principle. The 1,25 dihydroxyvitamin D concentration in plasma showed a 33-fold increase four h post treatment. Peak levels were recorded 12 h after dosing, decreased by half between 24–36 h and continued declining until 48 h. More recently, this plant has been proposed as a source of vitamin D activity (VDA) and thereby may contribute to improving Ca and P utilisation by animals and environmental care. The effects of different dietary levels of calcium (Ca) and phosphorus (P) over the range between commercial recommendations (control) and two thirds of NRC requirements (basal) as well as different sources of those minerals were therefore studied in experiments covering either a part or the entire breeding cycle of broilers through measurements of productive, nutritional, skeletal and biochemical parameters. Results indicated that birds fed diets deficient in these minerals exhibited skeletal responses but nevertheless showed better productive responses than those fed control diets. The high levels of vitamin D_3 employed in commercial farms (25 times NRC recommendations) could enable birds fed on deficient diets to increase synthesis of the active metabolite of the vitamin in order to partially overcome deficiencies in these minerals. On the other hand, such high levels of vitamin D_3 might have been unbalanced for optimal efficiency, at least under the experimental farm conditions of the present work.

1 Facultad de Ciencias Agrarias, Universidad Nacional de Lomas de Zamora, Llavallol, Buenos Aires, Argentina
2 Estación Experimental Agropecuaria-Pergamino, Instituto Nacional de Tecnología Agropecuaria. Pergamino, Provincia de Buenos Aires, Argentina
3 Grupo Pecuario, Centro Atómico Ezeiza, Comisión Nacional de Energía Atómica, Buenos Aires, Argentina

* Corresponding author: dallorso@agrarias.unlz.edu.ar

INTRODUCTION

Enteque Seco (ES), which affects grazing cattle in Argentina and other countries in South America, is caused by ingesting fallen leaves of the toxic weed *Solanum glaucophyllum (SG)* (Okada et al., 1977). It is characterised by loss of body weight, kyphosis, stiffness of the forelimbs, soft tissue calcification and modifications in Ca and P plasma concentrations (Worker and Carrillo, 1967). The active principle in *SG* is 1,25$(OH)_2$vitamin D_3-glycoside (Haussler et al., 1976). The economic relevance of *SG* could be seen on the one hand as being the causal factor of toxicosis of grazing cattle (negative value), and on the other, as a valuable source of vitamin D_3 active metabolites (positive value). Until now, most scientific work has dealt with the pathology and biochemistry of this calcinotic disease in experimental animals. Conversely, only a few publications have described quantitative studies about the vitamin D synthetic capability of the plant (Weissenberg, 1989), and only one report could be found about the environmental conditions that might influence vitamin D_3 production by the plant (Puche et al., 1980) in addition of the recent report of Dallorso et al. (2008).

Pharmacological applications of *SG* have been reported in human and veterinary medicine as well as in animal production. Potential veterinary uses include the prevention of milk fever, pseudo-vitamin D deficiency of pigs and acidosis in chicks (Weissenberg, 1989). The first assay applied in animal husbandry was about elucidating the effect of *SG* powder from leaves on egg shell quality in laying hens (Gallego et al., 1978/9). Recently, *SG* has been used as an additive in the feed of chicks to improve phosphorus utilisation (Cheng et al., 2004), and a further promising application would be the supplementation of finishing cattle with *SG* to improve meat tenderness (Paaren, 1999).

The present report summarises some unpublished results of research to improve the diagnosis of ES using a radioreceptor assay (RRA) to determine the concentration of 1,25$(OH)_2$vitamin D in plasma (1,25D) based on the knowledge that 1,25vitamin D_3-glycoside is cleaved by intestinal bacterial enzymes to 1,25D (Boland et al.,1987). This enabled us to apply our experience and expertise to the area of Ca and P nutrition in poultry.

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MATERIALS AND METHODS

Studies on ES Diagnosis

The experiment was performed to describe the fate of the active principle in *SG* on experimental cows.

Animals

Two non-pregnant, non-lactating adult Jersey cows without any clinical and pathological signs were used. Animals were housed indoors in individual pens and fed diets supplying 75 g Ca and 35 g P/d. Cows ('A' and 'B') were fed a diet composed of 65% corn silage, the remainder being a mixture of beet pulp, soybean meal, distillers grain and alfalfa hay with a commercial supplement. Each animal was fed 9 kg dry matter/d.

Vitamin D Compounds

The [^{3}H]–1,25D_3 was generously supplied by the National Animal Disease Center, (NADC), Iowa, USA. The 1,25D_3; 1,24,25$(OH)_3D_3$; 1,25,26,$(OH)_3D_3$ and 1a, 25$(OH)_2D_3$–26,23 lactone were kindly provided kindly by Dr. Milan Uskokovic (Chemical Research Department, Hoffmann-La Roche, Inc., N.J., USA.

Solanum glaucophyllum (SG)

The SG was provided by Dr. C. Corbellini (INTA, Argentina). The leaves were collected in Mercedes, Bs. As., Argentina, in May 1995.

Experiment

After 2 weeks on the experimental diet a single dose of 100 g of powdered dried leaves of *SG* was mixed with the silage and given to each of the two cows. Two blood samples were taken 24 h before feeding and just before the single dose of *SG* to obtain baseline physiological data. Sampling was then continued every four h during the first d, at 36 h, 48 h, 72 h, 96 h., and on d seven after dosing. Blood samples were taken from the jugular vein with heparinised syringes, centrifuged to obtain plasma, and kept frozen at –90 °C until analysed.

Determination of 1,25D_3

A one-step extraction procedure was used (Dallorso et al., 2001) followed by analysis using a non-HPLC charcoal binding assay using thymus gland receptor (Reinhardt et al., 1984). Radioactivity was quantified using a scintillation counter (Beckman L.S. 8 000).

Determination of 1,24,25$(OH)_3D_3$, 1,25,26,$(OH)_3D_3$ and 1,25$(OH)_2D_3$–26,23 Lactone

To elute the three more polar vitamin D_3 metabolites, prewashed SPE C18 cartridges were sequentially washed after sample application with double distilled water, methanol:water (50:50), hexane:methylene chloride (90:10), and hexane:isopropanol (99:1). Finally, these three metabolites were eluted with hexane:isopropanol (85:15). To obtain the three metabolites separately, the eluates were evaporated in a vacuum centrifuge, hexane/methylene chloride (2/1): isopropanol:methanol (46:50:4) added, injected onto a HPLC unit (Waters Corp.) and run through a silica column, with monitoring using an absorbance detector (Model 440) at 254 nm. The mobile phase used was hexane/methylene chloride (2/1): isopropanol:methanol (46:50:4). Each metabolite was collected in a different fraction by an ISCO Collector (Model 568) and all were analysed by radioimmunoassay (RIA, Hollis et al., 1996). The antibodies were raised in rabbits in the NADC. The [^{3}H]–1,25D_3 fraction was used to determine the recovery of all the vitamin D metabolites.

Experimental Studies on Calcium and Phosphorus Nutrition in Poultry

Intensive poultry production produces an excess of P in manure. When this is applied on fields as fertiliser, the surplus can cause eutrophication of water bodies (Sharpley et al., 1994). In order to contribute to environmental care through nutritional management the effects of different dietary levels and sources of Ca and P covering the range between commercial recommendations and near two-thirds of NRC (1994) requirements on productive, nutritional, skeletal and biochemical responses were studied in a series of experiments covering part or the entire breeding cycle of broilers. Two of these experiments are presented.

Animals

These were males Cobb–500 from Granja Tres Arroyos, Capilla del Señor, Buenos Aires, Argentina.

Trial 1

This covered the period from 7–20 d of age (initial weight [d 7] was 151 ± 17 g). The feed was mash composed of corn, soy meal (40% crude protein), soybean, soy oil, limestone, calcium phosphate, salt, methionine and a premix containing 5 000 IU vitamin D_3/ kg of diet and covering requirements for this strain of broilers except those of Ca and P in the basal diets. Animals were divided into two groups each of 25 broilers i.e. a control group that received Ca = 0.92% and P_a= 0.41%; and a group receiving a basal diet with 0.56% Ca and O.28% P_a.

Trial 2

This was carried out when animals were between 1 and 49 d of age. The feed was mash composed of corn, soy meal (45% crude protein, soybean, meat meal (41% crude protein), soy oil, limestone, salt, methionine, lysine, threonine and premix containing 5 000 IU of vitamin D_3/ kg diet and covering requirements for this strain of broilers except for Ca and P levels in basal diets. The control animals (five pens of 15 animals each) were on a starter ration of 0.9% Ca and 0.45% P_a, a grower diet with 0.88% Ca and 0.42% P_a, and a finisher of 0.84% Ca and 0.40% P_a; during the last week the diet had 0.78% Ca and 0.35% P_a.. Animals on the basal diet (five pens of 15 animals each) were on a starter ration containing 0.56% Ca and 0.28% P_a; a grower diet with 0.55% Ca and 0.26% P_a and finished on a diet with 0.52% Ca and 0.25% P_a. During the last week, the diet contained 0.49% Ca and 0.22% P_a.

Measurements

Animals were weighed and their feed consumption measured weekly and data were expressed as the average of each pen. At the end of each trial broilers from each pen (n = 5) were taken at random and maintained in cages until euthanised by complete bleeding to be used for some of the following determinations:

- Ca, P_i and 1,25D levels in plasma obtained from heparinised blood samples that were immediately centrifuged at 3 500 rpm for 10 min;
- Ca, P, and ash levels, morphology, weight, density and volume of right tibiotarsi; and
- Ca, P and ash in feed and manure.

Feed samples of each treatment were taken from the appropriate bags after preparation. Manure samples were collected in cages during a period of 8 h prior to euthanasia. Ca was determined by atomic absorption spectroscopy (AAS) on plasma diluted in a solution containing La_3O_2 0.5% and Pi by colorimetry (Fiske and Subbarow, 1925). Bone volume was determined by complete immersion of tibiotarsi in a fixed volume of water in a graduated tube (bone volume (mL) = water volume with bone inside the tube - water volume without bone inside the tube). Bone density was calculated as bone weight in air/bone volume. Tibiotarsi, feed and manure were dried in an electric stove at 80°C (DM), incinerated at 580°C and ash dissolved in HCl: H_2O (1:1) and filtered, and washed with water to a final volume of 50 mL.

[1,25D] was analysed by RIA (Gil and Dallorso, 2002) using plasma samples prepared as described previously (Dallorso et al., 2001). A radioreceptor assay (RRA) was replaced advantageously by RIA to determine the active vitamin D metabolite due to the fact that the binding capacity of the antibody raised against $1,25D_3$ remains constant when stored in solution at –20 °C while the intracellular vitamin D receptor (VDR) employed in RRA, although freeze-dried and stored at –80 °C needs to be prepared frequently, among other disadvantages. Although the antibody binds to 25(OH)vitamin D (25D) with five percent cross-reactivity (100% 1,25D), since 25D was undetectable in the analyte fraction (F2) and conversely, the analyte was not present in the 25D fraction (F1), this method was considered adequate for measurement of [1,25D].

Analysis of data

In trial 1, animals were considered experimental units (n = 25 animals in each group) for the analysis of individual variables under study. Values of body weight were studied in all 25 animals. The other determinations were made on a randomised sample of five animals. In trial 2, pens were considered experimental units (n = 5 pens in each group of 15 animals each). Values of body weight and feed consumption were obtained for all 15 animals in each pen and expressed as an average of each pen. The other determinations were made using a randomised sample of six animals from each pen.

Differences between control and basal groups were detected by Two-Sample t test (P-value ≤ 0.05) using the software Statistix SXW-Version 8.0.

RESULTS AND DISCUSION

Studies on ES Diagnosis

The evolution of the levels of vitamin D metabolites in cows 'A' and 'B' is shown in **Figures 1** and **2**. The 1,25D concentration showed a 45-fold increase to 3 345 pg/mL by 4 h post treatment in cow 'B' and a 20-fold increase to 5 200 pg/mL in cow 'A'. The maximum was observed 12 h after feeding in both cows, decreased to half its maximum between 24 h and 36 h and continued declining until 48 h. The 1α-lactone; $1,24,25(OH)_3D_3$ and $1,25,26(OH)_3D_3$ concentrations were quantified before and at 24, 48, 72, and 96 h and on day 7 post feeding. The $1,24,25(OH)_3D_3$ and **1α**-lactone levels reached a maximum at 24 h with 9.7 and 14-fold increases (1 930 pg/mL and 1 037 pg/mL) respectively in cow 'B' (see **Figure 2**). The $1,25,26(OH)_3D_3$ concentration was only slightly elevated compared with the other three vitamin D metabolites. All three metabolites returned to near baseline levels by 7 d. As expected, there was an increase in 1,25D and $1,24,25D_3$ levels after feeding cows with *SG* which is consistent with our previous findings (Dallorso et al., 1994). It was also observed that in addition to $1,24,25D_3$, the metabolite **1α**-lactone was also a major circulating $1,25D_3$ form. This is in agreement with reports of pharmacological doses of $1,25D_3$ to adult cows (Horst et al., 1983) and with experiments with radio-inert compounds in rats that showed injected $1,25D_3$ to be an efficient precursor of 1α-lactone (Horst et al., 1984). No significant elevation was recorded in plasma $1,25,26D_3$ levels. This situation is in accordance with previous work (Reinhardt et al., 1982). It seems reasonable to conclude that $1,24,25D_3$ and 1 α-lactone arose from further metabolism of the increased $1,25D_3$ levels emanating from the treatment with *SG* since these compounds circulate in low concentrations in normal cows (Reinhardt et al., 1981) and are found to be elevated in different species like the rat (Ohnuma and Norman, 1982), dog (Ishizuka et al., 1984) and cow

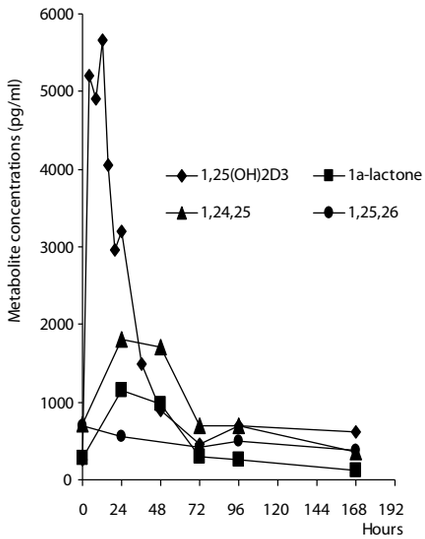

Figure 1. Plasma $1,25(OH)_2$ D_3; 1 alpha lactone: $1,24,25(OH)_3$ D_3 and $1,25,26(OH)_3$ D_3 concentrations after a single oral dose of 100 g *Solanum glaucophyllum* to cow 'A'.

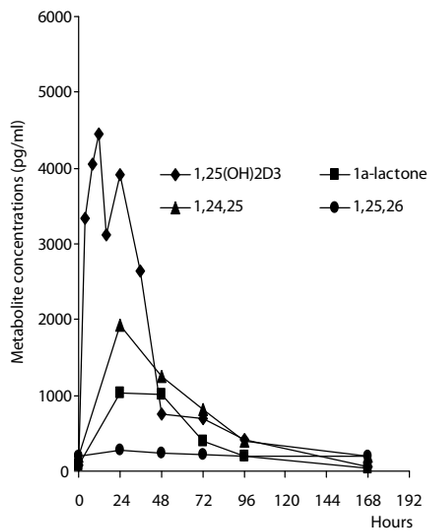

Figure 2. Plasma $1,25(OH)_2$ D_3, 1 alfa lactone; $1,24,25(OH)_2$ D_3 and $1,25,26(OH)_3$ D_3 concentrations after a single oral dose of 100 g *Solanum glaucophyllum* to cow 'B'.

(Horst et al., 1983) treated with vitamin D derivatives. The appearance of each metabolite paralleled the appearance of 1,25D.

The significantly augmented 1 α-lactone or 1,24,25D_3 values caused by the 1,25D_3 degradation recorded after the feeding period cannot be used to diagnose *SG* toxicity. In previous work with rabbits and ewes dosed orally with *SG* (Dallorso et al., 2000a), high plasma levels of the 1,25D metabolite occurred 1 h after administration and remained higher than control animals for up to 24 h after administration. Also, while rabbits dosed orally and subcutaneously showed increments in the plasma metabolite, highest values were recorded in those dosed orally. The calcinotic effects seen in rabbits dosed subcutaneously could have been caused by the 1,25D_3-glycoside without the intervention of enteric microbial enzymes (Dallorso et al., 2000b). This was suspected earlier (Barros et al 1981) who showed specific effects of this active principle in the aorta of rabbits 6 h after intravenous administration of aqueous extracts of *SG* leaves.

Experimental Studies on Calcium and Phosphorus Nutrition in Poultry

There were no significant differences (P = 0.6819) in body weight at 20 d of age between control (746 ± 62.47 g) and basal (736 ± 100.86 g)

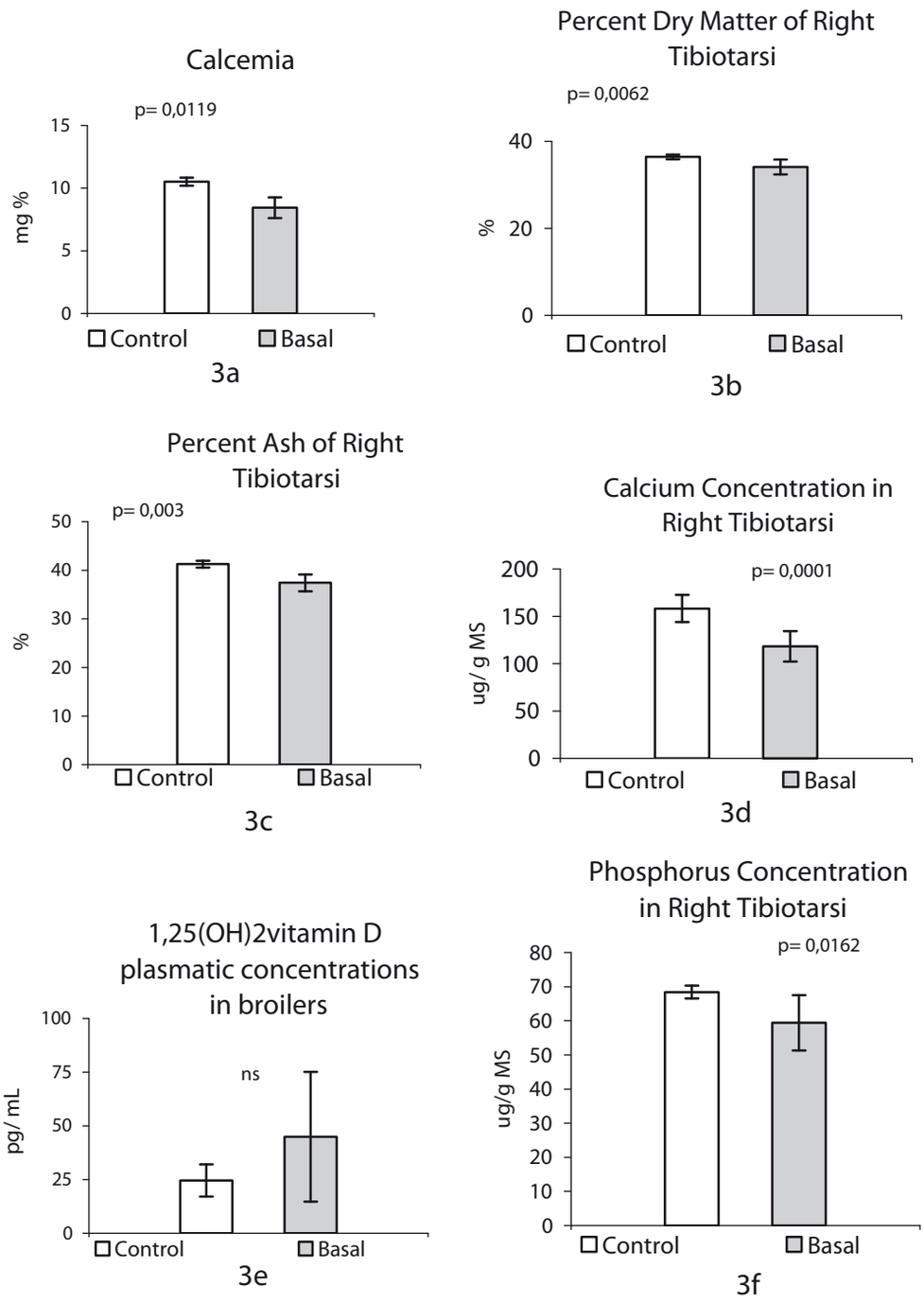

Figure 3. Biochemical and skeletal variables measured at 20 d of age in broilers (Trial 1).

Table 1. Body weight and feed conversion in broilers (Trial 2).

Treatment	Body weight (g)			Feed Conversion (g feed/g weight gain)		
Age(d)	21*	28*	35	21***	28***	35***
Control	750 ± 13	1 276 ± 29	1 901 ± 50	1 426 ± 11	1 516 ± 16	1 633 ± 9
Basal	824 ± 49	1 336 ± 52	1 966 ± 80	1 286 ± 24	1 423 ± 15	1 558 ± 14

Body weight (g) and feed consumption (g) were evaluated weekly and values in table are the mean of five pens (15 animals each).

Differences between control and basal groups within days of age: $^{*}P \leq 0.05$; $^{***}P \leq 0.001$.

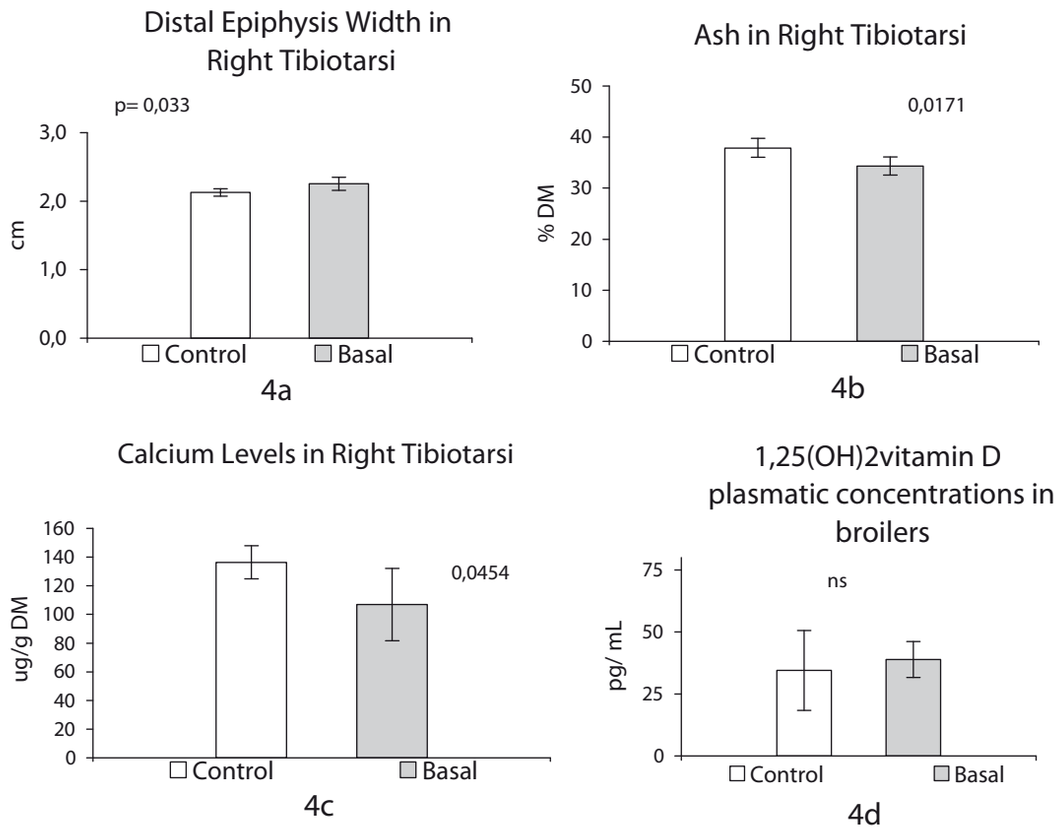

Figure 4. Biochemical and skeletal variables measured at 49 d of age in broilers (Trial 2).

groups in Trial 1 although the controls had higher body weights. Additionally, feed conversion values (g feed consumed / g weight gain) were lower in the controls (1 998 vs 2 099). At 20 d of age lower values ($P \leq 0.05$) were recorded in the basal groups for plasma Ca levels, percentage dry matter and ash, and for Ca and P levels of the right tibiotarsi (**Figures 3a-f**). Levels of vitamin D active metabolite, were twice as high in the basal than in the control group, but the difference was not significant ($P = 0.273$) (see **Figure 3e**). Neither were significant differences detected between the groups in plasma Pi concentrations, fresh and dry weight, and the volume and density of right tibiotarsi. Ca and P levels in manure paralleled those in feed (data not shown).

In Trial 2, although animals of the basal group were fed only two thirds of the recommended levels of Ca and P during their entire breeding cycle they showed significant and favourable differences in body weight at 21 d and 28 d of age, and in feed conversion at 21, 28 and 35 d of life (**Table 1**). Also, significantly lower values ($P \leq 0.05$) for ash and Ca levels accompanied by higher widths of the distal epiphysis and the right tibiotarsi were recorded at 49 d of age in these animals (**Figures 4a–c**). However, vitamin D active metabolite concentrations in plasma did not show significant differences ($P = 0.594$; $n = 5$) between the basal and control groups, but since the mean basal group value was slightly higher a greater number of experimental units should be used in future studies to ensure rigorous statistical analysis (**Figure 4d**). Other nutritional, biochemical and skeletal variables were similar in both groups.

The high levels of vitamin D_3 employed here (25 times the 1994 NRC recommendations) and in commercial farms (Barroeta et al., 2002) could enable birds fed on basal diets to increase synthesis of the active metabolite of vitamin D in order to ameliorate partially the effects of Ca and P deficiencies. Conversely, the diet with high levels of vitamin D_3 together with the recommended levels of Ca and P for lower levels of vitamin D_3 (NRC, 1994) might have been

unbalanced for optimal efficiency, at least in the experimental farm conditions of the present work.

CONCLUSIONS

The determination of plasma 1,25D levels in experimental cows contributed to knowledge about calcinosis of cattle in Argentina, named ES. The results obtained demonstrated the rapidity with which the active principle of *SG* is hydrolysed by digestive enzymes in ruminants, evidenced by the augmented levels of the free vitamin D_3 metabolite circulating in blood four h after oral administration.

The preliminary results obtained with feeding broilers suggest that it will be necessary to investigate the performance of commercial broiler chickens during the entire cycle with different combinations of vitamin D_3, calcium and phosphorus in order to determine the appropriate levels for optimum and deficient diets.

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Animal Health in Albania

A. Lika[1]*

ABSTRACT

The animal health service policy in Albania represents an integral component of overall governmental, social and economic policy in the field of agricultural and rural development, public health, food processing and import/export of animal products. In order to obtain the necessary political, economic and public support, the animal health service attempts to contribute effectively to the overall development of the country which aims at improving the standards of living of its inhabitants. Practical means of contributing to national development include reducing food loses due to animal morbidity and mortality, increasing the productivity of the livestock population, protecting human health against zoonotic diseases and ensuring humane treatment of animals. An animal health strategy contributes to the creation of conditions necessary for uninterrupted animal disease surveillance and control in the country. The main animal health problem in Albania is brucellosis in ruminants, caused by *B. melitensis*. This infection currently affects the entire country, reaching a prevalence of 10% in several districts. The latest and most severe outbreaks of classical swine fever were identified on 1996 when 5 515 animals were infected and 3 683 animals died. The circulation of bluetongue virus (BTV) was detected for the first time in Albania in 2002 with a seroprevalence of 15%. The evidence of BTV circulation in Albania and the absence of the main vector *C. imicola* suggest that other *Culicoides* species could be implicated in virus transmission. H5N1 avian influenza in Albania was confirmed in March 2006 in backyard flocks in the villages of Cuke and Peze-Helmes. In both villages there were no human cases. Rabies was of concern in Albania from 1928 until 1976. The disease re-emerged in March 2001 in the village of Morine in Kukes district affecting a domestic dog and three persons were bitten. Other cases have been reported in northern Albania.

INTRODUCTION

Agriculture plays an important role in Albania, although currently it is practised largely at a subsistence level. Albania's farming sector has been dominated by small private holdings since the collapse of the communist State in 1991, when peasant farmers disbanded the quasi-state collective farms. Agriculture subsequently became an important source of income support in rural areas, and is now undergoing a transition from a largely subsistence sector to a commercial one. Currently, the sector contributes 25% of GDP, which is high compared with neighbouring countries, while average gross income per farm is estimated at about US$ 1 800.

Around 40% of Albania's 28 748 km^2 land area is classified as agricultural land (24% arable and 15% pasture). The rest is divided between forest (36%) and other uses. Over 75% of Albania is hilly and mountainous, and much agricultural land is hilly. Albania is predominantly mountainous in the north and east, with agricultural land concentrated in the more densely populated coastal plains of the west (43% of arable land). A further 34% of agricultural land lies in river valleys; 23% is upland. Albania is located in the Mediterranean climatic zone and has short winters and hot, dry summers. It has abundant precipitation (1 430 mm annual rainfall) concentrated in autumn and winter, with frequent droughts in summer. It also has extensive underground water resources (World Bank, 2007).

The total number of farms in Albania is approximately 370 000, mostly dominated by small farms (average size 1.14 ha according to official statistics, or 0.8 according to HBS data (Albanian Agriculture, 2007). This is a much smaller average size compared with an average of 5 ha for Central and Eastern European countries and 27 ha for Western Europe. This is an important handicap to improving agricultural productivity and encouraging sustainable development of the agricultural sector.

MATERIALS AND METHODS

Identification of Gaps

The vision of the National Animal Health Programme (NAHP) is to improve the health and welfare of animals for meeting the needs of stakeholders, enable safe production of food, improve health of the public, sustain the rural society, and support the rural economy. Effective national food control systems are also essential to protect the health and safety of consumers. Food-borne diseases caused by microbiological contamination remain a major public health problem in Albania. The country is registering an increase in brucellosis, particularly in humans, transmitted either through contact with animal tissue or through the ingestion of contaminated milk and milk products (**Figure 1**).

The concept and requirements for working towards this vision are:

- the current veterinary services in the country, including the veterinary diagnostic institution, are very weak. The few resources in place are fragmented and reflect the historic paradigm of the previous regime with incomplete transition towards a market economy. The veterinary services in the 12 districts/regions appear to have been connected administratively but their field operations are neither connected with the national interest nor geared to the above vision;

1 Sector of Virology, Institute of Food Safety and Veterinary, Tirana, Albania

* Corresponding author: aldin_lika@yahoo.com

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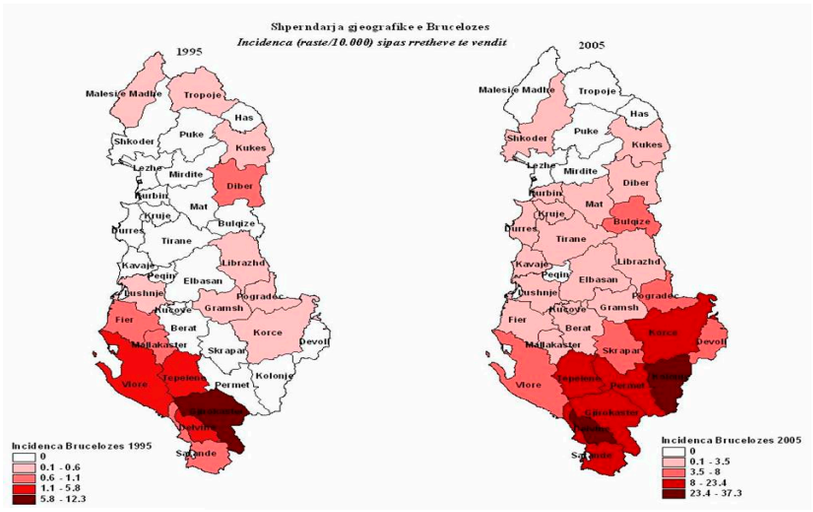

Incidence 1995 **Incidence 2005**

Figure 1. Geographic distribution of brucellosis. Incidence (cases/10 000 persons) according to district.

- there is a need to link food safety and zoonotic aspects to the public health sectors so that public interest and funding resources can be increased;
- the livestock sector is undeveloped and consumers do not have much influence or organisation. The veterinary service should therefore take this opportunity to present a comprehensive plan for national animal health with benefits for both consumers through safety food and the livestock sector itself through better production and trade;
- budgets need to be in place to meet all needs including coordination with many of the international organisations to secure funding for specific activities within the comprehensive NAHP;
- the foundation of a reliable NAHP is a scientifically based surveillance system in which contingency planning is incorporated for specific health events.

Data Collection

Our data originated from the Department of Animal Health in the Food Safety and Veterinary Institute and direct contact with District Veterinary Services throughout the country.

RESULTS AND DISCUSSION

The Main Animal Diseases

Brucellosis

The main problem is brucellosis in ruminants, caused by *B. melitensis*, which is widespread in several districts of the country. The infection reached its highest levels between 1960 and 1965, and subsequently decreased through the implementation of different control measures. In 1989, the country was proclaimed free of bovine brucellosis, with a low prevalence (0.002%) in small ruminants. During this period, compulsory herd testing and removal of reactors was successfully implemented and helped to eliminate positive animals. Moreover, application of the B–19 strain vaccine in cattle in combination with animal tracing yielded significant results. After the political and economic changes in the 1990s, the infection spread in animals throughout the country and reached its climax in 2000. The uncontrolled movement of animals, the failure to apply sanitary and quarantine measures as well as the low level of cultural and technical education of farmers, together with a limited budget for implementing an eradication strategy (total screening, total elimination of positive heads), led to this expansion across the country. This infection currently affects the entire territory of the country, reaching a prevalence of 10% in several districts such as Saranda and Gjirokaster. The number of persons affected by brucellosis is increasing, particularly in rural areas (Kakariqi, 2006).

A new strategy for the control of brucellosis in Albania using *B. melitensis* rev 1 strain vaccine was introduced in 2003, and is starting to have positive results.

Table 1. Number of pigs vaccinated against classical swine fever.

Year	Number of vaccinated pigs
1996	11 504
1997	13 702
1998	51 775
1999	37 927
2000	29 611
2001	7 115
2002	6 332
2003	51 524
2004	42 045
2005	20 055
2006	10 187
2007	17 679

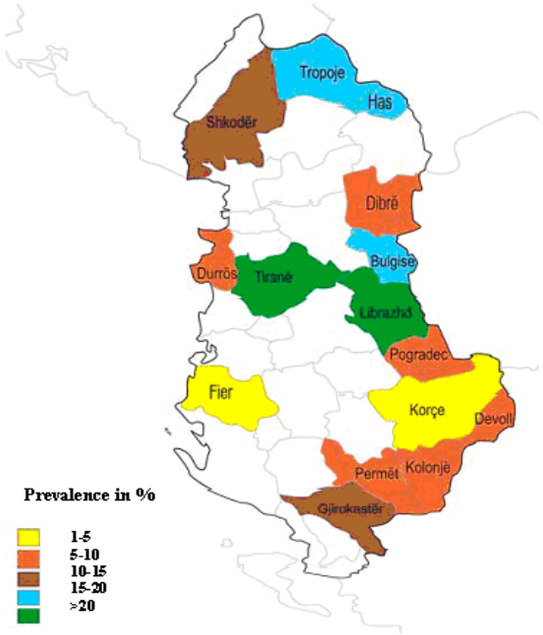

Figure 2. Seroprevalence of BTV in Albania in 2002.

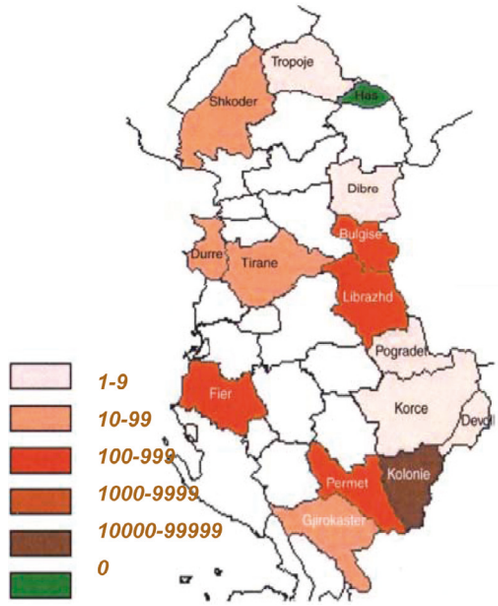

Figure 3. Abundance of *Culicoides* spp. in Albania (October-November 2002).

Classical Swine Fever

This was first diagnosed in 1966 in southeast Albania and outbreaks of the disease were later identified elsewhere based on clinical signs and postmortem lesions (it originated from swine imported from China). The disease was eradicated subsequently following drastic measures which included massive culling, quarantine, etc. The infection reappeared in 1975 in a reserve of wild boars in the south of Albania (Karaburun).

The latest and most severe outbreaks of the disease were identified in 1996 (using direct immunofluorescence test in the Food Safety and Veterinary Institute) when 5 515 animals out of 35 235 swine were infected and 3 683 animals died. The rest of the swine population was subjected to compulsory slaughtering and despite the very restricted financial possibilities, the State reimbursed the cost of the operation. The disease was kept under control by applying surveillance, quarantine, stamping out and vaccination

In general there are now only sporadic outbreaks of infection in the north of the country, although vaccination is still applied in old outbreak areas (**Table 1**).

Bluetongue Virus (BTV)

This was detected for the first time in Albania in 2002 (Di Ventura et al., 2004) with a seroprevalence of 15% (**Figure 2**) determined by competitive ELISA and virus neutralisation assays. During that year a survey for *Culicoides*, was also made. Twenty species were identified in the collections (**Figure 3**). The finding that serotype 9 of the virus was the only one involved suggested that BTV infection came from neighboring countries. However, evidence of BTV circulation in Albania and the absence of the main vector *C. imicola* suggest that other *Culicoides* species could be implicated in virus transmission. The high abundance of the Obsoletus complex, from which BTV was recently isolated in outbreaks where no specimens of *C. imicola* were captured, also suggest that probably the vector belong to this complex (Goffredo et al., 2003)

H5N1 Avian Influenza

The first case in Albania was confirmed on 7th March 2006 in a dead chicken from a backyard flock in the village of Cuke in the Sarande district of southern Albania, and a further case was confirmed on 21 March 2006 in the village of Peze-Helmes near Tirana. Dead chicks were sent to the Animal Health Laboratory in Tirana where avian influenza virus was isolated on SPAF eggs and detected using haemagglutination inhibition (HI) and agar immunodiffusion (AGID) tests. The isolate was sent to the OIE Reference Laboratory in Weybridge, UK which confirmed the presence of H5 with a final typing of H5N1.

In both villages outbreaks were confined to backyerd poultry and there were no cases in humans. Also, infections occurred on holdings with direct access to an adjacent floodplain and appear to have resulted from contact with infected migrating waterfowl. The level of contact may have been no more than some hens foraging on a pasture where waterfowl had grazed some time previously. The severity of the outbreak was limited by the fact that the majority of flocks in the village were either housed or confined within a walled garden. The response measures against AI during outbreaks in Cuke and Peze were well planned.

Following confirmation of the outbreak, the authorities put into operation the contingency plan which had been drawn up for such an eventuality. One of the first tasks was to conduct an awareness campaign among the villagers outlining the measures which would be implemented and the precautions that they should take. At this stage a preliminary appraisal was made of the number of poultry to be slaughtered and the manpower and resources required. A landfill site for the disposal of culled birds and contaminated material was identified and prepared. A number of meetings were held with per-

sonnel engaged in the operation where areas of responsibility were identified and delegated. Training was provided to hired workers on technical and safety aspects of culling and disinfection to ensure a professional and effective performance of these tasks.

Quarantine

A quarantine zone was established around the village with disinfection points for people and vehicles at the main access points. This action appeared to be very successful with a high level of compliance by all inhabitants. Within a three km zone, biosecurity measures were implemented including a requirement that all poultry must be housed and all illness and mortality in birds be reported to the veterinary service of the communes.

Depopulation and Disinfection

Culling and disinfection operations were carried out by three teams each under the supervision of a veterinarian. The teams consisted of a pickup truck and driver, a record keeper, two workers responsible for culling operations and one for disinfection. The teams had police support if required but most flock owners were very cooperative. Birds were killed by dislocation of the neck before being put in black plastic sacks. Initially it was debated whether it was necessary to kill the birds or let them die by suffocation. In practice killing proved both more humane and more efficient as it precluded any escape of birds from a torn bag in transit or at the landfill.

Disposal of Dead and Diseased Birds

The landfill was located about 2 km from the village. The site was well located being easily accessible from the village but isolated from any habitation. It was under constant supervision during the culling operation and was closed with topsoil at the end of each day to prevent scavenging by foxes or dogs.

Rabies

This was a disease of concern from 1928 until 1976. Cases were reported sporadically and most of them in wild animals (wolves, foxes, jackals, etc) and in stray cats and dogs. From 1976 up to 2000, rabies cases were minimal and the country was classified as free from disease, even if in neighbouring counties the disease was increasing. This status of 'freedom from disease' for that period can be explained by the fact that at the time the veterinary service was well organised and the border was protected by a permanent barrier called Clone[1].

The disease reoccurred in Albania in March 2001 in Morine village in the Kukes district affecting a domestic dog and three persons were bitten who forunately received the appropriate treartment quickly. Since then, further cases have been reported in northern Albania. Specifically:

- in November 2002 a fox was diagnosed positive in Qereti village in Puka district;
- in March 2003 a further two foxes were found positive in Gjorica village in Bulqiza district;
- in May 2004 a rabid wolf wandered around the villages of Perollaj, Helshan, and Zahrishte in the Has district. As a consequence of this incident, 21 animals of different species were bitten and about seven of them showed clinical symptoms; (**Figure 4**)
- since then, no cases of rabies have been reported;
- the rabies virus was identified by brain histo-pathological findings and immuno-fluorescence microscopy on fresh brain tissues.

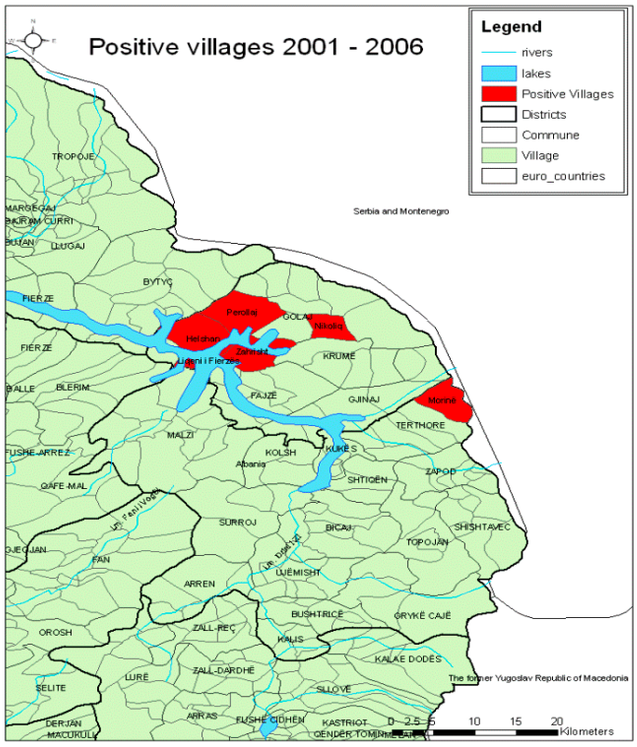

Figure 4. Villages with cases of rabies (in red) from 2001–2006.

Control measures comprise:

- vaccination of remaining dogs in the village;
- disinfection of likely contaminated areas;
- enforcement of quarantine measures;
- suspension of trade in live animals and by-products;
- strict surveillance of remaining dogs in the village;

Rabies monitoring in animals is performed by the Food Safety and Veterinary Institute. Laboratory confirmation of rabies virus is based on positive results obtained by the direct fluorescent antibody test (IFAT) using antirabies test serum from 'SIFIN'-Gmbh, histopathological examination of 'Negri' corpuscles and the mouse inoculation test (MIT). A large-scale rabies survey started in 1997 involving different geographical regions and focussing mainly on red foxes and other wild terrestrial carnivores; dogs, cats and bats are included as well. During the period 1997–2009, 1 220 animals, comprising 681 wild carnivores, 409 bats and 130 domestic animals were destroyed.

Unfortunately, Albania has not yet been able to introduce mass wildlife immunisation with oral vaccines due to lack of well-developed vaccination strategies based on prior ecological studies of target animals, appropriate planning, trained personnel and of course, adequate funding.

CONCLUSIONS

Government expenditures on agriculture have decreased in recent years, with the bulk of Ministry of Agriculture, Food and Consumer

1 Clone is a permanent barrier covering the entire Albanian national boundary and consisting of by a large densely fenced spine wire. This fence was constructed to prevent the illegal crossing of the border, but it also prevents the movement of wild fauna across the border.

Protection (MAFCP) expenditures (48% of the budget in 2005) going to investments in irrigation and drainage infrastructure. However, the 'Agricultural and Food Safety Inspections and Services and Consumer Protection' Programme (the third largest in MAFCP with 15% o f the MAFCP budget), is expected to see significant increases in the coming years to strengthen inspection services.

Given the limited absorptive capacity of relevant institutions, additional funding should be selectively applied and well prioritised. Establishing a national food safety system consistent with the EU Acquis will require increased levels of public expenditures on food safety, veterinary and phytosanitary activities. Establishing a national food safety system consistent with the Acquis is a priority and adequate financial resources should be allocated to support the creation of institutions and systems and to upgrade skills.

The challenge faced by a reliable National Animal Health Programme, is to sustain livestock production, including social needs, in the agricultural community, through modern economic approaches. The NAHP can be the core for this type of sustainability due to the trust of the agricultural community in the veterinary input. However, the NAHP should use police authority to implement necessary quarantine measures in case of tranbaundary diseases.

The overall capacity of the country's laboratories is deficient and should be further developed in order to provide food testing and analytical services that meet international standards and requirements. Although there are 36 laboratories throughout the country, most have inadequate or outdated equipment and infrastructure, a shortage of competent analytical and managerial staff, no official working methods or procedures or business plans, and poorly developed systems for recording test results, reporting, and information management. In some cases, laboratories have obtained sophisticated equipment under international projects, but analysts and technicians lack the necessary skills to operate and maintain them. Other challenges to be overcome include shortages in the power supply and lack of funds to meet operating costs. Many laboratories face difficulties in obtaining essential supplies of materials, reagents and services (disposable materials, reagents, gases, calibration and maintenance services), and lack access to technical support for calibration and reference testing. A further problem is the loss of qualified staff, including individuals trained by donor projects, either through dismissal or transitioning to other jobs.

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