Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

Animal Production Unit

Activities Report

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FAO/IAEA Agriculture & Biotechnology Laboratory
IAEA Laboratories Seibersdorf
International Atomic Energy Agency
Vienna, Austria

Executive Summary

The control of major transboundary diseases that affect many developing countries requires effective early and rapid diagnosis, utilizing sensitive and specific tests that identify infected livestock and enable appropriate treatments to be implemented. The Animal Production Unit (APU) has achieved a number of successes in developing diagnostic technologies that will contribute to this goal. One of these tests, that is in an advanced stage of development is an Enzyme Linked Immunosorbent Assay (ELISA) for the detection of infection with Contagious Bovine Pleuropneumonia (CBPP), a disease that is endemic in some 30 African countries and is a severe constraint on livestock productivity. Preliminary development in APU has established that this assay is specific for CBPP. These findings were provided to participants in an Animal Production and Health Sub programme, Collaborative Research Programme on CBPP and for further investigations into its diagnostic sensitivity and specificity using sera from naturally infected cattle, the test has been transferred to a Member State laboratory in Mali where the validation will be completed. APU strives to develop diagnostic technologies that can provide the basis for good animal health care to rural communities in developing countries. Some of these technologies may be used in well-equipped laboratories, but consideration is also given to tests that directly assist the small holder farmer by providing point-of-care diagnosis. Loop mediated isothermal amplification (LAMP) allows molecular technologies to be applied without sophisticated instruments as it can be carried out without a thermal cycler and the results can seen visually. LAMP technologies were developed for the use with Avian Influenza, CBPP and Peste des Petits Ruminants (PPR) and the findings suggest that it should be possible to develop rapid, quality assured tests that could be used at the penside.

Major advances have also been made in the diagnosis of capripox (CaPVs) viruses. Studies on the chemokine gene of CaPVs has shown that they grouped into three distinct lineages, namely sheep pox virus (SPPV), goat pox virus (GPPV) and lumpy skin disease virus (LSDV). Based on these findings, real time PCR rests have been developed that enable specific identification of SPPV, GPPV and LSDV.

Studies on the N protein of Peste des Petits Ruminants have identified a polypeptide that has a unique specificity for PPR. ELISA tests have been developed using this polypeptide as antigen and preliminary laboratory studies have been encouraging. The test requires further validation with serum samples from field to determine its diagnostic sensitivity and specificity. An alternative, competitive ELISA test is also being developed, targeting the H protein of PPR. In another approach to diagnosis, a real time PCR has been developed using a dual labelled fluorogenic probe and N protein primers. The test appears to detect all lineages of PPRV and will undergo further tests to determine its diagnostic sensitivity and specificity.

In animal genetics, the development of bioinformatics and genomics tools continues to form an integral part of the strategy for providing MS with the means for utilizing the genetic resources within their national herds. APU has developed a program to manage a Genetic Repository Bank on sheep, which can be expanded to include other livestock species. The application does not require software installation on user computers and can work on all web-browsers. APU has also worked extensively on the development of an RT-db (Real-Time database) for Quantitative Trait Loci (QTL)/Genes/DNA Sequences and Genetic characterization in small ruminants. The database will make available the genomic locations of QTL from all known studies on small ruminants. APU was involved in the genotyping of four sheep chromosomes and QTL expression was used to find the linkage map for a genome scan including these four markers. Comparative mapping was use to fine-map a chromosomal region to find genes responsible for parasite resistance. A total of 809 genes were identified and 149 genes involved in immune response pathways were selected as candidate genes for further analysis.

The APU is also involved in a project on Gene-based Technologies in Livestock Breeding: Characterization of Small Ruminant Genetic Resources in Asia, and a project on the Genomics of the Alpaca: Identification of Expressed Genes and Genetic Markers Associated with Productivity and Embryonic Mortality. On the animal health side, the Unit is involved in work on The Early and Sensitive Diagnosis and Control of Peste des Petits Ruminants and continues to support the work on Veterinary Surveillance of Rift Valley Fever.

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1. PROGRAMMATIC AND UNIT OBJECTIVES

The vision and goal of the Animal Production and Health (APH) Sub-programme are to minimized risks to livestock in FAO and IAEA Member States in order to increase food security, to fight hunger and to improve the livelihoods of the poor in FAO and IAEA Member States. To achieve this objective, two strategies guide the activities of the Sub-programme:

1. Capacity building within regions and countries.
Success in the control of highly infectious diseases relies on the capacity of early warning and early reaction, a capacity that is missing in many developing countries because of a lack of financial resources and also human and physical resources. Training of scientists is important for helping developing countries to manage different risks that are threatening their livestock production.

2. Promotion of applied research targeting areas that help alleviate risks for livestock in developing FAO and IAEA Member States.
This involves promoting the transfer of technologies, in particular, nuclear and nuclear-related techniques to developing countries, promoting and implementing applied research projects for the development of improved diagnostic tests and vaccines, for leading to better breeding strategies, and to improved farm management to optimize the use of animal feed resources and hence protect the environment.

The Animal Production Unit has worked very closely with the Section in all these activities.

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3. RESEARCH AND DEVELOPMENT ACTIVITIES

3.1. Animal Genetics

There is considerable genetic diversity among sheep and goat breeds in Asia, but there has been little attempt to exploit this diversity to increase small ruminant productivity. One reason for this is the lack of coherent organized breeding strategies. However, by enabling Member States (MS) to access the detailed genetic information available in various databases their capacity to realise the genetic potential of livestock would be improved. IAEA has transferred to several MS the technical capacity for DNA analysis and Marker Assisted Selection (MAS). The network through which this process is facilitated is illustrated diagrammatically in **Figure 1**. Small ruminants are a principal source of animal protein in many developing countries. Their biodiversity is often expressed in well-adapted traits that enable them to survive harsh, local environmental conditions or show resistance to endemic diseases where other, exotic breeds cannot thrive. Unfortunately, since such traits are not sufficiently characterized, they are underutilized in conventional breeding programmes and there is insufficient research on the ways to select breeds or individuals carrying the most advantageous traits. The characterization and mapping of genes controlling such traits – "quantitative trait loci" (QTL) and the subsequent use of this information in selection and breeding programmes, should make it possible to facilitate significant increases in small ruminant productivity.

The aim of the genetics group of the Animal Production Unit (APU) is to develop and deliver gene-based tools to MS for management of their indigenous livestock. There are two major project areas: the first is to identify chromosomal regions of sheep associated with resistance to intestinal parasites, particularly *Haemonchus contortus*; the second is the establishment of a bioinformatics database for analysis of the small ruminant genome.

Investigations undertaken by APU in collaboration with ILRI and USDA in the past five years on genetic resistance to parasitoses in crossbreeds of Red Maasai (parasite-resistant) and Dorper (susceptible) breeds of sheep has provided evidence of interesting QTL on several chromosomes. These results have provided the basis for increasing the technical capacity of participants in Technical Cooperation (TC) and Coordinated Research Projects (CRP) in MS to enable them to carry out DNA analysis on indigenous small ruminants. Further characterization of the QTL will provide the means to test local sheep and goat populations for genes linked to parasite resistance. These assays are based on single nucleotide polymorphisms (SNP) and use simple, low cost DNA technologies. In order to identify and develop assays relevant for a number of breeds, each SNP must be verified and characterized in different populations. A genetic repository for small ruminants, consisting of DNA and blood samples, has been established at the FAO/IAEA Laboratories Seibersdorf. Collaborators in a number of MS provided APU with samples from representative breeds and the repository now contains over 700 samples from 32 breeds of small ruminants from 12 countries.

Figure 1. Schematic overview of the animal health and genetic network.
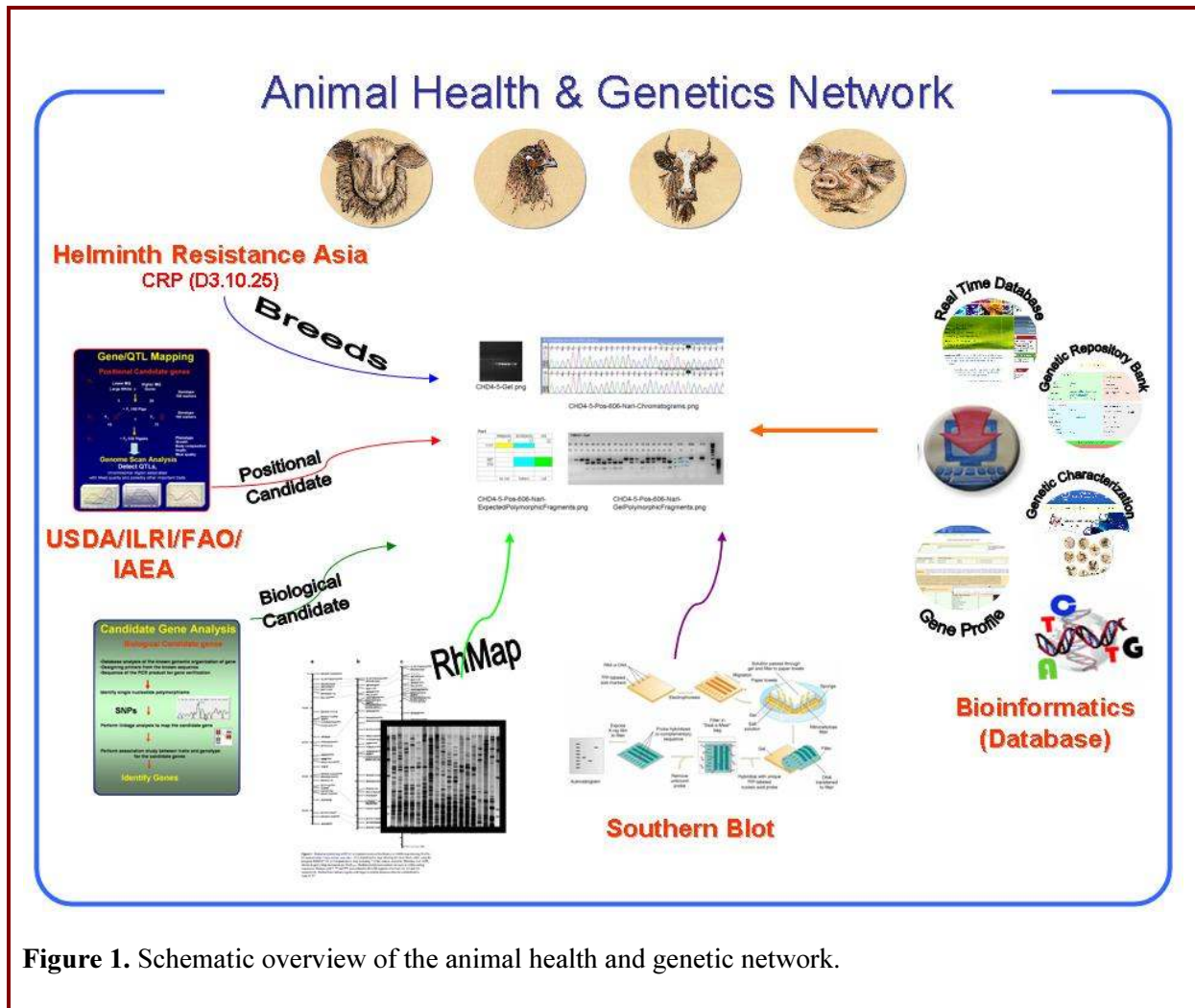
An integral part of our research programme is to ensure that we provide the means for increasing the technical capacity of MS in the use of bioinformatics. Several tools have been deployed to participating national research institutes. The first is an online program to manage a Genetic Repository Bank in sheep that will enable scientists to insert their own information into the database and graphically view the origin of samples through links to Google Map. This will mean that counterpart laboratories can liaise with APU and be provided with updated information, such as laboratory protocols, standard operating procedures, nuclear and related techniques, methodologies and procedures, detailed genome search and analysis tools, radiation hybrid map information and livestock molecular markers database.

The genotyping for all 18 microsatellite markers of the sheep has been completed at the Joint ILRI/CAAS Animal Molecular Genetics Laboratory in Beijing. The genotyping of 37 breeds of goats for 15 microsatellite markers has also been completed. The IAEA Collaborating Centre on Animal Genomics and Bioinformatics, located in Brazil, a contract holder in the CRP "Gene-based Technologies in Livestock Breeding: Characterization of Small Ruminant Genetic Resources in Asia" (D3.10.25) has been responsible for sequencing the mitochondrial DNA of a subset of individuals from Asian sheep and goat breeds, to reveal differences among them in the D-loop region of the mtDNA. The São Paulo State University (UNESP)

group, lead by Mr Fernando Garcia, has developed kits for DNA amplification, which were distributed to the nine counterparts. DNA sequencing work is underway in his laboratory and the data generated will be analysed in order to provide information about genetic diversity among sheep and goat breeds, facilitating decision-making on their conservation. Phenotypic and farming system information has been collected for each breed, and will be inserted into the Domestic Animal Diversity Information System (DAD-IS) of the FAO. Protocols for genotyping of SNP in various candidate genes that may influence traits of economic importance in small ruminants are in progress at APU. The genotyping and data analysis needed for basic genetic characterization is expected to remain a focus of our activities for the foreseeable future.

MS in Asia have been able to characterize approximately 100 breeds of sheep and goats. The characterization included analysis of both phenotypic and genetic data, including the genotypes of microsatellites from a standard FAO panel. The information will allow the participants to evaluate local biodiversity both within and across breeds. Currently, the genetics group at APU is building a web-accessible database for the CRP participants from which they will be able to view and download data. The eventual goal is to make this a global resource, with results from other characterization studies being included, together with data from new information generated during the course of the CRP. Breeds with unique genetic characteristics are considered to be more valuable for conservation programmes hence, by comparing the allelic frequency at common loci across breeds, MS will be able to compare the genetic profiles of indigenous breeds with those from surrounding countries and other regions. These activities will increase the genomic information on sheep and goats and disseminate it widely. It is likely that genomic tools for disease resistance, wool and meat quality and other traits will become available in the future. The use of these genetic markers will help to increase the speed and efficiency of increased productive performance in a population and assist in breeding genetically superior stock.

The APU is also participating in the Sheep HapMap project of the International Sheep Genomics Consortium (ISGC), Australia, in order to acquire detailed information regarding the location of genes affecting important productive traits in the African Dorper sheep.

3.1.1. *Identification of Host Candidate Genes for Parasite Resistance in Small Ruminants*

It is intended to identify sheep chromosome that are associated with parasite resistance, based on molecular genome scan analysis. It is likely that the majority of anonymous genetic markers will have no effect on performance traits themselves, but such markers do make it possible to identify areas of the genome containing important genes. Genes closely linked to the marker will generally be inherited with it. Genetic markers will help trace regions of chromosomes from parents to offspring. To refine these findings a positional candidate gene approach was used (**Figure 2**).
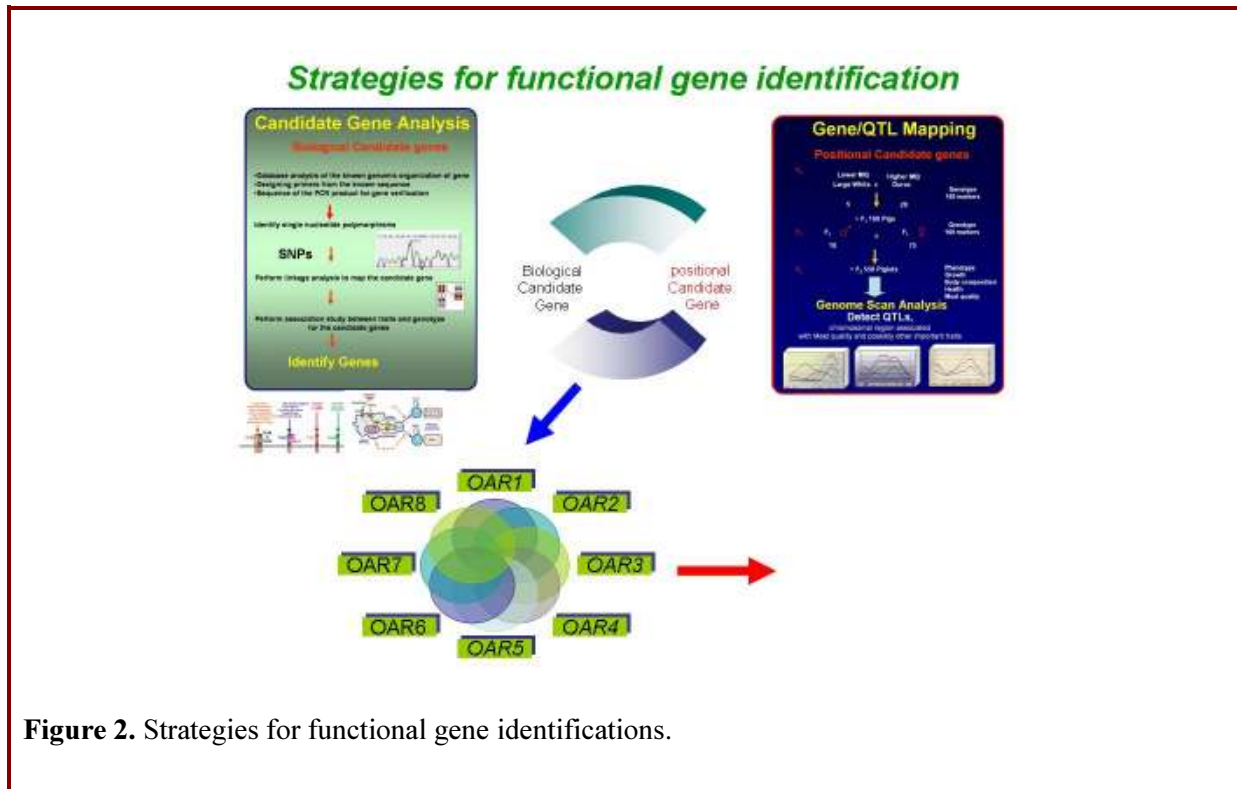
Figure 2. Strategies for functional gene identifications.

3.1.1.1. Sheep genome mapping (genome scan linkage analysis) for identification of parasite resistance markers

APU, in collaboration with ILRI and the USDA, analysed data from genome scan studies in small ruminants (both linkage and QTL analysis) to plan for the future direction of the programme. The aim of the sheep genome mapping project is to analyze and evaluate a genome scan based on a cross between Red Maasai and Dorper breeds. Preliminary analyses have provided evidence for QTLs on several chromosomes. Although genome scans can identify chromosomal regions that contain QTLs associated with parasite resistance and related traits, this approach has only limited mapping resolution, therefore, following the genome scan, a candidate gene approach was used in order to more fully refine the identity of the potential QTL.

3.1.1.2. Strategies for identification of parasite resistant markers in small ruminants

The development of DNA-based markers has had a significant impact on gene mapping, as they make it possible to exploit the entire diversity in DNA sequences that exist in any crossbreed. For this reason, high resolution genetic maps are being developed.

APU was involved in the genotyping of four sheep chromosomes (OAR21, OAR23, OAR25 and OAR26) as shown in **Figure 3**. QTL expression was used to find the linkage map for a genome scan including these four markers.
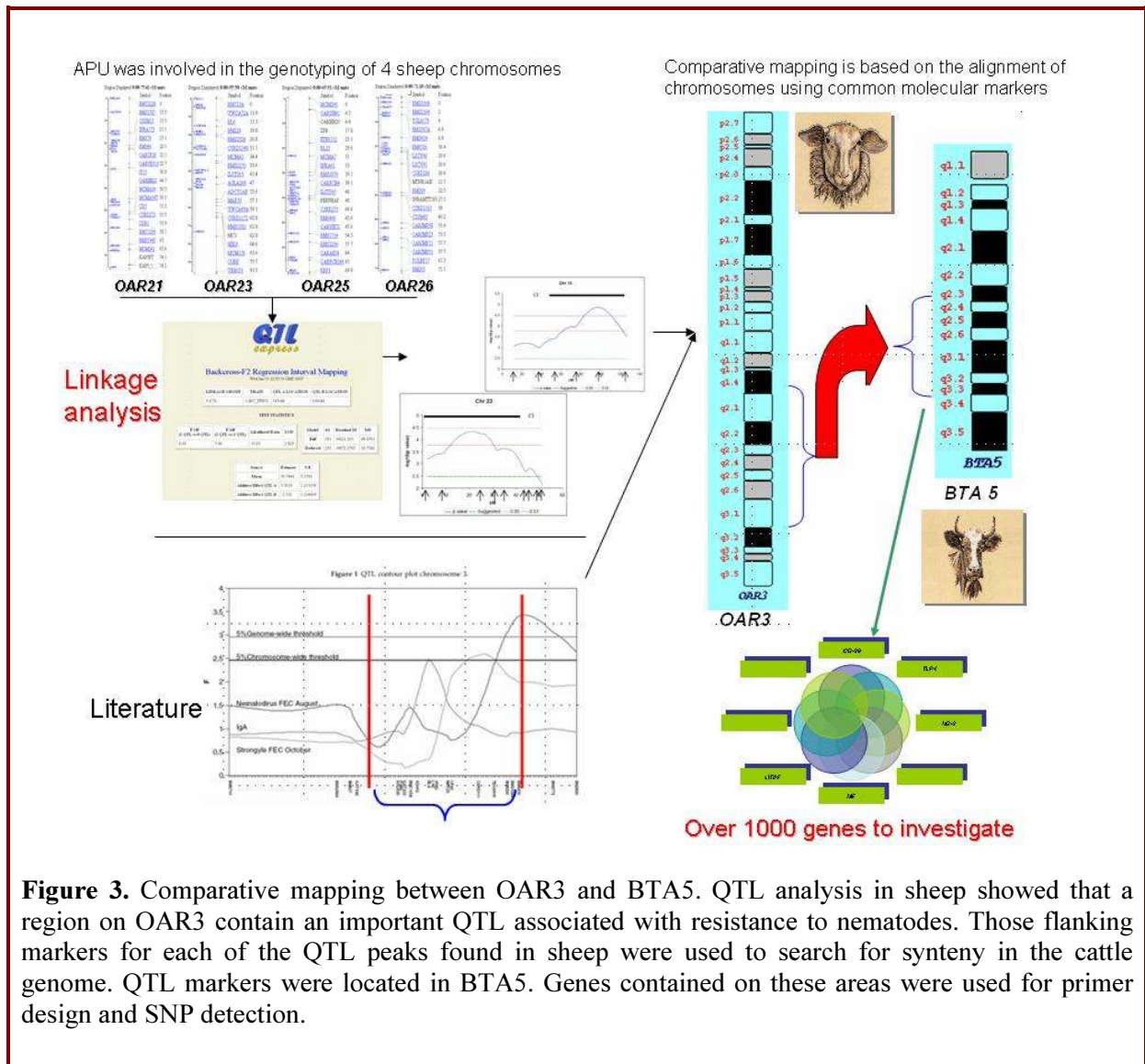
Figure 3. Comparative mapping between OAR3 and BTA5. QTL analysis in sheep showed that a region on OAR3 contain an important QTL associated with resistance to nematodes. Those flanking markers for each of the QTL peaks found in sheep were used to search for synteny in the cattle genome. QTL markers were located in BTA5. Genes contained on these areas were used for primer design and SNP detection.

Positional comparative mapping helps researchers interpret the results from the genomic map of one species based on those obtained from another, more extensively characterized, species. Since there is a high level of synteny (physical co-localization of genetic loci on the same chromosome) between the genomes of different animal species, it is possible to extrapolate characterized information on genes of interest, with another, in which only quantitative information (markers shared between those two maps) is available. Markers in this way operate as anchors that allow identification of regions on two different genomes that could share synteny. This tool helps investigations in sheep, where although insufficient information is available, molecular markers occur that are shared with cattle, in which the genome is more extensively characterized and mapped. Comparative mapping shows that cattle chromosome 5 (BTA 5) shares regions of homology with sheep chromosome 3 (OAR3) (**Figure 3**). The positional candidate gene approach allows us to combine information about a gene's chromosomal location for easier identification of a potential causative gene. It is assumed that candidate genes represent a large proportion of the QTL that has been previously identified from the parasite resistance genome scan. The positional candidate gene approach relies on a four stage process:

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1) Localizing the area of interest to a chromosomal sub-region, which is based on literature review and preliminary results from the parasite resistance genome scan

2) Searching databases for attractive candidate genes within the sub-region

3) Testing the candidate gene for causative mutations

4) Developing simple, inexpensive, and robust nuclear-based tests that can be used by MS in their own laboratories

As explained above, comparative mapping was use to address this issue, and to fine-map a chromosomal region (**Figure 3**) and improve the ability to find genes responsible for parasite resistance. A total of 809 genes were selected based on the cattle BTA5 region sharing synteny with markers on the OAR3 QTL region associated with parasite resistance on previous scans (**Figure 3**).

The selected genes were assessed for their putative function using the programme GeneCards (http://www.genecards.org/index.shtml). In this step, gene function, protein ID, and biochemical pathways were annotated for all genes. After clearance, 149 genes involved in immune response pathways were selected as candidate genes for further analysis. The next objective was the development of a test to detect Single Nucleotide Polymorphisms (SNPs) associated with parasite resistance in sheep for the 149 selected genes as an input into the current CRP on small ruminant genetic characterization in Asia (CRP.D.3.10.25).

The following steps were followed for the identification of SNPs in candidate genes:

1) Selection of a candidate gene

2) Search of databases for known protein function of the candidate gene and selection of genes involved in immune responses

3) Designing primers based on information from the known cattle sequences

4) Sequencing of the PCR products for gene verification

5) Amplification of pooled genomic DNA samples to find polymorphisms

6) Designing polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) tests to allow amplification and analysis of large numbers of individuals

7) Analysis of associations between traits of interest and genotypes for the selected candidate gene (**Figure 4**).
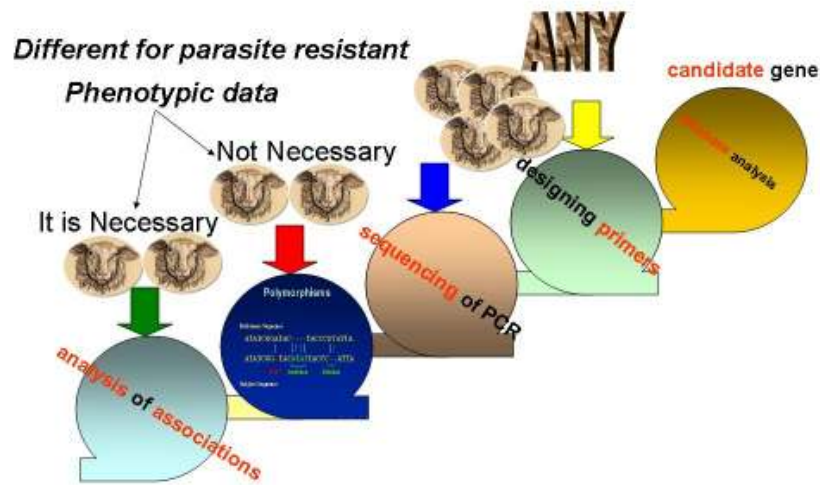
Figure 4. Processes involved in the identification of SNPs in candidate genes.

PCR primers from selected genes were developed for sheep based on published sequences of cattle from the US National Center for Biotechnology Information (NCBI). The primers were standardised with genomic DNA from a local Austrian breed of sheep. After primer standardization, over 200 primers were optimised for PCR and amplified using the same Austrian breed for sequence verification. After sequence verification, a subset of samples from the most representative breeds from different populations were pooled, amplified, and then sequenced again. Analysing this data allowed identification and selection of 100 SNPs on different genes.

3.1.1.3.   Sequence variation and PCR-RFLP

Restriction enzyme cut sites were selected from the polymorphisms following digestion of amplified DNA in order to genotype the alleles by PCR-RFLP in agarose gels. A primer set (**Table 1**) was used to amplify ten selected candidate genes. PCR-RFLP tests for selected genes (**Table 2**) were genotyped for 144 individual sheep from different populations in order to confirm these markers. Restriction endonuclease digestion of total genomic DNA was followed by hybridization with a labelled probe (short-lived radioisotopes) which revealed differently-sized hybridising fragments (**Figure 5**). An additional eight genes have been directly sequenced by barcode sequencing through an out-sourcing company (**Table 2**).

Table 1. Primer design for polymorphism identification of selected genes.

Gene	Project	Primer	Sequence	PCR product (bp)	Annealing Tm/Time/Mgcl$_2$
FGD6	RFLP-SB	Forward	5'-CTCCTGTCGCACCTAAACCT-3'	1051	55 ^{o}C / 1 min/1.5
		Reverse	5'-TTCACTTTGTCCACCTGTTCC-3'		
		SB Probe	5'-TGTCTTCATCAGACTGCTTT-3'		
PLXNC1	RFLP-SB	Forward	5'-CATTTGTTTCTCTGGCTGCT-3'	841	55 ^{o}C / 55 s/1.5
		Reverse	5'-CCTTTATTTACCCTTGGTGCT-3'		
		SB Probe 1	5'-TGTCTTCACATCAAGGCTGCA-3'		
		SB Probe 2	5'-TCCTGCTATCTCATAGATTGTT-3'		
OAR3-/C1R	RFLP-SB	Forward	5'-ATCCTACCACAAAGAAAAGATGC-3'	806	50 ^{o}C / 55 s/1.5
		Reverse	5'-GCGGATAGAAAAAGATAGATTG-3'		
		SB Probe	5'-TAGCTGTGTTTAGGCTATTACA-3'		
CSF2RB	RFLP-SB	Forward	5'-GTGAAGAGGCAGGGCAAG-3'	715	60 ^{o}C / 50 s/1.5
		Reverse	5'-AAGGGGGCAGTGACAAGTAG-3'		
		SB Probe 1	5'-TCCTGGACTGGCTAATGGGCCC-3'		
		SB Probe 2	5'-TCCCTCCAGCCACAGGCCAGTT-3'		
SMARCC2	PCR-SB	Forward	5'-TTCCCGAGTCCTCTCCTTT-3'	834	57 ^{o}C / 55 s/1.5
		Reverse	5'-ACTTTCCCTTTCTTGGTGG-3'		
		SB Probe 1	5'-TCAGCACCTCCATTTATAGTAA-3'		
		SB Probe 2	5'-TCATCTTACTTGTGGTTCCGAT-3'		
ARHGAP9	PCR-SB	Forward	5'-ACCTGGGAATTGTGTTTG-3'	769	57 ^{o}C / 55 s/2.0
		Reverse	5'-TTTGTGGCTATTCTGGATG-3'		
		Probe mid A	5'-TAAAGAGGAAGGTGGCTGGATT-3'		
		Probe 3end A	5'-TGAGGAAACTTCTCTAAAGAGGA-3'		
		Probe 5end A	5'-AAGGTGGCTGGATTAGCCCCT-3'		
		Probe mid G	5'-TAAAGAGGGAGGTGGCTGGATT-3'		
		Probe 3end G	5'-TGAGGAAACTTCTCTAAAGAGGG-3'		
		Probe 5end G	5'-GAGGTGGCTGGATTAGCCCCT-3'		
MYF6	PCR-SB	Forward	5'-ATACAAGCCCAAAGCCGAAAG-3'	938	55 ^{o}C / 55 s/1.5
		Reverse	5'-CAGACCCAAGCAAGAAAATGTG–3'		
		Probe mid AC	5'-TGCTTTCTACCCCCAGAGAGCT-3'		
		Probe mid AT	5'-TGCTTTCTATCCCCAGAGAGCT-3'		
		Probe mid CC	5'-TGCTTTCTCCCCCCAGAGAGCT-3'		
		Probe mid CT	5'-TGCTTTCTCTCCCCAGAGAGCT-3'		
PTPRB	PCR-SB	Forward	5'-GCAGTCCACAAGGTCACAA-3'	790	60 ^{o}C / 55 s/1.5
		Reverse	5'-TCAGAGCAAGGAAGTTACGG-3'		
		Probe mid C	5'-TGAGGGCCTTTCCCTCAAACCT-3'		
		Probe 3end C	5'-TGCCACTTTTTGAGGGCCTTTCC-3'		
		Probe 5end C	5'-CCTCAAACCTTTGGGGAGAA-3'		
		Probe mid T	5'-TGAGGGCCTTTCTCTCAAACCT-3'		
		Probe 3end T	5'-TGCCACTTTTTGAGGGCCTTTCT-3'		
		Probe 5end T	5'-TCTCAAACCTTTGGGGAGAA-3'		
SLC11A2	Sequencing	Forward	5'-TGAGGGAGATGTGTTGTTGG-3'	876	57 ^{o}C / 55 s/1.5
		Reverse	5'-TGACAGAGGCTGGAGGATTG–3'		
SLC11A2	Sequencing	Forward	5'-CATCAGAGCCAGTGTGTTTC-3'	1006	59 ^{o}C / 60 s/1.5
		Reverse	5'-CCAACGCTTACATCCAGAG-3'		

Gene	Project	Primer	Sequence	PCR product (bp)	Annealing Tm/Time/Mgcl$_2$
MARS	PCR-RFLP	Forward	5'-CCTGGAAACAAGGAGGTAAG-3'	721	55 °C / 50 s/1.5
		Reverse	5'-AGGCAAGAAGAAAAAGTGAAAG-3'		
CSF2RB	PCR-RFLP	Forward	5'-GGACCCTAAAGATGCCTGTG-3'	720	60 °C / 50 s/1.5
		Reverse	5'-TAGAGGGGACTGGGGAAACT-3'		
TIMP3	PCR-RFLP	Forward	5'-CCTGCTACTACCTGCCTTG-3'	967	57 °C / 55 s/1.5
		Reverse	5'-CACATTGCCTCCTGACCTC-3'		
ANKRD28	PCR-RFLP	Forward	5'-AGCATCACCCCTCTCTG-3'	702	57 °C / 50 s/1.5
		Reverse	5'-ACCCACCTTCTTCATCCA-3'		
CHD4	PCR-RFLP	Forward	5'-GCTACCTCTGTTGGCATC-3'	1117	60 °C / 60 s/1.5
		Reverse	5'-CTCGGCTCACCTGGAAG-3'		
CLEC1A	PCR-RFLP	Forward	5'-GTTAAGGCATTTTCGTGAG-3'	1030	57 °C / 60 s/1.5
		Reverse	5'-ACTTGGTCTATTCTCTGTAC-3'		
ITGA5	PCR-RFLP	Forward	5'-TGGAAGTGTTTGGGTGAGTG-3'	1166	60 °C / 60 s/1.5
		Reverse	5'-GAGCAGGGTGGTTTAGAGGA-3'		
ZBTB39	PCR-RFLP	Forward	5'-TCCAGAACCTCTTCCTCAAC-3'	946	54 °C / 60 s/1.5
		Reverse	5'-TTGCAGTTGCCAGTCAGTAG-3'		
SKR3	PCR-RFLP	Forward	5'-GCCTCCTTTTTGTCGCATAG-3'	885	50 °C / 55 s/1.5
		Reverse	5'-GAAACTCAGCAATGGTCTCC-3'		
FAM62A2	PCR-RFLP	Forward	5'-TCCTGGATGTCTTCTCTTACC-3'	803	54 °C / 55 s/1.5
		Reverse	5'-GCTCTTCTGTGACCAGTTCC-3'		
NAV3	PCR-RFLP	Forward	5'-CTTGCTTTCTCAGTGGTTATG-3'	826	52 °C / 55 s/1.5
		Reverse	5'-CATCTTCAGGGGTAAATGGT-3'		
RPS6KB2	PCR-RFLP	Forward	5'-GTTCTAGCCATGTGCTCTTCCT3'	1004	50 °C / 60 s/1.5
		Reverse	5'-ACCTTCCCTTTCGACTTTTGTT-3'		
KLRC1A	PCR-RFLP	Forward	5'-TCAGTTCACTTAGAGCAC3'	820	50 °C / 55 s/1.5
		Reverse	5'-ACAGGGAGTAATAACGATC-3'		

Table 2. PCR-RFLP assay for selected genes.

| Gene	SNP Location	Breed genotype	enzyme	Restriction (New England)	Buffer	Polymorphic products
FGD6	Pos. 910	Red Massai A/G Dorper G/G	AflIII 10 U	Buffer 3		Allele 1: 172 bp Allele 2: 145+27 bp
PLXNC1	Pos. 687	Red Massai C/T Dorper C/C	Tsp509I 10 U	Buffer 1		Allele 1: 584 bp Allele 2: 477+107 bp
OAR3-/C1R	Pos. 132	Red Massai A/G Dorper A/A	RsaI 10 U	Buffer 1		Allele 1: 754 bp Allele 2: 674+80 bp
CSF2RB	Pos. 340	Austrian C/T	MspI 10 U	Buffer 2		Allele 1: 462 bp Allele 2: 339+133 bp
SMARCC2	Pos. 501	Austrian G/C	HhaI 10 U	Buffer 4		Allele 1: 632 bp Allele 2: 499+133 bp
ARHGAP9	Pos. 318	Dorper G/G UK A/A	no found			Allele 1: 769 bp Allele 2: no
MYF6	Pos. 468 Pos. 469	RM, DOR A/C RM, DOR C/T	no found			Allele 1: 938 bp Allele 2: no
PTPRB	Pos. 588	RM, DOR C/T GB C/C	no found			Allele 1: 790 bp Allele 2: no
SLC11A2	Pos. 536	Red Massai C/C Dorper C/T	no found			Allele 1: C/C Allele2: T/T
SLC11A2	Pos. 228	Iran T/T Austrian C/C	no found			Allele1: T/T Allele2: C/C
MARS	Pos. 543	Red Massai A/A Dorper A/G	PflFI	Buffer 4		Allele 1: 721 bp Allele 2: 543 +178 bp
KLRD1	Pos. 179	All allele 2	ApoI	Buffer 3		Allele 1: 897 bp Allele 2: 718 + 179 bp
KLRD1	Pos. 281	RM, D allele 1 AU allele 1+2	SspI	Buffer 1		Allele 1: 897 bp Allele 2: 616 + 381 bp
KLRD1	Pos. 633	All allele 2	AccB7I	Buffer 1		Allele 1: 897 bp Allele 2: 633 + 264 bp
CSF2RB	Pos. 298	BG allele 1 KUR allele 2	PvuII	Buffer 2		Allele 1: 720 bp Allele 2: 425 + 295 bp
TIMP3	Pos. 787	IRAN allele 1 RM1 allele 2	MslI	Buffer 2		Allele 1: 967 bp Allele 2: 787 + 180 bp
ANKRD28	Pos. 129	IRAN allele 1 AU allele 2	AciI	Buffer 3		Allele 1: 702 bp Allele 2: 573 + 129 bp
CHD4	Pos. 606	DOR4 allele 1 RM1 allele 2	NarI	Buffer 1		Allele 1: 1117 bp Allele 2: 706 + 311 bp
CLEC1A	Pos. 701	AU allele 1 IR22 allele 2	AlwNI	Buffer 4		Allele 1: 1030 bp Allele 2: 701 + 329 bp
ITGA5	Pos. 111	All allele 2	MboII	Buffer 4		Allele 1: 1166 bp Allele 2: 856+ 310 bp
ZBTB39	Pos. 77	Red Massai C/T Dorper T/T	TaqI	Buffer 3		Allele 1: 946 bp Allele 2: 870+ 76 bp
SKR3	Pos. 441	Austrian C/A	BstYI	Buffer 2		Allele 1: 885 bp Allele 2: 441+ 444 bp
FAM62A2	Pos. 224	Red Massai C/T Dorper T/T	BpmI	Buffer 3		Allele 1: 803 bp Allele 2: 224+ 579 bp
NAV3	Pos. 486	Red Massai T/T Dorper C/T	BpuAI	Buffer 1		Allele 1: 826 bp Allele 2: 488+ 338 bp
RPS6KB2	Pos. 517	Red Massai A/G Dorper G/G	BspHI	Buffer 4		Allele 1: 1004 bp Allele 2: 515+ 489 bp
KLRC1A	Pos. 787	Dorper G/G	AciI	Buffer 3		Allele 1: 820 bp Allele 2: 787+ 33 bp
KLRC1A	Pos. 604	Dorper G/G	BcgI	Buffer 3		Allele 1: 820 bp Allele 2: 610+ 210 bp
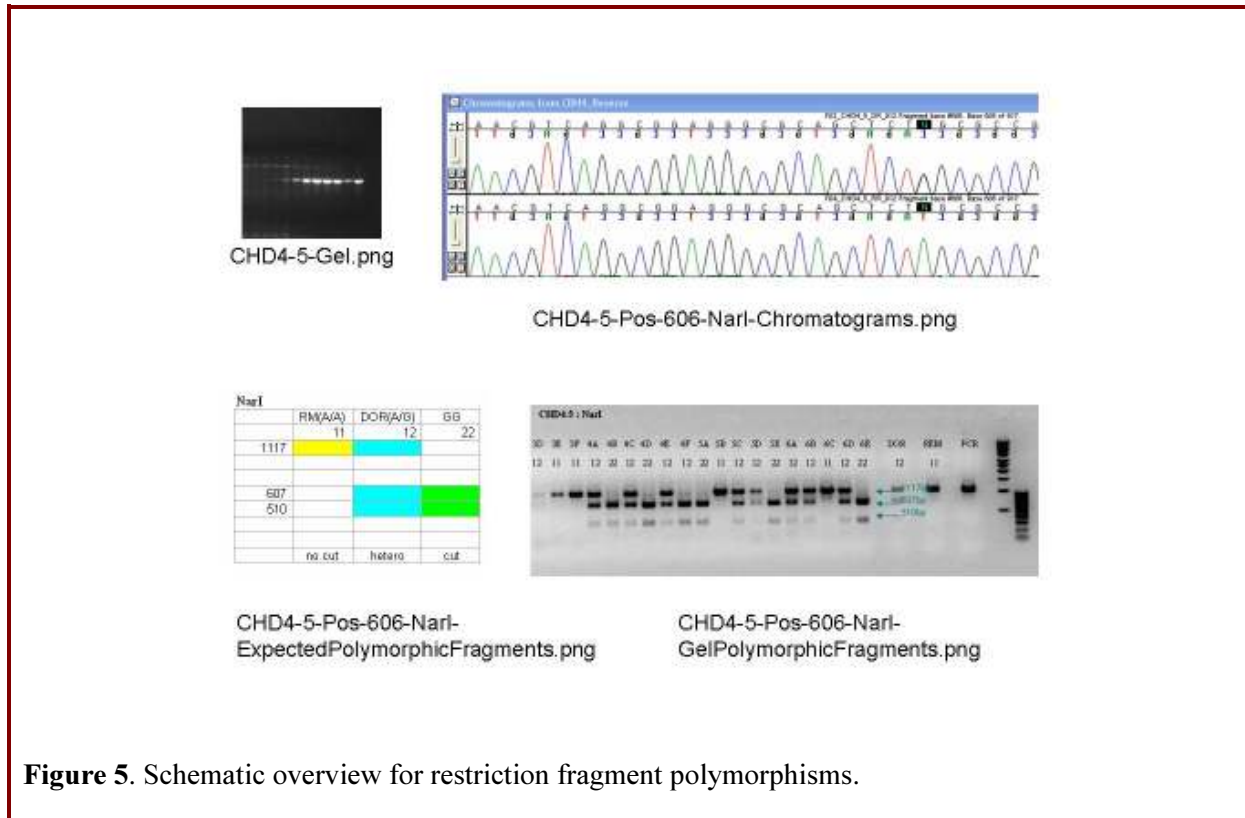
Figure 5. Schematic overview for restriction fragment polymorphisms.

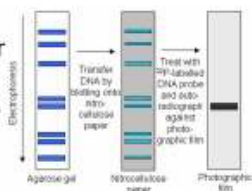*3.1.1.4.   Southern blot analysis with radioactive [A-32p] ATP labelling*

It was not possible to develop a test for PCR-RFLP in two situations a) where there was no enzyme to recognize the restriction site, b) where the enzyme cut multi-sites in which it is difficult to see a pattern. In order to overcome these problems we used Southern blot as a follow-up to single nucleotide polymorphisms (SNPs). Southern blots combine gel electrophoresis with transference of the separated DNA onto a filter membrane for probe hybridization. The probe DNA is labeled with a suitable radioactive isotope, so that it can be detected. The pattern of hybridization is then visualized by autoradiography. The various steps involved in this technique are shown in **Figure 6** and comprise the following: -

1) PCR amplification of candidate genes

2) Restriction endonuclease digestion of PCR products

3) Analysis of PCR products by agarose gel electrophoresis

4) Preparation of agarose gel for transfer to a nylon membrane

5) Transfer the DNA from a gel onto nylon membrane

6) Fixation of the DNA on the membrane by baking at 80☐ for 2 h (after this stage the membranes can be stored at 4°C before hybridization)

7) Design of specific probes for each gene in consideration of the following parameters (using Primer Express): a) proposal for direct detection of SNP; b) proposal for situation with multiple cuts

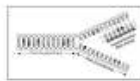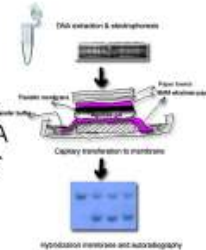8) Radioactive labeling of oligonucleotide probes

9) Purification of labeled probes for removing unincorporated radioactive nucleotides using Biospin 6 chromatography columns (Bio-Rad)

10) Southern blot hybridization

11) Exposure of the labeled membranes to a XAR-5 film in X-ray cassettes

12) Interpretation of the results

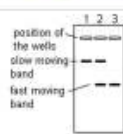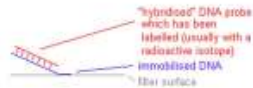**Figure 6.** Southern blot analysis with radioactive [α-32P] ATP labelling.

3.1.1.5.   Creating a DNA gene bank at the Agency's Laboratory for use by Member States

One objective for several current and planned TC and CRPs in the Animal Production and Health Programme is to transfer the technical capacity for DNA analysis and marker assisted selection to MS. In order to identify SNPs for parasite resistance in sheep and to develop assays to detect these, each SNP needs to be verified in different populations. Arrangements were made to acquire samples of blood and DNA from representative breeds from different populations. Blood samples were sent in two ways; blood mixed with Magic buffer in an evacuated tube, and blood deposited on Whatman FTA Classic cards. Both methodologies allow the long-term storage of the blood sample. Each MS was provided with material for blood collection, and samples from indigenous breeds were isolated and stored. These data were entered into a Genetic Resource Database (GR-*db*) that was created specifically for this purpose. At present this bank contains over 1000 samples from 32 breeds of small ruminants from 15 countries.

3.2. Bioinformatics and Genomics

3.2.1. *Development of New Modules and Databases required for the Web-based Laboratory Information Management System (ABL-LIMS)*

The current Laboratory Information Management System of the FAO/IAEA Agriculture & Biotechnology Laboratory (ABL-LIMS) is based on an open-source, independent platform that consists of a number of customised databases designed for the individual and specific needs of each organisational entity within ABL. The new platform is a transition between a traditional web application and a format that will provide improved usability and faster application. APU has expanded their existing LIMS with some modifications and new modules. A central database inventory of laboratory chemicals, serum bank management and appropriate records, for health, reproduction and genetics (phenotypic and genetic) data, will be gathered from different MS who have given their consent for their use at the IAEA. This database is web-accessible and housed at APU so it can be integrated with other available information. Users will be able to: -

1) Have access for data retrieval that they can also update at any time

2) Compare the results generated locally with those already in the database

3) Update any new data entry in real time

3.2.2. *Genetic Repository Bank (GRB) Database of Genetic Materials and Gene Profiling (GP) for Candidate Gene Information*

APU has developed a program to manage a Genetic Repository Bank on sheep, which can be expanded to include other livestock species. This facility will allow users to graphically view the location of samples with links to Google Map. In addition, the site will allow registered users to insert their own information, so that the site can remain continually up-to-date. This Genetic Repository Bank database (GRB-db) is a first step in providing MS with genomic information on small ruminants in a user-friendly format. The application does not require software installation on user computers and can work on all web-browsers. The PHP program with MySQL for the database will be used for platform compatibility (UNIX, Linux, Windows, and Solaris). Ajax (Asynchronous JavaScript and XML) will be used for data display in real time, which gives the database a unique format. The biggest advantage of this tool is the bandwidth usage because it generates HTML locally, within the browser, and does not require reloading the whole data after amending any portion of the programming. This tool will be used for sample traceability at DNA Repository Bank at Seibersdorf Laboratories of IAEA in support of CRP D3.10.25 and related IAEA projects.

3.2.3. *Development of Real -Time Databases (RT-db) for Genetic Information on Small Ruminants*

APU has worked extensively on the development of a Real-Time database (RT-db) for Quantitative Trait Loci (QTL)/Genes/DNA Sequences and Genetic characterization in small ruminants. The database will make available the genomic locations of QTL from all known studies on small ruminants. It will allow users to view graphically the positions of the QTL, filtered according to a number of criteria, such as trait name, chromosome number, and statistical significance. *RT-db* will be the first step in providing the MS with up-to-date genomic information on small ruminants in a user-friendly format. The database is now

available online and is fully functional. This work has been presented at an international conference (http://www.intl-pag.org/16/abstracts/PAG16_P08a_852.html).

3.3. Animal Health

3.3.1. Adapting Gene Technologies to Field Situations

Enhancing food security by providing effective control of infectious diseases in livestock requires major investment in developing diagnostic technologies of sufficient sensitivity and precision to accurately identify animal carriers of disease and to carry out appropriate measures for containing an outbreak. Diagnosis typically requires the identification of the infective organism, and this may be possible at point-of-care in certain instances where the disease agent can be identified microscopically from the various body fluids. However, in many cases, this method is not applicable and diagnosis requires laboratory backup to provide the information. Gene amplification methods provide a way of doing this without resorting to culture of viral or bacterial pathogens or collecting large amounts of biological samples. These methods, based on the Polymerase Chain Reaction are highly sensitive and specific, but incur a delay in transferring materials, analysing and providing the results. In some instances, this delay can be counterproductive in establishing an efficient disease control programme. For instance, in the Foot and Mouth Disease outbreak in the UK in 2001, delays in obtaining diagnosis meant that nearly 25% of farms declared infected on clinical grounds were actually free from disease. Being able to improve this decision-making gap would be an advantage, especially in the case of transboundary diseases, including Highly Pathogenic Avian Influenza, (HPAI) where suspected disease may occur in remote areas, far from the laboratory and where speedy diagnosis of a dangerous potential zoonosis is of paramount importance. Loop mediated isothermal amplification (LAMP) provides a means for applying molecular technologies to the penside as it does not require thermal cycling and the results can be visualized with the naked eye (**Figure 7**). Recently, APU investigated the potential of applying LAMP technologies to the diagnosis of Avian Influenza (AI), Peste des Petit Ruminants (PPR) and Contagious Bovine Pleuro-Pneumonia (CBPP). The work involved looking at technical modifications of the LAMP process to improve performance; these involved testing alternative polymerases, using PCR enhancers, optimizing reaction temperatures and testing lyophilized master mixes.

The Avian Influenza RT-LAMP was based on a published method to which various modifications were applied in order to evaluate potential improvements in its functionality. Originally the test results were read using a turbidimeter, but such equipment it is not routinely used in most laboratories. Hence, the first step was to change the method to a fluorometric one, by adding EVA-Green dye to the reaction mixture as an indicator. The amount of pyrophosphate precipitate that developed during the reaction was minimized by lowering the magnesium concentration, thereby solving the problems encountered in reading the fluorescence signal. Interestingly, lower magnesium concentrations had the added effect of making signal development faster. The dNTP
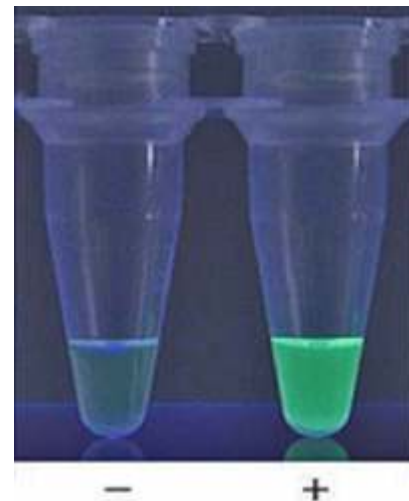

Figure 7. Colour changes in reaction tubes allow discrimination of positive and negative results when using LAMP-PCR.

and the primer concentration were then also optimised, leading to lower reagent usage and faster amplifications.

An alternative strand-displacing polymerase (Bsm), supplied by Fermentas was evaluated for use in the LAMP and found to be roughly comparable with another polymerase, Bst. As there was no optimized buffer system for Bsm available, the buffer system for Bst was used instead. This resulted in slightly quicker results (less time to signal development).

Betaine is an agent that has been used successfully for increasing yield and specificity of PCR products by reducing the formation of secondary structure caused by GC-rich regions, but it is rather expensive. Trehalose can also enhance the activity of certain enzymes, so its use in LAMP was evaluated. Betaine actually inhibited AI-RT-LAMP when using Bst, whereas Bsm needed a certain concentration of betaine to work. Trehalose could replace betaine in LAMP for both Bst and Bsm. The use of trehalose opened up another possibility; trehalose can preserve enzymatic activity when drying enzyme solutions. The dried master mix was found stable at room temperature, making it possible to prepare in advance large quantities for a whole series of experiments and for use over a long period, with possible benefits for standardization and quality assurance. It could even be possible for a central laboratory (e.g. Seibersdorf) to prepare master mix for supplying partner institutes, making it easier to compare inter laboratory results. This drying should at the same time reduce the risk of contamination. Isothermal conditions used for LAMP are usually between 60-65°C, with an optimum of 62.5°C. However, in our tests performed between 55-65°C it was found that optimum temperatures for both Bsm and Bst were below 60°C and gave faster than published positive results for AI-RT-LAMP (optimum around 57°C).

The formation of crystals by Mg-pyrophosphate makes it possible to read a positive result of LAMP using the naked eye, but at the same time inhibits the polymerase. Pyrophosphatase (PP) converts Mg-pyrophosphate to ortho-phosphate, liberating the Mg and reducing the inhibition of the reaction and the reading of the fluorescence. Hence, the inclusion of PP could make the PCR (and LAMP) more efficient. It was found that this enzyme does have an effect on LAMP, but only in the late phase of the reaction - the signal did not come earlier, but a stronger signal developed over time. Thus, the addition of PP could be of value if the signal to noise ratio needs to be influenced to give more stable results in a difficult LAMP test. An experimental PPR-LAMP was performed on plasmids with inserted sequences available in Seibersdorf. It was found that reaction varied greatly with different strains, from no detection to fast amplification. This was ultimately traced to the location of the primers on relatively variable sequence elements. Using this information, new primer sets, in less variable regions, will be designed and tested in the near future.

For the CBPP-LAMP, a set of primers was tested on CBPP DNA. While the outer primers were able to amplify DNA in a conventional PCR, for eight out of nine strains examined, a LAMP using both outer and inner primers failed to give a signal. Here too, new primer sets are under evaluation. The results obtained with the AI LAMP give promise that the LAMP can be further developed into a rapid and quality assured diagnostic system. The EVA green-based platform will help to better evaluate these tests with established laboratory equipment in our counterpart laboratories for the fitness for purpose and their future field application.

3.4. The Control of Contagious Bovine Pleuro Pneumonia in Sub-Saharan Africa

Although the occurrence of Contagious Bovine Pleuropneumonia (CBPP) was very much reduced during the Pan African Rinderpest Campaign (PARC), partly through the use of a combined vaccine for rinderpest (RP) and CBPP, it is still endemic in some 30 African countries. Furthermore, since the end of PARC, CBPP is continuing to spread as control programmes have had little effect due to local conflicts and unrestricted cattle movement. Other important factors that add considerably to the problem is the efficacy of the vaccine that provides inadequate protection, the difficulty in identifying vaccinated animals and the poor sensitivity of the recommended diagnostic tests. Our research therefore aims to improve diagnostic capacity for detecting CBPP by optimizing the available methods, by exploring new diagnostic challenges - for instance detecting infection in animals that are asymptomatic and can act as chronic carriers - investigating the use of molecular markers to assist in understanding the epidemiology of the disease and using gene-based diagnostics to enable rapid diagnosis.

A new serodiagnostic test has been developed in APU based on an Enzyme Linked Immunosorbent Assay (ELISA), using as coating antigen lipoprotein LppQ, which is specific to *Mycoplasma mycoides* subsp. *Mycoides* SC and does not therefore cross-react with other *Mycoplasma* species. Lipoprotein LppQ was expressed in *E. coli* identified and characterized by Western blot analysis (WB) and then purified using magnetic beads which bind to the *his*-tag of the protein. The purified antigen was used for coating microtitre plates to develop a prototype indirect ELISA (iELISA) for the detection of antibodies against CBPP in cattle. After a number of different brands of microtitre plates with different surface reactivity was tested using the purified antigen and standard ELISA protocols, Immulon 1B (Dynatech) plates were identified as being able to produce optimal adsorption of the antigen in the comparative tests. These initial studies confirmed that in order to optimize test parameters it was necessary to effect a reduction in background colour development. This therefore became a priority in the most recent work, aiming to reduce background "noise" due to unspecific binding, thereby enhancing the Binding Ratio (B/B0) and enabling more precise discrimination between positive and negative sera.

A number of commercial blocking buffers were used either non diluted, (Svanovir, Sigma B6429) or diluted in PBS Tween 20 (PBS-T) at different concentrations (Fish Gelatine Amresco at 1% and 5%, Roche Blocking Reagent 5% and Trehalose 5%). These buffers were compared with each other and PBS-T with skimmed milk (Fluka) at 5% and 10% (PBS-T-F5% and PBS-T-F10%) to see which was best suited to achieve a reduction in background. The antigen was coated at a dilution of 1/100 in phosphate buffered saline (PBS), the test serum samples were diluted 1/600 in a blocking buffer, and Horseradish Peroxidase (HRP) conjugate was diluted at 1/30000 dilution also in the homologous blocking buffer. The antigen coating stage and the serum and HRP incubation steps were carried out at 37°C for 1 hour in an orbital ELISA shaker. After substrate incubation (TMB/H_2O_2) at 37°C for 15 minutes the reaction was stopped with 1M H_3PO_4 and the optical density (OD) read at 450nm. Five of the buffers were rejected, (Fish Gelatine Amresco 5%, Sigma B6429 (1x), Roche Blocking Reagent 5%, Trehalose 5% and PBS-T-F5%), because they failed to meet the criteria for enhancing the binding ratio. The other three buffers were re-tested and the one that fulfilled the test criteria most completely was selected.
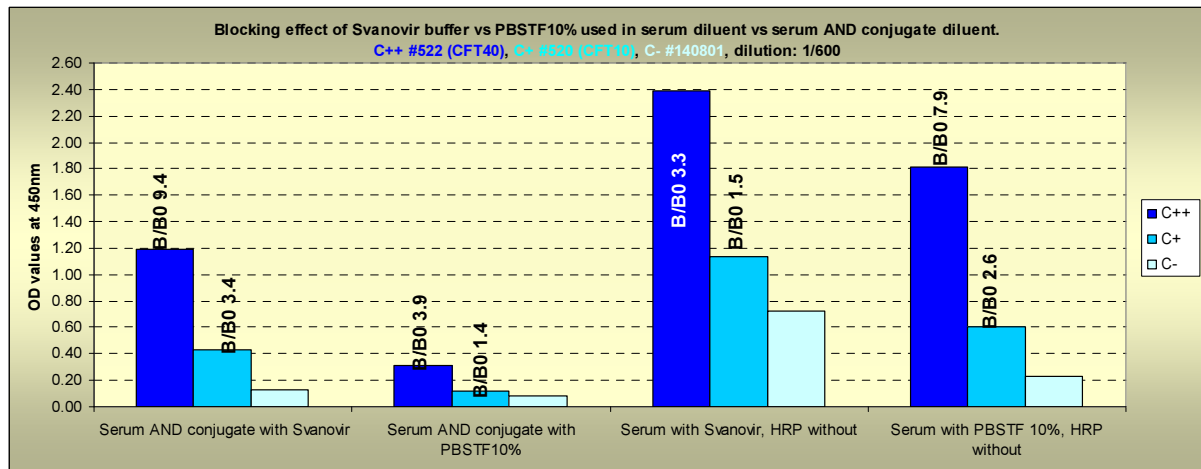
Figure 8. Example of blocking effect using two different buffers, Svanovir and PBST-F10%.

Skimmed milk in PBST at 10% reduced the total OD value of the reference high positive control serum (C++) drastically when used as a diluent for serum and HRP. When the PBS-T-F 10% was used for serum blocking the B/B0 ratio improved but the OD of the reference negative control serum (C-) was still too high (**Figure 8**).

Optimal binding capacities could be realized with the buffer from Svanovir; but the desired blocking effect was achieved only if the buffer was used for dilution of both serum and conjugate. If the buffer was used as serum dilution only, no blocking effect was observed. (**Figure 9**).

The results of this study were presented and discussed at the Second Research Coordination Meeting of the CRP (D3.20.25), held from 21 to 25 of April 2008. For further test validation procedures, all the necessary reagents and standards for this prototype LPPQ iELISA have been transferred to a Member State laboratory run by Dr. M. Niang, Head of the Diagnostic Department, Central Veterinary Laboratory of Mali (CVL). After further validation with field samples and quality assurance testing the assay will be made available to other MS participating in the CRP.

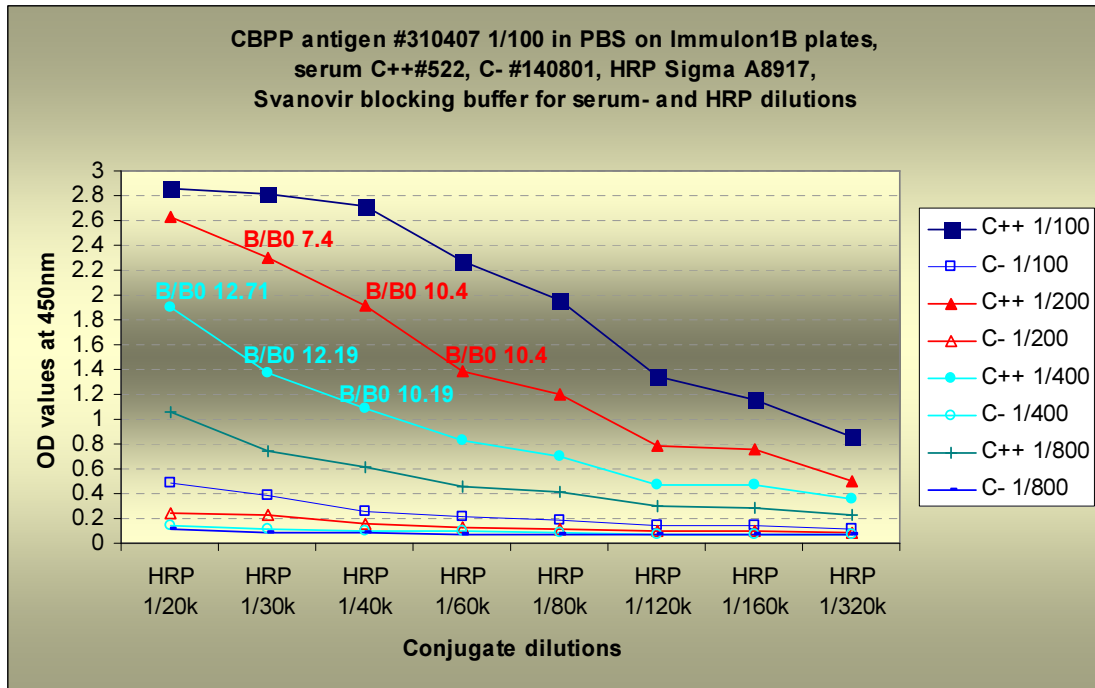**Figure 9.** ELISA checkerboard titration of different positive and negative reference sera versus different conjugate dilutions with Svanovir blocking buffer.

3.5.   Studies on Peste des Petits Ruminants: Analysis of the Antigenic and Immunogenic Properties of the N-Tail of the N Protein (PPRV N)

Peste des petits ruminants (PPR) is an infectious disease caused by a virus, the PPR virus (PPRV), belonging to the genus *Morbillivirus* in the family *Paramyxoviridae.* It is a highly contagious disease that affects sheep and goats and wildlife. It is endemic in Africa, the Arabian Peninsula, throughout most of the Near and Middle East, and in south-west Asia. Because of the high economic losses it can cause to small ruminant production, PPR is on the list of notifiable animal diseases requiring reporting to World Organization for Animal Health (OIE). PPR is an acute disease characterised by serous ocular and nasal discharges, severe pyrexia, and erosive lesions on different mucous membranes particularly in the mouth, diarrhoea and pneumonia. This latter sign can cause PPR to be confused with contagious caprine pleuropneumonia (CCPP) or pasteurellosis. Because of diarrhoea and mucosal erosive lesions, it was for a long time confused with rinderpest a disease caused by another morbillivirus, the rinderpest virus (RPV). RPV is closely related to PPRV and they cross react in serological test.

Morbilliviruses have six structural proteins, and one of them, the nucleoprotein (N), is the most abundant viral protein and is the one against which the majority of the antibodies induced by infection with PPRV are directed. This is why we have focused on this protein to develop a diagnostic test. The N gene of PPRV encodes a protein of 525 amino acids (aa). Sequence comparison studies of the nucleoprotein within the *Morbillivirus* genus have identified three main regions with different homologies:

- An N-terminal region of medium homology which has a small fragment not conserved between the different morbilliviruses

- A highly conserved central region

- A poorly conserved C-terminal domain (N-tail) covering the last 105 aa

Previous studies have shown that specific anti-N MAbs have their binding sites in non-conserved regions of the protein. Serological tests based on a competitive ELISA (cELISA) developed using this specific anti-PPRV N MAbs cross-react with sera from RPV-infected animals. As the objective was to develop a serological test specific to PPR, it was necessary to analyse the antigenic and immunogenic properties of PPRV N protein. For that, we used 62 overlapping synthetic peptides of N protein, the entire protein and deleted mutants expressed in a baculovirus system in an indirect ELISA (iELISA) to test PPR positive and negative sera obtained from goats. Preliminary results indicated that most anti-N antibodies developed by PPR-infected goats are probably conformational since the PPR positive sera failed to detect all the N peptides (see **Figure 10** for some examples). In contrast, the data show that antibodies raised in mice by injection of N protein recognized some linear epitopes (**Figure 10**).
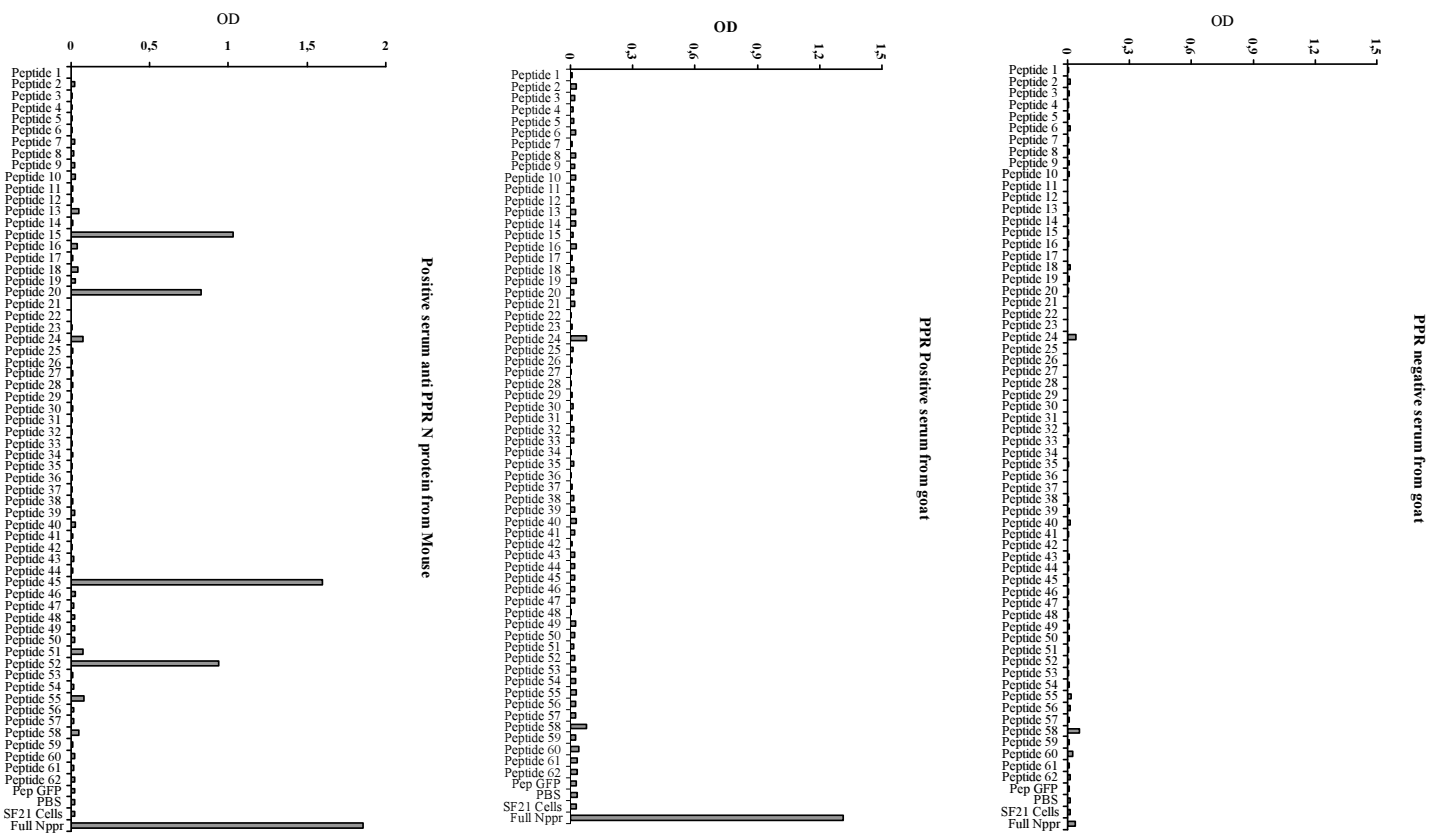
Figure 10. Reactivity of anti-N protein positive sera on the N protein peptide in iELISA. Figure 10A: Goat PPR negative serum; Figure 10B: Goat PPR positive serum; Figure 10C: Positive anti-PPRV N serum from mouse.
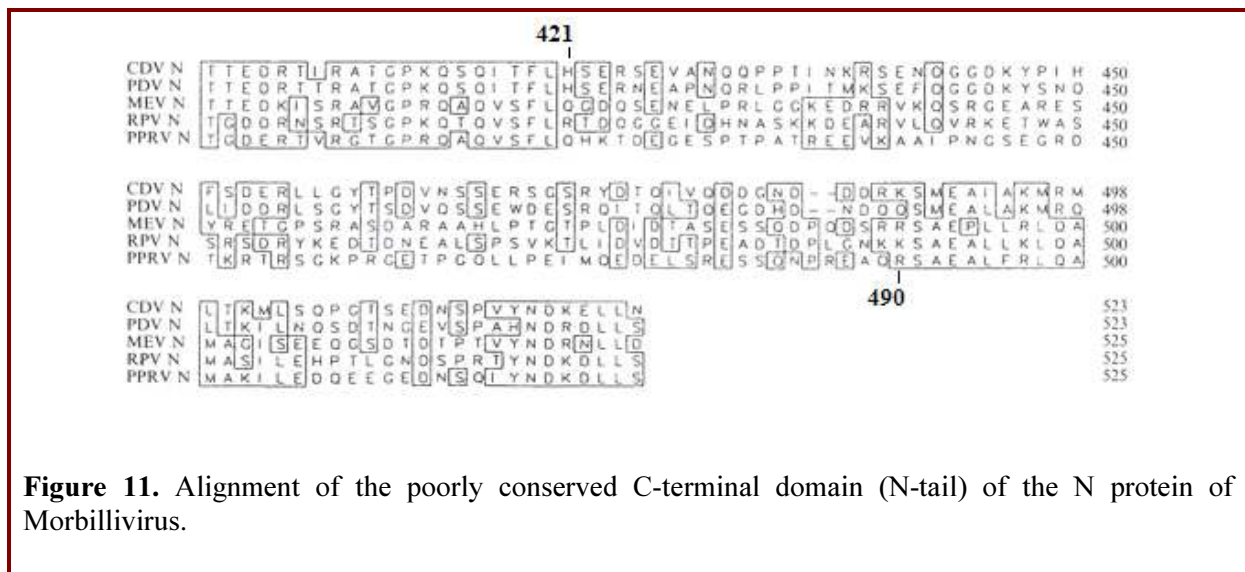
Figure 11. Alignment of the poorly conserved C-terminal domain (N-tail) of the N protein of Morbillivirus.

Since the data using N peptides in iELISA showed that probably most of anti-N antibodies from PPR-infected goats are conformational, we analyzed the reactivity of PPR and RP positive sera on the deleted PPRV N protein mutants. The work was focused on two N fragments, N421-490 and N421-525, from the N-tail of PPRV N protein (**Figure 11**) to identify antigen to detect specifically PPR antibodies by iELISA.

The nucleic acids corresponding to the genes of these two fragments were amplified by PCR, cloned, introduced into the genome of baculovirus by recombinant technology and expressed in insect cells. The expression of these two mutants and the entire N protein were analysed by Western Blot (WB) using a monoclonal antibody recognizing PPR N-tail, PPR positive, PPR negative and rinderpest positive sera from goats. The WB results obtained are presented in **Figure 12**. The results show that the sizes of the mutants N421-490 and N421-525 were 7 and 11 kDa respectively, as expected. The poorly conserved polypeptide N421-490, detected by the monoclonal antibody, is not recognized by either PPR- or RP-positive sera. This means that the hypervariable amino-acid sequence 421-490 of PPRV N protein is not immunogenic in goat since it is not recognized by PPR-positive goat serum. In contrast to N421-490, the polypeptide N421-525 shows good reactivity with PPR positive serum but not with RPV-positive serum. These data suggest that the epitope(s) in the N-tail recognized by the PPRV-positive serum from goats are composed of the amino acids located in the region from positions 491 to 525. It (they) seem(s) be specific to PPRV.
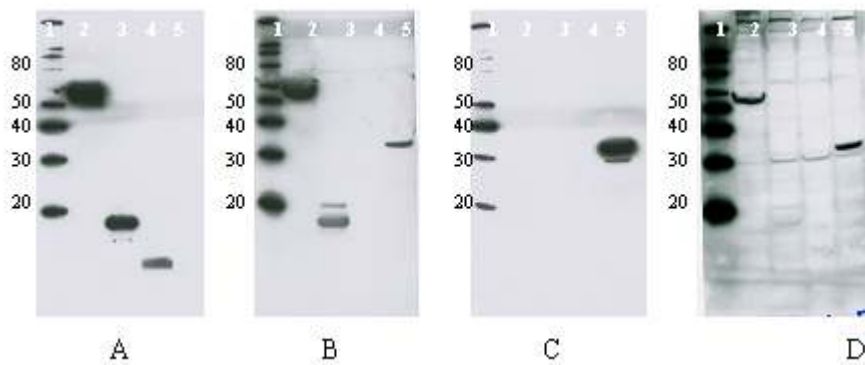
Figure 12. Western blot of reactivity of anti-N monoclonal antibody, PPR+ and RPV+ sera on full N-PPRV protein, polypeptide N421-525 and N421-490,. A: P4A3 MAb anti-N-PPRV; B: Goat serum PPR+; C: IVB2 anti-N-RPV MAb; D: Goat serum RPV+. Line 1: M: Protein Molecular Weight Markers, kDa (Invitrogen); line 2: N-PPRV protein; line 3: N421-525, line 4: N421-490, line 5: N-RPV protein (the protein size at 38 kDa indicates that it is digested by cells proteases).

The Western blot result was confirmed by iELISA in which the polypeptides N421-490, N421-525 and full N expressed in insect cells and insect cell lysate protein were used as antigens to coat 96-well microtitre plates. As seen in **Figure 13**, N421-525 reacts in iELISA in the same manner as full N with PPR-positive goat while N421-490 is negative with the same serum. Thus it can be concluded that the amino acids 491-525 contains the immunodominant epitope(s) recognized by the goat immune system.

Figure 13. Results of iELISA using the polypeptide N421-490 and N421-525 of PPRV N protein as antigen. SF21 non-infected cells and PPRV N protein were used as control antigens in the test. P4A3: monoclonal antibody from mouse directed against the N421-490. C++ and 9006 D15 are PPR positive sera from goat.

Rinderpest positive sera from cattle and goat were used to determine their reactivity with the polypeptide N421-525 in iELISA. The results (**Figure 14**) show that RP positive sera fail to detect the polypeptide N421-525. Since ruminants (goats) produce essentially conform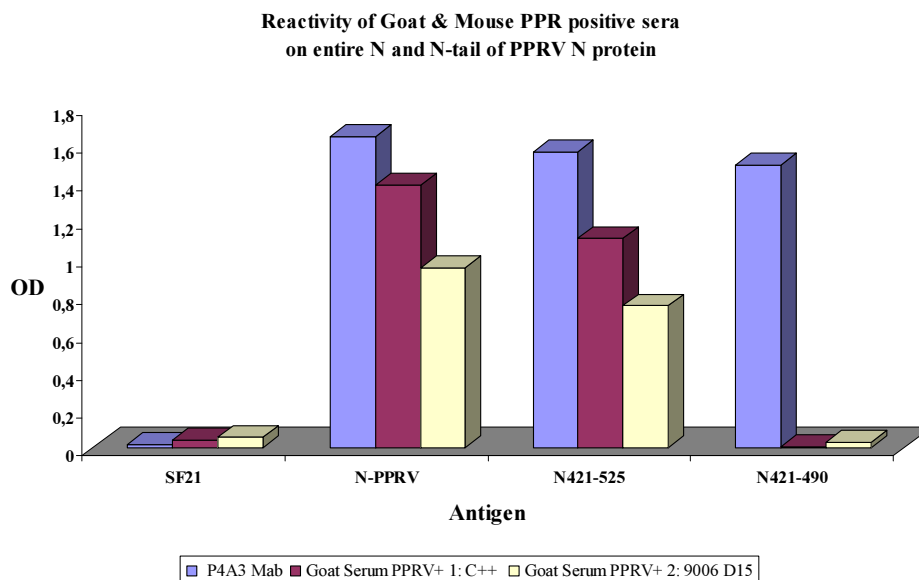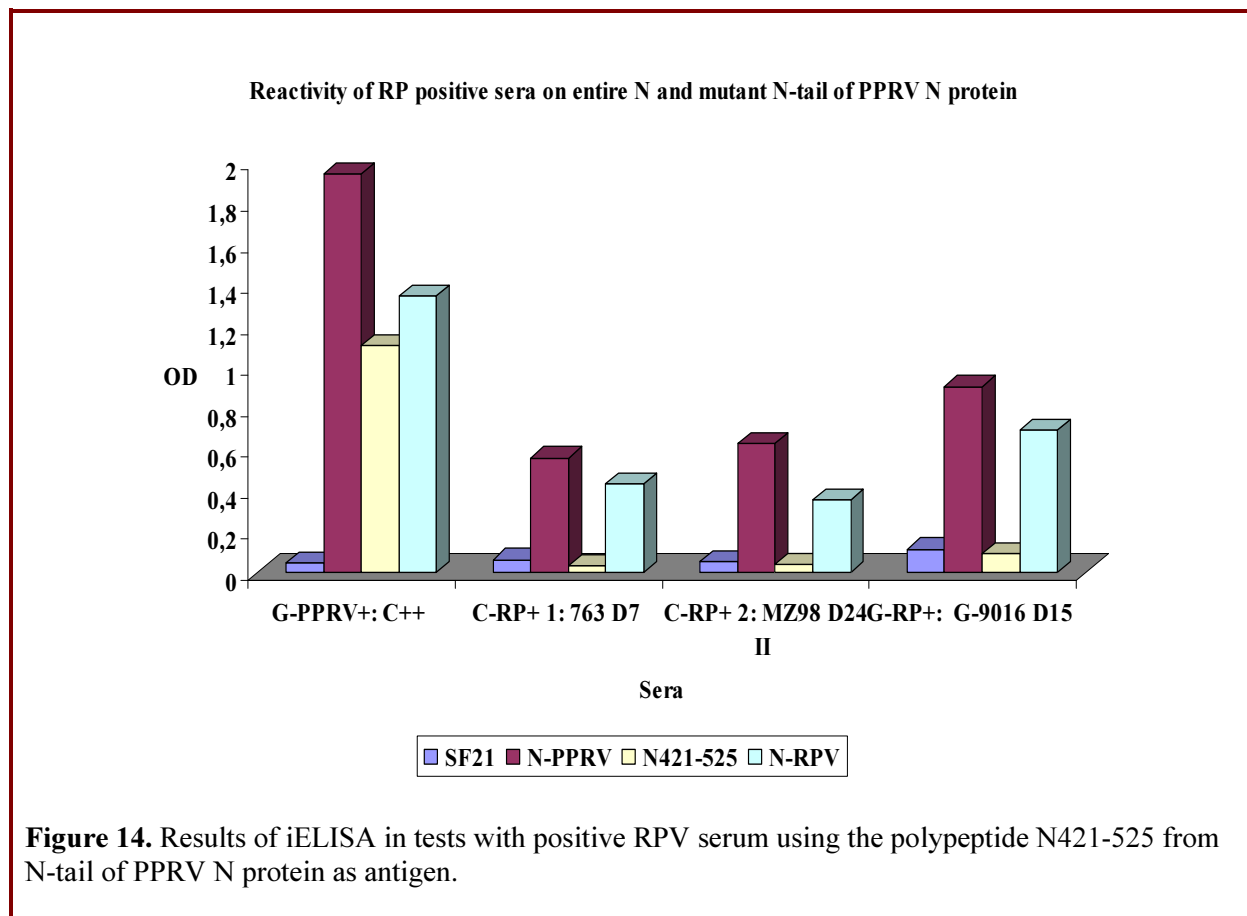ational antibodies directed against PPRV N protein, the detection of the polypeptide N421-525 may also be due to conformational antibodies and the entire N-tail seems be essential for these conformational epitope(s) which is (are) specific to PPRV.

Following the investigation of the antigenicity and immunogenicity of the N-tail of the PPRV N protein, the polypeptide N421-525 could be a good candidate as antigen in specific iELISA for PPR diagnosis. This polypeptide will be evaluated further in iELISA with a large panel of PPRV- and RPV-positive sera.

Reactivity of RP positive sera on entire N and mutant N-tail of PPRV N protein

Figure 14. Results of iELISA in tests with positive RPV serum using the polypeptide N421-525 from N-tail of PPRV N protein as antigen.

3.5.1.　*Analysis of the Domains of PPRV N Protein Interaction*

As part of our approach to develop a marker vaccine for PPR, the N protein of the virus was targeted for negative and positive markers. For that, it was necessary to identify the zones of N critical for the survival of virus and that cannot be deleted. This identification was done through functional mapping, i.e. identification of N zones involved in protein-protein interactions. Previous studies on N-N self interaction to form the nucleocapsid showed that the N-terminus region of N (from aa 1 to 241) is very important. The functional mapping of PPRV N was continued during the past year

3.5.1.1.   Analysis of N-N interactions with peptide

N-N domain interactions were analysed using a peptide. Sixty overlapping peptides of N were used to coat the plate for ELISA assay to analyse the extent of their binding with the N protein. The results are presented in **Figure 15**.

Nppr diluted in PBS-T+ 5% Roche: Plate 1
1. Coating the plate with 62 Nppr peptides.
2. Washing the plate and incubation with baculo Nppr diluted in PBS-T+% 5 BB Roche overnight at 4C.
5. Detecion of Nppr by Mab 38-4.

Figure 15. ELISA results for N-N interactions. ELISA plates were coated with 62 overlapping peptides from PPRV N protein to analyse their interaction with the entire N protein. The histograms show clearly those peptides that interacted strongly with N protein.

The N-N interactions analysed by peptide ELISA show that entire N protein bound both N-terminal and C-terminal peptides. The peptides which interact with N protein are mainly localised at the N-terminal. This confirms the results of previous studies that showed the N-terminal of N protein is essential for the self-interaction of the protein to form the nucleocapsid. Our new data show also that the C-terminal may participate in this N-N interaction, as a peptide in that region is highly reactive with N in the ELISA test. A study on N-tail (N421-525) has given an indication that the polypeptide might form dimer structures, a confirmation of the presence of an N-N interaction domain in that zone. The role of this C-terminal interaction domain in the nucleocapsid structure seems to be important since protease digestion of this region conducted by other authors on the measles virus (MV) N brings about a profound conformational change of the protein. Indeed, N protein of MV which was deleted at the C-terminal formed nucleocapsid helical structures which were more rigid than the full-length N nucleocapsid. This could indicate the role of the C-terminal N-N interaction domain in the final structure of the nucleocapsid to allow this flexibility.

3.5.1.2   Analysis of N-P interaction

The N and P proteins interaction was analysed by a combination of immuno-precipitation and a peptide ELISA test. To identify the binding site(s) of P on N protein, P protein and different N proteins, N full length and deleted mutants, were co-expressed in insect cells by recombinant baculoviruses. To detect the N-P protein complexes, the cell protein lysates were immunoprecipitated with anti-N MAb Proteins in the immune complexes were then separated upon electrophoresis on gel, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as shown in **Figure 16**). The proteins on the gel were transferred to PVDF membrane for Western blot analysis with a mixture of a mAb anti-N and a mouse serum anti-P. Before use, the specificity of the MAb and the serum was checked and confirmed (**Figure 17**). The results of immunoprecipitation and Western blot analysis (**Figure 18**), indicate that P interacts with all N proteins, entire protein or deleted mutants at the N-terminal or C-terminal. This suggests that the P protein possibly interacts with several domains on N protein localized in both N-terminal and C-terminal.
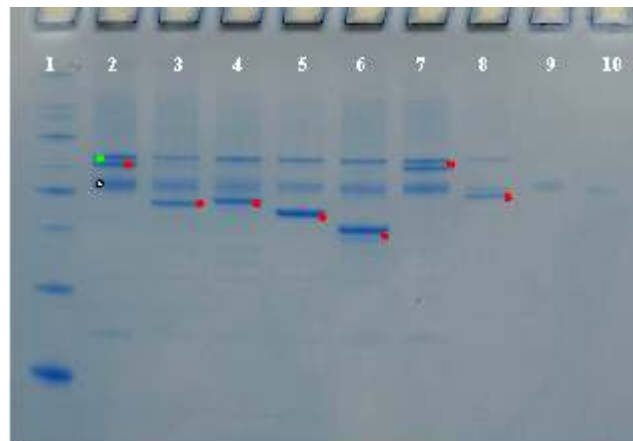
Figure 16. Analysis of the interaction between P and N proteins (full length and deleted mutants). Immunoprecipitated complexes which are obtained with anti-N mAb and lysates of cells co-infected with PPRV P and PPRV recombinant baculoviruses. Two Anti-N Mab was used, 38-4 (for entire N & N▲421-525) and P4A3 (for entire N, N▲1-145, N▲121-241, N▲121-277 & N▲121-319). The immune complex was separated by SDS-PAGE. :   N proteins;   P PPRV;   Ig heavy chain. Lane 1: Note: M: Protein Molecular Weight Markers (Invitrogen); Lane 2: mAb P4A3+P+Full N;, Lane3: mAb P4A3+P+N▲1-145; Lane 4: mAb P4A3+P+N▲121-241 ; Lane 5 : mAb P4A3+P+N▲121-277 ; lane 6 : mAb P4A3+N+NΔ121-319, lane 7 : mAb 38-4+P+N; lane 8 : mAb 38-4+P+ N▲421-525; Lane 9 : mAb P4A3+P and Lane 10: mAb 38-4+P.
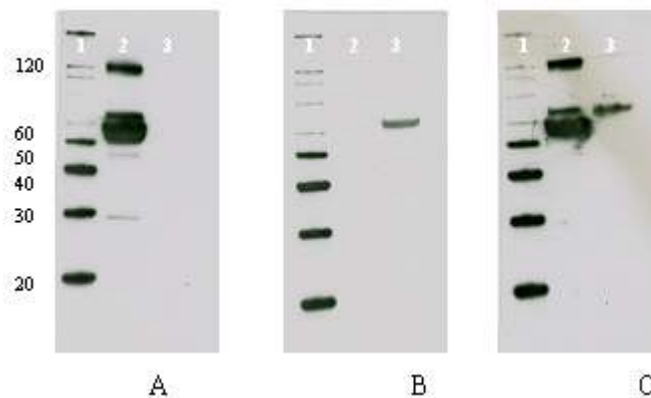
Figure 17. Specificity of each anti-N MAb and anti-P serum on their corresponding antigen. A: anti-N MAb; B: anti-P serum; C: Mix anti-N MAb + anti-P serum. Line 1: M: Protein Molecular Weight Markers (Invitrogen); line 2: N-PPRV protein (N presents monomer 60 Dka and dimmer 120 kda); Line 3: P-PPRV protein.
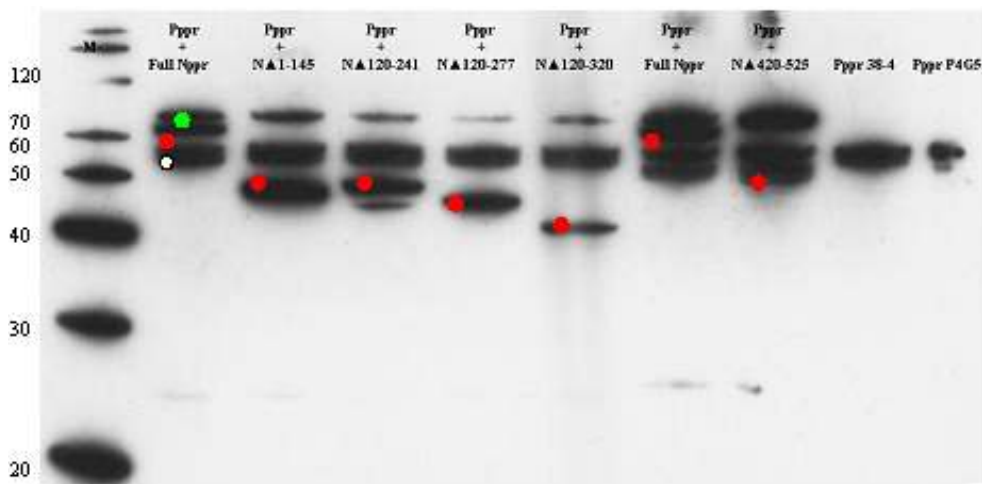
Figure 18. The immuno complexes were then analyzed by SDS-PAGE (see Figure 6) and transferred to PVDF membrane for Western Blot analysis, by using a mixture of anti-N MAb and anti-P antisera from raised in mice as primary antibodies: N proteins (Entire N: 60 Kda; N▲1-145: 45 Kda; N▲120-241: 47 Kda; N▲120-277: 42 Kda; N▲120-320: 38 Kda; N▲420-525: 48 Kda;), P PPRV (70 Kda); Ig heavy chain (55 Kda).

To define more precisely the domains of N protein that interact with P, we used 62 overlapping peptides of N in a protein-ELISA assay. The results obtained showed that two regions of N protein, one at N-terminal and the other at the C-terminal, interact with P (**Figure 19**).

Plate 2: P-PPRV 1/500 in PBS-T+Roche 10%

Figure 19. ELISA plates were coated with 62 overlapping peptides from PPRV N protein to analyse their interaction with P protein. The result is shown in the histogram and reveals interactions with P in two regions of the N protein, one at N-terminal and the other at the C-terminal end.
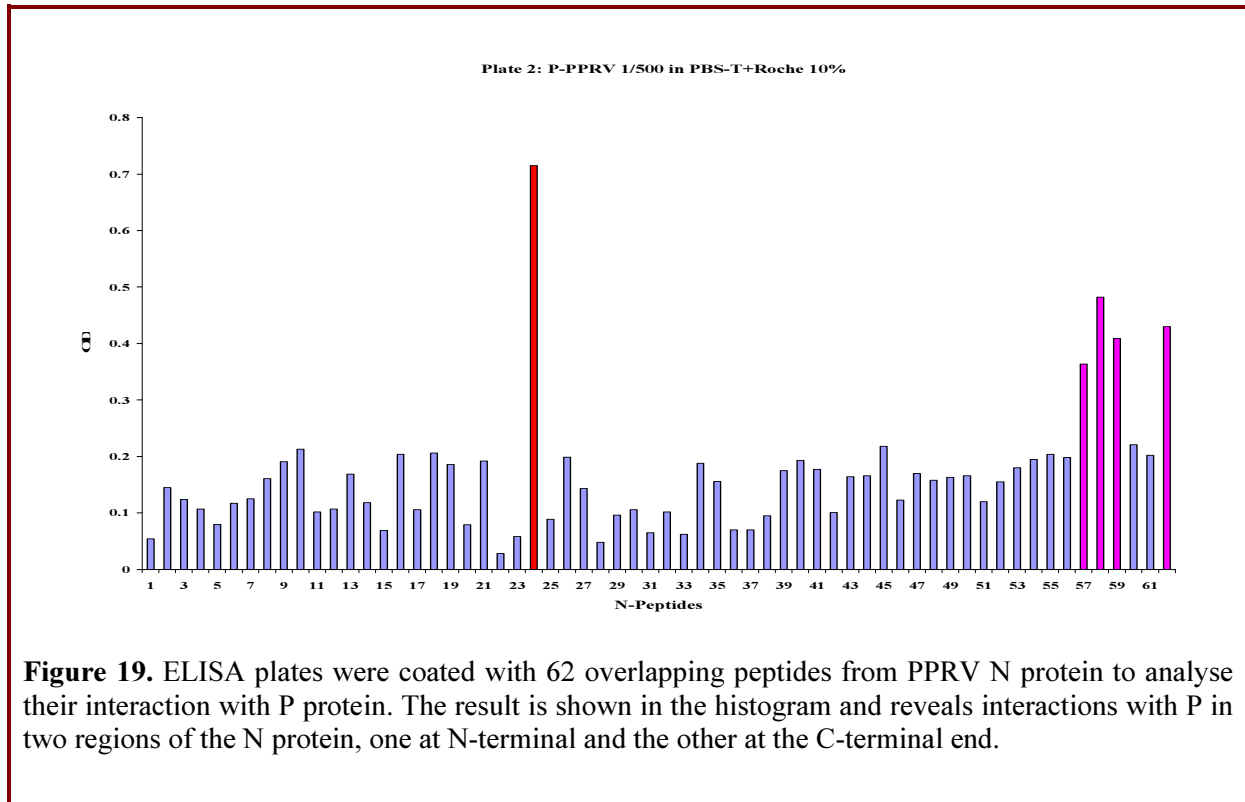
This investigation of the antigenicity and immunogenicity of PPRV N protein has shown that mice and ruminants did not recognize the same epitopes on this protein. While antibodies produced in mice can recognize a linear epitope on N, ruminants do not produce antibodies directed against those epitopes. The study also shows that the polypeptide N421-525 induces the production of specific anti- PPR antibodies in small ruminants and it could therefore be a suitable candidate antigen for a specific iELISA for PPR diagnosis. Analysis of the N protein interaction has showed that the C-terminal also participates in this N-N interaction. This interaction seems to be important since protease digestion of this region conducted for measles virus N brings about profound conformational changes in the protein. For N-P interaction the data suggest that the P protein possibly interacts with several domains on N protein localized in both N-terminal and C-terminal.

3.5.2. *Development of a Diagnostic Test for PPRV Based on the Viral H Protein*

Among the structural proteins comprising PPR, two are of particular interest for the diagnosis of Morbillivirus infection: the first is the N protein, which is the major viral protein and, also in addition, the most immunogenic, in spite of its internal location. The majority of antibodies induced by the virus are directed against this protein. The second is the attachment protein, H, to which are directed neutralizing antibodies produced by the infected host. Currently there are two diagnostic tests for PPR that utilise these proteins, both based on cELISA tests. Unfortunately, each shows cross reactivity with rinderpest virus, which is closely related to PPRV. Because of the cross-reactivity shown by currently available PPRV cELISAs, APU embarked on the development of a new cELISA with high specificity (supported by Wellcome Trust and European Commission Grants).

3.5.2.1.   Production of new MAbs against the HPPRV

For the development of a new cELISA, it was essential that new monoclonal antibodies were produced with different specificities from those currently available. In 2007, spleen cells from mice which had been immunized with the plasmid into which was cloned the PPRV H gene were fused with myeloma cells. Unfortunately, none of the hybridomas which were obtained were found to secrete anti-PPR H protein antibody. Further experiments were initiated to produce new batches of monoclonal antibodies after improvement of the protocol for immunizing mice. For this purpose, the full PPRV H gene cDNA was cloned into the pCineo plasmid but on this occasion the sequence upstream of the start codon of the gene translation was changed, with the objective of enhancing protein expression. Mice were then immunized with this new construct.

Before producing the new anti-PPPRV monoclonal antibodies H protein, the system for their selection needed to be improved. The PPRV H cDNA which had been cloned into the plasmid pCineo was transferred into a baculovirus expression vector. As can be seen in **Figure 20**, the insect cells infected by this recombinant baculovirus/PPRV H expressed the recombinant protein very efficiently, and it was detected optimally using an immunofluorescence test carried out with an antibody used in the current H-based cELISA.

A: Non infected, negative insect cells   **B: IF of cells infected with the recombinant baculovirus/PPRV H virus**

Figure 20. Immunofluorescence tests carried out with insect cells infected with recombinant baculovirus/PPRV H.
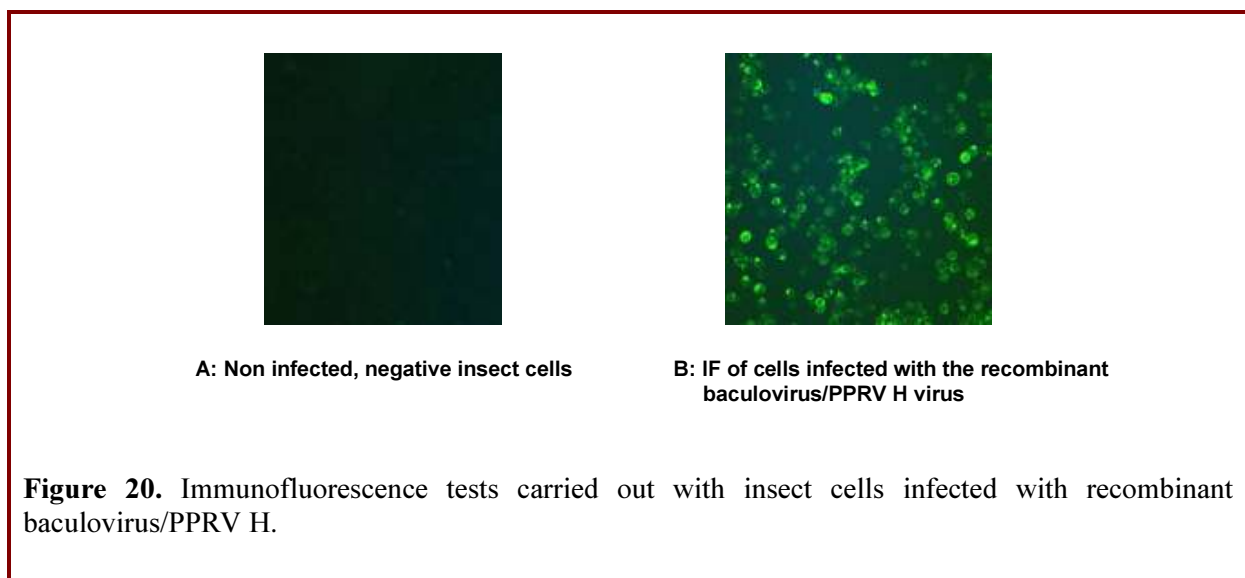
3.5.3.   Development of a Stable Eukaryotic Cell Line Constitutively Expressing Morbillivirus Receptor Signalling Lymphocyte Activator Molecule (SLAM) and Investigation of its Use for the Isolation of PPRV

Cellular receptors are one of the major determinants responsible for the host range and tissue tropisms of a virus. Recently it has been reported that human Signalling Lymphocyte Activator Molecule (SLAM), a membrane protein expressed on some lymphocytes and dendritic cells, is a receptor for morbilliviruses. It has been shown that isolation of wild type morbilliviruses (measles virus, rinderpest virus and canine distemper virus) in cell culture *in vitro* is enhanced when the cells are expressing this receptor. Currently, Vero cells and lamb cells are the cell culture systems used for PPRV isolation. This takes at least one to three weeks if successful. In order to facilitate the isolation of wild type PPRV from pathological samples collected in the field, it was decided to develop a cell line which expressed the virus

receptor constitutively.

3.5.3.1. Cloning and selection of the Synthetic Signalling Lymphocyte Activator Molecule Gene (SLAM) into a plasmid to use for Vero cell transfection

Bovine, sheep-goat, and canine synthetic SLAMs respectively named BTS, CHS and CLF were cloned into the plasmids pCineo and pCDNA5/FRT and were used to transfect Vero (pCineo derived recombinants) and Flp-CV-1 cells (pCDNA5/FRT derived recombinants). Those plasmids carry the gene for resistance to the following antibiotics: G-418 for pCineo and Hygromycin B for Flp-CV-1. Cells which have taken up the plasmid are selected for *in vitro* in the presence of these antibiotics. Cells which have survived after many passages in the selected medium are therefore presumed to have integrated the plasmid and, by extension, are expressing the SLAM protein. The characterization/identification of these cells was done in two ways: identification of viral RNA and immunofluorescence testing using an anti-SLAM antibody

3.5.3.2. Characterization of constitutionally expressing SLAM protein by identification of the RNA

If the SLAM gene has been integrated into the genome of the cell along with the gene which confers to such cells resistance to the antibiotic, then the corresponding messenger RNA should be found in those cells. Hence, messenger RNA, (mRNA), was extracted from the cells which were surviving in the selected medium. They were then reverse-transcribed into cDNA and amplified by PCR using specific primers to each SLAM or to a housekeeping gene, the β-actin, as an internal control and, as shown in **Figure 21**, the expression of SLAM

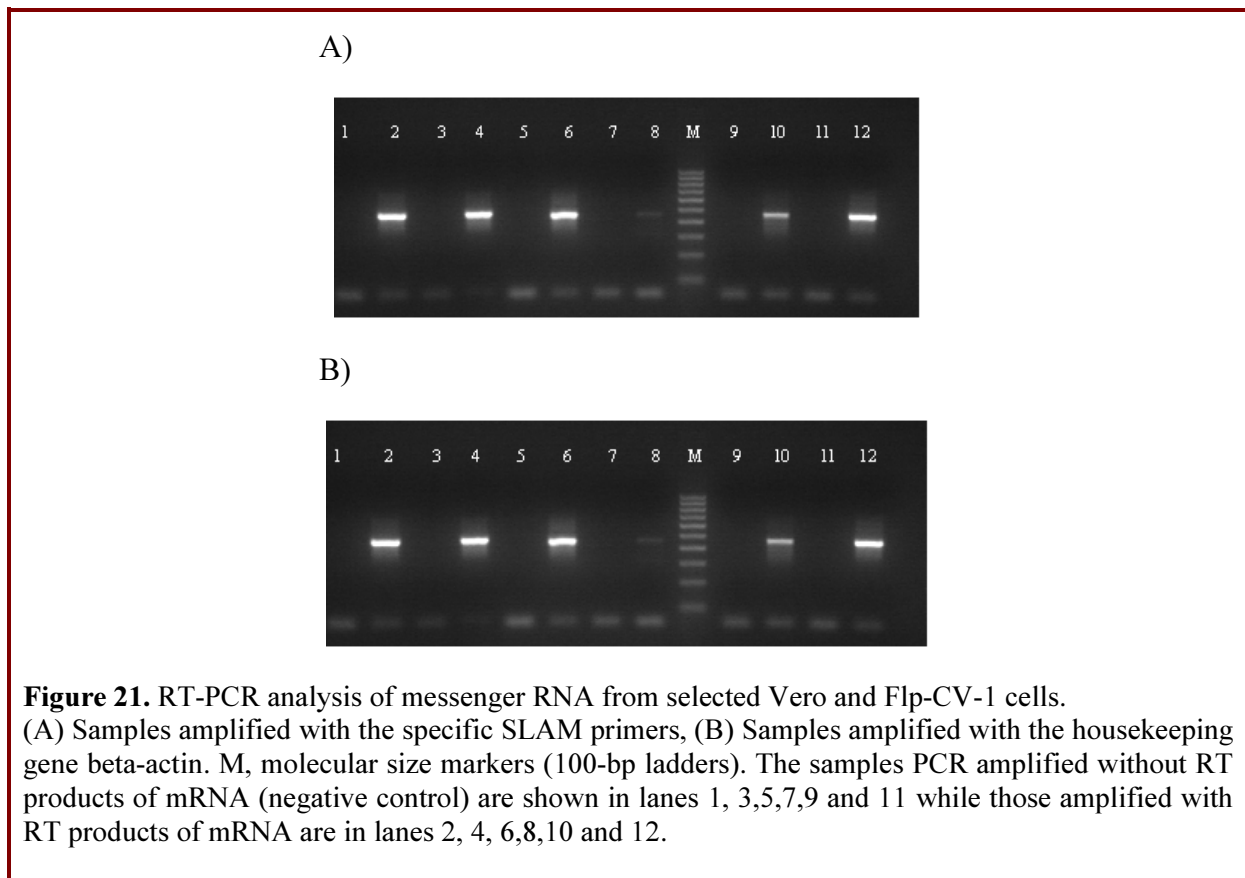**Figure 21.** RT-PCR analysis of messenger RNA from selected Vero and Flp-CV-1 cells.
(A) Samples amplified with the specific SLAM primers, (B) Samples amplified with the housekeeping gene beta-actin. M, molecular size markers (100-bp ladders). The samples PCR amplified without RT products of mRNA (negative control) are shown in lanes 1, 3,5,7,9 and 11 while those amplified with RT products of mRNA are in lanes 2, 4, 6,8,10 and 12.

was detected in samples 6 (BTS in Flp-CV-1) and 8 (CHS in Flp-CV-1).

3.5.3.3.   Characterization of constitutively expressed SLAM protein by an immunofluorescence (IF) test

The SLAM-specific mRNA expression of the two positive samples was analysed by immunofluorescence staining in paraformaldehyde (PFA), but without treating the cells with Triton. The expression of the SLAM protein was detected with two anti-SLAM MAbs that were raised against activated B cells (IPO-3) or T cells (A12) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin G (IgG). As can be seen in **Figure 22**, SLAM protein expression was confirmed in the two samples with those MAbs (C to F), but not on samples with the parental Flp-CV-1 cells (A and B).
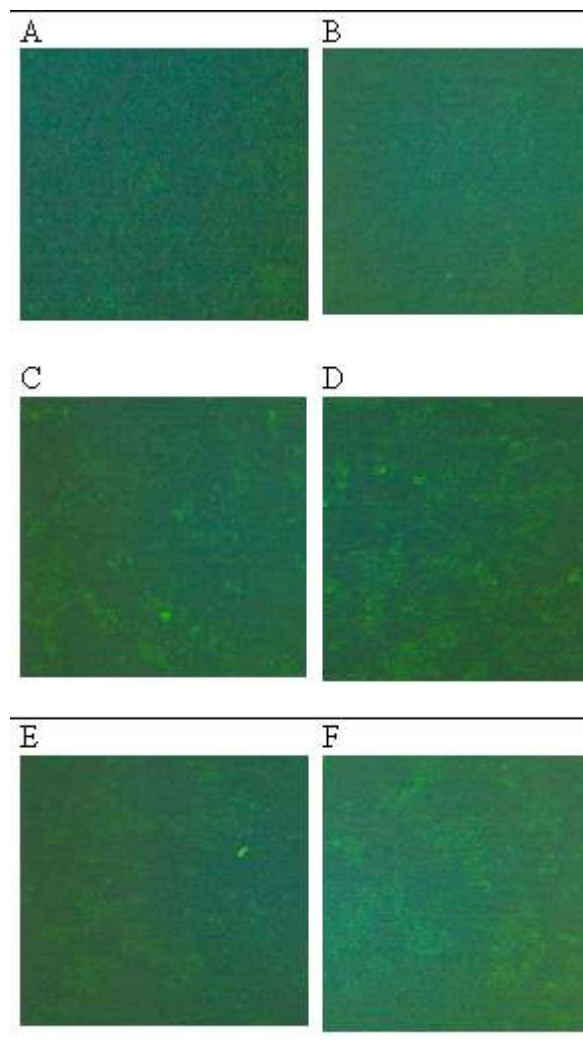

Figure 22. Detection of the SLAM gene protein expression by immunofluorescence staining with anti SLAM MAbs IPO-3 (C and E) and A12 (D and F) A and B, normal Flp-CV-1 cells.

These findings indicate that the BTS and CHS SLAMs are expressed in Flp-CV-1 cells. Therefore, they could be an ideal tool for the rapid isolation of PPRV. In 2009, these cells will be tested for their efficiency in isolating PPRV using positive field samples.

3.5.4.   Development of a Real-Time PCR for the Detection and Quantification of Peste des Petits Ruminants Virus

Nucleic acid recognition-based methods are powerful techniques for specific and rapid detection of pathogens. Currently, PPRV diagnosis based on this technology relies on a gene amplification technique using a classic PCR. For this purpose, the target proteins of the PCR are the nucleocapsid (N) or fusion (F) protein genes. The classical PCR, however, has several limitations of which the most important is the post amplification processing of the products with its concomitant high risk of contamination. This has driven scientists to seek alternative methods and there has been increasing focus on the development of assays relying on the closed vessel systems involved in real time PCR.

A real time PCR based on a classical TaqMan approach has already been described for the detection and quantification of PPRV. It uses the standard 3′ quencher fluorophore 6-carboxy-tetramethyl-rhodamine (TAMRA) to quench the 6-carboxyfluorescein (FAM) signal. At APU, we are developing a real time PCR for the detection of PPRV based on the use of a dual-labeled fluorogenic probe approach using TaqMan and dihydrocyclopyrroloindole tripeptide (MBG). Since in this method, a non-fluorescent quencher (NFQ) is used in the 3' position instead of the TAMRA and because the MBG allows the design of shorter probes, the fluorescence quenching is more efficient, thereby increasing sensitivity of the assay.

The amplification primers and the probe used in this test target the virus N gene. Since PPRV isolates are grouped into four discrete lineages, the design of the primers and probe was enabled by taking into consideration results from partial N gene sequencing obtained from a representative isolate of each group. Accordingly, they are expected to allow detection of any PPRV strain.

Preliminary evaluation showed that all four lineages of PPRV viruses could be detected. The assay linearity was determined by amplifying 10-fold serial dilutions of a plasmid containing the PPRV Nigeria vaccine strain (**Figure 23**). The dynamic range of the assay was found to be of 6 orders of magnitude (10^7 to 10^2) with an efficiency of the amplification of -3.25 (R2 = 0.998) as indicated by the slope of the standard curve obtained by plotting the Cycle threshold (Ct) values against the Log_{10} of the known input copy number (**Figure 24**). The limit of detection is below 10 copies; even 1 copy / PCR vial can be detected (results not shown). This test also has the advantage of being very fast as only a maximum of 40 cycles is needed (55 minutes with Bio-Rad CFX96).

Further evaluation will involve: -

- Checking specificity by comparing DNA or cDNA from other pathogens infecting ruminants.

- Determining quantitatively the limits of detection by probit regression analysis.

- Validating the assay by using a larger number of isolates of PPRVs from each of the four lineages.

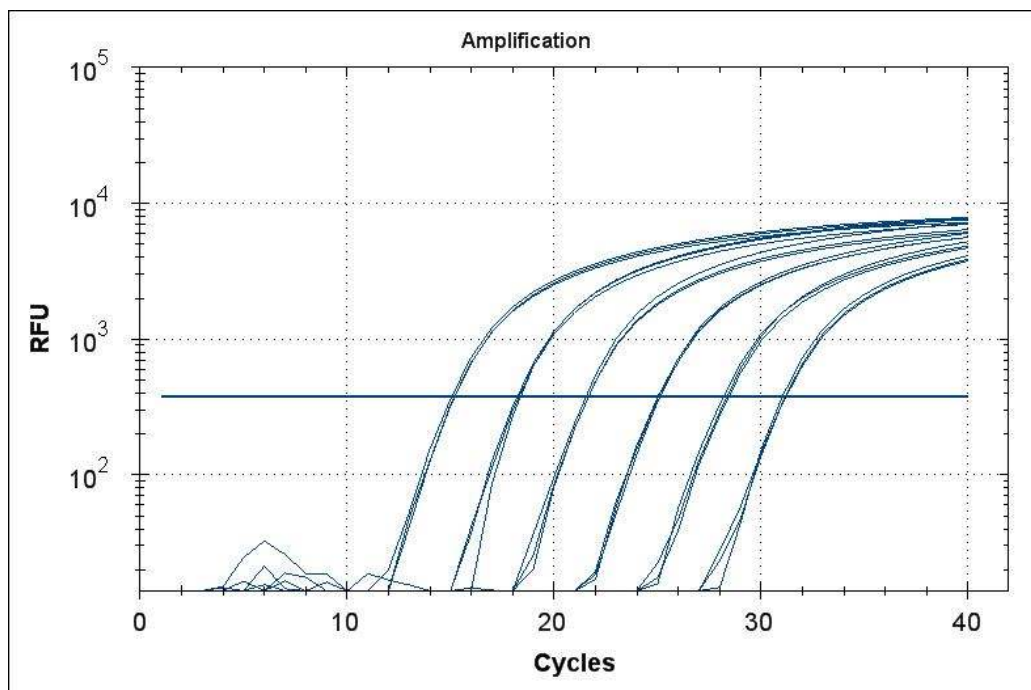**Figure 23:** Detection of PPRV by TaqMan MGB real time PCR. The amplification plots were realized on 10-fold serial dilutions (10^7 to 10^2 copies) assayed in triplicate using a plasmid containing the PPRV N gene.
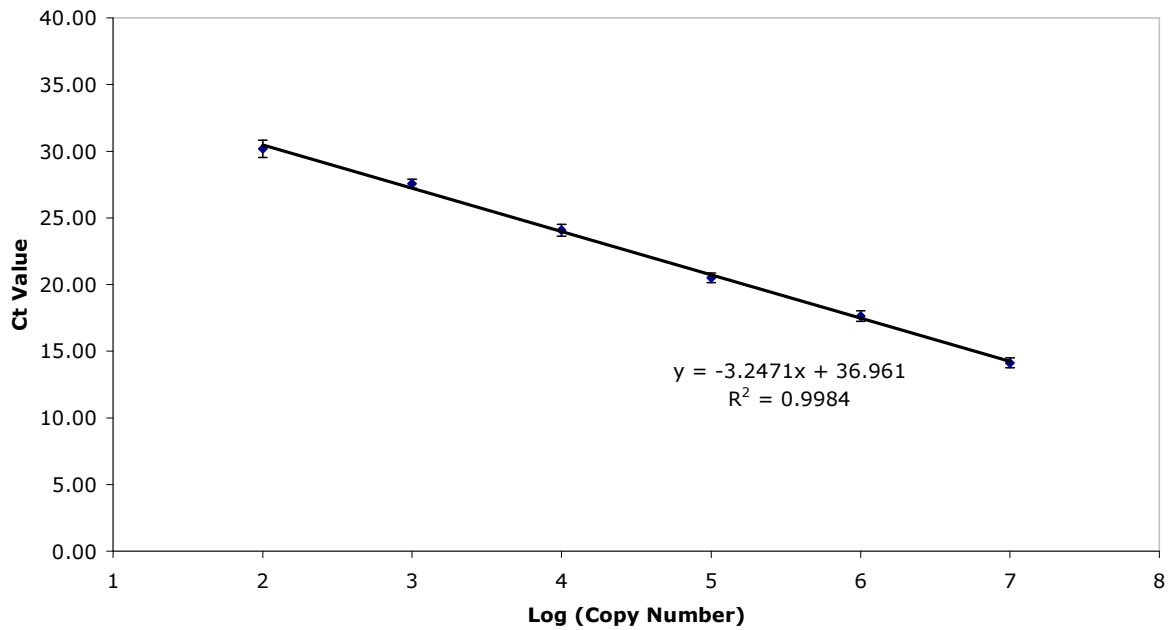
Figure 24. Linearity of the PPRV TaqMan MGB assay. The standard curve was generated by plotting the Cycle Threshold measured in triplicate during three separated runs against the Log of the input copy number.

3.6.   Studies on Capripox Virus

The *Capripoxvirus* (CaPV) genus of the *Poxviridae* family comprises *sheep poxvirus* (SPPV*)*, *goat poxvirus* (GTPV) and *lumpy skin disease virus* (LSDV) which are causative agents of highly contagious diseases of small ruminants and cattle characterized by fever and ocular and nasal discharge. Pox-like lesions appear on the skin, respiratory and gastrointestinal mucosa and the mortality can be very high. Together, these viruses are responsible for some of the most economically important diseases affecting sheep, goats and cattle in endemic areas in Africa and Asia. CaPVs are generally considered to be host specific, because virus isolates may preferentially occur or cause disease in only one host species. However, the ability of SPPV and GTPV strains to cross-infect, naturally or experimentally, and cause disease in both sheep and goats has been described. This apparent variability in SPPV and GTPV host range, together with the fact that infections they cause are indistinguishable clinically, and different strains of the viruses (including LSDV) cannot be differentiated serologically has been a stimulus to the development of new technologies to increase our understanding of the relationships amongst these diseases. Given their economic importance and potential to spread beyond regions where they normally occur, there has been interest in both the epidemiology of the diseases and the development of more effective means for their control. For instance, the criteria by which CaPVs are identified and upon which their nomenclature is based rely on the geographical origin and the host from which the virus has been isolated. Nonetheless, there have been reports, based on examination of a partial or full genome sequencing of CaPVs, to suggest that SPPV, GTPV and LSDV are genetically distinct from each other and can be grouped as three different species: goat poxvirus, sheep poxvirus and lumpy skin disease virus.

In order to clarify this problematic area, APU embarked on a project to produce new capripox virus-specific diagnostic tests and to attempt to resolve their epidemiological relationships. Financial support has been provided by the French Ministry of Foreign Affairs and there has been productive collaboration with partner laboratories in France and Africa. This has allowed us to acquire CaPVs samples from sheep and goats from various geographical origins. This study was made possible through an agreement between the Agency Laboratory and the Austrian Agency for Health and Food Safety (AGES). Infectious materials are received and handled at the high security laboratory of the AGES.

3.6.1.   *Molecular Epidemiology of CaPVs Based on G-Protein-Coupled Chemokine Receptor and RNA Polymerase Subunit of 30 Kda (Rpo30) Genes*

Our approach to understand more fully the epidemiology of CaPVs based on molecular methods as well as the development of tools for strain identification, initially involved the cloning and sequencing of the G-protein-coupled chemokine receptor (GPCR) gene of CaPVs from different geographical origins at APU and at CIRAD in Montpellier (France) in 2007. In 2008, at APU, further strains were included in the study: nine LSDV samples from Onderstepoort Veterinary Institute (OVI) in South Africa one LSDV and one SPPV isolate received from AGES and one additional SPPV isolate received from the Institute of Animal Health (IAH), UK. These findings were used together with those obtained in 2007 to generate a phylogenetic tree and search for distinctive molecular signatures for each isolate of CaPV. The complete phylogenetic tree was based on 58 CaPVs (including nine sequences retrieved from the gene bank) and confirmed the preliminary assessment of their phylogenetic relationships made in 2007:

- The CaPVs can be grouped into three distinct lineages; the SPPV lineage, the GTPV lineage and the LSDV lineage (**Figure 25**).

- With a few exceptions, this classification correlates closely with the host origin of CaPV isolates.
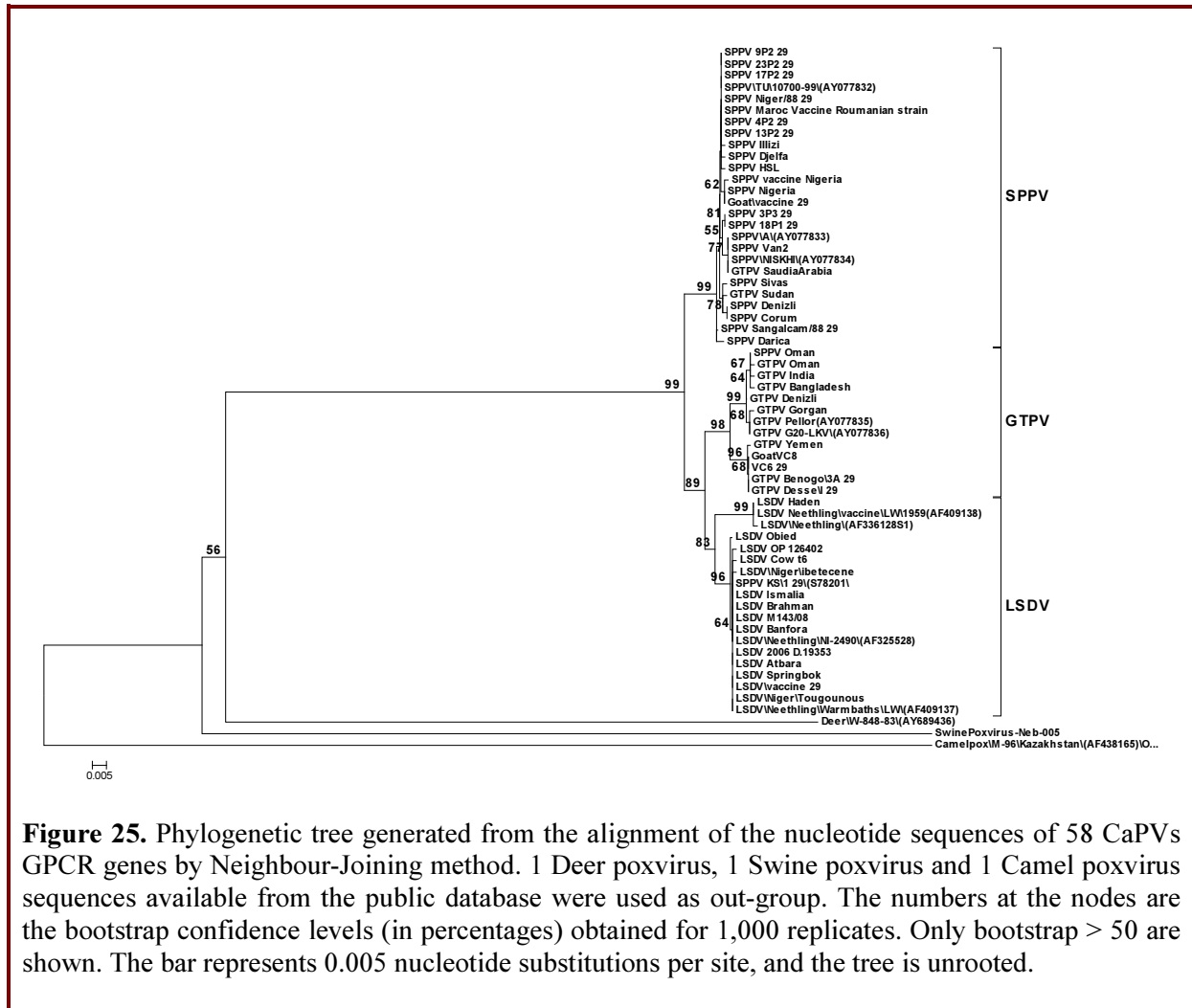

Figure 25. Phylogenetic tree generated from the alignment of the nucleotide sequences of 58 CaPVs GPCR genes by Neighbour-Joining method. 1 Deer poxvirus, 1 Swine poxvirus and 1 Camel poxvirus sequences available from the public database were used as out-group. The numbers at the nodes are the bootstrap confidence levels (in percentages) obtained for 1,000 replicates. Only bootstrap > 50 are shown. The bar represents 0.005 nucleotide substitutions per site, and the tree is unrooted.

In order to increase confidence in these conclusions, and also to assist in the development of molecular diagnostic tools with the capacity to differentiate strains, another CaPVs gene, the orthologue of the vaccinia virus RNA polymerase, subunit 30 (RPO30), was cloned and sequenced. In addition to the CaPV isolates already obtained from our collaborators in 2007, more DNA samples were provided by OVI (13 isolates) and CIRAD (12 isolates). The RPO30 genes of 29 isolates were successfully cloned and sequenced and the results were analysed together with those of eight isolates retrieved from the Gene bank. As was found with the GPCR gene, GTPV, LSDV and SPPV cluster clearly into three separate groups (**Figure 26**).

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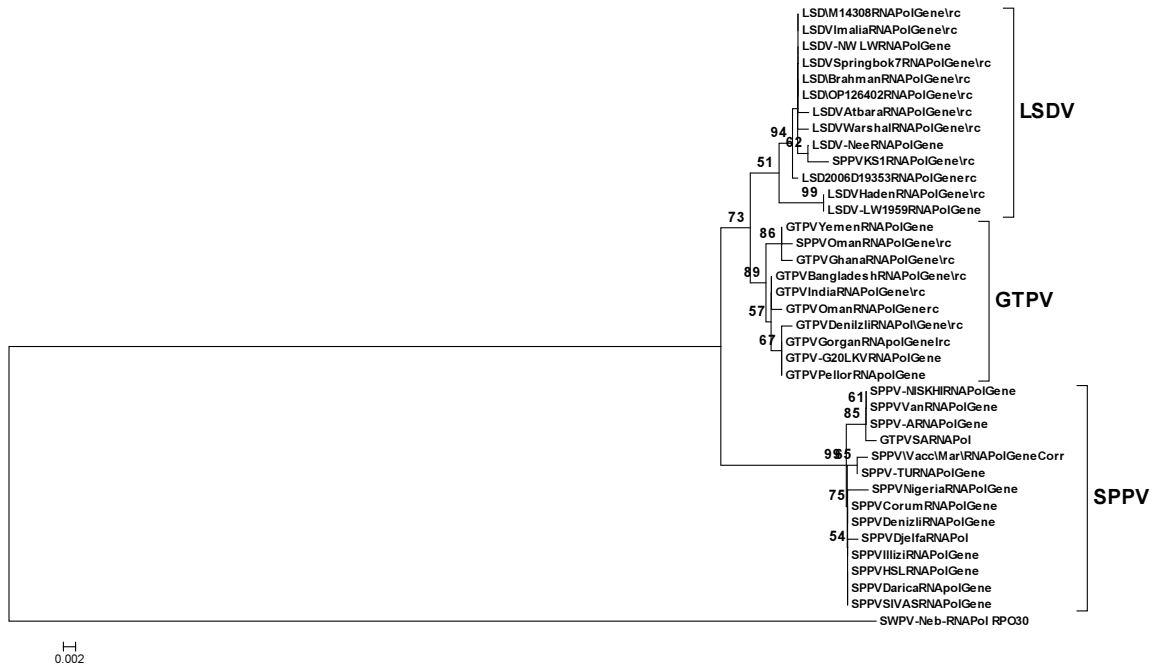
Figure 26. Phylogenetic tree generated from the alignment of the nucleotide sequences of 37 CaPVs RPO30 genes by Neighbour-Joining method. 1 Swine poxvirus sequence available from the public database was used as out-group. The numbers at the nodes are the bootstrap confidence levels (in percentages) obtained for 1,000 replicates. Only bootstrap > 50 are shown. The bar represents 0.005 nucleotide substitutions per site, and the tree is unrooted.

3.7. Development of Molecular Diagnostic Tools for Detection and Differentiation of CaPVs: Classical PCR for CaPVs Based on the RNA Polymerase Gene

In 2007 a classical PCR was developed to differentiate SPPV and GTPV based on a 21 nucleotide deletion found in the GPCR gene of SPPV strains. Unfortunately, the test failed to differentiate between SPPV and GTPV because some GTPV strains were also found to have this deletion in their GPCR gene. More recently, the alignment of nucleotide sequences following the cloning and sequencing of the CaPVs RNA polymerase gene (RNA Pol), has shown a constant 21 amino acid deletion in all SPPV genes. Neither GTPV nor LSDV virus isolates have presented this deletion (**Figure 27**). Therefore, primers were designed to amplify a region containing the deletion to give an amplicon of 172 bp for GTPV and 151 bp for SPPV (**Figure 28**).
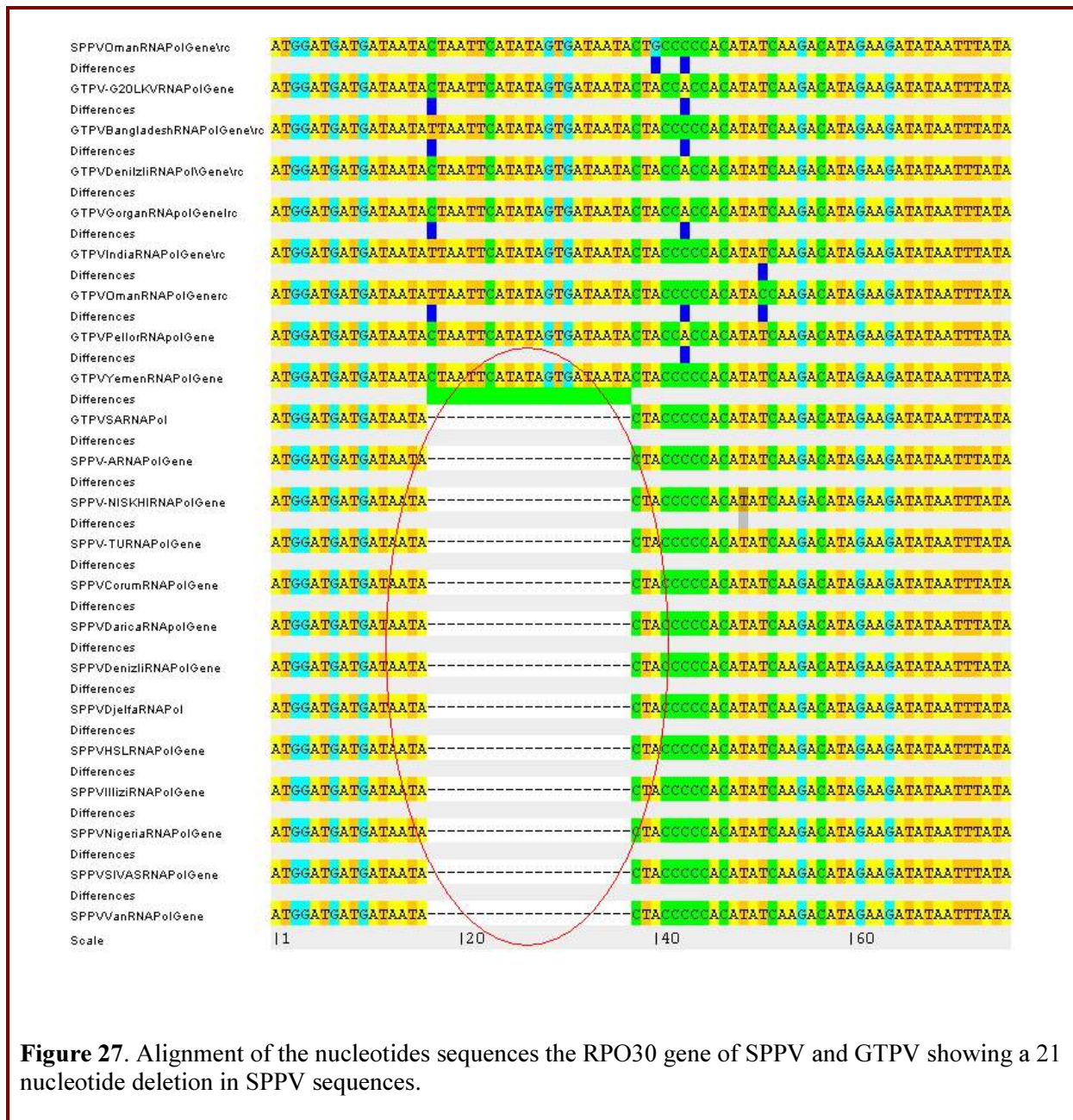

Figure 27. Alignment of the nucleotides sequences the RPO30 gene of SPPV and GTPV showing a 21 nucleotide deletion in SPPV sequences.
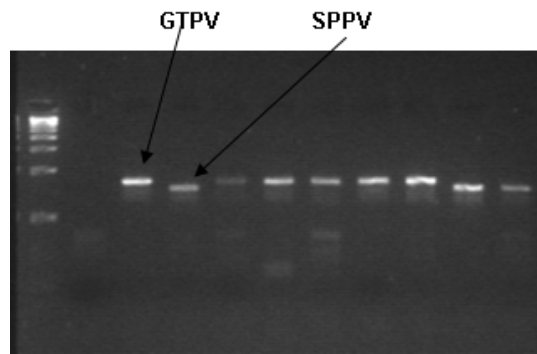
Figure 28. Classical PCR for GTPV and SPPV: SPPV and GTPV PCR product different lengths due to a deletion in SPPV RPO30 gene.

By using this technique it was possible to differentiate CaPVs containing the deletion in their RPO 30 gene from those without a deletion. This method will be suitable to differentiate SPPV from GTPV in sheep and goats, since these are the only two CaPVs capable of infecting small ruminants.

3.7.1.1.   *Real-Time PCR for CaPVs based on classical Fluorescence Resonance Energy Transfer (FRET)*

A real time PCR assay based on Fluorescence Resonance Energy Transfer (FRET) chemistry that was able to differentiate SPPV from GTPV was developed in 2007. This assay was further optimized and evaluated in 2008. It was found possible to detect and differentiate lumpy skin disease virus, the third member of CaPV genus. From the peaks obtained in the fluorescence melting curve analysis (FMCA) it was possible to readily distinguish individual isolates belonging to the SPPV (Tm = 52 °C), LSDV (Tm = 61 °C) and GTPV (Tm = 69 °C) groups (**Figure 29**).

The assay has now been validated using 50 CaPV isolates from different geographical origins. The results show that 23, 12 and 15 isolates showed melting peaks for SPPV, GTPV and LSDV respectively. The identification of each isolate in this FRET assay using its characteristic melting point is in agreement with what had already been determined by phylogenetic analysis using both the GPCR and the RPO30 genes. This confirms that this technique represents an easy and rapid method for genotyping CaPVs without the need for gene sequencing.

The linearity of the assay and the analytical sensitivity of detection were established for SPPV, GTPV and LSDV using plasmids containing GTPV, SPPV and LSDV GPCR genes. The limits of detection determined by probit regression analysis (**Figure 30**) were 15.08 (13.04 to 18.36), 16.38 (14.48 to 19.38) and 37.39 (30.03 to 51.24) copies per reaction for GTPV, SPPV and LSDV respectively.
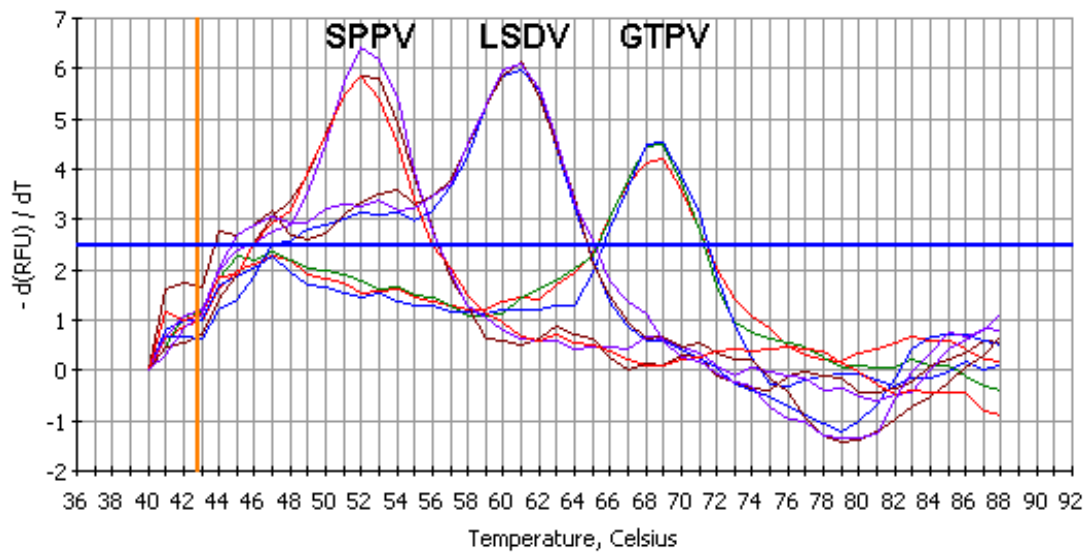
Figure 29. Melting curve of SPPV, LSDV and GTPV using FRET real time PCR.
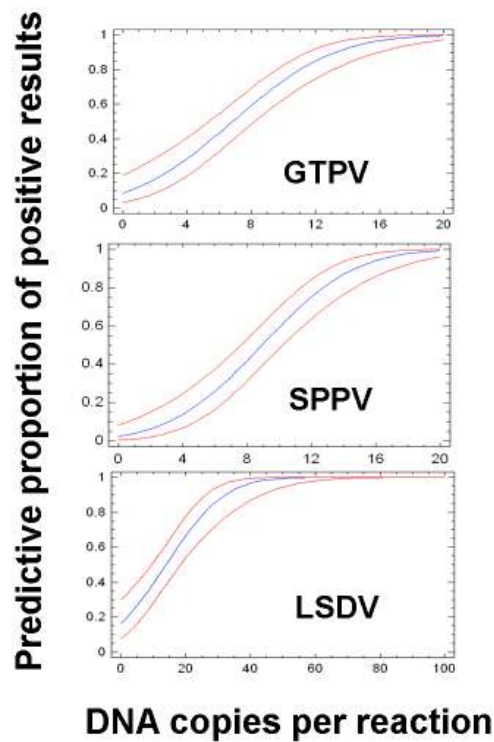
Figure 30. Determination of PCR detection limit by probit regression analysis.

The linearity of the assay was established by amplifying a 10-fold serial dilution of plasmid containing the full GPCR gene for each genotype. The resulting cycle threshold (Ct) values were plotted against the Log input copy number (**Figure 31**). The linearity range was from $2x10^7$ to $2x10^1$ for GTPV and SPPV (Slope = -3.33 and -3.46 respectively) and $2x10^7$ to $2x10^2$ for LSDV (Slope = -3.70).
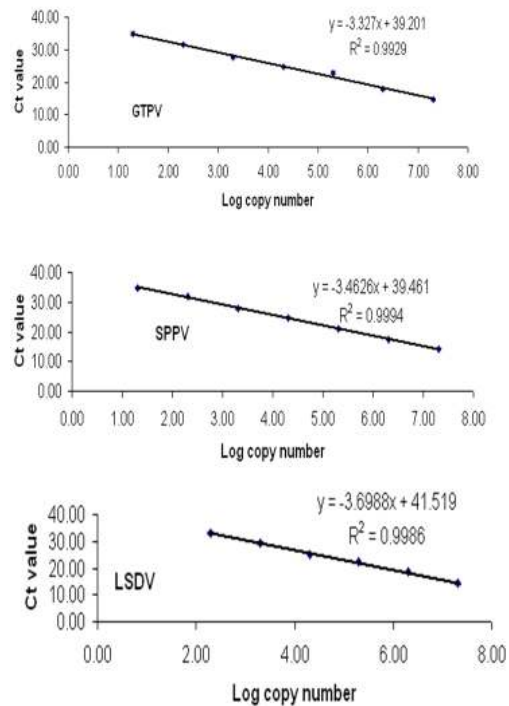
Figure 31. Linearity of the classic FRET assay. The standard curves were generated using 10-fold serial dilutions of plasmids containing SPPV GTPV and LSDV GPCR genes**.**

To see the performance of the quantification for the virus and not a plasmid with the single target gene, GTPV and SPPV genomic DNA was extracted from 10-fold serial dilution of infected cell culture and amplified. The corresponding Ct values were plotted against the Log tissue culture infected dose 50 (TCID$_{50}$)/mL (**Figure 32**).

Figure 32. Linearity of the classical FRET assay. The standard curves were generated using DNA extracted from 10-fold serial dilutions of SPPV and GTPV grown on cell culture.

The results shows good linearity from 10^{7} $(TCID_{50})$/mL to 10^2 $(TCID_{50})$/mL (slope -3.4, R^2 = 0.995 and slope -3.5 R^2 = 0.994 for GTPV and SPPV respectively). This real time PCR based on F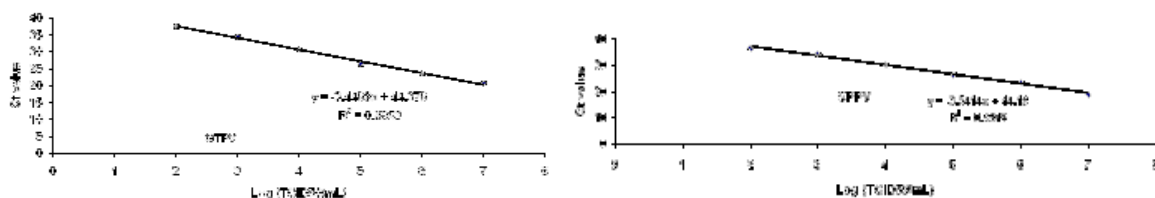RET chemistry is a powerful method for the simultaneous detection, quantification and genotyping of CaPVs. The main limitation of the FRET assay, however, is that it can only be performed on real time PCR machines which allow an asymmetrical positioning of the filters. Since some of our partners in the Member State laboratories do not have such equipment, an alternative method to the classical FRET, with similar performance characteristics, was developed for genotyping and quantification.

3.7.1.2.   Real-Time PCR for CaPVs using Black Hole Quencher (BHQ) on the 3' end of the Fluorescence Resonance Energy Transfer (FRET) donor probe

To enable the genotyping of CaPVs, a special method was designed at APU where the progress of the PCR reaction is monitored by following the decreasing intensity of the fluorescence signal. The same primers and probes sequences as for the normal FRET assay were used to target the GPCR gene of CaPVs. This new real time PCR method followed the same principle as the FRET assay except that instead of using a 3' fluorophore-labelled linear probe (6'- carboxy-fluorescein (FAM) in our classical FRET assay) to excite the acceptor probe, we have used a Black Hole Quencher (BHQ) to quench the signal from the 5'Cy5-labelled acceptor probe, consequently, the accumulation of PCR amplicons in this assay results in a decrease in the strength of the fluorophore signal. (**Figure 33**)
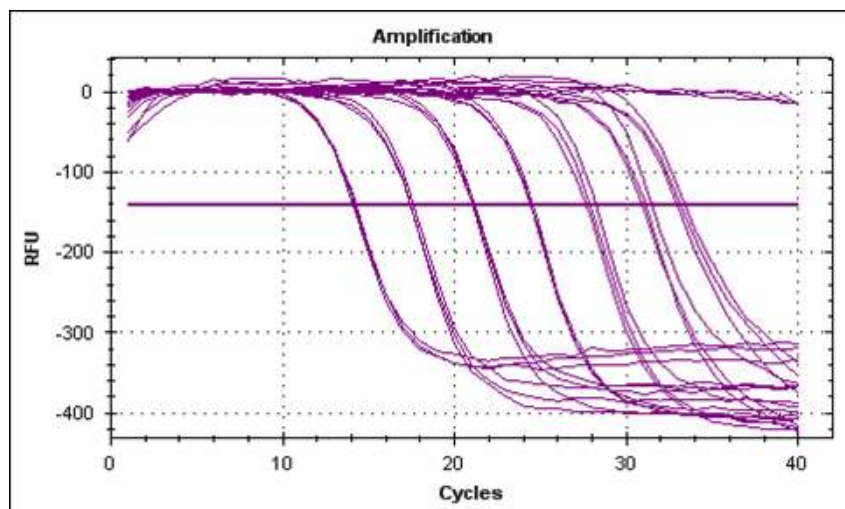
Figure 33. Amplification curves in BHQ-FRET assay. These curves show the relative fluorescence units (RFU) at each cycle of the reaction. The amplification threshold used to establish the cycle threshold (Ct) is indicated by a horizontal line across the graph.

A second consequence of this approach is that there is no need for asymmetric positioning of a filter in the PCR machine because the acceptor probe is excited using its own excitation filter, whereas in the normal FRET, the acceptor probe gains its excitation from the emission of a donor probe after it has been excited through it's own excitation channel. This method

has required considerable development and optimisation to obtain the correct quencher/fluorophore concentration so that the PCR amplification could be followed by the decrease in fluorescence. When FMCA is performed after the PCR, the melting peaks obtained are inverted compared to those obtained when using the normal FRET, but, as in the normal FRET, the melting curve analysis after the PCR allows to discriminate SPPV (49 °C) GTPV (65 °C) and LSDV (59 °C) (**Figure 34**).
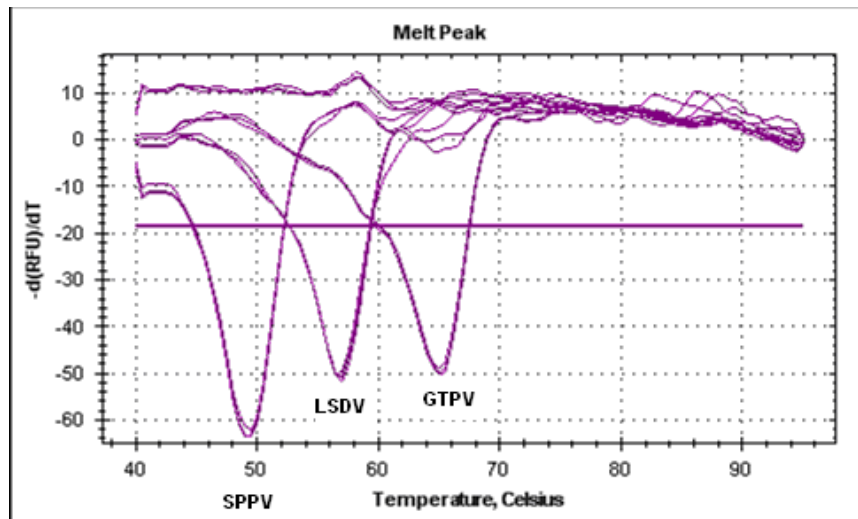
Figure 34. Thermal melting curve of the Cy5 labelled FRET probe with the SPPV, LSDV and GTPV sequences in the BHQ-FRET assay.

To see if this method could also be used for quantification, the data were exported via Excel and the Ct were calculated manually, because the currently available real time PCR software is unable to display Ct values obtained from decreasing fluorescence. The calibration curve for GTPV was generated from curve Ct against Log copy number using 10-fold serial dilutions of plasmid containing GTPV Denizli GPCR gene.

It was found that this method can also be used for gene quantification with good linearity and high sensitivity (slope = -3.26 and R^2 = 0.996) (**Figure 35**). This assay is currently being evaluated further to ensure that it can be performed on any q PCR machine. It is anticipated that this new technique will be transferred to our MS partners in Mali and Ethiopia in 2009.

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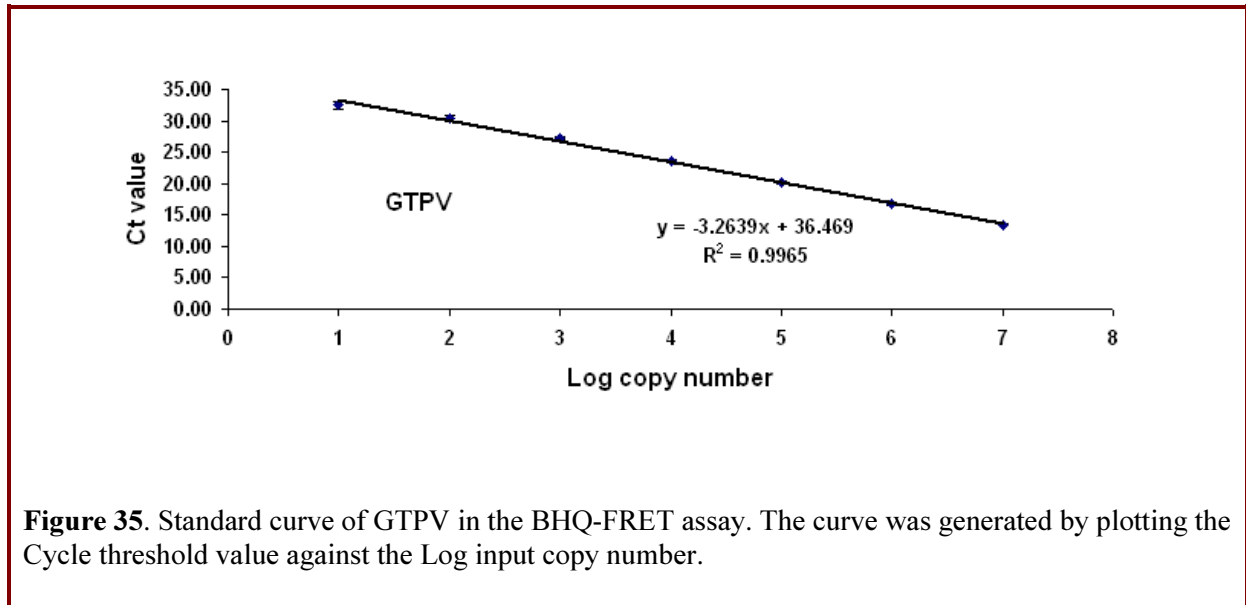
Figure 35. Standard curve of GTPV in the BHQ-FRET assay. The curve was generated by plotting the Cycle threshold value against the Log input copy number.

3.8. Sequencing of the Full Capripox Viral Genome

CaPVs infections are controlled in endemic areas by means of vaccination using live attenuated viruses. These vaccines are obtained from field isolates which have been attenuated by serial passage in cell culture or on the chorioallantoic membrane of embryonated hen's eggs. However, vaccine failures and adverse effects have been reported on many occasions after vaccination, indicating the need to improve their efficacy. This may be achieved when sufficient data are available on the full genome of CaPVs. The comparison of genomic data from field strains and vaccine strains within one member of the species and also the comparison of full genome data between representative numbers of CaPVs field strains may lead to the identification of genes which are involved in pathogenicity and host specificity. Rational deletion of such genes could attenuate virus infectivity and virulence, as has been found already with vaccinia virus. In order to understand the molecular basis of capripox virus pathogenicity and host species specificity, the genome of different capripox virus strains was sequenced. The same viruses will also be tested in animals in Africa. By analysis of these results and those already available in literature, it may be possible to develop a safe and efficient capripox vaccine. The capripox virus sequencing work started in 2007 and led to the sequencing of the full genome of one goat strain isolated in Turkey, the Denizli strain. In 2008, eight additional strains were purified and their genomic DNAs were extracted for full genome sequencing. These strains are: SPPV Denizli, SPPV Corum, GTPV Gorgan, GTPV Yemen, GTPV Oman, SPPV Oman, SPPV KS1, and SPPV Djelfa.

The data obtained were assembled into continuous sequences ranging from 147541 to 148093. The nucleotide composition of the strain is 75% A+T and each of them has 150 ORF as was also found for the SPPV and GTPV genomes available in the gene bank. Although 1000 to 2000 nucleotides are missing in the extremities, these full genome data were used to compare GTPV and SPPV strains on one hand and also to compare vaccine strains with field isolates in the case of SPPV and GTPV on the other hand. The Genome Annotation Transfer Utility (GATU) program (http://athena.bioc.uvic.ca/tools/GATU) was used to compare the genomes of all GTPV isolates to that of the GTPV Pellor (retrieved from the gene bank) (**Table 4**) and those of SPPVs to the genome of SPPV A (retrieved from the gene bank) used as reference. (**Table 5**)

Table 4. Comparison of GTPVs with GTPV Pellor as a reference.

Genome feature	GTPV Pellor	GTPV G20LKV	GTPV Denizli	GTPV Gorgan	GTPV Oman	GTPV Yemen	SPPV Oman
Number of genes with 100% similarity to GTPV pellor		146	92	131	76	37	39
Number of Genes with <100 to 98% similarity with GTPV Pellor		2	18	1	59	80	78

Table 5. Comparison of SPPVs to SPPV A taken as reference.

Genome feature	SPPV A	SPPV TU	SPPV Niskhi	SPPV Denizli	SPPV Djelfa	SPPV Corum	GTPV Yemen	SPPV Oman	SPPV KS1
Number of genes with 100% similarity to SPPV A		131	141	123	119	113	19	21	22
Number of Genes with <100 to 98% similarity with SPPV A		19	6	10	14	11	69	68	83

The results show that GTPVs genomes are more heterogeneous than SPPV and can be arranged into two groups:

Group 1: GTPVs with more than half of their genes displaying 100% similarity to GTPV Pellor and which contains GTPV Pellor, GTPV G20LKV, GTPV Denizli, GTPV Gorgan and GTPV Oman.

Group 2: GTPVs with less than half of their genes showing a similarity to GTPV Pellor and which contains GTPV Yemen and SPPV Oman. The heterogeneity of GTPVs is further distinguished by the fact that only 22 genes are entirely conserved between the different strains.

In contrast to GTPVs, all SPPV are highly similar to SPPV A with more than 110 genes out of 150 being fully conserved between each of them and SPPV A. Ninety three genes out of 150 are fully conserved between the different strains of SPPVs including that used for vaccine production. (**Table 5)**.

SPPV Oman and GTPV Yemen, with only 21 and 19 genes fully conserved from SPPV A, are more similar to GTPV than SPPV. When the threshold of this comparison is taken down to 98 % similarity, SPPV Oman and GTPV Yemen display 117 gene out of 150 similar to those of GTPV Pellor and 89 and 88 genes out of 150 similar to those of SPPV A, confirming that they are more closely related to GTPV. However, they also display high similarity to SPPV. These strains have similar pathogenicity in both sheep and goat. Such isolates may be responsible for the conflicting reports on CaPVs pathogenicity in sheep and goat. SPPV KS1, with only 22 genes fully conserved in SPPV A, appears to be a LSDV because 142 genes/158 are 100% similar to those of LSDV Nee. When the threshold value is taken down to 98%, the similarity to SPPV A becomes 105 genes out of 150. SPPV KS1, which is used as CaPV vaccine in small ruminants, has already been shown to be a LSDV, but its full genome was not sequenced. Within both GPTV and SPPV groups, the genomes of the vaccine strain and those of the field strains were compared. The result shows that:

For SPPV, the ankyrin repeat protein (SPPV 138) of SPPV Niskhi (SPPV vaccine strain) is below the threshold of 60% similarity to other SPPVs

For GTPV, the Kelch-like protein gene (GTPV_gp 016) of GTPV G20LKV (GTPV vaccine strain) is below the threshold of 60% similarity to others GTPV. Therefore, it appears that these genes may play a role in CaPVs pathogenicity and host specificity.

In 2009, we will sequence the missing fragments at the extremities of the nine CaPVs to obtain the entire genomes with all ORF. It is anticipated that the full genome sequencing project will continue and include the SPPV Romania vaccine strain and one GTPV field strain from Saudi Arabia. This will allow the comparison of more SPPV and GTPV genomes and provide consistent data on the genes involved in pathogenicity and host specificity.

3.9. IAEA Reference Serum Bank

The successful development and standardization of serological diagnostic tests requires the availability of standardized, equilibrated and validated reference serum samples of different animal species whose origin and infection status provide the means to identify causative agents of infectious disease. The APU is building up and maintaining an important resource of internationally accepted reference sera for such applications, providing material essential for internal quality control when standardizing serological assays. These sera are initially provided by Member States and, in addition, are made available to MS as standards in the development of assays, to act as reference reagents both internally in diagnostic testing, to establish quality control and to examine differences in the performance of different assays within and between laboratories.

The APU serum bank currently comprises gamma-irradiated infected cattle sera against the following serotypes:

Foot and Mouth Disease Virus Type SAT, 1 Strain KNP 196/91/1'

90t and Mouth Disease Virus Type SAT 1 Strain Nig 5/81 ZP02

Foot and Mouth Disease Virus Type SAT 2 Strain KNP 19/88

Foot and Mouth Disease Virus Type SAT 2 UGA 2/2002

Foot and Mouth Disease Virus Type SAT KNP 10/90

Foot and Mouth Disease Virus Type 0 SAR 11/00/0

Foot and Mouth Disease Virus Type Asia l 05/96

Foot and Mouth Disease Virus Type A GAM 52/98/ A

Foot and Mouth Disease Virus Type A Cruzeiro

Peste des Petits Ruminants 100 samples

4. TRAINING ACTIVITIES

4.1. Fellowships

Mr Nino Dante Arias Cruz

Mr Arias is from the Veterinary Faculty of the Universidad Peruana Cayetano Heredia (UPCH) in Peru. He joined APU in January 2008 for a five-month fellowship. His work at the Seibersdorf Laboratories was related to the Technical Cooperation Project PER5029 "Genomics of the Alpaca: Identification of Expressed Genes and Genetic Markers Associated with Productivity and Embryonic Mortality". The main focus of his work was in the area of alpaca genomics, namely to use the PCR to identify DNA markers for the Y chromosome. His training included the acquisition and use of archived information on public databases and pathways to select candidate genes. He also assisted APU in the implementation of Single Nucleotide Polymorphisms (SNP) analysis for sheep by developing Restriction Fragment Length Polymorphisms (RFLPs). He presented a paper entitled "DNA Markers for Sex Identification in South American Camelids" at the 16th International Congress on Animal Reproduction (ICAR) 2008, in Budapest, Hungary.

Ms Gisèle Marie Sophie Ouedraogo

Ms Ouedraogo is from Burkina Faso, and was sponsored by the IAEA Technical Cooperation Programme (BKF/07019). She stayed at the APU for three months, during which time she was involved in the preparation of new SNPs in candidate genes in sheep.

Mr Benson Mwenya

Mr Mwenya is an assistant researcher working on "The Genetic Characterization of Zambian Indigenous Livestock using DNA Microsatellite Markers"; he joined APU for a three-month fellowship. His work was sponsored by the Technical Cooperation Programme (ZAM/07002). During his stay Mr. Mwenya was involved in the identification of expressed genes and genetic markers associated with disease resistance in small ruminant. He received training in various techniques including real-time PCR, the use of public databases for candidate gene search and also assisted with routine experiments in APU.

Mr Vincent Simoongwe

Mr Simoongwe who is from Zambia was awarded a three months fellowship sponsored by the IAEA Technical Cooperation Programme (ZAM/08004).

Ms Argamjav Bayanzul

Ms Bayanzul, a parasitologist in the School of Veterinary Medicine and Biotechnology at the Mongolian State University of Agriculture, Ulaanbaatar, Mongolia, joined APU in March 2008 for a three-month training fellowship in the diagnosis of transboundary diseases using rapid and sensitive molecular diagnostic techniques. She is supported by Technical Cooperation Project MON5013, "*Diagnosis and Surveillance of Trans-boundary Diseases and Production of Diagnostic Reagents*"**.** Ms Bayanzul has received training in the use of the classical PCR, including extraction of DNA, target selection for PCR, primer design using appropriate software programs and the use of PCR for the differentiation of morbilliviruses. She has also used real time PCR for the diagnosis of Peste des Petits enzyme linked immunosorbent assays (ELISA) for the diagnosis of a number of infectious diseases.

4.2. Internships

Ms Malgorzata Nepelska

Ms Nepelska was awarded an internship for five months. She was trained in the identification of immune response genes involved in parasite resistance in sheep and the using various techniques. She also received training on radiation protection.

Mr Nemanja Milosevic

Mr Nemanja Milosevic was an intern from Serbia. He worked on the identification of parasite resistance genes in sheep involved in immune responses to helminth parasites. He also received training on the optimization and validation of protocols for using isotopes in Southern blot techniques.

Mr Mohsen FazeliNiaki

Mr FazeliNiaki was awarded an internship for two months. He received bioinformatics training and was directly involved in the development of the Knowledge and Technology Transfer (Katt) platform.

4.3.   Scientific visitors

Mr Zacarias Tusevo

Mr Tusevo, from Angola was awarded a scientific visit which was sponsored by the IAEA Technical Cooperation Programme (ANG/08016V). He stayed at the APU for two weeks, during his visit he worked on techniques for animal disease diagnosis and also spent some in Entomology, discussing aspects of *Glossina* control.

5. ACKNOWLEDGEMENTS

APU gratefully acknowledges the assistance of Dr Roland Silber and his colleagues at the Austrian Agency for Health and Food Safety (AGES) in screening samples imported from Africa and Asia, for potentially harmful pathogenic organisms and for enabling staff of APU to work on exotic pathogens in secure isolation premises in the high security laboratory.

6. APPENDICES

6.1. Staff Publications

1) **Bodjo, SC, M Lelenta**, E Couacy-Hymann, O Kwiatek, E Albina, D Gargani, G Libeau and A Diallo. Virus Research, 2008, 131: 23-32

2) E Couacy-Hymann, T Danho, D Keita, **SC Bodjo**, C Kouakou, YM Koffi, F Beudje, A Tripodi, P Benedictis G de and Cattoli. Zoonoses and Public Health, 2008, 56: 10 - 15

6.2. Travels

6.2.1. Staff

Staff Member	Destination	Period	Purpose of Travel
Malek, Massoud	PAG, San Diego	12-16 January	To attend the PAG-XVI international conference as a guest speaker on the subjects of "Goat Genomics at the International Atomic Energy Agency (IAEA)".
	Budapest, Hungry	12-13 July	Participated in Camelid Reproduction satellite meeting and presented the results of genetic markers developed for embryo transfer at Seibersdorf and related techniques.
	Lima, Peru	17-21 November	Review project development and upstream planning of TC PER5029

6.2.2. Consultants

Staff Member	Destination	Period	Purpose of Travel
Lamien, Charles Euloge	Montpellier, France	21-23 May	To attend the Steering Committee meeting (LABOVET project).
	Montpellier, France	18-19 December	To participate in LABOVET Steering Committee meeting and give a presentation on ongoing work on Capripox Viruses related to the LABOVET (FSP) project funded by French Ministry for Foreign Affairs.

6.3.   External Collaborations and Partnerships

Institution	Topic
Austrian Agency for Health and Food Safety (AGES), Vienna, Austria	Collaborative studies in the high security laboratories that APU is allowed to use for work on live viruses, including Peste des Petits Ruminants and Capripox.
Centre International en Recherche Agronomique pour le Développement (CIRAD), Montpellier, France	Partner in the Wellcome Trust supported research, '*Peste des Petits Ruminants (PPR) a serious and emerging plague of small ruminants in Developing countries : Epidemiology, transmission, host range and pathogenicity of the virus in West Africa*' and the project funded by the Fonds de Solidarité Prioritaire, '*Renforcement de cinq laboratoires de recherche vétérinaire en Afrique pour la surveillance et le contrôle de maladies animales*' for work on capripox viruses.
Institute of Animal Health (IAH), Pirbright, UK	Partner in the Wellcome Trust supported research, '*Peste des Petits Ruminants (PPR) a serious and emerging plague of small ruminants in Developing countries : Epidemiology, transmission, host range and pathogenicity of the virus in West Africa*'. Also provides isolates of capripox viruses for studies in APU.
Institut National de Médecine Vétérinaire (INMV), Algeria	Partner in the Wellcome Trust supported research, '*Peste des Petits Ruminants (PPR) a serious and emerging plague of small ruminants in Developing countries : Epidemiology, transmission, host range and pathogenicity of the virus in West Africa*' and the project funded by the Fonds de Solidarité Prioritaire, '*Renforcement de cinq laboratoires de recherche vétérinaire en Afrique pour la surveillance et le contrôle de maladies animales*' for work on capripox viruses.
Laboratoire National d'Appui au Développement Agricole (LANADA), Bingerville, Côte d'Ivoire	Partner in the Wellcome Trust supported research, '*Peste des Petits Ruminants (PPR) a serious and emerging plague of small ruminants in Developing countries : Epidemiology, transmission, host range and pathogenicity of the virus in West Africa*'

Institution	Topic
Laboratoire National Vétérinaire (LANAVET), Garoua, Cameroun	Partner in the project funded by the Fonds de Solidarité Prioritaire, '*Renforcement de cinq laboratoires de recherche vétérinaire en Afrique pour la surveillance et le contrôle de maladies animales*' for work on capripox viruses.
National Animal Research Health Center (NAHRC), Sabeta, Ethiopia	Partner in studies funded by the Fonds de Solidarité Prioritaire, '*Renforcement de cinq laboratoires de recherche vétérinaire en Afrique pour la surveillance et le contrôle de maladies animales*' for work on capripox viruses. This institue will also carry out experimental studies in sheep and goats using isolates of capripox characterized and provided by the APU.
University of Ankara, Ankara, Turkey	In collaboration with APU, the university has provided isolates of capripox viruses from cattle, sheep and goats for characterization.

6.4.   Trainees, Fellows and Scientific Visitors

Name	Country	Duration	Topic of Training
Fellows			
Cruz, Mr NDA	Peru	5 months	Alpaca Genomics
Bayanzul, Ms A	Mongolia	3 months	Disease Diagnosis
Mwenya, Mr B	Zambia	3 months	Genetic
Ouedraogo, Ms GMS	Burkina Faso	3 months	Preparation of SNPs
Simoongwe, Mr V	Zambia	2 months	Disease Resistance
Interns			
Nepelska, Ms M	Poland	5 months	Immune Response Genes
Milosevic, Mr N	Serbia	4 months	Immune Response Genes
FazeliNiaki, Mr M	Iran	2 months	Bioinformatics
Scientific Visitors			
Tusevo, Mr Z	Angola	2 weeks	Diagnostics and Tsetse Control

6.5. Coordinated Research Projects (CRP) and Technical Cooperation Projects (TCP)

CRP Title	Scientific Secretary
Gene-based Technologies in Livestock Breeding: Characterization of Small Ruminant Genetic Resources in Asia (2005 – 2009)	Malek, Massoud
TCP Title	**Technical Officer**
Improving the National Capacity in Diagnostics for Animal Diseases - Mauritania (2007 – 2008)	Luckins, Antony
An Integrated Approach for Improvement of Livestock Productivity – Niger (2007 – 2010)	Luckins, Antony
Genomics of the Alpaca: Identification of Expressed Genes and Genetic Markers Associated with Productivity and Embryonic Mortality – Peru (2007 – 2009)	Malek, Massoud
Maximizing Productivity on Goat Farms through Cost-Cutting and DNA-Based Technology in Selection for Breeding – Sri Lanka (2007 - 2009)	Malek, Massoud
Improving Sheep and Goat Production in Morocco through Genomic and Reproductive Physiology Characterization with the Help of Radio-immunoassay and Molecular Techniques – Morocco (2007 – 2009)	Malek, Massoud

6.6. Abbreviations

QTL	Quantitative Trait Loci
MS	Member States
6-FAM	6'-Carboxy-Fluorescein
AGES	Austrian Agency for Health and Food Safety
APU	Animal Production Unit
BHQ	Black Hole Quencher
CaPV	Capripoxvirus
CBPP	Contagious Bovine Pleuropneumonia
CIRAD	French Agricultural Research Centre for International Development
CRP	Co-ordinated Research Project
CWANA	Central and West Africa and North Africa
Ct	Cycle Threshold
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agricultural Organization of the United Nations, Rome, Italy
FMDV	Foot and Mouth Disease Virus
FMCA	Fluorescence melting curve analysis
FRET	Fluorescence Resonance Energy Transfer
GR-*db*	Genetic Resource Database
GTPV	Goat Pox Virus
GPCR	G-protein-coupled chemokine receptor
HRP	Horseradish Peroxidase
IAEA	International Atomic Energy Agency
ICARDA	International Center for Agricultural Research in the Dry Areas, Aleppo, Syrian Arab Republic
IIF	Indirect Immunofluorescence
ILRI	International Livestock Research Organization, Nairobi, Kenya
IPTG	Isopropylthiogalactoside
ISAG	International Society of Animal Genetics
LANADA	Laboratoire Central de Pathologie Animale de Bingerville, Cote d'Ivoire
LCV	Laboratoire Central Veterinaire, Bamako, Mali
LNA	Locked Nucleic Acid
LSDV	Lumpy Skin Disease Virus

MAI	Marker Assisted Introgression
MAS	Marker Assisted Selection
MGB	Minor Groove Binder
NCBI	National Center for Biotechnology Information, USA
NFQ	Non-fluorescent Quencher
NSP	Non Structural Protein
dNTP	Deoxynucleotide Triphosphate
OIE	World Organization for Animal Health, Paris, France
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PPR	Peste des Petits Ruminant
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescence Units
RPO30	RNA Polymerase Subunit of 30 kDa
RT-*db*	Real Time Database
SDS	Sodium Dodecyl Sulphate
SNP	Single Nucleotide Polymorphisms
SPPV	Sheep Pox Virus
TCID	Tissue Culture Infective Dose
TCP	Technical Cooperation Project
TMB	Tetramethylbenzidene
USDA	United States Department of Agriculture