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Use of Southern Blot with radioactive [α-^{32}P]ATP labeling in genetic marker analysis

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Background

Southern blot assay using [α-^{32}P]dATP-labeled probes was evaluated as new suitable method for SNP detection due to the following reasons:

1. For intensification of the signals in case when the DNA fragments exhibited only weak or questionable gelelectrophoresis bands (case 1: weak band, Fig. 1 and 4);

2. For the detection of a specific fragment in case the restriction enzyme cut the PCR product not only in a SNP position (case 2: multicut, Fig. 2 and 5);

3. For differentiation of nucleotides using a corresponding probe if no restriction enzyme cleavage site was identified in a SNP position (case 3: no cut, Fig. 3 and 6). In this case three different probes versus SNP were tested after additional sequence analysis for sample selection was performed.

Several candidate genes were chosen for establishing the Southern Blot method. PCR, digestion with restriction enzymes (if available) and gelelectrophoresis on selective DNA samples were carried out.

All steps connected to the work with the radioactive material were carried out under strict safety conditions and after the completion of a radiation course.

Materials and Methods

Gel preparation and capillary blotting

Following visualization of the DNA samples in the gel by UV light, the gels were submerged in depurination solution (0,25M HCL) for 10 min. Depurination was complete when the colour of the

bromophenol blue band turned yellow. Subsequently the gels were incubated in denaturation solution (1,5M NaCl; 0,5M NaOH) for 30 min. Denaturation was complete when the colour of the bromophenol blue band turned blue again. Thereafter, the gels were soaked in neutralization buffer (0,5M TrisCl; 1,5M NaCl) for 30 min and finally in transfer buffer (20xSSC) for 10 min pior to processing for blotting. Each step was carried out by shaking at room temperature; between each step the gels were rinsed throughly in distilled water.

Capillary blotting was carried out by employing a TurboBlotter from Whatman (including stack tray, GB004 and GB002 blotting papers and nylon membrane) according to the manufacturer's protocol. The capillary transfer of DNA fragments from the gel to the nylon membrane was performed overnight in transfer buffer. Afterwards the blots were baked at 80°C for 2h in order to fix the transferred DNA fragments (oven, PeqLab). Desiccated membranes could be stored at 4°C for several months for future use.

Design and radioactive labeling of oligonucleotides probes

For each investigated gene, 1-6 oligonucleotide probes were designed [Primer Express (AB), Primer Design (SECentral) or manual search]. The position of each probe on the gene was exactly calculated and graphically indicated (Fig. 1-3). For radioactive labeling of oligonucleotide probes 3' endlabeling procedure using terminal deoxynucleotidyl transferase (TdT, Fermentas) and α ^{32}P dATP (Perkin Elmer) was performed (Ref. 1). TdT catalyzes the repetitive addition of mononucleotides from a deoxynucleotide triphosphate to the terminal 3'-OH of a single- or double-stranded DNA initiator. 20 pmol of each probe was incubated for 60 min at 37°C in a total of 25 µl reaction mixture containing 5 µl 5x reaction buffer, 5 µl α ^{32}P dATP (10 mCi/ml) and 1 µl of 20 U/µl TdT. After heat inactivation for 10 min at 70°C, labeled probes were purified by Biospin 6 chromatography column (Biorad) for removal of unincorporated molecules. Cleaned probes could be stored at -20°C prior usage.

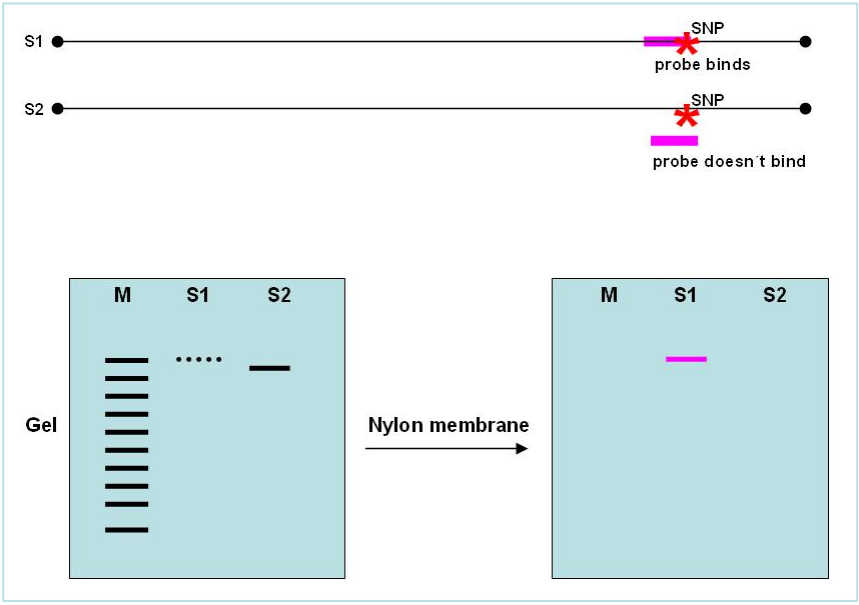

Fig. 1. Illustration of direct SNP detection. The corresponding probe binds to the specific complementary DNA fragment in case it is existing and intensifies the weak signal

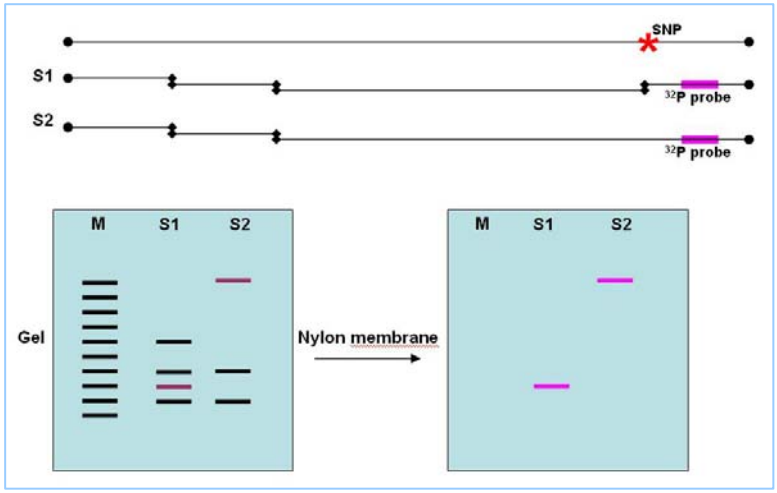

Fig. 2. Illustration of the case of multiple cuts. The corrresponding DNA probe binds only to the specific DNA fragment and exposes it from multiple bands

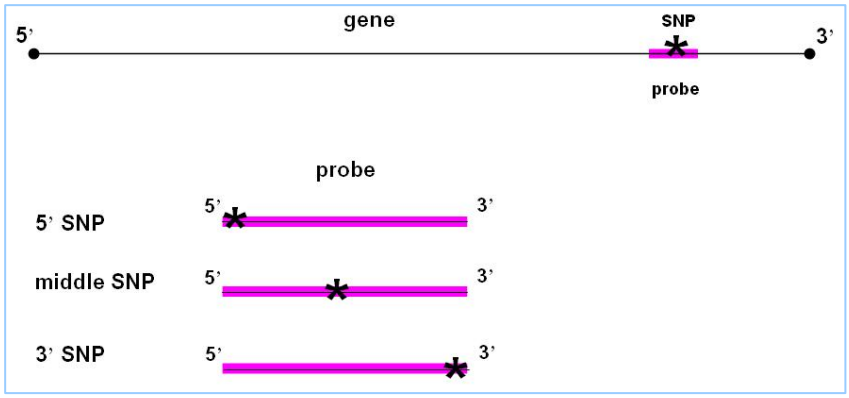

Fig. 3. Illustration of three different possibilities of probe location versus SNP in case if there is no restriction enzyme: SNP in the middle of probe as well at the 5´ and 3´ends

Southern hybridization with labeled probes

For hybridization the baked membranes were prehybridized for 45 min at 65°C in 20 ml prehybridization buffer (2x SSC; 0,5% SDS) and hybridized for at least 3 hours at 52°C in 10 ml hybridization buffer (6x SSC; 0,1% SDS) by addition of 10^6 cpm of the respective 32P-labeled oligonucleotide probe. These processes were carried out in 100 ml tubes (PeqLab) and an oven with rotator (PeqLab). After hybridization, the membranes were washed at 65°C in three different buffers (2 X SSC/0,1% SDS; 1 x SSC/0.1% SDS and 0,1 x SSC/0.1% SDS) for 30 min each, and then subjected to exposition by Kodak XAR-5 film (Sigma) in X-ray cassettes (Peq Lab) for 3h to overnight at -80°C. The achieved photographs were developed and fixed (developer and fixer from Sigma) in a dark room and were subsequently interpreted.

Results

For case 1 (weak bands) the gene PLXNC was chosen following digestion by the Tsp509I restriction enzyme (Fig. 4). After employing the ^{32}P labeled probe all previously weak signals were significantly intensified and allowed a clearcut interpretation of the results.

As an example for multicut, the gene CSF2RB-2 was used after digestion with the enzyme MspI (Fig. 5). This enzyme cuts PCR products not only in the SNP position (340) but also in two other positions, resulting in several DNA fragments after the DNA-RLFP investigation. The special located ^{32}P labeled probe attaches specifically to the 340 bp long DNA fragment in case the initial nucleotid is present,

and to the 462 bp long DNA fragment in case the other nucleotide shows up in this position. In case of heterozygotes two bands were detected.

For testing of "no cut" genes, the gene ARHGAP6 was used (Fig. 6). No restriction enzyme was found at the SNP position (318) thus all PCR products showed the same gelelectrophoresis pattern. The SNP evaluation was therefore carried out by sequencing. From the six investigated ^{32}P labeled probes only the probes with an "A" (Fig. 6B) or "G" (Fig. 6C) in the middle attached specifically to the corresponding complementary sequences. In case of heterozygotes both of the above-mentioned probes showed positive signals, thus the sequencing results could be confirmed. The probes with A and G at the 3`or 5`ends bound to all PCR products independently of the SNP (not shown).

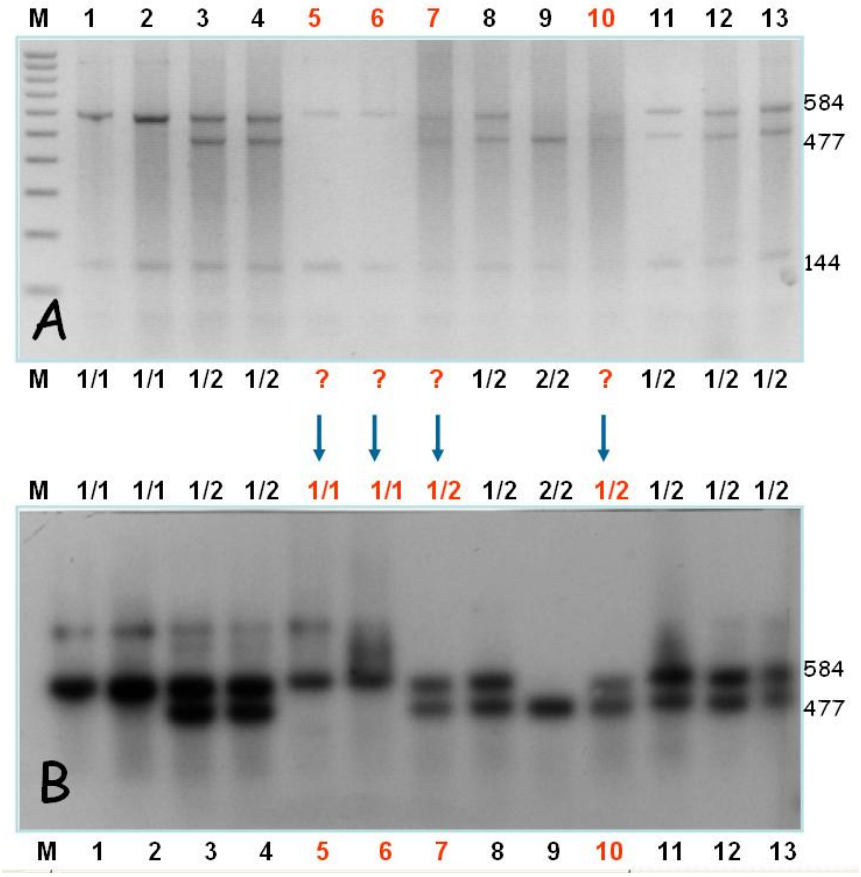

Fig. 4. (weak signal). Fig. 4A shows the DNA-RLFP picture of the gene PLXNC after digestion with the Tsp509I enzyme. The samples no. 5, 6, 7 and 10 are difficult to analyze. In Fig. 4B it is demonstrated that Southern blotting leads to the intensification of the initially weak signals, leading to improved interpretation of the results

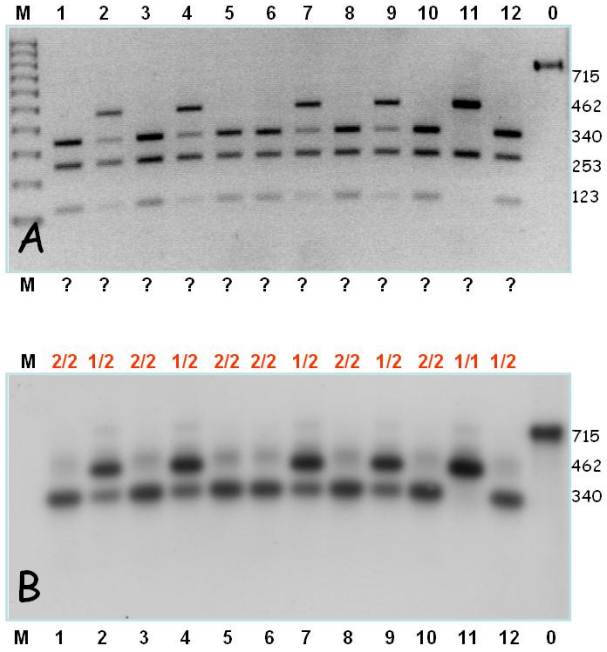

Fig. 5 (multicut). Fig. 5A shows the DNA-RLFP photograph of the gene CSF2RB-2 after digestion with the enzyme MspI. This enzyme cuts PCR products not only in the SNP position (340). SB-assay illustrated in Fig. 5B leads to the selection of important bands and allows the correct interpretation of results. Sample no. 0 constitutes an undigested PCR product

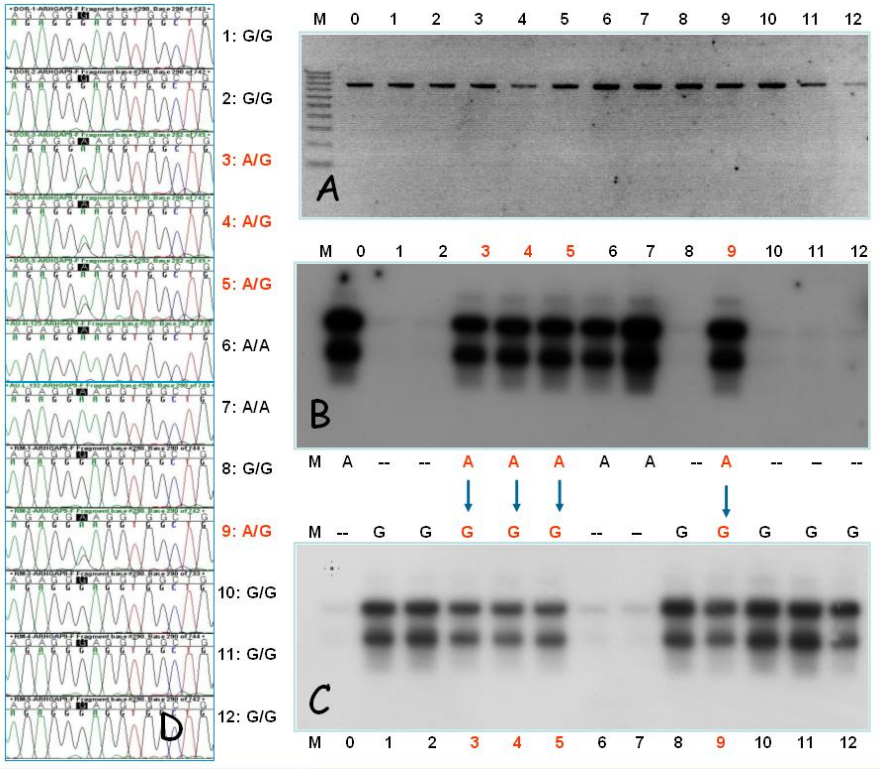

Fig. 6 (no cut). In Fig. 6A the PCR photo of the gene ARHGAP6 is shown. No restriction enzyme was found at the SNP position (318). The selected samples (selection was performed by sequencing, figure 6D) were Southern blotted and hybridized with 3 probes containing each SNP: in the middle as well as at the 5`and 3`end (altogether 6 probes). Only the probes with an "A" (fig. 6B) or "G" (fig. 6C) in the middle attached specifically to the corresponding complementary sequences. In case of

heterozygotes (samples no. 3, 4, 5 and 9) both mentioned probes showed positive signals, confirming the sequencing results. Sample no. 0 presents a control Austrain sheep DNA

Conclusions

A new Southern blot assay using isotope labeling and terminal deoxynucleotidyl transferase was successfully established to confirm or supplement the candidate gene results from the RLFP study. This method can be used for signal intensification if the PCR-RLFP bands are too weak, for detection of specific restriction fragments in case of multicut, and for detection and differentiation of SNPs if restriction enzyme cleavage sites are not identified in the SNP position. In the last case only DNA probes which contain a corresponding SNP in the middle are able to generate the expected results. By using this assay sequencing can be supplemented or even replaced.

Acknowledgements

The authors thank Christine Marizzi for advising on radioactive work and Tobias Benesch for carrying out the radiation course.

Reference:

[1] Aumaid Uthman, Detection of animal and human retroviral nucleic acid sequences in various tissues by using the PCR. Dissertation, Medical Faculty, University of Vienna 1995

SESSION 2: Effects of nutrition, reproduction, genetics, and environmental factors on animal productivity

IAEA-CN-174-232

Goat raising systems in Mexico's La Laguna region, and how they cope with the challenge of the economic crisis

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La Laguna region is noted for its high efficiency in farming and livestock productivity as a result of the use of technology. It comprises ten municipalities in the Mexican state of Durango and five in the state of Coahuila, located between the northern latitudes 26°51'00" and 24°22'48" and between meridians 101°51'36" and 104°48'36" west of Greenwich. It is 1096 meters above sea level, having a Bw(hw) climate defined as very dry, with a mean temperature of 21.7 ° C and summer rains averaging 226.9 millimeters a year.

La Laguna is the region with the largest goat milk production in Mexico, namely 81,117 liters of milk per year, or half of the production nationwide. In the rural areas goat raising is also the major source of income for thousands of families. The last decade has witnessed the establishment of dairy businesses with a high demand for goat's milk, which in turn encouraged businessmen and professional people to invest in goat farms (herd size = 500 goats), purchasing pedigree goats, lands, technology, setting up an infrastructure and hiring specialists. This intensive system is formed by 26 partners that have 5,000 goats, which produce 10,000 liters of milk per day, and are kept in open corrals with adequate feed. These

are primarily milk producing goats such as Saanen, Alpina and Toggenburg. However, the price increase in fuel and grain for animal feed lowered their profits and many investors could not continue operating. On the other hand, small rural producers (herd size = 60 goats) have kept operating much as their grandparents did. In this extended system the goats produce 0.900 liters of milk per day. Goats are put out to graze every day, facilities are rustic, and many farmers do not invest in formal vet-technical advice. Even though the extensive system has bad nutrition suplementation, bad health conditions and long pasture walks the goats take in search of native forage, which can be 8.6 hours and 9 kilometers long and consume much of there energy, it is still profitable do to the fact that it is a family activity which does not requier payed labor or professional services, feed has no cost, and diseases are rarely treated. Because of all these reasons costs are kept low. But goat sales during winter and milk sales through out the whole year, genarate enough income to support a goat raising family. For 88% of the producers, this is there only income. In the face of a sudden increase in expenses, these favorable factors have allowed these farmers to keep up production throughout the economic crisis of the last few years.

SESSION 3: Transboundary, emerging and zoonotic diseases

IAEA-CN-174-82

Preliminary Results of the C-ELISA for the Surveillance of Antibodies to Bluetongue Virus in Livestock in Mongolia

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ABSTRACT

Overall goal of the study was the prevalence of bluetongue virus antibodies using a competitive enzyme-linked immunosorbent assay /cELISA/.

The Mongolian economy is deeply rooted in the animal husbandry sector and livestock plays an irreplaceable role in the economy.

Bluetongue was never reported in Mongolia and until recently, the disease was limited to area between approximate latitudes 35^0S and 40^0 N.

The global warming and movement of Culicoides imicola to North side of the world increases the risk of bluetongue in our country and neighbouring countries.

Nearly 10,000 animal were tested from randomly stratified herds in the country.

The results presented here record the first confirmation of bluetongue virus antibody in sheep, goats and cattle in Mongolia. Prevalence of bluetongue virus antibody was detected in all aimags and cities, highest being recorded in goats 86% then in sheep 51% and cattle 9%.

Further studies are being undertaken to conduct more detailed investigation on bluetongue in near future and to determine the bluetongue virus serotypes that are and have been circulating in Mongolia.

SESSION 3: Transboundary, emerging and zoonotic diseases

IAEA-CN-174-281

Surveillance of Rift Valley Fever in the Republic of Guinea, Conakry

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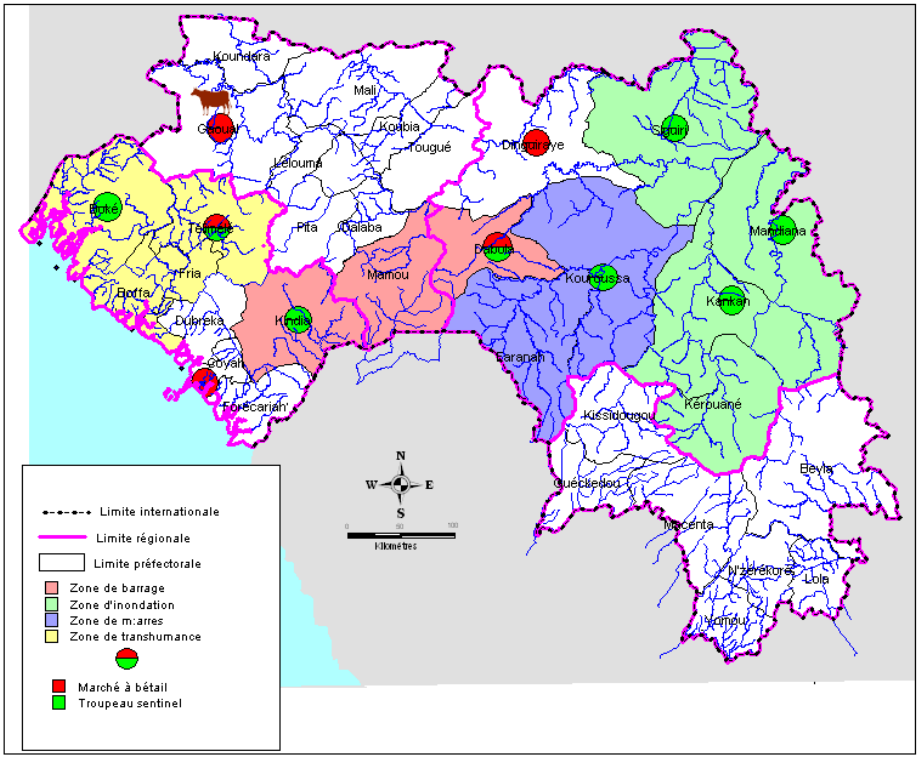
Abstract

To establish an estimate of the prevalence of Rift valley fever in Guinea an epidemiological survey for major clinic signs (abortion and mortality), sero surveillance for the infection rate, frequency and geographical distribution and according to the season, were carried out. Based on ecological consideration, breeding systems, to sanitary risk associated to the RVF emergence, sites has been chosen in four zones: hydroelectric dam, flooding zone and great installations, ponds and transhumance zone.

From 2005 to 2009, sampling of smaller ruminant has been executed in these zones at the level of cattle markets and herds sentinel. 2618 sera has been tested on IgG and 2660 on IgM for prevalence of 3.13% and 0.37% respectively. At the level of sampling zones, the transhumance has highest prevalence on IgG with 6.26%. According to specie the prevalence on IgG is highest to the sheep 8.48%. As the class of age, the prevalence is clearly significant at the level of transhumance zone 11.76% as animal to 2-3 years old.

Location	*IgG Prév %*	*IgM Prév%*
Dam	**3 ,13**	**1,19**
Flood areas	**2,11**	**0**
Ponds	**2,50**	**0**
Transhumance	**6,26**	**0**
Sentiel herds	**2,25**	**0**
Market survey	**3,86**	**0**

This survey has permitted to draw up a risk map for RVF infection.



It’s obvious that epidemiological surveillance is necessary in Guinea to prevent major outbreaks.