Surveillance of wildlife as a tool for monitoring rinderpest and peste des petits ruminants in West Africa

E. Couacy-Hymann [1], C. Bodjo [1, 3], T. Danho [1], G. Libeau [2] & A. Diallo [3]

(1) Laboratoire Central de Pathologie Animale, BP 206, Bingerville, Côte-d'Ivoire
(2) CIRAD-EMVT, UPR15, TA 30/G, Campus International de Baillarguet, 34398 Montpellier Cedex 5, France
(3) Animal Production Unit, FAO/IAEA Agriculture and Biotechnology Laboratory, IAEA Laboratories, A-2444 Seibersdorf, Austria

Summary
The authors provide a report on the surveillance of rinderpest virus (RPV) and peste des petits ruminants virus (PPRV) in the wildlife population in Côte d'Ivoire. For this purpose, 266 animals from nine different species, selected according to susceptibility and abundance, were captured and sampled from Comoé, Marahoué and Lamto Parks. Two hundred and forty seven sera and 214 nasal swabs were collected and analysed by competitive enzyme-linked immunosorbent assay (cELISA) and reverse-transcriptase polymerase chain reaction (RT-PCR) techniques, respectively. Serological data demonstrated that RPV was not circulating within the national Parks and estimated the PPR sero-prevalence to be less than 1%. The analysis of the nasal swabs revealed no cases of RPV infection, but PPRV infection was detected in four species, including buffalo. To minimise the cost of the study without affecting the sensitivity of the test, samples were pooled into different groups and submitted to RT-PCR using nucleoprotein gene specific primers. The RT-PCR used in this study, which was derived from the method developed by Couacy-Hymann *et al.* in 2002, was followed by a hybridisation step using internal specific probes to confirm the identity of the deoxyribonucleic acid product. When used in conjunction with a cELISA this method accurately demonstrated the absence of rinderpest viral persistence in Côte-d'Ivoire.

Keywords
Peste des petits-ruminants – Reverse-transcriptase polymerase chain reaction – Rinderpest – Wildlife.

Introduction

Rinderpest (RP) and peste des petits ruminants (PPR) are two major epizootic diseases found in Africa, the Middle East and Asia (10, 13, 17, 22, 23). Rinderpest is a devastating disease affecting large ruminants such as cattle and buffalo, while PPR predominantly affects small ruminants (10). In Asia, RP infection also occurs in small ruminants who may then transmit the infection to cattle and other large ruminants (2, 3), but in Africa, sheep and goats can remain unaffected depending upon the strain involved (7). These diseases are attributed to two closely related viruses, RP virus (RPV) and PPR virus (PPRV), respectively. Both are members of the *Morbillivirus* genus within the Paramyxoviridae family (14).

Intensive effort by the affected countries to control RP through mass vaccination during the Pan African Rinderpest Campaign, has reduced the disease to a few remaining reservoirs. Infection now only persists in East Africa in the southern Somali ecosystem, probably because the civil unrest in that region prevents the implementation

of an effective surveillance and vaccination programme. The persistence of rinderpest in East Africa is also related to the mildness of the strain circulating in this area, which has been demonstrated to be associated with African Lineage II RPV (4, 24). The disease usually affects young animals and can remain undetected within cattle populations that are well fed; in such cases the disease is only discovered when it is occasionally transmitted to wildlife, who remain very susceptible. It is now almost certain that wildlife are not important in the long-term maintenance of the disease and on the occasions that wild animals are found to be infected they are most likely to have been contaminated from a cattle source. Wildlife is therefore a key sentinel population in the monitoring of the remaining virus circulation in Africa.

Countries which have declared provisional freedom from RP, or freedom from RPV infection, have to implement intensive disease surveillance in all susceptible animal species, both domestic and wild, to prove the absence of RPV after the cessation of vaccination campaigns. Côte-d'Ivoire is among these countries; no RP has been reported in that country since 1986 and vaccination stopped in 1996. It was declared provisionally free from RP in 1997 and officially free of the disease in 2004 by the World Organisation for Animal Health (OIE). As part of the disease surveillance that is being implemented to fulfill the requirements to obtain and maintain the status of a country 'free from infection', the authors investigated the presence of RPV circulation among the wildlife population in Côte-d'Ivoire. Wildlife is recognised as a valuable sentinel population that can reveal previous cases of disease and recent occurrences of virus infection (11). The results of this investigation are reported here along with those of the concurrent study of PPRV, the closely related virus to which are attributed important losses in livestock in the country.

Material and methods

Wildlife and method of capture in the Comoé, Marahoué and Lamto Parks

The authors took samples when wild game were being transferred from the National Game Park in Comoé to Abokouamekro Park. The collection was completed with samples from the Marahoué and Lamto parks in order to have an overview of all the game parks in Côte-d'Ivoire. Comoé Park is located in the savanna zone (north-eastern region) while Marahoué Park is in an area of transitional habitat that incorporates forest and savanna, which is characteristic of the Sudan-Guinean zones. Lamto Park is located at the end of the tropical forest region. Comoé is the biggest park and contains a large number of mammal species including more than twenty species of artiodactyl.

The neighbouring villages are mostly reliant upon agriculture and hunting. Based on the availability and accessibility of animals at these parks, a total of 266 wild animals representing nine species were captured for sampling, as follows:

– 113 kobs (*Kobus kob*)

– 39 Defassa waterbuck (*Kobus defassa*)

– 56 buffaloes (*Syncerus caffer*)

– 19 bubal hartebeests (*Alcelaphus buselaphus*)

– 13 roan antilopes (*Hippotragus equinus*)

– 12 bushbucks (*Tragelaphus scriptus*)

– 5 red flanked duikers (*Cephalophus rufilatus*)

– 3 blue duikers (*Cephalophus monticola*)

– 6 warthogs (*Phacochoerus aethiopicus*).

Susceptible wildlife were first located from a vehicle then physically or chemically immobilised using nets or darts.

Samples collection

Blood was taken from the jugular of each animal using a 'vacutainer' system; it was then labelled and stored in a cool place until a clot was formed. The sera were decanted and transferred to a 1.8 ml Eppendorf tube and clarified by centrifugation in the laboratory. They were stored at –20°C until tested. Nasal swab were also taken, transported in refrigerated containers and stored at –80°C until their analysis. Some animals escaped before sampling, so out of the 266 animals that were captured a total of 247 serum samples and 214 nasal swabs were analysed.

Serological analysis

Monoclonal antibody-based competitive enzyme-linked immunosorbent assay (cELISA) for RP and PPR were used in the present investigation. These tests were developed using a virus neutralising monoclonal antibody against an epitope of the haemagglutinin protein specific for RPV or PPRV (2, 3). Positive serology for RPV, using a cut-off of 50% inhibition, was based on a test which reported specificity and global sensitivity of 95.5% (12) and 85% (20), respectively, and is one of the tests that the OIE recommends as an alternative to the virus neutralisation test. To interpret the results of the cELISA, the authors used ELISA Data Interchange (EDI) software developed by the Food and Agriculture Organization and the International Atomic Energy Agency (16).

Ribonucleic acid extraction

Genomic material was extracted from samples according to previously described protocols using the glassmilk

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ribonucleic acid (RNA) extraction technique, guanidine isothiocyanate and further alcohol precipitation (8, 25). Briefly, cotton swabs were placed in a 2.5 ml syringe and 250 µl of 1 × phosphate-buffered saline (pH 7.4-7.6) was added. The cotton swabs were squeezed three times and the expunged solution was collected in Eppendorf tubes. Glassmilk was mixed with 100 µl of this filtrate, then stirred slowly at room temperature for 5 min and spun at 2,000 rpm for 2 min in a microfuge. The supernatant was discarded and the pellet washed three times with 400 µl of ethanol-containing solution. The pellet was then vacuum-dried and the bound RNA was eluted from glassmilk in 50 µl of diethyl pyrocarbonate-treated (DEPC-treated) water and centrifuged at 13,000 rpm for 5 min. Fifty microlitres of RNA solution from five individual sample extracts were mixed to constitute one pool and 1 µl of RNase inhibitor (10 U/µl) was added. The 43 pools obtained, including pool number 9 that contained only 4 samples, were stored at –70°C until use.

Rinderpest virus and peste des petits ruminants virus primers and probes

The B12/B2 and P1/P2 primer sets were derived from the nucleoprotein (NP) gene of RPV and PPRV conserved sequences (6). SB1 and SP1 probes were labelled with digoxigenin-dUTP using a oligonucleotide labelling kit according to the manufacturer's instructions. The SB1 and SP1 probes were chosen to be located in the amplified sequence product of RPV and PPRV, respectively. The name, sequence, and location of the primers and probes are summarised in Table I.

Reverse transcription-polymerase chain reaction

The complementary DNA (cDNA) from the field samples was synthesised by reverse transcription (RT) reaction using a first strand cDNA synthesis kit as described by Couacy-Hymann *et al.* (8). A total of 7 µl of RNA extract was added to an RT mixture containing 1 µl of DTT (10 mM); 1 µl of RPV-, PPRV-specific primers (30 pmoles/µl of each forward and reverse primer); 1 µl of RNase inhibitor (10 U/µl); and 5 µl of cDNA synthesis mix (Amersham-Pharmacia-Biotech). Tubes were briefly spun at 2,000 rpm for 1 min and incubated at 37°C for 1 h.

For the polymerase chain reaction (PCR) amplification reaction, 2 µl of the cDNA was added to the PCR mixture, which contained 5 µl of dNTP mixture (200 mM of each dNTP); 1 µl of specific forward and reverse primers (30 pmoles/µl of each); 10 µl of 10 × Taq polymerase buffer; 1 µl of Taq polymerase (1.25 U/µl); and distilled water to a final volume of 100 µl. The tube was stirred and spun briefly, then the amplification reaction was carried

Table I

Sequence and position of primer sets and oligonucleotide probes

Primer	Gene	Position	Sequences
P1	PPR-N	1,302 > 1,327	5′ TCT CCT TCC TCC AGC ATA AAA CAG AT
P2	PPR-N	1,575 < 1,597	5′ACT GTT GTC TTC TCC CTC CTC CT
B12	RP-N	1,322 > 1,344	5′CAA GGG AGT GAG GCC CAG CAC AG
B2	RP-N	1,594 < 1,618	5′TAG GAA CAG CAA CAT ACG AGA GTC
Probes [a]			
Probe SB1	RP-N	1,463 > 1,482	5′ACT CTG ATT GAT GTG GAC AC
Probe SP1	PPR-N	1,442 > 1,459	5′CCC GGC CAA CTG CTT CCG

a) SB1 and SP1 are internal primers to the targets of B12/B2 and P1/P2 respectively
PPR: peste des petits ruminants
RP: rinderpest

out in a DNA thermal cycler (HYBAID Touch Down). The thermal programme consisted of a first cycle of 5 min at 95°C, followed by five cycles of 30 s at 94°C, 30 s of annealing at 60°C and 30 s of elongation at 72°C. The amplification process was then followed by 30 cycles in which the annealing temperature was reduced to 55°C. Elongation was extended to 10 min in the last cycle.

DEPC-treated-water and total RNA extracted from Vero cells infected with PPRV 75/1 (vaccine strain) served as negative and positive controls, respectively, and were included in all experiments.

The PCR products were visualised and sized by gel electrophoresis in 1% NuSieve, 1% Seakem GTG and 1 × Tris-borate-ethylenediamine tetra-acetic acid buffer (pH 8) containing ethidium bromide. The products were visualised by ultra violet (UV) fluorescence then photographed.

Hybridisation

The amplified DNA was transferred onto a positively charged nylon membrane overnight with 0.4 N NaOH. The membrane was dried, UV-irradiated for 5 min and prehybridised at 68°C for 30 min in 5 ml of hybridisation buffer (5 × SSC, 2% blocking buffer, 0.1% sarcosine, 0.02% sodium dodecyl sulphate [SDS]). The digoxigenin-labelled probe (SB1 or SP1), at a concentration of 10 pmol/ml, was then added to the hybridisation buffer. The membrane was incubated at 50°C for 30 min then washed twice at room temperature with a buffer composed of 2 × SSC, 0.1% SDS for 10 min. The membrane was then washed two more times for 10 min each at 50°C with a second buffer SSC 0.1%, 0.1% SDS. The presence of the probe on the filter was revealed by immunological detection with a phosphatase conjugated anti-dixogenin antibody.

Results

Rinderpest and peste des petits ruminants serology

Of the 266 samples taken at Comoé, Marahoué and Lamto Parks, 247 provided results that could be interpreted according to the OIE standards. None of the sera tested by cELISA for RP antibodies were positive. However, cELISA for PPR antibodies did detect two positive animals (a buffalo and a Defassa waterbuck) from Comoé Park. Overall prevalence of PPRV antibodies from the screened population, which may not represent the target population of Comoé Park, was < 1%. There were no virus neutralisation results from this study because none of the sera cross-reacted and the cELISA for RP is highly specific.

Rinderpest and peste des petits ruminants virology

To reduce the cost of the analysis the RNA samples were pooled into 43 groups. There were five samples in each group (except group 9, which was composed of four samples). Primers P1 and P2 were designed to specifically

amplify the PPRV N gene from the sequence information obtained earlier from wild-type strains of PPRV from Africa (6). This set of primers is located in the PPRV N gene sequence close to the position of the primer set NP3/NP4, which was designed for all known variants of PPR in endemic zones (8). The primers were tested by RT-PCR on pooled extracted RNA from swabs. DNA bands of the expected size (296 bp) were obtained for groups No. 5, 9, 30, 35, 65, 90 as represented in the photograph of the gel electrophoresis (Fig. 1a). Table II provides details of the composition of each group, including from which species and from which park the samples were collected. Positive samples were detected in four species: Defassa waterbuck, bubal hartebeest, buffalo, and kobs. The probe SP1, internal to the target sequence hybridised to all the six amplified cDNA when previously transferred onto a membrane by southern-blotting (Fig. 1b). This confirmed that the correct fragment was obtained. In contrast to this result, all attempts to amplify the RPV N gene from the different pools with the B12/B2 primer set followed by southern-blot technique and hybridisation with SB1 oligonucleotide, remained negative (data not shown).
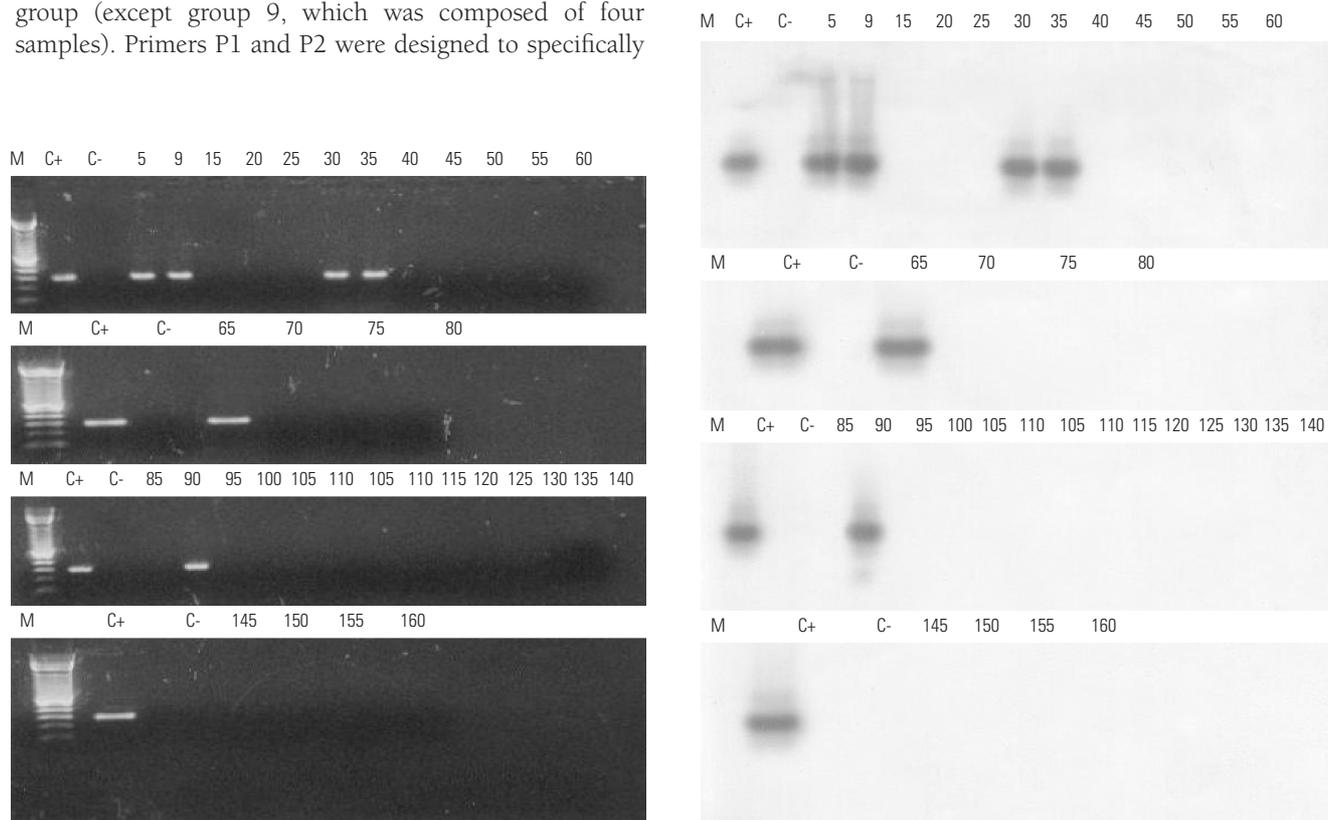
a) The amplified products were submitted to electrophoresis on agar gel, stained with ethidium bromide and visualised by ultra violet fluorescence

b) The amplified products were transferred from the same gel onto nylon membrane by southern-blot and probed with a digoxigenin labelled internal oligonucleotide SP1 (see materials and methods)

Fig. 1
Specific polymerase chain reaction amplification of the nucleoprotein gene fragment of different pools of samples with primers P1/P2
Lanes : M (DNA Ladder 100 bp, Phamacia Marker); C+ (positive control); C- (negative control); (5) Defassa waterbuck; (9) bubal hartebeest;
(30) buffalo; (35) buffalo; (65) kob; (90) buffalo; (15, 20, 25, 40-60, 70-85, 95-160) samples from negative pools

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Table II
Composition of peste des petits ruminants positive pool samples by reverse transcriptase-polymerase chain reaction technique

Pool identification number	Sample identification number	Species	Origin of sample
	1	Defassa waterbuck	Lamto Park
	2	Defassa waterbuck	Lamto Park
5	3	Defassa waterbuck	Lamto Park
	4	Defassa waterbuck	Marahoué Park
	5	Defassa waterbuck	Marahoué Park
	6	Bubal hartebeest	Marahoué Park
9	7	Bubal hartebeest	Marahoué Park
	8	Bubal hartebeest	Marahoué Park
	9	Bubal hartebeest	Marahoué Park
	26	Buffalo	Comoé Park
	27	Buffalo	Comoé Park
30	28	Buffalo	Comoé Park
	29	Buffalo	Comoé Park
	30	Buffalo	Comoé Park
	31	Buffalo	Comoé Park
35	32	Buffalo	Comoé Park
	33	Buffalo	Comoé Park
	34	Buffalo	Comoé Park
	35	Buffalo	Comoé Park
	61	Kob	Comoé Park
	62	Kob	Comoé Park
65	63	Kob	Comoé Park
	64	Kob	Comoé Park
	65	Kob	Comoé Park
	86	Buffalo	Comoé Park
	87	Buffalo	Comoé Park
90	88	Buffalo	Comoé Park
	89	Buffalo	Comoé Park
	90	Buffalo	Comoé Park

Discussion

It is hoped that rinderpest will be eradicated in Africa within the next few years. This goal is expected to be achieved by identifying areas of infection through comprehensive surveillance and then implementing intensive vaccination campaigns in those areas. Efficient and sensitive diagnostic tests are of great help in quickly providing evidence that RPV is not circulating in a free population. In the absence of a sequencing facility, this study was able to confirm the identity of the DNA product using RT-PCR followed by hybridisation with specific probes. This method, which stems from the method developed by Couacy-Hymann *et al.* (8), was used in conjunction with a cELISA to accurately detect reservoirs of viral persistence.

After 1996, the year in which the vaccination campaign against RP in Côte-d'Ivoire ended, an epidemiosurveillance was undertaken in the country to fulfill the requirements to obtain 'freedom from infection' status from the OIE. The present investigation was implemented to analyse the status of RP infection among wild ruminants. Sera and nasal swabs were collected and analysed by ELISA and RT-PCR, respectively, from 260 animals of different species. This study was subsequently extended to include PPR, a disease which is similar to RP and which is causing important losses in Côte-d'Ivoire.

The analysis of sera samples with cELISA techniques demonstrated that all were negative for the detection of RPV antibodies. However, sera from a Defassa waterbuck and a buffalo were positive for PPRV antibodies.

As the quality of the starting RNA material is a critical factor for the success of the RT-PCR technique, an efficient commercial kit for RNA extraction (the RNaid kit) was used to obtain RNA from the swab samples. For screening purposes, the samples were pooled into different groups to minimise the cost of the study without affecting the sensitivity of the test. A previous study to determine the optimum number of samples one pool could contain demonstrated that five samples is an appropriate number to carry out a RT-PCR technique with a significant result (Couacy-Hymann, unpublished data). Using this approach, six of the 43 groups of samples were positive by RT-PCR with PPRV NP gene specific primers while all the samples were negative with the RPV NP gene specific primers. The PPRV-positive samples were from four species: buffaloes, Defassa waterbucks, bubal hartebeests and kobs. In Africa, especially in enzootic areas, asymptomatic PPR cross-infection is known to occur in cattle in contact with sick goats (9, 19). Furthermore, in Asia, the isolation of PPRV from an outbreak in Indian buffalo (*Bubalus bubalis*) has been reported (15). On that occasion, cattle or buffaloes that developed sub-clinical symptoms to PPRV developed a protective immune response against both diseases. Buffalo are therefore a key species to monitor as part of a surveillance programme for RP as well as PPR infections.

Wildlife are important in RP surveillance because they are more vulnerable to the disease than cattle and because we know that any positive results are the result of infection rather than vaccination. However, there is still uncertainty about their role in maintaining RP reservoirs and in

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contributing to the spread of the virus in cattle populations. The data presented here support the current belief that RP is absent from West Africa and that wildlife in Côte-d'Ivoire are also still free of the disease 13 years after the last case of RP in this country and three years after the cessation of the vaccination.

This is consistent with results obtained from similar investigations carried out in three other African countries: Burkina-Faso, Chad and Central African Republic, under the Veterinary Wildlife Project initiated in 1998 as part of the Pan African Programme for the Control of Epizootics (PACE). In those studies, sera were analysed by cELISA and virus neutralisation test for the detection of antibodies to RPV and PPRV. The report related to those studies indicated that all sera collected from wildlife in the different countries were negative for RP antibodies while some sera, collected from bubal hartebeests, kobs and buffaloes, were positive for PPR antibodies (5). In 2003, at the PACE Regional Reference Laboratory, the authors analysed sixteen serum samples collected in Benin from wild game; all were negative for RP but three of them (all buffaloes) had antibodies for PPR (unpublished data).

In Cameroon, Planton (21) tested 110 wildlife serum samples using the virus neutralisation test. Out of these, three serum samples revealed specific antibodies against RP. However, two samples were collected from old animals which may have been in contact with the RPV during the epizootic of RP in 1983. The third positive serum sample was obtained from a two-year-old bubal hartebeest. The report showed the necessity for caution in interpreting this last result and the need for cross-neutralisation tests where RP is suspected. Since epidemiological evidence suggested that RPV could not survive among wildlife in Cameroon, the author concluded that the animal could have been infected with PPRV.

Those results, along with what is reported here seem to indicate that wildlife in Western and Central Africa does not play a role in the maintenance of RPV. However, PPRV is present in this population and a thorough investigation is needed to record the number of PPR disease cases. Indeed, PPR prevalence is very high in Côte-d'Ivoire: it is as high as 20% in the forest region (6) where no mass vaccination is conducted. This is consistent with the continued reports of PPR infection in other countries of West Africa and Central Africa (1, 18; Dr A. Sidibé, personal communication). In addition, in these countries, some villages dependent upon livestock breeding are very close to or are in some natural parks. Therefore, domestic animals and wildlife graze together in the same area, which facilitates the spread of PPR between the two populations. This regular contact was illustrated by a severe outbreak of PPR observed in a zoological collection in the United Arab Emirates that involved wild ungulates becoming infected by imported goats (13).

Nevertheless, the prevalence of PPR in wildlife in Comoé Park was seen to be extremely low. This data supports the hypothesis that the infection is not self-sustained and epidemics, if they occur, are the result of infection by small ruminants neighbouring the natural park.

Acknowledgements

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La surveillance de la faune sauvage en vue du suivi épidémiologique de la peste bovine et de la peste des petits ruminants en Afrique de l'Ouest

E. Couacy-Hymann, C. Bodjo, T. Danho, G. Libeau & A. Diallo

Résumé
Les auteurs présentent un rapport de surveillance sur le virus de la peste bovine (RPV) et le virus de la peste des petits ruminants (PPRV) dans les populations de faune sauvage en Côte d'Ivoire. À cette fin, des prélèvement ont été effectués sur 266 animaux de six espèces, choisis en raison de leur abondance et de leur susceptibilité, et capturés dans les parcs de Comoé, Marahoué et Lamto. Au total, 247 prélèvement sanguins et 214 écouvillons nasaux ont été prélevés et analysés en utilisant pour les premiers, le test immuno-enzymatique de

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compétition (cELISA) et pour les seconds, le test couplé de transcription inverse et d'amplification génique (RT-PCR). D'après les résultats sérologiques, le RPV est absent des parcs nationaux et la prévalence sérologique de la peste des petits ruminants est inférieure à 1 %. L'analyse des prélèvements nasaux n'a mis en évidence aucun cas d'infection par le RPV, mais la présence du PPRV a été détectée dans quatre espèces, dont le buffle. Afin de réduire le coût de l'étude sans altérer la sensibilité du test, les échantillons ont été regroupés et les différents groupes ont été soumis à la RT-PCR en utilisant des amorces spécifiques du gène de la nucléoprotéine. La RT-PCR appliquée ici est une variante de la méthode mise au point en 2002 par Couacy-Hymann et coll. Elle a été suivie par une phase d'hybridation à l'aide d'essais internes spécifiques visant à confirmer la nature du produit de l'acide désoxyribonucléique. Utilisée parallèlement à l'ELISA de compétition, cette méthode a démontré avec précision que le virus de la peste bovine est absent de Côte d'Ivoire.

Mots-clés

Amplification en chaîne de la polymérase-transcription inverse – Faune sauvage – Peste bovine – Peste des petits ruminants.

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Vigilancia de la fauna salvaje como medio de lucha contra la peste bovina y la peste de pequeños rumiantes en el África Occidental

E. Couacy-Hymann, C. Bodjo, T. Danho, G. Libeau & A. Diallo

Resumen

Los autores ofrecen un informe sobre la vigilancia de los virus de la peste bovina (PB) y la peste de pequeños rumiantes (PPR) en la fauna salvaje de Côte d'Ivoire. En los parques de Comoé, Marahoué y Lamto se capturaron un total de 266 ejemplares de nueve especies, seleccionadas por su abundancia y grado de susceptibilidad a dichas enfermedades. A continuación se procedió a la extracción de muestras, 247 de suero y 214 de exudado nasal en hisopo, que después se analizaron, respectivamente, con técnicas de ELISA de competición y de transcripción inversa acoplada a una reacción en cadena de la polimerasa (RT-PCR). Los datos serológicos demostraron la ausencia de virus de la peste bovina circulantes en los parques nacionales y permitieron cifrar en menos de un 1% la seroprevalencia de la PPR. El análisis de los exudados nasales no reveló ningún caso de infección por el virus de la PB, pero sí por el virus de la PPR en cuatro especies, entre ellas el búfalo. Para reducir en lo posible el costo del estudio sin pérdida de sensibilidad se mezclaron las muestras, divididas en varios grupos que después se sometieron a la RT-PCR utilizando cebadores específicos del gen de la nucleoproteína. La técnica de RT-PCR utilizada en el estudio es una variante del método descrito por Couacy-Hymann *et al.* en 2002. A la RT-PCR siguió una hibridación con sondas específicas internas que sirvió para confirmar la identidad de la cadena de ácido desoxirribonucleico. Utilizado en combinación con el ELISA de competición, este método demostró con gran exactitud la no persistencia del virus de la peste bovina en Côte d'Ivoire.

Palabras clave

Fauna salvaje – Peste bovina – Peste de pequeños rumiantes – Reacción en cadena de la polimerasa-transcriptasa inversa.

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