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Full Length Research paper

Diagnosis and surveillance of rinderpest using reverse transcription - PCR

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PCR technique was used as an alternative method to detect evidence of rinderpest virus for diagnosis and in epidemiological surveys. Viral RNA was purified in 20 to 30 min using a commercial kit (RNaid (BIO 101). Primers used mapped in the nucleocapsid protein gene of rinderpest virus and gave specific and sensitive amplification from pathological samples. The size of the amplified fragment was 297 bp and the result was confirmed using internal non-radioactive probe SB1. The specificity of the PCR products was also confirmed by cleavage using restriction enzyme Rsal to give a major band of 200 bp.

Key words: RPV, RT-PCR, rinderpest, PPRV, morbillivirus.

INTRODUCTION

Rinderpest (RP) is a highly contagious disease of cattle, buffaloes and some wild animals, and is caused by the rinderpest virus (RPV). The disease has a very high mortality (Scott, 1964; Plowright, 1968) and was one of the most economically important animal diseases in Africa, the Middle-East and Asia. Eradication campaigns have now almost completely eliminated the virus and rapid diagnosis and surveillance are keys to monitoring any possible foci and possible spread. Small ruminants can also be infected with RPV causing an acute disease (Shaila et al., 1989; Anderson et al., 1990), however some asymptomatic infections can occur (Couacy-Hymann et al., 1995). In the same geographical areas small ruminants can suffer from peste-des–petits-ruminants (PPR) which is caused by PPR virus (PPRV). There is a close relationship between the two viruses and they belong to the Morbillivirus genus of the paramyxoviridae family (Gibbs et al., 1979). In small ruminants, both diseases are clinically very similar, making it impossible to differentiate them in the field.

The rinderpest eradication programme has been very successful. In Africa, the Western and Central regions have ceased vaccination in all species and the countries are cerified free from rinderpest disease with the OIE. In May 2005, Benin, Eritrea, Senegal and Togo were declared free from rinderpest infection by the OIE. Epidemiological surveillance was launched in 2000 through the Pan African Control for Epizootic diseases programme (PACE) with the aim of proving the absence of rinderpest infection in the animal population. Detection of specific antigens and antibodies was needed to provide methods to clearly differentiate both RPV and PPRV during this phase.

Classical techniques for diagnosis and survey of rinderpest, such as virus isolation and agar gel immuno-diffusion, viral neutralisation test, are time consuming and not sensitive enough particularly for the complications inherent where mild strains of RPV are presented. Such strains produce minor or no clinical signs and only low levels of viral antigen from ocular secretions in infected animals (Wamwayi et al., 1995). The detection and elimination of such mild strains requires a new generation of diagnostic tests (Taylor, 1986). On the other hand, it is essential to differentiate clearly RPV from PPRV in small ruminants. Thus specific and sensitive techniques are needed to confirm any cases of rinderpest.

Molecular techniques have been developed to differentiate PPRV and RPV: Diallo et al., (1989) developed radioactive probes with the nucleoprotein (Np) genes of RPV and PPRV but these are applicable only in a well equipped laboratory; Pandey et al. (1992) develop-

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Table 1. Sequence and position of primers set and oligonucleotide probe.

Primer	Gene (Position)	Sequences
B12	RP-N (1322>1344)	5' CAA GGG AGT GAG GCC CAG CAC AG
B2	RP-N (1594<1618)	5' TAG GAA CAG CAA CAT ACG AGA GTC
Probe[a]		
Probe SB1 :	RP-N (1463>1482)	5' ACT CTG ATT GAT GTG GAC AC

ed non-radioactive probes and Libeau et al. (1994) differentiated RPV from PPRV by an immunocapture ELISA also based on the Np genes. However their sensitivity is lower than the radioactive ones. In such case, a specific and sensitive test to detect the virus in clinical samples is needed. This paper describes the investigation of a reverse-transcription – PCR (RT-PCR) technique for the specific diagnosis of RPV. The study uses the Np gene of RPV to distinguish all RPV strains from those of PPRV and also report on the use of the technique in epidemiological surveys for RP.

MATERIALS AND METHODS

Cells and viruses

Vero cells were grown in Eagle's MEM supplemented with 10% foetal calf serum and 1% antibiotic solution (GIFCO). Each 25 cm^2 flack received 10 ml of approximately 25,000 cells/ml and 200 µl of each of a specific RPV strain, and then incubated at 37°C (5% of CO_2 atmosphere). When confluent monolayer cells were established, the foetal calf serum was reduced to 1%. This medium was replaced every two days. The infected cells and supernatant were taken when the cytopathic effect (cpe) reached 50% (minimum) and the harvest was stored at –70°C until RNA extraction.

Origin of the RPV strains

Thirteen strains from CIRAD/EMVT (Montpellier, France) were used and RNA of each strain was extracted. These were RBOK (attenuated vaccine strain), RPVL (Japan, lapinized RPV strain), Buffalo Nigeria (Nigeria), Egypt, RGK (Kenya), RBT/1 (Tanzania), Sokoto (Nigeria), Iraq, Lebanon, Pakistan, Pendik (Turkey), Saudi (Saudi Arabia), Yemen.

The RNA from twelve PPRV strains was obtained under the same conditions, to check the specificity of primers. These were PPRV 75/1 (vaccine strain), PPRV 75/1 (wild strain), Burkina-Faso, Côte-d'Ivoire, Central African Republique, Egypt, Mieliq, Senegal, Sudan-Sennar, Dorcas, Ibri, Israel.

Extraction of RNA

RNA was extracted as described by Yamada et al. (1990) modified by Couacy-Hymann et al. (2002). Briefly, 10 µl of RNaid (BIO 101) were added to a mixture containing 100 µl of 6M-guanidine isothiocyanate (lysis solution), in a 0.5 ml tube. After mixing, this was stirred slowly at room temperature for 5 min and centrigfuged at 2000 rpm for 2 min in a microfuge. The supernatant was discarded and the pellet washed three times with 400 µl of ethanol solution (EtOH 50%, Tris-HCl 10 mM pH 7.4-7.6, EDTA 1mM, NaCl 50 mM). The pellet was vacuum dried and RNA bound to the matrix Rnaid was eluted by 30-75 µl of DEPC-treated water for 5 min at 55°C, followed by centrifugation at 15000 rpm for 5 min. The supernatant contained the total RNA and 1 µl of RNA inhibitor (10 U/µl) (Amersham-Pharmacia-Biotech) was added to RNA solution which was then kept at –70°C until the RT–PCR procedure. This method was used to extract RNA from experimentally infected animals and field samples such as lymph nodes, nasal discharges, lungs, mouth swabs.

RPV primers and probes

One set of specific primers was selected in the 3'-terminal region of the NRPV gene after alignment of both Np genes of RPV (Kamata et al., 1991) and PPRV (Diallo et al., 1994) to allow the amplification of a sequence of 297 bp of any RPV strains.

A specific probe was chosen in the fragment amplified by the set of primers B12/B2 and labelled with digoxigenin-dUTP using the non-radioactive DNA labelling kit (Boehringer) following the manufacturers' instructions. The name sequence and location of the primers and probe are summarized in Table 1. The oligonucleotides were synthesized on an oligo 1000 DNA synthesizer (BECKMAN)

Single-strand cDNA synthesis

Extracted RNA (1 µl) of each RPV strain was each mixed with 7 µl of DEPC-treated water and 1 µl of each primer (30 pmol/µl) in a 0.5 ml tube. The mixture was briefly stirred, centrifuged at 2000 rpm for a few seconds then incubated at 65°C for 10 min. The tube was chilled on ice and centrifuged briefly as before. Then 1 µl of RNase (10 U/µl) (RNaguard, Amersham-Pharmacia Biotech) and 5 µl of cDNA synthesis mix (first strand cDNA kit, Amersham-Pharmacia Biotech) was added, followed by a short centrifugation at 2000 rpm after which it was incubated for 1 h.

PCR reaction

The PCR reaction was made on the contents of the reaction in the tubes above, which received the following reagents: 10 µl of Taq polymerase buffer 10X, 0.8 µl of dNTPs (25 mM), 1 µl of each primers (30 pmol/µl), 71.2 µl of distilled water. The tube was stirred, centrifuged briefly, denatured at 95°C for 10 min; chilled on ice; centrifuged briefly at 2000 rpm and then promptly, 1 µl of Taq polymerase (2.5 U/µl) was added. The amplification reaction was made in a PHC3 thermal cycler (Techne). Temperature cycling was 60°C for 1 min, for annealing; 72°C for 30 s for extension; 94°C for 30 s for denaturation; repeated for 5 cycles followed by 30 cycles where temperature of annealing was reduced to 55°C. All samples were for 36 cycles including the final extension at 72°C for 10 min. To avoid false positive results, the good practices as described by

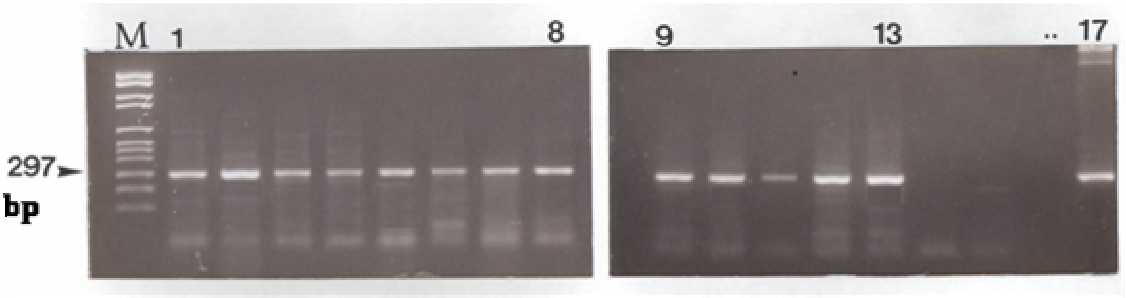

Figure 1. Specific PCR amplicafication of the NP gene fragment of different RPV isolates with primers B12/B2. The amplified products analysed by electrophoresis on 2% agarose gel stained with ethidium bromide are as follow: M (Marker, Boerhinger, Marker VI); (1) RBOK; (2) RPVL; (3) Buffalo Nigeria; (4) Egypt; (5) RGK; (6) RBT/1; (7) Sokoto; (8) Irak; (9) Lebanon; (10) Pakistan; (11) Pendik; (12) Saudi; (13) Yemen; (14) Negative control (Distilled water); (15) Negative control (uninfected Vero cells); (16) No sample; (17) Positive control (cDNA).

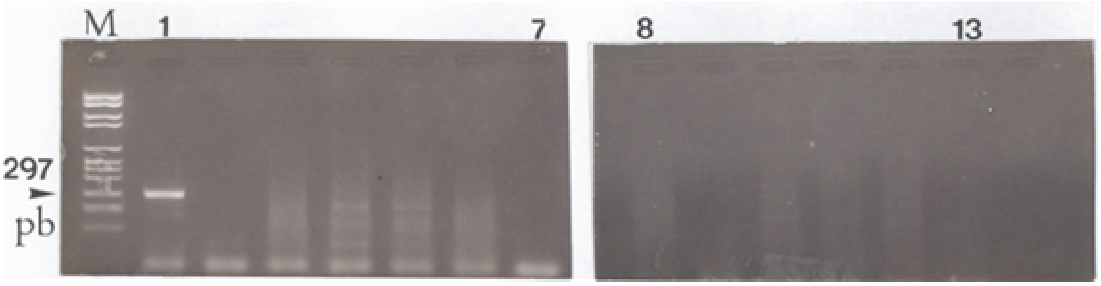

Figure 2. Attempt of specific PCR amplicafication of the NP gene fragment of different PPRV isolates with primers B12/B2. The amplified products analysed by electrophoresis on 2% agarose gel stained with ethidium bromide are indicated as follow: M (Marker, Boerhinger, Marker VI); (1) Positive control (RBOK, RPV vaccine strain); (2) PPRV 75/1 (Vaccine strain); (3) PPRV 75/1 (wild strain); (4) Burkina-Faso; (5) Côte-d'Ivoire; (6) Central African Republique; (7) Egypt; (8) Mieliq; (9) Senegal; (10) Sudan-Sennar; (11) Dorcas; (12) Ibri; (13) Israel.

Kwok and Higuchi (1989) were followed. Negative and positive controls were included in all experiments

Direct analysis of PCR-amplified products

Each amplified sample (10 µl) was electrophoresed on a composite 1% NuSieve and 1% Seakem agarose gel (FMC) in Tris-borate EDTA buffer 1X (0.089 M Tris base, 0.089 M boric acid and 0.02 M EDTA, pH 8) either directly or after digestion by restriction enzyme RsaI (Boerhinger). The size of the fragments was examined under UV illumination. The gel was transferred onto nylon filter positive (Boehringer) charged by capillary action overnight using 0.4 N NaOH as medium. Restriction enzyme digestion of PCR-products was made using 1 µl (10 U/µl) of RsaI at 37°C for 1 h following the manufacturer's instructions.

Southern-blot hybridisation

A nylon filter containing immobilised DNA was pre-hybridized in 5 ml of hybridisation buffer composed of SSC 5X, 2% blocking reagent (Boerhinger), 0.1 % sarcosine, 0.02% SDS, for 30 min and incubated at 68°C in an hybaid-hybridisation-oven. This was followed by a hybridisation buffer step with 2.5 ml of a new hybridisation buffer, 12.5 µl (about 10 pmol/ml) of the corresponding internal SB1digoxigenin–dUTP probe and incubated for 30 min at 50°C in the hybridisation oven. After hybridisation, the probe was recovered and frozen at –20°C for future use. The filter was washed with the washing buffer. Firstly, at room temperature with 10 ml of the washing buffer: SSC 2X, 0.1% SDS for 5 min (twice) and secondly, at 50°C with 10 ml of the washing buffer containing SSC 0.1% and 0.1% SDS for 10 min (twice). The presence of digoxigenin was revealed by immunological detection with phosphatase conjugated anti-digoxigenin antibody (Boerhinger)

RESULTS

The extraction method using the RNaid system gave RNA of good quality and yield in 30 min from diverse samples (infected cell culture, experimentally infected animals and field samples (data not shown). The primers B12/B2 were used successfully to specifically amplify the Np gene of RPV after extracting the RNA. An ethidium stained gel of Np RPV PCR-products from these isolates is shown in Figure 1. The size of amplified products is 297 bp as expected. Both negative controls (distilled water and uninfected Vero cells replacing RNA) remained negative as shown in lanes 14 and 15, respectively. After southern-blot transfer of all the PCR-products onto the membrane, the internal and non-radioactive probe SB1 served for the hybridisation. It detected the transferred cDNA products confirming that the correct fragment was obtained (data not shown).

The primers B12/B2 were unable to amplify RNA of PPRV obtained from infected cells (Figure 2). After diges-

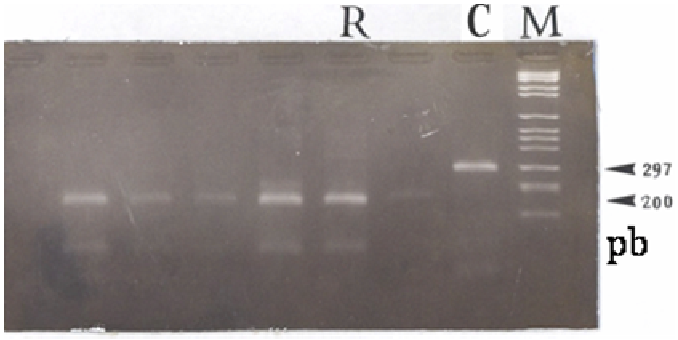

Figure 3. Specific PCR amplification of the NP gene of RPV isolates with primers B12/B2. The amplified products were then restricted with Rsa1 at 37°C for 1 h. There were 5 restricted RPV-amplified-products with a major band at 200 pb (R); Positive and unrestricted Control (C); M (Marker VI Boerhinger).

tion by RsaI, a major band of 200 bp was obtained. The expected fragments of 47 bp and 50 bp cannot be seen with this agarose gel (Figure 3). This study was also performed with the PCR-products of PPRV strains with primers P1/P2 and showed that RsaI was unable to cleave these amplified fragments (Couacy-Hymann, 1994).

RNA was extracted from lungs, nymph nodes, nasal discharges, mouth swabs, collected from experimental infected cattle and from controls were subjected to RT-PCR. The expected fragment of 297 bp appeared in each infected sample whereas the control ones remained negative (data not shown). Field samples since 1995 were all negative. This set of primers failed to amplify measles virus from samples collected from sick children from the National Pasteur Institute (Akoua-Koffi, unpublished).

DISCUSSION

The extraction of RNA for RT-PCR remains a critical factor in diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of testing. This step requires an efficient method to obtain a good quality material. The rapid procedure for RNA extraction described previously was very successful (Couacy-Hymann, 1994; Couacy-Hymann et al., 2002) and was simpler and easier for use in routine diagnosis than previous phenol-chloroform based extraction methods (Chomczynsky and Sacchi, 1987; Ausubel et al., 1993). The use of commercial kits for RNA purification and reverse transcriptase reaction avoids manipulation steps. The incorporation of guanidine isothiocyanate in the lysis solution efficiently inactives RNase to prevent RNA degradation. Samples in this solution do not require any cold chain which is very important in the field in Africa conditions.The specificity of the set of primers B12/B2 was confirmed as they did not amplify any Np from the PPRV and Measles virus genes. Additional confirmation came from the restriction enzyme data where RsaI cleaved the amplified fragments of RPV but not the Np of PPRV. The DSn of the RT-PCR applied to RPV diagnosis is equivalent to the PPRV tests for PPRV diagnosis where it was shown that RT-PCR was 1000 fold more sensitive than the classical titration test (Couacy-Hymann et al., 2002). This sensitivity could be increased with the non-radioactive probe SB1.

The RT-PCR technique followed by a hybridisation with a specific non-radioactive probe SB1 or by a cleavage by a specific restriction enzyme, RsaI, is a powerful tool for RPV diagnosis and differential diagnosis of RPV in the Morbillivirus genus, in particular between RPV and PPRV. Techniques involving RT-PCR for the diagnosis of RPV were first developed in 1992 and validated with reference to experimentally infected animals and on field samples. Using the fusion protein (Fp) gene of RPV, Barrett et al. (1993) designed a set of primers to diagnose RPV. Both RT-PCR techniques based on Np or Fp genes can be used for RPV diagnosis.

The PCR has contributed to investigation of rinderpest during the present eradication programme. The potentially very high DSn and DSp of the PCR make it ideal tool where the prevalence of RP is absent or very low, as is the case now in Africa. It must be stressed that if the RT-PCR is linked to statistical viable sampling frames and that when collection of samples is made into protective solutions to maintain nucleic acid activity, then the PCR offers an ideal test for establishing disease status. Many laboratories in Africa can implement the PCR technique when constraints such as rapid clearance of fragile reagents such as enzymes at airports are solved and when properly designed PCR laboratories and technicians are trained. There are good examples of the process of PCR equipment supply and training which began in about 1995 and are still being sustained by international organisations and the potential to perform PCR in control programmes is now present in many African countries.

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