



Discrimination between sheep-associated and wildebeest-associated malignant catarrhal fever virus by means of a single-tube duplex nested PCR

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ABSTRACT

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A single-tube duplex nested polymerase chain reaction (sdn-PCR) was developed for the detection of and discrimination between ovine herpesvirus-2 (OvHV-2) and alcelaphine herpesvirus-1 (AIHV-1). These viruses respectively cause sheep- and wildebeest-associated malignant catarrhal fever (SA-MCF and WA-MCF). In the first step of the sdn-PCR, two primers with high annealing temperatures based on conserved regions of the tegument genes were used for DNA amplification. In the second step, two primer sets based on variable regions of the respective OvHV-2 and AIHV-1 genes and with annealing temperatures > 11 °C below the primers used in the first step, were used. Internal regions of different sizes from amplicons produced in the first step were amplified. This single-tube test obviates the need for two separate assays to detect both viral types, thereby reducing time, labour and cost.

Keywords: Alcelaphine herpesvirus, malignant catarrhal fever viruses, ovine herpesvirus, single-tube duplex nested PCR

INTRODUCTION

Malignant catarrhal fever (MCF) (known in South Africa by its colloquial name “snotsiekte”) is a severe disease of domestic cattle, certain wild ruminant species and pigs. It is characterized by profuse mucopurulent oculo-nasal discharge, keratoconjunctivitis

with corneal opacity and peripheral lymphomegally. It generally occurs sporadically and has a worldwide distribution (Barnard, Van der Lugt & Mushi 1994; Loken, Alexandersen, Reid & Pow 1998). Two forms of the disease, sheep- and wildebeest-associated MCF, have been described and were so named after the reservoir ruminant species. The aetiological agents are two closely related viruses of the subfamily *Gammaherpesvirinae*, viz ovine herpesvirus-2 (OvHV-2 previously OHV-2) and alcelaphine herpesvirus-1 (AIHV-1) (Plowright, Ferris & Scott 1960; Baxter, Pow, Bridgen & Reid 1993). Outside Africa, the disease is usually associated with contact between sheep and susceptible species (Piercy 1952) although in New Zealand it was found that snotsiekte commonly occurs in farmed deer where no sheep are present (Oliver 1985, cited by Barnard *et al.* 1994).

The development of polymerase chain reactions (PCRs) for the detection of malignant catarrhal fever

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virus (MCFV) DNA has enabled the sensitive detection of low copy numbers of OvHV-2 (Baxter *et al.* 1993; Dungu, Bosman, Kachelhoffer & Viljoen 2002) and AIHV-1 (Hsu, Shih, Castro & Zee 1990; Katz, Seal & Ridpath 1991; Murphy, Klieforth, Lahijani & Heuschele 1994; Tham, Ng & Young 1994). In South Africa, both forms of the disease occur. Differential detection of low levels of virus present in animals infected with OvHV-2 or AIHV-1, generally requires two different nested PCR assays that are costly and prone to amplicon contamination. Recently, Fabian & Egyed (2004) developed a nested duplex PCR for the simultaneous detection of bovine herpesvirus 4 (BoHV-4) and AIHV-1 in a single nested PCR. We describe the development of a single-tube duplex nested PCR (sdn-PCR) (Shaio, Lin & Liu 1997; Tang, Bartlett, Smith & Lee 1997; Dungu *et al.* 2002) for the simultaneous detection of either OvHV-2 or AIHV-1 in field samples. This method is cost effective and could be especially useful for virus detection in large numbers of field samples.

MATERIALS AND METHODS

Samples

Whole blood samples in ethylenediaminetetra-acetic acid (EDTA) anti-coagulant or blood clots from infected and uninfected cattle were used. Positive control samples were bovine blood samples that tested positive by the single-tube nested PCR (stn-PCR) (Dungu *et al.* 2002) or by AIHV-1 PCR from suspected bovine field cases with typical clinical signs of MCF. The PCR products were authenticated by sequencing. Negative samples were from healthy bovines that tested negative using stn-PCR and AIHV-1 PCR.

Virus cultivation

Bovine herpesviruses 1, 2 and 4 were cultivated in MDBK cells grown in monolayers in Eagle's medium supplemented with 10 % bovine serum, penicillin (1 U/ml final concentration) and streptomycin (0.2 µg/ml final concentration) [both penicillin and streptomycin were from Novo Nordisk (Pty) Ltd]. Cells were harvested when the cytopathic effect (CPE) was at least 90 %.

DNA extraction

DNA extraction from bovine blood and cell cultures

DNA was extracted from blood and organs and from cell cultures using the Nucleon Bacc II kit (Amer-

sham, England) according to the manufacturer's instructions.

DNA isolation from agarose gel

To obtain purified amplicons for determination of the sensitivity of the sdn-PCR assay, MCFV-PCR was performed as described on DNA from bovines infected with either OvHV-2 or AIHV-1 [as determined by stn-PCR (Dungu *et al.* 2002) and AIHV-1-PCR]. Products were analysed by electrophoresis in a 1.5 % agarose gel according to standard procedures in 40 mM Tris-acetate, 2 mM EDTA buffer (pH 8) (Maniatis, Fritsch & Sambrook 1982). Gel bands containing amplicons were excised from gels. Amplicons were isolated using the QIAEX kit (QIAGEN, Germany). The same procedure was used to isolate DNA amplicons for sequencing.

Sequencing

The Big Dye terminator cycle sequencing kit (PE Biosystems, USA) was used in a Perkin Elmer 2400 thermocycler (PE Biosystems). Sequences were analysed using the ABI Prism 377 automated DNA sequencer (PE Biosystems). Primers used for sequencing were OVF and OVR for the OvHV-2 amplicon and WBN-1 and WR for the AIHV-1 amplicon (Fig. 1 and Table 1).

Polymerase chain reaction

Single-tube duplex nested PCR (sdn-PCR)

Reactions were performed in a Perkin Elmer 2400 thermocycler. Each 12.5 µl PCR mix consisted of the following: 1 x exTaq DNA polymerase buffer, 0.5 U exTaq DNA polymerase, 200 µM of dATP, dCTP, dGTP and dTTP, respectively (buffer, enzyme and dNTPs were from Takara), 0.02 µM of primers MF and MR and 0.8 µM of primers OVF, OVR, WBN1 and WR, respectively. Approximately 50–300 ng extracted total DNA containing template was normally added. The thermocycling conditions for the PCR were: 94 °C for 2 min followed by 20 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 30 s (first reaction) and 30 cycles of 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s (second reaction). Amplicons were analysed by agarose gel electrophoresis as described under *DNA isolation from agarose gel* (above).

MCFV-PCR

In order to determine the sensitivity of the sdn-PCR, pure MCFV-specific DNA originating from OvHV-2

and AIHV-1-infected animals was required. PCR amplicons were used for this purpose. MCFV-PCR was therefore performed on DNA extracted from OvHV-2 or AIHV-1 infected bovines. The MCFV-PCR reaction mixture was identical to that of the sdn-PCR [see *Single-tube duplex nested PCR (sdn-PCR)*] except that primers OVF and OVR as well as WBN-1 and WR were omitted and a higher concentration of primers MF and MR (0.8 μ M) was used. The PCR conditions were 94 °C for 2 min followed by 40 cycles at 94 °C for 30 s, 68 °C for 30 s and 72 °C for 30 s. The amplicons were isolated from the gel as described under *DNA isolation from agarose gel*.

AIHV-1 PCR

An AIHV-1-specific PCR was used for the initial selection of AIHV-1 positive samples. This PCR was performed in a volume of 12.5 μ l consisting of 1 x FastStart Taq DNA polymerase buffer (Roche, Switzerland) containing a final concentration of 2.5 mM MgCl₂, 0.5 U FastStart Taq DNA polymerase (Roche, Switzerland), 200 μ M of dATP, dCTP, dGTP and dTTP and 0.4 μ M of primers WBN1 and WR, respectively. Approximately 50–300 ng extracted total DNA containing template was normally added. PCR conditions were 95 °C for 7 min followed by 50 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s.

Primer sequences

Fig. 1 indicates the alignment of part of the tegument genes of OvHV-2 from a bovine field case propagated in a lymphoblastoid cell line (Baxter *et al.* 1993; Genbank S64565) and AIHV-1 isolate C500 (Ensser, Pflanz & Fleckenstein 1997; Genbank AF005370). The regions of the sequences on which the primers are based are underlined and in bold. The sequences of the primers used are indicated in Table 1.

RESULTS

Verification of the specificity of the sdn-PCR amplicons from DNA of a bovine infected with OvHV-2 or AIHV-1, was done by sequencing using primers OVF and OVR (OvHV-2) and WBN-1 and WR (AIHV-1) (results not shown). The PCR amplicon of the OvHV-2 sample showed a 100 % sequence identity over a 118 bp overlapping region with the homologous OvHV-2 gene sequence (Genbank S64565; Baxter *et al.* 1993). A 97 % identity of the AIHV-1-specific amplicon with the homologous gene of the C500 isolate of AIHV-1 (Genbank AF005370;

Ensser *et al.* 1997) was shown over a 150 bp overlapping region.

The specificity of the PCR amplicons was also demonstrated by performing sdn-PCR on DNA extracted from the blood of a previously confirmed positive AIHV-1 infected bovine and MDBK cells infected with bovine herpesvirus-1 (BHV-1), bovine herpesvirus 2 (BHV-2) and bovine herpesvirus 4 (BHV-4), respectively (Fig. 2). In order to confirm that the DNA extracted from the infected cell samples did not contain PCR inhibitors, duplicate samples were used and one of each was spiked with a positive control sample mentioned above and containing DNA isolated from the blood of a bovine infected with AIHV-1. All positive control samples gave an amplicon of the predicted size (241 bp). No amplicons of sizes predicted for AIHV-1 or OvHV-2 were observed when testing DNA extracted from BHV-1, 2 and 4 infected cells. In some instances, a weak DNA band of approximately 300 bp was observed with the BHV-4 sample (lane 7). When each of the six primers (Table 1) was aligned to the sequence of the BHV-4 tegument gene, using the National Center for Biotechnology Information BLAST algorithm (<http://www.ncbi.nlm.nih.gov>), no significant sequence similarity was identified. In some positive control samples a band of approximately 600 bp was also observed [Fig. 2 (lanes 1 and 8)] similar to that seen in Fig. 3 (lanes 7 and 10), Fig. 5 (lane 1) and Fig. 6 (lanes 1 and 2). These bands are probably non-specific and are usually seen in strongly positive samples. As they seem to be of the same size and are seen in the presence of both OvHV-2 and AIHV-1 DNA, it may be as a result of non-specific binding of the MF/MR primers to some conserved part of the virus genome or to bovine DNA.

Six clotted blood samples and one whole blood sample that tested OvHV-2 positive by single-tube nested PCR (Dungu *et al.* 2002), produced a 180 bp amplicon using sdn-PCR. Using the same procedure, a 241 bp amplicon was produced using DNA from 13 whole blood and one clotted-blood sample that tested positive by AIHV-1-PCR. In Fig. 3, amplicons produced from four OvHV-2 positive samples (lanes 1–4) and four AIHV-1 positive samples 1 (lanes 7–10) are indicated. Interim-sized PCR amplicons produced by primers OVF/MR (280 bp) and OVR/MF (207) as well as by WBN-1/WR (271 bp) and WR/MF (277 bp) were occasionally seen, but were usually not visible as a result of the low concentration of primers MF and MR. The extra band above the OvHV-2-specific band in Fig. 3 (lane 1) is probably the 307 bp band from the first PCR (primers MF and MR).

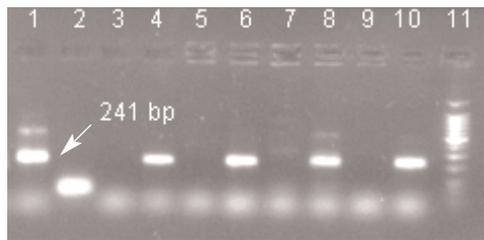


FIG. 2 An agarose gel showing electrophoretic separation of sdn-PCR products obtained from DNA extracted from the blood of bovines infected with AIHV-1 (lane 1), MDBK cell cultures infected with BHV-1 (lane 3), BHV-2 (lane 5), BHV-4 (lane 7), or from an uninfected bovine (lane 9). Samples were also spiked with DNA extracted from a bovine previously shown to be infected with AIHV-1 (from the DNA sample used in lane 1): thus BHV-1 and AIHV-1 (lane 4), BHV-2 and AIHV-1 (lane 6), BHV-4 and AIHV-1 (lane 8), DNA extracted from uninfected bovine and AIHV-1 (lane 10). Lane 2 indicates the product of a negative control sample where the template was replaced with water. Lane 11 contains a DNA size marker comprising a 100 bp ladder

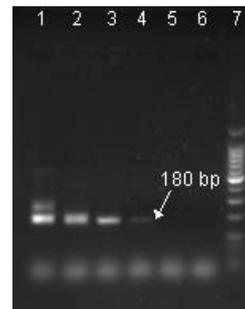


FIG. 5 Agarose gel electrophoresis of OvHV-2-specific sdn-PCR products. The PCR was performed using a two hundred-fold dilution series of template. The latter was 307 bp gel purified PCR amplicons obtained by performing MCFV-PCR on DNA extracted from an OvHV-2-infected bovine. Indicated are PCR products derived from 80 pg (lane 1); 0.4 pg (lane 2), 2 fg (lane 3), 0.01 fg (lane 4) and 0.05 ag (lane 5) starter amplicons as well as a negative control sample in which water was used as template (lane 6) 100 bp DNA ladder (lane 7)

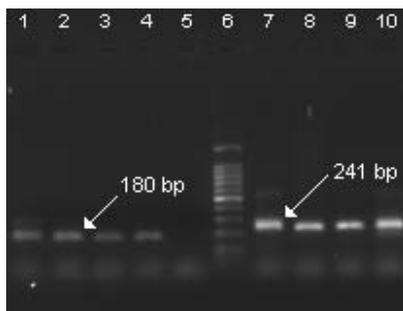


FIG. 3 An agarose gel showing electrophoretic separation of sdn-PCR products of DNA extracted from different field samples from the blood of bovines infected with OvHV-2 (lanes 1–4) or AIHV-1 (lanes 7–10), respectively. Lane 5 contains a sample of the PCR product from DNA extracted from the blood of an uninfected bovine and lane 6 contains a 100 bp DNA ladder

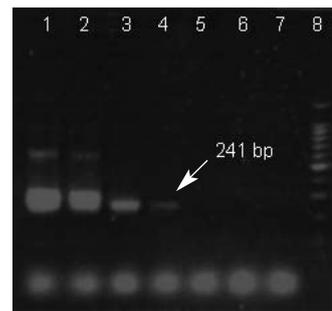


FIG. 6 Agarose gel electrophoresis of AIHV-1-specific sdn-PCR products. The reaction was performed on a 200-fold dilution range of template. The latter was 307 bp gel purified amplicons obtained by performing the MCFV-PCR on DNA extracted from an AIHV-1-infected bovine. Indicated are amplicons derived from 80 pg (lane 1); 0.4 pg (lane 2), 2 fg (lane 3), 0.01 fg (lane 4) 0.05 ag (lane 5) and 0.00025 ag starter amplicons (lane 6) as well as a negative control sample in which water was used as template (lane 7) 100 bp DNA ladder (lane 8)

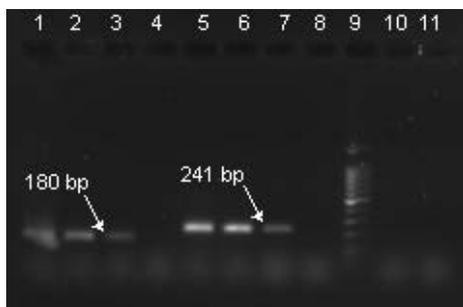


FIG. 4 An agarose gel showing electrophoretic separation of sdn-PCR products obtained from a five-fold dilution range of DNA extracted from whole blood of bovines infected with OvHV-2 (lanes 1–4) and AIHV-1 (lanes 5–8). [Lanes 1 and 5: 25 ng; lanes 2 and 6: 5 ng; lanes 3 and 7: 1 ng and lanes 4 and 8: 0.2 ng total DNA extracted from blood of the respective bovines.] Lanes 10 and 11 show negative control samples in which water was used as PCR template; lane 9: contains a 100 bp DNA ladder

The sensitivity of the PCR was determined by performing sdn-PCR on a dilution range of total DNA extracted from a whole blood sample from bovines previously shown to be infected with OvHV-2 or AIHV-1. Five-fold dilutions were made and a 2 μ l sample from each was analysed in a 12.5 μ l reaction by PCR. The smallest amount of total DNA extracted from infected bovine blood from which a specific amplicon could be amplified was 1 ng (Fig. 4).

MCFV-PCR was performed on DNA extracted from the blood of bovines infected with OvHV-2 or AIHV-1. The 307 bp DNA bands obtained were cut from

the gels and DNA was isolated as described under materials and methods. The sdn-PCR was performed on a 200-fold dilution series of the respective amplicons. The lowest amount that could be detected was 0.01 fg for both OvHV-2 and AIHV-1 amplicons (Fig. 5 and 6). This corresponds to 30 molecules of the 307 bp amplicon.

In samples containing a high concentration of AIHV-1 DNA, bands probably originating from primers MF/MR [Fig. 5 and 6 (lane 1)] and/or from primers MF/WR and MR/WBN1 [Fig. 6 (lanes 1 and 2)] were seen.

A faint band of approximately 450 bp was also produced in some samples [Fig. 5 (lane 2)]. This was probably a non-specific amplicon.

DISCUSSION

In South Africa both SA-MCF and WA-MCF are enzootic. Two different nested PCRs are usually performed on samples from each suspected field case for the detection of and discrimination between the different causative agents. Various effective PCRs for the detection of the viruses have been described and used previously for OvHV-2 (Baxter *et al.* 1993; Dungu *et al.* 2002) and AIHV-1 (Hsu *et al.* 1990; Katz *et al.* 1991; Murphy *et al.* 1994; Tham *et al.* 1994). In order to save reagents, time and labour, an sdn-PCR was developed. This method allows for the discrimination between OvHV-2 and AIHV-1 in a single tube and also avoids the problem of contamination associated with a two-tube nested PCR. Dungu *et al.* (2002) described a single-tube nested PCR for the detection of OvHV-2 only. We used the same principle and developed an sdn-PCR (Shaio *et al.* 1997; Tang *et al.* 1997; Dungu *et al.* 2002) which enabled us to detect either or both OvHV-2 or AIHV-1 from incoming field samples in a single reaction. Primers were based on a region of the OvHV-2 gene that have sequences with a significant identity (approximately 50%) with the BNRF1 and the EILF open reading frames (ORF) encoding the respective tegument genes of Epstein Barr virus and *herpesvirus saimiri* (Nicholas, Cameron, Coleman, Newman & Honess 1992; Baxter *et al.* 1993; Ensser *et al.* 1997). Part of the OvHV-2 genome sequence (Baxter *et al.* 1993) was aligned with that of the AIHV-1 genome (Ensser *et al.* 1997). Primers for detection of the MCFV group (MF and MR) were based on conserved regions of the OvHV-2 and AIHV-1 genes (Fig. 1) and were designed to have high midpoint annealing temperatures (T_m) so that primers of the second step would not anneal to the

template during the first PCR. MF and MR were used at a low concentration so that they could be depleted during the first step PCR. Type-specific primers OVF and OVR (OvHV-2) and WBN-1 and WR (AIHV-1) were based on variable regions of the genes (Fig. 1). They were designed to have a lower T_m (> 11 °C lower than that of the first PCR) and were used at a relatively high primer concentration. Most of the PCR amplicons produced in the second step were assumed to have been produced by the type-specific primers annealing to the respective amplicons produced in the first PCR, thus improving the specificity of the test.

The PCR was shown to be specific for bovine herpesviruses OvHV-2 and AIHV-1 when testing DNA obtained from cell cultures infected with BHV-1, BHV-2 or BHV-4 and from positive control DNA samples derived from OvHV-2 or AIHV-1 infected bovines. Furthermore, sequence analysis of the nested PCR amplicon of the AIHV-1 field sample indicated 97% identity with the published C500 strain (Ensser *et al.* 1997) over a 150 bp overlapping region. The PCR amplicon of the OvHV-2 sample showed a 100% identity with the published OvHV-2 sequence (Baxter *et al.* 1993) over a 118 bp overlapping region.

Baxter *et al.* (1993) made the assumption that one bovine cell nucleus contained 6.8 pg DNA. This they derived from the average genomic content per nucleus of various mammalian species determined by flow cytometry (Lee, Thornthwaite & Rasch 1984). Baxter *et al.* (1993) could detect OvHV-2 in 6.4 pg DNA from a lymphoblastoid cell line ICL 629 using hemi-nested PCR. They determined the corresponding viral genome copy number by extinction point determination using slot blot hybridisation analyses performed on dilutions of a plasmid containing a copy of the relevant part of the virus genome together with dilutions of DNA from the infected bovine. From this they determined that 35 viral targets could be detected per cell. The sdn-PCR could detect the viral DNA in 1 ng of total DNA extracted from the blood of both OvHV-2 and AIHV-1 infected bovines. In addition, the assay could detect 0.01 fg of OvHV-2 or AIHV-1-specific amplicons (Fig. 5 and 6). This corresponds to approximately 30 amplicon molecules. Assuming that 6.8 pg of genomic DNA corresponds to one bovine cell equivalent (Lee *et al.* 1984; Baxter *et al.* 1993), 30 viruses could be detected in 147 cells or one virus in five cells. With added advantages in terms of reduced labour, contamination and economics, the described sdn-PCR is an attractive method for surveillance studies in regions where both viruses are prevalent.

In a rare case where an animal was infected with both viruses, two bands of 180 and 241 bp could be seen (result not shown). In a large survey where samples are positive for AIHV-1, further testing for the presence of OvHV-2 (or vice versa) using primers for the group and the particular virus only, could be used to ensure that the presence of very low levels of one of the viruses is not obscured by high levels of the other.

The sdn-PCR for the simultaneous detection of and discrimination between OvHV-2 and AIHV-1, is economical and time-saving and can be useful in regions where both viruses are prevalent.

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