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Application of Conventional Reverse Transcription (RT) Polymerase Chain Reaction (RT-PCR) for Detection of African Horse Sickness Virus Serogroup

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Abstract

A conventional reverse transcriptase (RT) polymerase chain reaction (RT-PCR) for detection of African Horse sickness virus serogroup in cell culture was developed and evaluated. The sensitivity of the assay indicated that the RT-PCR produced a 240- base pair (bp) PCR product from all 9 serotypes of AHSV propagated in cell cultures. The specificity of the assay indicated that the PCR products were not amplified when the RT-PCR was applied to RNA from other related orbiviruses including Blue tongue virus (BTV) serotype 1, 2, and 4; Epizootic hemorrhagic disease virus (EHDV) serotype 1 and 2; Sudanese isolates of palyam viruses and total nucleic acid extracts from uninfected Vero cell control. The African horse sickness virus RT-PCR assay should be used as a rapid method for detection of AHSV RNA in cell culture. In addition, the assay could be optimized for rapid detection of AHSV in clinical samples during an epizootic of the disease among susceptible equines.

Key words: African horse sickness, virus cell culture, polymerase chain reaction.

المستخلص

تم تطويروتقييم تفاعل البلمرة المتسلسل التقليدي للنسخ العكسي (RT-PCR) للكشف عن المجموعة المصلية لفيروس مرض الخيل الأفريقي في الزرع الخلوي. أشارت حساسية فحص فيروس مرض الخيل الافريقي إلى أن RT- PCR أنتج منتج PCR مكون من 240 زوجًا أساسيًا (bp) من جميع الأنماط المصلية التسعة لـ AHSV المتكاثرة في مزارع الخلايا. أشارت خصوصية الفحص إلى أن منتجات PCR لم يتم تضخيمها عندما تم تطبيق RT-PCR على RNA من فيروسات أوربي الأخرى ذات الصلة بما في ذلك النمط المصلي 1 و 2 و 4 لفيروس اللسان الأزرق (BTV؛النمط المصلي 1 و 2 `منEHDV؛عزلات سودانية من فيروسات الباليامو مجموع مستخلصات الحمض النووي الضابط من خلايا فيروغيرالمصابة. يجب استخدام اختبار RT-PCR لفيروس مرض الخيل الأفريقي كطريقة سريعة للكشف عن الحمض النووي الريبي لـ AHSV في الزرع الخلوي . بالإضافة إلى ذلك، يمكن تحسين الفحص من أجل الكشف السريع عنAHSV في العينات السريرية أثناء الانتشارالوبائي للمرض بين الخيول المعرضة للإصابة.

الكلمات المفتاحية: مرض الخيل الأفريقي، الزرع الخلوى، تفاعل البلمرة المتسلسل.

Introduction

African horse sickness (AHS) is caused by African horse sickness virus (AHSV). The virus is a double- stranded (ds) RNA orbivirus of the family Reoviridae and is related to bluetongue virus (BTV), epizootic hemorrhagic disease of deer

virus (EHDV) and palyam serogroup of Orbiviruses (Borden *et al*., 1971; Fenner *et al*., 1974; Williams *et al*., 1998). Whereas BTV, EHDV and Palyam viruses cause clinical diseases in ruminants, the AHSV causes a fatal hemorrhagic infection in some breeds of horses particularly, Arabian horses. Donkeys and mules can amplify the virus and become infected with the disease but the infection is typically a symptomatic. There are nine serotypes of AHSV distributed worldwide, identified by serum neutralization and plaque inhibition tests. AHSV serotype 9 (AHSV-9) induced a fatal hemorrhagic infection in race horses in different parts of the Arab world including the Sudan. Serological evidence of AHSV infection in horses and donkeys is wide spread in the Sudan (Eisa *et al*., 1974; Aradaib *et al*., 2006; Aradaib, 2009). Sporadic infections with AHSV in Arabian horses were reported in different states of the Sudan based on clinical signs. The AHSV-9 was isolated from a naturally infected horse (Eisa., 1974). Since then, sporadic outbreaks of the disease were reported in different states of the Sudan. With the exception of the only one report described by (Eisa., 1974) virus isolation attempts were largely unsuccessful.

AHSV has a genome composed of 10 double-stranded (ds) RNA genome segments (Fenner *et al*., 1974). The genome segments code for the viral structural and non-structural proteins. Three nonstructural and seven structural proteins, are incorporated into the double layer protein coat (Borden et al., 1971). Group specific antigens and genome segments coding for these antigens have been defined (Maree and Paweska., 2005; Potgieter *et al*., 2003; Zientara *et al*., 1993a). Segment 3 and 7 probes of AHSV did not hybridize with BTV, EHDV or palyam virus dsRNA. A probe derived from genome segment 3 of AHSV-4 cross hybridized with the cognate genes of AHSV serogroup but not with other related orbiviruses (Moulay *et al*., 1995; Zientara *et al*., 1998; Moulay *et al*., 1995). The best hybridization signals with all AHSV isolates was obtained using a probe derived from genome segment 3 (L3), which codes for VP3 (Aradaib, 2009a). The major protein of the outer coat, viral protein 2 (VP2), coded for by genome segment 2, is associated with serotype specificity and induction of neutralizing antibody (Venter *et al*., 2000, Koekmoer *et al*., 2008; Martinez *et al*., 1994; Burrage *et al*., 1993; Zientara *et al* 1993).

The diagnosis of AHSV infection is currently conducted by serology, conventional virus isolation and molecular-based assays. Serology is complicated by cross reactions with other related orbiviruses and within the AHSV serogroup (Aradaib *et al*., 2006; Aradaib *et al*., 2009b). Virus isolation is time consuming, labor intensive and expensive. In previous studies, a simple, rapid, sensitive and specific RT-PCR-assay for detection of AHSV serogroup in cell culture was previously described (Aradaib *et al*., 2006). A nested RT-PCR assay was also developed and evaluated for detection of AHSV RNA in a variety of clinical samples (Aradaib, 2009b). The epidemiological studies indicated that AHS is endemic in sudan with AHSV-9 is being the most prevalent serotype. Therefore, the development of a rapid, sensitive, specific and inexpensive assay for detection of AHSV is urgently needed for clinical disease investigations. The developed assay will also facilitate molecular epidemiologic studies and would enhance vaccination campaigns and control programs in areas of endemicity. In addition, the assay will provide certification for international trade of equines and associated germplasm (Aradaib *et al*., 2009a). The objective of the present study was to develop a simple, rapid, sensitive and specific RT-PCR assay for detection of AHSV RNA in cell culture using primers derived from a highly conserve fragment of genome segment 3 of AHSV.

Materials and methods

Virus and cells:

The nine prototype vaccine strains of AHSV; the BTV prototype serotypes 1, 2, and 4; and the EHDV prototype serotypes 1 and 2, were obtained from the Institute of Animal Health, Pirbright, UK. Palyam virus isolates were recovered from sentinel cattle herds in Khartoum, Central Sudan. The viruses were processed as described previously (Aradaib *et al*., 2006). The virus serotypes and isolates were propagated on confluent mono-layers of Vero cells. The infectious material was harvested and centrifuged at 1,500 x g for 10 min and the supernatant was used for extraction of viral dsRNA.

Viral nucleic acid extraction from infected cell cultures:

The AHSV dsRNAs were extracted from supernatant of infected cell using QIAamp viral RNA kit (QIAmp, Humburg, Germany) as per manufacturer's instructions. Briefly, 140 µl of virus suspension were added to 560 ul AVL buffer containing carrier RNA into a 1.5 ml micro-centrifuge tube and mixed by pulse-vortexing for 15 sec. The mixture was incubated at room temperature for 10 min. 560 µl of absolute ethanol were added and mixed by pulse-vortexing for 15 sec. 630 µl of the mixture were transferred to QIAamp spin column mounted on 2ml collection tube and centrifuged at 6000 x g for one min. The column was then transferred to another collection tube and the remaining 630 µl of the mixture was again spin at the same speed. The column was then washed twice by 500 µl of washing buffers WB1 and WB2, respectively. Finally, dsRNAs were carefully eluted by 60 µl of AVE buffer equilibrated to room temperature. RNAs were quantified using a spectrophotometer at 260 nm wavelength. RNA Extracts were then kept at -20 0C till used for PCR amplification. Five µl of the nucleic acid were used for RT-PCR amplification.

Primer selection and synthesis of the probe:

It is well documented that AHSV genome segment 3, which codes for viral protein 3 (VP3), has a highly conserved nucleotide sequences among cognate genes of AHSV serogroup (Williams *et al*., 1998). Thus, selection of the primers was based on nucleotide sequence of VP3 of AHSV serotype 6 (AHSV-6) complete codons with Gene Bank accession number NC_002045. Primers were selected from the published nucleotide sequence of genome segment 3(L3) of AHSV-6 and used in the RT-PCR assays. A pair of primers 1 and 2 (P1 and P2) was selected for the synthesis of the primary PCR product. P1 included bases consisted of bases 281–300 of the positive strand (5)-GATAAACGCGCC ACCGAATT-(3). P2 was designed from the complementary strand between bases 501–520. (5)-TCTGGATCAGCAATCTCTGG-(3).

PCR amplification using P1 and P2 resulted in amplification of a 240-bp PCR product. All primers were synthesized on a DNA synthesizer and were received in lyophilized form from (Macrogen company, South Korea). The primers were diluted to a concentration of 100 picogram per microliter and used ion RT-PCR assay as per manufacturer's instructions.

Reverse transcriptase polymerase chain reaction (RT-PCR):

A single-tube RT-PCR amplification was carried out using One-Step Access RT-PCR system (QIA-Gen, California, USA). The RT-PCR was basically performed as described by Aradaib *et al*., 2009). Briefly, a standard 50 ul reaction mixture contained in final concentration of 1× enzyme mix reaction buffer, 5.0 ul of 10mMdNTP mix, 5.0 ul of 25mM MgCl2, 5.0U enzyme mix, 2.0_l of 20 pM of each outer primer (pal1 and pal2), 5.0_l of target RNA, were used. The total volume was brought to 50.0_l using RNase free water. Target genes were amplified in low-

profile 0.2 ml tube (MJ Research, California, USA). BTV and EHDV dsRNA templates were used as negative controls. The cycling program consists of a reverse transcription step at 50 ◦C for 30 min. A pre-denaturation step at 95 ◦C for 15 min was performed to inactivate excess RT enzyme and to activate the Taq DNA polymerase in the enzyme mix. This was followed by 40 cycles of denaturation at 94 ◦C for 1 min, annealing temperatures at 56 ◦C for 30 s each, extension at 72 ◦C for 45 s. The reaction mixture in each PCR tube was then subjected to a final extension step at 72 ◦C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ). Following amplification, 15 µ from each PCR amplification product were loaded onto 1% agarose gel and were electrophoresed. The gels were stained with ethidium bromide, and the AHSV PCR products were visualized under UV light.

Results

Sensitivity:

The RT-PCR provided sensitive and specific detection for all nine AHSV prototypes serotypes used in this study. The outer pair of primers (P1 and P2) produced a 240-bp primary PCR product from ≥100 fg RNA of AHSV1, 2, 3, 4, 5, 6, 7, 8, and 9. The primary PCR amplification products were visualized onto an ethidium bromide-stained agarose gels (Figure 1).

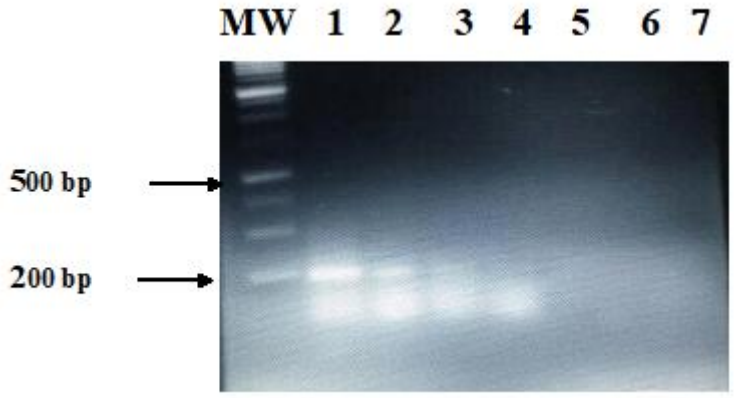

Figure 1. Sensitivity of the RT-PCR for detection of the primary 240 bp PCR product from AHSV prototype serotype 6. Lane MW: Molecular weight marker; lanes 1-6: (AHSV-6) 100 gp, 10 pg, 1.0 pg, 100 fg, 10 fg, 1.0 fg, 0.1 fg, respectively; Lane 7: non infected Vero cells (negative control).

Specificity:

The primary PCR products were not amplified from 1.0 pg RNA extracted from BTV prototypes serotypes 1, 2, 4 and 16; EHDV prototype serotypes 1 and 2; Sudanese isolates of palyam virus; or total nucleic acid extracts from non-infected Vero cells (Figure 2).

Detection of AHSV in cell culture:

Using 1 pg of RNA target, the specific 240- PCR products were detected in all AHSV serogroup. The identity of the PCR products was confirmed by real-time RT-PCR amplifications as described in chapter (Figure 3).

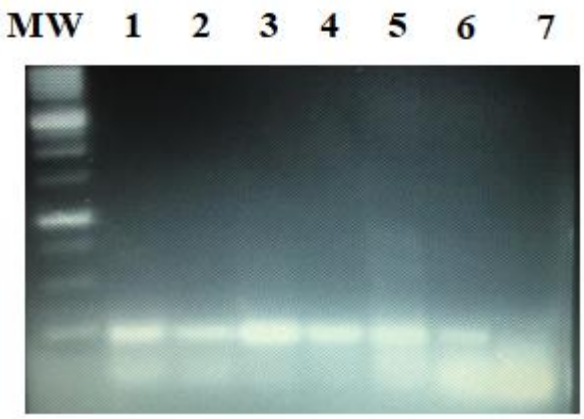

Figure 2. Visualization of AHSV specific 240-bp from as little as 0.1 pg of viral dsRNA from vaccine strains of AHSV serotypes 1, 2, 3, 4, 5. Lane MW: Molecular weight marker; lanes 1: AHSV (positive control); lanes 2: 10 pg of AHSV-1; lanes 3: 10 pg of AHSV-2; lanes 4: 10 pg of AHSV-3; lanes 5: 10 pg of AHSV-4; lanes 6: 10 pg of AHSV-5; Lane 7: non infected Vero cells (negative control).

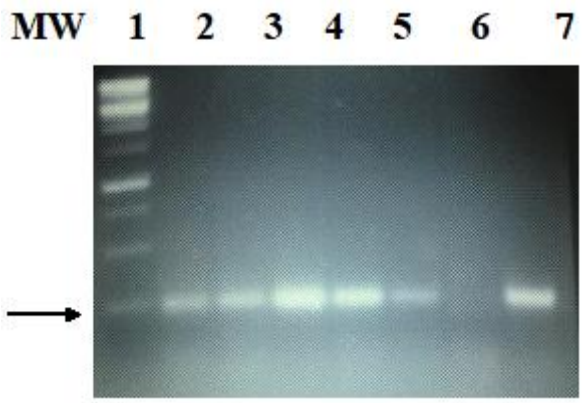

Figure 3. Visualization of AHSV specific 240-bp from as little as 0.1 pg of viral dsRNA from vaccine strains of AHSV serotypes 6, 7, 8, 9. Lane MW: Molecular weight marker; lanes 1: AHSV (positive control); lanes 2: 10 pg of AHSV-6; lanes 3: 10 pg of AHSV-7; lanes 4: 10 pg of AHSV-8; lanes 5: 10 pg of AHSV-9; Lane 6: non infected Vero cells (negative control). Lane 7: AHSV- infected Vero cells culture (positive control).

Discussion

African horse sickness virus (AHSV) causes an often fatal hemorrhagic disease, which usually results in death of susceptible breed of equines. The non-vaccinated thoroughbred and other horses have high susceptibility to infection with the virus. In addition, the virus has the potential for rapid spread and hence it is essential to confirm the identity and serotype of AHSV involved in an outbreak of the disease (Brown *et al*., 1994; Elhasnaoui *et al*., 1998; Fassi-Fihri *et al*., 1998; Sailleau *et al*., 2000). Conventional virus isolation and serology are time consuming and cumbersome. Definitive diagnosis of AHSV infection can be achieved rapidly and directly by the serogroup-specific ELISA (Hamblin *et al*., 1991; Laviada *et al*., 1992) or by serotype-specific RT–PCR (Zientara *et al*., 1995b). AHSV is enzootic in North and central and South Africa (Eisa. 1974). AHSV and other related orbiviruses are of concern to many governmental agencies because of the thread restriction on the international trade of race horses and associated germplasms to countries with no evidence of infection (Aradaib *et al*., 2009a). It is, therefore, becoming increasingly obvious that the development of molecular diagnostic techniques which provide rapid, sensitive, specific and inexpensive AHSV detection would be advantageous in a variety of circumstances including clinical disease investigation and epidemiological surveys (Aradaib *et al*., 2009a). The described RT-PCR assay using primers derived from segment 3 (L3) of AHSV-6, which codes for VP3, reproducibly and specifically detected AHSV-9 RNA in infected cell cultures and in clinical samples. Selection of the primers was based on the observation that L3 genome has the most conserved nucleotide sequences among cognate genes of AHSV serogroup. The primary 240-bp PCR products, visualized onto ethidium bromide- stained agarose gels were obtained from all the nine AHSV serotypes RNA samples tested. The sensitivity studies indicated that the RT-PCR protocol was capable of detecting the

amount of100 fg of total AHSV genomic dsRNA.

The specificity studies indicated that the 240-bp PCR product was not amplified from 1.0 ng of RNA from BTV, EHDV and Sudanese isolates of palyam viruses; or total nucleic acid extracts from Vero cell controls under the same stringency condition described in this study. This finding confirmed that VP3 gene is highly conserved among cognate genes of AHSV serogroup. In previous studies, detection of AHSV was made possible by direct sequencing techniques and digestion of AHSV-specific PCR products with restriction endonuclease enzyme. However, this method is tedious, laborious, time consuming, and expensive. RNA extraction was a simple procedure that takes only half an hour using QIAamp extraction kit. The time required for the primary amplifications was approximately 2 hours. The electrophoresis, staining of the agarose gel with ethidium bromide and visualization of the specific PCR products usually takes one hour. Confirmatory results of submitted samples could be obtained within the same working day. The described RT-PCR detected as little as 100 fg of viral RNA which is equivalent to 600 viral particles. The hybridization technique is tedious, laborious and time consuming and usually takes overnight. The RT-PCR assay is simple procedure that does not require sophisticated laboratory equipment such as DNA sequencer or hybridization facilities. Thus, the assay could be conducted in the African and other developing countries with minimal cost. The rapidity, sensitivity and specificity of the RT-PCR assay would greatly facilitate detection of AHSV infection during an outbreak of the disease among susceptible equines.

In previous studies, we described RT-PCR for detection of AHSV dsRNA in cell culture. However, in this study we evaluated the use of a different pair of highly conserved primers that reproducibly detected the specific 240 bp PCR product. The described RT-PCR could serve as an interesting alternative to serotyping and thereby reducing the time required for conventional virus isolation and identification. In addition, the assay would save the cost of maintaining reference sera and high quality cell culture supplies. Excellent correlation of results from the primary PCR amplification on ethidium bromide-stained agarose gels was obtained using this PCR-based assay. In addition, working with radioactive hybridization assay is hazardous, cumbersome and expensive. Real time PCR has advantages over conventional PCR for high thorough put detection of the virus during an outbreak of the disease in susceptible equines. The sensitivity of the real time PCR was demonstrated to be sufficient for use with dsRNA isolated directly from infected organ samples (Aradaib 2009b), making it potentially useful as a rapid diagnostic tool. In contrast, the sensitivity studies indicated that this RT-PCR protocol was capable of detecting the amount of 100 fg of total AHSV genomic dsRNA. This level of sensitivity is far less sensitive than real time PCR. Nevertheless, the RT-PCR described is a simple procedure that can be adopted for specific detection of AHSV in the African and other developing countries.

Conclusion

The described RT-PCR assay proved sensitive and specific for detection of AHSV serogroup. The assay could also be used as a valuable tool to study the epidemiology of the disease and incursion of the virus in a disease-free zone. In addition, the assay should be recommended for detection of AHSV in cell culture during an outbreak of the disease among susceptible equines.

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