Detection of Adenoviruses in cattle.

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The present thesis is a partial fulfilment of the requirements for a Master of Science Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Virology.

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To my dear family

Abstract

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The aim of the present work was to develop a sensitive and specific real-time PCR diagnostic system for the rapid detection of bovine adenovirus in cattle. A TaqMan real-time PCR assay capable of simultaneous detection of nine out of ten species of bovine adenovirus belonging to the genera *Atadenovirus* and *Mastadenovirus* was developed. The oligonucleotide primers and probe were selected from conserved sequences flanking the genome region coding for the Hexon protein. DNA from BAdV species 1 to 10 was isolated by standard phenol-chloroform extraction. Standard curve using a ten-fold dilution series of BAdV-8 positive DNA template was generated. The sensitivity of assay was determined using BAV-8 cloned PCR product and the detection limit was five copies of viral genome equivalents. The efficiency of PCR assay was 97%. Only BAdV-2 serotype was not possible to detect and BAdV-7 has shown low amplification rates and fluorescence, but anyway detected, due to four mismatches in the probe region. In conclusion, the TaqMan real-time PCR assay presented here is expected to be a good alternative tool to the traditional diagnostic methods like virus isolation, ELISA or conventional PCR in case of sporadic disease outbreak in which rapid, sensitive and specific diagnosis is required and when appropriate measures should be taken.

Contents

General background

Introduction

Adenoviridae is a well-defined virus family spread all over the world (Horwitz, 1990). Adenoviruses have been isolated from all vertebrate species including fishes, amphibians and reptiles (Russel and Benkö, 1999).

BAdV (Bovine adenovirus) were first isolated from cattle, their natural species, but later also from other animals including sheep, free-living buffalo, and fallow deer. After the human and fowl adenoviruses, BAdV comprises the third largest group of adenoviruses originating from one host species (Ursi at al., 2004). They appear to cause respiratory and enteric disease as well as conjunctivitis in cattle (Graham et al., 2005). BAdV infections are ubiquitous, easily transmitted and frequently unapparent. They are frequently found in association with other viruses or bacteria. BAdV infections cause sometimes-heavy losses in cattle husbandry. Sensitive methods are therefore required for the rapid detection of adenoviruses in clinical specimens.

Disease caused by adenoviruses

The members of the family Adenoviridae commonly infect humans and a wide variety of wild and domestic mammals, amphibians and birds. Adenoviruses are known to cause a variety of diseases such as respiratory, gastrointestinal, urinary, and ocular infections in human

(Adrian et al., 1987; Kinchigton et al., 1994) and hepatitis in dog. BAdV probably occur worldwide and have been isolated from both sick and apparently healthy cattle. Similarly to other adenoviruses, BAdV was reported as causative agents of respiratory and enteric tract infections (Burki, 1990; Fent et al., 2002; Graham et al., 2005). Disease of the respiratory and gastro-intestinal tract has been associated mostly with BAdV-3 (Stauber et al., 1986). Observations on naturally and experimentally infected cattle indicated that BAdV contribute to diarrhoea, enteritis, pneumonia, conjunctivitis, and so-called weak calf syndrome (Mattson, 1992). The role of BAdV as a cause of pneumonia is supported by virus isolation from calves with pneumonia (Reed et al., 1978) and experimentally produced mild pneumonia (Mattson et al., 1973). BAdV are considered among the many causes of bovine respiratory disease and may be the primary cause of some outbreaks of calf pneumonia (Lehmkuhl et al., 1999). BAdV 10 has been associated with acute and fatal episodes in cattle (Smyth at al., 1996). BAdV-3, 4 and 8 have been found in African buffalo calves. Lack of contact with cattle suggests a natural infection in this species (Baber and Condy, 1981). Deer may carry bovine adenovirus (Boros et al., 1985) and BAdV-2 variants are found in sheep (Belak, 1990).

Taxonomy

Adenoviruses represent a large family of double-stranded DNA viruses and over 100 distinct mammalian adenoviruses have been isolated. According to the official taxonomy, the family Adenoviridae consists of four genera comprising viruses isolated

from birds, mammals, amphibians and reptiles (Benkö and Harrach, 2003). The bovine adenovirus (BAdV) was first isolated from cattle (Klein, 1959), and later from other animals including sheep, buffalo and fallow deer (Belak et al., 1986). At least 10 serotypes (species) of bovine adenovirus are presently recognized (Lehmkuhl et al., 1999) and are designated bovine adenovirus serotypes 1 through 10. These serotypes were further divided into two subgroups, which have been identified on the basis of differences in their biological and serological properties. At present, the former BAdV subgroup I (BAdV-1 to 3, BAdV-9 and BAdV-10 species) belongs to the genus *Mastadenovirus*, and former BAdV subgroup II (BAdV-4 to 8 species) have been assigned to the new genus *Atadenovirus,* together with avian adenovirus, such as isolate 287 (OAV 287) and egg drop syndrome (EDS) virus (Both, 2002). DNA hybridization and phylogenetic studies have shown that BAdV subgroup I share similar genome arrangement with adenoviruses of human and other mammalian origin, while subgroup II is not (Benkö at al., 2000; Mayo, 2002). The latest accepted serotype BAdV-10 is distinct from the other BAdV species, but recent studies demonstrate that it has certain characteristics of *Mastadenovirus* (Matiz et al., 1998). Despite being assigned to this genus, BAdV-10 is very different from other members of *Mastadenovirus* and occupies a special place within the *Mastadenovirus* genus in a certain distance from the other members of this subgroup. (Smyth et al., 1996). For example, the degree of similarity exhibited between the BAdV-10 and BAdV-1, BAdV-2 protease was only 51,5%, BAdV-3 - 56,3%, BAdV-4 - 36,0%, BAdV-7 - 33,0% (Matiz et al., 1998). Probably, BAdV-10 is

not a genuine bovine adenovirus, but might originate from another species and is now in an adaptation process to a new host, cattle.

Genome organisation

Adenovirus virions are nonenveloped, 80-100nm in diameter, and exhibit icosahedral symmetry. Virions are composed of 252 capsomers: 240 hexons that occupy the faces and edges of the 20 equilateral triangular facets of the icosahedron and 12 pentons that occupy the vertices. From each penton projects a penton fiber 20 to 50 nm in length, with a terminal knob. The genome of BAdV consists of double-stranded DNA molecule, 36-44 kb in length. Virion DNA, in association with a 55K protein linked covalently to each 5`-terminus, is infectious. There are five early (E) transcriptional units: E1A, E1B, E2, E3, and E4, two intermediate units, IX and IVa2 and one late (L) from which five families of late mRNAs (L1 to L5) are transcribed. Each early region is under the control of a separate promoter whereas the late region uses a single promoter called the major late promoter. Previous classification of BAdV into two subgroups was supported by the significant difference in genome size between members subgroup I (ranging from 32 to 38 kb) and members subgroup II (ranging from 28-31) (Benkö et al., 1990).

Diagnostics

The diagnosis of BAdV infections is difficult because of the syndromes have been associated with many symptoms. Pneumoenteritis associated with BAdV must be differentiated from

bovine virus diarrhoea virus, bovine respiratory synsytial virus, rinotracheitis, and parainfluenza-3 has to be based on laboratory diagnosis (Belak, 1990). BAdV can be detected by different methods such as virus isolation, *in situ* hybridisation, ELISA, direct immunofluorescence, restriction enzyme and PCR (Sizov, 1982; Rosmanith and Horvath, 1988; Benkö et al., 1988; Giusti et al., 1998). PCR assays provide a highly sensitive and specific tool for investigating the significance of *Adenovirus* species (Allard et al., 2001), which are difficult to isolate and grow. At present, the most sensitive diagnostic tool for direct diagnosis is the nested PCR because it includes two rounds of amplification with two discrete primer sets, which increase drastically the limit of detection (Belak and Thoren, 2001). Virus isolation and *in situ* hybridisation are the most common laboratory method for detection of bovine adenoviruses, but both are time-consuming or in case of virus isolation, require expensive tissue culture system (Rusvai et al., 1992; Kiss et al., 1996).

The aim of the study

The aim of the study was to develop a sensitive and specific real-time PCR diagnostic system for the rapid detection of Adenovirus in cattle.

Conclusion

Amplification of nine out of ten positive BAdV species showed that designed real-time PCR assay was sensitive and specific for the detection of bovine adenoviruses. The predicted size of PCR product also confirmed specificity of the real-time PCR assay. The sensitivity of assay was determined using BAV-8 cloned PCR product and the detection limit was five copies of viral genome equivalent. The efficiency of PCR assay was 97%. Only BAdV-2 serotype was not detected and BAdV-7 has shown low amplification rates, but anyway always detected, due to four mismatches in the probe region. In conclusion, the real-time PCR assay presented here is expected to be a good alternative tool to the traditional diagnostic methods like virus isolation, ELISA or conventional PCR in case of sporadic disease outbreak in which rapid diagnosis is required and when appropriate measures should be taken.

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Research Report

Development of TaqMan real-time PCR assay for the rapid detection of bovine adenovirus species in cattle

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Keywords: Bovine adenovirus, real-time PCR, TaqMan, detection.

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Abstract

The aim of the present work was to develop a sensitive and specific real-time PCR diagnostic system for the rapid detection of bovine adenovirus in cattle. A TaqMan real-time PCR assay capable of simultaneous detection of nine out of ten species of bovine adenovirus belonging to the genera *Atadenovirus* and *Mastadenovirus* was developed. The oligonucleotide primers and probe were selected from conserved sequences flanking the genome region coding for the Hexon protein. DNA from BAdV species 1 to 10 was isolated by standard phenol-chloroform extraction. Standard curve using a ten-fold dilution series of BAdV-8 positive DNA template was generated. The sensitivity of assay was determined using BAdV-8 cloned PCR product and the detection limit was five copies of viral genome equivalents. The efficiency of PCR assay was 97%. Only BAdV-2 serotype was not detected and BAdV-7 has shown low amplification rates and fluorescence, but anyway always detected, due to four mismatches in the probe region. In conclusion, the real-time PCR assay presented here is expected to be a good alternative tool to the traditional diagnostic methods like virus isolation, ELISA or conventional PCR in case of sporadic disease outbreak in which rapid diagnosis is required and when appropriate measures should be taken.

Introduction

The adenoviruses, so named because initial isolations were from human adenoids (Enders et al., 1956), are associated with human adenoconjunctival-pharyngeal fever and other infections. Adenoviruses represent a large family of double-stranded DNA viruses and over 100 distinct mammalian adenoviruses have been isolated. According to the official taxonomy, the family Adenoviridae consists of four genera comprising viruses isolated from birds, mammals, amphibians and reptiles (Fauquet et al., 2005). The bovine adenovirus (BAdV) was first isolated from cattle (Klein, 1959), and later from other animals including sheep, buffalo and fallow deer (Belak et al., 1986; Boyle et al., 1994). At least 10 serotypes (species) of bovine adenovirus are presently recognized (Lehmkuhl et al., 1999) and are designated bovine adenovirus serotypes 1 through 10. These serotypes were further divided into

two subgroups. At present, the former BAdV subgroup I (BAdV-1 to 3, BAdV-9 and BAdV-10 species) belongs to the genus *Mastadenovirus*, and former BAdV subgroup II (BAdV-4 to 8 species) have been assigned to the new genus *Atadenovirus,* together with avian adenovirus and egg drop syndrome virus of fowl. The latest accepted serotype BAdV-10 is distinct from the other BAdV species, but recent studies demonstrate that it has similar genome arrangement characteristics of Mastadenoviruses (Matiz et al., 1998; Dan et al., 2001). Despite being assigned to this genus, BAdV-10 is very different from other members of *Mastadenovirus* and occupies a special place in a certain distance from the other members of this subgroup. (Smyth et al., 1996). For example, the degree of similarity exhibited between the BAdV-10 and BAdV-1, BAdV-2 protease was only 51,5%, BAdV-3 - 56,3%, BAdV-4 - 36,0%, BAdV-7 - 33,0% (Matiz et al., 1998). Probably, BAdV-10 is not a genuine bovine adenovirus, but might originate from another species and is now in an adaptation process to a new host, a cattle.

BAdV infections are ubiquitous, easily transmitted and frequently unapparent: they sometimes cause pneumonia enteritis and conjunctivitis. The delayed diagnosis may contribute to the spread of infections in cattle populations. Many diagnostic tests for BAdV have been developed and are based on *in situ* hybridisation, electron microscopy, fluorescent antibody assays, DNA restriction enzyme analysis, virus isolation, and enzyme–linked immunosorbent assay (ELISA) (Benkö and Harrach, 1990; Isakova et al., 1990; Adair et al., 1996; Smyth et al., 1996; Giusti at al., 1998). Virus isolation is the most common laboratory method for the direct detection of bovine adenovirus, but it is expensive and time consuming. ELISA is sensitive and specific, but it requires use of viral protein specific antibodies for detection (Rusvai et al., 1992). The rapid detection of viral infection is important for providing appropriate measures for controlling disease transmission among herds and treating according. PCR assays provide a highly sensitive and specific tool for investigating the significance of *Adenovirus* species (Allardet et al., 2001). At present, the most sensitive diagnostic tool for direct detection of different BAdV serotypes is PCR method (conventional, nested or real-time) (Kiss et al., 1996; Maluquer de Motes et al., 2004; Poddar, 1999). Although the nested PCR is undoubtedly effective, the practical application is rather complicated and it takes many steps to achieve the results: two rounds of amplification, gel

electrophoresis and photography. The repeated amplification not only delays the results, but also increases the risk of cross-contamination. Moreover, gel electrophoresis and photography in gel-based PCR include handling of ethidium bromide, which with its carcinogenic effects presents hazards to the health of laboratory personal. Real-time PCR is a good alternative detection method that is widely accepted due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry–over contamination. At present, the only real-time PCR for the detection of BAdV-3 based on molecular beacon probe is known (Poddar, 1999). For our knowledge, adenovirus real-time PCR assays developed in the past were directed to the detection mostly of human adenoviruses (Gu et al., 2003; Watanabe et al 2005).

The main aim of the present work was to develop a sensitive and specific real-time PCR diagnostic system for the rapid detection of adenovirus in cattle. The results indicated that real-time PCR could be useful for the rapid detection of BAdV species for tracking the origin and preventing a disease outbreak.

Materials and Methods

Samples. Cell cultures of BAdV species 1 to 10 were obtained from Veterinary Science Division, Stormont (Belfast, UK). Twenty-four random faecal samples were collected from calves with respiratory and/or diarrhoea signs from different farms in Sweden.

Isolation of DNA. DNA of cell culture samples was isolated by standard phenol-chloroform extraction. Briefly, 166 µl of BAdV cell culture was mixed with 33 µl of 6X Proteinase K buffer, 5µl of Proteinase K (Roche, Germany) and incubated at 56°C for one hour. DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), cleaned with 200 µl of chloroform and precipitated with two volumes of 95% ethanol in the presence of 0.3M sodium acetate (pH 5.2) overnight at -20°C. The precipitated DNA was centrifuged at 13500 X g for 30 min and the pellet was recovered in 50 µl of sterile water and stored at -20°C until further analysis.

DNA of faecal samples was isolated with Genovision M48 extraction robot and MagAttract Virus Mini Kit protocol (Qiagen) according manufacturer's instructions. The nucleic acid from each sample was eluted in 50 µl of elution buffer and stored at -20°C until use.

Primers and TaqMan® probe design. The oligonucleotide primers and probe against BAdV were designed from the published sequence of the Hexon gene (GenBank accession No. AF207658), collected from GenBank by NCBI Blast. Different BAdV species were aligned with Lasergene software (DNASTAR, Inc., version 5, Madison, WI, USA). Primers and probes for real-time PCR were selected with Primer Express™ software (version 1.0, Applied Biosystems, Foster City, CA, USA). The sequences of the primers were (5'-3'): BAdV-Hex-33F, AAGGGTCAAACATCTGCTTAACT (23 bp, forward), and BAdV-Hex-138R, TCTGCCTGWGGGAAAAAAGCTTAGAT (26 bp, reverse). The primers were ordered and synthesized at CyberGene AB (Huddinge, Sweden). The TaqMan® probe, termed BAdV-HEX-65-TaqMan, had the following sequence (5'-3'): AGAGCCTGCATTRTCACAATACCACC (26 bp). The probe was labelled with a TET fluorophore at the 5' end and with nonfluorescent Black Hole Quencher Dye at the 3' end (Biosearch Technologies, Novato, CA, USA).

Procedure and efficiency. Real-time PCR was carried out on a RotorGene3000 instrument (Corbett Research, Australia) and optimised by a titration series of primers, probe and magnesium ion concentration. The assay was in a total volume of 25 µl with 0.2 ml optical tubes (Corbett Research, Australia). Each 25 µl reaction mixture contained 2 µl of extracted DNA in sterile water, 0.9 µM of each BAdV primer, 0.4 µM of probe, 0.5 mM 4X dNTPs, 3.0 mM $MgCl_2$, 2.5 U/µl of TaqGold DNA polymerase (Applied Biosystems), 10X PCR Gold buffer and 2µl of 1 mg/ml of BSA. The final volume was adjusted to 25 µl with sterile water. The thermodynamic profile was 95°C for 10 min (initial denaturation) followed by 50 cycles of amplification: 95°C for 30 sec; 55°C for 30 sec (data collected); and 72°C for 45 sec.

Cloning, transformation, sequencing: PCR products of nine BAdV serotypes were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), cloned into the TA cloning

vector pCR®II-TOPO® (Invitrogen, Carlsbad, CA, USA) and transformed into E. coli INVαF´ cells. Recombinant plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen) and subsequently sequenced on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

Detection limit and analytical specificity: The plasmids with of BAdV-3, 7, 8 and 10 inserts were used in ten-fold dilution series experiment to determine detection limit of the assay. Concentration of the plasmids with insert was determined with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The number of copies in the stock solution was calculated using the molarity of the template and Avogadro's formulae. Ten-fold dilutions series from 10^9 to 1 copy were prepared in TE buffer (10 mM Tris-HCl, pH 8.0, 1mM EDTA) containing 10 µg/ml salmon sperm DNA (Ambion, Austin, TX, USA) to assure a constant amount of nucleic acids in the diluted samples. Dilution containing five copies of BAdV with insert was included in the sensitivity test as well. A standard curve was generated and PCR efficiency was calculated using RotorGene3000 instrument integrated software. All tests were performed in triplicate.

Specificity was tested using ten BAdV serotypes obtained from Veterinary Science Division, Stormont (Belfast, UK). Heterologous virus strains such as BCoV, BRSV, BVDV, and human RSV were included as well.

Results

Real-time PCR assay after optimisation indicated successful amplification of nine out of ten BAdV serotypes used. The PCR products were analysed by 2% agarose gel electrophoresis to confirm the predicted product size, which was, as expected, 106 bases. BAdV-7 serotype had low level of fluorescence and amplification rates. Sequencing of PCR products revealed four mismatches in the probe region (Fig.1). Nevertheless, BAdV-7 PCR product had only one specific band of correct size when tested by agarose gel electrophoresis (not shown).

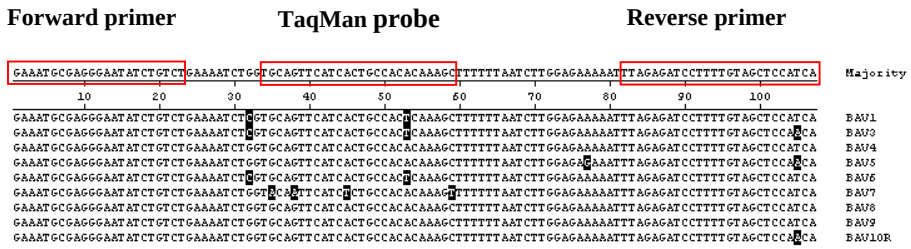

Figure 1. Conservative region and nucleotide mismatches at primer/probe sites of nine BAdV species used in the study. Frames show position of the primers and the probe on the genome sequence (Hexon gene). BAdV-7 strain has four mismatches in the probe region.

Unexpectedly, BAdV-4 showed delayed amplification with Ct value 34 (Fig.2A). The band of correct size and an extra unspecific weak band were detected when PCR amplified product were analysed by agarose gel electrophoresis and ethidium staining (Fig.2B).

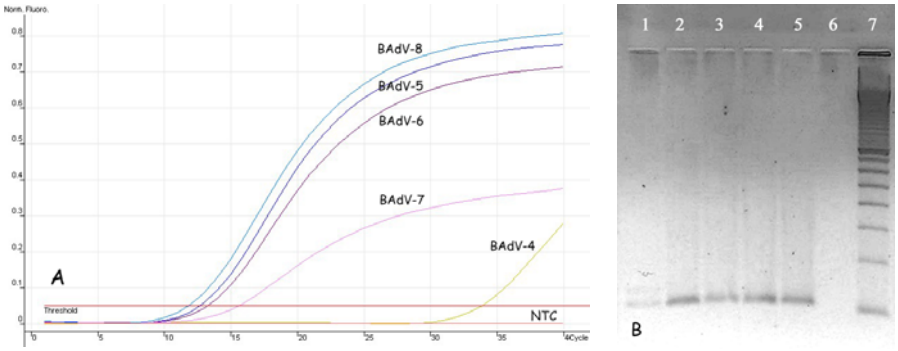

Figure 2. A. Amplification of all five BAdV species of genus *Atadenovirus*. NTC is a negative control (water) that stays below the threshold. **B.** The products of real-time PCR as shown by gel electrophoresis. Numbers 1-5 correspond to BAdV species 4-8; number 6 is negative control, number 7 is 100-base size marker. Predicted PCR product size is 106 bp.

BAdV TaqMan real-time PCR assay was tested on species of genus *Mastadenovirus* (BAdV-1, 2, 3, 9 and 10). All DNA, except BAdV-2, was amplified (Fig. 3). Predicted PCR product size was 106 bp (not shown).

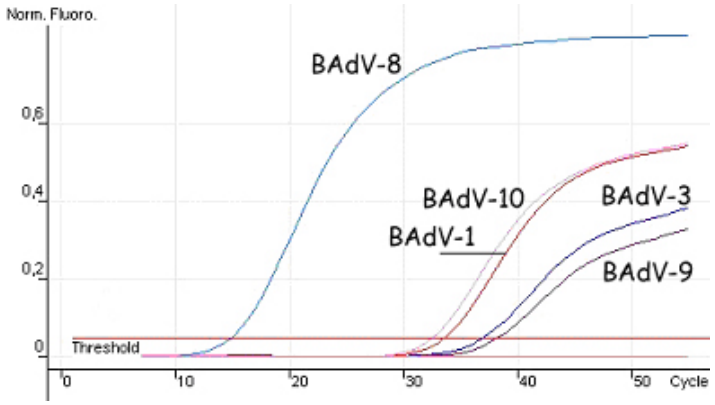

Figure 3. PCR amplification of 4 out of 5 species of genus *Mastadenovirus*. Only BAdV-2 was negative. BAdV-8 used as a PCR positive control.

The analytical sensitivity (detection limit) of TaqMan real-time PCR assay was tested in triplicate with ten-fold dilution series of BAdV-3, 8 and 10 with a range from 10^{-9} down to five copy of synthetic template. Dilutions down to one and 0,1 copy of viral genome equivalents were also prepared and tested in separate experiments, but showed negative results. Thus these dilutions were not included in the main experiments. Five copies of viral genome equivalents were included instead. Three strains listed above belong to two BAdV genera and have different number of mutations in the probe and/or reverse primer region (Fig. 1). Even though, the detection limit for BAdV-3, 8 and 10 was 5 copies of viral genome equivalents. Only results with BAdV-8 decimal dilution series are shown in the manuscript (Fig.4).

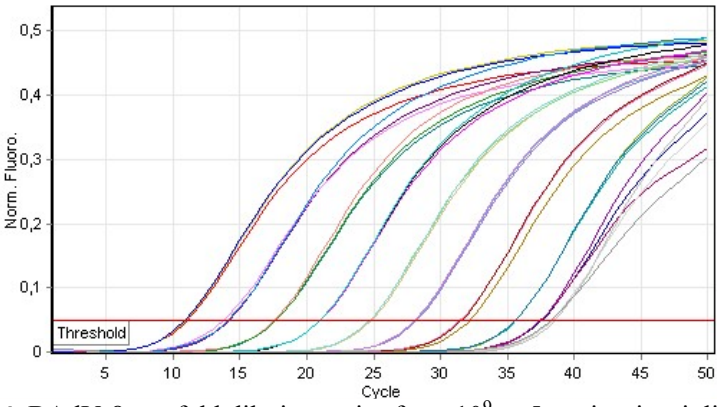

Figure 4. BAdV-8 ten-fold dilution series from 10^{9} to 5 copies, in triplicate. Detection limit is 5 viral genome equivalents.

A standard curve was designed after each dilution series experiment and the reaction efficiency (E) with correlation coefficient (R^2) calculated by integrated RotorGene3000 software were following: BAdV-3 (E =1,01; R^2 = 0,994; not shown), BAdV-8 (E =0,97; R^2 = 0,997; Fig.5); BAdV-10 (E =0,92; R^2 = 0,997; not shown).

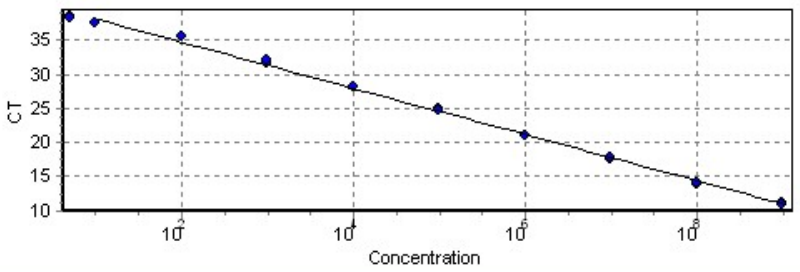

Figure 5. A standard curve based on 10-fold dilution series (from 10^9 to 5 copies, in triplicate) of BAdV-8 synthetic amplicon. Detection limit is 5 viral copies of genome equivalents.

In the specificity test nine out of ten BAdV serotypes were detected, while BCoV, BRSV, BVDV and human RSV were negative. PCR products were tested by a 2% agarose gel electrophoresis to confirm predicted product size, which was 106 bp, as expected (results not shown).

BAdV TaqMan real-time PCR assay was tested on random clinical samples (faeces) from calves obtained from outbreaks of respiratory and/or diarrhoea disease in different Swedish farms. In total, 24 clinical samples were tested among which 11 samples showed positive amplification for BAdV (results not shown). Earlier, 12 faecal samples from calves with respiratory symptoms were tested positive by BCoV antigen ELISA (Madeleine Tråven, personal communication). Six out of the same 12 samples showed positive amplification by BAdV real-time PCR (Fig. 6a). Predicted PCR products had correct size when tested by 2% agarose gel electrophoresis (Fig. 6b).

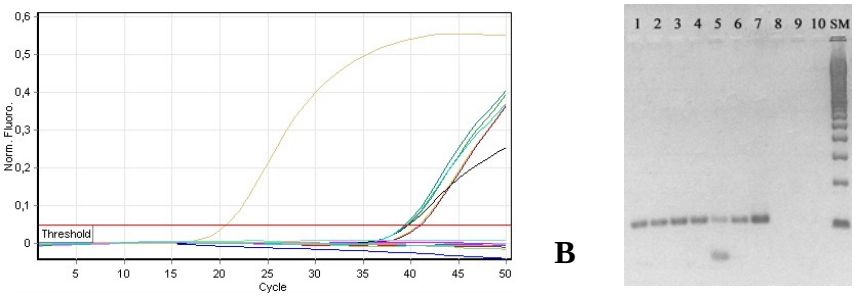

Figure 6. A. Six out of 12 faecal samples that were positive for BCoV antigen ELISA, were positive for BAdV, as well. Single amplification plot with Ct = 20,7 is a PCR positive control (BAdV-8, diluted 1:100). Lines below the threshold are three negative controls and 6 BAdV negative samples; **B.** 2% agarose gel electrophoresis of six BAdV positive samples (lines 1-6), positive control (line 7), negative control (line 8, water) and two random BAdV negative samples (lines 9-10). SM is a 100-base size marker. Amplicon size is 106 bp.

Discussion

Electron-microscopy and immunofluorescence are rapid diagnostic techniques for detection bovine adenovirus, but their sensitivity is not always satisfactory (Burki, 1990; Belak, 1990). Real-time PCR is faster than conventional PCR, as results from the former are available in approximately 3 h, compared to 5 h or more for the conventional PCR, which is the time required to run separate PCR and to visualize the PCR product by electrophoresis. PCR assay would be the best method the detection of adenoviruses, which are difficult to isolate and grow (Kiss et al., 1996).

It is important to stress that the TaqMan real-time PCR assay described in the paper was initially designed only against BAdV *Atadenovirus* species 4-8. Alignments of nucleotide sequences available from the NCBI database showed big variation between two genera - *Mastadenovisus* (BAdV species 1-3, 9 and 10) and *Atadenovirus* (BAdV species 4-8). Our first intension was design of two real-time PCR assays that allow detection of all 10 species of BAdV. However, the TaqMan assay described above worked well, after some optimisation steps, when tested with BAdV *Mastadenovisus* species 1, 3, 9 and 10, for exception BAdV-2. Amplification of 9 out of 10 BAdV serotypes showed that primers and probe initially designed to detect only the members of genus *Atadenovirus,* were specific for the members of genus

Mastadenovirus species, as well. The predicted size of PCR product also confirmed specificity of the real-time PCR assay.

In the future, a new primer set for the detection of BAdV-2 serotype will be designed. Alternatively, existing primers can be modified so that they could detect the last missing BAdV serotype. Unexpected results were obtained with BAdV-4 amplification. According to the alignment data obtained from the GenBank database (NCBI), BAdV-4 serotype should have only one mismatch at the 5' end in reverse primer region, which should not strongly affect on amplification, as the 3'-terminal sequence of the primer molecule is critical for the specificity and sensitivity of PCR. More over, sequencing of amplified BAdV-4 PCR product did not reveal any mismatches in the primer and probe region that could affect the amplification (see Fig. 1). Most probably, the low level of fluorescence could be a result of poor quality of extracted DNA. Similar, but expected results were observed during BAdV-7 serotype amplification, but it has four mismatches in the probe region, most of which are located close to the 5' end.

The present study suggests that TaqMan real-time PCR assay can sensitively detect BAdV DNA in clinical samples. Twenty-four bovine faecal samples tested gave additional evidence that the assay has good performance when applied for the detection of BAdV during outbreaks.

In conclusion, the real-time PCR assay presented here is expected to be a good alternative tool to the traditional diagnostic methods like virus isolation, ELISA or conventional PCR in case of sporadic disease outbreak in which rapid diagnosis is required and when appropriate measures should be taken.

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