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Development of a qPCR assay for the quantification of porcine adenoviruses as an MST tool for swine fecal contamination in the environment

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A B S T R A C T

The *Adenoviridae* family comprises a wide diversity of viruses that may be excreted for long periods in feces or urine. Previous studies have suggested that the detection of human and animal adenoviruses as well as human and animal polyomaviruses by PCR could be used as an index of fecal contamination of human and animal origin. In this study, quantitative PCR assays targeting specifically porcine adenoviruses have been developed and applied to fecal and environmental samples, including pig slurries, urban sewage, slaughterhouse sewage and river water samples. To develop real-time quantitative PCR for the detection and quantitation of porcine adenoviruses, primers and a TaqMan probe targeting a 68-bp region of the porcine adenovirus hexon gene were designed to amplify specifically porcine adenovirus, and the conditions of the reaction were optimized. The assay detected 1–10 genome copies per test tube and was specific in showing no positive results when samples containing human or bovine adenoviruses were analyzed. Fecal samples contained mean concentrations of porcine adenoviruses of 10^5 GC/g while slaughterhouse wastewater samples showed mean values of 10^3 GC/ml. The assay detected porcine fecal pollution in samples that were highly diluted and had been collected at a considerable distance from the input source, such as river water. In general, the data presented here provide a quantitative tool for the analysis of porcine adenoviruses as indicators of the presence of porcine contamination in the environment, and support the detection of porcine adenoviruses by real-time quantitative PCR as a promising and valuable tool for source-tracking studies.

1. Introduction

Microbial contamination of the environment poses a significant risk to human health through recreational exposure or consumption of water or contaminated food. According to the requirements of the Water Framework Directive and the United States Clean Water Act there has been a major shift from traditional point-source effluent quality regulations towards a catchment-wide approach (Stapleton et al., 2007). Given this new approach there is a need for new information on the microbial dynamics of catchments for the effective control of water quality at the point of use. Bacterial indicators including *Escherichia coli* and fecal coliform bacteria have been used historically to monitor water quality and safety. However, their major shortcoming is that they are often not correlated with viral pathogens or protozoan parasites (Gerba et al., 1979; Griffin et al., 2003; Pina et al., 1998). Fecal contamination may originate from point or nonpoint sources. Generally, point sources of fecal contamination include discrete sources such as discharges from large animal feeding operations, wastewater treatment outfalls and storms and combined sewers. The nonpoint sources are diffuse and include agricultural sources and livestock waste applied to agricultural land. Identifying the sources of microbial contamination plays a very important role in enabling effective management and remediation strategies and is known as microbial source tracking (MST). MST includes a group of methodologies that aim to identify, and in some cases quantify, the dominant sources of fecal contamination in the environment and, more specifically, in water resources (Field, 2004; Stoeckel and Harwood, 2007). Environmental and water contamination by the waste produced in pig farms is a source of microbiological and chemical contamination. The hepatitis E virus is a human viral pathogen that causes acute hepatitis and is highly prevalent in young pigs and present frequently in fecal samples, as well as in waste water and sludge produced in slaughterhouses dealing with pigs (Clemente-Casares et al., 2003; Pina et al., 1998). The identification of fecal contamination of porcine origin is important to pinpoint the source of contamination for remediation purposes and to evaluate the potential public health impact of fecal contamination in water.

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Molecular methods based on polymerase chain reaction (PCR) techniques for specific viruses such as human adenoviruses (HAdV) and polyomaviruses (JCPyV and BKPyV), porcine adenoviruses (PAdV) and bovine polyomaviruses (BPyV) (Bofill-Mas et al., 2000, 2006; Formiga-Cruz et al., 2003; Hundesa et al., 2006; Pina et al., 1998; Puig et al., 1994), bovine enterovirus and teschoviruses (Jimenez-Clavero et al., 2003, 2005) have been proposed in previous studies for detecting markers of contamination of human or animal origin in water and in shellfish. The high stability of viruses in the environment, host-specificity and the high prevalence of some viral infections over the year in the population support strongly the use of real-time quantitative PCR techniques (qPCR) for the identification and quantitation of specific viruses that can be used as source tracking tools. Quantitation of DNA viruses excreted persistently throughout the year would allow the development of cost-effective protocols with more accurate quantitation of contaminating sources compared to RNA viruses; this is due to the greater accuracy of qPCR quantitation and lower sensitivity to inhibitors, as reverse transcriptase is not used when amplifying DNA viruses.

PCR and nested-PCR (nPCR) protocols are used typically because of their high sensitivity but they are not quantitative and are subject to potential DNA cross-contamination which is solved normally by sequencing the PCR products. Real-time qPCR has emerged as a valuable technique because it reduces successfully the chances of laboratory contamination and time-consuming manipulations, and enables sensitive and rapid quantitation of small amounts of target DNA in biological and environmental samples. However, the fact that the application of genome amplification techniques does not provide information on the infectious capabilities of the viruses detected must be also considered.

The *Adenoviridae* family is the only known group of enteric viruses composed of double-stranded DNA genomes. This advantage is a result of the sequence conservation and quantitation efficiencies of DNA viruses. Human adenoviruses (HAdVs) are highly prevalent in sewage as well as in river water and shellfish with fecal contamination (Fong et al., 2005; Fong and Lipp, 2005; Formiga-Cruz et al., 2003) and are distributed conservatively in diverse geographical areas (Bofill-Mas et al., 2000). Adenoviruses are more stable than enteroviruses to UV irradiation and chlorination (Gerba et al., 2002; Thurston-Enriquez et al., 2003). As a specific group within the family, porcine adenoviruses (PAdVs) belong to the genus *Mastadenovirus*, are divided into six serotypes and have been reported to be abundant and highly prevalent in feces, slaughterhouse wastewaters and sludge (Hundesa et al., 2006; Maluquer de Motes et al., 2004). PAdVs are disseminated widely in the swine population but do not produce clinically severe diseases (Ishibashi and Yasue, 1984). These data underlie the interest in evaluating PAdVs as potential viral indicators of porcine fecal contamination. The purpose of the present study was to develop a qPCR assay with a specific primer/probe set for the quantitation of PAdVs in excreta, pig slurries and different types of waters. The data show clearly the applicability of PAdVs for the identification and quantitation of porcine fecal contamination in the environment, and demonstrate their validity as a potential tool for microbial source tracking.

2. Material and methods

2.1. Primer/probe set design for qPCR assay for PAdV

In order to optimize the specificity and sensitivity the primer/probe set was designed to amplify a small amplicon. Concentration of both primers and probe was optimized by assaying primer concentrations ranging from 0.4 to 0.9 μM and probe concentrations ranging from 0.225 to 0.9 μM for each reaction. Annealing temperatures were also optimized.

Table 1
Sequences and location of the primers/probe used in the PAdV qPCR assay.

Primers and probe set	Sequence and location[a]
Forward primer (Q-PAdV-F)	(20701) AACGGCCGCTACTGCAAG (20718)
Probe (Q-PAdV-P)	(20722) CACATCCAGGTGCCGC (20737)
Reverse primer (Q-PAdV-R)	(20768) AGCAGCAGGCTCTTGAGG (20751)

[a] Location of oligonucleotides refers to PAdV3 (GenBank accession number AJ237815).

The qPCR procedure is based on a TaqMan assay and uses two primers and a fluorogenic probe that recognizes a 68 bp fragment of the hexon gene of the PAdV genome. Primers and probe were designed specifically for the amplification of PAdV. PAdV DNA sequences from GenBank were aligned using the ClustalW Program (European Bioinformatics Institute, UK) and a 68 bp conserved fragment was selected. Using Primer Express software (Applied Biosystems) primers and probes were designed initially, however in order to obtain specific assays, the final selection of primers and probe was based on the study of aligned available sequences and the homology of all oligonucleotides was verified finally using Blast Search.

The probe was tagged with FAM (6-carboxyfluorescein) as the reporter dye at the 5′ end and BHQ-1 (Black-Hole Quencher 1) as the quenching dye at the 3′ end. The sequences of the primer/probe set and their location in the PAdV genome are given in Table 1.

2.2. Construction of qPCR standards for PAdV

Standard curves were generated by transforming *E. coli* JM109 cells (Promega, Madison, WI, USA) with a pGEM-T Easy plasmid (Promega) containing a 612 bp sequence of the PAdV-3 hexon. The transformation was carried out following the manufacturer's instructions. Colonies carrying the desired plasmid were checked by conventional PCR using vector primers to contain the target DNA, which was obtained with the QIAGEN Plasmid Midi kit (QIAGEN, Inc.) following the manufacturer's instructions. Once obtained and quantified using Genequant Pro (Amersham Biosciences), according to the molecular weight of the plasmid, approximately 10 μg of DNA was linearized with EcoRI, purified with the QUIAquick PCR purification kit (QIAGEN, Inc.) and then quantified again, before obtaining serial dilutions of 10^{-1}–10^{9} viral DNA molecules per 10 μl in TE buffer. The standard dilutions were then aliquoted and stored at −80 °C until use.

2.3. Nucleic acid extraction

In order to prevent inhibition in the PCR assays and generation of false-negative results, two different nucleic acid (NA) extraction methods were evaluated. Nucleic acids from viral concentrates were extracted using a procedure chosen in previous studies for its high sensitivity and efficiency (Puig et al., 1994). Basically, it is based on the method of Boom et al. (1990) and uses guanidinium isothiocyanate to denature viral capsids and silica particles to bind viral nucleic acids until their final elution in TE Buffer (Tris 10 mM, EDTA 0.1 mM, pH 7.4) and subsequent storage at −80 °C. The nucleic acid extraction kit NucliSens® (Biomérieux), based on the same principles and containing silica-covered magnetic beads, was also used in accordance with the manufacturer's instructions.

2.4. PAdV qPCR assay

For all experiments the PCR mix was prepared and dispensed in a working area dedicated to PCR reagent preparation; the samples were loaded in a pre-PCR laboratory and, finally, the plate was transferred to a separate laboratory for the addition of the qPCR