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Decription of a novel viral tool to identify and quantify ovine faecal pollution in the environment

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HIGHLIGHTS

- A new ovine specific polyomavirus (OpyV) was identified.
- A qPCR specific and sensitive assay for the quantification of OPyV was developed.
- The new ovine marker was detected in environmental samples from different European regions.

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ABSTRACT

Farmed animals such as sheep, cattle, swine and poultry play an important role in microbial contamination of water, crops and food, and introduce large quantities of pathogens into the environment. The ability to determine the origin of faecal pollution in water resources is essential when establishing a robust and efficient water management system. Animal-specific viruses have previously been suggested as microbial source tracking tools, but specific ovine viral markers have not been reported before now. Previous studies have shown that polyomaviruses are host-specific, highly prevalent and are commonly excreted in urine. Furthermore, they have been reported to infect several vertebrate species but not sheep. That situation encouraged the study of a new putative ovine polyomavirus (OPyV) and its use to determine whether faecal pollution originates from ovine faecal/urine contamination.

Putative OPyV DNA was amplified from ovine urine and faecal samples using a broad-spectrum nested PCR (nPCR). Specific nested PCR and quantitative PCR assays were developed and applied to faecal and environmental samples, including sheep slurries, slaughterhouse wastewater effluents, urban sewage and river water samples. Successful amplification by PCR was achieved in sheep urine samples, sheep slaughterhouse wastewater and downstream sewage effluents. The assay was specific and was negative in samples of human, bovine, goat, swine and chicken origin. Ovine faecal pollution was detected in river water samples by applying the designed methods. These results provide a quantitative tool for the analysis of OPyV as a suitable viral indicator of sheep faecal contamination that may be present in the environment.

1. Introduction

Most environmental waters, soil crops and foods are susceptible to faecal contamination of human or animal origin, representing significant impacts on human/animal health and environmental management. The ability to determine whether microbial indicators or pathogens present in the environment originate from human or animal sources would enable better management of water pollution problems. Human faeces are more likely to contain human-specific enteric pathogens, but animals can also serve as reservoirs of standard bacterial faecal indicators and human pathogens. Thus, faecal contamination source tracking is essential to ensure its elimination, to minimise its impact or to identify uncontrolled spills (Scott et al., 2002). Current environmental microbial quality assessment is based on combining rapid screening methods and detailed source tracking techniques (Roslev and Bukh, 2011). The most commonly used microbial source tracking (MST) tools are bacteria such as coliforms, coliphages such as F-RNA phages, *Bacteroides* spp., *Rhodococcus coprophilus* or bifidobacteria, phages

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such as *Bacteroides fragilis*, and more recently, human and animal viruses such as human adenoviruses (HAdV) and polyomaviruses (JCPyV), porcine adenovirus (PAdV), bovine polyomavirus (BPyV) and chicken/turkey parvovirus (Ch/TuPV) (Carratalà et al., 2012; Hundesa et al., 2006; Fong et al., 2005; Maluquer de Motes et al., 2004; Bofill-Mas et al., 2000; Leclerc et al., 2000; Pina et al., 1998).

Viruses are excreted in high concentrations not only by infected human or animal individuals but also by healthy populations (Girones and Bofill-Mas, 2012). HAdV and JCPyV have been reported to be widely disseminated and persistent throughout the year (Bofill-Mas et al., 2000). Their high stability, host-specificity and high prevalence in different geographical areas support the use of HAdV and JCPyV for the identification and quantification of faecal contamination in the environment (Bofill-Mas et al., 2011a,b; Girones et al., 2010; Albinana-Gimenez et al., 2009).

Livestock contribute large quantities of faecal pollution in the environment. There is a need to ensure proper management of livestock manure, and of all animal-based organic fertilisers in general, in order to avoid contamination of ground water or water streams that may later be used as drinking water sources or for crop irrigation (Regulation (EC) no, 1774/2002). In fact, recent studies have related the presence of high levels of nitrates in groundwater with the presence of pig slurries in the surroundings of the sampled wells (Bofill-Mas et al., 2011a,b). Despite the widespread perception that human faecal pollution presents greater risks than animal pollution, in Catalonia alone (7.5 million people), over half the land area is used for livestock farming, with approximately sixteen million swine, half a million cattle, about one hundred and sixty million fowl and nearly two million sheep (Health Protection Agency of the Catalonian Government, 2010).

Among the potential sources of animal viral pathogens, cattle, swine, poultry and ovine slurries, slaughterhouses or faeces/urine deposited on grazing land may represent the main sources of animal faeces in the environment. *Campylobacter* spp. *Brucella melitensis*, and *Listeria monocytogenes* are some of the suspected pathogens having zoonotic infection routes from sheep to humans (Gilpin et al., 2008; Ramos et al., 2008; Czuprynski, 2005).

Depending on the soil structure, viral indicators can be rapidly transported below the surface and leach from the soil following rainfall (Aislabie et al., 2011). Viral indicators have been proposed and described in previous studies as tools to survey animal faecal contamination in water and food. PAdV, BPyV and Ch/TuPV have been proposed as specific faecal markers of swine, bovine and poultry populations, respectively (Carratalà et al., 2012; Hundesa et al., 2010; Hundesa et al., 2009). However, no specific ovine viral faecal indicator has been described until now.

Several studies have reported potential ovine faecal indicators, including adenovirus (OAdV), noroviruses (NoV GIII), F + RNA bacteriophages or *Cryptosporidium* (Wolf et al., 2010; Chalmers et al., 2002), but the described assays have been reported to be prone to detecting bovine faecal pollution. Recent studies have suggested that each vertebrate species could host its own set of PyV (Orba et al., 2011; Wellehan et al., 2011), so we employed a nested broad-spectrum PCR (Johne et al., 2005) that uses degenerated primer pairs to screen sheep urine samples from five different domestic sheep breeds. Once we detected polyomavirus-related sequences, we designed a specific nested PCR (nPCR) and a quantitative PCR assay (qPCR) for the specific detection and quantification of a new putative ovine polyomavirus (OPyV) in the environment.

2. Material and methods

2.1. Faecal, urine and water samples analysed

A total of thirty-two ovine urine samples together with fifty straw-bed and eighteen sheep stool pooled samples were collected from farm soil in nine different ovine farming areas in three different countries over a two-year period (2010–2011). Sheep urine samples were collected directly from individuals, and wet straw-beds and wet stools (pooled samples) were collected from the soils of sheep farms in nine different locations: Catalonia (six farms in the north east of Spain), the Basque country (one farm in the north of Spain), Szolnok (one farm in central Hungary) and Patras (one farm in the south of Greece). The mean herd size was 40 animals, but herd sizes ranged from 10 to 200 animals. Samples (Table 1) were collected from breeding and fattening animals at different ages. Numbering over one billion animals, domestic sheep (*Ovis aries*) are the most numerous species of sheep in the world. For this study, different breeds were selected, located in different geographical areas. *Ripollesa* and *Xisqueta* are autochthonous breeds located in the north of Catalonia (Spain). These breeds originated by crossing native sheep from the central Pyrenees with merino-type individuals. *Latxa* is a breed of domestic sheep native to the Basque Country of Spain. *Merino* is the main breed in Hungary and *Serraika* and *Boutsko* are the native breeds of Greece.

Raw and effluent wastewater samples and biosolids were collected during summer 2010 from a slaughterhouse and from a downstream sewage treatment plant (STP) which processes 100,000 inhabitant equivalents. During the same time period, raw sewage samples were collected from a hospital with more than 1000 beds located in Catalonia, on the assumption that these samples would not contain any animal effluents.

River water samples were collected from two river basins: the Llobregat River in Catalonia and the Glafkos River in Greece. The Llobregat River crosses Catalonia from the Pyrenees to the Mediterranean Sea and receives several STP effluents along its course. In the case of the Glafkos River, large sheep herds live outdoors beside the river and may pollute the natural waterway.

Cow urine, pooled porcine faeces, pooled chicken faeces, raw wastewater from bovine, swine and avian slaughterhouses which do not sacrifice sheep and raw sewage from a STP with no ovine-related effluents were collected to perform the PCR specificity assays. A set of sixteen faecal samples from domestic goats (*Capra aegagrus*) was collected directly from individuals and six wet straw-beds from goat farms (pooled samples) were collected from four different locations in Catalonia and Greece (Table 2). All urine, stool, sewage, effluent and biosolid samples were kept on ice and processed within 24 h.

2.2. Sample processing and DNA extraction

Viruses were concentrated from 14 ml of bovine, goat and sheep urine samples by ultracentrifugation (110,000 ×*g* for 1 h at 4 °C).

Table 1
Detection and quantification of ovine polyomavirus in urine/wet-straw-beds/stool and environmental samples by nPCR and qPCR at the VP1 region.

Sample	Positive/tested samples (mean values in genome copies/l or g)	
	Ovine VP1 nPCR	Ovine VP1 qPCR
Sheep urine	9/13	5/5 ($1{,}56 \times 10^2$)[a]
Sheep wet straw-beds	21/42	5/5 ($1{,}34 \times 10^2$)[a]
Sheep stool	7/13	5/5 ($7{,}60 \times 10^1$)[a]
Ovine slaughterhouse raw sewage	3/4	3/4 ($9{,}81 \times 10^2$)
Ovine slaughterhouse treated effluent	1/2	1/2 ($4{,}85 \times 10^1$)
STP influent (downstream ovine slaughterhouses)	3/5	3/5 ($6{,}51 \times 10^1$)
STP effluent (downstream ovine slaughterhouses)	2/5	2/5 ($6{,}06 \times 10^0$)
STP biosolids (downstream ovine slaughterhouses)	1/1	1/1 ($2{,}36 \times 10^1$)
Llobregat river water (Catalonia)	NT	1/8 ($1{,}05 \times 10^2$)
Glafkos river water (Greece)	5/8	3/4 ($1{,}14 \times 10^1$)

NT: not tested.
[a] All quantified samples were previously positive for the specific nPCR.

Table 2
Specificity of the ovine polyomavirus nPCR and qPCR assays. Positive samples vs tested samples.

Sample origin	Sample type	N	Ovine VP1 nPCR	Ovine VP1 qPCR	Other polyomaviruses (genome copies/ml)
Bovine	Individual urine samples	5	0/5	0/5	BPyV1 5/5 (8.34 × 101)
	Slaughterhouse sewage	4	0/4	0/4	BPyV1 5/5 (7.75 × 102)
Chicken	Pooled faeces representing less than 5 individuals	5	0/5	0/5	
	Slaughterhouse sewage	2	0/2	0/2	
Goat	Pooled faeces representing less than 5 individuals	6	0/6	0/6	
	Individual urine samples	10	0/10	0/10	
	Wet straw-beds	6	0/6	0/6	
Human	Hospital raw sewage	10	0/10	0/10	JCPyV2 10/10 (1.61 × 102)
	Urban sewage without ovine slaughterhouse effluents	4	NT	0/4	JCPyV2 4/4 (3.33 × 103)
Porcine	Pooled faeces representing less than 5 individuals	5	0/5	0/5	
	Slaughterhouse sewage	4	0/4	0/4	

NT: not tested. 1 bovine polyomavirus, 2 human JC polyomavirus.

The pellet was resuspended in 100 μl of PBS, and stored at −80 °C. Forty grammes of wet straw-bed samples was concentrated by elution in 20 ml of 0.25 N glycine buffer (pH 9.5), kept on ice and shaken for 30 min before centrifugation (9200 ×*g* for 15 min). Lastly, the supernatant was concentrated by ultracentrifugation (110,000 ×*g* for 1 h at 4 °C) and viral particles were resuspended in 100 μl of PBS and stored at −80 °C.

Viral particles from faeces were concentrated as previously described (Maluquer de Motes et al., 2004). Briefly, 1 g of each sheep, goat, swine or chicken sample was eluted in 3.5 ml of 0.25 N glycine buffer (pH 9.5), kept on ice for 30 min and then centrifuged (9200 ×*g* for 15 min). Finally, the supernatants were concentrated by ultracentrifugation (110,000 ×*g* for 1 h at 4 °C) and viral particles were resuspended in 140 μl of PBS and stored at −80 °C.

Sewage and effluent samples were concentrated as previously detailed (Pina et al., 1998). Briefly, 42 ml of wastewater was subjected to ultracentrifugation at 110,000 ×*g* for 1 h at 4 °C to pellet all the viral particles with other suspended solid materials. The pellet was eluted with 4 ml of glycine 0.25 N at pH 9.5 and shaken for 20 min at 4 °C. Suspended solids were separated by centrifugation at 12,000 ×*g* for 15 min. Finally, viruses were concentrated by ultracentrifugation at 110,000 ×*g* for 1 h at 4 °C and resuspended in 140 μl of phosphate-buffered saline and stored at −80 °C until nucleic acid extraction was performed.

River water samples were concentrated as recently described (Calgua et al., 2013). Briefly, 10 l samples were carefully acidified to pH 3.5 using HCl 1 N and conductivity was adjusted to 1.5 mS. Then, a pre-flocculated skimmed milk solution was prepared and added to each of the previously conditioned samples to obtain a final concentration of skimmed milk in the sample of 0.01%. Samples were stirred for 8 h at RT and flocks allowed to sediment by gravity for 8 h. Supernatants were carefully removed and the final volume, about 500 ml, containing the sediment, was centrifuged at 7000 ×*g* for 30 min at 12 °C. Pellets were dissolved with 10 ml phosphate buffer at pH 7.5 and stored at −80 °C until nucleic acid extraction was performed.

Viral DNA was extracted from all samples using the QIAmp Viral RNA kit (Qiagen, Inc., Valencia, CA) with the QIAcube automated platform. Positive and negative controls were included in all the nucleic acid extraction procedures. Finally, NA eluates were stored at −20 °C until used.

2.3. Broad spectrum PCR

In order to identify a new polyomavirus infecting ovine, we screened seventy-four sheep samples using a broad-spectrum PCR assay targeted specifically to the VP1 gene (Johne et al., 2005). DNA samples obtained from thirty-two urine and thirty-two wet straw-bed samples and from ten faecal pools were analysed. Putative PyV-like sequences of approximately 240 bp in the VP1-encoding region were detected. PCR fragments were purified using a QIAquick purification kit (QIAGEN, Inc.) and the purified amplicons were directly sequenced using the ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems).

2.4. Specific nested-PCR amplification

A set of specific PCR primers was designed and tested in duplicate in urine and wet straw-bed and stool samples (Table 3). Amplification reactions were established in a volume of 50 μl, containing 1× Gold buffer at 50 mM, $MgCl_2$ 25 mM, 25 mM of each deoxynucleotide, primers at 0.25 μM, 1 U of the AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.) with 10 μl in the direct or 1 μl in the 1-fold dilution of the extracted DNA. The first denaturation cycle was performed for 10 min at 95 °C. The conditions for the 35-cycle amplification were as follows: denaturing at 95 °C for 1 min, annealing at the corresponding annealing temperature (Table 3) for 1 min and extension at 72 °C for 1 min. Amplifications were followed by a final 10-min incubation at 72 °C. PCR products were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide.

2.5. Quantitative-PCR (qPCR) primer and probe set design

Sequences obtained applying the nPCR described above were aligned using the ClustalX2 programme (European Bioinformatics Institute, UK) in order to choose the most conservative region and design a specific primer and probe set to perform a qPCR assay. Five urine, five wet straw-bed and five stool samples which had previously

Table 3
Oligonucleotide primers and probe used for the detection and quantification of ovine polyomavirus.

Primer	Sequence (5′-3′)	Annealing temperature (°C)	Product size (bp)
Ov_FI	AGATGGCCTCTTTCTCACA	52	227
Ov_RI	TTTATCTCCAGTCATGGCC		
Ov_nFI	AGACATTGTGGGCATGATTA	54	162
Ov_nRI	TTCCAATCTGGGCATAAGAT		
qOv_F	CAGCTGYAGACATTGTGG	60	168
qOv_R	TCCAATCTGGGCATAAGATT		
qOv_P	5′FAM-ATGATTACCAAGCCAGACAGTGGG-3′BHQ-1		

tested positive in the specific nPCR assay were selected to run the designed qPCR, which used two primers and a fluorogenic TaqMan probe to amplify a 168 bp fragment of the VP1 gene of the putative OPyV.

Standard curves were generated by transferring pGEM-T Easy plasmid (Promega, Madison, WI, USA) containing the 168 bp fragment of the VP1 gene into *Escherichia coli* DH5α cells (Invitrogen, Carlsbad, CA, USA). PCR was used to check that the transformed colonies contained the target sequence, and after purification with the QIAGEN Plasmid Midi kit (QIAGEN, GmbH Inc., Hilden, Germany), elutes were linearised with restriction enzyme EcoRI (Promega), and serial dilutions were performed with TE buffer to obtain standard dilutions ranging from 10^0 to 10^5 plasmid DNA molecules per 10 μl of sample in the qPCR reaction.

The designed 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 are given in Table 3. Annealing temperatures as well as primers and probe concentrations were optimised 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.

PCR master mix reagents were prepared in a DNA-free working area, samples were loaded in a pre-PCR working area and standards were finally added in a separate laboratory. Amplifications were performed in a mixture containing 10 μl of DNA and 15 μl of TaqMan® Universal PCR Master Mix, 0.4 μM of each primer (qOv_F and qOv_R) and 0.225 μM of fluorogenic probe (qOv_P).

TaqMan® Universal PCR Master Mix is supplied in a 2× concentration and contains AmpliTaq Gold® DNA polymerase, dNTPs with dUTP, passive reference, optimised buffer components and AmpErase® uracil-N-glycosylase. Following activation of the uracil-N-glycosylase (2 min, 50 °C) and activation of the AmpliTaq Gold for 10 min at 95 °C, 45 cycles (15 s at 95 °C, 20 s at 55 °C and 20 s at 58 °C) were performed with an MX3000P detector system (Stratagene, La Jolla, CA, USA).

Direct and one ten-fold dilution (1:10) of the extracted DNA was run in duplicate (4 runs/sample) for analysing environmental samples, while ten-fold serial dilutions of the qPCR standard were run in triplicate when quantifying viral genome copies (GC). In all the qPCRs carried out, the amount of DNA was defined as the mean of the data obtained. A non-template control was added to each assay. The faecal or urine samples tested by qPCR assays were quantified using duplicated undiluted and 10-fold dilution of the extracted DNA, producing more reliable results and minimising the effect of the potential inhibitors in the samples.

Depending on the starting volumes of water or the amounts of faecal matter analysed, and taking into account the several methods utilised, the quantity of sample analysed in one qPCR assay corresponded to 0.1 g of stools, 1 ml of urine, 3 ml of sewage or effluent and 17.5 ml of river water.

2.6. Specificity and sensitivity analysis

The specificity of the designed nPCR and qPCR assays was verified with samples collected from areas where no faecal contamination from ovine origin was expected to occur: bovine urine samples (n = 5) and bovine slaughterhouse wastewater (n = 4), chicken or turkey faecal pooled samples (n = 5), goat faeces (n = 6) and goat urine (n = 10) and wet straw-bed (n = 6) samples, hospital sewage (n = 10) and urban sewage (n = 4), porcine faeces (n = 5) and porcine slaughterhouse wastewater (n = 4). All samples suspected to be potentially positive for other animal-specific polyomaviruses were tested by the previously designed qPCRs. More specifically, bovine-related samples were tested for the presence of BPyV and human-related samples were tested for the presence of human JCPyV, both of which are already used as MST tools (Hundesa et al., 2010; Albinana-Gimenez et al., 2009). Known amounts of standard DNA containing 10^3, 10^2, 10^1, 10^0 and 10^{-1} OPyV GC/reaction were analysed ten times to determine the sensitivities of both specific nPCR and qPCR assays.

2.7. Sequence analysis

The amplicons obtained by nested PCR were purified using a QIAquick purification kit (QIAGEN, Inc.) following the manufacturer's instructions. After purification of the amplicons, both strains were sequenced using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase FS (Applied Biosystems), according to the manufacturer's instructions.

The obtained sequences were compared with the nucleotide sequences available in the Genbank using the BLAST algorithm from NCBI (National Center for Biotechnology Information), and were aligned with the ClustalX2 programme. The sequences reported in this paper have been submitted in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html).

3. Results

3.1. Identification of a novel OPyV in ovine samples by a broad-spectrum PCR

The broad-spectrum PCR developed by Johne et al. (2005) amplified the genome of a putative new polyomavirus in sheep urine, wet straw-bed and stool samples. After analysing several *O. aries* breeds of different origin (*Ripollesa* and *Xisqueta* from Catalonia, *Latxa* from the Basque country, *Merino* from Hungary and *Serraika* and *Boutsko* from Greece), polyomavirus-related sequences were detected by the broad-spectrum PCR in 31/64 (48.4%) of the urine and wet straw-bed samples and in 1/10 (10%) of the analysed stools.

3.2. Sensitivity and specificity of the nPCR and qPCR assays

Sensitivity and specificity were analysed in the nPCR and qPCR assays. One DNA copy was detected by nPCR in 7 of the 10 assays but only in 3 with the qPCR assay. Ten DNA genome copies were detected in 100% of the performed nPCR and qPCR reactions. Sensitivity did not vary even when high levels of exogenous but related viral DNA (samples with high levels of JCPyV) were added to the test tubes. The qPCR assay developed for the quantification of OPyV was shown to be specific both by the sequence analysis “in silico” of primers and probes considering nucleotide sequence databases (NCBI BLAST) and by experimental assays.

No positive samples were detected in any of the tested goat-related samples, urine, wet straw-bed or faeces (Table 2). All the hospital raw sewage samples also tested negative for the nPCR specific assay. No false positive results due to cross-reactivity with non-target DNA from the viruses infecting the various hosts analysed (bovine, chicken, goat, human and porcine) were detected with these assays.

3.3. Detection of the OPyV in sheep samples by the specific nPCR assay

An nPCR assay was developed in the polyomavirus VP1 region based on the sequences obtained by the broad-spectrum PCR. The developed nPCR assay proved to be specific for the new polyomavirus, which was detected in 9/13 (69.2%) of the urine and 21/42 (50%) of the wet straw-bed samples, and in 7/13 (53.8%) of the stool samples tested. The novel ovine polyomavirus was prevalent in all the geographical areas tested: 45% of the samples collected in Catalonia, 50% in the Basque country, 50% in Budapest and 62% in Greece tested positive.

3.4. Detection of OPyV in environmental samples by the specific nPCR assay

The nPCR was positive in three out of four sheep slaughterhouse wastewater samples and in one out of two tested samples of the same slaughterhouse effluent after a standard inorganic flocculation. When analysing downstream urban sewage treatment plant influents, 3/5 (60%) of the raw sewage samples and 2/5 (40%) of the treated effluents tested positive (Table 1). A very low level of inhibitors was observed in the undiluted samples.

3.5. Quantification OPyV from ovine and environmental samples

The qPCR was designed in the polyomavirus VP1 region based on the sequences obtained by the specific nPCR. The quantitative method was first used to quantify OPyV present in urine, wet straw-bed and stool samples which had previously tested positive in the nPCR. OPyV was also quantified in the slaughterhouse wastewater and effluents, as well as in urban sewage and secondary treatment effluents from the downstream STP. The qPCR assay amplified OPyV sequences in urine/faecal and slaughterhouse wastewater, STP sewage and effluent water samples. All mean concentrations and positive percentages are presented in Table 1. The analysis of environmental samples resulted in one positive result from the eight Catalan river water samples and five positive results from the eight Greek river water samples tested.

3.6. Nucleotide sequences and accession numbers

Eleven samples that tested positive in the Broad Spectrum nPCR assay were further studied by sequencing the obtained amplicons. No correlation was observed between either of the two sequences and the type, breed or geographical origin of the samples analysed. All the urine, wet straw-bed and stool sequences, obtained using the specific nPCR, from the Catalonian, Basque, Hungarian and Greek samples, as well as the sequences obtained from slaughterhouse wastewater (raw and effluent water), raw urban sewage with ovine effluents and the secondary effluent from the sewage treatment plant, resulted in a group of sequences with a shared identity of 99.5%. The positive river water samples were also sequenced and showed a 100% homology with the urine-related sequences.

A total of 23 samples were sequenced in this study, resulting in two groups of sequences differing by 4/215 nucleotides between them (98.1% similarity). The two sequences reported in this paper *Xisqueta* C1 and *Ripollesa* B5 presented high identities with other PyV. *Xisqueta* showed an identity of 74% with Bat PyV and 71% with goose haemorrhagic PyV and *Ripollesa* B5 81% identity with Bat PyV and 73% with the Chimpanzee PyV (*Pan troglodyte verus*). Both sequences have been deposited in the GenBank database under accession no. KC145150 for *Xisqueta* C1 and KC145151 for *Ripollesa* B5.

4. Discussion

A putative novel polyomavirus has been detected in sheep urine, wet straw-bed and stool samples by a broad-spectrum PCR assay. Samples tested in the study were collected from healthy animals, suggesting the asymptomatic presence and excretion of this virus in the sheep breeds studied (Hundesa et al., 2010; Bofill-Mas et al, 2000). The ICTV defines different polyomavirus species based on sequence identity; whole-genome nucleotide sequences with less than 81% identity can be classified as different species (Reimar et al., 2011). Therefore, further studies focused on genetic characterisation of the whole genome of the putative OPyV are being conducted at the moment to confirm that the detected virus constitutes a novel species within the Polyomaviridae family.

After developing specific and highly sensitive nPCR and qPCR, the putative novel OPyV was detected and quantified in sheep urine/faecal samples as well as in environmental samples. Both conventional and quantitative PCR assays were specific and able to distinguish ovine contamination but not other animal-originated contamination. Negative results were obtained when analysing slaughterhouse wastewater from bovine, porcine or chicken slurries, hospital sewage and urban sewage without any known ovine slaughterhouse effluent.

The DNA primer and probe sequences designed, in the polyomavirus VP1 gene region, appear to be highly specific and sensitive for OPyV. A high prevalence of OPyV was observed on the farms in all the geographical areas studied.

As expected, OPyV was detected in higher percentages in urine than in pooled stool samples, since the pattern of excretion identified in other polyomaviruses, such as the human JCPyV or the bovine BPyV, is persistently excreted in urine (Hundesa et al., 2010; Bofill-Mas et al, 2000).

With the developed assay, it was possible to trace ovine contamination from slaughterhouse raw wastewater (mean values of 9.81×10^5 GC/l) in the urban sewage treatment plant located downstream (mean values of 6.51×10^4 GC/l in raw sewage and mean value of 6.06×10^3 GC/l in the secondary effluent). Quantification of OPyV in slaughterhouse wastewater samples showed values, which were similar to human JCPyV concentrations detected in urban sewage in the same area (Bofill-Mas et al., 2011a,b). Similarly, the assay was able to detect ovine faecal contamination in the river water samples from an agricultural area in Greece receiving rainfall run-off and other discharges directly into the river basin (mean values of 1.14×10^4 GC/l). Depending on the recovery efficiency of the viral concentration method used for concentrating river water (Calgua et al., 2013) and the sensitivity of the detection methods designed, an amount of viral particles, ranging from 50 to 500 GC/l, should be present in river water samples in order to obtain a positive assay. On the Llobregat River, slaughterhouses are mostly located at the river mouth, so effluents are treated at STP located near the coast and the final effluent is discharged via an outfall to the sea. Most of the Llobregat River water samples tested negative since they were collected before receiving most of slaughterhouse discharges. Nevertheless, when sampling after Eid al-adha (feast of the sheep sacrifice) in which million sheep were slaughtered, OPyV was detected into the river water (1.05×10^2 GC/l).

5. Conclusions

The developed OPyV qPCR assay consistently detected the new viral marker in sheep urine samples, slaughterhouse wastewater and urban sewage, as well as in river water samples concentrated by applying a low cost and easy procedure (Calgua et al., 2013). The methods reported here have been proven to be specific and sensitive to detection of the specific ovine virus in environmental samples, being the first tools able to distinguish between ovine and bovine pollution. The sequences detected in different geographical areas were highly similar in their nucleotide sequence. Thus, the virus seems to be highly conserved. All these features indicate the potential use of the putative novel OPyV towards efficient microbial source tracking in countries where sheep is an important livestock.

The bovine polyomavirus (BPyV) is excreted in urine at mean concentrations of 2.21×10^4 GC/l and can be detected where intensive livestock and agricultural activities are present at concentration values of 3.06×10^2 GC/l (Hundesa et al., 2010). As the ovine marker is just as excreted in urine at mean concentrations of 1.56×10^5, we can presume that from the studied environmental samples it is a source tracking practical and effective marker for determining ovine faecal contamination.

This new MST tool, together with the previously described human and animal specific viral quantification tools, targeting human and porcine adenoviruses (HAdV and PAdV) and human and bovine polyomaviruses (JCPyV and BPyV) (Carratalà et al., 2012; Hundesa

et al., 2010; Hundesa et al., 2009; Bofill-Mas et al., 2000), represents a suitable toolbox to quantify human and animal livestock (porcine, bovine, poultry and ovine) faecal contamination in the environment.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2013.04.028.

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