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Viral tools for detection of fecal contamination and microbial source tracking in wastewater from food industries and domestic sewage

Melina Elizabeth Barrios[a,b,c], María Dolores Blanco Fernández[a,b], Robertina Viviana Cammarata[a,b], Carolina Torres[a,b], Viviana Andrea Mbayed[a,b,*]

[a] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Virología, Junín 956, 1113, Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Godoy Cruz 2290, 1425, Buenos Aires, Argentina
[c] Agencia Nacional de Promoción Científica y Tecnológica, Ministerio de Ciencia y Tecnología, Godoy Cruz 2370, 1425, Buenos Aires, Argentina

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ABSTRACT

Alternative indicators may be more suitable than thermotolerant coliform bacteria to assess enteric virus pollution in environmental waters and their removal from wastewaters. In this study, F-specific RNA bacteriophages (F-RNAPh) showed to be potential viral indicators of fecal contamination when they were quantified from domestic and food-industrial effluents containing human, chicken, swine or bovine wastes. In addition, they showed to be resistant to the primary and secondary treatments of the wastewater treatment plants. The viable F-RNAPh count showed correlation with viable thermotolerant coliforms but also with human polyomaviruses (HPyV) quantified by a new molecular method. In domestic effluents, F-RNAPh and HPyV indicators significantly correlated with a human viral pathogen, norovirus, while the bacterial indicator did not, being then better predictors of the behavior of enteric pathogenic viruses. In addition, we assessed human, bovine and fowl microbial source tracking markers, based on the molecular detections of human polyomavirus, bovine polyomavirus, and fowl adenovirus, respectively. The techniques implemented extend the range of viruses detected, since they target different viral types simultaneously. These markers could be applied when multiple source pollution is suspected, contributing to making decisions on public health interventions.

1. Introduction

Enteric viruses are among the main etiological agents of endemic waterborne diseases (Gall et al., 2015; Gibson, 2014). One of the major sources of environmental contamination are wastewater discharges, such as untreated and inadequately treated industrial and domestic sewage.

Thermotolerant coliform bacteria are used as a bacterial indicator of fecal pollution (FIB) and as process indicator during wastewater treatment. However, they may be native and multiply in some environments, they do not distinguish between fecal contamination sources and usually, they are less resistant to treatments than viruses and parasites (Hata et al., 2013; Lucena et al., 2004). The use of different viruses such as coliphages as indicators of pathogenic enteric viruses was proposed, based on their viral nature, similar size, structure and environmental survival characteristics (IAWPRC, 1991; Queensland Government 2005, n.d.). Experts gathered in the Coliphage Experts Workshop of the U.S. Environmental Protection Agency (USEPA) reviewed information to assess the use of F-specific and somatic coliphages as viral indicators (US Environmental Protection Agency, 2017). F-specific RNA bacteriophages (F-RNAPh) are a broad group of coliphages that infect Gram-negative bacteria, including *E. coli*, which possess a plasmid coding for an F, or sex pilus (Vinje et al., 2004). Replication of these viruses in environmental conditions is severely impaired, which constitutes an advantage for their use as a viral indicator of fecal contamination (Woody and Cliver, 1997). F-RNAPh are present in sufficiently high densities in raw sewage (US Environmental Protection Agency, 2017). They were morphologically similar and behave as human RNA viruses of concern, such as enteroviruses, caliciviruses, astroviruses, and hepatitis A and E viruses (Wadell Harrach et al., 2011). Several studies found a statistically significant relationship between F-RNAPh and gastrointestinal illness levels (Griffith et al., 2016; Lee et al., 1997; Wade et al., 2006).

However, a consensus on the use of different viruses as alternative indicators has not been reached yet, since some discrepancies were observed in the matter of correlations between the potential viral

* Corresponding author at: Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113, Ciudad Autónoma de Buenos Aires, Argentina.
E-mail address: vmbayed@ffyb.uba.ar (V.A. Mbayed).

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Table 1
Determinations performed on each sample.

Sample	Inflow (n)	Outflow (n)	Thermotolerant coliforms (MPN)	F-RNAPh (UFP)	HPyV (qPCR)	Nov (RT qPCR)	HPyV (PCR)	BPyV (PCR)	Fowl AdV (PCR)
Domiciliary WWTP A	2	2	x	x	x	x	x	x	x
Domiciliary WWTP B	1	5	x	x	x	x	x	x	x
Domiciliary WWTP C	3	3	x	x	x	x	x	x	x
Domiciliary WWTP D	8	0	–	–	–	–	x	x	x
Dairy Industry WWTP	5	5	x	x	–	–	x	x	x
Bovine slaughterhouse WWTP	10	10	x	x	–	–	x	x	x
Chicken slaughterhouse WWTP	2	1	x	x	–	–	x	x	x
Swine slaughterhouse WWTP	1	0	x	x	–	–	x	x	x
Horse slaughterhouse WWTP	1	1	x	x	–	–	x	x	x
Horse feces	9		x	x	–	–	–	–	–

The determinations practiced on each sample are indicated by "x". Not performed tests are indicated by "-".

indicators and the pathogenic enteric viruses (Jiang et al., 2007; Ogorzaly et al., 2009; Rezaeinejad et al., 2014). In addition, the occurrence and concentration of enteric viruses and coliphages vary at different sampling sites (Rezaeinejad et al., 2014), thus, the analyses of coliphages and other viral indicators at different geographical locations and matrices is needed to clarify their use for monitoring the microbial water quality.

Noroviruses (Nov) are the leading cause of waterborne gastroenteritis worldwide, and the risk of Nov infection and subsequent illness from waterborne and foodborne exposure is an emerging research topic (Gibney et al., 2017; Murphy et al., 2016; Soller et al., 2010; Van Alphen et al., 2014). Nov are non-enveloped, single-stranded RNA viruses that belong to the *Caliciviridae* family, genus *Norovirus* and are divided into six genogroups (I to VI). Some viral genotypes within genogroup II have been the most common cause of norovirus illnesses worldwide (Verhoef et al., 2015). Because of its importance as a human viral pathogen, noroviruses were compared with the different indicators analyzed during this study.

Host specific microbial markers have been proposed as microbial source tracking (MST) tools, which allow identifying the human or animal source of fecal contamination. Among them, different viruses have been suggested; however, most of the methods have been developed for the detection of only one viral target per host. Different authors have proposed polyomaviruses as microbial source tracking tools because they are stable in the environment, specific for human or animal host, cause persistent infections and are highly prevalent in different environmental water matrices (Bofill-Mas et al., 2000; Hundesa et al., 2010; McQuaig et al., 2006; Torres et al., 2016). Until now, fourteen human polyomaviruses (HPyV) (DeCaprio, 2017) and three bovine polyomaviruses (BPyV) have been described (Gräfe et al., 2017; Peretti et al., 2015), with differences in the prevalence of each viral type. However, in most of the cases, only the viral type identified as the predominant one in the samples has been used as target for MST, JCPyV for humans (Bofill-Mas et al., 2006; Rusiñol et al., 2014) and BPyV1 for bovines (Hundesa et al., 2006; Won and Xagoraraki, 2011). As an alternative methodology, other authors proposed the simultaneous detection of JCPyV and BKPyV by a TaqMan quantitative PCR (qPCR) assay based on the T antigen region (McQuaig et al., 2009).

Fowl Adenoviruses (FAdV) have been regularly isolated from healthy and sick domestic fowl. FAdVs are grouped into five different species (FAdV-A to FAdV- E) that comprise twelve serotypes (FAdV-1 to 8a and 8b to 11) (Hess, 2000; McFerran et al., 1972). Their simultaneous detection might give the opportunity to trace the fowl source of fecal contamination with higher success.

The aim of this work was to evaluate the suitability of alternative viral markers in wastewater samples to assess the fecal/urinary contamination and the performance of wastewater treatment, and to trace the source of human, bovine or fowl contamination. This analysis was performed on effluents from wastewater treatment plants (WWTP) belonging to the domestic sewage sanitation system and different food industries from Buenos Aires, Argentina, a temperate geographical region with high population density, where agriculture and food processing are important economic activities.

2. Materials and methods

2.1. Samples

Between August 2015 and February 2018, a total of 52 samples of raw and treated effluents were collected from wastewater treatment plants (WWTP) of slaughterhouses (bovine, equine), swine and poultry farms, a dairy industry and domestic sewage (WWTP A, B and C). Eight samples of domestic sewage (WWTP D) collected from April to November 2013 were added for the MST analyses. These WWTPs are located in the province of Buenos Aires, Argentina, and conduct primary and secondary treatments in stabilization ponds. Raw samples entering the WWTPs are named as inflow samples while samples collected after the treatments in the stabilization ponds are named as outflow samples. Additionally, freshly voided feces of nine horses from different farms from Buenos Aires province were collected.

Table 1 summarizes information about the samples and their analyses, according to the procedures described below.

2.2. Quantification of thermotolerant coliform bacteria and bacteriophages

Thermotolerant coliforms were enumerated by the most probable number (MPN) technique, according to the Standard Methods 9221E (American Public Health Association (1999)). F-specific RNA bacteriophages were quantified by the double-layer agar plaque assay according to the ISO 10705-1 standard (International Organization for Standardization, 2001), using the host strain *S. typhimurium* WG-49. Results were expressed in plaque-forming units (PFU) per ml. For the enumeration of thermotolerant coliforms and bacteriophages, sterile PBS was used as negative control and sewage samples previously analyzed, with countable coliforms or bacteriophages, were used as naturally polluted reference controls.

Bacteriophages and coliforms in the feces of horses were extracted as described before (Golomidova et al., 2007). Briefly, for bacteriophage enumeration, a homogeneous sample of 20 g of feces from each animal was suspended in 80 ml of phage solution [0.2 M NaCl (Merck, Darmstadt, Germany), 0.1 g sodium azide (Merck), 1 g Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) and water to a final volume of 1 L] and placed in an orbital shaker for 1 h at 120 rpm at room temperature, aliquoted and centrifuged for 2 min at 12,000 rpm. Bacteriophages in the supernatants were quantified according to the ISO 10705-1. For coliforms assay, another sample of 20 g of feces was suspended in 80 ml of a 0.9% NaCl solution, with a 20-min shaking time. Quantification was performed by the MPN technique.

When we were not able to enumerate bacteriophages, either in the effluent or feces samples, a qualitative (presence/absence) two-step

Table 2
PCR primers, probes and reaction conditions.

Primers and probes for Nested PCRs and qPCR	Cycling conditions
BPyV:	
Forward first round 1FBo12: CWGTAGGTGGYGAACCAC (from 2354 to 2371)[a] 1FBo3: CCATCATGGCAACATGCG (from 1753 to 1770)[a] **Reverse first round** 1RBo1: CAAATGTCTGCTGCTGTG (from 2708 to 2725)[a] 1RBo2: AAACTYGAAACTGGATATGGG (from 2819 to 2839)[a] AS-BP2: TCTAWAGGCYTCCCAAAC (from 2076 to 2093)[a]	**First round:** 2 min at 94°C, 10 cycles of 30 sec at 94°C, 30 sec at 45°C (with a decrease of 0.5°C per cycle), 40 sec at 72°C, 26 cycles of 30 sec at 94°C, 30 sec at 40°C, 40 sec at 72°C, and a final extension of 5 min at 72°C.
Forward second round 2FBo1: TGTGAGGATTTCAAAGCC (from 2390 to 2409)[a] 2Fbo2: MAAATACCCTAAGGGCAG (from 2409 to 2426)[a] 2FBo3: GGAAGAGGCAAGGTGACC (from 1779 to 1796)[a] **Reverse second round** 2RBo1: ATAGCTTGTCAGCTTTAC (from 2685 to 2703)[a] 2RBo2: GAGTWACATTAAAATATCTGGG (from 2773 to 2794)[a] AS-BS2: GCMTCAGGAATTTCAGGC (from 2031 to 2048) [a]	**Second round:** 2 min at 94°C, 8 cycles of 30 sec at 94°C, 30 sec at 42°C (with a decrease of 0.5°C per cycle), 40 sec at 72°C, 28 cycles of 30 sec at 94°C, 30 sec at 38°C, 40 sec at 72°C, and a final extension of 5 min at 72°C.
Fowl adenoviruses:	
Forward first round 52K-F: TGTACGAYTTCGTSCARAC[b] (from 13898 to 13916)[c] **Reverse first round** 52K-rv: AGCGCCTGGGTCAAACCGA[b] (from 14342 to 14360)[c]	**First round:** 2 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 52°C, 30 sec at 72°C and a final extension of 5 min at 72°C.
Forward second round 2FAdvF: GAGATGGCGTACATGATCGT (from 14056 to 14075)[c] **Reverse second round** 2RAdvF: CATGTASTCSGCGTCAGWCATC (from 14319 to 14340)[c]	**Second round:** 2 min at 94°C, 30 cycles of 30 sec at 94°C, 30 sec at 50°C, 30 sec at 72°C and a final extension of 5 min at 72°C.
HPyV (qPCR):	
S1-JC-BK: CTGCTGCTGCCACAGGATT(from 578 to 596)[d] AS2-JC-BK: CCTCTACAGTAGCAAGGGATGCA(from 673 to 652)[d] Probe-JC-BK: FAM-AGCAGCAGCCTCYCCAGCAGCAATTTCAGC-BHQ(from 633 to 604)[d]	10 min at 95°C, 40 cycles of 15 sec at 95°C, 60 sec at 60°C.

enrichment procedure was performed. Briefly, host strain *S. typhimurium* WG-49 was grown in 25 ml of tryptone yeast extract-glucose broth (TYGB) with 150 µl of $CaCl_2$ (1 M) (Merck) for 3 h at 36 °C, with gentle shaking. Then, 1 ml of the sample was added, and the incubation continued overnight. After that, 1 ml of the culture was transferred to a centrifuge tube; 0.4 ml of chloroform was added, mixed well and centrifuged at 3,000 g for 5 min. A drop of the chloroform-treated culture was placed on a 9 cm Petri dish with a bacterial lawn of *S. typhimurium* WG-49 supplemented with 300 µl of $CaCl_2$ (1 M). The plates were incubated face down at 36 °C for 18 h. A clear zone in the spotted area was indicative of the presence of coliphages in the original sample.

2.3. Concentration procedure of human and animal viruses in wastewater samples

2.3.1. Concentration method

To detect human and animal viruses, effluent samples were concentrated based on previously described protocols (Bofill-Mas et al., 2000). Samples (45 ml) were ultracentrifuged at 110,000 x g for 1 h at 4 °C. Viruses were eluted from the sediment by mixing with 4 ml of 0.25 N glycinebuffer (pH 9.5) on ice for 30 min; the suspended solids were separated by centrifugation at 12,000 x g for 15 min after the addition of 4 ml of 2X PBS. Viruses in the supernatant were finally pelleted by ultracentrifugation (110,000 x g for 1 h at 4 °C), suspending the pellets in 2 ml of PBS.

2.3.2. Inoculation of internal control viruses

To evaluate viral recovery during concentration, the samples were spiked with two internal control viruses: bacteriophage PP7 (ATCC 15692-B2) and a recombinant adenovirus, following the procedure previously described (Blanco Fernández et al., 2017). Briefly, control viruses were added to samples before the virus concentration, to globally evaluate the concentration, nucleic acid extraction, and amplification processes. The concentration achieved of each control virus in the initial samples was set as 1×10^6 genome copies (gc)/ml. To achieve this initial concentration, spiking volumes were calculated based on the known titers of virus stocks (2.7×10^{11} gc/mL of PP7 and 6×10^{11} gc/mL of rAdV) grown on a culture of *P. aeruginosa* or 293 A cells, respectively.

2.3.3. Calculation of viral recovery

To quantify both viruses in the initial and concentrated samples, nucleic acids were extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer instructions. PP7 viral RNA was retrotranscribed using random primers (Promega, Madison, WI, USA) and M-MLV retrotranscriptase (Promega). The PP7 bacteriophage was quantified by an RT TaqMan qPCR method previously described (Rajal et al., 2007b, 2007a). The rAdV was quantified by a TaqMan qPCR described by Blanco Fernández et al. (2017). Real-time TaqMan PCR assays were performed using the FastStart Universal Probe Master (ROX) mix (Roche Diagnostics) in an Applied Biosystems® 7500 Real-Time PCR system. Concentrations were estimated using a standard curve per run based on 10-fold serial dilutions (from 1 to 10^6 gc per reaction) of a plasmid DNA containing a partial sequence of the replicase gene of PP7, or a tag sequence of the hexon gene specific of the recombinant adenovirus developed by Blanco Fernández et al. (2017). The plasmid DNA was quantified by a fluorometric determination (Qubit® 3.0 Fluorometer, Life Technologies, USA) and the measure (in ng/ul) was converted to genome copies/ul according to the molecular weight of the clone (in Dalton). Samples were assayed in duplicate and no template controls (NTC) were included in each run.

The percentage of viral recovery (%VR) of the control viruses was calculated as the ratio of the total number of gc in the concentrate and the total number of gc in the initial sample:

$$\%VR = (gc\ in\ viral\ concentrate/gc\ in\ initial\ sample) \times 100$$

To estimate the median viral recoveries of PP7 and AdVr, four replicates of the concentration process were performed on an effluent sample.

2.4. Detection and quantification of human and animal viruses: development of new molecular tools

PCR based methods developed in this work or previously described have been used for identifying or quantifying the different human, bovine or avian viruses in the concentrated samples. PCR primers, probes and reaction conditions of the techniques developed in this work are described in Table 2. Nucleic acids extractions, retrotranscriptions and real-time TaqMan PCR assays were performed with the reagents described above, in Section 2.3.3.

2.4.1. Human polyomaviruses (HPyV)

2.4.1.1. Quantitative assay. A qPCR was developed to simultaneously quantify JCPyV and BKPyV, directed to the VP2 genetic region, Table 2. Primers and probe have been designed based on alignments of all reported sequences of both viruses in that genetic region on December 31 st, 2017. Primer properties and specificity were verified using OligoAnalyzer v3.1 (available at http://www.idtdna.com) and BLAST search. Concentrations were estimated using a standard curve per run based on 10-fold serial dilutions (from 1 to 10^5 gc per reaction) of a plasmid DNA containing a JCPyV VP2-VP1 sequence (subtype 2 A), quantified by fluorometric measure (Qubit® 3.0 Fluorometer). NTC were included in each run. The limit of detection (the lowest viral load that gives 95% of positive results (LOD95%)) has been estimated using the Probability of Detection (POD) model (Wilrich and Wilrich (2009)) implemented in the PODLOD calculation program v.9 (accessible at http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html). Briefly, 10–14 replicates containing 1.0, 2.5, 5.0 and 10.0 genomic copies of the JCPyV clone described above were tested and the number of positive values was recorded for each dilution. The LOD95% was estimated as 4.9 gc, (confidence interval 95% = 3.1–7.7). The limit of quantification (LOQ) has been estimated as the dilution that showed a coefficient of variation (CV%) lower than 35%. The LOQ was estimated as 5.0 gc (CV% = 33.0%).

2.4.1.2. Qualitative assay. The qualitative detection of HPyV was done by previously implemented nested-PCRs, (Torres et al., 2016) following an algorithm that included two steps: first, a broad-spectrum multiplex PCR was performed for the detection of JCPyV, BKPyV, MCPyV, TSPyV, HPyV9 and MWPyV (PCR A). When PCR A was positive, two nested PCRs were performed for the detection of JCPyV and BKPyV (PCR A1), and MCPyV, HPyV9 and MWPyV (PCR A2). The amplicon size of the PCR A1 allows identifying JCPyV and BKPyV. Positive controls, which were molecular clones previously sequenced, containing the VP1 gene of JCPyV (for PCR A and A1) or the VP1 gene of MCPyV (for PCR A2), and NTC were run simultaneously. The limit of detection of PCR A was previously estimated as 25–50 gc (Torres et al., 2016).

2.4.2. Human noroviruses

For the quantification of noroviruses genogroup II (GII), a RT-qPCR directed to the overlapping region of ORFs 1 and 2 was used, based on ISO_15216-1 (International Organization for Standardization, 2017). Concentrations were estimated using a standard curve per run based on 10-fold serial dilutions (from 2 to 2×10^5 gc per reaction) of a plasmid DNA containing the sequence of the ORFs 1 and 2 junction region (from a recombinant virus genotyped as GII.P7/GII6), quantified by fluorometric measure (Qubit® 3.0 Fluorometer). NTC were included in each run. The LOD95% was estimated as 8.3 gc while the LOQ was estimated as 10.0 gc.

2.4.3. Bovine polyomaviruses

A multiplex nested PCR directed to the VP1 coding region of the bovine polyomaviruses type 1, 2 and 3 was developed, Table 2. The primers were designed based on alignments of all currently reported sequences of the three viral types of BPyV in the GenBank database. Primer properties and specificity were verified using OligoAnalyzer v3.1 (available at http://www.idtdna.com) and BLAST search. The three viruses can be distinguished by the amplicon size (313 nt for BPyV1, 385 nt for BPyV2 and 269 nt for BPyV3). Positive controls, which were molecular clones previously sequenced, containing the VP1 gene of BPyV1 or BPyV2, and NTC (every four samples) were run in each assay. The limit of detection of this PCR was estimated as 5–10 gc.

2.4.4. Fowl adenoviruses

A nested PCR directed to the Hexon coding region of the group 1 (Gp1) of the genus *Aviadenovirus* was developed, Table 2. Primers were designed based on the genomic alignment of five species of fowl adenoviruses (FAdV A to E) comprised in the Gp1, retrieved from GenBank. Primer properties and specificity were verified using OligoAnalyzer v3.1 (available at http://www.idtdna.com) and BLAST search. Positive controls, which were molecular clones previously sequenced, containing the partial Hexon sequence of FAdV E, and NTC (every four samples) were run in each assay. The limit of detection of this PCR was estimated as 250–500 gc.

2.4.5. Calculation of polyomavirus and norovirus concentration in the original wastewater samples

The TaqMan PCR assays for HPyV and norovirus were performed in duplicate on each sample.

The recovery data calculated for PP7 and AdVr was used to correct norovirus and polyomavirus concentration results from (RT) qPCR methods described above.

The concentration of the viruses in the original wastewater samples was calculated with the following equations, based on that described by (Rajal et al., 2007a):

a For RNA viruses:

$$C_s = C_{qPCR} \times \left(\frac{V_{RT}^t}{V_{na}^{RT}} \times \frac{V_{na}^t}{V_c^{ext}} \times \frac{V_c}{V_s} \right) \times \frac{1}{VR}$$

- For DNA viruses:

$$C_s = C_{qPCR} \times \left(\frac{V_{na}^t}{V_c^{ext}} \times \frac{V_c}{V_s} \right) \times \frac{1}{VR}$$

Where C_s is the viral concentration in the sample (expressed as genomic copies per ml); C_{qPCR} is the mean concentration of each duplicated reaction, measured by qPCR (expressed as genomic copies per microliter of RT reaction); V_{RT}^t is the total volume of the RT reaction; V_{RNA}^{RT} is the volume of nucleic acids used in the RT reaction; V_{na}^t is the total volume of extracted nucleic acids; V_c^{ext} is the volume of viral concentrate used for extraction of nucleic acids; V_c is the volume of viral concentrate; V_s is the volume of the original sample and VR is the viral recovery (expressed as a fraction, not as percentage). Concentrations are expressed as genomic copies per ml.

2.5. Polyomavirus and adenovirus characterization

Species-specific PCR products from selected samples were sequenced. In the case of BPyV and based on the information of the viral type amplified in the screening reaction (different amplicon sizes were expected for the different viral types, see above) a new PCR was performed. For this new PCR not all the primers of the multiplex assay, but only the specific ones for the viral type identified, were used. A similar strategy was used to amplify the human polyomaviruses that were

detected in the bovine slaughterhouse, using only the primers specific for the virus identified by the amplicon size. In all the cases the PCR products were purified from the 3% agarose gel and sequenced using an Applied Biosystems 3730xl Genetic Analyzer (Applied Biosystems). These sequences were analyzed along with reference sequences obtained from GenBank. Sequences were aligned with ClustalX v2. (Thompson et al., 1997) and edited with BioEdit v7.0 (Hall, 1999). Phylogenetic trees were built using Maximum likelihood (ML) as phylogenetic inference methodology implemented in the PhyML 3.0 program (Guindon et al., 2010), using the substitution model estimated with the jModelTest v2.1 software (Darriba et al., 2015). A ML bootstrap analysis (1000 replicates) was used to evaluate the robustness of the phylogenetic groupings.

2.6. Treatment efficiency of industrial effluents and sewage

To determine the reduction of a specific indicator, the ratio between the load of the indicator flowing to the treatment plant (C_i) and the load of the indicator discharged from the WWTP (C_o) was established.

$$Reduction\ (log_{10}) = log_{10}(C_i/C_o)$$

2.7. Statistical analyses

Correlation between the titer of all the indicators and viral pathogens was evaluated using Pearson or Spearman correlation tests (Pearson *r* correlation coefficient, r, and Spearman's rank correlation coefficient, ρ, respectively), depending on the distribution of the data. Reductions of indicators by wastewater treatment were compared with Mann-Whitney or Kruskal-Wallis Tests ($p < 0.05$). All values below the detection limit for a given microorganism were included in the statistical analyses as half the value corresponding to the detection limit. The GraphPad Prism V5 software was used.

3. Results

The main objective of the study was to evaluate the suitability of alternative viral markers in wastewater samples. The study design comprised:

- The evaluation of alternative viral indicators of fecal/urinary contamination (F-RNA bacteriophages and human polyomaviruses) and their correlation with human enteric viral pathogens (noroviruses) and conventional indicators (thermotolerant bacteria);
- The use of the above-mentioned indicators to control the performance of wastewater treatment;
- The development of new microbial source tracking tools, to trace the human, bovine or fowl contamination, by means of multiplex PCR reactions that detect human polyomaviruses, bovine polyomaviruses or fowl adenoviruses, respectively.

3.1. Coliforms and bacteriophages as indicators of fecal contamination

Thermotolerant coliforms were detected in high titers in all the samples, exceeding the permissible limit of 10^3 MPN/100 ml, set out for the effluents disposal standards (Autoridad del Agua, 2003). Bacteriophages were quantified in domestic sewage and in industrial wastes derived from cattle, poultry, swine and dairy, obtaining 4.3×10^2 to 4.4×10^4 PFU/ml in inflows and 6.3×10^1 to 7.4×10^3 PFU/ml in outflows (Table 3). However, bacteriophages were undetectable in the equine slaughterhouse, except when a culture enrichment step with the bacterial host of the assay was performed.

To evaluate the presence of bacteriophages in horse samples, nine horse stool samples from different farms were evaluated and a wide range of viral titers was obtained, from no detection to 4.6×10^4 PFU/mg of feces, without correlation with the number of thermotolerant coliforms ($p > 0.05$), (Table S1, Supplementary Material).

3.2. Quantification of human polyomaviruses and noroviruses

Human polyomaviruses and noroviruses were quantified in domestic effluent samples. The viral titers were corrected according to the recovery efficiency previously estimated for the spiked viral controls PP7 and recombinant adenovirus, based on four replicate processes. The estimated median of the viral recovery was 12.27% (6.98–13.27) for PP7 and 12.08% (5.05–13.71) for the recombinant adenovirus. In a previous work performed on five of the sewage samples here analyzed, it was demonstrated that no significant differences were observed in the recovery between the two spiked control viruses and the enteric viruses naturally present in the samples, noroviruses and enteroviruses, (in all cases, $p > 0.05$) (Blanco Fernández et al., 2017). Then, the average of those close viral recovery values was applied in the calculations detailed in Materials and Methods, point 2.4.5.

The corrected titers of human polyomavirus and norovirus are presented in Table 3.

3.3. Correlations among the conventional and alternative indicators and the human pathogen norovirus

In the urban domestic effluents, we were able to evaluate the correlations among the bacterial and viral indicators as well as the correlation with the human pathogen norovirus GII. F-RNAPh, as a viral indicator, showed a high positive correlation with thermotolerant coliforms ($p < 0.0001$, $r = 0.861$) and with the other proposed viral indicator HPyV, which is specific of humans ($p = 0.038$, $\rho = 0.532$). On the other hand, HPyV showed a weak correlation with bacteria ($p = 0.042$, $r = 0.549$).

Both viral indicators showed a highly significant correlation with the human norovirus GII ($p < 0.0001$, $r = 0.852$ for HPyV; $p = 0.0064$, $r = 0.650$ for F-RNAPh) while the bacterial indicator did not correlate with this viral pathogen ($p = 0.0671$, $r = 0.484$).

Scatterplots of the correlations are shown in Fig. S1, Supplementary Material.

3.4. Reduction of coliforms, bacteriophages and human polyomaviruses by wastewater treatment

The reduction efficiency of thermotolerant bacteria and F-RNAPh due to the treatment was calculated from 10 to 12 paired inflow and outflow samples from the different WWTPs (Table 3). The median of the log reduction factor for each indicator from all sites, showed in Fig. 1, indicated a lower reduction of F-RNAPh (log R = 0.81) than bacterial indicator (log R = 1.45), although a non-significant difference was observed. For the calculation of the median of the log reduction factor, the values from the bovine slaughterhouse were not considered because its WWTP was under reconditioning and the treatment was highly deficient. The log reduction factors of this WWTP, which are even negative, can be seen in Table 3.

In a subset of five paired samples of domestic effluents we were able to evaluate the reduction of a human associated viral indicator, HPyV, quantified by qPCR. In these samples, HPyV showed a lower reduction than thermotolerant coliforms (log R = 0.30 vs log R = 2.00, $p < 0.01$) and F-RNAPh (log R = 0.30 vs log R = 0.83, $p < 0.05$), Fig. 1.

3.5. Polyomaviruses and adenoviruses as indicators of pollution source

We developed and evaluated techniques to detect human, bovine or avian markers for MST, based on species-specific nested-PCRs for polyomaviruses and adenoviruses.

Human polyomaviruses were found in all domestic sewage samples, with JCPyV and BKPyV as the most frequently detected viruses

Table 3
Quantification and reduction efficiency of thermotolerant coliform bacteria, bacteriophages, human polyomavirus and norovirus in industrial and sewage inflow and outflow samples.

Sample	Thermotolerant coliforms (MPN/100 ml)			Phages F-RNA (PFU/ml)			Human polyomaviruses (gc/ml)			Norovirus (gc/ml)	
	Inflow	Outflow	Reduction Log_{10} (Inflow/ outflow)	Inflow	Outflow	Reduction Log_{10} (Inflow/ outflow)	Inflow	Outflow	Reduction Log_{10} (Inflow/ outflow)	Inflow	Outflow
Domiciliary WWTP A											
Dec16_05	4.3×10^6	4.3×10^4	2.00	1.3×10^4	7.3×10^2	1.28	2.4×10^6	3.2×10^5	0.49	2.2×10^6	6.0×10^5
Dec16_06	2.4×10^6	4.3×10^5	0.75	3.4×10^3	7.2×10^2	0.67	8.2×10^5	4.4×10^5	0.27	1.2×10^6	4.0×10^5
Domiciliary WWTP B											
Dec17_05	nd	4.3×10^6	nd	9.3×10^3	1.6×10^3	0.76	2.6×10^6	9.0×10^5	0.46	3.1×10^6	1.4×10^6
Dec17_12	na	1.3×10^3	nd	na	7.6×10^2	nd	na	1.7×10^5	nd	na	1.6×10^6
Dec17_19	na	6.5×10^5	nd	na	2.5×10^3	nd	na	8.7×10^4	nd	na	U
Dec17_26	na	1.3×10^6	nd	na	1.2×10^3	nd	na	2.5×10^5	nd	na	1.4×10^6
Jan18	na	1.7×10^6	nd	na	1.3×10^3	nd	na	2.4×10^5	nd	na	4.5×10^5
Domiciliary WWTP C											
Jun16	2.4×10^6	$3.0 \times 10^*$	5.90	1.4×10^3	1.8×10^2	0.89	7.2×10^4	5.6×10^4	0.11	U	U
Ago16	4.3×10^6	4.3×10^5	1.00	6.4×10^3	5.6×10^2	1.06	2.5×10^5	2.0×10^5	0.10	7.8×10^5	U
Nov16	2.4×10^6	4.3×10^3	2.75	5.2×10^3	8.6×10^2	0.78	2.8×10^5	1.3×10^5	0.34	U	U
Dairy Industry WWTP											
Jun16	4.3×10^7	$4.0 \times 10^*$	7.03	4.3×10^2	6.3×10^1	0.84	nd	nd	nd	nd	nd
Ago16	2.4×10^8	4.3×10^3	4.75	1.1×10^3	2.7×10^2	0.62	nd	nd	nd	nd	nd
Jun17_05	1.5×10^5	4.3×10^4	0.54	4.8×10^2	9.6×10^1	0.70	nd	nd	nd	nd	nd
Jun17_04	2.4×10^5	9.3×10^4	0.41	6.2×10^2	1.2×10^2	0.71	nd	nd	nd	nd	nd
Ago17	9.3×10^6	7.5×10^5	1.09	6.7×10^3	9.0×10^2	0.87	nd	nd	nd	nd	nd
Chicken slaughterhouse WWTP											
Ago15	nd	nd	nd	4.4×10^4	4.8×10^2	1.96	nd	nd	nd	nd	nd
Oct17	9.3×10^6	na	nd	4.4×10^3	na	nd	nd	nd	nd	nd	nd
Horse slaughterhouse WWTP											
Sep15	1.5×10^7	2.4×10^6	0.80	U	U	–	nd	nd	nd	nd	nd
Swine slaughterhouse WWTP											
Sep17	2.4×10^8	na	nd	2.5×10^4	na	nd	nd	nd	nd	nd	nd
Bovine slaughterhouse WWTP											
Nov15	2.4×10^6	9.3×10^6	−0.59	4.8×10^2	3.8×10^2	0.10	nd	nd	nd	nd	nd
Dec15	4.3×10^6	1.5×10^7	−0.54	2.2×10^4	6.1×10^3	0.56	nd	nd	nd	nd	nd
Jan16	4.3×10^6	4.3×10^6	0.00	3.2×10^3	1.6×10^3	0.30	nd	nd	nd	nd	nd
Feb16	4.3×10^6	4.3×10^6	0.00	9.0×10^3	7.4×10^3	0.09	nd	nd	nd	nd	nd
Mar16	9.3×10^6	4.3×10^6	0.34	5.5×10^3	4.0×10^3	0.14	nd	nd	nd	nd	nd
Apr16	1.5×10^6	1.5×10^6	0.00	2.9×10^3	2.0×10^3	0.17	nd	nd	nd	nd	nd
May16	9.3×10^5	4.3×10^5	0.34	1.6×10^3	5.1×10^2	0.49	nd	nd	nd	nd	nd
Jun16	9.3×10^5	9.3×10^6	−1.00	1.4×10^3	1.6×10^3	−0.05	nd	nd	nd	nd	nd
Jul16	9.3×10^5	4.3×10^6	−0.66	9.4×10^2	5.7×10^2	0.21	nd	nd	nd	nd	nd
Dec16	4.3×10^6	4.3×10^5	1.00	4.8×10^3	6.1×10^2	0.89	nd	nd	nd	nd	nd

nd = not determinated; U = undetectable; na = not available samples.

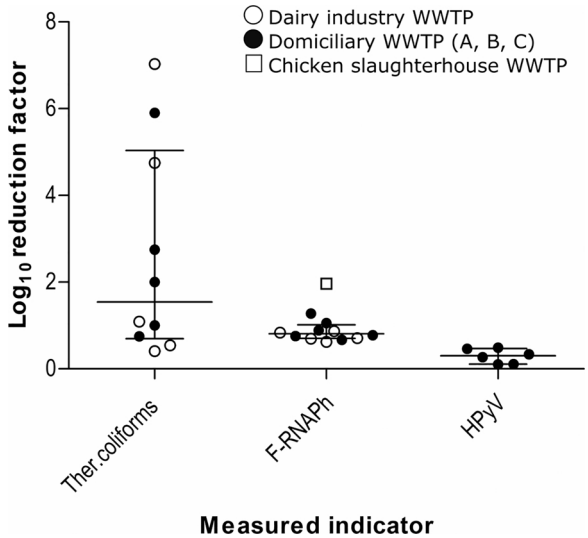

Fig. 1. Reduction of bacterial and viral indicators in WWTP.
Reduction of thermotolerant coliform bacteria, F-RNA bacteriophages and human polyomaviruses in several WWTP

(Table 4). To test HPyV as human-associated markers, we tried to detect them in the effluents from the different food processing industries, which collect wastes from agricultural animals that are nontarget hosts.

Six of 36 samples were positive, corresponding to outflow samples of the bovine slaughterhouse (5 of 20) and dairy industry (1 of 10), (Table 4). We were able to sequence four of these samples and phylogenetic analyses classified one of them as MCPyV and the other three as JCPyV, both human-associated viruses, demonstrating the specificity of the technique.

Bovine polyomaviruses were detected in all the inflow and outflow samples from cattle slaughterhouse (20/20) and most of the samples from dairy industry (7/10), with BPyV1 as the most frequently detected (27 of 30), followed by BPyV2 (17 of 30) and BPyV3 (1 of 30), Table 4. Nine of these viruses were sequenced and classified by phylogenetic analyses, showing that BPyV1 sequences from Argentina were related to a sequence from Spain while BPyV2 belonged to the cluster 2a and grouped with a sequence from Germany (Fig. 2a and b). None of the samples from poultry, swine and equine slaughterhouses were positive in the assay for bovine viruses. However, 2 of 24 samples of domestic sewage were weakly reactive in the assay.

Fowl adenoviruses were found in the inflow and outflow samples from chicken slaughterhouses (3/3). These viruses were sequenced, and phylogenetic analyses showed that one of them was related with the

Table 4
Results of molecular analysis of Human polyomaviruses, Bovine polyomaviruses and Fowl adenoviruses in food industrial wastes and sewage samples.

	Human Polyomavirus		Bovine Poliomavirus		Fowl Adenovirus	
WWTP Sample	Positive/ analyzed samples (%)	Polyomaviruses detected (n samples)	Positive/ analyzed samples (%)	Polyomaviruses detected (n samples)	Positive/ analyzed samples (%)	Adenovirus detected (n samples)
Human sewage	24/24 (100%)	JCPyV (23) BKPyV (23) HPyV A2[b] (14)	2[d]/24 (8.3%)	BPyV1 (2) BPyV2 (0) BPyV3 (0)	0/24 (0%)	U
Bovine slaughterhouse	5[a]/20 (25%)	JCPyV[d] (3) BKPyV (1) MCPyV[d] (1)	20/20 (100%)	BPyV1 (20) BPyV2 (10) BPyV3 (1)	0/20 (0%)	U
Dairy Industry	1[a]/10 (10%)	HPyV A[c] (1)	8[e]/10 (80%)	BPyV1 (8) BPyV2 (8) BPyV3 (0)	0/10 (0%)	U
Chicken slaughterhouse	0/3 (0%)	U	0/3 (0%)	U	3/3 (100%)	Gp1E (1) Gp1 (2)
Horse slaughterhouse	0/2 (0%)	U	0/2 (0%)	U	0/2 (0%)	U
Swine slaughterhouse	0/1 (0%)	U	0/1 (0%)	U	0/1 (0%)	U

JCPyV and BKPyV: were identified according to the amplicon size in PCR A1, except in samples from the bovine slaughterhouse, labeled as [(d)], which were sequenced and phylogenetically typed.
U: undetectable.
[a] Belonging to outflow samples.
[b] HPyV A: Positive samples in PCR A, which simultaneously amplify JC, BK; TS, H9, MW, MC.
[c] HPyV A2: Positive samples in PCR A2, which simultaneously amplify MC, H9, MW.
[d] Belonging to inflow samples.
[e] The two negative results belonged to outflow samples.

fowl adenovirus species E while the other two viruses, despite being more related to species A and C, did not cluster with any of the five defined species. Further characterization is needed to determine if they belong to a species not yet described within the Gp1 of the *Aviadenovirus* genus (Fig. 2c). The wastewaters from the other animal or human sources were not positive for FAdV.

4. Discussion

In this work it was shown that F-RNAPh were highly frequent in domestic sewage and food-industrial wastes, acting as potential viral indicators of fecal contamination from humans, chickens, swine and bovines, but not from horses. In domestic sewage, the F-RNAPh count showed correlation with FIB but also with another human-associated viral indicator, HPyV. As was expected, both viral indicators (but specially HPyV) significantly correlated with a human pathogen, norovirus GII, while the bacterial indicator did not, being then better predictors of the behavior of enteric pathogenic viruses.

It should be noted that, although we found a positive correlation between human noroviruses and the broad group of F-RNAPh, which theoretically includes viruses from different animal species, a better correlation should be expected with a host-specific indicator (such as human polyomaviruses). Other authors found that in river waters with pollution from multiple origins, the broad group of F-RNAPh correlated with norovirus GII. However, a stronger correlation was found with a subgroup of the F-RNAPh, identified by molecular methods as genotype GII, which are human-associated coliphages (Vergara et al., 2015).

Regarding the absence or low levels of F-RNAPh in horse stool samples and in wastewater from horse slaughterhouse, this could be associated to the non-suitability of host bacteria strains used according to the ISO procedure for their detection. Then, other organisms that better fit the host range of bacteriophages infecting horse gut bacteria might be evaluated.

The use of molecular techniques that quantify viral genomes without information about viability of the viruses has been of concern. However, in this work we observed a good correlation among a proposed viral indicator quantified by a molecular method (HPyV) and another viral indicator detected by infectivity (F-RNAPh); and both also correlated with a viral pathogen detected by a molecular technique (norovirus GII). The method developed for the simultaneous quantification of JCPyV and BKPyV has shown a reliable performance and presents an expanded target than techniques only focused on the detection of JCPyV and a higher sensitivity than a method previously proposed to target both viruses (McQuaig et al., 2009) . These two viruses are the most prevalent human polyomaviruses, according to the results of this work and previous reports in environmental samples from the same studied location and from other geographic regions (Barrios et al., 2016; Bofill-Mas et al., 2000). In this study, the concomitant use of two determinations resulted in a more complete description of the water contamination with viruses: a human-associated viral molecular marker gave valuable information about the accurate source of contamination while the enumeration of F-RNApH provided a measure of viral infectivity.

Regarding the evaluation of wastewater treatment performance, viable F-RNAPh could be more resistant indicators than thermotolerant coliforms in most of the ponds systems analyzed in this work, as others also observed (Davies-Colley et al., 1999; Hata et al., 2013; IAWPRC, 1991). Meanwhile, it was clearly shown that viral genomes of HPyV persisted more than thermotolerant coliforms.

Viruses and bacteria could have different susceptibility to the distinct processes causing microbial decay. The two viral indicators used in this work (bacteriophages and polyomaviruses) could be more conservative indicators of removal of human and animal viruses than conventional FIB for different wastewater treatments evaluation. However, the detection of the viral genomes can be less affected by the treatment than the viral viability, since the reduction of polyomavirus genomes was significantly lower than the reduction of the F-RNAPh.

We also evaluated MST markers developed in this work, and other previously designed by our group, that increased the number of viral targets in the assays to detect human, bovine and fowl contamination.

As a human-associated marker, the multiplex broad-spectrum PCR for HPyV will increase the probability of viral detection by targeting other polyomaviruses in addition to JCPyV and BKPyV, such as MCPyV and HPyV6, frequently found in environmental and sewage samples in different geographical regions but specially in the location studied in this work (Calgua et al., 2013; Torres et al., 2018, 2016). This technique might result in a sensitive MST tool with a reliable performance when multiple source pollution is present, since it was able to detect a minor

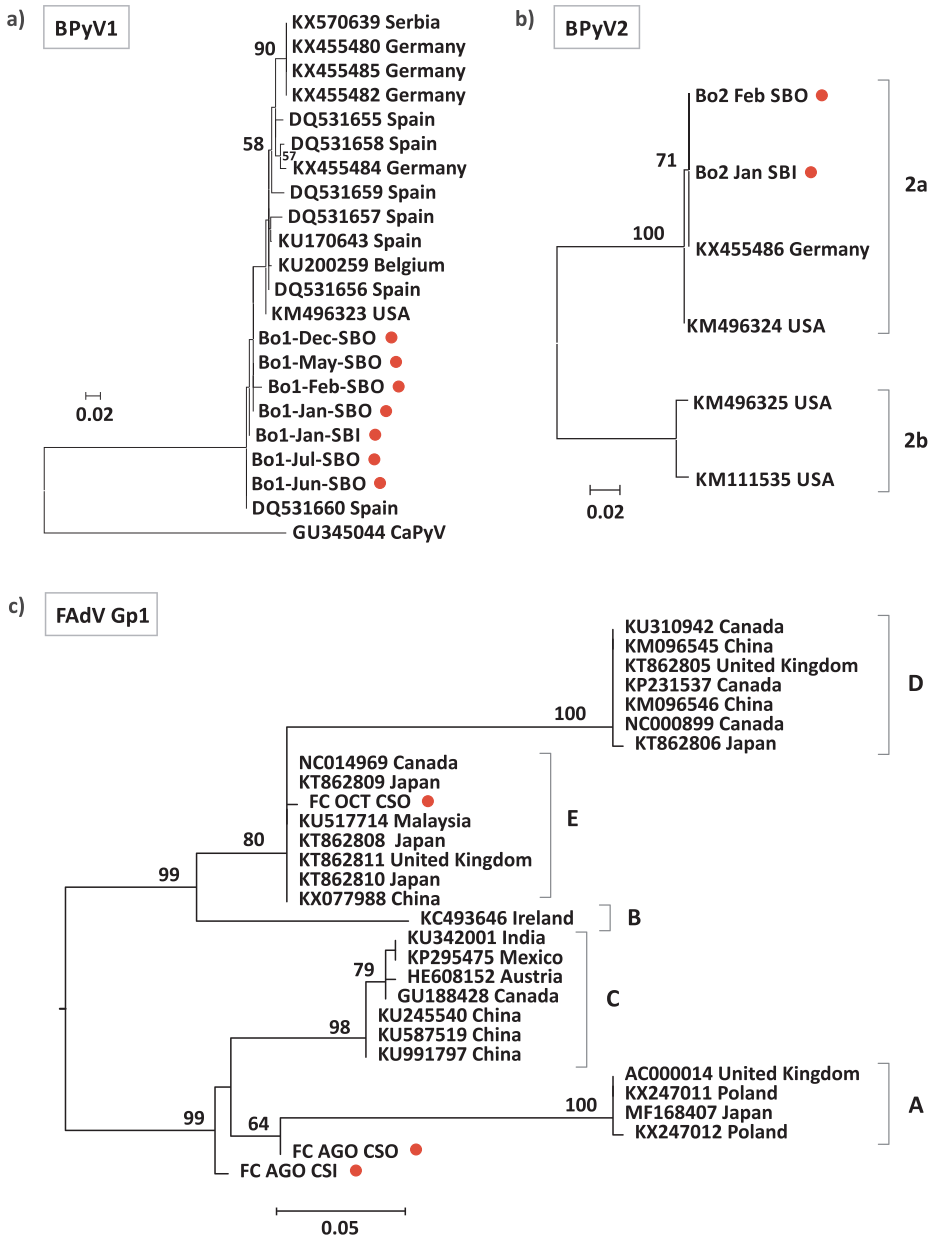

Fig. 2. Maximum likelihood phylogenetic trees of Bovine Polyomavirus and Fowl Adenovirus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
a) Maximum likelihood phylogenetic tree of VP1 partial sequences of BPyV1 (313 nt) (CaPyV was used as outgroup); b) Maximum likelihood phylogenetic tree of VP1 partial sequences of BPyV2 (385 nt); c) Maximum likelihood phylogenetic tree of hexon partial sequences of FAdv. Sequences from Argentina are indicated with red dots. Sequences presented in this work: GenBank accession numbers MF993028-37 and Table S2, Supplementary Material.

contamination with human viruses in wastewater samples from the bovine slaughterhouse and dairy industry. A leak from the human sewage pipes of the companies to the ponds systems treating wastes of animal source, could explain the mixed contamination.

Regarding the use of an indicator to trace bovine fecal/urine contamination, different authors proposed the detection of BPyV1 (Corsi et al., 2014; Hundesa et al., 2006). However, in our work not only BPyV1 but also BPyV2 were highly prevalent in bovine wastes reinforcing the applicability of the novel method to increase the probability of detection of bovine contamination. In the bovine slaughterhouse monitored over a year, one, two or the three viral types were co-detected in different months. The slaughterhouse received animals from different livestock farms and each viral profile could be associated with the different origins of the animals. The technique was able to detect bovine viruses in the effluents from the domiciliary sewage pipes, which should be minor pollutants among the major human viruses. Effluents discharged by the WWTP of the urban meat industries into the sewage pipe of the city could be the source of the bovine virus detected. In addition, the molecular analyses presented in this work showed that Argentinean BPyV are close related with European ones, although very few sequences of these viruses have been reported worldwide.

About tracing pollution originated in poultry, we developed a technique able to detect the five species of fowl adenoviruses (FAdV A to E) of group 1 of the genus *Aviadenovirus*. Viruses found in this work were unrelated and different origins could be traced. Fowl AdV Gp1 has been worldwide found in both sick and healthy chicken (Brown Jordan et al., 2018). Their detection and characterization could be useful as microbial source tracking as well as to study the circulation of these viruses in poultry farms.

A revision carried out on behalf of the US Environmental Protection Agency (USEPA) stated the need for a viral indicator of recreational waters quality and WWTP performance, such as coliphages, that resemble the behavior of human pathogenic viruses (US Environmental Protection Agency, 2017). Information in this field from different geographic regions with differences in viral epidemiology, social conditions, industrial development, wastewater practices and climate scenarios will be helpful in the development of universal water quality criteria. This work provides valuable information about the presence and behavior of F-RNAPh and human viruses in comparison with traditional FIBs in the context of the secondary treatment of wastewaters

from different domiciliary and industrial activities in Argentina, where this has been scarcely investigated. In addition, we developed human, bovine and fowl MST tools that simultaneously target several viral types, which could be applied when multiple source pollution is suspected, contributing to making decisions on public health interventions.

Declarations of interest

None

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.10.002.

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