Specific Viruses Present in Polluted Groundwater Are Indicative of the Source of Nitrates and Faecal Contamination in Agricultural Areas

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Abstract Microbial source tracking (MST) tools are used to identify sources of faecal pollution to accurately assess public health risks and implement best management practices. Many different viruses are excreted by humans and animals and are frequently detected in water contaminated with faeces or/and urine. Because of the large degree of host specificity of each virus and the substantial stability of many excreted viruses in the environment, some viral groups are considered to be accurate MST indicators. The Laboratory of Virus Contaminants of Water and Food at the University of Barcelona has proposed the use of viral indicators as well as cost-effective methods for the concentration of viruses from water. The developed procedures have been used to determine the levels of faecal pollution in environmental samples as well as for tracing the origin of faecal contamination. Such tools were recently used by the Catalan Water Agency to identify nitrate contamination sources in groundwater.

Human adenoviruses, human polyomavirus JC, porcine adenoviruses, bovine polyomaviruses, chicken/turkey parvoviruses, and ovine polyomaviruses can be quantified in samples using molecular methods (qPCR). The selected DNA viruses specifically infect their hosts and are persistently excreted in faeces and/or urine throughout the year in all geographical areas studied. The procedures that have been developed to quantify these viruses have been applied to bathing, coastal, surface and groundwater. In this study, the source of nitrate contamination in groundwater was identified by analysing viral markers, thereby demonstrating the usefulness of

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the selected viruses for the identification of sources of contamination in water. This methodology can be used to provide information to guide the proper application of measures in place to protect water from pollution caused by nitrates from several sources and thus to facilitate the accurate application of the 91/676/EEC Directive, which is mainly focused on agricultural sources of water contamination.

Keywords Adenovirus, Faecal contamination, Microbial source tracking, Nitrate contamination, Polyomavirus, Viral markers

Contents

Abbreviations

ACA Catalan Water Agency (in Catalan)
CRBD Catalan River Basin District
WFD Water Framework Directive

1 Faecal Contamination in Groundwater

Humans, as well as farmed animals, play an important role in the microbial contamination of water, crops and food and introduce large quantities of pathogens into the environment through their excretions.

Although most pathogens could be removed if sewage, manure and slurry were appropriately treated, many are discharged into receiving waters or may be disposed of in biosolids on land. Pollutants enter the water environment from two main types of sources: point sources, which are single and identifiable sources of contamination, and nonpoint sources, which are more diffuse sources of contamination. Nonpoint sources of contamination may release pollutants intermittently and may be attributable to infiltration from farmland treated with pesticides and fertilisers. Examples of point sources are landfills, leaking gasoline storage tanks, leaking septic tanks and accidental spills. Both point and nonpoint sources of contamination may affect groundwater, and several waterborne disease outbreaks

that are believed to have had viral aetiologies have been attributed to the consumption of polluted groundwater [1, 2]. Viruses (23–80 nm) are much smaller than bacteria (0.5–3 μm) and protozoa (4–15 μm) and thus move more easily through soil pores. They are highly stable at low temperatures in the darkness and survive for long periods in groundwater environments. However, relatively limited data on the level of viral contamination in groundwater are available compared with other environmental water matrices [3].

Detailed knowledge about sources of contamination is needed to develop efficient and cost-effective waste management strategies to minimise faecal contamination in watersheds and food, to evaluate the effectiveness of best management practices and to conduct system and risk assessments as part of water- and food-safety plans, as recommended by the World Health Organisation. Faecal sources of contamination have high nitrogen content, and both pathogens and nitrates present in groundwater polluted with faeces may pose a risk to human health when such groundwater is used as a source of drinking water.

Nitrate is the most widespread groundwater quality problem in many countries, and it is the most frequent cause of a groundwater body failing to meet good status under the WFD in some EU countries (http://ec.europa.eu/environment/water/water-nitrates/reports.html), including Catalonia [4]. The principal nitrogen inputs into groundwater are derived from manure, fertilisers, sewage sludge and crop residues from agricultural areas [5]. In the environment, several forms of nitrogen (NO_2, NH_4, NH_3) can potentially be transformed into nitrate (NO_3). Various activities may cause nitrate groundwater pollution in agricultural areas. The use of synthetic nitrogen fertilisers as well as the use of organic fertilisers, such as manure and slurries, is the main cause of this pollution. In some areas, high levels of nitrates in groundwater used as a source of drinking water are a consequence of the increase in livestock production that has occurred in recent years. Moreover, an absence of slurry, manure tanks or storage facilities may also contribute to this problem. The disposal of municipal or industrial effluents by spreading sludge on fields may also be a diffuse source of nitrate pollution in groundwater.

Other sources of nitrate pollution in groundwater include the following: interactions between groundwater and surface water, nitrogen-rich effluents, poorly constructed wells that allow water to be exchanged between polluted and nonpolluted aquifer layers, old and badly designed landfills, septic tanks and leaking sewerage systems (http://www.who.int/water_sanitation_health/dwq/chemicals/en/nitrateschap1.pdf).

The intensification of livestock production results in an increase in the amount of animal waste that must be managed. Catalonia, with a population of nearly 7.5 million people, has an important meat industry, with 6.8 million pigs, 0.5 million cattle and 0.6 million sheep [6]. A total of 19 out of 53 (36%) groundwater bodies in Catalonia have been classified as being of poor chemical quality as a result of high nitrate levels. Most of the affected groundwater bodies are located in agricultural areas, although not all stresses on groundwater result from agricultural activities. In some cases, urban wastewater leakage may also contribute to this problem. However, to date, agricultural sources and manure applications on fields, in particular, have been the main causes of pressure on groundwater. Together with nitrogen

compounds, faecal microorganisms are released into the environment in manure in holding ponds or storage areas or are applied to pastures to fertilise crops. Most livestock manure is disposed of on the ground, depending on the crop type, and annual quantities of nitrogen that are applied per hectare are specifically restricted in vulnerable areas [7, 8]. However, microorganisms and especially viruses can still, in some cases, infiltrate groundwater. The survival, fate and transport properties of viruses in the environment vary based on the type of virus, viral inactivation kinetics at high temperatures, UV exposure, filtration or adsorption in porous media or sediments and deposition and resuspension of sediments [9, 10].Survival is likely shorter in surface water than in groundwater because of UV exposure, higher temperatures (depending on the time of year and the location) and the opportunity for more interactions with other organisms that can inactivate viruses [11] in superficial water. Tracing and identifying the sources (human and/or animal) of faecal contamination in water are therefore essential, both to improve waste management and to assess risks to human health.

2 Development of Microbial Source Tracking (MST) Techniques

Faecal pollution is a primary health concern in the environment, in water and in food; for this reason, bacterial faecal indicators have been analysed widely to assess the microbiological quality of water, and such assessments are required by water safety regulations. The use of index microorganisms, whose presence points to the possible occurrence of a similar pathogenic organism, and indicator microorganisms, whose presence represents a failure affecting the final product, to assess the microbiological quality of water or food is well-established and has been practised for almost a century.

Classic microbiological indicators, such as faecal coliform bacteria, *Escherichia coli* and enterococci, are most commonly analysed to evaluate the level of faecal contamination. However, whether these bacteria are suitable indicators of the occurrence and concentration of pathogens such as viruses and protozoan cysts has been questioned for the following reasons: (1) indicator bacteria are more sensitive to inactivation by treatment processes and sunlight than are viral or protozoan pathogens; (2) indicator bacteria may not originate exclusively in faecal sources; (3) indicator bacteria may have an ability to multiply in some environments of interest; (4) it may not be possible to identify the source of faecal contamination; and (5) the presence of indicator bacteria may be poorly correlated with the presence of other pathogens. Thus, various authors have concluded that these indicators could fail to predict the risk of contamination with waterborne pathogens, including viruses [12–16]. Therefore, the team at the Laboratory of Virus Contaminants of Water and Food at the University of Barcelona has proposed that quantitative tests of specific viruses be used as complementary indicators of faecal contamination in water.

Methods for detecting and identifying the source of faecal pollution in the environment are known as microbial source tracking (MST) tools [17, 18]. These methods mainly focus on detecting a microorganism that is intrinsically related to faeces and that thus indicates the presence of contamination and hence of potentially excreted pathogens, such as bacteria, viruses and parasites. MST can assist health and environmental agencies with the identification of sources of faecal contamination. MST tools can also be employed to help make decisions related to the management of drinking water sources, shellfish-growing waters and recreational waters.

A large body of work has been developed in the MST field over the past several years. The first reviews listing the available methods for identifying indicators of faecal pollution in water were published in 2002 [19, 20]. Three years later, the US Environmental Protection Agency published the first guide document [21], and since then, several authors have published newer methods and have compared their applicability with existing methods [18, 22–26]. MST tools can be classified into several broad categories: genotypic versus phenotypic analyses of either cultivated target organisms or indicators or cultivation-independent approaches in which samples from the environment are analysed directly.

Some of the MST methods proposed in the literature lack environmental stability, host specificity and/or global prevalence. Moreover, some MST methods are laborious; they require large and suitable databases for each context and good statistical tools to allow meaningful interpretation of the results [18]. These limitations can be overcome using molecular methods to detect and quantify host-specific viral faecal indicators in water and food. Molecular techniques, specifically nucleic-acid amplification-based assays, provide sensitive, rapid and quantitative analytical tools for studying pathogens, newly emergent strains and indicators that are examined for microbial source tracking. Such methods are used to evaluate the microbiological quality of water [27], the efficiency of virus removal in drinking water and wastewater treatment plants [28–30].

3 Viruses Used for Tracing the Sources of Contamination in Water

Considering the limitations of current standard bacterial faecal indicators, selected viral groups have been proposed as alternative or complementary indicators to improve control of the microbiological quality of water and to reduce microbiological risk. Viruses are more stable than common bacterial indicators in the environment and are usually highly host-specific; because they are host-specific, their detection helps to trace the origin of faecal contamination. The viruses most commonly used for MST to detect faecal pollution are bacteriophages and DNA viruses (Table 1).

These viruses are recognised as important waterborne pathogens that are present in faeces, and new viruses that produce both symptomatic and asymptomatic infections are currently being described by metagenomic techniques [60]. Many orally transmitted viruses produce subclinical infections, and symptoms due to

Table 1 Summary of the proposed viral MST tools for the detection of human and animal faecal contamination

Host	Viral MST tools	Genome	References
Human	Bacteriophage RNA F-specific (FRNAPH)	RNA	[31–37]
	Bacteriophage of *B. fragilis* spp.	dsDNA	[38–40]
	Adenovirus (HAdV)	dsDNA	[41–44]
	Polyomavirus (JCPyV, BKPyV)	dsDNAc	[44, 45]
	Enterovirus (HEV)	ssRNA	[46, 47]
	Tobamovirus (PMMoV)	ssRNA	[48]
Cattle	Bacteriophage RNA F-specific	RNA	[33, 34, 36]
	Adenovirus (BAdV)	dsDNA	[49, 50]
	Polyomavirus (BPyV)	dsDNAc	[50–52]
	Enterovirus (BEV-2)	ssRNA	[46, 53, 54]
Swine	Adenovirus (PAdV)	dsDNA	[49, 55]
	Circovirus (PCV2)	ssDNAc	[56]
	Teschovirus (PTV)		[53, 57]
Sheep	Polyomavirus (OPyV)	dsDNAc	[58]
Avian	Parvovirus (Ch/TyPV)	dsDNA	[59]

dsDNA double-strand DNA, *ssDNA* single-strand DNA, *dsDNA*/*ssDNAc* double- or single-strand circular DNA

these viruses are only observed in a small proportion of the population. However, some viruses may give rise to life-threatening conditions, such as acute hepatitis in adults, as well as severe gastroenteritis in small children and the elderly. Some of the most important faecal viral pathogens are noroviruses, enteroviruses, adenoviruses, rotaviruses and the hepatitis A and E viruses. Human and animal viruses, such as adenoviruses [41, 42, 52], polyomaviruses [44, 55, 61] and parvoviruses [59], are frequently asymptomatic in immunocompetent hosts and often cause persistent infections. Moreover, they are highly host-specific, highly stable in the environment and resistant to disinfection [42, 62, 63]. Thus, the identification and quantification of specific viruses using molecular assays can be used for MST [42, 44].

3.1 Adenovirus

The *Adenoviridae* family has a double-stranded DNA genome of approximately 35,000 base pairs (bp) surrounded by a 90–100 nm, non-enveloped, icosahedral capsid with fibrelike projections from each vertex. Adenovirus infection may be caused by consumption of contaminated water or food or by inhalation of aerosols from contaminated waters, such as those used for recreational purposes. HAdV comprises 7 species with 57 types, which are responsible for enteric and respiratory illnesses and eye infections [64–66]. Among animal adenoviruses, porcine adenovirus (PAdV) may cause gastroenteritis symptoms such as diarrhoea, anorexia or

dehydration in piglets, while sows can suffer multifactorial respiratory diseases and even abortion [67].

- *Excretion pattern*: HAdV particles may be excreted in faeces for months or even years [49, 68]. Fifty per cent of the population has asymptomatic AdV infections at some time, and gastroenteritis occurs in 60% of children under 4 years of age [69]. HAdV40 and 41 serotypes of HAdV can be excreted at high concentrations in faeces (10^{11} viral particles per gram) and transmitted via the faecal-oral route. Other adenoviruses, such as HAdV-1, HAdV-2, HAdV-5, HAdV-7, HAdV-12 and HAdV-31, are related to respiratory diseases and have also been detected in contaminated water and shellfish [70, 71]. PAdV infections can also be asymptomatic and are detected in nearly 70% of swine faeces, with most isolates being closely related to serotype 3 [49].
- *Prevalence*: Human and porcine adenovirus (HAdV) have been detected in contaminated water samples throughout the year in all geographical areas studied [29, 44, 49, 55, 72]. HAdV has been found in nearly 100% of urban wastewater samples tested, including those from cities in Africa, the USA, Central and South America and Europe. Adenoviruses are also frequently detected in shellfish, including samples that met current safety standards based on levels of faecal bacteria [73].
- *Stability*: Adenovirus is inactivated only after 2 h at 85°C [74]. With moist heat, the time and temperature of inactivation are slightly reduced; exposure to 65°C for 30 min is then sufficient to inactivate adenovirus particles [75]. Chlorine treatment, which is very commonly used to disinfect and purify water, oxidises viral protein shells and nucleic acids [76]. Nevertheless, infectious HAdV can still be detected after chlorine treatment for 30 min (2.5 mg/L), although its concentration drops by approximately 2.7 $\log_{10}$ [77, 78].

3.2 Polyomavirus

Polyomaviruses are small, icosahedral viruses that have circular, double-stranded DNA genomes approximately 5,000 bp in length and that infect several species of vertebrates. The first human polyomaviruses, JC and BK (JCPyV and BKPyV), were identified in clinical samples from immunocompromised patients [79, 80]. The pathogenicity of JCPyV is commonly associated with progressive multifocal leukoencephalopathy (PML) in immunocompromised states, and infections with this virus have attracted new attention because of JCPyV reactivation and pathogenesis in some patients with autoimmune diseases who are being treated with immunomodulators [81, 82]. Among the known animal polyomaviruses, bovine polyomaviruses (BPyV) does not cause significant pathogenicity in cattle, and no disease has as yet been ascribed to this agent.

- *Excretion pattern*: Both human and animal PyVs are excreted in urine by healthy individuals [52, 83, 84]. JCPyVs have been detected in 40–80% of the population, and BPyV has been detected in 30% of the bovine urine samples analysed

[52, 61]. Polyomaviruses are transmitted by an unknown mechanism, although it is speculated that respiratory, cutaneous and faecal-oral routes could be involved in their transmission.

- *Prevalence*: Human JCPyV is distributed worldwide, and specific antibodies have been detected in over 80% of humans [85]. JCPyV and BKPyV were first described in environmental samples in 2000 [44]. JCPyV is frequently detected in river water, seawater, reclaimed water [72], drinking water [86] and shellfish grown in waters affected by sewage [61]. These viruses are present in nearly 100% of all sewage samples from different geographical areas [72]. BPyV has been identified as the cause of a widely disseminated infection in bovines, and it is a frequent contaminant of commercial bovine serum. BPyV has been detected in river water samples near slaughterhouses, farms and grazing areas [72].
- *Stability*: Polyomaviruses, such as SV40, are only significantly affected by exposure to a temperature of 95°C for 1 h [74]. Numbers of JC polyomavirus Mad4 viral particles were reduced by 1.5 to 1.1 $\log_{10}$ GC, as measured by qPCR after 30 min of contact (2.5 mg/L), although no infectivity assays were conducted for this virus in these studies [77].

3.3 Parvovirus

The *Parvoviridae* family comprises small animal viruses with 5 kb linear, single-stranded DNA genomes with two large open reading frames. This family of viruses is divided in two subfamilies: the *Parvoviridae*, which mainly infect vertebrates, and the *Densoviridae*, which infect arthropod hosts.

- *Excretion pattern*: Human parvoviruses have been detected in stool samples, but their transmission pathways remain unclear [87, 88].
- *Stability*: These viruses have shown high resistance to temperature and low pH [89, 90] and have been found in commercial meat samples [91]. Bovine parvovirus was not significantly affected by exposure to 95°C for 2 h [74].
- *Prevalence*: When sewage water, as a representative matrix that can be used to test large populations, was monitored, a high prevalence (81%) of parvovirus was observed [92]. Avian parvoviruses are excreted in poultry faeces and have been reported in studies from different countries [59].

4 Methods for the Use of Viral Markers for MST in Groundwater

Viruses are present in the environment in low concentrations and are distributed unevenly. To detect viruses in the environment, it is essential to collect a significant volume of sample and to concentrate the viral particles before employing a detection assay. Detection of viruses in minimally or moderately polluted waters requires

that viruses from at least several litres of water be concentrated into a much smaller volume (Fig. 1).

There are several concentration methods available, and many of them include two concentration steps in series, which will affect the recovery efficiency of the whole process. The development of cost-effective methods for the concentration of viruses from water and of cost-effective molecular assays, as well, facilitates the use of viruses as indicators of faecal contamination and as MST tools. The first methods used were based on the detection of viral indicators by PCR [41, 42, 44, 49]; more recently, quantitative PCR techniques have been developed that allow not only the detection but also the quantification of these viruses in environmental samples [29, 52, 58, 59] (Fig. 2).

It has been proposed that HAdVs and JCPyVs be quantified to trace human faecal contamination. HAdVs are present in sewage samples from all geographical areas

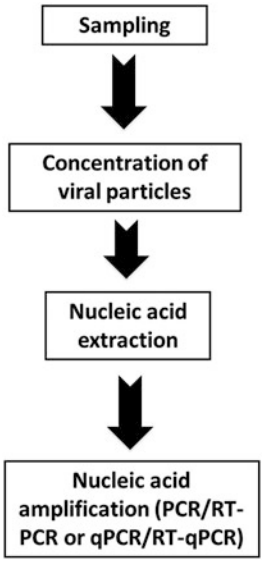

Fig. 1 Flowchart of the method used to detect and quantify viruses in water samples by PCR-based methods

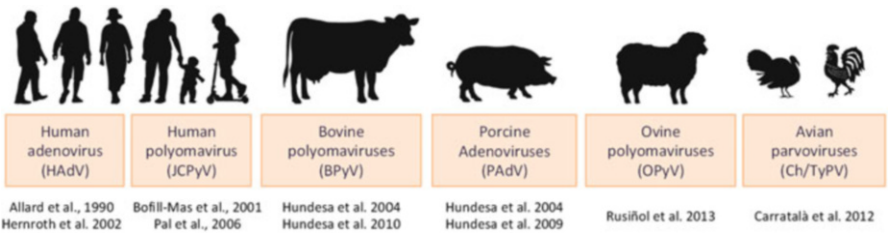

Fig. 2 Human and animal MST methods constituting a toolbox for identifying sources of faecal contamination

Table 2 Review of MST studies using HAdV to trace human sources in the environment

Country	References	qPCR	Main results
USA	[94]	[95]	16% (18/114), 1E+02-1E+04 GC/I
Japan	[96]	[95]	45% (29/64)
USA	[95]	[95]	S: 80% (4/5) 4.3E+04 GC/I; SW: 100% (11/11), 8.1E+06GC/I
Spain	[86]	[43]	R: 93% (13/14), 4E+02 GC/I; SW: 100% (10/10), 1.4E+07 GC/I
Spain	[29]	[43]	100% (6/6), 3.8E+07 GC/I
Spain	[28]	[43]	R: 90% (102/114), 1E+01-1E+04 GC/I
New Zealand	[97]	[95, 98]	R: 83% (5/6), 1.70E+01-1.19E+03 GC/I; SW: 100% (10/10), 1.87E+05GC/I
Germany	[99]	[98]	97.5% (40/41), 1.0E+04-1.7E+06 GC/I
France	[100]	[43]	100% (42/42), 1.0E+04G/I
Spain	[101]	[43]	R: 100% (7/7), 3E+03 GC/I; SW: 100% (7/7), 3.2E+06 GC/I
Japan	[102]	[95]	61.1% (11/18), 3.6E+03-1.38E+05 GC/I
Germany	[103]	[98]	96.3% (193/190), 2.9E+03-7.3E-7.3E+05GC/I
Brazil	[104]	[43]	SW 64.2% (54/84) 1E+07 GC/I
Brazil	[105]	[43]	100% (12/12); 5E+04-1.3E+07 GC/I
Spain	[106]	[43]	100% (7/7), 1E+01-1E+06 GC/I
Ghana	[107]	[98]	GW: 0% (0/4), SW: 22% (2/9)
Chad	[108]	[43]	GW: 0%, R:6% (1/16)
Germany	[109]	[98]	R: 9.3% (108/111), 3E+03 GC/I; SW: 100% (12/12), 1.0E+07 GC/I
Greece	[110]	[43]	45.8% (22/48)
Europe	[63]	[43]	R: 41% (381/928) S: 27% (132/482)
Brazil	[111]	[43]	100%, 1E+07 GC/I
Brazil	[112]	[43]	96% (46/48)
Spain	[113]	[43]	100% (44/44), 8.32E+03 GC/I
Brazil	[112]	[43]	69% (25/36) 1E+05 GC/I, 52.7% infective
Brazil	(114)	[43]	100% (24/24), 1E+05-lE+06 GC/I
New Zealand	[115]	[43]	R:86% HAdV (30/35) and 63% HAdV F (22/35), 1E+02 GC/I; S: 60% (9/15), 2.8E+02 GC/I; SW: (37/37)1E+05 GC/I
Uganda	[116]	[43]	GW: 0%; R: 70% (29/41), 2.65E+04 GC/I
Australia	[117]	[98]	91% (21/23) after sewerage overflow
Australia	[118]	[98]	100% (30/30), 1E+05-1E+06 GC/I
China	[119]	[98]	100% (24/24), 2.28E+04 GC/I
USA	[120]	[98]	40% (26/65), 2.2E+04 GC/I
Spain, Brazil, Hungary, Greece, Sweden	[72]	[43]	Spain 1.5E 03 GC/I (50/61), Greece 4.8E+02 GC/I (18/80), Brazil 3.9E+05 GC/I (253/276), Hungary 1E+04GC/I (108/109), Sweden 1.6E+02GC/I (12/108)

G groundwater, *R* river water, *S* seawater, *SW* raw sewage

that have been studied, while JCPyV is a less abundant but highly human-specific virus [93]. For this reason, the analysis of both viruses to determine the extent of human faecal pollution of environmental samples is a good approach that has a specificity of 100%. Both viruses have been evaluated in various studies in different water matrices, and their utility in MST has been demonstrated (Tables 2 and 3).

Porcine adenoviruses (PAdVs) and bovine polyomaviruses (BPyVs) have been proposed as porcine and bovine faecal indicators [49, 51], and several studies have

Table 3 Review of MST studies using JCPyV to trace human sources of contamination in the environment

Country	References	qPCR	Main results
Spain	[86]	[45]	R: 100% (9/9), 2.7E+04 GC/I
Spain	[29]	[45]	SW: 100% (6/6), 6.11E+06 GC/I
Spain	[28]	[45]	75% (18/27), 7.4E+02-1.3E+03 GC/I
United States	[121]	[121]	100% (41/41), 3.07E+07 GC/I
Germany	[99]	[122]	97% (40/41), 2.4E+04 GC/I
United States	[123]	[121]	50% (40/40)
Australia	[124]	[121]	R: 25% (5/20), 1E+03GC/I; SW: 100% (40/40), 1E+05GC/I
Spain	[71]	[45]	SW: 85% (6/7), 1E+05 GC/I; R: 100% (7/7), 1E+03 GC/I
Brazil	[125]	[45]	96% (6/7), 1.2E+06 GC/I
Japan	[102]	[45]	11% (1/18), 7.91E+02-3.42E+03 GC/I
Germany	[103]	[122]	68% (129/188), 1.4E+04 GC/I
Germany	[109]	[122]	R: (73/111) 1E+03 GC/I; SW: 100% (12/12) 1E+08 GC/I
United States	[126]	[121]	S: 3% (1/32); SW: 100% (15/15)
Greece	[110]	[121]	68% (33/48)
United States	[127]	[121]	1% (2/35), 1E+04 GC/I
Brazil	[112]	[121]	21% (10/48)
United States	[128]	[121]	12% (90/752)
Spain	[113]	[45]	100% (6/6), 5.44E+05 GC/I
United States	[129]	[121]	20% (26/130), SE+02-3.55E+05 GC/I
United States	[130]	[121]	61% (15/25)
Spain and Brazil	[131]	[45]	R: 100% (12/12), 9.38E+03 GC/I; SW: 100% (12/12), 1.05E4 GC/I
New Zealand	[115]	[121]	R: 51% (18/35), 1E+03GC/I; 5: 67% (7/15), 1E+03GC/I; SW: (36/37), 1.5E+06GC/I
Brazil	[114]	[45]	100% (24/24), 1E+05-1E+06 GC/I
Australia	[117]	[121]	52% (12/23)
Spain, Greece, Brazil, Hungary, Sweden	[72]	[45]	Spain 1.8E+03GC/I (41/61), Greece 5.6E+02 GC/I(15/80), Brazil 4.6E+03 GC/I (190/276), Hungary 2.1E+04GC/I (76/109), Sweden 7.2E+01GC/I (10/108)

G groundwater, *R* river water, *S* seawater, *SW* raw sewage

shown that these viruses are widely disseminated in swine and bovine populations, respectively, without producing clinically severe disease (Table 4) and are thus useful MST tools.

More recently, the quantification of ovine polyomaviruses and chicken/turkey polyomavirus has been suggested for tracing ovine and poultry faecal pollution, respectively [58, 59]. Quantification of each of these viruses has been used to trace the origins of nitrate pollution in groundwater in some areas of Catalonia, as described in the next section.

Table 4 Studies describing the detection of bovine and porcine faecal pollution using BPyVs and PAdVs as MST tools

Virus	References	qPCR	Matrices analysed	Main results
PAdV	[55]	[55]	River, slaughterhouse and urban sewage	100% positive samples in slaughterhouse sewage (1.56E+03 GC/L) and 100% in river (8.38 GC/L)
	[133]	[133]	River	50% positive river-water samples
	[56]	[55]	Manure	66% of the samples collected in the SMTS positive and 78% of the samples collected in the manure treatment system positive
	[135]	[55]	Manure	PAdVs were more prevalent than other viruses and may possibly be considered indicators of manure contamination
	[136]	[55]	Influents and effluents from swine manure biodigester	60% (24/40) samples positive
BPyV	[134]	[134]	Sewage	100% positive for manure and wastewater samples, 5.6% positive for faecal samples
	[52]	[52]	River, slaughterhouse and urban sewage	91% positive samples in slaughterhouse sewage (2.95E+03 GC/L) and 50% in river (3.06E+02 GC/L)
	[132]	[52]	Groundwater	1/4 well-water samples positive for BPyV (7.74E+02GC/L)
OPyV	[58]	[58]	River, slaughterhouse	75% (3/4) slaughterhouse samples positive 20% (1/8) river water samples positive

5 Case Study: Identification of the Sources of Nitrate Contamination in Catalonian Groundwater

Virus-detection assays have been used to detect viruses in groundwater samples from diverse areas in which nitrate levels exceeded >50 mg/L [137, 138] to trace the origins of nitrate pollution, as a collaborative study with the Catalan Water Agency and the Laboratory of Virus Contaminants of Water and Food from the University of Barcelona.

To ensure the designation of vulnerable zones according to the Directive against pollution caused by nitrates from agricultural sources [138], a total of 14 different monitoring stations were evaluated (Table 5). This study aimed to determine whether the pollution sources in these areas were manure, urban wastewater sludge or chemical fertilisers applied for agricultural uses. From three to five samples were taken per well for later analysis for the presence of different human and animal viruses. Samples were assayed for the presence of human adenovirus (HAdV) and human polyomavirus (JCPyV) to detect human pollution sources (from urban wastewater sludge used in agriculture or from sewage leaks); samples were assayed

Table 5 Human and animal viruses in groundwater from wells with nitrate contamination

Groundwater monitoring station	Type	Depth (m)	*N*	Human pollution		Animal pollution		NO_3^- mg/L
				HAdV	JCPyV	PAdV	BPyV	
				Genome copies/100 mL				
Font través (ClarianaCardener)	Spring	0	5	ND	ND	ND	ND	>110
Pou Casa Lloch (Olius)	Well	5	5	ND	ND	7.74E +01	ND	>100
Pou de Ca l'Arnau (Solsona)	Well	9	5	ND	ND	ND	ND	>100
Pou Ardèvol (Pinós)	Well	40	5	ND	ND	ND	9.53E +02	30–40
Mina del Sanou (Sta Coloma Queralt)	Gallery	0	4	7.00E +02	ND	ND	ND	24–46
PouBudell (Forès)	Well	6	4	ND	ND	ND	ND	70.9
PouNou (Conesa)	Well	30	4	1.42E +02	ND	ND	ND	5.1
Pou de les Escodines (Forès)	Well	11	4	ND	ND	ND	ND	150.1
Mina Aiguadolç (Sta Coloma Queralt)	Gallery	0	4	ND	ND	ND	ND	24–46
Font de la Freixa (Argençola)	Spring	0	4	8.47E +01	ND	ND	ND	45–54
Pou de Biure (Les Piles)	Well	0	4	8.01E +02	ND	ND	ND	76
Pou de les Piles (Les Piles)	Well	65	4	1.59E +02	ND	ND	ND	100–140
PouGuialmons (Les Piles)	Well	12	4	1.19E +02	ND	ND	ND	117–122
Pou de Sant Gallard (Les Piles)	Well	40	4	ND	ND	ND	ND	80–100

for the presence of porcine adenovirus (PAdV) to detect porcine sources of pollution (from the application of pig manure); and samples were assayed for the presence of bovine polyomavirus (BPyV) to detect bovine sources of pollution (from cow manure applications; Table 5).

Viruses were concentrated using the procedure described by Calgua and coworkers [131], based on flocculation with skimmed milk. After viruses were concentrated from 10 L water samples, viral nucleic acids were extracted. Then, qPCR assays specific for human adenoviruses (HAdV), JC polyomavirus (JCPyV), porcine adenoviruses (PAdV), bovine polyomaviruses (BPyV), ovine polyomaviruses (OPyV) and chicken/turkey parvoviruses (Ch/TuPV) were used to determine the relative quantities of each of these viruses in the samples and hence to determine the source of faecal contamination. The source of faecal

contamination determined in this way is then indicative of the source of nitrates in the groundwater from which samples were taken [43, 45, 52, 55].

The results obtained by qPCR were further confirmed by nested PCR and sequencing, as previously described [44, 49, 51]. The results obtained are summarised in Table 5.

The results show that in one area (Olius), faecal/urine contamination of porcine origin is clearly present (4/4 replicates tested positive), strongly suggesting that the application of swine slurries could be a significant source of nitrate contamination in the groundwater at that location. In the Pinós area, for which low levels of nitrate were measured, sporadic bovine contamination was detected (1/4 replicates tested positive), and diffuse contamination or the application of bovine manure was considered to be the potential source of the viruses that were detected. Finally human faecal pollution was detected as the main source of contamination in 4 other studied areas; further investigation is needed to identify the sources of contamination in these areas. This methodology has been tested in areas where nitrate concentrations are above the statutory limit (i.e. >50 mg/L) and thus where the use of groundwater as drinking water is compromised. A greater number of samples would be required to determine whether a relationship exists between the concentration of nitrates and the presence of the virus.

This study determines the origins of contamination of nitrates in groundwater, so that their sources (urban, animal or inorganic fertiliser use, in the case that viruses were not detected) could be established. The conclusions of this study could have implications for the future management of water in the region.

6 Conclusions and Future Trends

Groundwater is a vital source of water that provides, in Europe alone, drinking water for 300 million inhabitants. In Catalonia, over 587 hm^3/year of groundwater is used, and this amount represents close to 20% of the total water used in the region. Today, high nitrate levels in groundwater remain an important target for pollution reduction worldwide, with implications for human and environmental health [139]. Nitrate and pesticide pollution from agricultural sources are major, well-known problems with groundwater quality, and increases in water demand and population density will increase the probability of faecal contamination of groundwater. Moreover, falling groundwater levels will further endanger the quality of groundwater and its ability to clean itself. In addition to these problems, overabstraction has already begun to induce saltwater intrusion along most stretches of the Mediterranean coast, rendering the groundwater in those areas useless for drinking and most other purposes. Appropriate management of water resources and more specifically of groundwater resources requires the reliable evaluation of water quality and the identification of sources of contamination. These measures are needed to prevent further contamination, to implement remediation measures and especially to provide information that can be used to institute

measures to protect waters from pollution caused by nitrates from agricultural sources [138].

Currently, microbiological quality assessments of environmental waters largely rely on detecting faecal indicator bacteria. Although this approach has clearly reduced health risks in many countries, the faecal indicator approach may be combined with monitoring of more environmentally stable viral indicators specific to human and animal sources of contamination [27, 93]. The viral MST tools developed in this study can be used to track faecal contamination of human, bovine, ovine, porcine and avian origins using specific individual assays or, in the near future, using multiplex assays. Multiplex diagnostic tools are already available, and multiplex quantitative PCR assays for MST have been described previously in a study that examined diverse human and animal viruses [133].

Human (HAdV, JCPyV), bovine (BPyV), porcine (PAdV) and ovine (OPyV) viral markers have been shown to be useful for identifying the origin of faecal contamination in river water and seawater in Brazil, Sweden, Spain, Hungary, Greece and New Zealand [58, 72]. However, more information on the environmental stability and distribution of viruses in diverse geographical areas and water matrices will be needed to validate some animal viral markers, including new viral MST tools that may be developed in the future for other animals representing other sources of contamination.

Routine quantitative PCR assays for viral indicators may also be improved and standardised, in light of new methods that have been developed that allow the absolute quantification of genome copies without requiring that independent calibration curves be generated [140]. Other technical improvements that can be expected include advances in microfluidics and nanobiotechnology, as a result of which miniaturised systems for the detection of viral indicators could be developed that are based on microchips. Several such approaches have been described [141, 142]. New technologies, such as high-throughput mass sequencing, have been used to analyse urban sewage from diverse geographical areas and have produced a wealth of data about the viruses present in wastewater [60]. However, further development of NGS techniques are still needed to provide more sensitive and affordable assays that could potentially be used for routine analyses.

Cost-effective methods for using specific DNA viruses as markers of the source of faecal (or nitrate) contamination in water have been developed and validated. These methods may be standardised to acceptable levels of cost, feasibility, sensitivity and repeatability, especially in the case of the DNA viruses selected in our MST studies. The sampling strategies should also be considered carefully to obtain samples that best represent the water in question. Ideal sampling strategies could involve the use of hydrological and physicochemical sensors and time- and flow-integrating automated sampling devices.

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