Universidade de Aveiro Departamento de Biologia
Ano 2009

Andreia Marques Ribeiro

DETECÇÃO DE VÍRUS ENTÉRICOS EM FEZES HUMANAS E ÁGUAS RESIDUAIS: COMPARAÇÃO DE MÉTODOS DE CONCENTRACÇÃO E DE DETECÇÃO

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Detecção de vírus entéricos em fezes humanas e águas residuais: comparação de métodos de concentração e de detecção

Detection of enteric virus in human faeces and residual waters: comparison of concentration methods and detection methods

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Maria Adelaide Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro e Doutor Newton Carlos Marcial Gomes, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro

Outside a cell, they were nothing more than a collection of particles no more biologically active than a crystal of salt or a diamond. But inside a cell, these “inanimate” particles underwent a remarkable transformation. They become “alive”.

McKane & Kandel (1996)

o júri

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palavras-chave

Rotavírus, Adenovírus, gastroenterites, métodos de concentração, ultracentrifugação, floculação, métodos de detecção, nested PCR, RT-PCR, fezes humanas, águas residuais.

resumo

A presença de microrganismos patogénicos que provêm do esgoto e que são libertados nas águas ambientais determinam a qualidade da água e consequentemente afectam a saúde pública. A gastroenterite viral constitui uma das doenças humanas mais comuns em todo o mundo sendo transmitida feco-oralmente através do consumo e utilização de águas contaminadas com vírus entéricos como o rotavírus, norovírus e adenovírus. Por esse motivo, é epidemiologicamente relevante documentar a presença dos principais vírus entéricos em águas superficiais e fezes humanas. A detecção dos agentes etiológicos responsáveis pelas gastroenterites virais em amostras de águas, de modo a avaliar o risco da sua libertação no meio ambiente, requer a prévia concentração dos vírus presentes nas amostras. Com o advento das técnicas moleculares tornou-se possível a eficaz detecção de vírus presentes em amostras de águas e fezes. Actualmente, em amostras clínicas são geralmente utilizados testes imunocromatográficos como VIKIA® Rota-Adeno (BioMérieux) que permitem a detecção qualitativa dos vírus.

Para testar a sensibilidade e especificidade do método imunológico VIKIA® Rota-Adeno utilizado na dupla detecção de rotavírus A e adenovírus em amostras de fezes de pacientes com sintomas gastrointestinais do Hospital Infante D. Pedro, em Aveiro, entre Dezembro de 2008 e Julho de 2009, 18 amostras foram analisadas recorrendo ao método imunológico VIKIA® Rota-Adeno e moleculares (PCR). A detecção de Rotavírus A usando o kit VIKIA® Rota-Adeno, revelou a sua presença em 61% das amostras, enquanto a realização de RT-PCR permitiu a sua detecção em 56% das amostras, revelando uma concordância nos resultados obtidos pelos dois métodos. Relativamente à identificação de Adenovírus, a utilização do kit imunocromatográfico permitiu a sua detecção em apenas 6% das amostras, enquanto por nested PCR foi possível detectar a sua presença em 89% das amostras analisadas, demonstrando uma diferença significativa entre a capacidade de detecção de adenovírus usando as duas metodologias. A comparação dos resultados obtidos usando ambas as abordagens indica que, quando se usou métodos moleculares o adenovírus foi o agente que ocorreu com maior frequência, enquanto que utilizando o kit imunocromatográfico o rotavírus A foi o mais frequente. A sequenciação dos produtos amplificados por PCR das amostras para as quais os resultados através do kit imunocromatográfico foram negativos, permitiu a confirmação da presença de rotavírus humano do grupo A e de adenovírus humano serotipo 41 em algumas amostras de fezes. Através destes resultados observou-se que o kit VIKIA® Rota-Adeno é um método rápido e simples de diagnóstico de infecções por rotavírus A, no entanto poderá não ser suficientemente sensível e específico para detectar adenovírus, por esse motivo a utilização de métodos moleculares como o PCR parecem ser a alternativa mais eficiente.

Neste trabalho observou-se também a presença simultânea de rotavírus A e adenovírus em 50% das amostras de fezes analisadas, quando detectadas por métodos moleculares, levanta a questão se um único vírus é responsável pela doença ou se a sua etiologia é provocada pela presença dos dois vírus.
Estudos passados demonstraram que rotavírus A e adenovírus são agentes causadores de gastroenterites, neste estudo observou-se esse facto. Neste trabalho analisou-se também 467 amostras de fezes de pacientes com sintomas gastrointestinais através do kit imunocromatográfico, onde foram detectadas positivamente rotavírus A em 12.6% amostras, enquanto apenas 1% das amostras foram positivas para adenovírus. De modo a avaliar a incidência de gastroenterites virais através de informações recolhidas em 474 casos, foi possível concluir que a maioria dos pacientes com sintomas gastrointestinais tinha menos de 5 anos de idade e que o menor número de casos ocorre nos meses de Verão (entre Julho e Setembro). No que diz respeito à infecção por rotavírus concluiu-se também que a maioria das infecções ocorreu em crianças com idade pré-escolar e apresenta sazonalidade nos meses de inverno (de Dezembro a Março). No que diz respeito à infecção por adenovírus só foi possível observar que a maioria dos casos também ocorre em crianças com idade pré-escolar.
Para a detecção de vírus em águas residuais foram utilizados dois métodos de concentração (ultracentrifugação e floculação), tendo sido a sua eficiência de recuperação determinada por quantificação das partículas virais obtidas em cada um dos métodos por microscopia de epifluorescência. As amostras de água residual foram recolhidas em Março e Julho de 2009 na zona de tratamento secundário da ETAR Sul de Aveiro. A detecção da presença de rotavírus A e adenovírus por métodos moleculares (RT-PCR e nested PCR, respectivamente) em todas as amostras de águas analisadas, foi possível após a concentração prévia dos vírus. Com base na simplicidade, rapidez e eficácia de recuperação, a ultracentrifugação (68% de eficiência de recuperação) revelou ser o melhor método de concentração de vírus comparativamente com a floculação em que a taxa de recuperação média registada foi de 38% e o procedimento tem uma duração de pelo menos 16 horas. O facto do protocolo da ultracentrifugação ser relativamente rápido e não envolver a adição de reagentes, permite uma visão ecológica global dos vírus presentes nas amostras de água, recorrendo-se posteriormente, por exemplo, a abordagens metagenómicas ou de pirosequenciação. No entanto na floculação é adicionado leite desnatado, HCl e NaOH à amostra podendo afectar a comunidade viral.

keywords

Rotavirus, Adenovirus, gastroenteritis, concentration methods, ultracentrifugation , flocculation, detection methods, nested PCR, RT-PCR, human faeces, residual waters.

abstract

Wastewater-borne pathogenic microorganisms that are released into the environmental waters affect water quality, and consequently human health. Viral gastroenteritis is one of the most common human illnesses worldwide, contracted by human consumption and use of polluted waters contaminated with enteric virus, namely rotavirus, norovirus and adenovirus. Therefore, it is epidemiological relevant to document the presence of etiological agents of these diseases in environmental waters and human faeces. In order to evaluate the risk of sewage discharges into the environment it is necessary to detect pathogenic viruses, and for that, it is required a virus concentration from superficial waters before the detection. The advent of molecular analysis allows an effective and more sensitive detection of viruses in samples like water or faeces. In clinics are usually used immunochromatographic assays such as VIKIA® Rota-Adeno (BioMérieux) that allows qualitative virus detection.

To test the efficacy of immunochromatographic kit in virus detection, between December 2008 and July 2009, 18 faecal samples of gastroenteritis symptomatic patients from Hospital Infant D. Pedro, in Aveiro, were analyzed using simultaneously the immunologic method VIKIA® Rota-Adeno and molecular methods (PCR). The presence of rotavirus A was detected in 61% using the VIKIA® Rota-Adeno kit and in 55% of the samples using RT-PCR, demonstrating a similarity in the sensitivity of both methods for rotavirus A detection. In the case of adenovirus, 6% of the samples were positive when detected by VIKIA® Rota-Adeno kit, while the nested PCR revealed a positive result in 89% of the samples, indicating a significant difference in the detection power of the two methods. Through the analysis of this results, using the immunochromatographic method, rotavirus A were the most frequent while using molecular methods the most frequent was adenovirus. The presence of the viruses in positive amplifications was confirmed by sequencing analysis indicating the presence of human rotavirus A and human adenovirus 41 in all samples that were positive through PCR. The results obtained also indicate that the VIKIA® Rota-Adeno kit is a rapid and efficient diagnosing method, however might not be sensitive enough. Consequently, PCR approach might be the best detection method, since is a higher specific method but is more time consuming. In this work it was also observed simultaneous detection of rotavirus A and adenovirus in 50% of the samples analyzed using molecular methods, raising the question of whether a single virus is responsible for the illness or wither both viruses are involved causing the disease.

To evaluate the incidence of viral gastroenteritis, two of the main enteric viruses implicated in gastroenteritis were detected in faecal samples of patients with gastroenteritis symptoms. From January 2006 to July 2009 were collected 474 faecal samples of patients with gastroenteritis symptoms. Among 474 samples 467 were analyzed by VIKIA® Rota-Adeno kit and was detected rotavirus A infections in 12.6% samples while only 1% of the samples were positive for adenovirus. Through the clinical information of the 474 cases was possible to demonstrate that the majority of the patients with gastroenteritis symptoms had less than 5 years and the number of cases was lower in summer months (from July to September). Rotavirus infection also had a higher incidence in children younger than 5 years old and the peak of the cases occurred in winter months (between December and March). Relatively to adenovirus infection, was just possible to conclude that the majority of the cases occur in children with pre-scholar age.

To detect viruses in residual waters two concentration methods were compared (ultracentrifugation and flocculation), using the total viral number determined by epifluorescent microscopy before and after water concentration by both concentration methods. The samples were collected in March and July 2009 at a wastewater secondary treatment in Aveiro. Rotavirus A and adenovirus were positively detected by molecular analysis (RT-PCR and nested PCR, respectively) in all residual water samples analyzed after virus concentration. The results based on simplicity, rapidity and recovery rate, the ultracentrifugation was the best method with 68% of virus recovery, while flocculation had 38% of recovery and took at least 16 hours of procedure. The rapidity of ultracentrifugation method could avoid structural changes in viral community allowing the analysis of the sample by metagenomics or pyrosequencing, giving a global ecologic view of the viruses present in water samples. In flocculation method the viral community could be more affected, since was added skimmed milk to form flakes and was added HCl and NaOH to low and high the pH.

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ABBREVIATIONS

WHO: World Health Organization

EIA: Enzymoimmunoassays

PAGE: Polyacrylamide Gel Electrophoresis

PCR: Polymerase Chain Reaction

RT-PCR: Reverse Transcriptase – Polymerase Chain Reaction

n: number of total cases

sec: seconds

min: minutes

h: hour

L: liter

mL: milliliter

µL: microliter

mM: milimolar

µM: micromolar

mm: mililimeter

nm: nanomillimeter

bp: bases pairs

rpm: revolutions per minute

CHAPTER 1

INTRODUCTION

OBJECTIVES

Enteric viruses

1. Viruses transmission

Viruses are the most abundant and genetically diverse “life form”, representing the main pathogens of planktonic organisms and consequently impacting nutrient and energy cycle in aquatic ecosystems in a significant way. As well, they are pathogens of higher organisms and there are good evidences that some viruses move between aquatic and terrestrial reservoirs (Suttle, 2005).

The rapid growth of the population in urban areas, the expansion of industrial parks and agriculture wastes increases the production of sewage that contains high levels of microorganisms and organic matter, that need to be remove through wastewater treatments (Willey *et al*., 2008). Wastewater treatment involves a number of steps spatially segregated, that are divided in primary, secondary and tertiary treatment. This treatment induces the reduction of harmful microorganisms, such as bacteria, viruses and protozoa (Costa, 2007), minimizing the environmental impacts of wastewater discharges (Marincas *et al.*, 2009). However, the common water treatment does not ensure the complete removal or inactivation of the viruses, allowing these agents to become contaminants of the aquatic environment. Nevertheless the biggest problem is when wastewater is discharged without any treatment (Percival *et al.*, 2004; Muscillo *et al.*, 2008; Rodríguez-Díaz *et al.*, 2009). The presence of enteric viruses have been reported in sewages, wastewater and recreational waters, representing a serious public health risk, since, even at low concentrations, viruses can cause illness when ingested. Viral gastroenteritis is the main causes of outbreaks of virus-associated diseases worldwide (Lopman *et al*., 2003). Outbreaks of this nature occur in areas of natural catastrophe, military bases, cruise ships, schools, kindergartens, nursing homes and hospitals, mainly because of norovirus (Grotto *et al.*, 2004; Matson, 2005; Svraka *et al.*, 2007; Braham *et al.*, 2009). The main risk group are immunocompromised individuals, transplant patients, prematurely babies and children under 5 years (Allard *et al.*, 2001; Aminu *et al.*, 2007; Huppertz *et al.*, 2008).

The recognition that viruses plays a major role in aquatic marine ecosystems and in last instance in public heath, adds a significant new dimension to the understanding of virological process (Suttle, 2005). However it is still difficult to quantify and identify the number and type of virus present in environmental and wastewaters, but it is known that viruses vary from season to season, from year to year and between different geographical locations (Percival *et al*., 2004).

1.1. Transmission by contaminated water

Waterborne viruses are an important cause of disease, being responsible for 14% of outbreaks and 38% of illnesses associated with drinking water in the USA from 1999 to 2002 (Lee *et al.*, 2002; Yoder *et al.*, 2004).

Waterborne viral pathogens have a large socioeconomic and ecologic impact in industrialized and developing countries, being the magnitude of its impact more severe in world regions with highly polluted environments (Hamza *et al*., 2009; Rodríguez-Díaz *et al*., 2009), representing a severe health problem that causes significant mortality in susceptible people as well as an economic problem due to disease-related non-productive time (Verheyen *et al.*, 2009).

Water can be contaminated directly by faecal matter and vomits or indirectly by exposure to contaminated surfaces. In the environment, the fate of enteric virus can take several routes, such as irrigation with contaminated water from sewage, recreational activities in polluted water and the consumption of contaminated drinking water (Kovac *et al.*, 2009). Furthermore, confirmation of the presence of norovirus in different brands of European mineral water indicates that bottled water could also be an important source of viral infection (Beuret *et al.*, 2000; Beuret *et al.*, 2002). Verheyen *et al.* (2009) detected 12.9% positive samples for adenovirus and 2.1% of rotavirus in a total of 541 samples of drinking water, revealing the importance of appropriate decision support systems in developing counties.

Outbreaks have been recorded in a wide variety of types of recreational water like water terms, swimming pool, lakes, beaches and rivers, however many are not published (Begier *et al.*, 2008; Sinclair *et al.*, 2009). Sinclair *et al.* (2009) determine that 28% of the outbreaks were associated with swimming pools, 56% resulted from lakes and 8% from rivers. The exact risk of disease by enteric viruses after exposure to contaminated water is difficult to quantify, the assessment of the virus abundance and consequently the risk level to human health is crucial (Bosch, 1998).

Several studies recommend the development of more efficient water treatments and the monitoring of viral contamination in environmental waters (Bosch *et al*., 2008). However, the European Bathing Water Directive (2006/07/EC) regulating the quality control of recreational waters does not take in consideration the presence of viruses. To obviate these gap european projects like Virobathe had the objective of developing methods for the detection of adenoviruses and noroviruses in European Bathing Waters in order to instigate the revision of the Bathing Water Directive 76/160/EEC.

1.2. Transmission by contaminated food

Food has been recognized as a potential carrier of disease since the beginning of recorded history, being responsible for more than 60% of nonbacterial gastroenteritis outbreaks (Inouye *et al.*, 2000). However, improved conditions of food production and better laboratory detection techniques in recent years have changed substantially in industrialized countries, reducing the frequency of foodborne diseases (Hall *et al.*, 2005; Willey *et al.*, 2008).

The transmission of enteric virus through food can be due to the washing of vegetables and fruits with contaminated water or due to plant growth in contact with contaminated water, since contaminated water is often used to irrigate and to fertilize crops (Wheeler *et al.*, 1999; Widdowson *et al.*, 2005b; Guyader *et al.*, 2008). Filter-feeder bivalve shellfish, such as oysters, cockles and mussels raised in polluted water are also frequently associated with foodborne gastroenteritis, by filtering particulate matter that may include faecal material and consequently viruses (Sanchez *et al.*, 2002; Percival *et al.*, 2004; Guyader *et al.*, 2006). Many studies of foodborne outbreak have been associated with oysters consumption, and in one of these studies five enteric viruses (aichi virus, norovirus, astrovirus, enterovirus and rotavirus) were actually detected in the oysters analyzed (Guyader *et al.*, 2008).

1.3. Other pathways of virus transmission

Other important way of enteric virus transmission, besides the water and food, is the transmission person to person by contact, sharing objects and through sneeze and cough (Hutson *et al.*, 2004; Webby *et al.*, 2007). Likewise, lack of socio-economic conditions and hygiene, as well as environmental problems linked to municipal sewage discharges and lack of investment in wastewater treatment systems or the failure of existing ones are additional factors that contribute to virus propagation (Mead *et al.*, 2000; Froggatt *et al.*, 2004; Wit *et al.*, 2004; Goodgame, 2006; Oh *et al.*, 2009; Rodríguez-Díaz *et al.*, 2009).

A significant number of waterborne diseases could be avoided if several measures were implemented by each individual as by the authorities. It would be enough to change hygiene habits, consumption of safe water, better sanitary conditions and different practices of cooking certain foods to reduce the number of infected individuals (McKane and Kandel, 1996; Bosch *et al.*, 2008).

Some enteric viruses can cross-infect, that meaning be transmitted from vertebrate animals to humans, an infection designated as zoonoses. Rotaviruses have been detected in livestock

animals, most notably in young calves and piglets (Percival *et al.*, 2004; Martella *et al.*, 2009). In norovirus case is know the possible role of swines as reservoirs for norovirus that could potentially infect humans (Wang *et al.*, 2005; Almanza *et al.*, 2008). On the other hand, Halaihel *et al*. (2009) affirmed that human norovirus are unlikely to be circulating in the porcine population.

2. Characterization of enteric viruses

Enteric viruses replicate in cells of the human gastrointestinal tract, whose main transmission is faecal-oral, and have a great human public health importance because of social and economic problems that developed (Hurst *et al.*, 1997).

Enteric viruses have a range of important attributes that distinguish them from other pathogenic microorganisms, such as low infectious dose and high survival in environmental water (Percival *et al.*, 2004). The number of infectious enteric viral particles declines after being released into the environment, some enteric viruses may survive for long periods depending on the environmental conditions (Percival *et al.*, 2004). Viral particles comprise a protein coat that protect nucleic acid from degradation by faecal organic material and consequently from adverse factors in the environment, being frequently destroyed in the environment by desiccation, UV light, heat above 56°C, digestion by other microorganisms and by predation (Percival *et al*., 2004).

Viral gastroenteritis outbreaks have a short incubation period (few days) and are caused by a large number of viruses. The most implicated enteric viruses in gastroenteritis includes rotavirus, adenovirus, sapovirus, norovirus and astrovirus (Clark and McKendrick, 2004; Bosch *et al.*, 2008). However, in 40% of acute viral gastroenteritis the etymologic agent remains unidentified, and it has been suggested that aichi virus, bocavirus (Schildgen *et al.*, 2007), parechovirus (Li *et al.*, 2009), picornovirus and picobirnovirus might be possible responsible viruses (Wakuda *et al.*, 2005; Meqdam and Thwiny, 2007; Schildgen *et al.*, 2007; Victoria *et al.*, 2009).

Rotavirus A is the major known etiologic agent of severe diarrhoea in infants and young children in most areas of the world, and norovirus is currently the most commonly recognized causes of epidemic viral acute gastroenteritis worldwide (Wu *et al*., 2008). Adenovirus is an important emerging enteric virus whose infection is mostly associated with contaminated water (Wilhelmi *et al.*, 2003).

2.1. Rotavirus

Rotavirus is member of the *Reoviridae* family and is divided in seven groups (A through G). They are nonenveloped viruses with 60-85 nm in diameter and contain 11 segments of double-stranded RNA (Adler *et al*., 2005; Kittigul *et al*., 2008). Most rotavirus infections are caused by rotavirus A, that is classified into genotypes G (14 types) and P (20 types), according to the antigenic variation of structural proteins VP7 and VP4 (Portella, 1997; Alfieri *et al.*, 2004). Various studies have shown that rotavirus have a physical resistance to inactivation when subjected to various temperatures, and the increased of humidity is accompanied by the loss of human rotavirus infectivity (Ansari *et al*., 1991).

Rotavirus displays seasonal peaks, the annual season starting mid November, peaked between February and March and reached the baseline by May (Rendi-Wagner *et al*., 2006). This virus is easily transmitted from person-to-person or indirectly via food or water, and is just need 10 virus particles to infect a young child (Adler *et al*., 2005; Kittigul *et al*., 2008).

Several epidemiological studies have demonstrated that rotavirus are the leading cause of gastroenteritis in children and young animals, in industrialized and developing countries (Parashar *et al*., 1998). Worldwide, every year, rotavirus is the main cause of death in children under 5 years of age. From all rotavirus related death, 80% occurs in developing nations (Parashar *et al.*, 2006). Comparing studies conducted between 1986-1999 and between 2000-2004 has revealed a decrease in the proportion of diarrhea hospitalizations attributable to rotavirus, compared with other causes of severe diarrhea in children. This decrease is probably due to the improvement of hygiene conditions (Parashar *et al.*, 2006; Rendi-Wagner *et al.*, 2006). Rotavirus A causes acute gastroenteritis in children and asymptomatic illness in adults. Rotavirus are responsible for 30-50% of hospital admissions in Thailand and about 39% in USA (Maneekarn and Ushijima, 2000; Jiraphongsa *et al.*, 2005; Parashar *et al.*, 2006). Due to the magnitude of these values, the implementation of a safe and effective vaccine, such as Rotarix (RIX4414) is important (Vesikari *et al.*, 2007), allowing children immunization (Santos *et al.*, 2008).

The presence of rotavirus in water and in faecal samples has been detected using different techniques. Consequently, the results obtained differ significantly (detection of 5% to 90%) (Table 1). In faecal samples, immunological tests (EIA) are usually used for rotavirus detection, while their detection in environmental waters has been performed by PCR. The percentage of positive samples with rotavirus in environmental waters is higher (63% to 90%) than the observed in faecal samples (10% to 37%) (Table 1).

Table 1. Incidence of rotavirus in faecal samples and environmental waters in different locations in the world.

Rotavirus					
Samples	Viral detection	Number of samples	Method of detection	Countries	Reference
Faeces	10%	458	EIA	Guatemala	(Cruz *et al.*, 1990)
	16%	284	EIA	Saudi Arabia	(Meqdam and Thwiny, 2007)
	16%	262	PCR	Thailand	(Kittigul *et al.*, 2009)
	20%	3768	EIA*	Brazil	(Carraro *et al.*, 2008)
	23%	380	EIA + PAGE	Brazil	(Andreasi *et al.*, 2007)
	37%	3270	EIA + PAGE	Brazil	(Costa *et al.*, 2004)
River water	90%	41	PCR	Germany	(Hamza *et al.*, 2009)
Polluted river water	77%	30	PCR	Venezuela	(Rodríguez-Díaz *et al.*, 2009)
Residual water	63%	396	PCR	Spain	(Portella, 1997)
Sewage water	67%	357	PCR	Spain	(Villena *et al.*, 2003)
Sewage water	86%	35	PCR	Egypt	(Villena *et al.*, 2003)

EIA: enzymoimmunoassays
PCR: polymerase chain reaction
PAGE: polyacrylamide gel electrophoresis
* VIKIA® Rota-Adeno kit

2.2. Norovirus

Norovirus is a group of noncultivable viruses that belong to the family *Caliciviridae*. They are single-stranded RNA, nonenveloped, viruses with 35-40 nm in diameter and with approximately 7.5 Kb (Atmar and Estes, 2001; Green *et al.*, 2001; Hutson *et al.*, 2004; Haramoto *et al.*, 2005). Norovirus is a genetically diverse group classified into five distinct genogroups (GI to GV) based on the diversity of capsid sequences. Human norovirus strains include members of the GI, GII and GIV groups and are further subdivided into at least 8 genotypes for GI and 19 genotypes for GII (Khamrin *et al.*, 2007; Webby *et al.*, 2007; Kittigul *et al.*, 2009). Genogroup II, genotype 4 (GII.4) strains are the most commonly associated with outbreaks of human acute gastroenteritis worldwide (Chen *et al.*, 2009; Zheng *et al.*, 2009).

Norovirus affect people of all ages and are recognized as the major cause of outbreaks as well as sporadic cases of acute gastroenteritis, causing more than 90% of nonbacterial and approximately 50% of all causes of epidemic gastroenteritis worldwide (Koopmans *et al.*, 2002; Widdowson *et al.*, 2005a; Svraka *et al.*, 2007; Braham *et al.*, 2009).

Norovirus is highly infectious, being as low as 10-100 virus particles sufficient to established disease. Their high transmissibility represents a serious public health problem and economic impact. Currently, candidate vaccines are being tested for use in the prevention of norovirus disease (Hutson *et al.*, 2004).

Norovirus were found to be one hundred time more abundant in winter than in summer in wastewater treatment, but this seasonal profile is not consistent, since high numbers of norovirus have also been detect in summer (Katayama *et al.*, 2008), suggesting that these viruses circulate in human environment throughout the year. The survival strategy of norovirus in summer is important from the epidemiological point of view, and might be a key to reduce the outbreaks in winter (Katayama *et al.*, 2008).

The main routes of norovirus transmission are not consensual. Some studies indicate that person-to-person direct contact is the main route, while others indicate that the consumption of contaminated food and water might be more important (Hutson *et al.*, 2004; Webby *et al.*, 2007; Guyader *et al.*, 2008). Recent studies have demonstrated that norovirus infection was undervalued before mid 1990s because of the poor sensitivity of the available diagnostics methods at the time such as electron microscopy and antigen detection assays (Patel *et al.*, 2008). However, because novel assays are not usually available outside of reference laboratories, the true global prevalence and potential economic impact remains unrecognized (Lopman, 2006). Furthermore, viral carriers can be asymptomatic and still passing on the virus to other people, difficulting source tracking of the infection. The mechanism of pathogenesis of norovirus infection can damage the intestinal epithelium, as a result of viral replication and the severity of the symptoms may be related with the presence of high viral concentration (Phillips *et al.*, 2009).

Recent researches based on molecular biology suggest that Norovirus (GII.4) are very resistant to free chlorine disinfection, while rotavirus are completely inactivated under the same disinfection condition (Espinosa *et al.*, 2008). Nevertheless, procedures for the better disinfection of waters are being tested in order to reduce the risk of norovirus gastroenteritis from drinking water exposure (Shin and Sobsey, 2008).

Though the detection of norovirus in faecal samples is not high (between 7% and 36%), the detection can be 100% in sewage water or 16% in seawater (Table 2).

Table 2. Incidence of norovirus in faecal samples and environmental waters in different locations in the world.

Norovirus

Samples	Viral detection	Number of samples	Method of detection	Countries	Reference
Faeces	7%	173	PCR	India	(Monica *et al.*, 2007)
	12%	50	PCR	France	(Bon *et al.*, 1999)
	16%	669	PCR	Netherlands	(Wit *et al.*, 2001)
	21%	50	PCR	Germany	(Oh *et al.*, 2003)
	22%	201	PCR	Taiwan	(Wu *et al.*, 2008)
	31%	248	PCR	Peru	(Oh *et al.*, 2003)
	36%	2422	PCR	England	(Amar *et al.*, 2007)
Seawater	16%	19	PCR	Italy	(Rosa *et al.*, 2007)
Sewage water	83%	18	PCR	Venezuela	(Rodríguez-Díaz *et al.*, 2009)
Sewage water	100%	9	PCR	Japan	(Ueki *et al.*, 2005)
Sewage water	100%	10	PCR	Italy	(Rosa *et al.*, 2007)
River water	44%	64	PCR	Japan	(Haramoto *et al.*, 2005)
River water	45%	58	PCR	South Korea	(Lee and Kim, 2008)
River water	67%	8	PCR	Japan	(Ueki *et al.*, 2005)

PCR: polymerase chain reaction

2.3. Adenovirus

Human adenovirus belong to the *Adenoviridae* family, which is divided in 51 different serotypes classified into six species (A through F), on basis of biophysical, biochemical and genetic properties (Benkö and Harrach, 2003a; Shimizu *et al.*, 2007). These enteropathogens are double-stranded DNA viruses without envelope (Dey *et al.*, 2009). The double-stranded DNA genome confers exceptional resistance to purification and disinfection processes; and allows the virus to use the host-cell enzymes during replication to repair damage in the DNA caused by the UV light or chlorine (Nwachcuku and Gerba, 2004; Heerden *et al.*, 2005). Due to this repair mechanism the adenovirus are included as candidates in the list of contaminants of the United States Environmental Protection Agency (Heerden *et al.*, 2005; Xagoraraki *et al.*, 2007). However adenovirus are sensitive to inactivation by oxidizing (Hijnen *et al*., 2006).

The most commonly adenovirus associated with gastroenteritis in young children are species F (serotype 40 and 41) and species A (serotype 12, 18 and 31) (Aminu *et al*., 2007). Several studies indicate that serotype 41 was predominant compared to serotype 40 (Fukuda *et al.*, 2006; Katayama *et al.*, 2008). Adenovirus 40 and 41 are unculturable viruses (Dey *et al.*, 2009a).

Adenovirus are mainly responsible for respiratory infections, however adenovirus infection also includes dehydration, abdominal pain and vomiting associated with gastroenteritis infections (Dey *et al.*, 2009a).

Most studies indicate that adenovirus does not display seasonality (Wong *et al.*, 2008), consequently, have been suggested as a viral indicator of human faecal contamination of water (Muscillo *et al.*, 2008; Albinana-Gimenez *et al.*, 2009). However, some studies showed that adenovirus incidence was higher in the winter/rainy season and in spring (Carraro *et al.*, 2008; Dey *et al.*, 2009a).

The incidence of adenovirus is higher in contaminated environmental waters than in faecal samples (Table 3), but these differences can be associated with the use of different detection method.

Table 3. Incidence of adenovirus in faecal samples and environmental waters in different locations in the world.

Adenovirus

Samples	Viral detection	Number of samples	Method of detection	Countries	Reference
Faeces	2%	917	PCR	Bangladesh	(Dey *et al.*, 2009a)
	2%	3060	EIA + PCR	Brazil	(Filho *et al.*, 2007)
	3%	892	EIA	Hiroshima	(Fukuda *et al.*, 2006)
	8%	337	PCR	Japan	(Shimizu *et al.*, 2007)
	22%	458	EIA	Guatemala	(Cruz *et al.*, 1990)
	23%	282	EIA	Nigeria	(Aminu *et al.*, 2007)
	27%	60	PCR	Sweden	(Allard *et al.*, 1990)
Sewage water	76%	25	PCR	Portugal	(Duarte, 2007)
Sewage water	100%	60	PCR	Spain	(Puig *et al.*, 1994)
River water	22%	188	PCR	South Africa	(Heerden *et al.*, 2005)
Polluted river water	50%	12	PCR	Venezuela	(Rodríguez-Díaz *et al.*, 2009)
River water	97%	41	PCR	Germany	(Hamza *et al.*, 2009)
River water	100%	60	PCR	Spain	(Puig *et al.*, 1994)
Tap water	32%	188	PCR	South Africa	(Heerden *et al.*, 2005)
Tap water	39%	23	PCR	Korea	(Lee and Kim, 2002)

EIA: enzymoimmunoassays
PCR: polymerase chain reaction

2.3. Other viruses

Astrovirus are important enteropathogens associated with worldwide diarrhea outbreaks in all ages. It belongs to the *Astroviridae* family (Matsui and Greenberg, 2001; Mitchell, 2002; Simpson *et al.*, 2003), being single-stranded RNA and nonenveloped viruses (Matsui and Greenberg, 2001).

Human bocavirus was recently described as new specie belonging to the *Parvoviridae* family (Ma *et al.*, 2006; Vicente *et al.*, 2007). The genus *Bocavirus* is constituted by human bocavirus, bovine parvovirus and canine minute virus (Foulongne *et al.,* 2006*)*. Bocavirus is a double-stranded DNA virus without envelope and has been associated with upper and lower respiratory tract disease and gastroenteritis worldwide (Arden *et al.*, 2006; Chow and Esper, 2009).

Sapovirus has positive sense single-stranded RNA virus, belonging to the *Caliciviridae* family and is a causative agent of gastroenteritis in children and adults, responsible for a few outbreaks (Katayama *et al.*, 2004; Hansman *et al.*, 2007; Zhang *et al.*, 2008).

The aichi virus is a small round virus with a single-stranded positive sense RNA that has been recently integrated in *Picornaviridae* family, genus Kobuvirus (Pham *et al.*, 2007). This virus is associated with gastroenteritis in humans in world (Yamashita *et al.*, 1998; Pham *et al.*, 2007).

The human enterovirus are divided into 65 serotypes classified into four species (species A to D), based on phylogenetic analysis of multiple genome regions (Oberste *et al.*, 2006). Enterovirus are small, icosahedral particles with approximately 27 nm in diameter (Percival *et al.*, 2004). Is a group of nonenveloped RNA viruses that belong to the *Picornaviridae* family (Oberste *et al.*, 2006; Simmonds and Welch, 2006). They can cause widespread infections in human and other mammalian populations. Enterovirus are genetically highly variable, and recombination within and between serotypes contributes to their genetic diversity (Simmonds and Welch, 2006).

Hepatitis A is a viral liver disease that can cause mild to severe illness. It is a nonenvelope, simple positive stranded RNA virus (WHO, 2008; Barrella *et al.*, 2009). Hepatitis A occurs sporadically and in epidemics worldwide (WHO, 2008). Its organization is typical of picornoviruses, but is sufficiently distinct to be classified in its own genus Hepatovirus A (Percival *et al.*, 2004). The virus replicates in the hepatocytes of the liver causing local necrosis and a marked response by lymphocytes (Percival *et al.*, 2004). The virus remains viable for months in water, sewage and shellfish (Sobsey, 1989).

The most recently recognized viral cause of hepatitis is now known as hepatitis E virus, usually resulting in a self-limited disease. Hepatitis E is a waterborne disease (Daniels *et al.*, 2009). Its genome is single-stranded, positive sense RNA and is nonenveloped virus (Percival *et al.*, 2004).

3. Human enteric viruses detection

The basic steps of virological analysis of environmental waters are sampling, virus concentration, purification and detection.

3.1. Concentration methods

Virus concentrations in environmental samples are low and, consequently, the direct detection is not easy, being necessary to concentrate water samples before virus detection (Hurst *et al.*, 1997; Bosch *et al.*, 2008; Verheyen *et al.*, 2009). This leads to a need of large sample volume that is reduced by concentration to a small volume.

Viral concentration techniques have an inherent inefficiency which results in some virus loss. Also, some virus inactivation may occur during concentration processes as a result of changes in pH and addition of chelating chemicals (Hurst *et al.*, 1997). A major problem of concentration techniques is the addition of PCR inhibitors (skimmed milk) and the concentration of pre-existing inhibitors (fulvic acids, humic acids and metals) in the samples. So a variety of nucleic acid purification procedures have been developed to solve this problem (Percival *et al.*, 2004; Bosch *et al.*, 2008).

A good virus concentration method should fulfil several criteria: it should be technically simple, fast, provide high virus recovery rate, concentrate a large range of viruses, do not alter viral community structure, provide a small volume of concentrate, be inexpensive, be capable of processing large volumes of water and be repeatable (within a laboratory) and reproducible (between laboratories). However, there is not a method that fulfils all these requirements (Percival *et al.*, 2004; Bosch *et al.*, 2008; Albinana-Gimenez *et al.*, 2009).

There are several types of methods for virus concentration from environmental water samples. These methods include adsorption-elution, precipitation, ultracentrifugation, lyophilisation, ultrafiltration and magnetic beads (Table 4). The choice of the concentration technique takes in account the volume of the final concentrate and the characteristics of the sample. Usually, these methods are not used alone, rather, two concentration techniques are normally used. However, the techniques are often modified to take greater profitability and lower costs.

Table 4. Methods used to concentrate viruses in environmental waters (from Bosch, 2008).

Method	Principle	Advantages	Limitations
Adsorption-elution			
Negatively charged filters	Ionic charge	Good recoveries	Requires sample preconditioning
Positively charged filters		Good recoveries	Costly
Glass powder		Cheap. Good recoveries	Fragile apparatus
Glass wool		Good recoveries	Differences depending on manufactures
Precipitation			
Organic flocculation	Chemical precipitation	Efficient for dirty samples or as secondary concentration	Beef extract is inhibitory to RT-PCR enzymes
Ammonium sulfate		Efficient for dirty samples or as secondary concentration	High cytotoxicity. Inhibitory to RT-PCR enzymes
Polyethylene glycol		Efficient for dirty samples or as secondary concentration	Intra-assay variability
Ultracentrifugation	Physical sedimentation	Efficient as secondary concentration	Costly
Lyophilisation	Freeze-drying	Efficient for dirty samples or as secondary concentration. May remove RT-PCR enzymes inhibitors	Costly. Time-consuming
Ultrafiltration	Particle size separation	Good recoveries for clean samples	Costly. Time-consuming
Magnetic beads	Immunoaffinity	Good recoveries for small volumes	Requires specific assay for each virus. Costly. Little data available

The adsorption elution method is a method based on sample filtration where the positive or negative filters adsorb the viruses because of electrostatic and hydrophobic interactions. Small volumes of alkaline buffers are used to elute the viral particles from the filter, resulting in a concentrated solution (eluate) containing the virus (Roepke *et al.*, 1988).

Ultrafiltration is a process that uses anisotropic semi-permeable membranes to separate particles like virus on the basis of the size. The type of purification or concentration depends of the membranes (Cheryan, 1998).

Precipitation (organic flocculation) is a method in which viruses in sample solution are adsorbed into flakes by the addition of beef extract or skimmed milk (protein solutions). The flakes are recovered by centrifugation and the viruses are retrieved by solubilizing the flakes in small volumes of phosphate solutions (Katzenelson *et al.*, 1976; Shields and Farrah, 1986). This method involves drastic changes in pH to produce flocculation of proteins. The utilization of a protein solution for virus adsorption gives a high recovery but does not allow readsorption and can cause inhibitory effects on PCR detection (Katzenelson *et al.*, 1976; Abbaszadegan *et al.*, 1993; Schwab *et al.*, 1995; Shieh *et al.*, 1995; Jaykus *et al.*, 1996).

Ultracentrifugation as secondary concentration is a method capable of concentrate virus in a sample providing sufficient g force and time. Differential ultracentrifugation allows separation of different virus types (Percival *et al.*, 2004). The ultracentrifugation of viruses has been shown to be the better concentration method (Satoh *et al.*, 2003). The advantages are that the viral community is not altered, since the samples are not manipulated (the pH is not changed), the method is fast and simple, and with a low volume of sample it is possible to reach high recovery rates. The disadvantage is the need to have expensive equipment, an ultracentrifuge.

After virus concentration it is necessary to determine the recovery rate by viral quantification, which can be done by plaque assay, electron microscopy, addition of a known concentration virus, epifluorescence microscopy or quantitative PCR. Quantitative PCR and epifluorescence microscopy are the preferred methods for counting viruses in environmental waters because of the higher accuracy and precision (Suttle, 2005). However, it is necessary to take into account that quantification by quantitative PCR and by microscopy does not provide information about the infectivity of the viruses. The quantification by plaque assays on cell cultures allows the evaluation virus infectivity but it is an expensive and time consuming method.

Detection and quantification are conveniently considered together, since for many virus they are performed simultaneously, particularly where the virus multiplies in culture and infectivity assays are done (Percival *et al.*, 2004).

3.2. Detection methods

The virus can be detected by cell culture, antigen and nucleic acid detection (Hurst *et al.*, 1997). The efficiency of virus detection is the result of two factors well related: the recovery efficiency of the concentration technique and the purity of the recovered viruses (Bosch *et al*., 2008).

Nowadays techniques for amplification of nucleic acids (PCR, Reverse Transcriptase PCR and Real Time PCR) are the most used to detect viruses in water. These techniques allows to obtain important epidemiological information from the genome, particularly with regard to vaccination programs (Bosch *et al*., 2008). Molecular techniques are sensitive, specific, rapid and cost effective, but do not allow the evaluation viral infectivity, while virus detection by cell culture allows the evaluation viral infectivity, but has low sensitivity, is time consuming, laborious and expensive. However, the biggest problem is that some viruses cannot be easily cultivated and others are not even cultivable (Gilgen *et al*., 1997).

Among the immunological methods, enzymoimmunoassays (EIA) are the most used due to higher sensitivity and specificity. The method of immunochromatography, is a simple and fast procedure that does not require specific equipment. Its specificity and sensitivity is similar to enzymoimmunoassays (Flewett *et al.*, 1989; Wilhelmi *et al.*, 2001; Bon *et al.*, 2007). These immunological techniques are available for many viruses and can be used when large amounts of viral antigens are present in a sample. They have been used successfully in clinical microbiology for the detection of viruses in clinical samples and for the detection of enteric viruses in water samples. However, these methods are less sensitive and specific than the molecular ones and are very susceptible to interference from external material in environmental concentrated samples. Moreover, these techniques do not provide information about virus infectivity (Hurst *et al.*, 1997).

The objectives of this work were:

- To evaluate the fiability of the immunological test VIKIA® Rota-Adeno (BioMérieux) to detect rotavirus A and adenovirus in human faecal samples.

- To evaluate the seasonality and distribution by age group of patients with gastroenteritis symptoms and rotavirus A and adenovirus infection.

- To compare two methods, ultracentrifugation and organic flocculation, for virus concentration in residual waters, using the total viral number to evaluate the recovery rate.

- To evaluate the suitability of the two concentration methods to detect enteric viruses (rotavirus A and adenovirus) by molecular methods (RT-PCR and nested PCR).

CHAPTER 2

Incidence of Rotavirus and Adenovirus: detection by molecular and immunological methods in human faeces from gastroenteritis episodes

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ABSTRACT

Rotavirus and adenovirus are among the main responsible for gastroenteritis worldwide and, consequently it is imperative to know their impact in the human population. With the optimization of molecular and immunologic methods, the comparison between the efficacy of these two detection methods of enteric viruses is important. This study compares the ability of the immunological method VIKIA® Rota-Adeno (BioMérieux, France) and molecular methods (nested PCR and Reverse Transcriptase-PCR) to detect rotavirus A and adenovirus in human faecal samples of patients with gastroenteritis symptoms from Hospital Infant D. Pedro of Aveiro (Portugal). From December 2008 to July 2009, 18 faeces samples were analyzed using immunologic and molecular methods. Eleven (61%) of the 18 samples were positive for rotavirus using the kit VIKIA® Rota-Adeno and 10 (56%) using RT-PCR. The adenovirus was detected using VIKIA® Rota-Adeno kit in 1 (6%) sample, while by nested PCR 16 (89%) of the samples were positive. These results showed that the rotavirus is the most frequent virus using the immunologic kit VIKIA® Rota-Adeno. Although using molecular methods adenovirus is more frequent in gastroenteritis episodes. Sequence analysis of PCR amplification confirmed the presence of human rotavirus A and human adenovirus 41 in 15 faecal samples analyzed, where the results of VIKIA® Rota-Adeno kit were negative. So, PCR it seems to be a good detection method of rotavirus A and adenovirus. Through PCR results, 50% of the 18 samples analysed had the simultaneous presence of rotavirus A and adenovirus. From January 2006 to July 2009, 474 faecal samples were collected from individuals with gastroenteritis symptoms In 467 samples the presence of rotavirus A and adenovirus was detected using the immunochromatographic VIKIA® Rota-Adeno kit. Among these, 59 (12.6%) samples were positive for rotavirus A and 5 (1%) were positive for adenovirus. Suggesting that rotavirus A and adenovirus are epidemiologic agents of gastroenteritis. The incidence of viral gastroenteritis, as the incidence of rotavirus infection, showed a clear seasonal and age patterns of variation with higher incidence during the cold months and in children under than 5 years old.

Key words: Rotavirus A, Adenoviruses, gastroenteritis, faeces, nested PCR, RT-PCR, VIKIA® Rota-Adeno, seasonal variation, age incidence.

Introduction

Despite the improvement of sanitary conditions, diarrhoea remains the leading cause of disease worldwide, and the viruses has been responsible for most outbreaks of gastroenteritis (Patel *et al.*, 2008; Braham *et al.*, 2009). Worldwide the main enteric virus responsible for gastroenteritis is rotavirus A, astrovirus, adenovirus, sapovirus and norovirus, and young children are particularly affected. The contact person-to-person, ingestion of contaminated water,

consumption of contaminated food and contact with polluted recreational water are the main transmission ways (Mead *et al.*, 2000; Oh *et al.*, 2003; Froggatt *et al.*, 2004; Wit *et al.*, 2004; Goodgame, 2006).

The incidence of sporadic gastroenteritis and acute infectious diarrhoea is very different among countries. This can be, in part, associated to the fact that the confirmation of these cases is rare because a large fraction of the patients do not seek medical attention (Goodgame, 2006) and also only a small number of laboratories are prepared to detect virus, namely in developing areas (Weitzel *et al.*, 2007). Recent studies suggest, however, that children mortality associated with diarrhoea has decreased in the past 20 years and the number of hospitalized people has declined between 2000 and 2004, due to an improvement in sanitary conditions, hygiene habits and less consumption of contaminated water and food (mainly shellfish) (Parashar *et al*., 2006).

Rotavirus A and adenovirus are among the most commonly recognized causes of epidemic viral acute gastroenteritis worldwide (Wu *et al*., 2008). Rotavirus A are considered the major etiological agents of acute diarrhoea in infants and young children (Andreasi *et al*., 2007). It is a nonenveloped double-stranded RNA virus (Dey *et al.*, 2009b). Rotavirus A infection displays a tendency for seasonality in temperate areas where peaks occur predominantly in winter, but their infection occurs throughout the year in tropical areas (Tiemessen *et al.*, 1989; Inouye *et al.*, 2000). Adenovirus is considered a very significant enteric virus. They are gastroenteritis-associated emerging virus, responsible for a higher number of outbreaks in nurseries, schools and hospitals (Chiba *et al.*, 1983; Van *et al.*, 1992; Mulholland, 2004; Akihara *et al.*, 2005; Muscillo *et al.*, 2008). Human adenovirus belong to the *Adenoviridae* family and are nonenveloped double-stranded DNA virus. To date more than 51 human adenovirus serotypes have been identified, and classified into six species (A to F) based on biophysical and biochemical criteria (Benkö and Harrach, 2003b; Davison *et al.*, 2003). Adenovirus is associated with gastroenteritis in children, namely the species F (serotypes 40 and 41) and species A (serotypes 12, 18 and 31). Serotypes 40 and 41 are the first responsible for gastroenteritis, being the most frequent serotypes detected in hospitalized children (Uhnoo *et al.*, 1984; Aminu *et al.*, 2007; Shimizu *et al.*, 2007).

The most frequently used method to detect rotavirus and adenovirus in clinical samples are enzymoimmunoassays (EIA), since they are simple and fast (Flewett *et al.*, 1989; Wilhelmi *et al.*, 2001; Bon *et al.*, 2007). Nowadays, techniques like PCR have been introduced as a convenient and powerful method to confirm the diagnosis. PCR is an highly sensitive and specific method for virus detection (Logan *et al.*, 2006). Additionally, genome amplification by PCR allows further characterization of the viruses by sequence analyses (Takeuchi *et al.*, 1999; Phan *et al.*, 2004). It

has been reported that the detection rate of enteric viruses by molecular methods is higher than that of immunological tests (Pring-Akerblom *et al.*, 1997; Sen *et al.*, 2000; O'Neill *et al.*, 2002). Logan *et al*. (2006) showed that the detection rate of rotavirus A by RT-PCR is 111% superior to that obtained by EIA, and also report that the detection of adenovirus by PCR was 175% increased relatively to EIA detection.

The main objective of this work was to compare the detection of rotavirus and adenovirus by molecular techniques (RT-PCR and nested PCR) and immunological test (kit VIKIA® Rota-Adeno) in human faeces of individuals with gastroenteritis symptoms. Additionally, the seasonal variation and the distribution by age of the two viruses were also studied.

Material and methods

Faecal samples

A total of 474 faecal samples were collected from babies, children and adults with gastroenteritis symptoms in Hospital Infant D. Pedro, Aveiro city, Portugal, between January 2006 and July 2009.

Detection of rotavirus A and adenovirus by VIKIA® Rota-Adeno

Among 474 faeces samples, 467 were subjected to immunochromatographic analysis using the VIKIA® Rota-Adeno (BioMérieux) for double detection of rotavirus A and adenovirus according to the manufacturer´s instructions. This kit is based on the dual detection of rotavirus and adenovirus. It is a rapid three step qualitative test based on the association of monoclonal antibodies specific to rotavirus A and genus adenovirus (BioMérieux, 2003; Téllez *et al.*, 2008).

Nucleic acid extraction

From all 18 samples collected, a faecal suspension was prepared diluting small aliquots of sample in 500 µL of distilled water that was vortexed and centrifuged at 5000 x g for 15 min. The viral nucleic acids were extracted from the supernatant using geneMAG-RNA/DNA kit (Chemicell® Germany). The magnetic RNA/DNA purification kit was used according to the manufacturer´s instructions.

Detection of rotavirus A by RT-PCR

RT-PCR was performed on RNA extracted using the OneStep RT-PCR kit (Qiagen®Germany) according to the manufacturer´s recommendations. Detection of rotavirus A was performed using the primers described by Villena *et al.* (2003) that correspond to an highly conserved region of group A rotavirus: VP6-3 (5´ GCT TTA AAA CGA AGT CTT CAA 3´; positions 2 to 23 of human strain) and VP6-4 (5´ GGT AAA TTA CCA ATT CCT CCA G 3´; positions 187 to 166 of human strain). The RT-PCR was performed at 42°C for 30 min, 95°C for 15 min; followed by 40 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light. The PCR was considered positive when specific band products with 186 bp were observed.

Detection of adenovirus by nested PCR

For adenovirus detection a nested PCR approach was conducted using the primers described by Allard *et al.* (2001). The primers hex1deg (5´ GCC SCA RTG GKC WTA CAT GCA CAT C 3´; S=C+G; R=A+G; K=T+G; W=A+T) and hex2deg (5´ CAG CAC SCC ICG RAT GTC AAA 3´; I=deoxyinosine) created a 301 bp products. The nested primer pair, nehex3deg (5´ GCC CGY GCM ACI GAI ACS TAC TTC 3´; Y=C+T; M=A+C) and nehex4deg (5´ CCY ACR GCC AGI GTR WAI CGM RCY TTG TA 3´) produced a 171 bp products. The amplifications were carried out in 20 mL reaction mixtures containing 1x Taq Buffer with KCl (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 0.28 mM dNTP (Fermentas), 0.4 µM of each primer and 1 U/µL of Taq DNA polymerase (Fermentas). To reaction mixture of first PCR was added 5 µL of sample and 2 µL to the nested PCR. The first PCR was performed at 95°C for 10 min, immediately followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 5 min. The second amplification was performed at 94°C for 3 min, immediately followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 5 min.

PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide and visualized under UV light.

Positive and negative controls

Rotavirus A and adenovirus 41 suspensions, with unknown concentrations, were used as positive controls. Viral nucleic acids were extracted and stored at -20°C.

Negative controls were also realized, adding RNase free water to reaction mixture, instead the sample.

Nucleotide sequence analysis

PCR amplification products were subjected to sequence analysis in BigDyeTerminator v1.1 from Applied Biosystems, using forward primer VP6-3 for rotavirus A identification and the forward primer Nehex3deg for adenovirus identification, to confirm the results from PCR.

Results

Incidence of patients with gastroenteritis symptoms

Through the observation of the 474 patients with gastroenteritis symptoms was possible to see that the number of gastroenteritis was similar during the period of study (2006-2009) (Table 5). It was also observed a clear seasonal pattern of variation with low incidence in the summer months, between July and September (Figure 1).

Table 5. Incidence of cases with gastroenteritis symptoms between 2006 and 2009 by month.

	2006 (n=103)	**2007** (n=108)	**2008** (n=166)	**2009** (n=97)	Total of cases	Average of cases
January	8	2	15	13		10
February	7	10	9	17		11
March	12	18	20	12		16
April	4	8	12	19		11
May	8	12	14	19		13
June	13	16	13	17		15
July	9	9	10	-		9
August	10	9	8	-		9
September	8	3	9	-		7
October	11	6	20	-		12
November	5	9	15	-		10
December	8	6	21	-		12

n: number of total cases by year

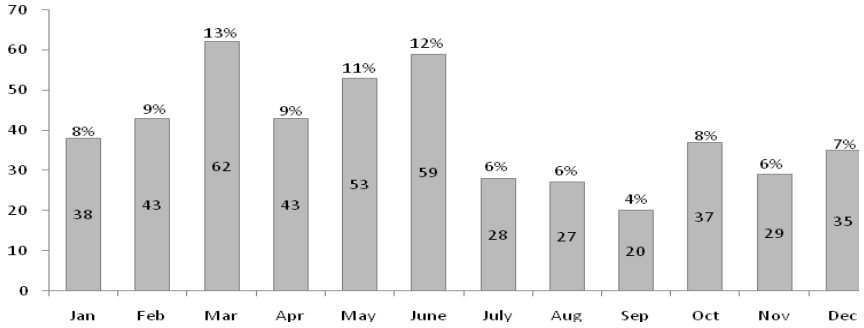

Figure 1. Average of seasonal variation in gastroenteritis cases during the study period.

Regarding the distribution of gastroenteritis cases, it was observed that 73% of the cases occurred in children less than 5 years old, with particular incidence in the group under one year (Figure 2).

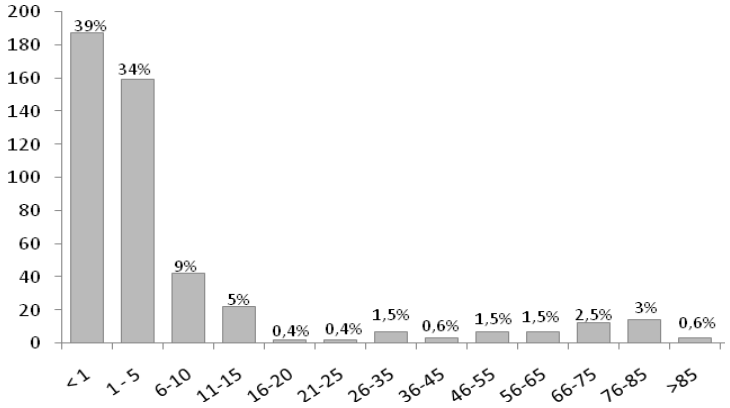

Figure 2. Gastroenteritis cases by age group during the study period.

Incidence of rotavirus A and adenovirus

Through the observation of 467 cases, rotavirus A infection occurred in 59 (12.6%) patients. The incidence of viral gastroenteritis caused by rotavirus was higher in the winter months, being 42 (71%) cases registered between December and March (Figure 3). The rotavirus incidence by age group was higher (56 cases; 95%) in children under 5 years old, particularly in babies less than 1 year (Figure 4).

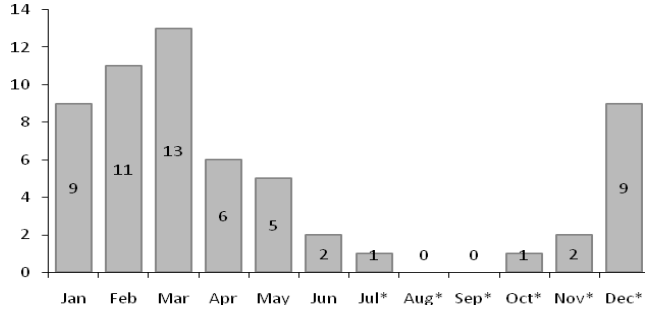

Figure 4. Seasonal incidence of rotavirus A during the study period.

*Months that does not include 2009.

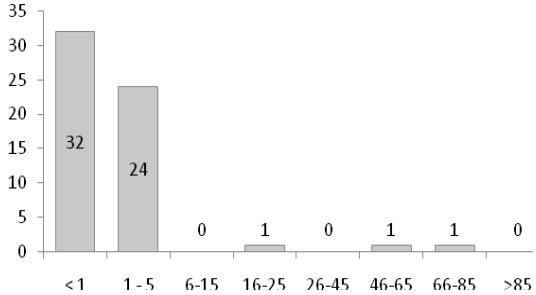

Figure 4. Incidence of rotavirus A by age group during the study period.

Adenovirus infection detected by VIKIA® Rota-Adeno between January 2006 and July 2009 was much lower, with only 5 (1%) positive cases, so did not allow to conclude about adenovirus annual distribution. Nevertheless in the 5 cases observed 4 of them occurred in children with pre-scholar age (Table 6).

Table 6. Incidence of gastroenteritis cases caused by adenovirus during the study period.

	Nº of cases	Month	Sex	Age group
2006	1 case	July	M	45-55
2007	1 case	March	F	<1
2008	3 cases	May	M	<1
		October	M	<1
		December	M	1-5

Detection of rotavirus A and adenovirus by molecular and immunological methods

Between December 2008 and July 2009, rotavirus A and adenovirus infection were also detected by RT-PCR and nested PCR, respectively (in a total of 18 samples). From the 18 samples analyzed, 11 (61%) were positive for rotavirus A detected by VIKIA® Rota-Adeno and 10 (56%) were positive for RT-PCR (Table 7). For adenovirus infection 1 (6%) sample was positive using VIKIA® Rota-Adeno kit and 16 (89%) samples were positive using nested PCR (Table 7). Regarding to rotavirus A the molecular and immunological methods had a concordance of 83%, while for adenovirus the agreement of both methods it was only 17%.

In the 18 samples analyzed, 9 (50%) were positive to both virus using PCR as detection method.

Table 7. Rotaviruses and Adenoviruses detected by VIKIA® Rota-Adeno and PCR in faecal samples.

		Samples																		Total
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Rotavirus	VIKIA® Rota-Adeno	+	+	+	+	-	+	+	-	+	-	-	-	-	+	+	-	+	+	11
	RT-PCR	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	+	+	10
Adenovirus	VIKIA® Rota-Adeno	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
	nested-PCR	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	16

Sequence analysis

The results from PCR and immunochromatographic kit that were not concordant were subjected to sequence analysis. The sequencing results showed that the positive results of PCR were due to the presence of the viruses and were not false positives. The sequencing result for the sample 8 with positive PCR and negative VIKIA® Rota-Adeno kit showed that the sample was positive for rotavirus A that was identified as human rotavirus A with 97% homolog identity. In the 15 samples of adenovirus witch the PCR and VIKIA® Rota-Adeno kit were not concordant (samples 1, 3, 4, 6, 7,

8, 10, 11, 12, 13, 14, 15, 16, 17, 18) showed the presence of human adenovirus specie F serotype 41, being the lower percentage of identity 91%.

Discussion

Molecular epidemiological studies are increasingly popular in the field of clinical viruses research (Phan *et al.*, 2004). The developing of molecular diagnosis has allowed the detection of enteric viruses previously undetected due to their inability to grow in conventional cell culture systems (Braham *et al.*, 2009) and has made possible to detect enteric viruses in negative results using immunological tests (O'Neill *et al.*, 2002; Logan *et al.*, 2006).

This study showed that in 18 faecal samples analysed, using VIKIA® Rota-Adeno kit and RT-PCR, rotavirus A infection was detected in 11 (61%) and 10 (56%) cases, respectively, while adenovirus was present in 1 (6%) detected by VIKIA® Rota-Adeno kit and 16 (89%) detected by nested PCR. The results of VIKIA® Rota-Adeno kit clearly suggest that rotavirus A is the most frequent virus associated with gastroenteritis symptoms. Although the molecular analysis indicates that adenovirus is more frequent. All the results that were negative for VIKIA® Rota-Adeno kit and positive for molecular methods were submitted to sequencing identification of the amplification product that confirmed the presence of human rotavirus A in one sample and the presence of human adenovirus 41 in 15 faecal samples analyzed.

Therefore since the percentage of rotavirus A detected using RT-PCR and VIKIA® Rota-Adeno kit was similar and the immunochromatographic test is rapid and simple, so the VIKIA® Rota-Adeno kit seem to be a good method to diagnostic rotavirus A infections. On the other hand, Tellez *et al.* (2008) also compared VIKIA® Rota-Adeno with RT-PCR for rotavirus A detection and the results showed that the concordance between the two methods was juts 30%. The study also conclude that the sensibility of the kit was high (100%) but the specificity was low (24.2%). So, it is possible to conclude that molecular methods like PCR even being more time consuming than immunological tests, at the moment, are the best to detect rotavirus A and adenovirus in clinical samples, because of his higher specificity. Nevertheless, the low results obtained in adenovirus identification using VIKIA® Rota-Adeno kit can be explained by the possibility to occur false negative results when the number of viral particles is too low (BioMérieux, 2003; Téllez *et al.*, 2008).

Other important evidence in this work, is the fact that 50% of the 18 samples analyzed had simultaneously the presence of rotavirus A and adenovirus, suggesting that both viruses can act together causing gastroenteritis (Aminu *et al.*, 2007).

The analysis of 467 faecal samples from patients with gastroenteritis symptoms, using the immunochromatographic kit, suggests that rotavirus A and adenovirus are causative agents of gastroenteritis, being rotavirus A responsible for 59 (12.6%) of the cases and adenovirus for just 5 cases (1%). Analysis of medical records of 467 patients with gastroenteritis symptoms indicates that the occurrence of gastroenteritis episodes all over the year, with lower incidence between July and September, which is in accordance to previous studies (Jothikumar *et al.*, 2005; Kittigul *et al.*, 2009). Furthermore, 73% of the cases occurred in children less than 5 years old. The results also demonstrated the high incidence of rotavirus A and adenovirus infection in children with pre-school age (Breitbart *et al*., 2008). Rotavirus cold seasonality also meets with the expected (Rendi-Wagner *et al.*, 2006).

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CHAPTER 3

Ultracentrifugation versus flocculation methods to concentrate viruses in residual water

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ABSTRACT

Water quality, and consequently human health, is affected by the presence of pathogenic microorganisms that comes from sewage and that cause several infections such as viral gastroenteritis and hepatitis. Due to the low levels of some pathogenic viruses in aquatic environments (e.g. human enteric viruses), it is fundamental to develop efficient approaches to concentrate viral particles from large volumes of water. Despite the advances in concentration and detection methods of viruses, it is still a challenge to find the ideal concentration technique to recover viruses from environmental samples. The main goal of this work was the comparison of two methods (ultracentrifugation and flocculation) for virus concentration in residual water, using the total viral number to determine the recovery efficacy. The presence of two enteric viruses (rotavirus A and adenovirus) was determined in concentrated water samples in order to evaluate the problems associated to PCR detection for both concentration methods. The samples were collected in March and July 2009, after secondary wastewater treatment. The results indicate that ultracentrifugation is a better concentration method, in comparison with flocculation, since the virus recovery was 68% and less than 40%, for organic flocculation. Total viral number was determined by epifluorescent microscopy before and after water concentration by the two methods. Rotavirus A was detected by RT-PCR and adenovirus by nested PCR. Consequently the detection of the both enteric virus is possible using molecular methods after virus concentration through the two methods. The higher recovery rate of viruses by ultracentrifugation as well as the simplicity and rapidity of the method make it more practical than flocculation to concentrate viruses in residual waters. The rapidity of this method could avoid structural changes in viral community, making this concentration procedure appropriate, not only to detect specific human health relevant viruses, but also to study the total viral community in order to get a global ecologic view of the pathogenic viruses transmitted by environmental waters.

Key words: Concentration methods, ultracentrifugation, flocculation, total viral number, Rotavirus A, Adenovirus, residual water.

Introduction

The contamination of recreational waters with pathogenic virus is a concern of human health and an ecological issue. The main source of pathogenic viruses in the environment is the discharges of residual waters not properly treated (Hamza *et al*., 2009; Rodríguez-Díaz *et al*., 2009).

In residual waters can be found more than 140 types of viruses that can cause hepatitis, gastroenteritis, meningitis, fever, influenza, respiratory disease, conjunctivitis, among others diseases (Puig *et al.*, 1994; Bosch *et al.*, 2008; Hamza *et al.*, 2009). However, only a small number of these viruses are epidemiologically relevant (Bosch, 1998). The most relevant viral pathogens

found in water, from an epidemiological point of view, are the enteric viruses: norovirus, rotavirus, adenovirus, astrovirus, enterovirus, polyomavirus, parvovirus and hepatovirus (Bosch *et al*., 2008; Rodríguez-Díaz *et al*., 2009).

Viruses are the most abundant biological component in aquatic systems (about 10^7 viruses mL^{-1}), although the number of health significant viruses in water is low (Wommack and Colwell, 2000; Suttle, 2005; Miki and Jacquet, 2008). However, even at low concentrations these pathogenic viruses can cause diseases when ingested. Consequently, it is required large volumes of water to detect enteric viruses, which imply to concentrate a large volume of water to a few milliliters before viruses detection (Bosch, 2007). A good viral concentration method should be simple, fast, adequate to large types of samples and viruses, produce a small volume of sample, avoid changes in viral community structure and, of course, provide high virus recovery (Bosch *et al.*, 2008; Albinana-Gimenez *et al.*, 2009). There is several virus concentration methods, some of them are directed for large volumes of sample and other to small volumes. Methods like adsorption-elution and flocculation are used to concentrate large volumes of water, while ultracentrifugation and ultrafiltration are more appropriate to concentrate viruses from smaller volumes or as a second step reconcentration method (Percival *et al.*, 2004; Bosch, 2007).

The adsorption-elution method is based in virus contact from the samples with a solid matrix that adsorbs the viruses at determine conditions of pH and ionic strength. After adsorption, viruses are eluted from the solid support into a smaller volume. The selection of the matrix, eluting fluid and the type of sample will determinate the efficiency of the method (Percival *et al*., 2004). Ultracentrifugation is a “catch all” method since it is able to concentrate all the virus in a sample using sufficient g force during a certain period of time The viral particles have a relatively high molecular weight, sedimenting by ultracentrifugation when in suspension (Percival *et al*., 2004). The flocculation method is based on the ability of proteins to flocculate at acid pH, getting the virus trapped in the protein flakes, which are then released after dissolution of the flakes (Percival *et al*., 2004). Ultrafiltration is a method that concentrates viruses by size, being retained by a membrane with pore sizes that allow the passage of water but not the virus (Percival *et al*., 2004).

In the last 20 years, several studies have been conducted using different concentration methods and with several types of environmental water samples. The results of these studies showed that there is no perfect concentration technique; all concentration methods have advantages and disadvantages. However, during the last years an effort has been done to develop methods with high recovery efficiency (Table 8). The comparison among these studies is,

however, difficult because it is necessary to take into account many variables, as the type of sample, the volume of water and the methods used to determinate de recovery efficacy. In general, for environmental waters, the concentration method that allows higher virus recovery rates is flocculation (Table 8).

Table 8. Recovery efficacy of the main methods used to concentrate viruses in environmental waters.

Method	Water quality	Recovery	Used allow?	Reference
Adsorption-elution	river	60%	Yes	(Puig *et al.*, 1994)
	tap water	62±16%	Yes	(Guttman-Bass and Armon, 1983)
	seawater	34-95%[a]	No	(Katayama *et al.*, 2002)
	wastewater	80±42%[b]	No	(Katayama *et al.*, 2008)
	river	23±19%[b]	No	(Katayama *et al.*, 2008)
	river	21-100%[a]	No	(Hamza *et al.*, 2009)
Ultrafiltration	clean water	good	Yes	(Bosch, 2007)
	tap water	>80%	Yes	(Haramoto *et al.*, 2004)
	tap water	75%	Yes	(Vilaginès *et al.*, 1993)
	surface water	56±32%[b]	Yes	(Haramoto *et al.*, 2005)
	drinking water	70%	Yes	(Lambertini *et al.*, 2008)
Ultracentrifugation	clean water	medium	Yes	(Bosch, 2007)
	artificial seawater	50%	No	(Muscillo *et al.*, 2008)
	bottled water	30-37%	No	(Kovac *et al.*, 2009)
Flocculation	seawater	46%	No	(Calgua *et al.*, 2008)
	natural waters	53%	Yes	(Shields and Farrah, 1986)
	tap water	61%	Yes	(Guttman-Bass and Armon, 1983)
	tap water	74%	No	(Katzenelson *et al.*, 1976)
	tap water	97%	Yes	(Guttman-Bass and Nasser, 1984)
	lake	96%	Yes	(Guttman-Bass and Nasser, 1984)
	seawater	63%	Yes	(Guttman-Bass and Nasser, 1984)

[a] recovery rates between this two values

[b] The mean and standard deviation

Nowadays, molecular techniques like PCR are the most common worldwide way to detect viruses in concentrated waters. These techniques are more specific and sensible than the traditional ones, giving more relevant information about virus genotypes and providing also important epidemiological information (Villena *et al.*, 2003; Hovi *et al.*, 2007; Pinto *et al.*, 2007). However, there are some problems of incompatibility between molecular procedures and concentration methods because not only are concentrated viruses but also inhibitors substances that could affect the PCR. Consequently, procedures to remove inhibitor from virus concentrate must be applied before virus detection by PCR (Haramoto *et al.*, 2005). So, besides the degree of efficacy of the concentration method, it is also important that the concentration method avoids

introduction of more inhibitors to PCR in addition the inhibitors that already exist in the sample, like some organic compounds (Percival *et al.*, 2004; Haramoto *et al.*, 2005; Bosch *et al.*, 2008).

The objective of this study was the comparison of two methods (ultracentrifugation and flocculation) for virus concentration in residual waters, using the total viral number to determine the recovery efficacy. The suitability of the two concentration methods to detect enteric viruses by PCR analysis was also evaluated.

Material and methods

Water sampling

The water samples were collected at a wastewater treatment plant of Aveiro (South ETAR) after secondary treatment on two dates, March and July 2009. The residual waters were collected in sterile bottles that were kept on dark at 4°C until the concentration.

Virus concentration by ultracentrifugation

Three sub-samples of 500 mL each were filtrated by a glassfibre prefilter (142 mmø; Sartorius AG) and 0.22 µm membrane (142 mmø; Milipore Durapore) at low pressure (<200 mm Hg) using a filter system (A.E.B., S.R.L. Druck Ablassen, Italy). The filtrate was centrifuged (Beckman Optima^{TM}, LE-80K Ultracentrifuge, rotor 50.2 Ti) at 29000 rpm for 1 h at 20°C. The supernatant was removed and the pellet was resuspended in 200 µL of PBS. The concentrated samples were stored at -20°C until analysis.

Virus concentration by skimmed milk flocculation

The pH of each sub-sample (1 L) was adjusted to 3.5 by the addition of HCl 1 M. Fifty milliliters of pre-flocculated skimmed milk (0.1%) were added to each sub-sample. Water samples were stirred for 8 h at room temperature and the flakes sedimented by gravity for another 8 h. The supernatants were carefully removed without disturbing the flakes. The final volume was centrifuged at 7000 x g for 30 min at 4°C, the supernatant was carefully removed and the pellet was resuspended in 10 mL of PBS. The pH of water samples was adjusted to 7.5 by the addition of NaOH 1 M. The concentrated samples were stored at -20°C until analysis.

Quantification efficacy of the total viral number before and after concentration

The efficacy of virus concentration was evaluated through the quantification of the total viral number before and after concentration. To determine the number of total viruses in the water samples was used the method of Noble and Fuhrman (1998) modified. Water samples were filtered by a 0.2 µm polycarbonate membrane and then by a 0.02 µm Al_2O_3Anodisc, which were then stained in the dark for 20 min with Sybr Gold (2X). The enumeration of total virus number was made by epifluorescent microscopy using a Leitz Laborlux K microscope with a HBO 50 W, 450-490 nm, 515 nm filter mercury lamp. At least 200 viruses or 20 microscope fields were counted in each duplicate replica of the three sub-samples.

Nucleic acid extraction and purification

Viral DNA and RNA were extracted from the concentrated samples using the geneMAG-RNA/DNA (Chemicell® Germany) purification kit, according to the manufacturer´s instructions. This kit allows the isolation of total RNA/DNA using magnetic silica beads.
For DNA and RNA purification the GeneClean Kit (MP Biomedicals, LLC), was used according to the manufacturer´s instructions. After purification the nucleic acids were diluted 1:15 with TE and stored at -20°C until analysis.

Detection of rotavirus A and adenoviruses

The identification of rotavirus A was made by the RT-PCR technique according to the protocol described by Villena *et al.* (2003), using the OneStep RT-PCR kit (Qiagen®Germany) according to the manufacturer´s instructions. The primers used are based in a high conservated region of all rotavirus from A group (Table 9). The reverse transcription was carried out for 30 min at 42°C and 15 min at 95°C. The amplification was performed during 40 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. A final extension step was performed at 72°C for 7 min.

The identification of adenoviruses was made by nested PCR technique using the protocol described by Allard *et al.* (2001). The outer par of primers Hex1deg and Hex2deg (Table 9), and the nested primers Nehex3deg and Nehex4deg (Table 9) detect all the 51 adenovirus serotypes based on conserved region of the hexon gene. The first reaction mixture used consisted of 1x Taq Buffer with KCl (Fermentas), $MgCl_2$ 1.5 mM (Fermentas), dNTP 0.28 mM

(Fermentas), 0.4 µM of each outer primer and 0.1 U/µL of Taq DNA polymerase (Fermentas), to a final volume of 25 µL. The amplifications were carried out at 95°C for 10 min, followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, and a final extension at 72°C for 5 min. Two microliters of the first reaction were added to 23 µL of reaction mixture in the same conditions of first PCR. The second PCR was performed at 94°C for 3 min, followed by 45 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension step at 72°C for 5 min.

PCR products were analyzed by electrophoresis in a 2% agarose gel stained with ethidium bromide, and visualized under UV transiluminator. Positive PCR results were determined by visualization of specific band product (Table 9).

Table 9. Primers used for detection of rotavirus A and adenovirus.

Virus	Primer	Sequences (5´-3´)	Size (bp)	References	Degenerated bases
Rotavirus	VP6-3	GCT TTA AAA CGA AGT CTT CAA C	186	Villena *et al.*, 2003	
	VP6-4	GGT AAA TTA CCA ATT CCT CCA G			
Adenovirus	Hex1deg	GCC SCA RTG GKC WTA CAT GCA CAT C	301	Allard *et al.*, 2001	S=C+G W=A+T
	Hex2deg	CAG CAC SCC ICG RAT GTC AAA			R=A+G K=T+G
	Nehex3deg	GCC CGY GCM ACI GAI ACS TAC TTC	171		Y=C+T M=A+C
	Nehex4deg	CCY ACR GCC AGI GTR WAI CGM RCY TTG TA			I=deoxyinosine

Results

Viral recovery rate from the two concentration methods

The viruses recovery rate was calculated based on the determination of the total number of viruses before (Table 10) and after (Table 11) wastewater sample concentration, through an epifluorescent microscopy. The ultracentrifugation method showed an average recovery rate of 67.5% (Table 11) and the flocculation method of 37.5% (Table 11).

Table 10. Number of total virus in wastewater before concentration.

Sampling date	Replicates	Viruses in non-concentrated water (particles/ml)
March	1	$8{,}27 \times 10^7$
	2	$6{,}65 \times 10^7$
July	1	$8{,}50 \times 10^7$
	2	$1{,}66 \times 10^8$

Table 11. Number of viruses after concentration by flocculation and ultracentrifugation method and recovery rate.

Concentration method	Sampling	Sub-samples	Average of viral (particles/ml)	Recovery rate
Ultracentrifugation	March	1	$1{,}15 \times 10^{11}$	71%
		2	$1{,}18 \times 10^{11}$	
		3	$1{,}65 \times 10^{11}$	
	July	1	$1{,}79 \times 10^{11}$	64%
		2	$1{,}86 \times 10^{11}$	
		3	$2{,}39 \times 10^{11}$	
Flocculation	March	1	$2{,}29 \times 10^{9}$	37%
		2	$2{,}39 \times 10^{9}$	
		3	$3{,}57 \times 10^{9}$	
	July	1	$5{,}28 \times 10^{9}$	38%
		2	$4{,}45 \times 10^{9}$	
		3	$4{,}63 \times 10^{9}$	

Detection of rotavirus A and adenovirus

The water samples concentrated by the two methods were tested for the presence of rotavirus A and adenovirus, by RT-PCR and nested PCR, respectively. These two viral groups were present in all wastewater samples after concentration by ultracentrifugation and flocculation methods. However it was necessary to purify and dilutes the water samples before detection because of the inhibitors.

Discussion

The success of virus detection by PCR is the result of two factors, the recovery efficacy of the concentration method and the degree of purity of the concentrated sample (Bosch *et al.*, 2008). Both concentration methods are effective for virus recover, although the ultracentrifugation method shows a higher recovery of viruses in residual waters. Moreover, the simplicity and rapidity of the ultracentrifugation method makes it more practical than flocculation to concentrate viruses in residual waters. The virus recovery rate of the ultracentrifugation (68%) method in residual waters, compared with organic flocculation (38%), was 30% higher. Besides that, comparatively to ultracentrifugation, in flocculation the water samples are more manipulated since it requires the addition of HCl in water samples, in order to adjust the pH of the water to 3.5, NaOH to increase the pH 7.5 and skimmed milk solution to allow virus absorption.

It is, however, well known that during the concentration method the inhibitory substance are concentrated along with viruses and the addition of HCl, NaOH and skimmed milk may increase

the inhibitory effect when PCR analysis is used to detect viruses. In ultracentrifugation, only the non-added inhibitors substances are concentrated. Although for residual water this may not be very important, wastewaters have large concentrations of inhibitors, always requiring purification of the concentrated samples before molecular analysis (Guo *et al.*, 2009; Moussavi *et al.*, 2009).

The incubation period of about 16 hours in the flocculation method may also affect the structure of the viral community. Since bacteriophages represent a large fraction of virioplankton (Suttle, 2005; Bettarel *et al.*, 2008; Miki and Jacquet, 2008) and that their life cycle is short, frequently less than 1 hour (Madigan *et al.*, 2006; Bettarel *et al.*, 2008), allowing these viruses undergo several replications during water concentration by flocculation. On the other hand, viruses that infect eukaryotic cell have approximately 40 hours long life cycles (Wommack and Colwell, 2000) and, consequently, it is improbable that they replicate during the incubation period. Although this fact may not be important if the concentration method is used to detect specific enteric viruses by PCR using specific primers, however if the concentrated water samples are to be used to study the structure of viral community through, for instance, through pyrosequencing, the results may not reflect the natural community structure. In the ultracentrifugation method, water samples are filtered by 0.2 µm membranes in order to remove bacteria before centrifugation, all the viral hosts are removed and viral replication is avoided, even for bacteriophages that have short life cycles. However, the water filtration before ultracentrifugation can cause viral loss through membrane colmatation. The replacement and/or the employment of large membranes during filtration can overcome this problem. Moreover, when ultracentrifugation is used to detect specific enteric viruses, water filtration is not necessary and, consequently the loss of viruses by membrane colmatation is avoided. Therefore, the concentration by ultracentrifugation allows a more realistic picture of viral community structure than flocculation methods.

The recovery rate obtained with both methods was similar to that achieved in other studies using specific enteric viruses to determine the rate of viruses recovery by ultracentrifugation and flocculation methods (Katayama *et al.*, 2008) (Table 8). The determination of the total number of viruses by epifluorescent microscopy is a good alternative to determine the rate of recovery for the concentration methods. The enumeration of the total number of viruses by epifluorescence is a simple, fast and a cost-effective approach comparatively to traditional plaque assay approaches.

The results show that both methods produce for viral concentration are suitable for molecular-based detection of enteric viruses in wastewater, as revealed from the presence of rotavirus A

and adenovirus in all samples concentrated by the two methods. However, before PCR detection is required purification and dilution of the samples.

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CHAPTER 4

GENERAL DISCUSSION

Considering the wide distribution and frequent occurrence of waterborne outbreaks worldwide, it is epidemiological relevant to document the presence of the main pathogenic enteric viruses implicated in these outbreaks in different geographic areas. These viruses reach the environmental waters by sewage discharges, so it is important to evaluate the efficiency of the wastewater treatment in the inactivation of these viruses. Moreover, to evaluate the risk of environmental contamination by sewage discharge, a simple, fast and efficient method to concentrate enteric virus has to be used before virus detection. Besides, to determine the recovery rate of the concentration methods, a practical approach applicable to all type of enteric viruses must be used. The methods used to evaluate which viruses are implicated in gastroenteritis outbreaks and to detect their presence in environment waters must be specific and precise.

Human rotavirus A and adenovirus are important pathogens associated with waterborne outbreaks of acute gastroenteritis in children and adults (Lopman et al., 2003). These viruses, together with norovirus, sapovirus and astrovirus, are estimated to be the main causative agents of nonbacterial gastroenteritis all over the world (Lopman et al., 2003; Victoria et al., 2009). The results of this study confirm that rotavirus A and adenovirus are important agents of gastroenteritis. From the 467 faecal samples analyzed through immunochromatographic assay, 59 (12.6%) were positive for rotavirus A and 5 (1%) were positive for adenovirus.

As far other studies, (Rendi-Wagner *et al.*, 2006; Shimizu *et al.*, 2007), in the present work the incidence of viral gastroenteritis was higher during the colder months and in pre-scholar age children . From the 474 faecal samples analyzed by immunochromatographic assay, a clear pattern of seasonal variation was observed, with lower incidence of gastroenteritis cases in the summer months, between July and September. The incidence of gastroenteritis in children under 5 years old was also observed. The incidence of rotavirus infection was also clearly higher in children with pre-scholar age and a seasonal distribution of rotavirus-associated gastroenteritis was observed with a peak in December and occurring continually through March. In respect to adenovirus infections the reduced number of positive cases was too low to support solid conclusions. However, the occurrence of most cases in infants and children under 5 years old was observed.

The presence of rotavirus A and adenovirus was also detected in residual waters by molecular methods (RT-PCR and nested PCR). Both viruses were detected in every sub-sample of the two sampling moments, for the two concentration methods tested (flocculation and centrifugation). This confirms that sewage discharges into surface waters, even after secondary treatment, are

important ways of virus dispersion to environmental waters. Since the impact of virus discharges on human health is high, there is a need to establish regulation for the monitoring of residual waters before their release in the environment (Katayama *et al.*, 2008). Moreover, in the environment viruses can survive for weeks to months in water or by attaching to particles matter and accumulating in sediments (Muscillo *et al.*, 2008). The actual legislation for treated wastewater that is discharged in the environment do not consider microbiological monitoring, no even bacterial parameters.

The efficacy of the detection/quantification of enteric virus in environmental waters depends on the concentration procedures applied (Bosch, 2007). Therefore, it is important to use an efficient method to concentrate virus, but also a simple and fast method that does not alter the structure of the viral community. In this study, the ultracentrifugation method, usually used as a secondary step of virus concentration, was compared with organic flocculation, most typically used to concentrate viruses from environmental waters due to its efficiency (table 8). The results of this work showed that ultracentrifugation is the best method to concentrate virus in residual waters, since the virus recovery using ultracentrifugation (68%) was higher than in flocculation (38%). Moreover, in the ultracentrifugation method the samples are less manipulated than in the flocculation procedure. The samples concentrated by ultracentrifugation are only filtrated by 0.2 µm membranes, to remove bacteria, while in the flocculation procedure HCl, NaOH and skimmed milk are added, and the samples incubated for, at least 16 hours. Beside, such long incubation periods in the flocculation procedure might also cause changes in viral community structure, since viruses with short life cycles can replicated several times, while eukaryotic viruses do not have enough time to undergo replication. Although this may not be relevant for the PCR detection of particular viruses, since specific primers are used, but it can be important when the concentrated samples are used for metagenomic or pyrosequencing studies. During the 16 hours of incubation some viruses can be inactivated, namely RNA viruses that are less resistant to adverse conditions like low pH. The selection of the concentration method is based on the recovery rate. However, the approaches used to determinate the recovery rate are not practical, are expensive and do not evaluate the concentration of all viruses. Usually, a suspension of viruses of known concentration is added to the sample and after concentration the number of viruses is enumerated and the recovery rate is determinate. In this study the recovery rate was determinate by calculation of total viruses present in the sample before and after concentration through the two methods by epifluorencent microscopy. These seems to be a simple, rapid, cost effect and precise way to

determinate the recovery rate. Moreover, using this approached, all the viruses present in the sample are used to determine the recovery rate, reflecting the recovery for all type of viruses.

The determination of recovery rate is just for direction, since the efficiency of a concentration method depends on many variables, such as the quantity of viruses present in the sample and the nature and volume of the sample (Albinana-Gimenez *et al.*, 2009). It is also necessary to take into accounts the way to determinate the recovery rate. The differences between the recovery rates present in Table 8 and the recovery rate determinate in this study can probably be related with the turbidity and concentration of organic matter in the sample.

The development of immunological and molecular diagnostic technique to detect viruses, has allowed the detection of enteric viruses in clinical and in environmental samples that previously were not detected due to their inability to grow in conventional cell culture systems. Immunochromatographic techniques detect the antigens of the virus capsid, while PCR detects the presence of viral DNA/RNA. In principle, immunological techniques are less sensitive than PCR, but also depend on the methods of nucleic acid extraction, the primers used, the absence of nonspecific inhibitors of enzymatic reactions and stability of viral nucleic sample (Téllez *et al.*, 2008).

The immunological method is frequently used to detect enteric viruses in clinical samples and the detection by PCR is the most frequently used method to detect enteric viruses in environmental waters. However the specificity and the sensibility of these two method are different, which difficult the comparison of viral concentration between clinical and environmental samples, and, consequently the interpretation of epidemiological data. On the other hand, it has been know that the immunological methods are less effective to detect enteric viruses, as rotavirus A and adenovirus (Hurst *et al*., 1997). The results of this study confirm this tendency for the detection of adenovirus in clinical samples. In this study rotavirus A and adenovirus were detected simultaneously in 18 faecal samples using VIKIA® Rota-Adeno , an immunologic assay, and molecular methods (PCR and nested PCR). The results showed that immunological test is effective to detect rotavirus A but is not appropriate to detect adenovirus. In the 18 samples tested, 11 were positive for rotavirus with VIKIA® Rota-Adeno and 10 were positive with PCR detection. However, for adenovirus only 1 sample was positive with VIKIA® Rota-Adeno but with PCR the adenovirus was detected in 16 samples. The identity of positive PCR products that were not concordant with VIKIA® Rota-Adeno assessed by sequencing analysis, which verified the presence of human rotavirus A in faecal sample 8 and confirming the presence of human adenovirus 41 in 15 samples. Viral nucleic acid sequencing also allows a genetic

characterization of both viruses, suggesting that adenovirus 41 was the most implicated in the gastroenteritis cases analyzed. The VIKIA® Rota-Adeno kit is useful for a rapid diagnosis of rotavirus but is not the good option to detect adenovirus when his concentration is low. Although, PCR, relatively to the immunologic test is time consuming and requires specific equipment.

This study also showed rotavirus A and adenovirus co-infection observed in 50% of infected patients, the dual infection observed in this study raise the question of whether a single virus is responsible for illness or whether two viruses act in synergy (Aminu *et al*., 2007).

The results of this research emphasize the importance of the introduction of rapid molecular methods to routine clinical hospital laboratories to provide definitive diagnosis. An accurate diagnosis of acute gastroenteritis would facilitate appropriate management of patients and reduce extensive or unnecessary use of antibiotics in the treatment of gastroenteritis-associated viral infections.

CHAPTER 5

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ANNEXES

Annex I – Tampon and Reagents

TE	
Tris-HCl	1M
EDTA	0.5M
H_2O miliQ	
Filter and autoclave	

TBE 10x	
Tris-Base	108g
Boric ácid	55g
EDTA	40mL
H_2O miliQ	960mL

PBS 1X	
H_2O miliQ	1L
CaCl2 2H2O	0.1325g
MgSO4	0.0592g
KCl	0.2g
KH_2PO_4	0.2g
ClNa	8g
NaHPO4	1.15g
Adjust pH 7.4 and autoclave	

Ethanol 70%	
Ethanol 96%	73mL
H_2O miliQ	27mL

Artificial seawater	
Sea salt	33.3g
Deschlorinated tap water	1L

Skimmed milk 0.1%	
Artificial seawater	100mL
Skimmed milk (VWR)	1g
Adjust pH 3.5	

Sybr Golg 0.25%	
Sybr Golg (Invitrogen)	40µL
TE	1960µL
Store -20ºC	

H_2O Rnase free	
H_2O miliQ	1L
DEPC (Aldrich)	1mL
Stir and autoclave	

Electrophoresis gel 2%	
Agarose (Vivantes)	2g
TBE 1X	100mL
EtBr	8µL

Reagents	
Buffer Taq 10x + KCl - $MgCl_2$	Fermentas, Life sciences
dNTP 2mM	Fermentas, Life sciences
Geneclean kit	MP Biomedicals, LLC
geneMAG-RNA/DNA kit	Chemicell
Leadder DNA 100bp	Fermentas, Life sciences
$MgCl_2$ 25mM	Fermentas, Life sciences
OneStep RT-PCR kit	Quiagen
Primers	StabVida
Rnase OUT	Invitrogen, Life technologies
Taq DNA Polymerase	Fermentas, Life sciences

Annex II – Results

Electrophoresis Gel

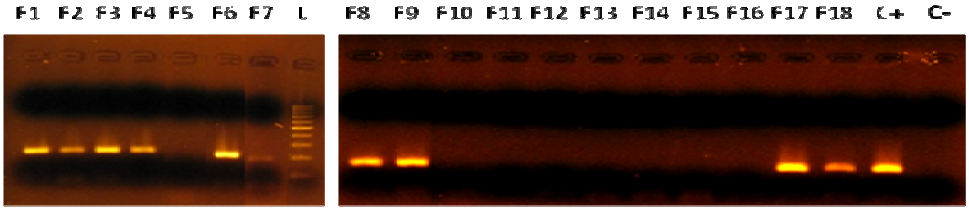

Figure 5. Electrophoresis result for Rotavirus RT-PCR in faecal samples. Agarose gel 2%, 20min, 80V.

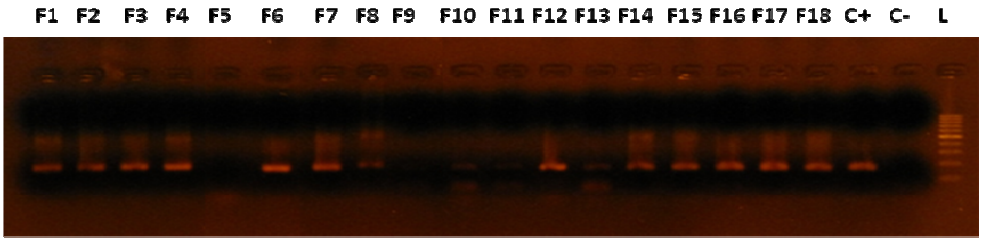

Figure 6. Electrophoresis result for Adenovirus nestedPCR in faecal samples. Agarose gel 2%, 20min, 80V.