New technique for direct fluoroimmunomagnetic detection of rotavirus in water samples

Raquel A. Villamizar-Gallardo, Johann F. Osma and Oscar Orlando Ortíz

ABSTRACT

A new rapid, sensitive and selective method for rotavirus detection in water samples is described in this paper. Amino pink magnetic microparticles were functionalized with monoclonal antibodies and used to capture, concentrate, separate and detect infectious rotavirus particles in distilled and drinking water samples. The fluorescence of the microparticles was used to determine the presumptive presence of rotaviruses by using confocal microscopy. Atomic force microscopy and transmission electron microscopy were used to confirm the presence of the anti-rotavirus antibodies attached to the surface of the magnetic microparticles as well as that of viruses attached through the antibody. In addition, RNA extraction, quantification and amplification were carried out to validate the microscopic observations. The selectivity of the microparticles was tested in a sample containing a mix of enteric viruses. It was concluded that functionalizing fluoromagnetic microparticles with anti-rotavirus monoclonal antibodies constituted a fast, simple and reliable technique for detecting as low as 10 Rotavirus particles in 1 L of artificial or real water in just 2 hours.

Key words | enteric diseases, fluoromagnetic microparticles, rotavirus, water

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INTRODUCTION

Human enteric viruses are highly prevalent among a huge number of waterborne pathogens, and rotavirus is one of the most frequently studied groups. In 2013, these pathogens caused the deaths of around 215,000 children aged 5 years and below (WHO 2016). Worldwide research has reported the presence of this virus in different water types including sewage (Kiulia *et al.* 2015), river water (Haramoto *et al.* 2010) and drinking water (Räsänen *et al.* 2010; Siqueira *et al.* 2010). A recent publication of the Colombian National Health Institute reported that at least one type of enteric virus was present in 50.7% of water samples from 102 towns. Among these samples, rotaviruses were second most frequent (20.5%) after hepatitis A. In addition, researchers reported that viral content was similar in both raw and drinking water (Peláez *et al.* 2016). In 2011, the World Health Organization (WHO) established that drinking water should not contain more than 1 rotavirus in 90,000 L (WHO 2011). However, in Colombia, as in many other developing countries, no regulation specifies tolerable limits for viruses in either raw or drinking water.

One of the reasons for this omission is probably the lack of easy-to-handle methods. From an analytical point of view, viruses are hard to detect due to their low concentration in water (1 to 10^3 viral particles per liter); therefore, sensitive methods are required for their detection. Polymerase chain reaction (PCR)-based techniques (e.g., reverse transcription (RT)-PCR, qPCR, real time PCR) are some of the most frequently used because of their high sensitivity (Kiulia *et al.* 2015). Nevertheless, there are limitations, such as the absence of correlation between the detected viral genome and its level of infection, which makes it necessary to apply additional methods like cell culture, which improves

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identification. Although cell-culture-based methods are highly sensitive, they are also laborious and expensive. In addition, not all viruses produce a cytopathic effect, which makes the process more difficult. Finally, for some types of enteroviruses (e.g., noroviruses), there are no propagation cell lines (Hamzaa *et al.* 2011).

The use of serological methods is also very frequent, the enzyme-linked immunosorbent assay (ELISA) being among the most common. However, an important drawback is the detection limit, requiring sample pretreatment to concentrate the viral content (Kiulia *et al.* 2015). The immunomagnetic separation (IMS) method has proven to be effective in the detection of several virus types, namely adenoviruses (Haramoto *et al.* 2010), rotaviruses (Yang *et al.* 2011) and the causal agent of hepatitis A (Ha *et al.* 2014), all of them detected in water samples obtained from different environments. By using this technique, it is possible to eliminate background interference, including PCR inhibitors. Besides this, the use of an antibody as molecular receptor ensures that the captured antigen corresponds to an intact infectious particle. Thus, based on the IMS, the current study introduces a new fluoroimmunomagnetic technique for the concentration and separation of rotavirus particles present in water samples. The magnetic properties of the microparticles allow separation and concentration of viral particles from water, while fluorescence facilitates conduction of the process through microscopic techniques.

MATERIALS AND METHODS

Reagents

Rotavirus monoclonal antibodies at a concentration of 100 μg/mL were obtained from AMSBIO (UK). They were diluted in phosphate buffer saline (PBS) to a final concentration of 10 μg/mL (pH 7.2) and stored at −20 °C until use. PBS was obtained from Sigma-Aldrich (USA). Pink amino magnetic microparticles with an average size of 0.5 μm and 1% w/v were purchased from Spherotech Inc. (USA). They were suspended in distilled water and stored at room temperature under dark conditions until use. A QIAamp viral RNA extraction kit was purchased from QIAGEN (Germany).

Equipment

A Zeiss LSM 510 confocal microscope was used to characterize the fluorescent magnetic microparticles before and after the functionalization with antibodies and exposure to the virus. Images of the magnetic microparticles and the microparticle–antibody–virus complexes were obtained with a Park Unveils NX20 (Korea) atomic force microscope (AFM) operated under non-contact mode and with a Tecnai F20 Super Twin TMP transmission electron microscope (TEM). TEM images were obtained by applying a voltage of 80 kV. A Handi-Mag separator (Spherotech Inc., USA) was employed to separate and concentrate the rotavirus particles in the water samples. A NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was used to quantify the viral RNA. A Gel Doc^{tm}XR+ system (BIO-RAD, USA) was used to visualize the gel electrophoresis.

Virus preparation

Rotateq pentavalent oral vaccine for children (Merck, USA) was obtained from the Health Department Institute of Norte de Santander (Colombia). The vaccine combines serotypes G1, G2, G3 and G4, plus a series of G serotypes containing P1 (e.g., G9). Dilutions were prepared in PBS to produce samples with final concentrations ranging from 10 to 10^6 viral particles per milliliter (VP/mL). The dilutions were stored at 5 °C until use.

Functionalization of the magnetic particles

Amino pink fluorescent microparticles were functionalized as follows. First, the particles were incubated at 37 °C in a 10 μg/mL solution of anti-rotavirus antibodies in 10% PBS for 1 h. Then, the particles were rinsed with a 0.15 mM solution of PBS (pH 7.4) containing 0.1% bovine serum albumin (BSA) and finally with distilled water (Villamizar *et al.* 2009) (Figure 1(a)). The particles were stored at 4 °C until use.

Fluoroimmunomagnetic separation of rotavirus from distilled water and sterilized drinking water

Five hundred microliters of the functionalized microparticle solution was added to 1 L of distilled water containing

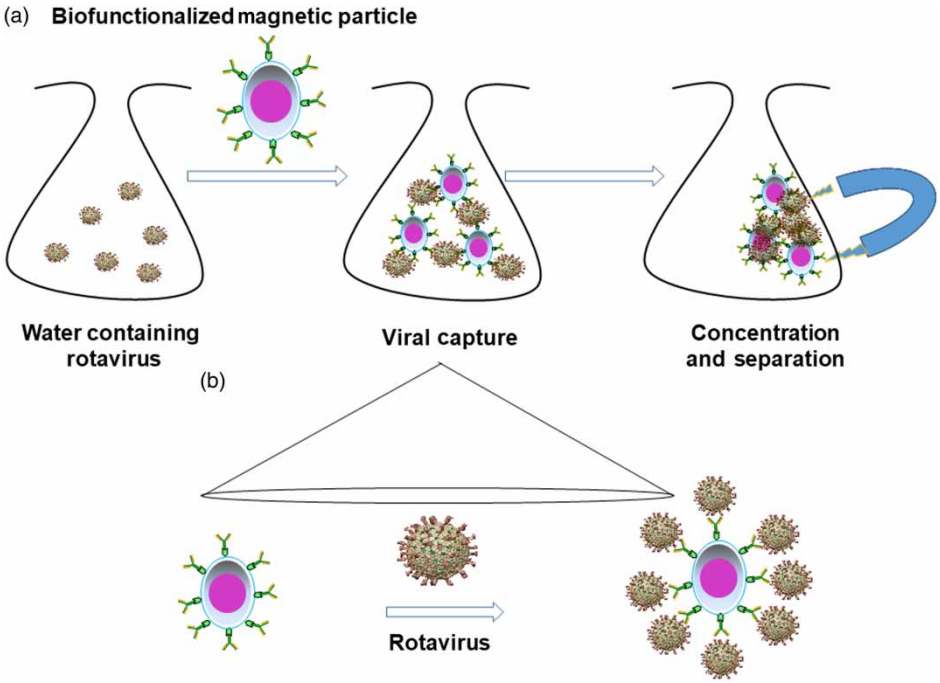

Figure 1 | (a) Experimental setup for the capture of rotavirus with magnetofluorescent microparticles functionalized with rotavirus antibodies. (b) Detection of rotavirus by antigen–antibody interaction.

increasing concentrations of rotavirus (10 VP/L to 10^6 VP/L). The water samples were gently agitated at room temperature for 2 h. Then, a magnetic field was applied in order to concentrate and separate the particle–antibody–rotavirus complex (Figure 1(a) shows a scheme of the experimental setup for the IMS of the rotavirus particles). The magnetized sample was stored in an Eppendorf tube and subsequently characterized. The same procedure was performed in sterilized drinking water. The process was repeated at least three times.

Molecular characterization

The antigen–antibody binding was broken by heating the complex at 95 °C for 5 min and immediately cooling at 4 °C. Subsequently, the microparticles were centrifuged at 10,000 rpm for 2 min (Yang *et al.* 2011). The supernatant was used to carry out RNA extraction with the extraction kit (Qiagen). Following the manufacturer's instructions, a 140 μL supernatant sample was used for the process, which yielded a final volume of 60 μL of RNA extract. The eluted RNA was then quantified by means of a NanoDrop spectrophotometer.

Rotavirus detection by RT-PCR

Because the rotavirus genome is based on double-stranded RNA, prior to RT-PCR, it was denatured at 95 °C for 5 min and immediately placed on ice for 5 min. The SuperScript™ III One-Step RT-PCR System (Invitrogen) was used to amplify rotavirus RNA. The initial incubation was at 55 °C for 30 min and then at 85 °C for 5 s in order to inactivate the enzyme. The primer pair sense (5′-TTGCCACCAATTCAGAATAC-3′) and antisense (5′-ATTTCGGACCATTTATAACC-3′) was used to amplify a 211 bp region of the rotavirus viral protein (VP6) gene segment. The PCR amplification was carried out through 40 preheating cycles at 95 °C, of 15 s each, annealing at 62 °C for 1 min and extension at 68 °C for 5 min (Tate *et al.* 2013). The products were analyzed using agarose gel electrophoresis (1.8% w/v).

Selectivity of the functionalized microparticles

Selectivity was examined in the presence of common enteric viruses also found in water (e.g., poliovirus, adenovirus and hepatitis virus). The functionalized microparticles were first

suspended in 1 L of distilled water artificially spiked with 1 ml of a stool sample provided by the Colombian National Health Institute containing a mix of the above-mentioned viruses. Capture, separation and characterization were performed as described above.

Non-specific binding assay

In order to see if rotavirus could display affinity for amino-groups present on the surface of the microparticles, the latter were exposed to 1 L of water previously spiked with Rotateq vaccine (10^6 VP/L), prior to functionalization (without antibodies). Samples of this solution were gently agitated at room temperature for 2 h. Afterwards, a magnetic field was applied in order to concentrate and separate the microparticle–rotavirus complex from the rest of the solution. Microscopic and molecular characterization was performed as previously described.

Rotavirus detection in real samples

Finally, fluoroimmunomagnetic concentration and separation were performed in 1 L samples of drinking water from the distribution phase of each of four water treatment plants (hereafter called PA, PB, PC and PD) located in the Department of Norte de Santander (Colombia). After adding functionalized microparticles to the samples, the immunoreaction was carried out for 2 h at room temperature under gentle agitation. Later, a magnetic field was applied in order to concentrate and separate the particle–antibody–rotavirus complex as shown in Figure 1. The magnetized sample was stored in an Eppendorf tube and subsequently characterized both microscopically and molecularly. The process was repeated at least three times per treatment plant.

RESULTS

Microscopic characterization

Non-functionalized fluorescent magnetic microparticles are shown in Figure 2(a). They are uniformly dispersed in the medium, revealing electrostatic repulsion forces acting between the positive charges of the amino groups present on the surface of the particles. Figure 2(b) shows microparticles functionalized with the rotavirus antibodies. Some small clusters, mainly containing two or three microparticles, can be observed due to electric charge differences in the amino acid base containing residues of anti-rotavirus antibodies. These slight differences in the superficial charges of the microparticles decrease their net surface charge and, hence, their colloidal stability. Finally, Figure 2(c) shows the functionalized microparticles after exposure to high concentrations of the virus (10^6 VP). In this case, large clusters ranging from 5 to more than 30 microparticles can be seen as a consequence of the virus–microparticle interactions. These interactions promote the self-assembly of clusters whose size is highly dependent on the concentration of the virus in the sample, as previously reported (Perez *et al.* 2003).

AFM was used to validate the effect of the virus on the formation of clusters of functionalized microparticles. Through this technique, the diameter of the microparticles was measured as 562 nm (Figure 3(a)), thus corresponding

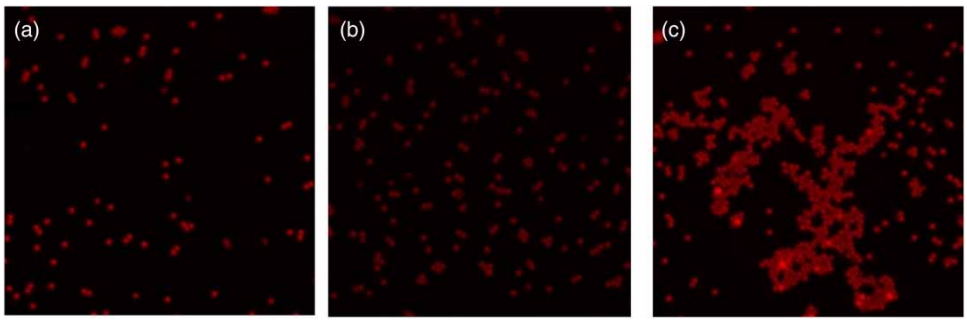

Figure 2 | Fluoromagnetic microparticles: (a) without antibodies; (b) coated with anti-rotavirus antibodies; (c) lusters of functionalized particles after exposure to 10^6 VP/ml of rotavirus.

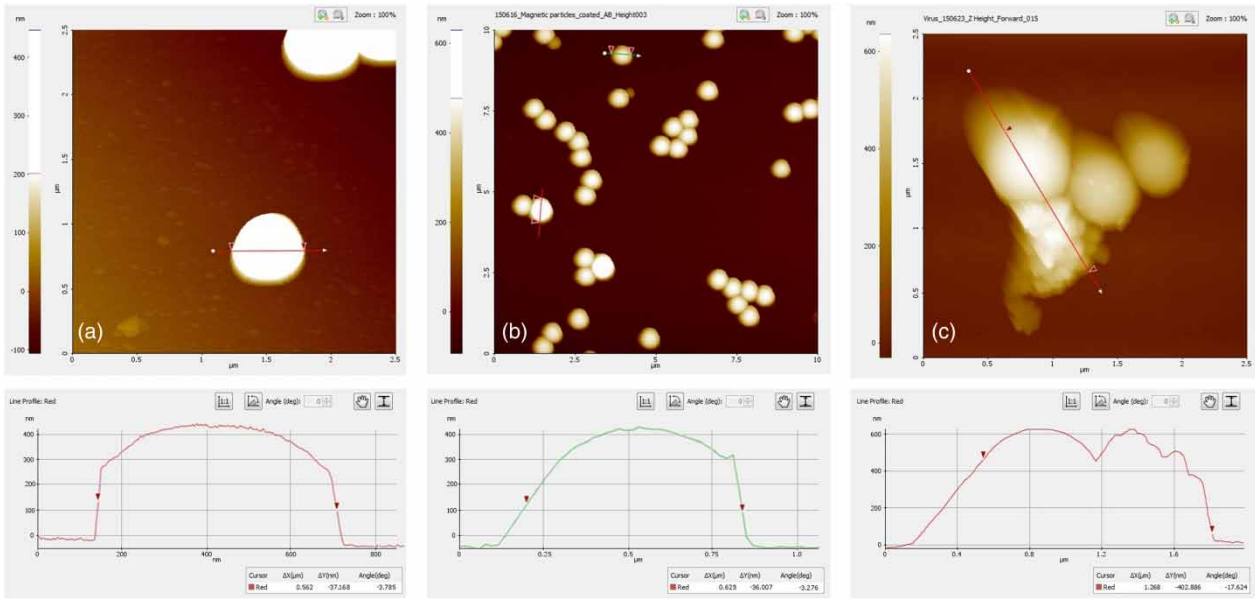

Figure 3 | AFM images: (a) fluoromagnetic particles; (b) functionalized particles; (c) biofunctionalized magnetic particles after exposure to 10^6 VP/L of rotavirus.

with the manufacturer's technical description. Once the microparticles had been functionalized with the rotavirus antibodies, their diameter increased by 10 nm (Figure 3(b)). The vertical length of one IgG is about 12 nm, assuming the Fab region points towards the rotavirus. However, due to adsorption mechanisms of the amine groups, rotational movements of the biomolecule can take place, thus generating variations in length (Villamizar 2010). This was the case in the present experiment, in which length values of 12 to 10 nm were recorded. After exposure of the functionalized microparticles to rotavirus, their diameter increased dramatically. Figure 3(c) shows the AFM analysis of a microparticle cluster formed by effect of the virus. Clusters >1 μm can be easily identified. Smaller structures of about 100 nm can be observed, which suggests the presence of the virus around the microparticles.

Further software analysis with a specialized tool for establishing the number of particles per cluster, the area occupied by the microparticles of each sample (i.e., microparticles, functionalized microparticles and rotavirus exposed microparticles) was carried out. Figure 4 describes the distribution of the size of clusters in terms of the number of microparticles for each sample. An area of 900 μm^2 was analyzed in each sample to have comparable results. When functionalized microparticles were exposed to the virus (Figure 4, gray bars), about 92% of them formed clusters, showing about 64% chance of forming clusters of more than 40 microparticles and about 28% chance of forming clusters of fewer than 20 microparticles. In contrast, non-treated microparticles generated clusters mainly of two to four microparticles about 40% of the time (Figure 4, black bars), while functionalized microparticles generated clusters mainly of two to four microparticles 65% of the time (Figure 4, hatched bars).

Equation (1) was used to determine the difference between the diameters of the functionalized and non-treated microparticles, thus:

$$OA = n \times A = n \times \pi \times r^2 = n \times \pi \times (d/2)^2 \quad (1)$$

where OA is the area occupied by all the measured microparticles; A, the average area of a microparticle; n, the number of particles in the studied area; and r and d, the average radius and diameter of the microparticles, respectively. The functionalized microparticles occupied an area of 22 μm^2 (2.4% of the total area), while the non-treated ones occupied 59 μm^2 (6.6% of the total area). These differences represent an 8.8% area increment and a 1.0% microparticle diameter net increment (approximately 15 nm), both values being attributable to the presence of antibodies on the surface of the microparticles.

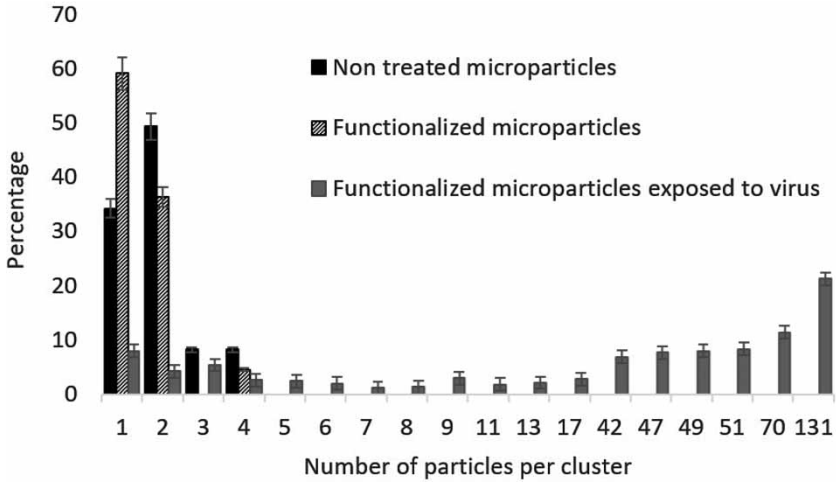

Figure 4 | Cluster size frequency distribution of non-treated microparticles, functionalized microparticles and functionalized microparticles exposed to a 10^6 VP/L concentration rotavirus solution.

A TEM analysis was also carried out in order to verify the results of both the functionalization and the microparticle–virus interaction. Figure 5(a) shows functionalized microparticles interacting with viral ones at 10^6 VP/L, which resulted in >1 μm long clusters, as observed before with the AFM analysis. These clusters evidenced the superficial interaction of the viral particles with the antibodies and their consequent clustering. Figure 5(b) shows the general structure of a non-treated microparticle, the diameter of which falls within the same range as the one obtained through the AFM analysis. Figure 5(c) details rotavirus particles bound to microparticles, showing an approximate size of 80–90 nm.

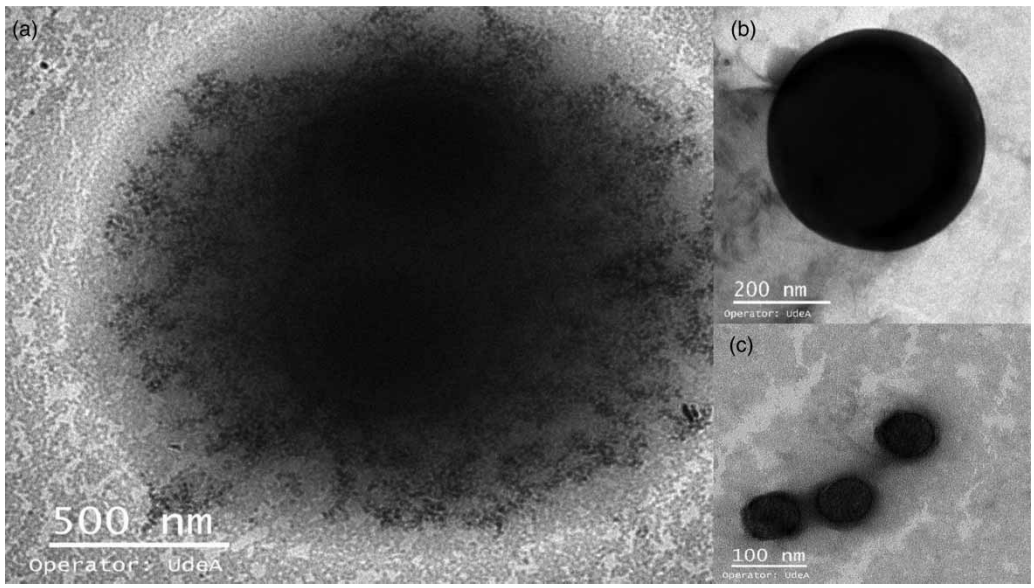

Figure 5 | Negative staining TEM images of: (a) magnetic microparticles after being exposed to a 10^6 VP/L rotavirus solution; (b) pristine magnetic microparticles; (c) rotavirus particles attached to the surface of magnetic microparticles.

Molecular characterization

Molecular characterization allowed confirmation of the presence of rotavirus. Figure 6 shows the calibration curve obtained by recovering rotavirus RNA at different concentrations ranging from 10 to 10^6 VP/mL. The (□) solid line shows RNA amounts extracted directly from the undiluted Rotateq vaccine stock. The (▲) and (x) dotted lines show RNA obtained from the same stock diluted in 1 L of distilled water and in 1 L of sterilized drinking water, respectively. In all cases, the higher the concentration of rotavirus, the higher the amount of RNA. According to these results, the best-fitting models corresponded to R values greater than 0.92. In the same way, it was determined that fluoroimmunomagnetic detection allowed average virus recovery percentages of 80.0% with distilled water and comparatively of 52.7% in sterilized drinking water. This suggests that the sensitivity of the microparticle–antibody complex was reduced by almost 20.0% and 47.3% respectively, due to the likely presence of interfering substances in the water matrix. Although the amount of organic matter in this kind of matrix is low, the reduction in the recovery percentage can be attributed to the loss of fluoroimmunomagnetic particles during the separation process, due to the volume of the analyzed sample (1 L). The current recovery percentage is higher than those reported by Haramoto *et al.* (2009), who obtained only 15% when using an absorption-elution technique for concentrating norovirus from river water, and by Ha *et al.* (2014), who recovered 6.2% of hepatitis A virus and 8.5% of norovirus from minimally processed foods. In the present case, the fluoroimmunomagnetic technique handled an acceptable level of rotavirus concentration, probably due to the microparticle functionalization process, which allowed the antibody to be immobilized and oriented directly towards the antigen (with the Fab region facing the antigen), thus increasing the sensitivity of the detection process.

In order to verify if the extracted RNA actually corresponded to the rotavirus, a RT-PCR was performed and the genomic material was then amplified. Agarose gel was used to visualize the RNA amplified from both distilled water and drinking water (Figure 7); the origins of the samples in lanes A to H are detailed in the figure legend. A negative control (C−) and positive control (C+), corresponding to the 211 bp segment of the gene for protein VP6 of the rotavirus capsid, were used. The results demonstrate that the previously extracted and quantified RNA belonged to rotavirus and that the fluoroimmunomagnetic technique can be equally effective for viral concentration in both distilled and drinking water from 10 to 10^6 VP/L.

The results obtained from the selectivity test and the non-specific binding assay are presented in Figure 8. The extraction of the genetic material led to a total viral RNA concentration of 16.7 ng/μL, which might imply slight cross-reactivity of the anti-rotavirus antibody. In order to

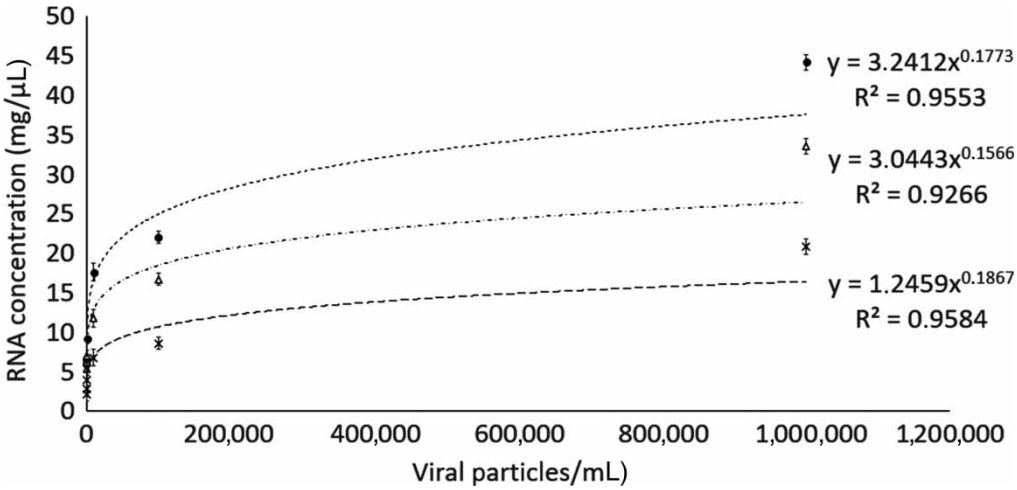

Figure 6 | RNA extracted from: (□) Rotateq vaccine stock; (▲) distilled water artificially spiked with Rotateq; and (x) drinking water artificially spiked with Rotateq.

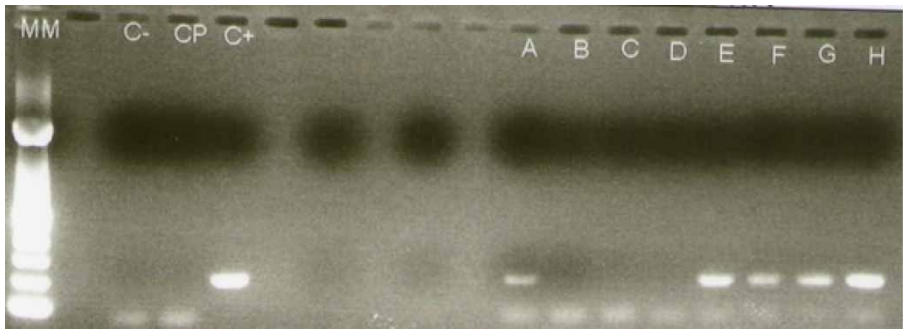

Figure 7 | Agarose gel electrophoresis showing the amplification of a region of the VP6 gene. Lanes A–D correspond to samples from water treatment plants (PA, PB, PC, PD). Lanes E and F correspond to Rotateq stock concentrations of 10 VP and 10^6 VP diluted in 1 L of distilled water. Lanes G and H correspond to the same viral concentrations but diluted in 1 L sterilized drinking water, separated and concentrated by fluoromagnetic functionalized microparticles.

check this suggestion, RNA amplification products were separated by electrophoresis in 1.8% agarose gel. This assay was carried out in triplicate. Lanes 1 to 3 correspond to the selectivity test, where lane 3 corresponded to the 211 bp segment of capsid protein VP6, thus confirming that the RNA extracted from the complex belonged to rotavirus present in the analyzed liquid stool sample. However, in lanes 1 and 2 the amplification is slight, and the band cannot be seen clearly, maybe due to some handling mistake. By contrast, the high specificity of the system was evident in the non-specific absorption assay. This is due to the fact that, in the absence of the molecular recognition element (i.e., the anti-rotavirus antibody), no viruses were attached to the system. The concentration of RNA extracted and quantified was only 0.6 ng/μL. The amplification and electrophoresis results (Figure 8, lanes 4 to 6) show that in the absence of anti-rotavirus antibodies, no specific absorption took place on the complex. This can be attributed to the use of PBS as buffer with BSA, which acts as a blocking agent that avoids non-specific binding events. Finally, lanes 7 to 10 correspond to environmental water samples.

Finally, fluoroimmunomagnetic separation and concentration were performed in real samples without any treatment. For this test, 1 L drinking water samples from the distribution phase of each of four water treatment plants located in the Department of Norte de Santander (Colombia) were analyzed. The fluoromagnetic microparticle–antibody–virus complex was characterized by using confocal microscopy. Cluster formation was observed in only one of the four plants studied after the complex was exposed to the drinking water sample (Figure 9).

The concentration of RNA extracted and quantified for PA water treatment plant was 10.6 ng/μL. For the other plants, the value was 0.0 ng/μL or below the limit of detection. Figure 7 shows the RNA amplified from both distilled water and drinking water, where lanes A–D correspond to water treatment plants PA, PB, PC and PD. The results confirmed the presence of rotavirus in PA drinking water treatment plant. Extrapolating the values of extracted RNA

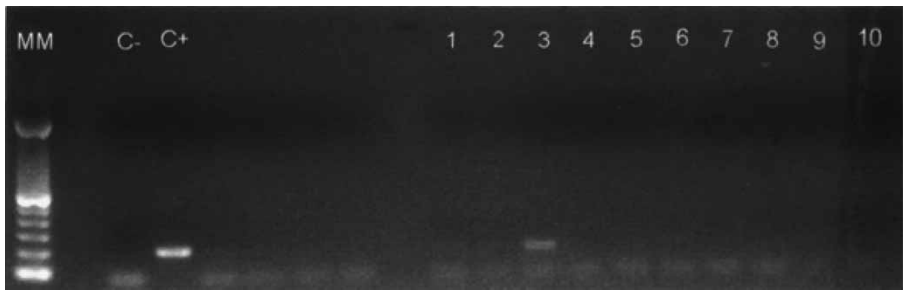

Figure 8 | RNA amplification in 1.8% agarose gel electrophoresis. RNA was obtained through selectivity and non-specific binding tests performed on fluoromagnetic particles functionalized or non-functionalized with an anti-rotavirus antibody. Lanes 1 to 3: selectivity test; lanes 4 to 6: RNA amplified from the non-specific binding; lanes 7 to 10: environmental water samples.

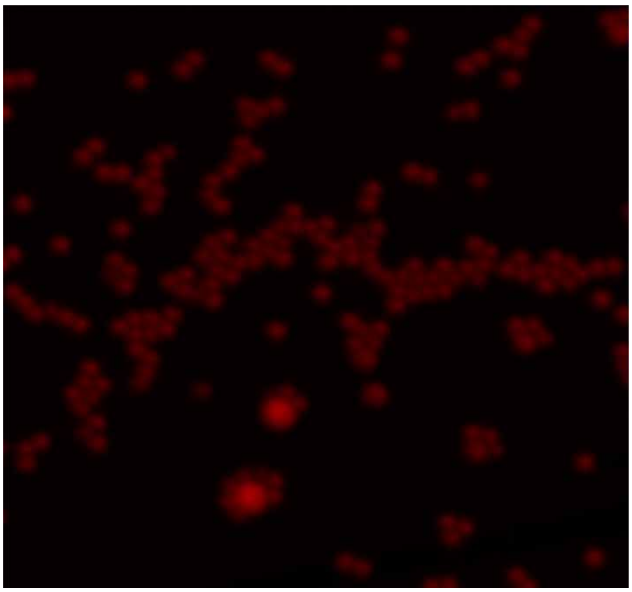

Figure 9 | Confocal characterization of the potential presence of rotavirus in a drinking water sample taken from water treatment plant PA of the department of Norte de Santander, Colombia.

in the calibration curve (gray line corresponding to sterilized drinking water), it can be concluded that by using fluoroimmunomagnetic separation it was possible to detect around 2.4 VP/L in real samples.

For practical reasons, it is difficult to analyze 90 L of water, as recommended by the WHO. However, the current study proves that it is possible to concentrate viral particles present in small amounts of water in a short time. Besides this, fluoroimmunomagnetic particles allow capture of intact (and thus potentially infectious) viral particles, as also reported by Yang *et al.* (2011). An adequate functionalization process allows the sensitivity of the detection process to be increased. Therefore, our technique has displayed good performance in real samples, since it allowed the detection of rotavirus in drinking water from a treatment plant.

CONCLUSION

Fluoroimmunomagnetic microparticles can be used as an effective and highly sensitive technique for the concentration, separation and qualitative detection of rotaviruses in drinking water. The whole process requires only 1 L of water sample and takes no more than 3 hours, including molecular characterization. By using microscopic techniques, it was possible to monitor the separation and concentration process and, therefore, the efficiency of the system. In addition, by using the appropriate molecular recognition stock, it is possible to detect any enteric virus present in a given water sample.

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