

# Rapid and sensitive detection of MS2 coliphages in wastewater samples by quantitative reverse transcriptase PCR

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## SUMMARY

Coliphage MS2 is used in place of pathogens in many studies and is considered one of the indicators of pathogenic viruses in wastewater. We developed a quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay to quantify MS2 coliphages in treated wastewater samples. The format used was SYBR Green. The assay included an internal control to disclose the presence of PCR-product inhibitors. The method had a wide dynamic range (8 logs) with a correlation coefficient of 0.999 and is capable of detecting as few as  $4 \times 10^2$  genome equivalents/100 ml of wastewater sample. The method was validated by using artificially contaminated water samples. The validated method was then applied to naturally contaminated samples collected in a wastewater treatment plant and the results were compared with those obtained by a plaque assay. In comparison with the plaque assay the PCR-method yielded viral counts about 1.5 orders of magnitude higher. The entire detection method, including sample processing and real-time PCR amplification, was completed within 4 hours, making it a rapid single-day method.

**KEY WORDS:** Coliphages, MS2, Indicators, Real-time SYBR Green quantitative RT-PCR, Coxsackievirus, Wastewater

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## INTRODUCTION

Faecal pollution of water resources is a problem of increasing concern. Human population growth, inadequate sanitation, mismanagement of animal wastes and surface run-off from agricultural land contribute to the escalating faecal pollution of both surface and underground water resources (Schaper *et al.*, 2002).

The direct consequence of this phenomenon is more scientific interest in simple and rapid meth-

ods for the detection of waterborne infectious agents with pathogenic potential for man and animals because this represents the first step in the management of outbreaks caused by these pathogens (Baggi *et al.*, 2001).

Human enteric viruses have long been associated with outbreaks of waterborne nonbacterial gastroenteritis (Kukkula *et al.*, 1999; Schvoerer *et al.*, 1999; Koopmans, 2005; Reuter *et al.*, 2006), although these viruses have rarely been isolated or directly detected either in clinical or water samples. In fact, the isolation and subsequent identification of viruses by cell culture assays is very laborious, time-consuming and is not practical for fast routine diagnosis or for non cultivable viruses. Furthermore, culture-based methods lack the sensitivity and specificity required for unequivocal detection of viruses. Because of these limitations, certain groups of bacteria have been used as indicators for the possible presence

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of enteric pathogens in waters. Routine water quality assessments still rely on detection or enumeration of bacterial indicator organisms (American Public Health Association, 1998; Pianetti *et al.*, 2000; Savichtcheva and Okabe, 2006). However, the reliability of these indicators is controversial. They may in fact be present in cases where faecal contamination is absent and may be absent from waters where pathogens are detected. Furthermore, the increased resistance of various pathogenic agents (i.e. viruses and some parasites) to water treatment processes compared to the bacterial indicator microorganisms is now well recognised (Allwood *et al.*, 2005).

Therefore better approaches for monitoring the performance of water treatment processes for viral contamination are needed. With the use of DNA technologies, it is possible to detect the microorganism of interest directly even if present at low levels (Tsai *et al.*, 1993; Severini *et al.*, 1993; Le Guyader *et al.*, 1994; Egger *et al.*, 1995; Gilgen *et al.*, 1997; Chapron *et al.*, 2000; Casas and Sunen, 2001; Zanetti *et al.*, 2003; Di Pinto *et al.*, 2004; Le Cann *et al.*, 2004; Cricca *et al.*, 2006; Jimenez-Clavero *et al.*, 2005; Bonvicini *et al.*, 2006).

However, because of technical difficulties, potential new viral indicator agents such as somatic coliphages and F-specific RNA bacteriophages have been proposed (Wentsel *et al.*, 1982; Stetler, 1984; Toranzos, 1991; Hernandez-Delgado *et al.*, 1991; IAWPRC Study Group on Health Related Water Microbiology, 1991; Tartera *et al.*, 1992; Jofre *et al.*, 1995; Araujo *et al.*, 1997; Zanetti *et al.*, 2006; Zanetti *et al.*, 2007).

Bacteriophages that infect coliform bacteria have been considered possible indicator agents for enteric viruses in surface and groundwater contaminated with faecal material. Although they occur in fewer numbers than the bacterial indicators in which they replicate, they are relatively hardier and persist longer in the environment, making them candidates for indicator and index purposes for enteric viruses (IAWPRC Study Group on Health Related Water Microbiology, 1991; Havelaar *et al.*, 1990; Havelaar *et al.*, 1993; Sobsey *et al.*, 1996).

The aim of the present work was the standardisation of a rapid and sensitive quantitative SYBR Green based real time RT-PCR to detect and quantify the MS2 coliphage in wastewater treated samples as indicator of faecal pollution.

## MATERIAL AND METHODS

### *MS2 coliphage preparation and enumeration*

*Escherichia coli* bacteriophage MS2 (ATCC 15597-B1 strain) was cultivated and enumerated with the double agar layer technique according to UNI EN ISO 10705-1 (2001) using the host strain *Escherichia coli* C3000. A filtered portion of MS2 stock suspension with a concentration of  $10^{11}$  plaque-forming units (PFU) per millilitre was used for serial 10-fold dilutions as PFU per millilitre.

### *Development of the qRT-PCR assay*

A real time qRT-PCR assay was developed to detect MS2 RNA coliphage. The format used was SYBR Green. The selected specific primers (O'Connell *et al.*, 2006) were able to amplify a 115 bps fragment located in the region of the lysis protein (nucleotides 1693 to 1807, GenBank Accession Number NC-001417).

One-step RT-PCR reaction was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen S.p.A., Milano, Italy). The qRT-PCR reaction was carried out in 25  $\mu$ l final volume containing 12.5  $\mu$ l of (2 $\times$ ) QuantiTect SYBR Green RT-PCR Master Mix, 0.5  $\mu$ M of each primer, 0.25  $\mu$ l of QuantiTect RT Mix and 10  $\mu$ l of extracted sample containing  $1.7 \times 10^6$  copies of internal control (IC). Since SYBR Green lacks discrimination capability between two different targets, the amplification reactions for MS2 coliphage and IC were conducted separately in two different tubes.

The qRT-PCR cycling program included a reverse transcription at 55°C for 30 min, a denaturation at 95°C for 15 min followed by 40 cycles at 94°C for 15 s, at 52°C for 30 s and at 72°C for 30 s. Since the melting temperatures of MS2 coliphage and IC were 80°C and 85°C respectively, the fluorescence measurements were performed at the extension temperature (72°C) to exclude the possible signal arising from non specific products. Thermal cycling and fluorescence detection were carried out using the Rotor-Gene Instrument (RG-3000, Corbett Research).

The MS2 coliphage standard curve was created from serially 10-fold diluted MS2 plasmid assaying  $10^8$  down to  $10^1$  copies per reaction. The target dilutions were tested in triplicate and in three different runs. There was a linear relationship between the threshold cycle value plotted against

the log of the copy number over the entire range of dilutions tested.

The assay included an internal control that allowed the inhibition of qRT-PCR to be quantitatively assessed. The selected IC was the human coxsackievirus B4 (CVB4) and the specific primers were designed using the Clone Manager Professional Suite Software (version 8.0). The forward primer was 5'-TGCGGCTAATCCTAACTGCG-3' (nucleotide 464 to 483, GenBank Accession Number X05690) and the reverse 5'-GGAAACACGGACACCCAAAG-3' (nucleotide 549 to 568). The primer pair amplify a fragment of 105 bps located in the region of 5'UTR.

The IC standard curve was created from 10-fold serially diluted purified CVB4 RNA, assaying from  $1.7 \times 10^8$  to  $1.7 \times 10^4$  copies per reaction. The purified RNA was obtained from a suspension of CVB4 titrated by a plaque assay.

The amounts of both MS2 and CVB4 RNA copies in the samples were determined by linear extrapolation of the crossing point values ( $C_p$ ) using the equation of the corresponding regression line obtained from the standard curves. The amount of MS2 RNA was expressed as number of RNA copies per 100 ml of sample.

Each run of the qRT-PCR assay included both positive and negative controls. The positive control was an amount of  $10^3$  copies of MS2 RNA and the negative control was a 100 ng pool of whole genomic bacterial DNA from strains of *Escherichia coli* (ATCC 700928D-5), *Enterobacter cloacae* (ATCC 13047D), *Providencia stuartii* (ATCC 33672D), *Citrobacter freundii* (ATCC 8090D) and *Enterococcus faecalis* (ATCC 700802D-5) and 10 pg pool of viral nucleic acids from 5 different virus strains (*Reovirus type 1*, ATCC VR-230; *Hepatitis A virus*, ATCC VR-1402; *Echovirus 1*, ATCC VR-1038; *Human coxsackievirus B4*, ATCC VR-184 and *Human rotavirus*, ATCC VR-2417) extracted by QIAamp UltraSens Virus Kit (Qiagen S.p.A., Milan, Italy).

#### *Development of MS2 plasmid as standard for the qRT-PCR assay*

To quantify the number of MS2 phage genomes by the qRT-PCR assay, a plasmid was constructed by cloning nucleotides 1470 to 2000 (GenBank, accession Number NC\_001417). The 531 bps-fragment encompassed the 115-bps MS2 product of qRT-PCR. The primers for both reverse tran-

scription and cDNA amplification were obtained using Clone Manager Suite Professional Software (version 8.0). The forward primer was 5'-ACCTGTAGCGTTCGTCAGAG -3' (nucleotides 1470 to 1489) and the reverse primer 5'-ATTCGCCTCCCGTAGCTTAG -3' (nucleotides 1981 to 2000).

The first step was a one-step RT-PCR, performed with extracted MS2 coliphage RNA acting as the template for reverse transcription and cDNA amplification. Both reactions were performed sequentially in a single tube using the C. therm. Polymerase One-Step RT-PCR System (Roche Diagnostics S.p.A., Milano, Italy). The reaction was carried out in 50  $\mu$ l final volume containing 0.4 mM of each dNTP, 7% of DMSO, 5mM of DTT solution, 0.3  $\mu$ M of each primer, 10  $\mu$ l of (5 $\times$ ) RT-PCR buffer, 2  $\mu$ l of C. therm. A 30-min step of reverse transcription at 60°C and a 2-min step of denaturation at 94°C were followed by two rounds of cycles: the first 10 cycles included a denaturation step at 94°C for 30 sec, a primer annealing step at 55°C for 30 s and a chain elongation step at 72°C for 60 s, the following 30 cycles were similar to the first except for an increment of 5 s at each elongation step. The final elongation was then prolonged by 7 min.

The resulting RT-PCR product was visualized, under low UV transillumination gel on a GelDoc 2000 imager (Bio-Rad Laboratories S.r.l., Milano, Italy) in a 1.6% agarose-1 $\times$ TAE gel containing 1  $\mu$ g/ml of ethidium bromide. The visualized 531-bps band was excised, gel purified using Gel Extraction Kit (Qiagen S.p.A., Milano, Italy), cloned into a pCR@2.1 vector (TA cloning kit, Invitrogen S.r.l., Milano, Italy) and transformed into One-Shot TOP10 chemically competent *Escherichia coli* (Invitrogen S.r.l., Milano, Italy) according to the manufacturer's instructions. Plasmid DNA (pCR-MS2) was isolated from positive clones using the Plasmid Midi Kit (Qiagen S.p.A., Milano, Italy) and sequenced bidirectionally.

#### *Development of the internal control for the qRT-PCR assay*

Human coxsackievirus B4 (ATCC VR-184 strain) was employed as internal control in qRT-PCR assay to disclose PCR inhibitors.

CVB4 was purified from a viral suspension titrated by the plaque assay (O'Connell *et al.*, 2006; Brilot *et al.*, 2002; See and Tilles, 1992) and was used to construct an external standard curve, in

particular 10-fold diluted viral suspension from  $1.7 \times 10^8$  to  $1.7 \times 10^4$  copies/reaction.

A fixed amount of  $10^7$  CVB4 particles were added to the samples prior to RNA extraction procedure. 1/6 of elution volume ( $1.7 \times 10^6$  copies per reaction) was tested in qRT-PCR.

#### *Recovery of MS2 coliphages particles from wastewater samples*

From April to October 2006, 14 wastewater samples were collected in sterile 500 ml polyethylene containers at the exit of secondary treatment in an activated sludge treatment plant and kept at 4°C for less than 8 h until the MS2 bacteriophages were quantified by plaque assay (UNI EN ISO 10705-1, 2001) and by qRT-PCR analysis. This plant treats 650.000 population equivalents of wastewater. Fifty ml of treated wastewater samples were filtered by using 0.22 µm filters (Millipore S.p.A., Milano, Italy), and were then concentrated by centrifugation at 3.500 rpm for 45 min at 4°C using Amicon® Ultra-15 concentrator (Millipore S.p.A., Milano, Italy).

#### *Nucleic acid extraction*

One milliliter volume of the concentrated wastewater samples was employed for the RNA extraction using the QIAamp Ultrasens procedure (Qiagen S.p.A., Milano, Italy). Total RNA was extracted and purified according to the manufacturer's instructions and RNA was eluted in 60 µl volume.

## RESULTS AND DISCUSSION

#### *Specificity of the qRT-PCR assay*

The primers for MS2 bacteriophage were tested against a panel of DNA from non target microorganisms to determine the specificity of the assay. The panel included the whole genomic DNA from *Escherichia coli*, *Enterobacter cloacae*, *Providencia stuartii*, *Citrobacter freundii* and *Enterococcus faecalis*. The specificity of MS2 primers was also tested against nucleic acids from 5 different viruses (*Reovirus type1*, *Hepatitis A virus*, *Echovirus1*, *Human coxsackievirus B4* and *Human rotavirus*). The non target nucleic acids were tested in separate reactions by qRT-PCR and none of them produced a signal.

#### *Sensitivity and reproducibility of qRT-PCR assay*

To allow the quantification of MS2 RNA a standard curve was generated by amplification of 10-fold dilution series of the pCR-MS2 plasmid from  $10^8$  to  $10^1$ .

Cycle threshold values ( $Ct_s$ ) were determined in triplicate and in three different runs and plotted against the plasmid copy number. The dilution series enabled template quantification over eight orders of magnitude with  $Ct_s$  between approximately 7 and 34 PCR cycles for  $10^8$  and  $10^1$  copies, respectively. Amplification plots were highly reproducible between triplicate samples in the same experiment and in three different runs (Table 1). The standard curve of the pCR-

TABLE 1 - Inter-assay variability of MS2 qRT-PCR.

| Copies per reaction | Maximum $Ct^1$ | Minimum $Ct$ | Average | $SD^2$ $Ct$ | $CI^3$ $Ct$ |
|---------------------|----------------|--------------|---------|-------------|-------------|
| 1.00E+08            | 7.36           | 6.67         | 7.07    | 0.36        | 6.18-7.95   |
| 1.00E+07            | 11.34          | 11.09        | 11.2    | 0.13        | 10.88-11.51 |
| 1.00E+06            | 15.37          | 15.13        | 15.21   | 0.14        | 14.87-15.55 |
| 1.00E+05            | 19.8           | 19.54        | 19.67   | 0.13        | 19.34-20.00 |
| 1.00E+04            | 23.53          | 22.93        | 23.18   | 0.31        | 22.41-23.96 |
| 1.00E+03            | 27.25          | 26.85        | 27.07   | 0.21        | 26.56-27.58 |
| 1.00E+02            | 31.26          | 30.74        | 31.04   | 0.27        | 30.37-31.72 |
| 1.00E+01            | 36.14          | 33.79        | 34.66   | 1.29        | 31.44-37.87 |

<sup>1</sup>Ct: threshold cycle; <sup>2</sup>SD: Standard Deviation; <sup>3</sup>CI: 95% Confidence Interval.

TABLE 2 - Suitability of pCR-MS2 plasmid as DNA standard for quantification of MS2 RNA.

| Expected values<br>(copies/ reaction) | Experimental values<br>(copies/reaction) | Max Var (%) <sup>1</sup> | Min Var (%) <sup>2</sup> | Av Var (%) <sup>3</sup> | SD Var (%) <sup>4</sup> | CI Var (%) <sup>5</sup> |
|---------------------------------------|--|--------------------------|--------------------------|-------------------------|-------------------------|-------------------------|
| 3.33E+07                              | 3.19E+07                                 | 12.80                    | 0.50                     | 6.80                    | 6.20                    | -0.22-13.82             |
| 3.33E+06                              | 3.71E+06                                 | 33.30                    | 8.60                     | 18.10                   | 13.30                   | 3.05-33.15              |
| 3.33E+05                              | 2.55E+05                                 | 20.20                    | 3.60                     | 10.00                   | 8.90                    | -0.07-+20.07            |
| 3.33E+04                              | 2.66E+04                                 | 6.20                     | 4.60                     | 5.20                    | 0.90                    | 4.18-6.22               |
| 3.33E+03                              | 2.75E+03                                 | 7.45                     | 3.20                     | 6.37                    | 2.80                    | 3.21-9.53               |
| 3.33E+02                              | 3.13E+02                                 | 12.45                    | 6.32                     | 12.50                   | 6.20                    | 5.48-19.52              |
| 3.33E+01                              | 3.99E+01                                 | 18.00                    | 13.47                    | 19.02                   | 6.13                    | 12.08-25.96             |
| 3.33E+00                              | 4.89E+01                                 | 43.40                    | 8.00                     | 28.13                   | 18.19                   | 7.54-48.72              |

<sup>1</sup>Max Var (%): Maximum percentage of variance; <sup>2</sup>Min Var (%): Minimum percentage of variance; <sup>3</sup>Av Var (%): Average percentage of variance; <sup>4</sup>SD Var (%): Standard Deviation percentage of variance; <sup>5</sup>CI Var (%): 95% Confidence Interval percentage of variance.

MS2 plasmid had a correlation coefficient ( $r$ ) of 0.999. The suitability of pCR-MS2 plasmid as DNA standard for quantification of MS2 RNA was evaluated using 10-fold dilution series of an MS2 viral suspension of  $2 \times 10^8$  PFU/ml. The viral RNA was extracted as previously described and 1/6 elution volume was used for the qRT-PCR assay. As showed in Table 2, the experimental values obtained by plotting  $C_t$  values of MS2 RNA in the MS2 plasmid standard curve presented a standard deviation (SD) of variation comprised between 0.90% and 18.19% in relation to expected values. The detection limit was 3.3 copies per reaction corresponding to  $2 \times 10^1$  PFU/ml.

#### Efficiency and sensitivity of MS2 coliphage concentration method from wastewater samples

The efficiency of MS2 concentration method from water samples was determined as follows: six sterile water samples were spiked with serial dilutions of MS2 coliphage ( $2 \times 10^6$  to  $2 \times 10^1$  PFU/ml) and a fixed amount of IC ( $10^7$  PFU/ml) into a volume of 50 ml. Filtration, concentration, extraction and amplification were performed as previously described and the virus recovery was measured by the qRT-PCR protocol. The detection limit of the recovery method was  $4 \times 10^2$  PFU/100 ml and the mean percentage of recovery efficiency was 75%.

#### Correlation between plaque assay and qRT-PCR assay

The genome copy number of MS2 coliphage was determined in 14 wastewater samples using our assay in parallel with the standard plaque assay. All the samples gave positive results with both techniques. In comparison with the plaque assay results, real-time PCR methods yielded viral counts of about 1.5 orders of magnitude higher (Table 3).

The relationship between the copy number of viral genomes and PFU was inconsistent and could be affected by many factors such as sample preparation and culture conditions. Therefore, it is important to emphasize that the health implications of detecting viral genome by PCR has multiple complications. It is likely that molecular analysis-based methods tend to overestimate the quantity of viruses, while the culture-based methods tend to underestimate them (He and Jiang, 2005).

#### CONCLUSION

MS2 coliphage is very stable in the environment and has thus been proposed as a useful indicator of water and environmental contamination as well as a potential surrogate for other highly infectious enteric viruses to optimize extraction and

TABLE 3 - Comparison of MS2 quantification by qRT-PCR and plaque assay in wastewater samples.

| Sample no. | qRT-PCR<br>(viral genome copies/100 ml) | Plaque assay<br>(PFU/100 ml) | qRT-PCR/Plaque assay |
|------------|---|------------------------------|----------------------|
| 1          | 3.51E+04                                | 6.00E+02                     | 5.85E+01             |
| 2          | 8.15E+03                                | 1.35E+03                     | 6.04E+00             |
| 3          | 3.47E+04                                | 1.90E+03                     | 1.83E+01             |
| 4          | 2.22E+04                                | 3.70E+03                     | 6.00E+00             |
| 5          | 4.33E+04                                | 3.85E+03                     | 1.12E+01             |
| 6          | 3.15E+04                                | 4.15E+03                     | 7.59E+00             |
| 7          | 1.19E+05                                | 4.30E+03                     | 2.77E+01             |
| 8          | 2.56E+04                                | 4.95E+03                     | 5.17E+00             |
| 9          | 3.08E+04                                | 5.35E+03                     | 5.76E+00             |
| 10         | 4.37E+04                                | 6.90E+03                     | 6.33E+00             |
| 11         | 3.42E+04                                | 1.02E+04                     | 3.35E+00             |
| 12         | 2.10E+04                                | 1.18E+04                     | 1.78E+00             |
| 13         | 1.97E+04                                | 1.20E+04                     | 1.64E+00             |
| 14         | 7.61E+04                                | 1.21E+04                     | 6.29E+00             |

detection procedures for the contamination of water.

We developed a reproducible and reliable real-time PCR method for MS2 coliphage quantitation in wastewater samples. This method enabled the detection of 400 copies per 100 ml of sample with an excellent linearity of 8 logs. The major advantages of the real time RT-PCR assay lie in the ability to avoid laboratory contamination and to provide rapid results. Since there were no post-PCR manipulations, the virus could be detected within approximately 4 hours following receipt of the sample. The real-time RT-PCR protocol was validated on artificially contaminated water samples then evaluated on natural samples. MS2 coliphage genomes were detected in all samples collected at the exit of secondary treatment in an activated sludge treatment plant with a mean value of  $3.89 \times 10^4$  MS2 genomes per 100 ml.

Moreover, samples used in this study were collected in a relatively large plant able to treat ef-

fluents from 650.000 inhabitants. This is a major concern for public health, especially concerning agricultural reuse and recreational areas located downstream from the sewage treatment plants. In conclusion, we optimized a rapid, reproducible, highly specific and sensitive MS2 RNA detection procedure that, by combining a simple water concentration step with a real-time RT-PCR, allows detection of as little as 4 MS2 RNA molecules/ml of wastewater tested.

These features make it an excellent large-scale screening tool to evaluate the efficacy of the different treatments used in sewage treatment plants, monitor the level of MS2 coliphage release in the environment and thus to clarify the epidemiology of enteropathogenic infections.

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