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NOTE TO THE EDITOR

Statistical correlation between enterovirus genome copy numbers and infectious viral particles in wastewater samples

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Abstract

Aims: Classic virological tests are time consuming and labour-intensive; real-time RT-PCR has proven to be a fast method to detect and quantify enterovirus genomes in clinical and environmental samples. This method is unable to discriminate between infective and noninfective enterovirus particles; few clinical studies have compared real-time RT-PCR and viral culture. We wondered if the enterovirus genome quantification could be correlated to the infectivity.
Methods and Results: We used the statistical approach to verify our hypotheses to correlate data, obtained by the standard method (most probable number of cytopathic units—MPNCU) and molecular test (real-time RT-PCR), on wastewater treatment plant samples. Chi-squared test was used, considering several cut-off values ('50'-'100'-'200' genome copy numbers), to determine statistical significance in comparison of the two methods. Chi-square value was not significant when cut-off of 50 ($P = 0{\cdot}103$) and 100 ($P = 0{\cdot}178$) was assumed but was significant with cut-off of 200 ($P = 0{\cdot}044$).
Conclusion: This limit, 200 genome copy, could be used as cut-off value to indicate enterovirus survival in environmental monitoring.
Significant and Impact of the Study: To introduce a fast procedure that is able to compensate for disadvantages of cell culture method for viral environmental analyses.

Introduction

Microbiological quality of wastewater effluents is currently evaluated by bacterial indicators; however, their presence does not correlate with the number and viability of enteric viruses (Havelaar *et al.* 1986). It is well known that bacterial indicators are less resistant to environmental factors than viruses, and therefore the real viral risk can be underestimated (Skraber *et al.* 2004). Agricultural reuse of treated wastewater is a common practice, and the related potential spread of pathogens represents a true infectious risk factor for the public health.

Virological tests are based on the quantification of viral particles in cell cultures or, more recently, on genome detection by molecular methods. The cell culture propagation procedure is still the best method to enumerate viruses and demonstrate their infectivity.

The classic method of culturing enterovirus particles uses different types of cells, as buffalo green monkey (BGM) cells, and the quantification is based on plaque count or on the most probable number of cytopathic units (MPNCU). Both tests are time consuming and labour intensive; besides, their sensitivity decreases with a low number of viral particles. Moreover, they are unsuitable for the detection of hepatitis A virus, noroviruses and other enteric viruses, for which appropriate cell cultures are not available or their growth is limited.

Molecular tests have been successfully applied on environmental samples, allowing a rapid and specific detection of human enteric viruses (Divizia *et al.* 1999; Donia *et al.* 2005). More recently, real-time RT-PCR has proven to be a fast way to detect and quantify enteric viral genomes in clinical and environmental samples (Nijhuis *et al.* 2002; Monpoeho *et al.* 2004). Although widely

applied, there are some important limitations that slow down the introduction of this method as a routine test for environmental screening. The main issue is the impossibility of discriminating between infective and noninfective viral particles; indeed, nucleic acids can persist for long periods in the environment (Skraber *et al.* 2004).

The diagnosis of enterovirus infections in clinical analysis can be obtained by real-time RT-PCR in a few hours, with a higher sensitivity compared to standard methods (Nijhuis *et al.* 2002). This quantitative method has been used successfully to monitor the progression of viral diseases and to evaluate the response to antiviral therapies with difficult-to-cultivate viruses (Jung *et al.* 2000).

There is a need to introduce a fast and reliable procedure, able to compensate for disadvantages of cell culture method in viral environmental monitoring.

Previous studies have compared real-time RT-PCR and viral culture mainly to assess the meaning of viral genome persistence followed by active viral replication during illness (Falsey *et al.* 2003; Botero *et al.* 2008) or to improve sensitivity in virus detection (van Elden *et al.* 2001).

A statistical study, aimed at evaluating the correlation between results from molecular and culture tests in clinical specimen, showed a high level of sensitivity and specificity employing a reference cut-off value (Cheng *et al.* 2004).

Some authors suggest that viral genome can be used as an indicator of viral survival in river water, as reported by Skraber *et al.* (2004).

Considering the above-mentioned studies, we wondered whether the amount of enterovirus genome in wastewater could be correlated with infectivity, and thus represent a useful indicator of viral risk in environmental analysis.

We used the statistical approach to verify the validity of our hypotheses regarding the possible significance, in terms of infectivity, of enteroviral genome quantity in environmental samples, to adequately address the health risks. Wastewater samples from two treatment plants were collected at various treatment steps, on the occasion of another study, aimed at evaluating removal efficiencies of enteric viruses and bacterial indicators, and the reuse potential of final effluent (Petrinca *et al.* 2009). In this study, the cultivable enteroviruses were always found as the most abundant viruses isolated on BGM cell lines, where they showed a clear cytopathic effect. Enteroviruses were detected both with conventional (MPNCU) and molecular methods (real-time RT-PCR), and the results were compared.

Sample concentration was performed as previously reported (Petrinca *et al.* (2009)). Infective enterovirus particles were counted on BGM cells grown in 96-well plates. Each sample dilution was inoculated in triplicate, incubated at 37°C in 5% CO_2 and checked daily for 5 days. The presence of cytopathic effect was confirmed by a secondary passage, and results were expressed in MPNCU per litre of sample (Petrinca *et al.* 2009).

Real-time RT-PCR was performed using SYBR Green I fluorescent dye strategy: an aliquot corresponding to 1 l of concentrated wastewater sample was used for enteroviral RNA extraction by Trizol–LS Reagent (Life Technologies, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction and amplified with a Bio-Rad iCycler. Primers used were selected in the 5′-noncoding region of enteroviruses (poliovirus, coxsackievirus, echovirus) using the Beacon Designer 3.01 software, by Bio-Rad (http://www.premierbiosoft.com/molecular beacons/index.html) (Donia *et al.* 2005).

Extraction and amplification efficiency were evaluated using ARMORED EV RNA control, at known concentration supplied by Ambion Diagnostics ($1{\cdot}05 \times 10^5$ copies μl^{-1}). The Syber Green I chemistry method had provided good results in terms of sensitivity: the best detection limit was one copy of viral RNA in 50% and ten copies in 100% of assays. This method offered the best amplification efficiency, and the amplicons specificity was confirmed by melting curve analysis with reproducibility of the method (Donia *et al.* 2005).

The results of the statistical test are summarized below.

Irrespective of the detection method used, 83% (69/83) of samples were positive for enteroviruses; real-time RT-PCR detected specific viral genome copies in 76% (63/83) of samples, of which only 52, 38% (33/63) were positive for infectivity (Table 1).

The absence of enteroviral genome always coincided with the absence of infectious enterovirus particles, except in six cases where the MPNCU test showed cytopathic effect in the absence of specific viral genome. This evidence could be because of the presence of polymerase chain reaction inhibitors or of RNA inactivating enzymes in these samples.

The chi-squared test was applied to compare the results obtained with the two methods; sensitivity and specificity were calculated on 2×2 tables (faculty.vassar.edu/lowry/tab2x2.html), considering the MPNCU test as the gold standard. Statistical significance was assumed at $P \leq 0{\cdot}05$.

Several cut-off values ('50'-'100'-'200', expressed in genome copy numbers per litre) were tested to evaluate statistical significance.

Our statistical analysis showed that sensitivity of real-time RT-PCR decreased from 59 to 48% with the increase in cut-off values, whilst the specificity of the molecular method improved from 61 to 75% as cut-off values were increased (Table 1).

Chi-square value was not significant when cut-off values of 50 ($P = 0{\cdot}103$) and 100 ($P = 0{\cdot}178$) copy numbers

Table 1 Distribution of positive and negative samples with statistical analysis

	Cut-off >50 c.n. l^{-1}		Cut-off > 100 c.n. l^{-1}		Cut-off > 200 c.n. l^{-1}	
Test	Positive (CC)	Negative (CC)	Positive (CC)	Negative (CC)	Positive (CC)	Negative (CC)
Real-time positive	23	17	19	14	19	11
Real-time negative	16	27	20	30	20	33
Chi-square	2·65		1·81		4·06	
P-value*	0·103		0·178		0·044	
Sensitivity	59%		48%		48%	
Specificity	61%		68%		75%	

c.n., copy numbers; c c, cell culture (MPNCU method).
*Statistical significance $P \leq 0{\cdot}05$.

were used but it was significant with a cut-off value of 200 ($P = 0{\cdot}044$) (Table 1).

It is worth to point out that 47·6% of total samples showed a positive molecular test but were negative for the cell culture method. When cut-off values of '50' and '100' were applied, the above proportion slightly decreased to an average of 42%.

This may be because of the presence of the enteroviral genome as fragments, unable to replicate, and the resulting loss of infectivity (Ma *et al.* 1994), or it can depend on the different effectiveness of the BGM cells, used in this study, in detecting the various enterovirus types (Terletskaia-Ladwig *et al.* 2008).

The best correlation between the two methods was obtained when the cut-off value was set at 200 ($P = 0{\cdot}044$). In these statistical conditions, the share of samples positive for enteroviral genome but negative for infectivity decreases to 36·6%. In all of cases, the real-time RT-PCR efficiency was not greater than 62% (on average).

Some possible reasons for these results can be hypothesized.

The first can be ascribed to the low number of samples, in total 83. The second can be recognized in the heterogeneity of samples, taken at different treatment steps. In these samples, the presence of various anionic and cationic detergents, under certain pH conditions, may be destructive or protective to enteroviruses (Berg *et al.* 1988). Detergents, as demonstrated by Richards (1999) and Ma *et al.* (1994), may damage viral capsid proteins, compromising the infectivity of enteroviral particles without causing damage to specific genome. Other commonly practiced treatments, such as UV disinfection, have been documented as a cause of the nucleic acid fragmentation (Ma *et al.* 1994; Richards 1999).

Although based on limited data, these preliminary results show the existence of a relationship between the quantity of enteroviral genome, determined by real-time RT-PCR, and infectivity (MPNCU) in wastewater:

The lower statistically significant limit of infective enteroviral particles was found to be at 200 genome copy numbers per litre. This limit could be used as cut-off value to indicate enteroviral survival in environmental monitoring.

Further studies, with a larger number of samples and more homogeneous experimental conditions, are required to validate the model, to evaluate the infectivity risk in wastewater treatment plants and to extend this model to different environmental matrices.

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