Rapid Detection of Enteroviruses in Sewage Samples by Real-Time Reverse Transcriptase PCR for Enhanced Viral Surveillance in Milwaukee, Wisconsin

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ABSTRACT

Objective: To survey the presence and concentration of enteroviruses (EVs) in Milwaukee sewage samples by real-time PCR.

Methods: Enteroviruses were isolated using BGM, Caco-2 and HEp-2 cell lines from samples collected during 2001-2005. Concentration of viruses in sewage samples was accomplished by the EPA Information Collection Rule (ICR) organic flocculation procedure using most probable number (MPN) calculations. Viral RNA was extracted and detected by quantitative real-time qRT-PCR assay.

Results: A total of 60 samples were assayed over a period of 5 years from the Milwaukee area waste water treatment plant. Viruses (enteroviruses, reoviruses or adenoviruses) were detected in 58 of 60 (96.6 %) samples. EVs were detected in 46 of 60 (76.6 %) samples with a highest titer of 3152 MPN/liter detected in July 2002. All culture positive samples were re-tested by qRT-PCR, and quantified with standard curves. Several samples were diluted for optimal amplification and to avoid possible inhibition from sewage components. Periodically, sewage and clinical EV serotypes were identical.

Conclusion: The combination of virus isolation and qRT-PCR are effective and complementary approaches to tracking circulating EVs in the local environment. Along with sequence analysis, these techniques could offer a better understanding of the natural history and relationships among the clinical and environmental EV serotypes circulating in Milwaukee.

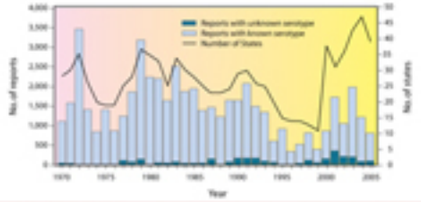
INTRODUCTION

Human enteroviruses (EV) belong to *Picornaviridae* family and include over 60 serotypes divided among five groups (coxsackieviruses A & B, echoviruses, enteroviruses and polioviruses). EVs are estimated to cause millions of infections annually in the US and are the most common cause of aseptic meningitis and neonatal enteroviral sepsis[1]. EVs are small (30 nm), non-enveloped, single-stranded RNA (7.5 kb) viruses consisting of four structural proteins (VP1- VP4). Most of the molecular assays utilize the 5' non-translated regions (NTR) as targets which are relatively conserved among the serotypes[2].

The National Enterovirus Surveillance System (NESS) is a voluntary, passive system that has monitored trends in circulating enteroviruses since 1969 (Fig 1)[1]. The City of Milwaukee Health Department Laboratory (MHDL) routinely identifies and tracks EV serotypes in the influent of local sewage treatment plants[3] as well as community wide clinical isolates. A rapid PCR assay has recently been introduced by MHDL for enhanced viral surveillance.

Figure 1

Number of Enteroviruses Reported in National Enterovirus Surveillance System in the United States during 1970-2005



MATERIALS AND METHODS

Sewage collection. Influent samples were collected monthly from the Jones Island wastewater treatment plant and concentrated by organic flocculation procedure (EPA ICR water-monitoring protocol)[4]. The concentrates were tested and remaining pellets stored at -70° C for future use.

Virus isolation & host range analysis. EVs were isolated on BGM, Caco-2 and HEp-2 cells. In addition, HFS, MRC-5 and MK cells were used for clinical samples. The non-polio EV isolates were typed using LBM microneutralization test (A-H pool, Statens Serum Institut, Copenhagen, Denmark). The most probable number (MPN) for the ICR was calculated using U.S. EPA (Cincinnati, OH) software.

Extraction of nucleic acid. Total nucleic acid was extracted from clinical samples or frozen pellets using the NucliSens easyMAG extraction system (BioMerieux, Durham, NC).

Real-time RT-PCR assay. Viral RNA was amplified by Taqman RT-PCR assay on the SmartCycler II (Cepheid, CA) using SuperScript™ One-step kit (Invitrogen, CA) and EV Analyte Specific Reagents (ASR) with TaR-labeled internal/inhibition controls (IC) with Cycle threshold (Ct) default setting at 30. Sixty three different EV serotypes are targeted using Alexa-532 and FAM probes in the EV ASR-based assay (Cepheid, CA) (Table 1).

Sequence analysis of virus isolates. The VP1 sequences of five isolates were amplified and sequenced# at the CDC Enterovirus laboratory using 3130xl sequence detection system (Applied Biosystems, Foster City, CA).

Specificity, sensitivity and predictive values. Measurements for cell culture and PCR assays were calculated using Bayesian analysis.

Table 1 Significant Enterovirus Serotypes Targeted in Cepheid EV ASR Assay

Groups	Probe labels	Serotypes
1	Alexa-532	Coxsackie A2-A6, A10, A12, A14, A16, EVT1
		Coxsackie A9, B1-B6, echo1-7, 9, 11-21, 24-27, 29-33, EV69
2	FAM	Coxsackie A11, A13, A15, A17-22, A24
		EV68, EV70
		Poliovirus 1-3

Table 2 Sewage Enteroviruses Isolated on Various Cell Lines (2001-2005)

	Number of EV isolates			
Cell types	Coxsackievirus A	Coxsackievirus B	Echovirus	Total isolates
BGM	0	47	4	51
Caco-2	1	31	321	353
HEp-2	0	14	8	22
Total	1	92	333	426

RESULTS

Percent EV and other culturable viruses from sewage samples. Of the 60 sewage samples analyzed, adenovirus and reovirus were detected in 38 (63.33%) and 56 (93.3%) samples respectively for a total of 58 (96.6%) positive samples (Fig. 2). EVs were detected in 46 (76.6%) samples with a minimum and maximum titer being 20 and 3152 MPN/L in March and July 2002 respectively.

Cell sensitivity of sewage EV isolates. Table 2 indicates the cell sensitivities of the sewage isolates.

Seasonal patterns. Clinical EV cases increased during summer and fall, with peaks during June-August as did sewage isolates during the same months (Fig. 3).

EV PCR assay. Of 60 sewage samples tested, 48 were PCR positive with an average Ct 20.56 (Ct range: 15.42 - 29.00), indicating fairly high titer of EVs in sewage and clinical samples. All the culture positive samples were confirmed by PCR. In addition, PCR results were in complete agreement with 26 blinded validation samples. However two clinical HRV isolates cross reacted in the EV PCR assay. On several occasions both probes (each of multiple serotypes) were positive for a single sewage sample, indicating more than one serotype present in a given sewage sample.

Amplification efficiency. The Ct values were plotted against RNA dilutions (Fig. 4). A linear relationship between RNA dilutions and Ct values, with a correlation coefficient (R^2) greater than 0.99, indicates efficient amplification (Fig 5). Virus concentration in sewage (8560 virus copies/L) and clinical (4440 virus copies/L; EV only) samples were calculated by extrapolating Ct value with the dilutions and known RNA concentration.

Sensitivity & specificity. For sewage samples the PCR assay was 100% specific and 96% sensitive compared to cell culture (Table 3) with positive and negative predictive values 1.0 and 0.60 respectively. There was no cross reaction with FluA (H1 & H3), FluB, RSV, HSV (1 & 2), VZV and Norovirus.

Sequence analysis. Serotypes of five isolates were confirmed at CDC by pairwise comparison of the VP1 amplicon sequences to EV, human rhinovirus (HRV) and Picornavirus databases and BLAST searched for other microbes (Table 4). Comparison of cell culture, PCR and sequencing for identification of clinical and sewage isolates is presented in Table 5.

Table 3
Comparison of the Cell Culture and RT-PCR Assay

N= 60	PCR +	PCR -
Culture +	46	0
Culture -	2	10

Figure 2 Percent Virus Isolated in Sewage (2001-2005) (n=60)

Figure 3 Comparison of Clinical Enterovirus Cases with Sewage Enterovirus Titers: January 2001 through December 2005

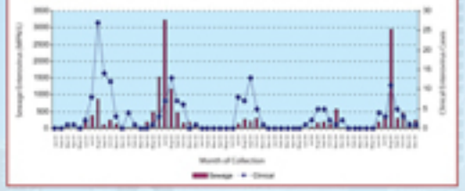
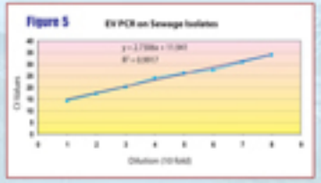


Figure 4 Multiplex Real-time RT-PCR Assay Using EV ASR

Figure 5 EV PCR on Sewage Isolates



CONCLUSIONS

1. Real-time PCR was more sensitive in rapidly identifying the majority of EVs allowing rapid screening of larger numbers of samples.
2. PCR can potentially detect multiple serotypes, or non-culturable EVs in single sewage sample.
3. Infrequent HRV cross-reactivity with the EV PCR assay is likely due to high viral load or major sequence resemblance of certain HRV serotypes.
4. Frequency of clinical and sewage EVs (and serotypes*) often tracked together following similar seasonal trends.
5. Sequencing of virus isolates confirmed findings and clarified discrepancies.
6. Rapid EV PCR and sequence-based typing, as a surrogate for neutralizing epitopes of both clinical and environmental isolates, will likely play a role in a better understanding of the dynamics and epidemiology of EV.

* data not presented

Table 4 Partial Sequence Match of the Sewage and Clinical Isolates for Viral Identification

Virus ID	Partial sequences (100 bp)	Match (%)
Cox B3	CAAGTTGTTCCAGGTGACACTATGCAAACGCGCCACCGTGAAAAACTATCACTCAAGATCTGAATCAACTATTGAGACTTTGTGTGCAGGTCTGCGTGTG	91 %
HRV1-B	ACTTCTCTTCAACTTGAGGACATGATTGAAACTAGCTATCTATCATCACAGCCAAACAAGGCATGAAACTACTATTGAAACTTTTTGGCAAGCATTGCTT	91 %
HRV- 16-like	AATCTACACTAGAGGACATGCTCAAACAAGCTATCTCATCACATCATGAAATGAGTATTGAGACTTTTTGGCAAGCATTGCTGATTCAGCAGTGCTA	94 %
HRV 1-2	AATCTACACTAGAGGACATGATGAACAAGCACGCTATCTCTTAAATTCACAAACTAGAGATCAAATGAGCATTTTTGGCAAGCATTGCTTCTA	92 %
HRV-B5	CAGACTATGGGATGAAATGACTCTAGAGAGTTCTTTAGGAAGATCTGGGTCATCCACATCTACATACTGTGAATAATAGCTTAAATATGATGAGA	90 %

Table 5
Comparison of Cell Culture, Real-time PCR and Nucleotide Sequencing

MHDL Isolates	Source	Cell culture-CPE (ID)	EV PCR ID	Sequence ID
407	Clinical	HFS/MRC-5 (Rhinos)	-	HRV108
444	Clinical	HFS/MRC-5 (Rhinos)	-	HRV-Hanks
458	Clinical	HFS/MRC-5 (Rhinos)	-	HRV33
486	Clinical	HFS/MRC-5/MK (Rhinos*)	-	HRV86
1339	Sewage	HEp-2, BGM, Caco-2 (EV)	+	Coxsackie B3

*Atypical CPE

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