

## Quantitative detection of enteroviruses in activated sludge by cell culture and real-time RT-PCR using paramagnetic capturing

D. Pusch, St. Ihle, M. Lebuhn, I. Graeber and J. M. López-Pila

### ABSTRACT

We have compared in extracts of activated sludge the number of enteroviruses detectable with buffalo green monkey (BGM) cell-cultures versus the number of enteroviral genomes determined by reverse-transcription quantitative real-time PCR (RT-qPCR). In order to find conditions adequate for quantifying enteroviral RNA isolated from (waste)water we have investigated affinity capture of RNA with polystyrene beads (Dynabeads). The capture efficiency strongly depended on the genomic region chosen for the affinity binding. Capture of the RNA by its 3'-tail was most efficient (almost 100%); other regions within the genome yielded variable but lower results. Indirect capture (first hybridization of the RNA to the oligonucleotides, then attachment of the duplex molecules to the beads) was much more efficient than direct capture (attachment of the oligonucleotides to the beads first, then binding of the RNA), and resulted in RNA capture of maximally 60–80%. At least partly, this was due to incomplete hybridization of the RNA to the complementary oligonucleotides.

No correlation was found between the number of cytopathic effects (CPE) determined by cell culture and the number of genomes quantified by RT-qPCR; RT-qPCR values were consistently much higher than the number of CPE. This points to overestimation of infectious enteroviruses by RT-qPCR and/or underestimation by the cell culture approach.

**Key words** | activated sludge, dynabeads capturing, enteroviruses, poliovirus Sabin 1, quantitative real-time RT-PCR

### INTRODUCTION

Most viral pathogens present in polluted water cannot be detected using cell cultures. The detection of virtually all (pathogenic) organisms and viruses has become feasible by nucleic acid hybridization and amplification, provided that adequate RNA or DNA sequences are known.

Until very recently, studies have been limited to qualitatively demonstrate the presence or absence of the nucleic acid of a (pathogenic) organism or virus, quantitative approaches were difficult or unreliable. However, there are a number of important issues in environmental and health related microbiology which can be addressed only if quantitative estimates of pathogens in the water sources can be carried

out. Among those is the estimation of the ratios of fecal indicators to pathogens and the enumeration of pathogens which pollute recreational water or other water used for human purposes. Once the concentration of pathogens can be reliably quantified in environmental water, it will be feasible to derive risk assessments values from the estimated amount of pathogens ingested. Besides the significance for health related issues, quantitative ecological studies of non-cultivable microorganisms would be considerably boosted by quantitative procedures (Holland *et al.* 1991; Becker *et al.* 2000; Schvoerer *et al.* 2001).

A major bottleneck in the quantitative estimation of the concentration of pathogens in environmental samples such as (waste)water is the unknown, variable or poor nucleic

D. Pusch

St. Ihle

I. Graeber

J. M. López-Pila (corresponding author)

Federal Environmental Agency

(Umweltbundesamt)

Department II 2.4. Microbiology and Parasitology,

Corrensplatz 1,

Berlin, D-14195,

Germany

Phone: ++49-30-89031394

Fax: ++49-30-89031830

E-mail: [juan.lopez-pila@uba.de](mailto:juan.lopez-pila@uba.de)

M. Lebuhn

Technical University of Munich Institute of Water

Quality Control and Waste Management,

Am Coulombwall,

Garching, D-85748,

Germany

E-mail: [M.Lebuhn@bv.tu-muenchen.de](mailto:M.Lebuhn@bv.tu-muenchen.de)

acid recovery efficiency of extraction methods currently available (Lebuhn *et al.* 2003). The presence of substances which inhibit the enzyme activities required for reverse transcription PCR (RT-PCR) can also considerably affect the quantitative determination of pathogens (Lebuhn *et al.* 2003). Quantification of minute amounts of pathogens in environmental samples by PCR based techniques hence requires maximum extraction efficiencies and highest purity of the extract to avoid false negative results.

Isolation and concentration of viral RNA from environmental samples by hybridization to complementary oligonucleotides attached to magnetic beads has been reported (Gilgen *et al.* 1995; Jacobsen 1995; Regan & Margolin 1997; Loisy *et al.* 2000; Maher *et al.* 2001). This procedure is very promising since separation of the hybridized beads from the liquid phase in the sample may simultaneously remove potential inhibitors and non-target nucleic acids from the target sequences.

The objective of this study was to optimize conditions influencing the attachment of nucleic acids to either poly-dT or to streptavidin-coated polystyrene beads. Using the poliovirus 1 genome as a model target nucleic acid, the efficiency of oligonucleotides complementary to different regions of the poliovirus 1 genome for binding of the RNA to the paramagnetic beads was compared.

The sensitivity achieved with our optimized RNA extraction, reverse-transcription quantitative real-time PCR (RT-qPCR) approach was compared with the sensitivity achieved with a cell culture procedure for the detection of enteroviruses. In order to avoid any additional procedure for the concentration of enteroviruses which may have rendered the viruses non-infectious and bias the comparison of the RT-qPCR with the cell culture procedure, we carried out the analysis with unconcentrated extracts from activated sludge.

## MATERIAL AND METHODS

### Wastewater samples, extraction of activated sludge for cell culture infectivity test and real-time PCR

Grab samples of 100 ml were taken from the activated sludge basin of a Berlin wastewater treatment plant, brought

to the laboratory, and each mixed with 10 ml chloroform. After pouring the sample in an Erlenmeyer flask, it was shaken vigorously for 60 minutes at room temperature in an end-over-end shaker, and left standing until the chloroform had settled down (usually 5 to 10 minutes). The upper water phase was centrifuged at 47,000 g for 10 minutes (Sorvall SS 34) in order to sediment the coarse particulate matter but leave the viruses in suspension. The supernatant was transferred to a sterile vacuum flask with a silicon rubber stopper equipped with a cotton-plugged inlet and an outlet. Air was blown in through the inlet until no chloroform smell was detected at the outlet. This step was necessary to sufficiently remove the chloroform which might otherwise have damaged the cell cultures.

For the inoculation of cell cultures, 5 ml of the wastewater extract was mixed with 5 ml of twice concentrated minimal essential medium (MEM) containing 2% fetal calf serum (FCS). Additionally 20  $\mu\text{l}$  of an antibiotic mix was added yielding the following final concentrations: penicillin, 500 I.U.  $\text{ml}^{-1}$ ; streptomycin, 0.5 mg  $\text{ml}^{-1}$ ; kanamycin, 0.05 mg  $\text{ml}^{-1}$ ; nystatin 25 I.U.  $\text{ml}^{-1}$ .

One hundred  $\mu\text{l}$  of this suspension (i.e. the mixture of activated sludge extract, MEM and antibiotics) were distributed in each well of a 96-wells microtitre plate which had been seeded the previous day with  $c \times 3 \times 10^4$  buffalo green monkey (BGM) cells per well in 100  $\mu\text{l}$  MEM (2% FCS). This medium was not removed before the addition of the extract, so that the total final volume per well was 200  $\mu\text{l}$ , containing 1.5% FCS.

After 7 days of incubation at 36°C the wells were examined for cytopathic effects (CPE). When more than ten wells displayed a CPE, the cell culture assay was repeated with less than the 100  $\mu\text{l}$  extract volume used before, and the volume difference was made up with MEM. The number of positive wells of the second assay was then set equal with the number of infectious units in the volume applied. Although a more accurate approach, such as a most probable number (MPN) procedure, would have been more appropriate, we decided not to do so because the cell toxicity of the extracts was too high when the amount of wastewater or activated sludge exceeded 50% in the cell medium to which the cells were finally exposed. This fact prevented us from using cell medium with a higher fraction of wastewater or activated sludge, and subsequent dilutions

thereof, in order to implement an MPN dilution system. Instead, we used only the dilution described in the previous paragraphs and set the number of infectious units equal to the number of CPE-positive wells. In order to reduce the error due to doubled-infected wells (which of course would score only as one infectious unit) we repeated the test with a smaller amount of extract whenever the number of CPE-positive wells in a titration plate exceeded ten. In this way the probability of doubled-infected wells was as an average kept below 10/96. Using this procedure we are aware of underestimating the concentrations of viral units by *c.* 10%, a margin that should not bias the results substantially.

For the real-time PCR, aliquots of the centrifuged grab samples were pelleted for 90 min in a Beckman ultracentrifuge with the Ti 45 rotor at 40,000 rpm, and the pellet containing the viruses was resuspended in 300 µl DEPC treated water. This suspension was used for the hybridization.

### Dynabeads, oligonucleotides and determination of the hybridization efficiency

Dynabeads were supplied by Deutsche Dynal GmbH, Hamburg. Before the binding experiments, the beads were separated from the bulk of the fluid by means of a magnetic particle concentrator from Dynal, and washed twice with 0.2 ml buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 2 M NaCl, using the MX 1 sample mixer from Dynal.

Optimum primer and probe positions were calculated from a manually refined Clustal alignment containing all 5'-UTR *Enterovirus* and *Rhinovirus* sequences available in publically accessible databases (GenBank, EMBL) using programme 'Signature' (Lebuhn and Majewski, unpublished). The qPCR system of reverse primer PVK 577–597 (oligo 11 in Table 1), forward primer Pvi 450–468 (oligo 12 in Table 1) and hydrolysis probe TaqPVK 532–552 (oligo 13 in Table 1) was developed for the genus *Enterovirus*

**Table 1** | Primers and capture oligonucleotides

No.	Oligonucleotide*	Sequence (5' → 3')
1	PVK 7424–7400	AAT TTA CCC CTA Cag Cag TAT gAC C
2	Biotin-(dg) <sub>10</sub> (dT) <sub>20</sub>	Biotin-(dg) <sub>10</sub> (dT) <sub>20</sub>
3	PVK bio (A) <sub>20</sub> 510–534	Biotin-(A) <sub>20</sub> C CgC CAC ggA CTT gCg CgT TAC gAC
4	PVK (A) <sub>20</sub> 510–534	(A) <sub>20</sub> C CgC CAC ggA CTT gCg CgT TAC gAC
5	PVK bio (A) <sub>20</sub> 3431–3450	Biotin-(A) <sub>20</sub> g CCA AgT ggT AgT TgC AAA T
6	PVK (A) <sub>20</sub> 3431–3450	(A) <sub>20</sub> g CCA AgT ggT AgT TgC AAA T
7	PVK bio (A) <sub>20</sub> 4431–4450	Biotin-(A) <sub>20</sub> g TTA ATA gTA TgC TCT AgT T
8	PVK (A) <sub>20</sub> 4431–4450	(A) <sub>20</sub> g TTA ATA gTA TgC TCT AgT T
9	PVK 490–509 (probe)	Agg CCA ATC ACT ggT TTg Tg
10	PVK bio (A) <sub>20</sub> 577–597	Biotin-(A) <sub>20</sub> AT TgT CAC CAT AAg CAg CCA
11	PVK 577–597	AT TgT CAC CAT AAg CAg CCA
12	Pvi 450–468	gCC CCT gAA TgC ggC TAA T
13	Hydrolysis probe PVK 532–552	FAM-CAC CCA AAg TAg TCg GTT CCg-TAMRA

\*The numbers indicate the position in the *Human Poliovirus* Sabin 1 genome

including *Poliovirus*, *Human Enterovirus A-D*, new and unclassified *Enterovirus* species, and porcine enteroviruses. Bovine enteroviruses, swine vesicular disease viruses and *Echovirus 1* were predicted not to react. *In-silico*-specificity tests (BLASTN, FASTA, MatInspector) predicted that under stringent PCR conditions no other known nucleotide sequence would test positive, except eventually for two sequences, AF108177 and AF108187, deposited as *Rhinovirus*. However, these sequences do not contain the binding region of oligo 11. Sequence AF108187 clustered with Enterovirus D in a neighbour joining analysis (not shown) and may hence be mis-assigned. This would leave only *Rhinovirus* sequence AF108177 as a potential mismatch candidate. From the total of 353 *Enterovirus* sequences tested *in silico*, 8.5% (sequence X87588, AY055151, AJ417542, AJ417543, AJ417544, AJ312089, S76769, AF447484, U11705, X80059, U11709, AF405325, X90722, AF447482, X92886, AF405319, AF510747, AF412370, X05690, U30916, S76767, AJ314833, AF179613, AF447479, AF447483, L76400, AF132497, X87604, AF029859 and X89531) presented one or several mismatches with one of the primers or the probe. The corresponding enterovirus may hence not react under stringent PCR conditions, but the sequences may well have not been determined correctly.

Specificity of the fluorogenic primer/probe system 11, 12, 13 was also tested *in vitro*. Positive reactions were obtained for inactivated cell culture supernatants of the following enterovirus types: *Human Coxsackievirus A16*, B1, B2, B3, B4, B5, B6, *Human Echovirus 6*, 7, 11, 30, and *Human Poliovirus 1* (strains Sabin, E3). Negative results were obtained for Norwalk viruses N13, N14, N18, N23 and for alantoic liquid containing *Influenza A virus Wisconsin* (all inactivated).

Oligonucleotides (Table 1) were 3' labelled using the kit purchased from and following the procedures of Boehringer, Mannheim.  $^{32}\text{P}$ -alpha-labelled ddATP and  $^{32}\text{P}$ -UTP for the transcription were purchased from Applied Biosystems and Amersham.

Submarine electrophoresis was performed in 2% agarose gels as described in Kopecka *et al.* (1993). After blotting the gel using standard procedures, the nylon membrane was hybridized overnight at 45°C against  $1-3 \times 10^7$  cpm of the labelled probe in 5x SSC, 35% formamide, 3x Denhardt's

solution, 0.5% SDS and 5 mM potassium phosphate buffer, pH 7.5. After washing out unbound probe in 2x SSC / 0.1% SDS at 45°C for 10 min twice and once for 20 min at 61°C, the membrane was dried and incubated with a Kodak Film X-OMAT AC at -80°C in the presence of an intensified screen from Dupont. Where stated, the areas to be counted were identified by superposition of the corresponding X-ray film, cut, and their radioactivity was counted in a Packard liquid scintillator counter.

### Transcription and labelling of RNA, capturing conditions with dynabeads

RNA transcripts were obtained from the plasmid pHK8, a gift of Dr H. Kopecka, Institut Pasteur, Paris. This plasmid contains the cDNA of the complete *Human Poliovirus 1* strain Sabin genome, except for the cDNA corresponding to the poly-A tail at the 3' end. The transcripts lack therefore the poly-A tail. Standard transcription conditions were followed as described in Kopecka *et al.* (1993), including  $^{32}\text{P}$ -UTP in the transcription mixture. For the transcripts used in the direct capture experiments the specific activity of the transcription mixture was  $3 \times 10^9$   $\mu\text{Ci}$  per  $\mu\text{mol}$  UTP. These conditions yielded RNA with  $5.6 \times 10^6$  cpm per pmol genome. The indirect capture experiments and the experiments evaluating the different genome binding regions (Figure 2) were carried out with RNA transcribed with  $6 \times 10^6$   $\mu\text{Ci}$  per  $\mu\text{mol}$  UTP yielding RNA with  $10^4$  cpm per pmol genome.

The volume of the beads suspension used for each binding experiment was, unless stated otherwise, 100  $\mu\text{l}$ . This volume corresponds to 1 mg beads and contained 100 pmol streptavidin or, according to our own estimations using  $^{32}\text{P}$ -labelled poly-dA-tailed oligonucleotides, at least 200 pmol poly-dT side chains, respectively. The amount of RNA (for direct capture) or RNA-oligonucleotide duplexes (for indirect capture) introduced in the binding assays never exceeded 10% of the theoretical binding capacity of the beads.

### Liquid-phase hybridization

For the standard liquid-phase hybridization we incubated 0.5 pmol RNA and 1.5 pmol oligonucleotides in 250  $\mu\text{l}$

hybridization buffer, containing 1.0 M LiCl, 0.1 M Tris-HCl buffer, pH 7.5, 8 mM EDTA, and 0.1% SDS. Standard hybridization was carried out for 3 hours at 65°C.

The effect of the hybridization temperature on RNA stability was tested with radiolabelled pHK8-derived RNA and Dynabeads streptavidin for 10 min at room temperature and at 50–90°C (intervals 5°C). After RNA separation from the beads (see below), electrophoresis and radioblots were performed as described above.

### Lysis conditions for poliovirus Sabin 1

An amount of  $2.3 \times 10^9$  tissue culture infectious dose 50% (TCID<sub>50</sub>) poliovirus 1 was added to 100 µl lysis buffer containing 100 mM Tris-HCl (pH 7.5), 5 mM EDTA and 50 µg ml<sup>-1</sup> proteinase K. This mixture was incubated at 40°C and after 0, 15, 30 and 60 minutes, 5 µl (containing *c.*  $1.15 \times 10^8$  TCID<sub>50</sub> viruses) were withdrawn and mixed with 20 µl of 1.25x hybridization buffer containing 0.04 pmol oligonucleotide 3 (Table 1), labelled with 10<sup>7</sup> cpm per pmol <sup>32</sup>P. This mixture was incubated for 3 h at 65°C. As a control, an amount of  $1.15 \times 10^8$  TCID<sub>50</sub> viruses, suspended in 5 µl lysis buffer without proteinase K, was added to 20 µl of 1.25x hybridization buffer and incubated for 3 h at 65°C.

Five µl of all hybridization mixtures, plus a negative control containing only ultracentrifugation supernatant, were separated by electrophoresis, blotted and radioactive areas were quantified as described above.

### Detachment of RNA from the beads

For this experiment, we used Dynabeads streptavidin which had been coupled with oligonucleotide 3 and hybridized with radioactive pHK8 transcripts. The RNA loaded beads were washed three times at room temperature with 0.2 ml distilled DEPC-treated water using the MX 1 sample mixer. Equal fractions of the beads were resuspended in parallel in 100 µl bidistilled water, 10 mM Tris-HCl buffer (pH 8.0) or PCR buffer, heated at 90°C for 5, 10 or 30 min, and immediately immersed in ice-water. The beads were then separated from the supernatant at 4°C with the MX 1 sampler mixer placed in a refrigerator. The radioactivity released into the supernatant was determined.

### Reverse transcription (RT) and quantitative real-time PCR (qPCR)

Reverse transcription was carried out in 25 µl, containing PCR buffer (50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl<sub>2</sub>), 2 µl of the supernatant, 0.75 µM primer 11 (Table 1), 300 µM of each dNTP, 1 U per µl MuLV reverse transcriptase (RT) and 1 U per µl RNase inhibitor. cDNA synthesis was carried out at 25°C for 10 minutes and 42°C for 30 minutes. The reaction was stopped by heating at 99°C for 5 minutes. Twenty µl of RT-reaction were taken for the PCR.

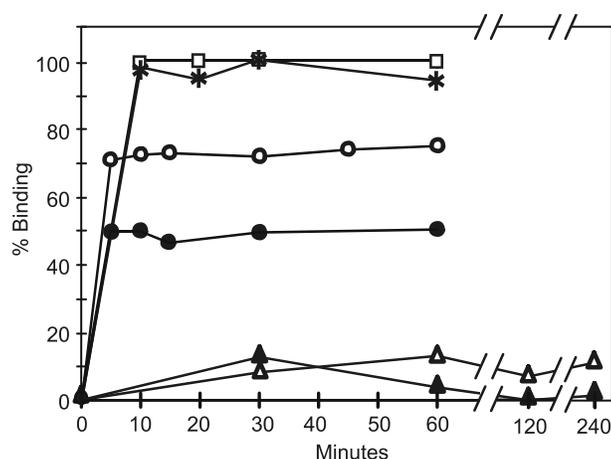
An AbiPrism 7000 from Applied Biosystems was used for qPCR. The PCR reaction contained in 50 µl: universal mastermix (Applied Biosystems catalogue number 4304437), 0.6 µM primer 12 (Table 1), 1.2 µM primer 11 (Table 1), 0.3 µM hydrolysis probe 13 (Table 1).

qPCR conditions were: 10 min 95°C (hot start), 50 cycles of: 30 s 95°C, 30 s 60°C, 30 s 72°C. Serial dilutions of Sabin 1 genomes were taken for establishing the standard curves. The concentration of Sabin 1 genomes was calculated similarly as described above: a complementary <sup>32</sup>P-labelled oligonucleotide of known concentration and specific radioactivity was hybridized to the viral RNA, the hybridization mixture subsequently separated by electrophoresis, membrane-blotted and the membrane exposed to an X-ray film. The radioactivity corresponding to the RNA-hybridized and the non-hybridized oligonucleotide was counted and from their ratio the number of gene copies was calculated.

## RESULTS

### Capture efficiency of Dynabeads in dependence of the binding position

A suspension of poliovirus 1 Sabin was lysed and the viral RNA simultaneously hybridized to the <sup>32</sup>P-labelled oligonucleotide 1 (direct capture, Table 1) in order to tag the RNA. The hybridization mixture containing the duplex molecule was incubated with Dynabeads (dT)<sub>25</sub>, separated magnetically, and the radioactivity of the supernatant was measured at different times after the onset of incubation. Binding was already complete after 10 min (empty squares in Figure 1). Indirect capture by biotinylated oligonucleotide 2 and Dynabeads streptavidin was almost equally complete (asterisks in Figure 1). The curves were corrected for the



**Figure 1** | Capture of Sabin 1 RNA or pHK8-transcripts by Dynabeads targeting different genomic regions. Capture of viral RNA by Dynabeads targeting the 3' region of the Sabin 1 genome: capture by Dynabeads oligo (dT)<sub>25</sub> (asterisks) or by Dynabeads streptavidin (white squares); capture of radiolabelled pHK8-transcripts by Dynabeads coated with oligonucleotides 3 (Dynabeads streptavidin, white triangles) or 4 (Dynabeads oligo (dT)<sub>25</sub>, black triangles), complementary to the 510–534 genomic region; capture of radiolabelled pHK8-transcripts by either Dynabeads streptavidin (white circles) or Dynabeads oligo (dT)<sub>25</sub> (black circles) after prehybridization with oligonucleotide 3 or 4, respectively.

unbound amount of radioactive oligonucleotide in the supernatant. This amount was calculated by determining the percentage of unhybridized radiolabelled oligonucleotide in the mixture after gel electrophoresis, hybridization and blotting.

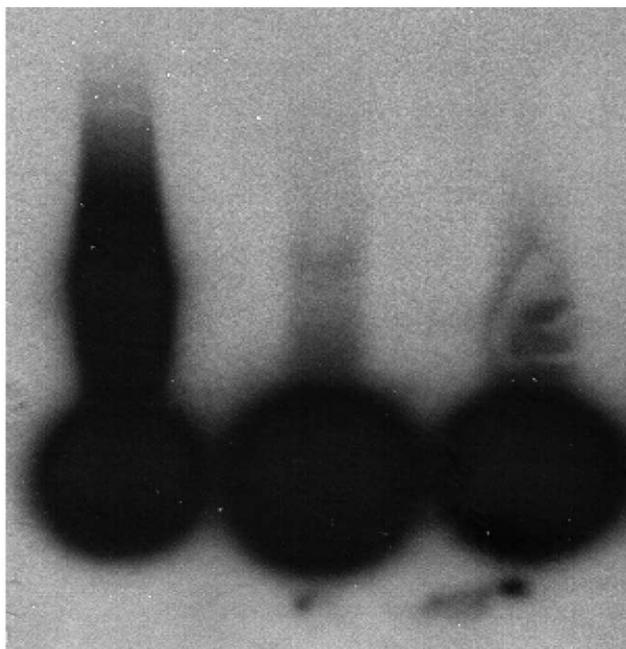
In order to find out if internal domains of the viral RNA were as efficient as the 3' poly-A stretch and the 3'-end of the genome for mediating the binding of the viral RNA to the beads, six oligonucleotides, complementary to the regions 510–534, 3431–3450 or 4431–4450, were either attached to Dynabeads streptavidin (oligonucleotides 3, 5 and 7) or to Dynabeads (dT)<sub>25</sub> (oligonucleotides 4, 6 and 8). Radioactive transcripts, which lacked poly-A (plasmid pHK8 lacks the 3' poly-dT stretch and therefore the 3' poly-A tail is missing in its transcripts), were then exposed to the Dynabeads-oligonucleotide-hybrids, and the radioactivity of the supernatants was monitored at different intervals. Figure 1 (triangles) shows that less than 15% of pHK8 transcripts were bound by region 510–534. The other two regions bound only 10% of the transcripts (not shown).

We next wanted to find out if the capture efficiency could be improved by first hybridizing the oligonucleotides to the viral RNA and subsequently exposing the duplex molecules to the beads (indirect capture, as opposed to the direct

approach of the previous experiment). <sup>32</sup>P-labelled RNA transcripts were hybridized to oligonucleotides 3–8 and the hybridization mixtures then exposed to Dynabeads streptavidin (oligonucleotides 3, 5 and 7) or Dynabeads (dT)<sub>25</sub> (oligonucleotides 4, 6 and 8). The RNA which had been hybridized to the oligonucleotides complementary to the regions 3431–3450 (oligonucleotides 5 and 6) or 4431–4450 (oligonucleotides 7 and 8) still did not attach efficiently to the beads, with bound amounts less than 10% (not shown). The RNA which had been hybridized to the oligonucleotides complementary to the region 510–534 (oligonucleotides 3 and 4) was captured by the beads with an efficiency of 50% for Dynabeads(dT)<sub>25</sub> and 70% for Dynabeads streptavidin (Figure 1, closed and open circles, respectively).

In order to see if the failure of the RNA which had been incubated with oligonucleotides 5–8 to become attached to the beads was because the oligonucleotides had not hybridized to RNA, we determined the degree of hybridization (standard hybridization conditions) of <sup>32</sup>P-labelled oligonucleotides 4, 6 and 8 to transcripts of plasmid pHK8. Aliquots of the hybridization mixture were separated by electrophoresis, blotted, the membrane developed by autoradiography and radioactive areas counted. Figure 2 shows that pHK8 RNA had hybridized to oligonucleotide 4 only by 20%, and to oligonucleotides 6 and 8 only by 2% and less. Very poor hybridization of oligonucleotides 5–8 with RNA was hence the reason for the very low RNA-associated radioactivity in the respective supernatants in Figure 1.

In order to find out why the RNA was not bound completely by oligonucleotides 3 and 4, we hybridized first pHK8 RNA transcripts to radiolabelled oligonucleotides 3 and 4. The hybridization solutions were then exposed to Dynabeads streptavidin (oligonucleotide 3) or Dynabeads (dT)<sub>25</sub> (oligonucleotide 4). Before and after hybridization and after exposure of the hybridization solution to Dynabeads, samples were taken, separated by electrophoresis, and the radiolabelled RNA was quantified. Figure 3(a) shows that 80% of the radioactivity in the supernatant consisted of RNA-oligonucleotide 3 duplex molecules and were removed by Dynabeads streptavidin, and that 20% of the label corresponded to unreacted oligonucleotide or unspecific binding lane. When this supernatant was again treated with Dynabeads, practically no additional radioactivity was removed, showing that exhaustion of the beads' binding capacity was



	Lane 1	Lane 2	Lane 3
Radiolabelled oligonucleotide	4 (PVK bio (A) <sub>20</sub> 510–534)	6 (PVK bio (A) <sub>20</sub> 3431–3450)	8 (PVK bio (A) <sub>20</sub> 4431–4450)
cpm in oligonucleotide bound RNA	6.110 (20%)	770 (1.5%)	0.711 (2%)
cpm in free oligonucleotide	24.009 (80%)	53.800 (98.5%)	37.291 (98%)
Total cpm	30.119 (100%)	54.750 (100%)	38.002 (100%)

**Figure 2** | Hybridization of weakly radiolabelled pHK8 RNA to radiolabelled oligonucleotides targeting different genomic regions.

not the reason for the incomplete capture. **Figure 3(b)** shows the result for oligonucleotide 3. Since 64% of labelled RNA remained in the supernatant, 36% of the label was removed by the polyA Dynabeads capture.

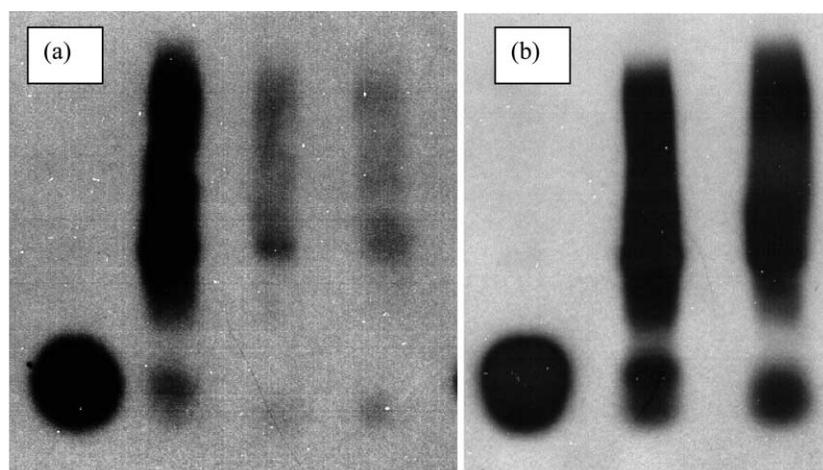
#### Detachment of bound RNA from the Dynabeads

Detachment of RNA from complexes of Dynabeads streptavidin with oligonucleotide 3 and radioactive pHK8

transcripts was tested at different conditions. Best RNA release was obtained when the complexes were treated with PCR buffer at 90°C for at least 5 min (**Table 2**).

#### Effect of the hybridization temperature on RNA stability

Radiolabelled pHK8 RNA was hybridized to Dynabeads streptavidin for 10 min at different hybridization temperatures. The radioblots showed that at room temperature and



1	2	3	4	1	2	3
cpm in RNA						
-	10,406 (100%)	2,106 (20%)	1,932 (19%)	-	7,931 (100%)	5,074 (64%)

**Figure 3** | Capture of RNA-oligonucleotides duplex molecules by (a) Dynabeads streptavidin or (b) Dynabeads oligo (dT)<sub>25</sub>. Transcribed pHK8-RNA was liquid-phase hybridized with <sup>32</sup>P-labelled oligonucleotide 3 or <sup>32</sup>P-labelled oligonucleotide 4, and the hybridization mixtures were exposed to (a) Dynabeads streptavidin or (b) Dynabeads oligo (dT)<sub>25</sub>, respectively. 1, hybridization mixture prior to hybridization; 2, hybridization mixture after liquid-phase hybridization; 3, supernatant of 2 after exposure to Dynabeads streptavidin; 4, supernatant of 3 after re-exposure to fresh Dynabeads.

50°C no visible RNA breakdown had occurred. With increasing temperature, however, RNA fragmentation was visible (accelerated radiolabelled RNA), leaving almost no intact RNA in the supernatant at 75°C and higher hybridization temperatures. This indicated that substantial RNA fragmentation may occur during RNA extraction from viral and environmental samples, and suggested that capture

and RT-qPCR regions should not be separated and ideally should be identical.

### Lysis conditions for poliovirus Sabin 1

We tested whether our standard lysis/hybridization conditions suffice for lysing strain Sabin 1, or if the addition of proteinase K which possibly might damage the RT or PCR enzymes, may be necessary. With and without proteinase K (50 µg ml<sup>-1</sup>) and irrespective of the lysis time, 37–46% of the oligonucleotide radioactivity was retarded due to hybridization with RNA. No radioactivity was found in the negative control (ultracentrifugation supernatant).

Taking into account that each hybridization tube contained 0.04 pmol oligonucleotide, the amount of lysed viral genomes present was estimated to be *c.* 0.016 pmol, equivalent to *c.* 10<sup>10</sup> viral genomes in 1.1 × 10<sup>8</sup> TCID<sub>50</sub>.

**Table 2** | Release of radioactive pHK8 transcripts from Dynabeads after heating at 90°C for different time periods

	Time at 90°C		
	5 min	10 min	30 min
Tris HCl, pH 8.0	3%	2%	3%
H <sub>2</sub> O	48%	62%	51%
PCR-buffer	60%	60%	61%

### Enterovirus quantification by RT-qPCR and comparison with cell culture

Various grab samples from the activated sludge basin of a wastewater treatment plant were assayed for the presence of infectious enteroviruses by cell culture and enteroviral genomes by RT-qPCR. Table 3 shows that there was no correlation between quantification by cell culture and RT-qPCR, and that cell culture values were consistently considerably lower than the RT-qPCR values.

### DISCUSSION

We investigated the capture of enteroviral RNA by Dynabeads using specific sequences of the *Human Poliovirus* Sabin 1 genome as a model. The efficiency of capturing depended strongly on the region of the Sabin 1 genome chosen for attaching the RNA to the beads (Figure 1). This may be due to different accessibility of the specific RNA domain chosen for binding. Sabin 1 genomes were captured with almost 100% efficiency by its 3'-poly-A tails by both the poly dT and the streptavidin beads. The very high capture efficiency was not specifically confined to the poly-A tail, but to the 3'-end of the target sequence. If RNA transcripts from pHK8 plasmids without poly-A tail were hybridized to

biotin coupled oligonucleotide 1 (complementary to the 3'-end of the genome) the capture efficiency was almost as good as with the genuine Sabin 1 genome (Figure 1).

Capture by the chosen three internal domains was very poor when the direct approach was applied, that is, by binding the RNA to beads which had beforehand been coated with complementary oligonucleotides. If the capture oligonucleotides had been successfully hybridized to the RNA (indirect capture), a substantial percentage (45–75%) of the RNA-oligonucleotide adducts was captured by the beads (Figure 1, open and closed circles). The radioactivity which remained unbound in the supernatant corresponded mainly to unattached duplex molecules (Figure 3(a) and (b), lane 3), not to other spurious sources (e.g. incomplete removal of <sup>32</sup>P-dATP after the labelling of the oligonucleotide), nor was the incomplete capture a consequence of insufficient binding capacity of the beads. If the unbound molecules were again exposed to fresh beads, practically no binding increase was observed (Figure 3(a), lane 4). The sterical conformation of a fraction of the duplex molecules might not have allowed the attachment, and this fraction may have remained in the supernatant.

The different degree of hybridization observed with oligonucleotides complementary to different regions of the Sabin 1 genome may reflect different accessibilities of these genome sites. The efficiency to elicit fluorescence with

**Table 3** | Comparison of viral units in cell culture with enteroviral genomes quantified by RT-qPCR in activated sludge samples

Sample	Genomes per ml	Viral units per ml	Sample	Genomes per ml	Viral units per ml
1	83,000	0.1	10	684	0.1
2	44,983	0.2	11	631	0
3	32,424	0.5	12	460	0.1
4	27,154	0.1	13	351	0.8
5	17,491	0.4	14	189	0
6	16,980	0.1	15	125	0.3
7	1,643	0	16	<100	0.8
8	883	0.1	17	<100	0.1
9	773	0.4	18	<100	0

oligonucleotides complementary to certain regions of the *E. coli* 16S and 23S ribosomal RNA subunits was very low, probably as a consequence of poor accessibility of these sites (Fuchs *et al.* 2001). Therefore, when attempting the quantitative estimation of RNA from environmental samples, one should first evaluate a suitable attachment and RT-qPCR site and optimize the hybridization conditions of the chosen oligonucleotide, and then assess the efficiency of the duplex molecule to attach to the beads.

It seems advisable to isolate enteroviral RNA by a capture domain within or in very close vicinity of the region used for RT-qPCR analysis. During lysis of the viruses and RNA processing, substantial fragmentation of the RNA appears to occur: *Human Poliovirus* Sabin-1 extraction via capture by the domain chosen for RT-qPCR (510–534) was approximately 400-fold more efficient than extraction via capture by a distant domain (poly-A stretch), and considerable RNA breakdown occurred in the hybridization experiment with different temperatures (data not shown). When the poly-A stretch was captured, regions of the fragmented genome that were not in the vicinity of the poly-A stretch probably were washed out and caused substantial losses of the RT-qPCR target region. It was hence more efficient to capture the enteroviral genomes via the internal RT-qPCR targeted region, although the hybridization efficiency of the Sabin 1 RNA with 3'-end and poly-A targeted Dynabeads was higher.

As further processing of environment-isolated RNA or DNA typically encompasses PCR or other amplification methods which involve enzymatic reactions, it is essential that the nucleic acid isolation procedure removes or inactivates not only substances capable of inhibiting these enzymes, but also agents such as RNA or oligonucleotide degrading nucleases and proteinases that might attack streptavidin and counteract the attachment of RNA to the beads. We tested therefore whether proteinase K, which is robust and resistant and hence frequently used in extracting nucleic acids from environmental samples, is required to lyse the virions. No difference was found between the samples with proteinase K incubation for different times and those without proteinase K. The ratios of cpm in RNA to cpm in the free oligonucleotides were very similar for all variants (not shown), indicating that the radioactive oligonucleotide had hybridized equally well at all

conditions. Proteinase K was therefore not necessary to release RNA from the virions. The viruses had not been lysed prior to the hybridization, because no radiolabelled RNA was found for the supernatant of the ultracentrifugation step. We conclude that our standard hybridization conditions cause enteroviral lysis, making addition of proteinase K unnecessary.

The number of enteroviral genomes found in the activated sludge samples was surprisingly high in comparison with the number of BGM-cytopathogenic viruses (Table 3), even if it is well established that the ratio of physical to infectious particles is high (0.01–0.03) in polioviruses (Schaffer & Schwerdt 1959) and presumably in other enteroviruses as well and that only a fraction of the enteroviruses is cytopathogenic for the BGM cells. A substantial amount of the viruses might have been present not as individual virions but as conglomerates of viral particles containing thousands of genomes, and possibly, the majority of viral particles in the activated sludge may have been in an advanced stage of deterioration with damaged coat rendering them incapable of entering and infecting cells. Such particles, however, most probably still possess an (almost) intact genome, still able to generate an amplicon in RT-PCR. Literature data have similarly shown that a part of environmental samples with negative results in cell culture were positive in PCR analysis (Enriquez *et al.* 1993; Margolin *et al.* 1993; Borchardt *et al.* 2003). Our results indicate that intact and infectious viruses in environmental samples are overestimated by RT-qPCR with respect to the cell culture assay, but on the other hand, cell culture detection, as no single cell line is able to sustain development – and achieve detection – of all enteroviruses, most probably results in underestimation.

A comparison of our results with the results of Monpoeho *et al.* (2000) underscores the importance of the careful choice of extraction procedures for viruses and RNA, and of using uniform, standard procedures. These workers also determined the ratio of cell culture-detected enteroviruses to the amount of genome copies in different types of sludge. The results reported for the three samples of activated sludge that were analysed show huge differences against ours. Whereas the yield of infectious units was approximately ten times lower than ours, which might be explained with a lower virus content of their sludge, the

yield of enteroviral genome copies was *c.* 1,000 times less than ours. This discrepancy is much too high to be accounted for by true differences of concentration. Presumably the different methods used for extracting the viral RNA, along with the slightly different choice of primers, are primarily responsible for the discrepancies found. It must be kept in mind, too, that most workers use viral cDNA instead of viral RNA for their calibration curves or positive controls in their RT-PCR measurements. This introduces another error possibility in the detection system, which might lead to substantial additional discrepancies. Further work will help to find out which procedures do yield optimal and consistent quantitative results.

## CONCLUSION

We would like to stress that using a Dynabeads-based approach, the proper choice of the capture oligonucleotide with respect to sufficient accessibility of the target RNA region and location of the capture sequence within or very close to the RT-qPCR-targeted region allowed the quantification of viral genomes in environmental samples. The choice of suitable conditions of lysing the viruses and the separation of viral RNA from inhibitors was also important for good performance. By radiolabelling we were able to monitor and optimize the hybridization conditions, and to show that indirect capture was much more efficient than direct capture. With our optimized approach, we were able to recover *c.* 60% of viral RNA, further optimization may result in higher yields. With respect to hygienic considerations, our results indicate that infectious enteroviruses are overestimated by RT-qPCR and/or underestimated by the cell culture approach.

## ACKNOWLEDGEMENTS

We thank Dr I.v. Truchseß (Institute of Virology, Technical University of Munich) for providing the Coxsackie-echo and influenza viruses, and Dr M. Bayer (Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Oberschleißheim) for providing the Norwalk viruses and enterovirus E3.

## REFERENCES

- Becker, S., Böger, P., Oehlmann, R. & Ernst, A. 2000 PCR bias in ecological analysis: a case study for quantitative Taq nuclease assays in analyses of microbial communities. *Appl. Environ. Microbiol.* **66**, 4945–4953.
- Borchardt, M. A., Bertz, P. D., Spencer, S. K. & Battigelli, D. A. 2003 Incidence of enteric viruses in groundwater from household wells in Wisconsin. *Appl. Environ. Microbiol.* **69**(2), 1172–1180.
- Enriquez, C. E., Abbaszadegan, M., Pepper, I. L., Richardson, K. J., Margolin, A. B. & Gerba, Ch. P. 1995 Comparison of poliovirus detection in water by cell culture and nucleic acid hybridisation. *Wat. Sci. Technol.* **27**, 315–319.
- Fuchs, B. M., Syutsubo, K., Ludwig, W. & Amann, R. 2001 In situ accessibility of *Escherichia coli* 23S rRNA to fluorescently labelled oligonucleotide probes. *Appl. Environ. Microbiol.* **67**, 961–968.
- Gilgen, M., Wegmüller, B., Burkhalter, P., Bühler, H. P., Müller, U., Lüthy, J. & Candrian, U. 1995 Reverse transcription PCR to detect enteroviruses in surface water. *Appl. Environ. Microbiol.* **61**, 1226–1231.
- Holland, P. M., Abramson, R. D., Watson, R. & Gelfand, D. H. 1991 Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl Acad. Sci. USA* **88**, 7276–7280.
- Jacobsen, C. 1995 Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridization and PCR amplification assay. *Appl. Environ. Microbiol.* **61**, 3347–3352.
- Kopecka, H., Dubrou, S., Prevot, J., Marechal, J. & López-Pila, J. M. 1993 Detection of naturally occurring enteroviruses in waters by reverse transcription, polymerase chain reaction, and hybridisation. *Appl. Environ. Microbiol.* **59**, 1–7.
- Lebuhn, M., Effenberger, M., Gronauer, A. & Wilderer, P. 2003 Using quantitative real-time PCR to determine the hygienic status of cattle manure. *Wat. Sci. Technol.* **48**(4), 97–103.
- Loisy, F., Le Cann, P., Pommepuy, M. & Le Guyader, F. 2000 An improved method for the detection of Norwalk-like caliciviruses in environmental samples. *Lett. Appl. Microbiol.* **31**(6), 411–415.
- Maher, N., Dillon, H. K., Vermund, S. H. & Unnasch, T. R. 2001 Magnetic bead capture eliminates PCR inhibitors in samples collected from the airborne environment, permitting detection of *Pneumocystis carinii* DNA. *Appl. Environ. Microbiol.* **67**, 449–452.
- Margolin, A. B., Gerba, Ch. P., Richardson, K. J. & Naranjo, J. E. 1993 Comparison of cell culture and a poliovirus gene probe assay for the detection of enteroviruses in environmental water samples. *Wat. Sci. Technol.* **27**, 311–314.
- Monpoeho, S., Dehé, A., Mignotte, B., Schwartzbrod, L., Marechal, V., Nicolas, J.-C., Billaudel, S. & Ferré, V. 2000

- Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR. *BioTechniques* **29**, 88–93.
- Regan, P. M. & Margolin, A. B. 1997 Development of a nucleic acid capture probe with reverse transcriptase-polymerase chain reaction to detect poliovirus in groundwater. *J. Virol. Methods* **64**, 65–72.
- Schaffer, F. L. & Schwerdt, C. E. 1959 Purification and properties of poliovirus. *Adv. Virus Res.* **6**, 195–204.
- Schvoerer, E., Ventura, M., Dubos, O., Cazaux, G., Serceau, R., Gournier, N., Dubois, V., Caminade, P., Fleury, H. J. A. & Lafon, M. 2001 Qualitative and quantitative molecular detection of enteroviruses in water from bathing areas and from a sewage treatment plant. *Res. Microbiol.* **152**, 179–186.