Discussion
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Microbiological contamination of water has long been a concern to the common public and contamination of water resources intended for use by general population with enteric viruses is a great public health problem. Though presence of human enteric viruses has been demonstrated in water bodies as well as drinking water supplies throughout the world and several outbreaks of enteric viral diseases attributed to either contamination of drinking water or vegetables and seafood grown in such waters have been recorded (Croci et al., 2000), but routine examination of water samples for the presence of enteric viruses is not carried out in both developed and developing countries.

The National Institute of Virology, Pune is involved in applied and basic research in prevention and control strategies for viruses of public health importance and investigation of epidemics of suspected viral etiology is an important mandate of the institute. Over the years, the institute has investigated a large number of waterborne outbreaks. For example, more than 150 epidemics of enterically transmitted viral hepatitis were investigated, etiologic diagnosis provided, contamination source identified in most cases and control measures were suggested. Similarly, outbreaks of diarrheal diseases are also investigated. Based on the study of sporadic cases, endemicity of these diseases was proved beyond doubt. Though water was incriminated as the cause of enormous number of cases, no test was being performed to show the presence of virus in the water and an absolute necessity for the development of an assay for virological evaluation was felt. Generation of data on the safety of drinking water supplied to the masses was another immediate requirement. With this background, we planned to evaluate the water supply system and the water available for consumption for common public. For obvious reasons, Pune was chosen, the city in which the institute is situated.

Pune is the eighth largest city in India and the second largest in the state of Maharashtra, after Mumbai. Situated 560 meters above sea level on the Deccan plateau at the confluence of the Mula and Mutha rivers. Punekars
use the waters of the Mutha from the Khadakwasla reservoir. Dams at Panshet, Warasgaon and Temghar supplement the storage capacity of Khadakwasla. Water treatment plants have been built by Pune Municipal Corporation at Parvati, Pune Cantonment, Holkar Bridge Warje (new and old), Wagholi and Wadgaon. Water is supplied to different parts of the city through a network of pumping stations and pipelines. We selected three water purification plants supplying drinking water to major part of the city. Raw water for all three plants is supplied from Khadakwasla dam.

The pre-requisites for our study were

1. Development of a virus concentration method for concentration of viruses from large amount of water samples.

2. An efficient, sensitive, quick and relatively inexpensive protocol for simultaneous detection of Hepatitis A Virus (HAV), Hepatitis E Virus (HEV), entero and rotaviruses in water samples.

We also intended to determine the prevalence of these enteric viruses in Mutha River that flows through Pune city. Water purification at the household level is practiced by majority of the population and several point-of-use water purification devices are available to the consumers, hence we thought to evaluate these purification devices for their efficiency to remove the virus from seeded water samples.

Development of a virus concentration method:

Development of a virus concentration protocol for simultaneous concentration of HAV, HEV, Entero and Rotaviruses from 40 litres of water sample was an important step in our study. As described earlier that enteric viruses are present in very low concentrations in the water environments, hence it becomes necessary to concentrate them into a smaller volume making their detection possible.

Following a thorough literature survey and considering suitability and yield, we decided to opt for membrane filtration based methodology. Earlier, we had evaluated membranes developed by one of the Indian national laboratories. One of the membranes (Poly Acrylo Nitrile membrane) gave very promising...
results. Therefore, PAN membrane was explored as the first choice. Other inherent advantages include:

1. Use of positively charged membranes alleviates the need for pre-conditioning of water sample to be concentrated, since enteric viruses present in the water samples are negatively charged at neutral pH while the membranes are positively charged.

2. The viruses are adsorbed on to the membrane when the water sample containing viruses is passed through the membranes.

3. Also PAN membranes have the ability to remove the PCR inhibitors, which may interfere in the detection of viruses by PCR (Quieroz et al., 2001).

Spirally wound flat sheet PAN membranes were used for the initial concentration and this arrangement provided a large surface area for filtration enabling us to concentrate enteric viruses from large volume of water in minimal time so that the loss of viral particles with time and at room temperature could be minimized. The problem of membrane clogging due to presence of particulate matter in the water samples was overcome by applying tangential flow filtration (TFF) technology. Amicon dead-end ultrafiltration with PAN membrane disc was used for secondary concentration and we were able to concentrate 40 litres of water sample to 3-4 ml in ~3 hrs.

Selection of a suitable eluent is a critical factor for efficiency of membrane filter based virus concentration. Although beef extract has been the eluent of choice for the past 15 years, it contains reverse transcription polymerase chain reaction (RT-PCR) inhibitors (schwab et al., 1995). A solution of 4 M urea buffered at pH 9 with 0.05 M lysine was able to elute greater than 60% of the virus adsorbed to each of the several filters tested (Farrah and bitton, 1979). However, the recovery was different for different filter membranes. Since we were using indigenously developed PAN membrane for our concentration procedure and it was important to select an appropriate eluent, we examined the recovery efficiency of 4 M urea and 0.05 M glycine either singly or in combination at different pH values. We found that the combination of 4 M urea and 0.05 M glycine at pH 9 was giving best
recovery of the seeded HEV and this combination was selected for further use.

Electropositive filters have been used previously for concentration of viruses from water samples. Chang et al. (1981) used positively charged Zeta Plus filters to concentrate enteroviruses from 19 liters of effluent from activated sludge units. They reported that neither the addition of salts nor the acidification of the effluent was required for adsorption of viruses to the filters. Viruses adsorbed to the filters were eluted by treating the filters with a solution of 4 M urea buffered at pH 9 with 0.05 M lysine. Eluted viruses were concentrated into final volumes of 1 to 2 ml by using a two-step concentration procedure that employed inorganic and organic flocculation. Approximately 50% of the viruses added to effluents could be recovered in the final sample.

In a study by Ma et al. (1994) two electropositive filters, the MK and the 1MDS filters were compared for the recovery of poliovirus 1 (PV1) and coxsackievirus B3 (CB3) from 378 liters of tap water. Viruses were eluted from the filters with 3% beef extract buffered with 0.05 M glycine (pH 9.5) and re-concentrated via organic flocculation. At high virus inputs (approximately \(10^6\) PFU), the overall recovery (after elution and re-concentration) of PV1 and CB3 from tap water with the MK filter was less than that achieved with the 1MDS filter (P < 0.05). The recoveries of PV1 from tap water with the MK and 1MDS filters were 73.2% ± 26% (n = 5 trials) and 90.2% ± 5.9% (n = 5 trials), respectively. The recoveries of CB3 from tap water with the MK and 1MDS filters were 32.8% ± 34.5% (n = 4 trials) and 95.8% ± 12.0% (n = 4 trials), respectively. This study indicated that the MK filter consistently provided lower recovery, with wider variability, of PV1 and CB3 from tap water than the 1MDS filter.

In contrast to the use of enteroviruses in the above studies, we used HEV (27-32 nm in diameter) as the model virus for the determination of efficiency of the concentration protocol. Though, the protocol standardized for one virus may not be applicable to other viruses, it was difficult to use each virus for standardization of the protocol mainly because quantitation assays needed to be standardized. Importantly, the basis for concentration was
mainly molecular weight based and the diameter of the model HEV (27-32 nm) was similar or smaller than other viruses (entero/HAV – 27-30 nm, Rota – 100 nm) In our study the overall recovery rate of the seeded HEV in 40 liters of water sample varied between 67% (for 10^3 HEV RNA copies) and 87% (for 10^5 HEV RNA copies). The recovery of seeded virus in our protocol is greater than that reported by chang et al. while comparable to that reported by Ma et al. using 1MDS filters. The spirally wound PAN membranes were reused after disinfection and washing. One membrane was used for concentration of 10 water samples (40 liters each), thus effectively 400 liters of water was concentrated by one membrane before it showed reduction in flow rate indicating deterioration of membrane. In the secondary concentration step one disc of PAN membrane was used only for one sample and discarded after use. The total time required for concentration of 40 liters of water sample up to ~3-4ml was ~3hrs, and no chemical treatment like pH adjustment, flocculation etc was required. Development of the two-step method of virus concentration allowed us to proceed further with the study.

**Real time PCR for quantification of HEV, the model virus for the study**

Development of a real time PCR assay for quantification of Hepatitis E virus was a pre-requisite since we planned to use HEV as the seed virus in our virus concentration and domestic water purification unit evaluation experiments, and the virus does not grow efficiently in-vitro or laboratory animals except primates. Real time PCR assay developed in this study was able to detect and quantitate HEV in both clinical and environmental samples including sewage samples. The assay had a wide dynamic range of detection, i.e. 10^{10} to 10^1 copies/reaction. The highest values of coefficient of variation for intra-experimental and inter-experimental variability were 2.16% and 3.48%, respectively, indicating the reproducibility of the assay.

Choice of the molecule to be used for generation of the standard curve for the target quantification plays a critical role in accurate quantification. It is well known that the most critical step in an RT-PCR is the RT reaction rather than the PCR, hence use of in vitro transcribed ssRNA was preferred over cloned ds DNA molecule. Specificity and sensitivity are important measures
for evaluation of a real-time TaqMan RT-PCR assay. Absence of amplification signals with non-HEV RNA and healthy negative controls confirmed the specificity of the assay. The sensitivity of the real-time RT-PCR and nested PCR were comparable and real-time RT-PCR was preferred over nested RT-PCR.

Nucleic acid amplification techniques are extremely sensitive to presence of PCR inhibitors and may lead to either false negative result or under estimation of viral copy number. Although extraction controls and positive/negative RT-PCR controls give information on the validity of the extraction and RT-PCR procedure in general, it does not monitor PCR performance of individual samples. The control for an RT-PCR should correspond to the viral target and the amplification of the control as well as the viral target should be achieved by the same pair of primers under exactly the same conditions and with the same efficiency. It was shown that by using appropriate internal amplification controls the number of HEV positive pig farms increased from 33% to at least 55%, because of reduction of the number of false negative results (Rutjes et. Al., 2007). The concentration protocols required for detection of viruses in water samples may also simultaneously concentrate the PCR inhibitors. Inclusion of internal positive control in our real time PCR assay ensured detection of PCR inhibition. Same primers carried out amplification of the internal positive control and HEV RNA. The optimum concentration of IPC was found to be $10^6$ copies/reaction.

Considering that hepatitis E is an enterically transmitted disease and large-scale outbreaks have been recorded following consumption of contaminated drinking water, a rapid and highly sensitive real-time RT-PCR assay for detection and quantification of HEV in environmental samples is of immense importance. Real time RT-PCR assay developed in this study for HEV will provide a rapid and sensitive method for detection and quantification of HEV in environmental, food and clinical samples. Inclusion of internal control would help in detecting PCR inhibitors, giving false negative results. Assay will be useful for tracking of environmental contamination sources during outbreaks & for routine monitoring of efficiency of sewage/water treatment plants. Since HEV RNA is the first marker of early infection and it
appears in feces and bile during the first week of infection, real time PCR assay may prove to be a valuable tool for diagnosis of HEV infection during epidemics and in persons consuming water from a source suspected to be contaminated with HEV.

**Multiplex PCR for detection of enteric viruses**

Detection of specific viruses in the concentrated samples was the next step. Traditionally, detection of enteric viruses in environmental samples is done by cell culture approach involving propagation of viruses in a susceptible cell line producing cytopathic effects (CPE) (Zurbriggen et al., 2008). However, it is important to emphasize that a single cell line can not suffice for propagation and detection of all or even some of enteric viruses, because each virus behaves differentially in a particular cell line (Rodríguez et al., 2008; Lee et al., 2004; Chonmaitree et al., 1988). Therefore, use of multiple cell lines becomes essential, making the process time-consuming, laborious and expensive. Also some enteric viruses having significant epidemiologic potential, such as hepatitis A virus (HAV), hepatitis E virus (HEV), norovirus and adenovirus 40 and 41 do not grow efficiently in cell culture (Emerson et al., 1991; Divizia et al., 1999; Cromeans et al. 2008; Duizer et al., 2004). Owing to these limitations nucleic acid-based methods such as PCR and hybridization have proved to be important tools. PCR was shown to be more effective than cell culture technique for detection of enteric viruses (Abbaszadegan et al., 1999). However, the use of PCR for detection of multiple viral targets in environmental samples is limited due to high cost and sometimes the availability of adequate test sample volume for several individual reactions. Unlike single PCR, multiplex PCR (Chamberlain et al., 1988) with different pairs of specific primers for amplifying different viral genomes in one reaction tube enables detection of two or more targets in a single test, hence making the later more cost-effective and less time consuming. Multiplex PCR has been used for simultaneous detection of enteric viruses from environmental samples and food samples (Fout et al., 2003; Tsai et al., 1993)
We developed multiplex PCR for the simultaneous detection of HAV, HEV, Entero and Rotavirus in a single PCR reaction. The primer pairs were able to amplify specific products respective to each virus and no non-specific amplification was observed in multiplex PCR when tested with duplex, triplex and quadruplex PCR in all possible combinations. The specificity was confirmed by sequencing the amplified product. We optimized different parameters affecting the PCR reaction such as amount of AMV RT and dNTPs during cDNA preparation and concentration of Taq polymerase and dNTP during 1st and 2nd PCR. Comparison of the visual images of amplification utilizing individual primer sets and multiplex primer sets revealed that multiplex PCR methods were as specific and efficient as individual PCR methods. Development of this multiplex PCR enabled us to detect all four viruses in a limited sample volume in a cost-effective and less time consuming manner. With this the second objective i.e. development of an appropriate detection system for enteric viruses was achieved.

**Application to drinking water samples**

After having the methodologies in place, we examined the drinking water supply to Pune city. For this Drinking water samples (n=662) collected during January 2007 to December 2007 were concentrated and subjected to multiplex PCR for the detection of HAV, HEV, Entero and Rotavirus. To our surprise (but feeling elated) only two samples showed the presence of enterovirus. Apart from these two samples, all other samples were negative for all four viruses. To assess the possibility of less sensitivity of multiplex PCR being responsible for such a low positivity, we did test representative samples (n=8) employing individual PCR for all the four viruses For understanding possible association of PCR inhibitors with negativity, for negative samples, known viruses were added & subjected to PCR with positive results.

These results make us believe that the water supply by Pune Municipal Corporation is reasonably safe with respect to enteric viruses. It may be pertinent to note here that no epidemic of these enteric diseases was reported.
during the study period, though sporadic cases continued to occur: reflecting other sources of transmission of these viruses.

One of our aims was to evaluate the efficacy of three water treatment plants. However, as none of the water samples collected from the inlet points of these plants were contaminated with enteric viruses and the source water samples collected from Khadakwasla dam were also negative, it is not possible to critically comment on the relative performance of these plants.

**River water samples**

Screening of river samples led to shocking findings. Multiplex PCR results showed the presence of enteric viruses in most of the samples. This shows that the river water is heavily contaminated with enteric viruses and down-stream populations using river water is at high-risk of these infections. On one side, the drinking water and the source water were almost free of these viruses, high prevalence of all the four viruses in river was other extreme situation. This reflects discharge of sewage (treated & untreated) into the river at different points. The river does not have lots of water (except during monsoon months), hence the dilution effect is also not seen.

It is therefore important to take utmost care in treating the sewage before discharging into river. We have earlier observed (Vaidya et al., 2002) that the sewage treatment plant was non-functional for 5 months for mechanical reasons and untreated water was being flown into river. The treatment protocol may not eliminate all the viruses. In addition to outlets of sewage treatment plants, several unauthorized untreated sewage outlets are also discharged in the river. The corporation needs to strictly prohibit such activities.

One important fact is the rise in the prevalence of HAV and HEV over the years. In 2007, HAV RNA and HEV RNA positivity in samples collected from Mutha River was 76.56% and 25% respectively. As compared to 1999 (sewage analysis) (Vaidya et al., 2002) percent positivity rose from 11% to 25% (p<0.02) for HEV and from 24.42% to 76.56% for HAV (p<0.001), during the next 7 years. This itself is alarming. The river samples were tested without a concentration step, this was primarily because we intended to examine
presence of enteric viruses in river with respect to discharge of sewage in the river, however the possibility of further increase in prevalence upon concentration of samples can not be ruled out. Thus, sewage treatment & discharge were identified as the most important issues to be tackled urgently in the interest of the public health.

**Virological evaluation of domestic water purification units**

Use of household water purification systems is practiced by majority of the Indian families who can afford the same, especially in the urban and semi-urban areas. Many Non-Government Organizations (NGOs) have come forward to provide such units in the schools from rural areas as well. This definitely indicates the concern of the population about the quality of drinking water available and efforts to remove the disease causing microbes at their level. The manufacturers routinely follow testing of such units for the removal of bacteria, as it is easily available and less expensive. Though enteric viral diseases are of immense public health importance, due to the difficulties in concentrating large volumes of water as well as non-availability of rapid and highly sensitive techniques for the quantification of these viruses, evaluation of domestic water purification units for the removal of viruses is rarely practiced in India. Moreover, no guidelines are provided for certification of these units as virus-free. We therefore thought that it would be extremely important to evaluate the different domestic water purification units available in the Indian market.

Since these were controlled experiments under laboratory conditions, 5 liters of distilled water was fixed as the water volume to be passed through every unit after addition of a known amount of the model virus, HEV. The “so called” purified water was concentrated to ~3ml employing two-step membrane-filtration method. The entire protocol takes only 2 hours and the concentrate is kept on ice thereby minimizing the possibility of loss of virus with time and at room temperature.

Development of a highly sensitive and specific real time PCR assay provided us the most needed handle for the quantification of HEV RNA, an integral part of the evaluation protocol. With both methodologies in place, we
were able to clearly document that only 2 of the 8 units were able to remove HEV completely as evidenced by real time PCR. In the absence of a cell-culture system or a small laboratory animal (except monkeys), it was not possible to assess the infectivity of the virus recovered from the various units. However, the fact that we were able to amplify almost the entire genome from all these virus positive concentrates, we tend to believe that the PCR positivity did not indicate partially degraded virus but the intact virus. There are evidences to demonstrate that signals generated after RT-PCR amplification of viral genome correlates well with the presence of infectious virus in the sample. Viral samples inactivated by heat or UV treatment produce significantly lower signal strength that paralleled infectivity of the sample in cultured cells (Bhattacharya et al., 2004).

Three units (1, 2 and 5) were able to remove two logs of seeded HEV particles and two units (3 and 4) were showing one log reduction. Performance of unit 6 was poorest showing no reduction at all. Thus, only units 7 and 8 were able to fulfill the log reduction value requirements laid by USEPA (4 log reduction). These results indicate superiority of the techniques employed for designing unit 7 (hollow fiber membrane filtration) and unit 8 (combination of Polyester nonwoven fiber, chlorine dispenser, silver-impregnated granular activated carbon) as against those employed for other units. It may be noted that ultra violet irradiation, activated carbon filtration and other filtration techniques were not able to either remove or destroy seeded HEV particles beyond two logs.

These purification units were evaluated under controlled laboratory conditions and distilled water spiked with HEV was used as test water, which does not mimic in situ conditions where the feed water quality is poor. The maintenance and the frequency of the replacement of filter medium of purification units may not also be adequate. In Practice, these limitations may result in even poorer performance of the units. Employing Male-specific MS2 coliphage as a surrogate marker for enteric viruses, Clasen et al., (2006) evaluated Pureit™ water purifier developed by Hindustan Lever Limited (HLL) and three other units (Clasen et al., 2007). The removal of the phage was 99.9% with the former unit whereas none of the three later devices achieved
4.0 Log Reduction Value. The Pureit™ water purifier (unit 8) and one of the three units tested subsequently (unit 1) were also evaluated in the present study employing hepatitis E virus as the test virus. Though the methods employed were absolutely different, the results obtained were similar; complete virus removal by unit 8 and the LRV of 2 for the unit 1. These results suggest that the method developed by us for the evaluation of water-filters for virus removal using hepatitis E virus as an enteric virus is in accordance with the accepted protocol, employing surrogate for enteric viruses. The possibility of use of actual enteric virus in place of a surrogate organism in evaluation programs may be considered following extensive comparison of both methods.

As a rule, viruses persist longer than enteric bacteria in water environments (Bosch, 1998). It is therefore absolutely unsafe to rely on bacteriological standards to assess the virological quality of water. Importantly, waterborne outbreaks related to potable water that met bacteriological standards have been reported (Bosch et al., 1991; Hejkal et al., 1982). During an outbreak of infectious hepatitis among a military community, HAV, rotaviruses and enteroviruses were detected in water samples that were consistently free of indicator bacteria (Bosch et al., 1991). These same samples showed free and total chlorine levels that were adequate to ensure proper elimination of bacterial contaminants, but were unable to remove pathogenic viruses. These reports clearly document a definite need for a separate, well-defined virological standard for drinking water as well as for the evaluation of water treatment plants and domestic water purifiers. The minimum standards established by USEPA were not designed for developing countries where the microbiological quality of public water supply may not be as good as in developed countries. India and other developing countries should formulate their own standards and ensure strict adherence by all those concerned. This will help both manufacturers and consumers to be quality conscious with respect to drinking water, a basic need for every population and the major source of a variety of infectious diseases taking heavy toll every year in all the under-developed and developing countries. It would be worthwhile judging the performance of the domestic units in field i.e. houses, with respect to water
quality, adherence to the recommended maintenance of the units as well as time period of usage.

We would like to point here that we have evaluated one unit of each type. The batch-to-batch or unit-to-unit variation was not evaluated. This is a limitation of this study and needs to be extended to several units from one batch as well as different batches. Our study suggests that even with the limitation of the study pointed above, the results indicate that 6/8 units tested (one unit/type) do not confirm to USEPA standards and emphasizes the need for a definite national policy for the evaluation of such devices by the regulatory authorities as well as at factory level. Such an exercise will ensure availability of quality assured domestic water purification units to the community thereby reducing the burden of water-borne infections. It is desirable to set up our own national virologic standards as well as evaluation of the protocol developed by us in several laboratories followed by strict adherence to the method accepted and approved by the regulatory authorities.

Overall, this study has tried to address a few very important issues with respect to contamination of enteric viruses in water in a large city from a developing country. While doing so, we have been able to standardize requisite methods that can be applied elsewhere i.e. other cities, villages and most importantly, military establishments and schools, especially in rural areas.

We sincerely wish that similar studies are conducted at multiple sites and data generated be used to improve quality of water being supplied to the masses.