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Evaluation of a simple and cost effective filter paper-based shipping and storage medium for environmental sampling of F-RNA coliphages

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Male specific RNA (F-RNA) coliphages are used as indicators of fecal contamination and for source tracking. However, collecting fecal samples for analysis from remote sites is problematic due to the need for an uninterrupted cold chain to guarantee sample suitability for downstream molecular detection of these coliphages. Here, we investigated the feasibility of using filter paper as a collection and storage vehicle for F-RNA coliphages. Various concentrations (10^1 to 10^4 pfu) of two F-RNA coliphages, MS2 and Qβ, were prepared in lambda buffer or a 10% bovine manure slurry, spotted onto filter paper disks, dried, and maintained at 37 °C for up to 37 days. Nucleic acids were extracted from the spotted filter paper disks at 0, 6, 13, and 37 days post inoculation and analyzed by real time RT-PCR. F-RNA coliphages at concentrations of 10^2 pfu/filter paper unit were readily detected, and only a slight decrease in nucleic acid detection was observed over time. Furthermore, the sensitivity of real time RT-PCR detection of the F-RNA coliphage RNA was similar between the developed filter paper sampling methodology and traditional cold storage. These results indicate that filter paper is a suitable storage and transport medium for F-RNA coliphages when refrigeration is not possible.

1. Introduction

The family *Leviviridae* comprises a group of single stranded, positive sense RNA bacteriophages (F-RNA coliphages) that share structural characteristics with pathogenic human enteric viruses including caliciviruses, hepatitis A and E viruses, enteroviruses and astroviruses (Jofre et al., 2011). These F-RNA coliphages do not naturally proliferate in environments other than the gastrointestinal tracts of warm-blooded animals (Grabow, 2001), and their constant presence in human and animal feces make them suitable indicators of fecal contamination (Havelaar et al., 1993; Leclerc et al., 2000). The United States Environmental Protection Agency recognizes F-RNA coliphages as one of the three groups of microorganisms useful as fecal indicators (EPA, 2000), and they have been proposed as an index of the presence and risk of Norovirus in oysters and other bivalves (Doré et al., 2000), and as indices and indicators of viral contamination on animal carcasses (Flannery et al., 2009; Jones and Johns, 2012).

Based on their serological and genetic properties, F-RNA coliphages are classified into four genogroups within two genera: genogroups I and II are comprised within the genus *Levivirus*, and genogroups III and IV within the genus *Allolevivirus* (Leclerc et al., 2000). The presence or absence of particular F-RNA coliphage genogroups can be used to track the origin of fecal pollution. F-RNA coliphage genogroups II and III are primarily associated with human sources of fecal contamination, while genogroups I and IV are predominantly associated with animal feces (Schaper et al., 2002b). However, some reports indicate that F-RNA coliphage/host associations are not absolute. Genogroups II and III were found in poultry, cattle, swine and dogs feces, and genogroups I and IV have been detected in human waste waters (Jofre et al., 2011; Noble et al., 2003; Scott et al., 2002).

A potential problem of this source tracking approach is the possible differential survival and recovery rates of different F-RNA phage genogroups in the laboratory. Highly sensitive and culture-independent molecular techniques based on RNA detection have been developed (Friedman et al., 2011; Kirs and Smith, 2007) to overcome these problems, but for such methods to be successful, shipment of the fecal samples from remote field settings and low resource regions to laboratory facilities must be adequate to maintain the suitability of specimens for molecular detection. Standard collection procedures of fecal material require fresh or frozen samples (Nechvatal et al., 2008), not only in order to avoid nucleic acid

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degradation but also to preserve the original profile of the sample microbiota (Cardona et al., 2012).

Filter paper is an appropriate alternative for collection, shipment and storage/preservation of virus-containing clinical samples. Several studies have described the use and efficacy of different types of filter paper (Maw et al., 2006; Michaud et al., 2007; Vilcek et al., 2001) to collect various clinical samples including blood, urine, and feces (Nozawa et al., 2007; Solmone et al., 2002) for detection of a wide variety of human and animal viruses including hepatitis A virus (Desbois et al., 2009), hepatitis B (Lira et al., 2009), norovirus (Wollants et al., 2004) and rotavirus (Rahman et al., 2004). In general, viruses stored on filter paper have been shown to remain suitable for molecular detection for long periods of time. Nevertheless, molecular detection of some viruses is differentially impacted by storage time and/or temperature (Johansson et al., 1997; Katz et al., 2002; Rahman et al., 2004). To the best of our knowledge, filter paper has not been evaluated as a medium for collection, storage/transport, and detection of low concentrations of F-RNA coliphages from feces.

The objective of this study was to compare the performance of filter paper as a storage and transport medium for F-RNA phages in feces to traditional cold storage transportation, and to determine the efficacy of filter paper as a simple and inexpensive method of sampling for the presence of F-RNA phages in remote areas and low resource settings. MS2 and Qβ were chosen for these analyses because they each represent one of the two genera (*Levivirus*, MS2; *Allolevivirus*, Qβ) of the family *Leviviridae* and are present in animal (genogroup I) or human feces (genogroup III), respectively.

2. Materials and methods

2.1. Coliphages and bacterial strains

F-RNA coliphages, MS2 (genogroup I) (ATCC 15597-B1) and Qβ (genogroup III) (ATCC 23631-B1), were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and amplified in *Escherichia coli* HS (pFamp)R (ATCC 700891). Briefly, cultures of *E. coli* were grown in tryptic soy broth (supplemented with 50 μg/ml of ampicillin and streptomycin) to logarithmic phase and infected with coliphages at a multiplicity of infection (MOI) of 5–10. Infected cultures were incubated with shaking for 18–24 h, treated with chloroform (10%), and centrifuged at 6000 × *g* for 25 min at 4 °C to remove unlysed bacteria and cellular debris. The coliphage-containing supernatants were filtered through a 0.22 μm low protein-binding filter (PALL Life Sciences, Ann Arbor, MI) and stored at 4 °C. Resulting coliphage stocks were enumerated using the double agar overlay plaque assay (Kropinski et al., 2008).

2.2. Inoculation of F-RNA coliphages in liquid samples and onto filter paper

Serial, ten-fold dilutions of coliphage stocks were performed in lambda buffer (liquid buffer samples) (0.58% NaCl, 0.2% $MgSO_4$ heptahydrate, 0.01% gelatin, and 0.05 M Tris–HCl, pH 7.5) or in 10% bovine manure slurry (liquid manure samples), made by homogenizing bovine manure (previously confirmed to be negative for the presence of F-RNA coliphages by real time RT-PCR) in lambda buffer. Liquid samples were stored at 4 °C, and 10 μl aliquots were obtained at 0, 6, 13 or 37 days (three replicates were tested for each time point and treatment) for further RNA isolation.

Filter paper samples were prepared by spotting portions (10 μl) of each coliphage concentration from either the liquid buffer samples (filter paper buffer) or from the liquid manure samples (filter paper manure) onto 6 mm diameter, precut, Whatman No.1 filter paper disks (Whatman Inc., Clifton, NJ). The filter paper samples were dried at room temperature for 1 h, and packed into standard business paper 3 5/8 × 5 1/2 inches envelopes. Envelopes were sealed in a plastic bag and stored in an incubator at 37 °C, to simulate transportation of the samples in a tropical region where refrigeration is unavailable. As with the liquid samples, three replicates of each filter paper treatment were tested on 0, 6, 13, and 37 days post-incubation.

2.3. Environmental sampling

In addition to laboratory studies aimed at comparing storage methods, the use of filter paper as a storage medium for environmental fecal samples was also evaluated. Ten fresh bovine fecal specimens were obtained from a dairy farm in Northern China (Autonomous region of Xinjiang). Individual samples were placed in a plastic bag, diluted (1:10) in peptone water and 200 μl of the slurry was spotted on qualitative grade cellulose filter paper, equivalent to Whatman No.1 (New star, Hangzhou Fu Yang special paper Industry Co., Ltd., Hangzhou, Shanghai, China), dried overnight at room temperature, and stored in envelopes contained in plastic bags. After 10 days, RNA was isolated as described below, except that 140 μl of ultrapure water was added to a pool of three 6 mm filter paper disks punched out from the slurry spot. For negative controls, portions of clean filter paper areas surrounding the slurry spot were punched out and eluted as described ($n = 3$ pools). Real time RT-PCR for all four coliphage genogroups was performed.

2.4. RNA isolation

RNA was obtained from equivalent amounts of coliphage liquid samples (10 μl) or from filter paper samples (one disk), to allow further direct comparison of results. Nucleic acid isolation was performed using the QIAmp viral RNA kit© (Qiagen, Valencia, CA) according to the manufacturer's instructions. When liquid samples were used, 10 μl of each sample was mixed with 130 μl of water to attain the sample volume recommended by the kit manufacturer (140 μl). Alternatively, when filter paper samples were used, coliphages were eluted from filter paper prior to RNA purification by immersing each paper disk in 140 μl of nuclease free water and incubating it at room temperature for three minutes. The eluate was transferred to a clean 1.5 ml tube to start RNA purification. Final elution of RNA was accomplished using 60 μl of AVE buffer.

2.5. Nucleic acid detection

For the artificially inoculated samples, F-RNA coliphages were detected by real time RT-PCR using genogroup specific primers (Table 1) described previously (Kirs and Smith, 2007). The reaction was modified to employ SYBR Green and OneStep real time RT-PCR kit enzymes (Qiagen, Valencia, CA) using the StepOne Plus thermocycler (Applied Biosystems, Foster City, CA). Real time RT-PCR reactions (15 μl) contained 5 μl of RNA, 0.6 μl of enzyme mix, 3 μl of 5× buffer, 100 nM of each forward and reverse primer, 0.4 mM (each) of deoxynucleotide triphosphates, 1.2 U RNAse inhibitor (Qiagen, Valencia, CA), and 0.3 μl of 10× SYBR Green solution (Sigma–Aldrich, Saint Louis, MO). Retrotranscription and thermocycling conditions were as follows: 30 min at 50 °C, 15 min at 95 °C, 40 cycles of 1 min at 95 °C, 30 s at 60 °C and 1 min at 72 °C. Amplicon fidelity was evaluated through melting curve analysis. Reaction products with the same melting temperature as purified coliphage controls (87.4 °C for MS2 and 84.6 °C for Qβ) were considered positive.

Real time RT-PCR detection of F-RNA coliphages from the naturally contaminated fecal samples was achieved using a probe-based assay (Table 1) specifically designed to detect a wide range of environmental F-RNA coliphages (Friedman et al., 2011).