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Full Length Research Paper

Efficiency of filtration technique for isolation of leptospires from surface waters: Role of different membranes with different pore size and materials

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The objective of this research focused on evaluation of membrane filters application for isolation of *Leptospira* from surface water samples. The filter materials evaluated included nitrocellulose (0.22 and 0.45 µm pore diameters), polyvinylidene fluoride (Durapore 0.22 and 0.40 µm pore diameters), nylon mesh (37 µm), and glass fiber (1.0 µm). Millipore polyvinylidene fluoride filter (0.22 µm) was examined by scanning electron microscopy to verify that leptospires were present following filtration. Our results suggest for isolate nearly 100% of *Leptospira* from water samples, the optimal pore diameter should be less than 0.45 µm (a standard pore size used to detect indicator microorganisms in 100 ml of water). Although filtration method can be used to isolation leptospires from surface water samples, it is unclear whether this is a useful method for detection of all leptospires exist within surface water samples. Accordingly, a large proportion of leptospires can be retained by membrane filter with a pore diameter commonly used to isolate leptospires from surface water samples (0.45 µm pore size).

Key words: *Leptospira,* filtration technique, leptospirosis.

INTRODUCTION

Leptospirosis is considered a reemerging disease (Levett, 1999) that infects people who have contact with contaminated water, soil or urine from infected animal hosts (Levett, 2001).

The disease is commonly associated with flooding and is prevalent in flood-prone areas (Morshed et al., 1994).

Leptospirosis is characterized by some researchers as the most common waterborne illness in the world (Bharti et al., 2003).

Leptospirosis is caused by serovars of at least eight species of spirochetes from the genus *Leptospira* (Slack et al., 2006), which are the environmentally transmitted form of the pathogen.

Common pathogenic serogroups within *Leptospira interrogans* include Canicola (associated with infected dogs) and Copenhageni strain M20 (also classified as Icterohaemorrhagiae and associated with infected rodents). *Leptospira biflexa* is also found in the environment but is nonpathogenic. Because *L. interrogans* and *L. biflexa* species are morphologically similar, they cannot be differentiated with microscopy (Levett, 2001).

Leptospira species spirochetes are helical and motile with dimensions of approximately 0.2 - 0.3 µm in diameter by 6 - 30 µm in length. Pathogenic leptospires belong to any of more than 200 known serovars, which are organized into at least 23 serogroups. Each serovar may be adapted to infect a particular reservoir host that sheds leptospires primarily in urine (Levett, 1999; Ward, 2002).

Common serogroups identified in patients with leptospirosis in Hawaii between 1974 and 1998 include (in descending order of prevalence) Icterohaemorrhagiae, Australis, Ballum, Bataiae, Sejroe, and Pomona (Katz et al., 2002).

The leptospires survive well in fresh water, soil, and mud in tropical and temperate climates (C.D.C, 1998).

Research has focused on efficient techniques for isolating and detecting *Leptospira* spirochetes from surface

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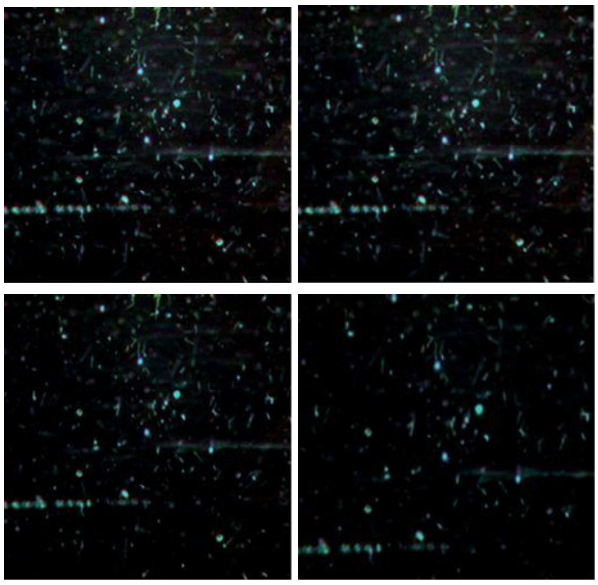

Figure 1. Petroff-Hausser bacterial counting chamber at 100x, with *Leptospira* spirochetes appearing as bright curvilinear objects.

water, bodily fluids and tissue samples (LeFebvre et al., 1985, 1987; Faber et al., 2000; Levett, 2001; Bunnell et al., 2003). Although antibodies for serovars of *Leptospira interrogans* have been developed as clinical diagnostic and research tools, they have not been applied for water sample analysis. Culturing methods for *Leptospira* are also available, but the recommended incubation periods are exceedingly long (16 - 26 weeks) (Wilson and Fujioka, 1995).

The primary challenges for carrying out water sampling for *Leptospira* spirochetes include isolation, concentration, and quantitative detection of small numbers of target microorganisms in water. The hypothesis that guided this research was that we could effectively isolate leptospires from surface waters using filtration, either with a nested approach (involving removal of debris and sediment with a coarse filter followed by a finer filter) or with a simple membrane filtration approach similar to that used to isolate indicator organisms from surface waters. So, the objectives of this research were to:

1.) Develop a method and evaluation of current filtration method to concentrate *Leptospira* spirochetes from water samples.
2.) Using this technique, assess the occurrence of *Leptospira* water samples.

MATERIAL AND METHODS

Culture method

Suspensions of pure culture were prepared using liquid and semi-solid Ellinghausen and McCullough medium as modified by Johnson and Harris (EMJH) with *Leptospira* (*Leptospira interrogans* serovar Icterohaemorrhagiae) obtained from the Pasteur Institute of Iran, Tehran, Iran. The semi-solid EMJH medium (DIFCO™ EMJH) was prepared with 0.2% noble agar (weight/ weight) using triple-filtered (1.0, 0.45, 0.22 µm filters) distilled, deionized water and was supplemented with 200 µl/ml 5-fluorouracil (Acros Organics, Catalog No. 228440050) to suppress the growth of bacterial contaminants. Liquid EMJH was prepared as above but without noble agar. Inoculated EMJH was stored in the dark at 21°C (70°F) for 3 - 5 weeks (Levett, 2001). When cultures in the semi-solid medium began to present the characteristic cloudy, compressed layer of leptospires approximately 1.5 cm below the surface (the Dinger's ring), an aliquot of the leptospires was withdrawn from the ring and from the well-mixed liquid medium to be sure that leptospires were present in both types of culture.

The leptospires suspension density was determined using a Petroff-Hausser counting chamber, observed at magnification of 100x on a microscope equipped for darkfield microscopy (Nikon Model) (Figure 1). Leptospires were bright curvilinear objects, approximately 20 µm long, often flexing or spinning along their long axes in suspension. Ten replicate counts were averaged to determine each suspension density.

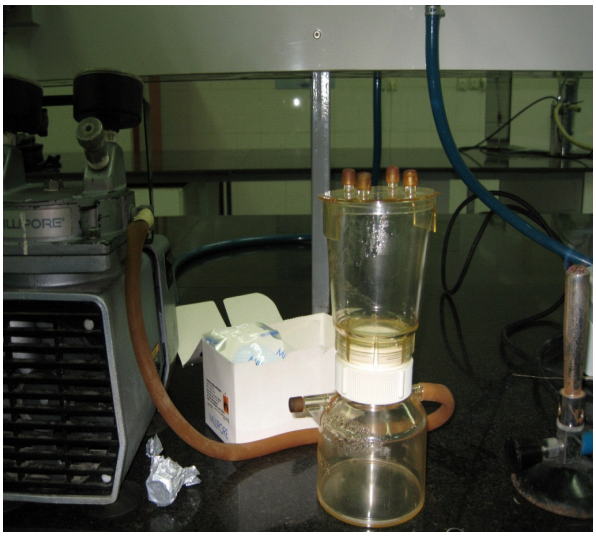

Figure 2. Filtration device used in this study.

Filtration trials

Fifty ml autoclaved, prefiltered experimental suspensions from surface water samples were prepared that have had inoculated approximately 1.5×10^6 leptospires per ml. Also to ensure no impact on water samples debris our results, fifty ml experimental suspensions were prepared containing approximately 1.5×10^6 leptospires per ml in autoclaved, filtered 0.01 Molar phosphate buffered saline (PBS) solution. Filtration trials used 30 ml of the surface water suspension, with several filters (see Table 1) mounted in a 47-mm filter holder (Millipore model PFC0004703), with a vacuum of 5 inches of Hg to draw the sample into a 50-ml tube (Figure 2). The number of leptospires retained on the filter was estimated by comparing the average of ten replicate 10 µl aliquots of filtrate with the numbers of leptospires present in the stock suspensions of surface water samples.

The filter materials evaluated (Table I) included nitrocellulose (0.22 and 0.45 µm pore diameters), polyvinylidene fluoride (Durapore0.22 and 0.40 µm pore diameters), glass fiber (1.0 µm),

Table 1. Filter types tested to determine recovery efficiencies of surface water suspensions containing *Leptospira interrogans* serovar Icterohaemorrhagiae.

Filter (Catalog number)	Type	Material	Pore diameter
GE nitrocellulose-mixed esters of cellulose Membrane (E02WP04700)	Hydrophobic membrane for water sampling	Nitrocellulose	0.22 µm
Millipore durapore membrane filter (HTTP04700)	Hydrophobic	Polyvinylidene fluoride	0.40 µm
Fisher (09-719-555)	Hydrophobic membrane for water sampling	Nitrocellulose	0.45 µm
Small parts Inc (CMN-0040)	Hydrophobic nylon mesh sheet	Nylon mesh	37 µm
Millipore durapore (GVWP)	Hydrophobic membrane for liquid purification	Polyvinylidene fluoride	0.22 µm
Millipore isopore (HTTP)	Hydrophilic membrane for filtration of biological liquids	Polycarbonate	0.40 µm
Millipore (AP1504700)	Hydrophilic prefilter for coarse debris removal	Glass fiber	1.0 µm

and nylon mesh (37 µm).

Imaging with scanning electron microscope

Millipore Durapore polyvinylidene fluoride filters (0.22 µm) were examined by scanning electron microscopy to verify that leptospires were present following filtration. Specimens were prepared by passing 0.200-ml aliquots from undiluted liquid EMJH cultures through filters at low vacuum (~ 5 inches Hg), followed by 200-µl of fixative (Karnovsky's Fixative, Electron Microscopy Services Catalog No. 15720, prepared as 16% paraformaldehyde, 50% electron microscopy grade glutaradehyde, 0.2 M sodium phosphate buffer, with distilled water, per manufacturer's instructions) to preserve microorganism structure. The fixative was added with vacuum off. After 20 min., excess fixative was drawn through the filter to waste with vacuum. The specimens were vacuum freeze-dried (- 0.133 mBar, - 40°C, with a Labconco Freeze Dry System - Freezone 18) mounted on a 1.6-cm diameter carbon stage on a bed of dessicant (anhydrous calcium sulfate - Drierite©) for 24 h. A multimolecular platinum layer was applied to the filters by sputter-coating using an EMITECH model K575x Turbo Sputter Coater with 30 mm platinum target, sputter cycle of 20 s, under ultrahigh purity argon gas at 85 mA, in a vacuum of at least 10^{-5} mBar. The samples were examined using a Field Emission Scanning Electron Microscope (FE-SEM) Hitachi model S-4700 type II, operated at a voltage of 10 kV.

RESULTS

The filtration results are displayed in Figure 3. These results suggest several important aspects of using filters to isolate leptospires from water samples. First, in order to isolate nearly 100% of leptospires from sampled volumes, the optimal pore diameter should be less than 0.45 µm (a standard pore size used to detect indicator microorganisms in 100 ml of water) (Clesceri et al., 1998). Second, the results suggest that the filter material itself may affect recovery rates. For example, with pore diameters of 0.4 µm (hydrophilic polyvinylidene fluoride Durapore filters) and 0.45 µm (Fisher nitrocellulose filters), flow through recovery rates varied from <32% - <10%, respectively.

This effect is also seen in the results from glass fiber filters and nylon mesh filters. The results from trials with glass fiber filters may be biased in part, because glass fiber filters are very similar in appearance to leptospires, which likely led to false positive results. Given the difficulties of counting spirochetes microscopically, this suggests that glass fiber filters would be unsuitable as prefilters for surface water samples. This is because, using microscopy, glass fibers could be mistakenly identified as leptospires.

For surface water samples, in order to sample volumes of water that are at least comparable to those used for detecting indicator microorganisms, it may be best to prefilter the sample using either a glass fiber filter or a nylon mesh filter to remove large pieces of debris prior to working with the 0.22 µm pore diameter filters. It may be possible to use 0.45 µm pore diameter filters (especially the nitrocellulose filters) to isolate leptospires from surface samples, given that approximately 90% of leptospires appear to be isolated on or within the filters.

Scanning electron microscopy results (Figure 4) support, in part, the results presented in Figure 3. In Figure 4, leptospires are visible on the 0.22 µm pore diameter filter (Durapore® 0.22 polyvinylidene fluoride filters). The image of the filter suggests that a small number of pores are spaced closely enough to slightly overlap, such that the resulting pore diameter could be approximately equal to the diameter of a spirochete. As a consequence, a pressure gradient across the membrane could force leptospires through these large pores, leading to passage through filters that should retain them. In fact, a small proportion of leptospires passed through filters that were expected to completely retain leptospires under the experimental conditions [the 0.22 µm pore diameter nitrocellulose and 0.22 µm Durapore® filters (Figure 3)].

Filter performance could be determined by more than one process, including hydrophobic bonding and mechanical retention. The tests performed on the filter by the manufacturer do not differentiate between the mechanisms of retention. Accordingly, it is possible that even though pore size appears to be highly variable, in some cases larger than the 0.45 µm specified for these filters, an additional factor related to sorption, such as hydrophobic binding, could retain leptospires on or within the filter. The second factor, depth of the filter, suggests that leptospires that could not be found on the surface of the filter were retained out of the field of focus within the filter

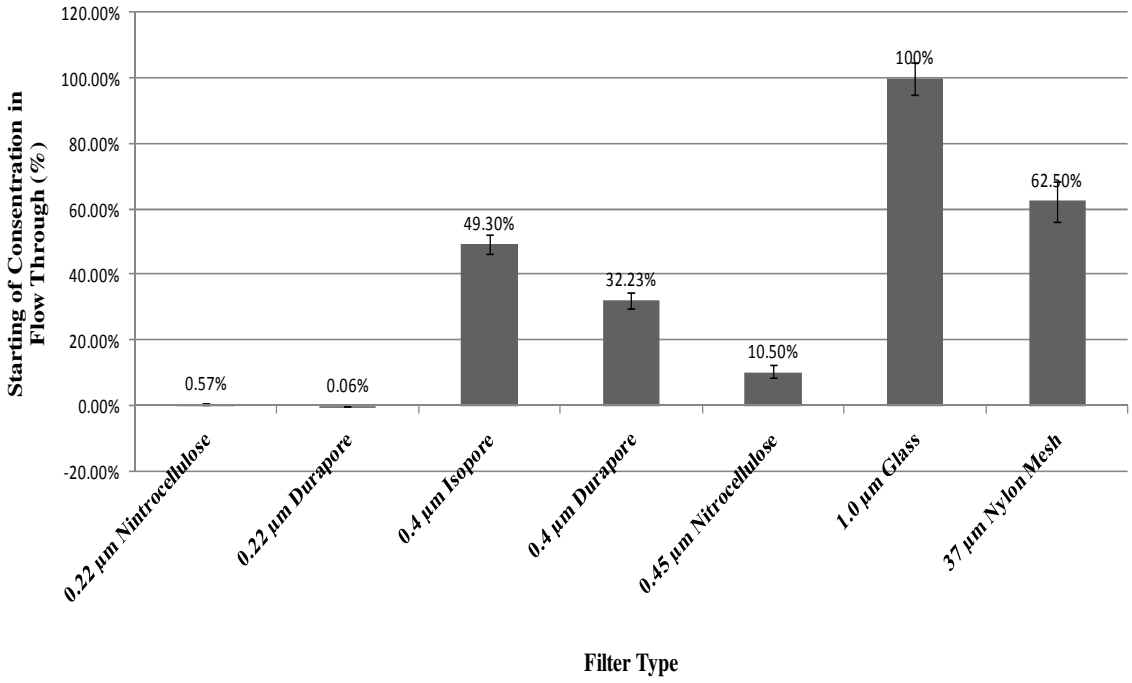

Figure 3. Average percent of starting numbers of leptospires in stock suspensions of water samples that were found in filtrate, with 95% confidence intervals displayed.

itself. Third, the variation of pore geometry and orienttation may also enhance retention, because leptospires may be forced into contact with the filter material due to the tortuosity of flow paths. This could enhance the likelihood of contact with the filter matrix, which would increase the opportunities for sorption. Finally, the actual surface area of the filters is much larger than the 958-mm² surface presented in the filter holder. This would enhance the likelihood of sorption, if hydrophobic binding occurs.

Overall, although filtration can be used to isolate leptospires from surface water samples, it is unclear whether this is a useful method with respect to detection. Although a large proportion of leptospires can be retained by filters with a pore diameter commonly used to isolate indicator microorganisms from water samples (0.45 µm pore diameter), the filters must be processed to recover spirochetes. Given that sample volumes are likely to be small (100 ml or less if waters have significant suspended sediment content) and that under ideal conditions a maximum of 90% of leptospires in liquid filtered by a 0.45 µm pore diameter filter will be retained, additional inefficiencies will be introduced during filter processing. This suggests that rather than relying on processing techniques to obtain leptospires from filters, it may be useful to apply a detection technique to the filter itself. In order to increase efficiency of isolating leptospires from liquid, it may be appropriate to work with filters that have a smaller pore diameter (e.g., the polyvinylidene fluoride filters with 0.22 µm pore diameter).

DISCUSSION

The first characteristic of these filters is important with respect to expected retention of spirochetes. The pore diameter of this type of filter is determined by retention of *Serratia marcescens*, a rod -shaped bacteria that has a size range of 0.5 - 0.8 µm in diameter by 0.9 - 2.0 µm in length. Product certification for pore size is based on overall retention of the *S. marcescens*, rather than direct examination of the filter surface. The results of trials indicate that a substantial proportion of leptospires in surface water samples and pure suspensions can be isolated from water with a 0.45 µm pore diameter nitrocellulose filter. The filter is readily available and is commonly applied to determine whether indicator microorganisms, including *E. coli* and, more generally, fecal coliform, are present in water samples (Clesceri et al., 1998).

One of the concerns about field application of this method is whether interfering debris and sediment will limit the volume sampled to less than 50 ml. In several trials with soil/water slurries (~1 g soil/100 ml water, representing ~10 mg/l total suspended solids), filters clogged and failed before relatively small volumes (<10 ml) could be processed. It is unclear whether such a concentration of total suspended solids will be often equaled or exceeded in surface waters (Henry and Johnson, 1978; Wilson and Fujioka, 1995; Ganoza et al., 2006).

However, during high-flow events, especially in erosion

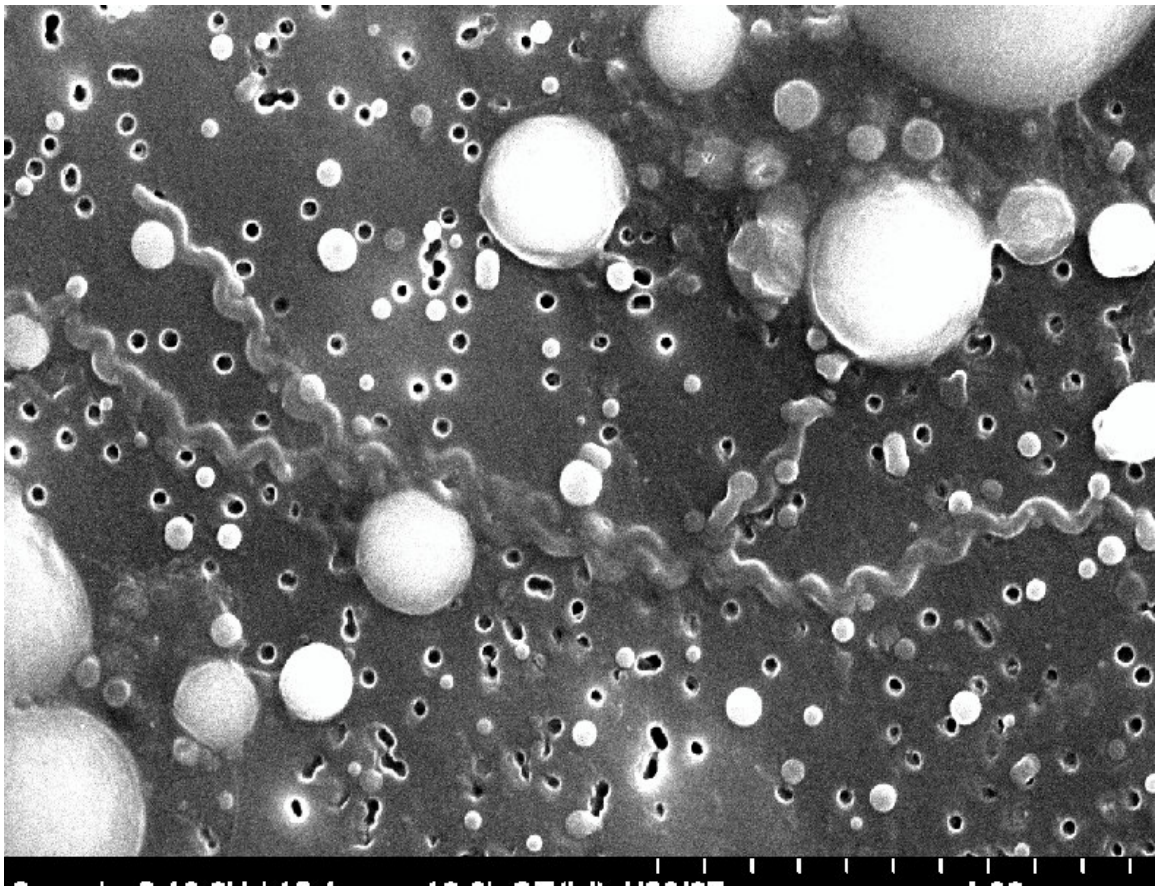

Figure 4. *Leptospira* spirochetes on the surface of a 0.22-µm pore diameter filter (Durapore Polyvinylidene fluoride membrane). Surface of filter indicate overlapping pores that could be large enough to allow passage of leptospires under vacuum (Bar, 4.0 µm).

prone watersheds with unstable, steep headwater areas, it is possible that total suspended solid concentrations could reach and exceed this level. Given the link between flooding and outbreaks of leptospirosis, such events may be important to sample. However, it may be most efficient to concentrate sediment and leptospires in samples collected from such events directly by centrifugation of volumes of 50 - 250 ml. In either case, whether the sample is concentrated on a filter or pelleted by centrifugation, the concentrate will be a compact sample that likely can be transported without significant loss during shipping, especially if genomic DNA from pathogenic spirochetes is the analytic target rather than direct examination of the filter using microscopy.

One promising technique that could be explored further is use of a water DNA isolation kit (for example, the UltraClean™ water kit, Catalog No. 14800-10, MoBio Laboratories, Inc.) These kits are designed for extracting microbial DNA from filters used to isolate microorganisms from water samples and have been in use for several years but have not been applied for use with *Leptospira* spirochetes.

In addition to establishing a laboratory limit of detection for a sampling method, it will be important to carry out field trials with surface waters to quantify expected limits of detection in the presence of naturally occurring chemical and biological compounds. This includes humic acids and sediments, both of which may be important in surface waters (Clesceri et al., 1998).

Conclusion

The current standard method for collecting and processing samples for *Leptospira* spirochetes [method 9260 I (Clesceri et al., 1998)] specifies culturing and animal inoculation as potential analytic endpoints, with a clear statement that successful cultures may contain a mixture of saprophytic and pathogenic leptospires, as well as other microbial contaminants. It does not provide expected limits of detection for either field or laboratory methods. A complete description of a field method that describes application of filters, or centrifugation, to isolate and concentrate leptospires, followed by a filter processing, would be useful, especially if accompanied by guidelines about expected sensitivity and specificity of

the entire field and laboratory manual. This study was able to analytic detection limit for isolating leptospires and optimized of filtration method for isolating *Leptospira* spirochetes from water samples.

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