Comparative analysis of efficiency of pore size and materials of filters for the isolation and purification of leptospires

Prabhusaran Nagarajan[1,*], Natarajaseenivasan Kalimuthusamy[2], Joseph Pushpa Innocent Danialas[3]

[1]Associate Professor, Dept. of Microbiology, Chennai Medical College Hospital & Research Centre, (SRM Group) Tiruchirapalli, India, D.Sc. Research Scholar, The Tamilnadu Dr. M.G.R. Medical University, Chennai, India, [2]Professor, Dept. of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirapalli, India, [3]Professor, Dept. of Microbiology, Karpaga Vinayaga Institute of Medical Sciences, Madurathagam, Kancheepuram, India

***Corresponding Author:**
Email: leptoprabhu@gmail.com

Abstract

Filtration is a major procedure performing in the culture media preparation and avoiding contamination during sub culturing of leptospiral strains. The main objective of this study is to determine the effective filter material and pore size by compare among various filters. The filter materials evaluated in this study are glass fiber (1µm pore diameter), millipore polyvinylidene fluoride (0.22µm and 0.45µm) nitrocellulose (0.22 and 0.45µm pore diameters) and nylon mesh (37µm). The leptospiral cultures in aqueous was filtered by using all filters for the determination of effective filtration of leptospires. All the filtrates were examined by dark field microscopy and the effective filters were selected for further analysis. The filtrate also inoculated in bacterial and fungal culture media to find out any contamination found. Our results suggested that the isolation of 100% leptospires using millipore polyvinylidene fluoride and the optimal pore diameter should be 0.45µm from EMJH broth with well cultured leptospires. Although filtration method can be applicable for isolation and purification of leptospires, it is unclear whether this is a useful method in all situations. This study concluded with the statement "large proportion of leptospires can be filtered by membrane filter at the pore size of 0.45µm".

Keywords: *Leptospira*, filtration technique, pore size, EMJH broth

Introduction

Among the spirochaetal bacterial diseases, leptospirosis is considered as a re-emerging infectious disease, that mainly affecting and infecting humans accidentally when have contact with contaminated environment including soil, rainy mud, sewage and stagnant water etc., where the infected rodents and other animals excrete urine.[1,2] Globally many reports highlighted that leptospirosis is characterized as most common water borne illness and this anthropozoonotic disease is commonly associated with flooding and other water related natural disasters.[1,2,3]

The common pathogenic species is *Leptospira interrogans*; whereas *L. biflexa* is a nonpathogenic environmental leptospirae. Microscopically, the pathogenic and non-pathogenic species of leptospirae cannot be differentiated and are morphologically similar.[4] The pathogenic leptospirae belong to any of more than 230 serovars and are organized into 23 serogroups. Each serovar may be adopted to infect a particular reservoir host that sheds leptospires primarily in urine and almost all the serovars infecting humans.[5] Common pathogenic leptospiral serogroups are Australis, Autumnalis, Canicola, Grippotyphosa, Hebdomadis, Icterohaemorrhagiae, Javanica, Pomona, Pyrogenes, Sejroe etc.

This spirochete survives in fresh water, soil and rainly mud in both tropical and temperate climates. This spirochete is helical and highly motile with 0.2 to 0.3µm in diameter and 3 to 30µm in length.[2,6] In most cases, the leptospiral cultures and isolates are contaminated with environmental and commensal microorganisms respectively. For separation and purification of the isolates and cultures, an efficient technique is required. The direct dark field microscopy of the clinical specimens and cultures may be easily determined but cannot be differentiated among the species and serovars.[6] Eventhough the detection of antibodies by enzyme linked immunosorbant assay (ELISA) for *Leptospira* have been developed as clinical diagnostic tool, it may sometime provides false positive and false negative. Thus, microscopic agglutination test (MAT) is a gold standard serological test thereby it requires expertise and live leptospiral serovars for confirming the infectious state.[7]

Meanwhile the need of culturing leptospires in Ellinghausen, McCullough, Johnson and Harris (EMJH) semisolid medium and observing the clinical samples directly under wet mount dark field microscopy supporting the diagnosis.[8] Thus the requirement of maintenance of contamination free leptospiral serovars in the diagnostic and research laboratory is mandatory.[9] The primary challenges for performing the culturing of *Leptospira* include isolation, concentration and quantification. The hypothesis that described in this study is to maintain leptospires in the laboratory effectively using filtration techniques either by nested approach – removal of debris and sediments with coarse filter followed by finer or with a simple membrane filter approach with appropriate material and pore size.

Based on the hypothesis, the main objective of this study is to evaluate the effective filtration material and appropriate pore size to concentrate *Leptospira* serovars from samples or cultures.

Materials and Method

Routine leptospiral culturing: The suspension of pure culture of *L. interrogans* serovar *Grippotyphosa* was prepared using liquid EMJH culture media. The leptospiral cultures were obtained from National Leptospirosis Research Centre, Regional Medical Research Centre (RMRC), Port Blair, Andaman and Nicobar islands, India. The liquid EMJH medium was prepared using distilled and deionized water without adding 0.2% agar and 5-fluorouracil (5-FU). A control culture in EMJH semisolid medium with 0.2% agar and 200µl/ml 5-FU was done.

Further, the inoculated EMJH culture media were incubated in dark condition at room temperature for 2 to 3 weeks. Periodic observation was done to check the formation of Dinger's ring (bacterial cell concentrate just 5mm below the surface) in semisolid control medium and cloudy, well mixed turbid are confirmed the leptospiral growth present in the culture medium.

Filtration trials

Pre-requisites: Approximately 25ml of well grown leptospiral cultures were used in this study. Before performing filtration, all the test cultures were observed under dark filed microscopy for determining the contamination status. The active leptospiral cells (75%) with other microbial contamination found in the culture media is the primary eligibility criteria for selection to perform filtration.

Filters used in this study: The filter materials evaluated in this study are glass fiber (1µm pore diameter), millipore polyvinylidene fluoride (0.22µm and 0.45µm) nitrocellulose (0.22 and 0.45µm pore diameters) and nylon mesh (37µm). Except nitrocellulose mixed with esters of cellulose membrane, all other filters included in this study are Millipore (Table 1).

Table 1: Types of filters used in this study

Filter	Type of membrane	Material	Pore diameter
Nitrocellulose mixed esters of cellulose membrane	Hydrophobic for water	Nitrocellulose	0.22µm
Millipore durapore membrane filter	Hydrophobic	Polyvinylidene fluoride	0.45µm
	Hydrophobic	Nitrocellulose	0.45µm
	Hydrophobic nylon mesh	Nylon mesh	37µm
Millipore durapore	Hydrophobic for liquid	Polyvinylidene fluoride	0.22µm
Millipore isopore	Hydrophobic for biological liquid	Polycarbonate	0.45µm
Millipore	Hydrophobic for coarse debris removal	Glass fiber	1µm

Filtration: Filtration trials was done using 25ml of liquid well grown *L. interrogans* serovar *Grippotyphosa*, with several filters mounted in a filter holder with a vacuum of 5 inches of Hg to draw the sample in the bottom flask (Fig. 1). The entire filtration unit was autoclaved before initiating the procedure. Initially all the filters are analyzed for its efficiency by filtering the sterile phosphate buffered saline (PBS) solution.

Fig. 1: Membrane filter unit (Duran)

Every time, the entire filtration unit was washed and sterilized for using various filters. After filtration, the filtrate was collected in sterile boiling test tubes for further evaluation and finds the efficient filter materials and pore size. The observation of contamination after

filtration was assessed by direct dark field microscopy and growth in bacterial and fungal culture media.

Results

The cultures used for this study *L. interrogans* serovar *Grippotyphosa* showed approximately 30% contamination by other microorganism by observed under dark field microscopy with minimum of 10 fields. The filtration results suggested important aspects of using filters to purify leptospires from culture media. Three major observations were incorporated in this study:

1. For obtaining the entire volume of cultures as filtrate
2. Suggesting the filter material which not affecting the recovery rate
3. Pore size of the filter material where the flow recovery rates vary

The effect of recovery rate of the filtrate was analyzed among various filters used with different pore size. The filtration results are impregnated in Fig. 2. These results suggested that several important aspects of using various filters to isolate, separate and purify the leptospires from liquid cultures.

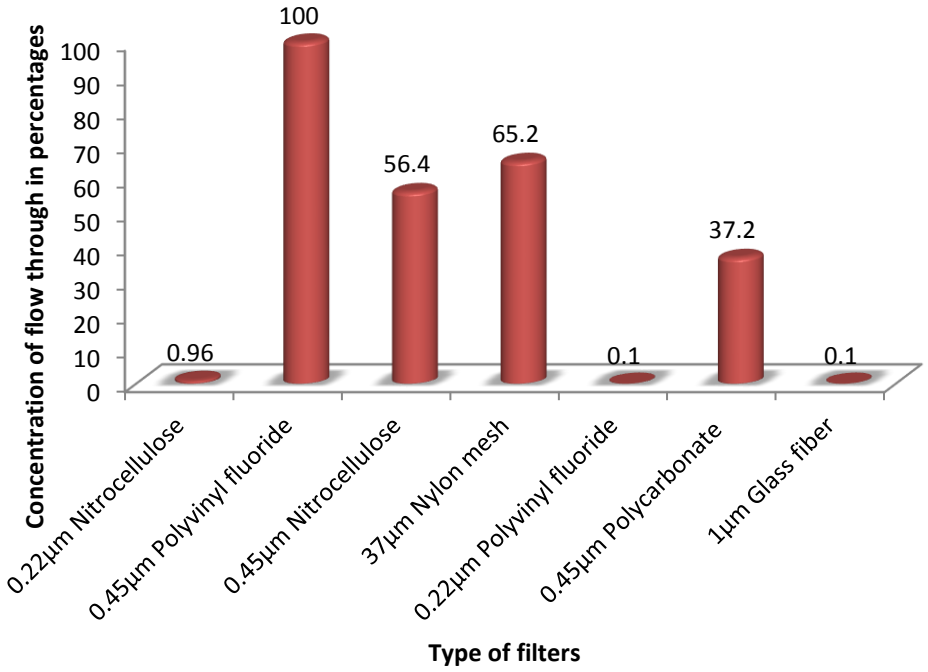

Fig. 2: Average percentage of leptospires in cultures that were found in filtrate with 95% confidence intervals displayed

The flow through rates varied from filter to filter, where the flow rate was very high in glass fibers and nylon mesh filters. The observation with glass fiber filters may be biased because glass fiber filters are closely resemblance like leptospires, which likely led to false positive microscopy. The filtrate obtained in all filters was assessed for its count of leptospires and artefacts or other microbial contaminants. On comparing with other filters 0.45µm pore sized and polyvinyl fluoride filter material are highly effective in filtering leptospires alone without any contamination. Whereas other filters tested in this study with varied pore size showed differences in percentage of rate of filtration and leptospires.

The dark field microscopy revealed the variations among the filters used for the contamination state of the filtrate. The percentage of observation of filtrate about the presence and absence of contamination using EMJH liquid cultured leptospires was also noted. On comparing with seven filters tested in this study highlighted the polyvinyl fluoride filter at the pore size of 0.45µm showed filtrate free from contamination (only leptospires present). The Table 2 highlighted the recovery time of the culture filtrate and contamination state among the filters used in this study.

Table 2: Recovery time of leptospiral culture filtrate and contamination rate

Filter type	Pore size (in µm)	Recovery time (in minutes) of 100ml of leptospiral broth culture verses Contamination rate (in %)			
		Without suctioning		With suctioning	
		Recovery time	Contamination rate*	Recovery time	Contamination rate*
Nitrocellulose mixed esters of cellulose membrane	0.22	25	0	22	20
Polyvinylidene fluoride Millipore durapore membrane filter	0.45	21	0	17	0
Nitrocellulose Millipore durapore membrane filter	0.45	23	0	20	20
Nylon mesh Millipore durapore membrane filter	37	8	100	5	100
Polyvinylidene fluoride Millipore durapore	0.22	29	0	26	20
Polycarbonate Millipore isopore	0.45	23	0	19	30
Glass fiber Millipore	1	15	80	12	100

(*Contamination rate is determined by observing the bacterial cells other than leptospires in 10 fields under dark filed microscopy where every field depicted 10 percentages)

Further the filtrates were plated in the Nutrient and Saboroud's Dextrose agar for determining bacterial and fungal growth respectively. Results revealed that the filtrate obtained through nylon mesh Millipore durapore membrane filter and glass finer Millipore showed growth of bacterial and fungal contaminations. The growth of contamination also found in less CFUs in suctioned filtrates of nitrocellulose mixed esters of cellulose membrane, nitrocellulose Millipore durapore membrane, polyvinylidene fluoride Millipore durapore (0.45µm) and polycarbonate Millipore isopore. No growth was observed when using polyvinylidene fluoride Millipore durapore (0.22µm) membrane filters.

Discussion

The major characteristics of using the membrane filters are for the retention of leptospires without contamination. In general, the culture media that are using for the cultivation of leptospires (EMJH) both semisolid and liquid are more sensitive of getting contamination due to the presence of vitamins and peptides. If any contamination found, the cultures should be filtered for getting the contamination free leptospires for maintenance, diagnostics and other purposes.[10,11] The results of trials for the filtration indicated that a substantial proportion of leptospires in culture of both semisolid and broth can be separated by using 0.45µm pore diameter nitrocellulose filter.

Filters that are used for the purification of the leptospires, whose pore size ranged from 0.22 to 0.45µm that are challenged with leptospiral strains. The results of this study indicated important findings including affirming visual quantification using the microscopic chamber work using dark field microscopy for leptospires in pure solution which is already done preliminarily.[8]

Future studies of leptospires may benefit by using this method, pore sizes do affect filter performances with leptospiral suspensions that may have been low due to a pattern of similar performance levels with similar pore sizes and although literature and previous work has demonstrated selectivity is influenced by filter materials, no pattern was observed between materials of similar sizes. Recovery rate also determined in the study with different filter make and pore sizes 0.22µm - 0.45µm which was already determined in the study using deionied water supply.[11]

Conclusion

Thus, this study concluded that the leptospires are difficult to filter due to its long and spiral and may tangle or damage easily on rough filter surfaces. Also this study highlighted the membrane types including nitrocellulose, glass and nylon are negatively charged, and are likely to hold onto bacterial surfaces.

References

1. Knibbs LD. Occupational hazards to the health of professional gardeners. Int J Environ Health Res 2014;24:580-9.
2. Sequi RJD et al. The identification of *Leptospira* strains of different origins. Rev Cubana Med Trop 1992;44:33-6.
3. Stoddard RA et al. Viability of *Leptospira* isolates form a human outbreak in Thailand in various water types, pH and temperature conditions. Am J Trop Med Hyg 2014;9:1020-2.
4. Wynwood SJ et al. Leptospirosis from water sources. Pathog Glob Health 2014;108:334-8.
5. Rubin SJ et al. Isolation of *Leptospira biflexa* from commercially prepared deionized water labelled sterile for tissue culture. J Clin Microbiol 1980;12:121-3.
6. Kaboosi H et al. Efficiency of filtration technique for isolation of leptospires from surface waters: role of different membranes with different pore size and materials. Afr J Microb Res 2010;4:671-6.

7. Prabhu N et al. Retrospective analysis of leptospirosis among children – a clinico microbiological and therapeutic aspects for the cases. Clin Rev Opin 2010;2:31-4.
8. Prabhusaran N et al. Appearance of black pigments in the EMJH semisolid medium – cultural confusion. Eur J Pharm Med Res 2015;2:864-72.
9. Wilson R, Fujioka R. Development of a method to selectively isolate pathogenic *Leptospira* from environmental samples. Water Sci Tech 1995;31:275-82.
10. Turner LH. Leptospirosis. 3. Maintenance, isolation and demonstration of leptospires. Trans R Soc Trop Med Hyg 1970;64:623-46.
11. Brendle JJ, Alexander AD. Contamination of bacteriological medial by *Leptospira biflexa*. Appl Microbiol 1974;28:505-6.