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Evaluation of decontamination methods for obtaining pure cultures of leptospires from mixed samples with *Spirillum* spp.

Israel Barbosa Guedes[1], Juliana Fernandes de Paula Castro[2], Gisele Oliveira de Souza[3], Paulo Vitor Takano[4], Matheus Burilli Cavalini[2], Juliana Aizawa[5], Marcos Bryan Heinemann

1. Médico Veterinário. Doutorando no Programa de Epidemiologia Experimental Aplicada às Zoonoses.Laboratório de Zoonoses Bacterianas, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil.
israel.guedes@usp.br *http://lattes.cnpq.br/8896537060491073* *http://orcid.org/0000-0002-9843-1452*
2. Médico(a) Veterinário(a). Mestrando(a) no Programa de Epidemiologia Experimental Aplicada às Zoonoses.Laboratório de Zoonoses Bacterianas, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil.
julianafpcastro@yahoo.com.br *http://lattes.cnpq.br/0369212657146523* *http://orcid.org/0000-0002-1468-658X*
matheus.cavalini@usp.br *http://lattes.cnpq.br/6662746537440211* *http://orcid.org/0000-0003-0550-8615*
3. Nutricionista. Doutora em Ciência Animal. Laboratório de Zoonoses Bacterianas, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil.
ginutri2011@gmail.com *http://lattes.cnpq.br/5593865933793746* *http://orcid.org/0000-0002-4604-6034*
4. Médico Veterinário. Laboratório de Zoonoses Bacterianas, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil.
paulo.takano@usp.br *http://lattes.cnpq.br/1304138436581586*
5. Médica Veterinária. Doutoranda em Laboratory of Microbiology, Parasitology and Hygiene, Department of Pharmaceutical Sciences, University of Antwerp, Wilrijk, Belgium.
juliana.aizawaportodeabreu@uantwerpen.be *http://lattes.cnpq.br/9253164527932978* *http://orcid.org/0000-0003-1778-9469*
6. Médico Veterinário. Professor Doutor. Laboratório de Zoonoses Bacterianas, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil.
marcosbryan@usp.br *http://lattes.cnpq.br/2008760316007525* *http://orcid.org/0000-0001-6444-9788*

ABSTRACT

It is challenging to obtain pure cultures of *Leptospira* from environmental samples, mainly due to the presence of accompanying microbiota that can contaminate cultures, highlighting bacteria of the genus *Spirillum* that have characteristics similar to leptospires. Thus, cannot be eliminated by the most common decontamination techniques during leptospires isolation process. The purpose of this study was to evaluate five decontamination methods to obtain pure cultures of *Leptospira* from mixed samples with *Spirillum* spp. We collected ten samples from untreated domestic sewage dumped of to streets in Macapá city – Brazilian Amazon, and submitted to bacteriological culture for *Leptospira* isolation. The presence of leptospires was confirmed by visualization under dark-field microscopy and by 16S PCR. In the samples in which *Spirillum* was observed, five methods of decontamination were tested: 1 – Membrane filtration (0.20 µm and 0.22 µm); 2- Culture in selective medium for *Leptospira* spp.; 3- Centrifugation; 4- Serial dilution and 5- Plating on solid EMJH medium (Ellinghausen, McCullough, Johnson and Harris). Out of all 10 samples, five were positive for leptospires and in three there was the simultaneous detection of leptospires and *Spirillum*. Plating on solid EMJH medium (5) was the most efficient method, decontaminating all three mixed cultures. Only one culture was decontaminated by the technique of culture in selective medium for *Leptospira* spp. (2), and the other methods were ineffective. In conclusion, plating on solid EMJH medium method can be used to obtain pure cultures of *Leptospira* spp. from environmental samples containing *Spirillum* spp.

Keywords: *Leptospira*; decontamination; mixed cultures; *Spirillum*.

Avaliação de métodos de descontaminação para obtenção de culturas puras de leptospiras a partir de amostras mistas com *Spirillum* spp.

RESUMO

É um desafio obter culturas puras de *Leptospira* a partir de amostras ambientais, principalmente devido à presença de microbiota acompanhante que pode contaminar culturas, destacando bactérias do gênero *Spirillum* que possuem características semelhantes às leptospiras. Assim, não pode ser eliminado pelas técnicas mais comuns de descontaminação durante o processo de isolamento das leptospiras. O objetivo deste estudo foi avaliar cinco métodos de descontaminação para obter culturas puras de *Leptospira* a partir de amostras mistas com *Spirillum* spp. Foram coletadas dez amostras de esgoto doméstico não tratado descartado nas ruas da cidade de Macapá - Amazônia Brasileira, e submetidas à cultura bacteriológica para isolamento de *Leptospira*. A presença de leptospiras foi confirmada por visualização em microscopia de campo escuro e por 16S PCR. Nas amostras em que *Spirillum* foi observado, foram testados cinco métodos de descontaminação: 1 - Filtragem por membrana (0,20 µm e 0,22 µm); 2- Cultura em meio seletivo para *Leptospira* spp.; 3- Centrifugação; 4- Diluição em série e 5- Plaqueamento em meio sólido EMJH. Das 10 amostras, cinco foram positivas para leptospiras e em três houve a detecção simultânea de leptospiras e *Spirillum*. Plaqueamento em meio sólido EMJH (5) foi o método mais eficiente, descontaminando as três culturas mistas. Apenas uma cultura foi descontaminada pela técnica de cultura em meio seletivo para *Leptospira* spp. (2), e os outros métodos foram ineficazes. Em conclusão, o método de plaqueamento em meio sólido EMJH pode ser usado para obter culturas puras de *Leptospira* spp. de amostras ambientais contendo *Spirillum* spp.

Palavras-chave: *Leptospira*, descontaminação, culturas mistas, *Spirillum*.

Introduction

Leptospirosis is a zoonosis of worldwide distribution caused by pathogenic bacteria of the genus *Leptospira* (LEVETT, 2001). Although the disease is considered cosmopolitan, the incidence is increasing in tropical and subtropical regions, especially in developing countries in which this increase is generally associated with precarious sanitary conditions (SILVA et al., 2016; SANTOS et al., 2017).

Water plays an important role in leptospirosis outbreaks that can occur in cases of environmental disasters, aquatic sports and occupational exposure (WYNWOOD et al., 2014). When water is contaminated by the urine of infected animals (BHARTI et al., 2003) the bacteria can disseminate and remain viable for long periods in the environment (LEVETT, 2001). Leptospires (saprophytic and pathogenic) have been isolated and studied from environmental samples such as water and soil (MENY et al., 2017; MASUZAWA et al., 2018; SCIALFA et al., 2018), and in urban environments, where effluents and sewage drains with presence of pathogenic strains may represent important sources of infection for humans (BENACER et al., 2013; MOHD ALI et al., 2018).

Obtaining pure clonal cultures of *Leptospira* is essential for genetic studies involving virulence and phylogenetic analysis of

these microorganisms (THIBEAUX et al., 2018a, 2018b). However, one of the major difficulties in isolating leptospires from environmental samples is the presence of accompanying microbiota that contaminates the culture media (WYNWOOD et al., 2014). *Spirillum*, naturally found in these environmental samples, especially water, can be among the contaminants from these sources (HYLEMON et al., 1973). The presence of this microorganism is also reported in clinical samples, resulting in mixed cultures of leptospires and *Spirillum* (RAGUNATH et al., 2018).

Bacteria of the genus *Spirillum* are flexible, mobile and resemble spirals when observed under dark-field microscopy. They have catalase and oxidase activity, use peptone, sodium pyruvate, succinic acid, chlorides and sulfates as metabolism sources, which are incorporated into bacteriological media for their cultivation and maintenance (HYLEMON et al., 1973). These characteristics are very similar to the genus *Leptospira* (FAINE et al., 1999; LEVETT, 2001).

During the process of isolating *Leptospira*, some traditional techniques are employed to reduce or inhibit contaminants in samples or cultures, the most common are membrane filtration (PRAMEELA et al., 2015; CHÁVEZ et al., 2018; SCIALFA et al., 2018) and seeding in selective media with inhibitors, generally 5-fluorouracil (AZALI et al., 2016; LALL et al., 2016; MOHD ALI et al., 2018) or 5-fluorouracil associated with a mixture of antimicrobials (CHAKRABORTY et al., 2011; SAITO et al., 2013; TANTENGCO; GLORIANI, 2017). Other techniques are also described for isolation or purification in clinical and environmental samples such as centrifugation (ESCÓCIO et al., 2010), serial dilution (BROD et al., 2005) and plating on solid medium (THIBEAUX et al., 2018a).

Up to now there are few environmental studies involving leptospires detected in large urban centers in the Brazilian Amazon, as well as a method to enable them by decontaminating mixed samples with microorganisms such as *Spirillum* spp. Thus, the aim of this study was to evaluate five decontamination methods to obtain pure cultures of *Leptospira* from mixed cultures with *Spirillum* spp.

Materials and Methods

The city of Macapá (state of Amapá) was the study area in the present work. It is situated in the extreme north of country and it is one of the Brazilian Amazon capitals in which the Amazon River flows through (**Figure 1.**). Crossed by the equator line, the city has humid megathermal equatorial climate characterized by high annual rainfall (2,000 mm to 2,500 mm), with a short dry period of 3 to 4 months and rainy period from December to June. The minimum temperature is around 23 °C and the maximum temperature is around 33 °C, with relative air humidity above 80% (PINEDO-VÁSQUES; RABELO, 1999). Extreme weather events such as heavy rain and floods are becoming more common in this city (TAVARES, 2014).

Figure 1. Geographical location of the City of Macapá in Brazil. / **Figura 1.** Localização Geográfica da Cidade de Macapá no Brasil.

For isolation of *Leptospira* we collected approximately 500 mL of untreated domestic sewage from the streets of Macapá in different districts, totaling 10 different samples. Sampling was by convenience and the collection point was preferably chosen where rubbish, debris, animal carcasses and/or rodents were observed. The samples were left to settle for 20 to 30 min, and then 5 mL of the supernatant was filtered (0.22 µm) to reduce contamination and seeded in liquid EMJH medium (Ellighausen, McCullough, Johnson and Harris). The tubes were incubated in a bacteriological incubator at approximately 29 °C for a period of six weeks.

When the turbidity of the medium was found, the confirmation of the presence of leptospires was carried out by visualization under dark-field microscopy and polymerase chain reaction (PCR). For the performance of this last technique, DNA extraction and purification from the cultures was performed using a PureLink® Genomic DNA Mini Kit (Invitrogen) following the manufacturer's protocol. The PCR amplification of *Leptospira* spp. was performed with the primers Lep1 and Lep2, which amplify a 330-bp region of the 16S rRNA gene (*rrs*) using GoTaq™ Green Master Mix (Promega, Brazil). The reaction occurred under the following conditions: an initial denaturation of 5 min at 94°C, 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and a final primer extension of 72°C for 5 min (MÉRIEN et al. 1992).

Cultures with leptospires and helical shape cells with rapid motility, characteristic of the genus *Spirillum* (HYLEMON et al., 1973), were submitted to the five methods commonly used for decontamination and obtaining pure samples of *Leptospira*. Due to rapid bacterial growth of the *Spirillum* genus, in 24-48 h incubation, it was possible to observe growth macroscopically (turbidity of the culture medium) as well as microscopically (dark-field microscopy), this parameter was used to evaluate the decontamination methods.

Membrane Filtration (0,20 µm and 0,22 µm)

The cultures were filtered using 0.20 µm and 0.22 µm porosity membranes, and then 1 mL of the filtrates was seeded in liquid EMJH medium. The medium was incubated in a bacteriological incubator at approximately 29 °C for 24 h and then examined under dark-field microscopy for detection of *Spirillum*. In the presence of *Spirillum*, cultures were filtered again at 24, 48 and 72 h incubation, re-seeded in EMJH and Fletcher media, and incubated in a bacteriological incubator at 29 °C for a maximum period of six weeks. They were monitored daily for turbidity of the media, and when there was turbidity in the medium, the evaluation was carried out using dark-field microscopy. The efficiency of the technique was determined by the single observation of leptospires and total absence of *Spirillum.*

Culture in selective medium for Leptospira spp.

Initially, 0.5 mL of mixed cultures were seeded in EMJH medium added with a selective supplement for *Leptospira* (nalidixic acid 50 mg / L, cycloheximide 100 mg / L, chloramphenicol 5 mg / L and neomycin 5 mg / L) for decontamination. The media was incubated in a bacteriological incubator at approximately 29 °C for a period of 12, 24, 48, 72 and 96 h. After this period, 0.5 mL of media of each incubation period was seeded into new EMJH and Fletcher media without the selective supplement. The media was then incubated in a bacteriological incubator at 29 °C for a maximum period of six weeks and monitored daily for turbidity, and when there was turbidity in the medium, the evaluation was carried out using dark-field microscopy. The efficiency of the technique was determined by the single observation of leptospires and total absence of *Spirillum.*

Centrifugation

The cultures were initially centrifuged at 5000 g for 15 min at room temperature and 1 mL of the supernatant was seeded in EMJH and Fletcher. The media was incubated in a bacteriological incubator at 29 °C for a maximum period of six weeks and monitored daily for turbidity, and when there was turbidity in the medium, the evaluation was carried out using dark-field microscopy. The efficiency of the technique was determined by the single observation of leptospires and total absence of *Spirillum.*

Serial dilution

The cultures were diluted 1:9 (10^{-1}) in Sörensen saline (pH 7.4) and then further diluted in four 10-fold serial dilutions (10^{-2} to 10^{-5}). Subsequently, 1 mL of the supernatant of each dilution was seeded in EMJH and Fletcher media. The media was incubated in a bacteriological incubator at 29 °C for a maximum period of six weeks and monitored daily as turbidity of the media, and when there was turbidity in the medium, the evaluation was carried out using dark-field microscopy. The efficiency of the technique was determined by the single observation of leptospires and total absence of *Spirillum.*

Plating on solid EMJH medium

The cultures were diluted 1:9 in Sörensen saline (pH 7.4) then 0.1 mL (100 µL) of this dilution was plated into petri dishes containing the solid EMJH medium (agar 12 g/L). Surface plating was performed with the aid of a drigalski spatula. The plates were incubated in a bacteriological incubator at 29 °C for a period of two weeks and were examined daily for the presence of small and bright isolated colonies. Verification was performed by dark-field microscopy. After confirmation of the colonies as *Leptospira*, they were seeded in liquid EMJH medium to obtain a clonal culture per sample.

Ethic committee

This work was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science (Universidade de São Paulo) – CEUA/FMVZ nº 9534211118.

Results and Discussion

After four days incubation period, leptospires were detected in five of the 10 samples (50%). This reveals the bacteria are in the domestic sewage and may present a risk to population health. The intense human population growth in Macapá favors irregular occupation, increasing urban solid waste generation in the city (CARDOSO et al., 2015), which faces difficulties in basic sanitation, with only 26.8% of adequate sanitary sewage (IBGE, 2017). This directly influences the occurrence of infectious diseases, such as leptospirosis, reported between the years of 2011 and 2015 with an average of 53 cases per year in Macapá (REZENDE et al., 2016). The pathogenicity of the bacteria was not evaluated in this study, and leptospires isolated could be saprophytes, as the growth time was fast (FAINE et al., 1999). In three of the five positive cultures for *Leptospira* there was also the detection of *Spirillum* spp.

When membrane filtration was applied (1), *Spirillum* remained present in all cultures filtered both by 0.20 µm membrane and 0.22 µm membrane, regardless of incubation time. These same results were reported by Anderson and Heffernan (1965) that isolated *Spirillum* from seawater after filtration using membranes of 0.45 µm and 0.22 µm. The spiral form morphology of *Spirillum*, as the one of leptospires, may facilitate passage through the filter membranes (WANG et al., 2008).

This method, widely used to obtain pure cultures of *Leptospira,* is not restricted to environmental samples and contaminated cultures. It is also used to reduce contamination and perform isolation of leptospires from clinical samples such as urine (SILVA et al., 2015; GUEDES et al., 2019). The technique is simple and eliminates many environmental contaminants present in samples (STERN et al., 2010) that are not able to cross the membranes pores. Moreover, the membrane pore size is ideally inferior to 0.45 µm, thus allowing the passage of leptospires (KABOOSI et al., 2010).

When the technique of culture in selective medium for *Leptospira* spp. was used (2), in 12, 24 and 48 h of exposure to selective medium, there was no elimination of *Spirillum* in any of the 3 mixed cultures. Nevertheless, after 72 h of exposure, in one of the cultures *Spirillum* was eliminated. Using the same method, it was observed that in cultures submitted to selective medium for 96 h it was not possible to recover viable leptospires. Four inhibitory agents to suppress accompanying microbiota and allow *Leptospira* growth were applied, including chloramphenicol (5 mg / L), an antibiotic that has been described as able to completely inhibit *Spirillum* cell multiplication (MCELROY et al., 1967; AJAYI et al., 2017).

Another cocktail of selective agents known as STAFF (Sulfamethoxazole, Trimethoprim, Amphotericin B, Fosfomycin and 5-Fluorouracil) has been successfully used in sample decontamination for *Leptospira* isolation (SAITO et al., 2013; LOUREIRO et al., 2016; ALBUQUERQUE et al., 2017). This cocktail was proposed by Chakraborty et al. (2011) after testing five drugs against 16 possible microbial contaminants during the process of *Leptospira* isolation and used, in the same study, in environmental samples of water and soil. However, the efficiency of this cocktail was not evaluated for bacteria of the Spirillum genus. In the present study 5-fluorouracil was not used, considering that *Spirillum* can grow in *Leptospira* culture media containing 5-fluorouracil (CODY, 1968).

The centrifugation technique (3) used in this work did not eliminate *Spirillum* from the contaminated cultures even at rotation of 5000 x g for 15 min (ESCÓCIO et al., 2010). This goes against Kumar et al. (1974) description that *Spirillum* cells were obtained in the pellet of a centrifuged culture at 1000 x g for 5 min. The same was observed in the serial dilution technique (4), even using 10^{-1} to 10^{-5} dilutions (BROD et al., 2005) *Spirillum* continued to be observed in all dilutions.

The method of plating in solid EMJH medium (5) was the most efficient. It was possible to obtain pure cultures of *Leptospira* from all three samples. A disadvantage of this method was the long incubation time, of approximately seven days, until visualization of suspected leptospires colonies in the plates. During this period, swarming motility of *Spirillum* in plate's surfaces was observed and some colonies of leptospires were covered, making it difficult to obtain isolated colonies (Figure 2). Nevertheless, the use of solid media for leptospires isolation has the major benefit of successfully attaining isolated colonies from the different species that may be in liquid media, mainly from environmental samples (CHAKRABORTY et al., 2011; THIBEAUX et al., 2018b).Moreover, it can be used for antimicrobial susceptibility testing (WUTHIEKANUN et al., 2013).

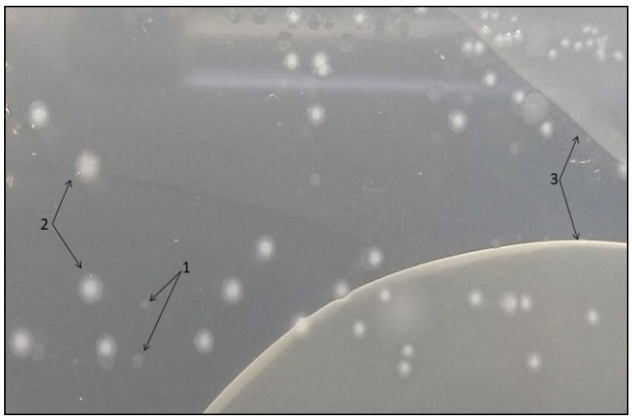

Figure 2. Mixed culture plated on solid EMJH medium, eight days incubation. (1) Isolated colonies of *Leptospira*. (2) *Spirillum* colonies. (3) Swarm behavior exhibited by *Spirillum.* / **Figura 2.** Cultura mista plaqueada no meio EMJH sólido, oito dias de incubação. (1) Colônias isoladas de *Leptospira*. (2) Colônias de *Spirillum*. (3) Comportamento de enxame exibido por *Spirillum.*

Conclusion

Leptospira spp. was isolated from untreated domestic sewage dumped of to streets in Macapá city, a situation still little explored in the region. The elimination of *Spirillum* from mixed cultures with *Leptospira* using decontamination methods for the isolation or purification of leptospires is little discussed in literature and is not easy to perform in practice. Therefore, we described that plating on solid EMJH medium method (5) can be useful to obtain pure cultures of *Leptospira* spp. from environmental samples containing *Spirillum* spp. as accompanying microbiota.

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