Isolation of *Leptospira* spp from dogs, bovine and swine naturally infected

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**ABSTRACT**

Leptospira isolation allows definitive diagnosis of the infection. Contamination by microorganisms is one of the inconveniences of the culture. The objective of this study was to describe the isolation of leptospira from dogs, bovine and swine naturally infected. Urine samples from 14 dogs and three bovines, and kidney, liver, ovary, and uterus body samples from 36 slaughtered sows with unknown health history, were used. The urine and organ samples were cultured in culture medium. Modified Ellinghausen-McCullough-Johnson-Harris medium (EMJH) culture medium was used with addition of 5-fluorouracil, chloramphenicol, vancomycin, nalidixic acid and neomycin. Incubation was performed at 28°C for 24 hours, followed by subculture in modified EMJH without antibiotics. The cultures were assessed weekly for up to eight weeks for the dog and swine samples and for up to 16 weeks for the bovine samples. With this methodology, *Leptospira* spp could be isolated from 11 dogs, two bovines and liver fragments from two sows.

**Key words:** Leptospirosis, diagnostic, culture, cattle, dog, swine.

**INTRODUCTION**

Leptospirosis is a worldwide zoonosis and is considered as a re-emergent disease in some countries (BOLIN, 1996). Besides economic losses caused by this bacterium to animal production, its zoonotic character makes it an important public health problem (MYERS, 1985; FAINE et al., 1999). The clinical diagnosis of leptospirosis is inconclusive due to the different clinical signs that can be attributed to other pathogenic agents (VASCONCELLOS, 1979). The microscopic seroaglutination test (SAM) is considered the reference test among the several serological methods for leptospirosis diagnosis (SANTA ROSA, 1970; FAINE et al., 1999). However isolation and identification of the microorganism allow definitive diagnosis and
provides epidemiological and prophylactic studies of this disease. Isolation of leptospirosis from tissues and fluids both in animals of economic interest and pets has been simplified with the availability of culture mediums, antibiotics and improvement of sample handling and dilution techniques (SCHÖNBERG, 1981; THIERMANN, 1984; ADLER et al., 1986).

ADLER et al. (1986) stated that the main problem in culturing leptospirosis is contamination with other microorganisms, especially when attempting to culture from non-sterile sources such as urine and fetal tissues. The inclusion of antibiotics to inhibit the contamination in the culture medium has been recommended, but inhibitory substances had a detrimental effect on the multiplication phase of the leptospirosas (SCHÖNBERG, 1981). THIERMANN (1984) stated that the most important factors for isolation of leptospirosa are aseptically collected material, quick processing, culture medium suitability and selective antibiotics.

The objective of this study was to describe the isolation of leptospirosa from dogs, cattle and swine with natural infection.

MATERIAL AND METHODS

Isolation attempts were carried out between April 2000 and November 2001 in the Leptospirosis Laboratory at the Department of Preventive Veterinary Medicine (DMVP) of Londrina State University (UEL) - Brazil, using urine samples from 14 dogs and three bovines classified as positive for leptospirosis in the direct examination under dark field microscopy, all naturally infected. Dog urine samples were collected by cystocentesis, and bovine urine samples by directed bladder puncture on the slaughterhouse eviscerating table. Kidney, liver, uterus body and ovary fragments were also used, without macroscopic lesions, from 36 slaughtered sows with unknown health history. These organ fragments were collected on the slaughterhouse eviscerating table.

The Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (Difco-USA) was used for leptospirosa isolation modified with the addition of 10% rabbit serum enriched with calcium chloride and magnesium chloride (ALVES, 1995). This culture medium was prepared in two formulations, one without antibiotics and the other with the addition of 5-fluorouracil (400mg/L; Sigma-USA) (HEER et al., 1982), chloramphenicol (5mg/L; Sigma-USA), nalidixic acid (50mg/L; Inlab-BR), neomycin (10mg/L; Sigma-USA) and vancomycin (10mg/L; Acros-USA) (SCHÖNBERG, 1981).

The dog and bovine urine samples were cultured in duplicate in modified EMJH medium added with antibiotics and incubated at 28°C for 24h, followed by subculture in duplicate in the same culture medium but without antibiotics. Inoculates used for isolation and subcultures corresponded to 10% of the volume of the culture medium cultured. The kidney, liver, body and ovary fragments obtained from the sows were triturated and diluted at 1:10 (p/v) in sterile phosphate buffer solutions (PBS) pH 7.4, about two hours after collection. After this dilution, the same cultivation methodology used for the dog and bovine urine samples was carried out.

The cultures were assessed weekly for up to eight weeks for the dog and swine samples (THIERMANN, 1980) and up to 16 weeks for the bovine samples (ELLIS et al., 1982). When the presence of leptospirosa was observed, a subculture in duplicate was carried out in modified EMJH medium without antibiotics. The tubes that presented contamination in the weekly assessment had a new subculture in modified EMJH with antibiotics and after 24h incubation at 28°C they were returned to the culture medium without antibiotics. Leptospirosas isolated from dogs and swine were weekly subcultured in modified EMJH medium without antibiotics. For maintenance of leptospirosas isolated from bovine the Tween 80/40/LH culture medium (ELLIS et al. 1985) was also used.

RESULTS

Leptospirosa was isolated from 11 of the 14 samples cultured of dog urine and two from the three bovine samples. Leptospirosa could only be isolated from the liver of two animals of the total of fragments cultured from 36 sows (Table 1). The growth of leptospirosa was observed during one week after culture in some dog samples, after two weeks in the sow samples and up to 12 weeks in those from bovines.

The Tween 80/40/LH medium used for the maintenance of leptospirosas isolated from bovine samples allowed their recovery when they became unviable in the modified EMJH medium without antibiotics. The growth of contaminant microorganisms only occurred in tubes that were cultured with eight swine samples, being five from liver and three from uterus. It was not possible to isolate leptospirosa from the subcultures that used these contaminated tubes.

DISCUSSION

Leptospirosis can be diagnosed by several laboratory methods, of which the serological methods are the most used, but the isolation allows the definite diagnosis of individual infections and also provides epidemiological and prophylactic studies of regional and national interest.

In Brazil, there are few researches related to isolation of leptospires from naturally infected animal, as the majority of data is limited to serology. ADLER et al. (1986) and BOLIN et al. (1989) recognized the difficulty in isolating leptospires, despite the presence of leptospires in samples. MOREIRA (1994), using 420 urine samples from bovines naturally infected, corresponding to 2100 cultured tubes, obtained two leptospiras isolation. The large number of isolations obtained from dogs and bovine is expressive when the total of cultured urine samples was compared with that of the isolation samples. The results obtained with the sow organ fragments are also expressive, as the organs did not present macroscopic lesions and were collected from randomly chosen animals.

The isolation techniques are fastidious, require skill and experience and it is difficult to obtain positive results in natural infection samples for several reasons, including, the need for a long incubation period, the presence of contaminating microorganisms and the interval between obtaining and processing the samples (SANTA ROSA, 1970; THIERMANN, 1984).

SCHÖNBERG (1981) states that the contaminant microorganisms make the isolation difficult because they multiply quickly and consequently impede leptospiroa growth. FAINE (1982) and ADLER et al. (1986) stated that undesirable microorganism growth could be inhibited by the addition of antibiotics to the culture medium without modifying the leptospiroa cell multiplication. The Leptospirosis Laboratory at DMVP-UEL used only four of the seven antibiotics tested by SCHÖNBERG (1981) plus 5-fluorouracil at the concentration recommended by HEER et al. (1982). These antibiotics added to the culture medium did not inhibit the contaminant growth in eight swine samples, but in other samples, the contamination did not occur. Probably, the antibiotics controlled the possible contaminant microorganisms, resulting as a fundamental point in this study for the 15 leptospiroa isolations from naturally infected animals. SCHÖNBERG (1981) observed a detrimental effect of antibiotics on leptospiroa multiplication after two-day incubations. FAINE et al. (1999) stated that subcultures should be made within 48h to minimize the inhibitory effect of the selective agents on leptospires. All the cultured urine and organ fragment samples were kept in culture medium with antibiotics for up to 24h in this study to prevent the detrimental effect observed by SCHÖNBERG (1981).

The short time between obtaining and processing the samples was probably also important for contaminating microorganism control but mainly for the leptospiroa viability. THIERMANN (1980) and FAINE (1982) reported that the acidic pH in the urine hinders isolation whereas it inactivates and lyses the leptospiroa cells in less than three hours. GRÉGOIRE et al. (1987) observed that the collection of kidney tissue samples under aseptic conditions associated with immediate processing are probably the factors of greatest importance in leptospiroa isolation.

The EMJH culture medium has been used successfully in the isolation of leptospiroa (ELLIS et al., 1982; GRÉGOIRE et al., 1987; FAINE et al., 1999) but in this study the two isolations obtained from bovine urine decreased in cell concentration and motility after the first subculture in modified EMJH culture medium without antibiotics. A subculture in Tween 80/40/LH culture medium allowed the recovery of the cell concentration and also their motility. ELLIS & THIERMANN (1986) and LEONARD et al. (1992)

<table>
<thead>
<tr>
<th>Animal Species</th>
<th>Samples</th>
<th>Number of samples</th>
<th>Dark-field examination</th>
<th>Leptospira spp isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine urine</td>
<td>14</td>
<td></td>
<td>positive</td>
<td>11</td>
</tr>
<tr>
<td>Bovine urine</td>
<td>03</td>
<td></td>
<td>positive</td>
<td>02</td>
</tr>
<tr>
<td>Swine kidney</td>
<td>36</td>
<td></td>
<td>NP</td>
<td>00</td>
</tr>
<tr>
<td>Swine liver</td>
<td>36</td>
<td></td>
<td>NP</td>
<td>02</td>
</tr>
<tr>
<td>Swine uterus body</td>
<td>36</td>
<td></td>
<td>NP</td>
<td>00</td>
</tr>
<tr>
<td>Swine ovary</td>
<td>36</td>
<td></td>
<td>NP</td>
<td>00</td>
</tr>
</tbody>
</table>

NP = Not performed.
showed better results with the use of Tween 80/40/LH medium in the isolation of fastidious serovars such as hardjo and bratislava.

The methodology used in this study was shown to be efficient in the isolation of leptospira from dogs, bovine and swine naturally infected. The identification of these isolations will allow new epidemiological and prophylactic studies of leptospirosis in Brazil.

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REFERENCES


