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

Isolamento de *Leptospira* spp de cães, bovinos e suínos naturalmente infectados

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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.*

RESUMO

O isolamento da *leptospira* permite o diagnóstico definitivo da infecção. Um dos inconvenientes do cultivo é a contaminação por microrganismos. O objetivo deste trabalho foi descrever o isolamento de *leptospiras* de cães, bovinos e suínos naturalmente infectados. Foram utilizadas amostras de urina de 14 cães, três bovinos, e amostras de rim, fígado, ovário e corpo de útero de 36 matrizes suínas de descarte sem histórico sanitário. As amostras de urina e tecidos foram semeadas em meio de cultura. Foi utilizado o meio de cultura Ellinghausen-

McCullough-Johnson-Harris (EMJH) modificado acrescido de 5-fluorouracil, cloranfenicol, vancomicina, ácido nalidixico e neomicina. A incubação foi realizada a 28°C por 24 horas, seguida de subcultura em EMJH modificado sem antibiótico. Com esta metodologia, foi possível o isolamento de *Leptospira* spp da urina de 11 cães e dois bovinos e de fragmentos de fígado de dois suínos.

Palavras-chave: *Leptospirose, diagnóstico, cultura, bovino, cão, suíno.*

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 seroagglutination 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

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provides epidemiological and prophylactic studies of this disease. Isolation of leptospira 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 leptospire 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 leptospiras (SCHÖNBERG, 1981). THIERMANN (1984) stated that the most important factors for isolation of leptospira are aseptically collected material, quick processing, culture medium suitability and selective antibiotics.

The objective of this study was to describe the isolation of leptospira 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 leptospira 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, uterus 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 leptospira 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. Leptospire isolated from dogs and swine were weekly subcultured in modified EMJH medium without antibiotics. For maintenance of leptospire isolated from bovine the Tween 80/40/LH culture medium (ELLIS et al. 1985) was also used.

RESULTS

Leptospira was isolated from 11 of the 14 samples cultured of dog urine and two from the three bovine samples. Leptospira could only be isolated from the liver of two animals of the total of fragments cultured from 36 sows (Table 1). The growth of leptospira 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 leptospire 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 leptospira from the subcultures that used these contaminated tubes.

Table 1 - Isolation of *Leptospira* spp from dogs, cattle and swine naturally infected, from April 2000 to November 2001.

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

NP = Not performed.

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 leptospires 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 leptospira 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 leptospira 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 leptospira isolations from naturally infected animals. SCHÖNBERG (1981) observed a detrimental effect of antibiotics on leptospira 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 leptospira viability. THIERMANN (1980) and FAINE (1982) reported that the acidic pH in the urine hinders isolation whereas it inactivates and lyses the leptospira 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 leptospira isolation.

The EMJH culture medium has been used successfully in the isolation of leptospira (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)

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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