

EMJH MEDIUM WITH 5-FLUOROURACIL AND NALIDIXIC ACID ASSOCIATED WITH SERIAL DILUTION TECHNIQUE USED TO RECOVER *LEPTOSPIRA SPP* FROM EXPERIMENTALLY CONTAMINATED BOVINE SEMEN

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ABSTRACT

Bovine semen experimentally contaminated with *Leptospira santarosai* serovar Guaricura was submitted to the modified EMJH medium with 5-fluorouracil (300mg/L) and nalidixic acid (20mg/L), named as “selective medium” and using the serial dilution technique, in order to evaluate the percentage of recovery of the added microorganism. The selective EMJH medium was found with higher percentage of recovery of leptospires and minor losses of samples due to contamination with opportunistic microorganisms than the non-selective EMJH medium: 151/376 (40.0%) of positive growth; and 38/376 (10.0%) contamination and 58/376 (15%) and 129/376 (34.0%), respectively. These results were statistically significant ($p < 0.0001$; Fisher). Differences were found when the frequencies of positive leptospires recovery have been compared in the serial dilution technique (10^{-1} to 10^{-4}) between the selective and non-selective media at different dilution factors. At 1/10th dilution the percentages found were (0%, 0/80) and (38%, 30/80), at 1/100th dilution, (3%, 2/80) and (49%, 39/80) and at 1/1,000th dilution, (25%, 20/80) and (50%, 40/80), respectively. The percentage of recovery of leptospires was found to be directly proportional to the dilution used. The methodology of the serial dilution technique (setting at least three dilutions) and the use of selective EMJH medium have been found to be efficient for the isolation of leptospires from the bovine semen samples.

Key-words: Leptospires, Culture media, antimicrobial, isolation, semen

INTRODUCTION

Leptospires are slow growing organisms having a generation time of approximately 24 hours at 30°C and the requirement for a rich medium at neutral pH predisposes the cultivation of those microorganisms sometimes troublesome, particularly from natural sources (Adler *et al.*, Cousineau & Mckiel, Hussaini & Ruby, Johnson & Rogers) (1,3,7,8).

The isolation of leptospires from animal and human tissues, urine and semen has often been difficult due to the presence of contamination, once strictly aseptic methods of collection are not always possible under field conditions (Hussaini e Ruby) (7).

Schönberg and Schönberg *et al.* (17,18) observed that the isolation of leptospires from material with high bacterial count is difficult once the fast growth of the opportunists leads to the death of leptospires.

The inclusion of antibiotics in the culture medium, which is used to isolate leptospires and the serial dilution technique had been suggested because these proceedings were found effective for inhibiting the growth of contaminants (Adler *et al.*, Cousineau & Mckiel, Ellis & Little, Johnson & Rogers, Myers, Santa Rosa, Schönberg, Turner) (1,3,5,8,12,15,16,19).

Johnson and Rogers (8) showed that 5-fluorouracil is lethal to various microorganisms, but not to leptospires, in

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concentrations of 200–400 µg/mL. This substance is widely used as an additional medium, with serial dilutions of inocula, to obtain pure primary culture of isolates, however, some strains of *Leptospira*, may be inhibited by 5-fluorouracil in primary isolation cultures. Therefore, it is advisable to use batches of medium, in parallel, without this substance (Turner) (19).

Russeau *et al.* (14) observed that the use of 5-fluorouracil at concentrations of 50–1,000 µg/mL, in semi-solid EMJH medium, with the addition of 10% rabbit serum, did not inhibit the growth of Australis, Canicola and Pomona serovars, when the same was incubated at 30°C for seven days.

Myers and Varela-Diaz (12) using the media described by Fletcher (1928) and Ellinghausen and McCullough (1965) with the addition of 200 µg/mL 5-fluorouracil, indicated the need to subculture later into an antibiotic containing medium, for total elimination of surviving contaminants.

Ris (13) demonstrated that 5-fluorouracil had a bacteriostatic effect on leptospires from bovine urine when used in Johnson and Rogers' medium (1964), containing bovine albumin, Tween 80, aminoacids, purine and pyrimidine bases, but not in Fletcher's medium.

Hussaini and Ruby (7) concluded that the incorporation of 5-fluorouracil at concentrations of 100–150 µg/mL in Korthof's medium was found to be effective in inhibiting *Staphylococcus aureus* and *Escherichia coli* without affecting leptospires (Wolffi serovar) growth when cultures were incubated at 30°C or at room temperature (from 15°C to 22°C). It was concluded that Korthof's medium, containing 5-fluorouracil at these concentrations was a useful selective medium for the isolation of leptospires when incubated at appropriate temperatures.

Adler *et al.* (1) demonstrated that Hardjo and Pomona serovars growth in semi-solid EMJH medium with bovine albumin and Tween 80, modified by the addition of actidione (100 mg/L), bacitracin (40 mg/L), 5-fluorouracil (250 mg/L), neomycin sulphate (2 mg/L), polymyxin B (0,2 mg/L) and rifampicin (10 mg/L) would be recommended for inhibiting the opportunistic microorganisms, but these same serovars did not grow in selective EMJH medium enriched with rabbit serum.

Schönberg (17) found that pure cultures of Icterohaemorrhagiae, Canicola, Pomona, Grippotyphosa and Tarassovi serovars, in Korthof' medium with rabbit serum, vancomycin (10mg/L) and nalidixic acid (50mg/L) produced the lowest adverse effects on the multiplication phase of leptospires.

Schönberg *et al.* (16) described that the suppressing of contaminants growth for the recovery of leptospires (Icterohaemorrhagiae, Canicola, Pomona, Grippothyphosa, Tarassovi and Hardjo) from swine semen, had been found in EMJH medium with 5-fluorouracil (100 mg/L) or with the combination of nalidixic acid (50 mg/L), vancomycin (10 mg/L) and polymyxin B (5000 UI/L). The last combination was better than the 5-fluorouracil, however the need for subcultures was

found in a medium free of the inhibitory substances two days after the primary culture.

Freitas *et al.* (6) described the leptospires isolation of dogs, bovine and swine naturally infected by leptospires. The urine samples from 14 dogs and three bovines and also kidney, liver, ovary and uterus body samples from 36 slaughtered sows with unknown health records were used. The urine and organs samples were cultured in EMJH modified medium, with the addition of 10% rabbit serum in two formulations, one without antibiotics and the other one with the addition of 5-fluorouracil (400 mg/L), chloramphenicol (5 mg/L), nalidixic acid (50 mg/L), neomycin (10 mg/L) and vancomycin (10 mg/L). Incubation was performed at 28°C during 24 hours, followed by subculture in modified EMJH medium without antibiotics. *Leptospira spp* could be isolated from urine samples from 11 dogs, two bovines and liver fragments from swines.

The aim of this study was to evaluate the efficacy of EMJH semi-solid and modified medium with 5-fluorouracil and nalidixic acid associated with the serial dilution technique for the isolation of *Leptospira santarosai* serovar Guaricura from bovine semen experimentally contaminated.

MATERIAL AND METHODS

The Ellinghausen, McCullough, Johnson and Harris - EMJH (Becton-Dickinson Biosciences/DIFCO/Detroit/USA) modified, semi-solid medium was prepared with the addition of 15% rabbit serum and enriched with L-asparagin (3%), calcium chloride (1%), magnesium chloride (1%), pyruvate sodium (1%) and 0.2% agar¹ (w/v). This culture medium was prepared in two formulations, one without antibiotics (non-selective) and the other, with the addition of 5-fluorouracil² (300 mg/L) and nalidixic acid³ (20 mg/L), named as the selective medium (Ellis *et al.*) (5).

The inoculum was *Leptospira santarosai* serovar Guaricura, Sejroe serogroup, M04-98 strain, isolated from the urine of buffalo (*Buballus bubalis*) (Vasconcellos *et al.*) (20), typed by monoclonal antibodies technique (Terpstra *et al.* 1985) and cultivated in semi-solid EMJH modified medium (Alves *et al.*, Turner) (2,19).

The concentration of leptospires was adjusted to contain 20 to 30 live spirochetes when observed under the dark field microscopy, with 200x objective (15) and the standard volume of 2,0 mL of leptospires culture with 1,0 mL of semen diluted in egg yolk-citrate extender. These mixtures were incubated at 28° to 30°C by 30 min., for the contact between the leptospires and the diluted semen. The next step, four ten -fold serial dilutions

¹ Bacto-Agar – DIFCO (Detroit USA)

² 5-Fluorouracil - ICN Pharmaceuticals

³ Nalidixic Acid - SIGMA

⁴ DIFCO (Detroit USA)

(10^{-1} to 10^{-4}) were performed in modified EMJH, without agar and antibiotics (2,19). Each dilution was cultured in five tubes with selective EMJH in proportion at 1:10 (v/v). After 24h incubation at 28° to 30°C, these dilutions were sub cultured in the same proportion in EMJH, without antibiotics, and then incubated at 28° to 30°C for six weeks. Eight repetitions were carried out. Cultures were examined weekly under dark field microscopy and tubes showing contaminants were discarded. The control of microorganisms in semen was carried out by cultivation in appropriate culture media. Seven frozen semen samples and a fresh one (from each repetition) were cultivated in BHI⁴ (brain heart infusion) broth and after 24h at 37°C, they were cultivated in 5% sheep blood agar, MacConkey agar and Saboraud-dextrose agar. Blood and MacConkey agar plates were incubated at 37°C, 24 to 72h. Saboraud plates were kept at room temperature for at least seven days. Microorganisms that were isolated have been identified by macro and microscopically and by biochemical characteristics, and were classified according to Krieg and Holt Murray *et al.* (9,10).

RESULTS AND DISCUSSION

In Table 1, there are significant differences shown ($p < 0.0001$; Fisher) in the percentage of recovery of leptospires by cultivation, under selective EMJH medium, with 40% growth of leptospires (151/376) and non-selective EMJH with 15% growth of leptospires (58/376). Significant differences ($p < 0.0001$; Fisher) were found in the percentage of contamination by microorganisms, 10,0% (38/376) of samples from selective EMJH medium were contaminated and 34,0% (129/376) of samples from non-selective EMJH medium. These results were according to Adler *et al.*, Cousineau & Mckiel, Hussaini & Ruby, Johnson and Rogers, Schönberg *et al.* (1981) and Schönberg (1980) (1,3,7,8,16,17) who observed that the isolation of leptospires from material with high bacterial count was difficult, once the fast growth of the contaminant microorganisms led to leptospires death.

In Table 2, there are illustrated the results of bacterial cultures from semen with eight repetitions. Six out of seven samples of frozen semen presented growth of *Corynebacterium sp* (6/7), four of *Bacillus sp* (4/7), two of *Streptococcus sp* (2/7) and one of *Micrococcus sp* (1/7), suggesting that microorganisms present in the environment affected the samples preparation, because from the fresh semen sample, only *Proteus mirabilis* (1/1) was isolated. There was no growth of molds and yeasts. The antibiotics, at the concentrations used in the EMJH medium, were found to be able in inhibiting contaminants but not leptospires. Myers and Varela-Diaz (12) found that subcultures were needed further on into an antibiotic containing medium, for total elimination of surviving contaminants after 24h, differently from what was described by Freitas *et al.*, Turner, and Schönberg *et al.* (6,16,19), that sub cultured after two days.

Table 1. Number of samples with positive leptospires cultures* and contaminated cultures according to the type of EMJH medium used.

Culture Medium	Positives	Contaminated	TOTAL
EMJH (non-selective)	58 (15.0%) ^a	129 (34.0%) ^a	187 (50.0%)
EMJH (selective)	151 (40.0%) ^b	38 (10.0%) ^b	189 (50.0%)
TOTAL	209 (56.0%)	167 (44.0%)	376 (100.0%)

* incubation at 28°-30°C and assessed weekly for up to six weeks through dark field examination; **a**, **b**: different superscripts, in the same column, significant difference; FISHER: $p < 0.0001$.

Table 2. Microorganisms other than leptospires isolated from semen samples in each repetition.

Repetition	Isolated Microorganisms
01 ⁽¹⁾	<i>Corynebacterium sp</i>
02 ⁽¹⁾	<i>Corynebacterium sp</i> / <i>Streptococcus sp</i>
03 ⁽¹⁾	<i>Bacillus sp</i> / <i>Corynebacterium sp</i>
04 ⁽¹⁾	<i>Bacillus sp</i> / <i>Corynebacterium sp</i>
05 ⁽¹⁾	<i>Bacillus sp</i> / <i>Corynebacterium sp</i>
06 ⁽¹⁾	<i>Bacillus sp</i> / <i>Corynebacterium sp</i>
07 ⁽¹⁾	<i>Micrococcus sp</i> / <i>Streptococcus sp</i>
08 ⁽²⁾	<i>Proteus mirabilis</i>

(1) frozen semen (2) fresh semen.

In Table 3, positive and negative cultures to leptospires are shown according to the type of EMJH medium and the serial dilution used. Differences were found between the selective EMJH and the non-selective at the dilutions 10^{-1} , 10^{-2} : and 10^{-3} . The percentage of leptospires recovered was found to be directly proportional to the dilution applied in two media formulations and the growth percentage was higher in selective EMJH at the three dilutions used. These results agree to those of Adler *et al.*, Cousineau & Mckiel, Ellis & Little, 1986; Johnson & Rogers, Myers, Santa Rosa, Schönberg, Turner (1,3,5,8,11,15,18,19) who showed that the incorporation of antibiotics in the culture medium and applied for the isolation of leptospires, using the serial dilution technique, was found to be effective in inhibiting the growth of contaminant microorganisms.

In Fig. 1, it is presented the percentage of growth of leptospires in bovine semen samples, experimentally contaminated with *Leptospira santarosai* serovar Guaricura and according to the serial dilution and EMJH medium. The selective EMJH was found with high percentages of recovery of leptospires than non-selective EMJH in all of dilutions used.

Table 3. Number of samples with positive leptospire cultures* according to EMJH medium type and dilution applied.

DILUTIONS	NON-SELECTIVE EMJH MEDIUM		SELECTIVE EMJH MEDIUM [#]		TOTAL
	POSITIVE	NEGATIVE	POSITIVE	NEGATIVE	
10 ⁰	0 (0.0%)	40 (50.0%)	4 (5.0%)	36 (45.0%)	80 (100.0%)
10 ⁻¹	0 (0.0%) ^c	40 (50.0%) ^d	30 (38.0%) ^c	10 (13.0%) ^d	80 (100.0%)
10 ⁻²	2 (3.0%) ^e	38 (48.0%) ^f	39 (49.0%) ^e	1 (1.0%) ^f	80 (100.0%)
10 ⁻³	20 (25.0%) ^g	20 (25.0%) ^h	40 (50.0%) ^g	0 (0.0%) ^h	80 (100.0%)
10 ⁻⁴	36 (45.0%)	4 (5.0%)	38 (48.0%)	2 (2.0%)	80 (100.0%)

* incubation at 28°-30°C and assessed weekly for up to six weeks through dark field examination; **c, d, e, f, g, h**: different superscripts, in the same column, significant difference; [#] 5-fluorouracil and nalidixic acid; FISHER: p<0.0001.

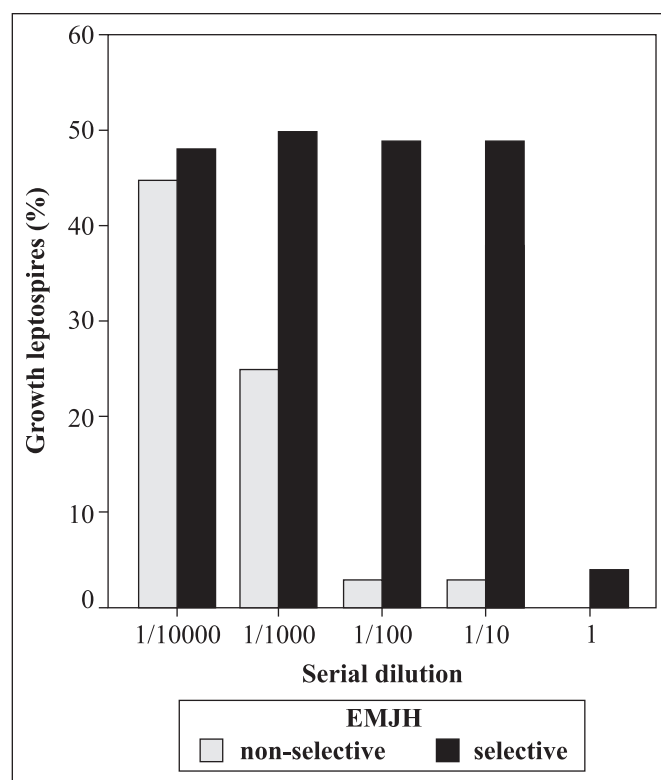


Figure 1. Growth of leptospires (%) in bovine semen samples, experimentally contaminated with *Leptospira santarosai* serovar Guaricura, according to the serial dilution using EMJH medium type.

The association of the serial dilution technique (at least three dilutions) with the use of the selective EMJH medium under the test conditions of the experiment were found to be an efficient procedure for the recovery of leptospires from the bovine semen.

RESUMO

Meio de EMJH com 5-fluorouracil e ácido nalidíxico associado a técnica das diluições seriadas usados para recuperar *Leptospira spp* do sêmen bovino experimentalmente contaminado

Sêmen bovino experimentalmente contaminado com a estirpe *Leptospira santarosai* Sorovar Guaricura foi utilizado para verificar a porcentagem de recuperação de leptospiras, utilizando o meio de EMJH com 5-fluorouracil (300 mg/L) e ácido nalidíxico (20 mg/L), denominado “meio seletivo”, associado à técnica das diluições seriadas. O meio seletivo EMJH foi encontrado com porcentagem de recuperação mais elevada e com menos perda de amostras devido à contaminação com microorganismos oportunistas quando comparado com o meio EMJH não seletivo, que foram encontrados, respectivamente, com 151/376 (40.0%) de crescimento positivo e 38/376 (10.0%) de contaminação, 58/376 (15%) e 129/376 (34.0%). Estes resultados foram estatisticamente significantes (p<0,0001; Fisher). Diferenças foram encontradas quando as freqüências de leptospiras recuperadas foram comparadas com a técnica de diluição seriada (10⁻¹ a 10⁻⁴) e entre os meios seletivo e não-seletivo, em diferentes níveis de diluição. À diluição de 1/10, as porcentagens encontradas foram (0%, 0/80) e (38%, 30/80); à diluição 1/100, (3%, 2/80) e (49%, 39/80); e à diluição 1/1000, (25%, 20/80) e (50%, 40/80), respectivamente. A porcentagem de recuperação de leptospiras foi diretamente proporcional às diluições empregadas. A metodologia das diluições seriadas (estabelecendo pelo menos três diluições) e o uso de meio EMJH seletivo mostraram ser eficientes no isolamento de leptospiras presentes nas amostras de sêmen bovino.

Palavras-chave: Leptospiras, meio de cultura, antimicrobianos, isolamento, sêmen.

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