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COMPARISON OF FOUR ANTIBIOTICS FOR INACTIVATING LEPTOSPIRES IN BULL SEMEN DILUTED IN EGG YOLK EXTENDER AND EXPERIMENTALLY INOCULATED WITH *LEPTOSPIRA SANTAROSAI* SEROVAR GUARICURA

Fabiana Miraglia; Zenaide Maria Morais; Adriana Cortez; Priscilla Anne Melville; Maria Fernanda Vianna Marvullo; Leonardo José Richtzenhain; José Antonio Visintin; Silvio Arruda Vasconcellos*

Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brasil

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ABSTRACT

Inactivation of leptospires in pools of semen from three Holstein Friesian bulls, collected in an artificial vagina, was investigated. Spermatic concentration was adjusted in egg yolk citrate extender, submitted to the following treatments: A (control; without antibiotics); B (penicillin, 1,000 UI/mL - streptomycin, 1,000 µg/mL); C (amoxicillin, 1,000 µg/mL); D (ceptiofur sodium, 1,000 µg/mL); E (amoxicillin 1,000 µg/mL - ceptiofur sodium 1,000 µg/mL). Leptospires (2.0×10^6 leptospires/mL) were added into the diluted semen. Recovery of leptospires was obtained in modified EMJH semi-solid medium with and without antibiotics. The antibiotics in the concentrations used did not affect means of percentage of progressive motility and individual progressive motility of spermatozoids. Penicillin-streptomycin presented the best results in leptospire inactivation (97.1%). Amoxicillin, ceptiofur sodium and their combination at the concentrations studied presented poor results: 59.29%; 32.5% and 60.36% of inactivation, being less effective in leptospire inactivation than penicillin-streptomycin.

Key words: Leptospires, semen, antibiotics, extender

INTRODUCTION

Bovine leptospirosis is spread worldwide and has been considered an important cause of economic losses in beef and dairy cattle industries (42).

Reproductive disorders include: abortion (11,18,21), stillborn calves (11,12,19,26,47), premature calving (12,19), calving of weak calves (13), metritis and retained placenta (39); infertility, also in bulls (29), and sterility (29,43).

Serovars of leptospires isolated, in Brazil, from cattle were: pomona (16); icterohaemorrhagiae (33); guaricura (44), goiano (46); hardjo and georgia (44).

Jones (22) and Sleight and Willians (37) mentioned leptospirosis transmission by artificial insemination or coitus. Ellis *et al.* (14) confirmed the presence of serovar hardjo in male and female bovine genital tract. Viability of leptospires in infected semen during cooling (5ºC) and freezing (-196ºC) was confirmed by Amatredjo and Campbell (7); Jones (22) and Kiktenko *et al.* (23).

Heinemann *et al.* (20) examined 20 bulls and found 80% of *Leptospira* spp positive samples using Polimerase Chain Reaction (PCR). All these samples were negative for leptospire when analyzed by culture, but most of them presented growth of microorganisms other than leptospires. Forty-five percent of these animals were positive by microscopic agglutination test (MAT). The authors concluded that bulls negative by MAT may not be free of leptospire infection even if semen cultures did not present any leptospire growth.

Considering that leptospirosis may be transmitted by artificial insemination and because of possible failures in the diagnosis in the bulls, research in semen treatment is needed to identify conditions that inactivate leptospires, but do not affect spermatozoa.

* Corresponding author. Mailing address: Departamento de Medicina Veterinária Preventiva e Saúde Animal, Av. Prof. Dr. Orlando Marques Paiva, 87. 05508-000, São Paulo, SP, Brasil. Fax: (+5511) 3091-7928. E-mail: savasco@usp.br

MATERIALS AND METHODS

Animals

Semen donors were three Holstein Friesian bulls (two-years old) clinically normal and without evidences of leptospiral infection either by MAT with 24 serovars in blood serum and seminal plasma (32) or by PCR in semen (20).

Semen extender and antibiotics

Egg Yolk Citrate (EYC) extender (27) prepared with different antibiotics was used and concentrations were based on Almquist *et al.* (2,3,4,5), Arriola and Foote (8); Back *et al.* (9); Varner *et al.* (41). Experimental groups were as follows: A - EYC without antibiotics (control); B - EYC, penicillin (ICN Biomedicals) (1,000 UI/mL) - streptomycin (FARMAVET) (1,000 µg/mL); C - EYC, amoxicillin (ICN Biomedicals) (1,000 µg/mL); D - EYC, ceptiofur sodium (Excenel – UPJHON) (1,000 µg/mL); E - EYC, amoxicillin (1,000 µg/mL)-ceptiofur sodium (1,000 µg/mL).

Semen samples

Obtained by artificial vagina and mixed soon after collection in order to make a pool, maintained in a water bath at 38ºC. The number of spermatozoa was counted using a Newbauer chamber. The pool was diluted in EYC prepared with the above mentioned antibiotics, in order to achieve 8 x 10^7 spermatozoa/mL.

Spermatozoa control

Percentage of Progressive Motility (PMP) and Individual Progressive Motility (MPI) were evaluated soon after semen dilution and again after 24 h of storage at 5ºC. Comparison of these values was performed using ANOVA (18).

Experimental inoculation of extended semen samples with Leptospires

One millilitre of the extended semen pool was added to 2 mL of leptospire culture (10^6 leptospires/mL) cultivated in modified EMJH liquid medium (6,40). The extended semen pool experimentally contaminated with *L. santarosai* serovar guaricura (44) was stored at 28-30ºC for 30 minutes and then five ten-fold serial dilutions were prepared in modified EMJH (Difco Laboratories-USA) liquid medium.

Trial for Leptospire isolation

Five tubes of modified EMJH semi-solid (0.2% agar without antibiotics) and other five tubes of modified EMJH semi-solid with antibiotics (selective) (28) (0.2% agar-5-fluorouracil - 300 mg/L and nalidixic acid 20mg/L) were inoculated with each dilution of semen sample plus leptospires. For each culture tube the proportions of inoculum and medium were, respectively, 0.5 and 5mL. Incubation was performed at 28 to 30ºC. After 24h, samples in EMJH with antibiotics were subcultured in antibiotic free medium. Leptospire growth was confirmed by microscopic examination under darkfield ilumination. Cultures were maintained for six weeks and tubes presenting growth of microorganisms other than leptospire were discarded. The proportion of tubes presenting leptospire growth was compared by Fisher's Test (18).

Control of microorganisms other than Leptospire in semen

Eight pools of non-diluted frozen bovine semen were cultivated in BHI broth, and after 24h at 37ºC, they were cultivated on 5% sheep blood agar, MacConkey agar and Saboraud-dextrose agar. Blood agar and MacConkey plates were incubated at 37ºC and bacterial growth was observed after 24 and 72h; Saboraud plates were kept at room temperature for at least seven days. Microorganisms were identified by macro-microscopical morphology and biochemical characteristics and were classified according to Murray *et al.* (30) and Krieg and Holt (31). A fresh pool of semen was cultivated in 5% sheep blood agar, MacConkey agar and Saboraud-dextrose agar, and microorganisms isolated were identified as described for the pools of frozen semen.

RESULTS

Table 1 presents the results of spermatozoa pool examination according to the groups of antibiotics included in the EYC extender. Mean PMP and mean MPI (0-5) were evaluated soon after semen dilution (T0h) and again 24h after semen dilution (T24h). There were no significant differences between the five treatments in the different evaluation times.

Table 2 presents the isolation of leptospire according to the media used and the antibiotics included in EYC. The best results were obtained with EYC with penicillin-streptomycin and media with antibiotics for isolation in the first 24 hours after sample seeding.

Fig. 1 presents the percentages of leptospire growth reduction in the different treatments, considering as 100% of growth the number of positive tubes in the samples diluted in EYC without antibiotics (A). It was possible to observe that results were, in decreasing order, B: 97.11%; E: 60.36%; C: 59.29% and D: 32.50%.

Table 3 presents microorganisms other than leptospire, isolated from eight batches of bovine semen. From seven frozen batches examined, five presented growth of *Corynebacterium* sp; four presented *Bacillus* sp; two, *Streptococcus* sp; and one, *Micrococcus* sp. In one culture of fresh semen, growth of *Proteus mirabilis* occurred. There was no growth of molds and yeasts.

MAT (24 serovars) for anti-leptospire agglutinines in seminal plasma of all semen pools investigated presented negative results.

Table 1. Mean percentage of progressive motility (PMP) and mean progressive individual motility (MPI) of spermatozoa from semen pools, according to the time of evaluation and to the type of antibiotic added to the egg-yolk-citrate extender. São Paulo, 2003.

Groups	PMP mean ± SD		MPI mean ± SD	
	T0h	**T24h**	**T0h**	**T24h**
A	77.27 ± 10.09 [a]	64.54 ± 12.13 [b]	3.63 ± 0.50 [c]	2.90 ± 0.70 [d]
B	74.09 ± 9.17 [a]	66.81 ± 8.44 [b]	3.72 ± 0.46 [c]	3.00 ± 0.63 [d]
C	72.72 ± 10.80 [a]	66.36 ± 13.80 [b]	3.18 ± 0.75 [c]	2.90 ± 0.70 [d]
D	76.36 ± 10.74 [a]	58.18 ± 23.79 [b]	3.72 ± 0.46 [c]	3.36 ± 0.50 [d]
E	77.72 ± 9.84 [a]	60.45 ± 22.41 [b]	3.81 ± 0.40 [c]	2.81 ± 0.87 [d]

A: EYC without antibiotics (control); B: EYC with penicillin-streptomycin; C: EYC with amoxicillin; D: EYC with ceptiofur sodium; E: EYC with amoxicillin-ceptiofur sodium; [a] [b] [c] [d] [e]: means with same superscripts, in the same column, do not differ (P>0.01) / ANOVA: P=0.7415; ANOVA: P=0.7099 / KRUSKAL-WALLIS: P=0.9729; ANOVA: P=0.0566; ANOVA: P=0.3952, respectively.

Table 2. Number of cultures performed for leptospire isolation in bull semen samples experimentally contaminated with the *L.santarosai* serovar guaricura, according to the type of isolation medium and the antibiotics included in the egg-yolk-citrate extender. São Paulo, 2003.

Groups	**EMJH** [1]	**EMJH selective** [1] [2]
	Porcentage of positive cultures (positive number/total)	**Porcentage of positive positive cultures (positive number/total)**
A	87.6 (99/113) [x]	89.0 (178/200)
B	5.1 (7/136)	0.0 (0/212)
C	43.8(63/144) [y]	28.1 (55/196) [z]
D	78.8 (100/127) [x]	45.4 (99/218)
E	44.0 (62/141) [y]	26.1 (57/218) [z]

A: EYC without antibiotics (control); B: EYC with penicillin-streptomycin; C: EYC with amoxicillin; D: EYC with ceptiofur sodium; E: EYC with amoxicillin-ceptiofur sodium; (1) modified medium with 0.2% agar; (2) with nalidixic acid and 5-Fluorouracil; [x] [y] [z] same superscripts, in the same columns, do not differ; P<0.0856, P=1.000, P=0.7397, respectively.

Table 3. Microorganisms other than leptospire, found in bovine semen, according to the batch. São Paulo, 2003.

Semen pool	**Microorganisms**
01 [1]	*Corynebacterium* sp
02 [1]	*Corynebacterium* sp / *Streptococcus* sp
03 [1]	*Bacillus* sp / *Corynebacterium* sp
04 [1]	*Bacillus* sp / *Corynebacterium* sp
05 [1]	*Bacillus* sp / *Corynebacterium* sp
06 [1]	*Bacillus* sp / *Corynebacterium* sp
07 [1]	*Micrococcus* sp / *Streptococcus* sp
08 [2]	*Proteus mirabilis*

(1) frozen semen samples; (2) fresh semen sample.

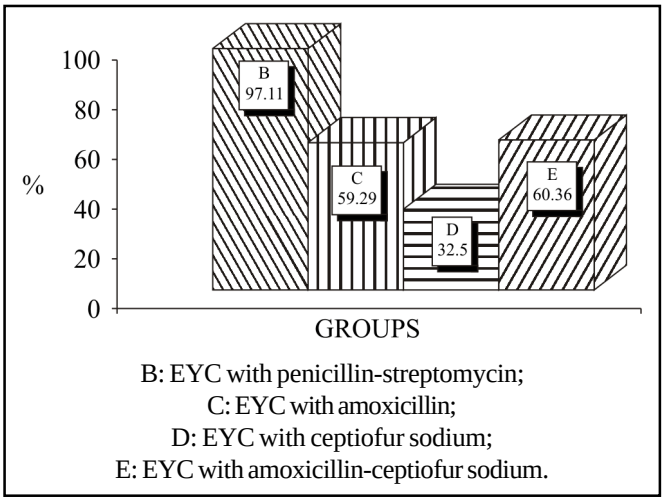

Figure 1. Reduction of leptospiral growth taking group A (EYC free of antibiotics) as 100% of growth, according to the treatment.

DISCUSSION

Mean PMP and MPI, determined in EYC extenders containing antibiotics in the concentrations tested, presented no significant differences. EYC with penicillin-streptomycin presented results in agreement with those by Almquist (2,3,4,5) who did not find that these antibiotics could cause any spermatozoa impairment. Arriola and Foote (8) reached the same conclusion with equine semen diluted in egg-yolk-tris extender including penicillin-streptomycin or amikacyn. Varner (41) applied the milk-glycose extender to cooled equine semen including ceptiofur sodium and other antibiotics. Back (9) obtained similar results with equine semen diluted in egg-yolk-tris extender including gentamicin, kanamycin, lincomycin, penicillin and streptomycin.

For leptospire isolation, treatment with penicillin-streptomycin presented better results than the other treatments. The inactivating ability of these two antibiotics was also

reported for other serovars, such as pomona (7,22,23,31). The efficiency of streptomycin alone was also reported for serovar sejroe (45). Hoag and Bell (21) described the growth of pure cultures of the serovars sejroe, pomona, grippotyphosa and icterohaemorrhagiae in media prepared with streptomycin.

Although the inclusion of penicillin-streptomycin in EYC inactivated all leptospire cultures seeded in selective isolation media (0/212), the occurence of 5.1% (7/136) positive tubes in non-selective media shows that semen collected from infected animals and diluted in extenders prepared with these antibiotics may contain live leptospires. Bryan and Boley (10) also found surviving leptospires in bull semen diluted in extender prepared with penicillin, streptomycin and sulfanilamide.

Although amoxicillin (1,000 µg/mL) showed poor ability to inactivate leptospire serovar guaricura in bull semen, other concentrations of this antibiotic should be investigated. Smith (38) has described a strong effect of amoxicillin on the "*in vivo*" treatment of kidney carriers, in cattle infected by the serovar hardjo.

The treatment of bull semen samples with ceptiofur sodium (1,000 mg/mL) was not effective for leptospire inactivation in 57.7% (199/345) of positive cultures. Alt and Bolin (1) did not find any results using cephalosphorin in the control of kidney carriers in swine infected by serovar pomona. However, in hamsters experimentally infected with the same serovar, Santos *et al.*(34) observed that this antibiotic presented a strong ability to control the disease.

The presence of positive leptospire cultures in semen samples diluted in extender prepared with penicillin (1,000 UI/mL) and streptomycin (1,000 µg/mL) would suggest that other trials with higher concentrations of these two antibiotics should be performed. However, Almquist *et al.* (3,4,5) and Arriola and Foote (8) observed that concentrations higher than these led to significant losses in the progressive motility of bull spermatozoa.

Other antibiotic associations must be investigated, like those done by Golsteyn-Thomas *et al.* (17), who got good results in the inactivation of serovars pomona and hardjobovis with an extender that included gentamicin, tylosin, lincomycin and spectinomycin.

The number of tubes presenting leptospire growth in bovine semen samples experimentally contaminated with serovar guaricura was higher in selective modified EMJH semi-solid medium than in the non-selective medium. This result was attributed to the natural presence of microorganisms other than leptospires in semen samples. This was also observed by Heinemann *et al.* (20); Schönberg (35) and Schönberg *et al.* (36). In the present study, in fresh individual semen samples, positive cultures for *Proteus mirabilis* were observed. In frozen pools, the microorganisms isolated were *Corynebacterium* sp, *Bacillus sp, Streptococcus* sp and *Micrococcus* sp, suggesting that environmental microorganisms may enter the samples during the preparation of semen pools.

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RESUMO

Comparação de quatro antibióticos para inativar leptospiras em sêmen bovino diluído em gema-citrato e experimentalmente contaminado com *Leptospira santarosai* sorovar guaricura

A inativação de leptospiras em misturas de sêmen, obtidas através de vagina artificial, de três touros holandeses, foi estudada. A concentração espermática foi ajustada em diluidor gema-citrato utilizando os seguintes tratamentos: A (controle; sem antibióticos); B (penicilina, 1000 UI/mL - estreptomicina, 1000 µg/mL); C (amoxicilina, 1000 µg/mL); D (ceftiofur sódico, 1000 µg/mL); E (amoxicilina 1000 µg/mL - ceftiofur sódico 1000 µg/mL). Leptospiras ($2,0x10^6$ leptospiras/mL) foram adicionadas ao sêmen diluído. A recuperação das leptospiras foi obtida em meio EMJH modificado semi-sólido, com e sem antibióticos. As médias da porcentagem de motilidade progressiva e a de motilidade individual progressiva dos espermatozóides não foram afetadas pelos antibióticos nas concentrações usadas. Penicilina-estreptomicina apresentou os melhores resultados na inativação das leptospiras (97.1%). Amoxicilina, ceftiofur sódico e suas combinações, nas concentrações estudadas, apresentaram resultados insatisfatórios: 59.29%; 32.5% e 60.36% de inativação, sendo menos efetivos na inativação das leptospiras do que penicilina-estreptomicina.

Palavras-chave: Leptospiras, sêmen, antibióticos, diluidor

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