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Effects of Tiamulin, Neomycin, Tetracycline, Fluorophenicol, Penicillin G, Linco-Spectin, Erythromycin and Oxytetracycline on Controlling Bacterial Contaminations of the River Buffalo (*Buballus bubalis*) Semen

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Abstract: In order to investigate the effects of tiamulin, neomycin, tetracycline, fluorophenicol, penicillin G, Linco-Spectin (0.15 mg mL^{-1} lincomycin + 0.3 mg mL^{-1} spectinomycin), erythromycin and oxytetracycline on controlling bacterial contaminations of the river buffalo semen, 120 mL diluted buffalo bull semen (diluted by tris-egg yolk extender) was divided into 5 mL tubes after initial evaluation and before (control sample) and at 0, 2, 6, 12 and 24 h after adding each of the above antibiotics at the recommended dose (D) and twice the recommended dose (D×2) to the semen samples, each sample was cultured 4 times on Muller-Hinton agar medium and the results were recorded after 18 h incubation at 37°C. Tiamulin, tetracycline, neomycin and fluorophenicol were ineffective. Oxytetracycline was effective in both D and D×2 ($p<0.001$). Penicillin G in both D and D×2 was effective ($p<0.001$). Linco-Spectin was effective, though not significant, in D at 2 h and in D×2 at 0 h only. Erythromycin in D was not significantly effective, but, in D×2 was effective ($p<0.001$). Duration of the antibiotic exposure had no significant effect on the antibiotic potentials except for Linco-Spectin at 2 h ($p<0.014$). The biochemical tests identified the contaminant bacteria as being a member of *Arcanobacter* (*Corynebacterium*) sp. In the next step, the semen sample of the same bull was taken, semen quality tests were carried out and the semen was diluted with the same extender (tris-egg yolk) + 7% glycerol, containing a double dose (D×2) of these antibiotics and semen quality tests were carried out immediately after dilution, 18 h after storage at 4°C and after the semen was packed in the straws, frozen in liquid nitrogen (-196°C) and later thawed in 37°C water bath to investigate whether these antibiotics have any adverse effect on the spermatozoa during the process of freezing and thawing. The comparison of the results with those of the control group (the sample undergone the same process without adding antibiotics) indicated that oxytetracycline adversely affected sperm motility at 0 and 18 h, all the antibiotics had a lower percentage of sperm abnormal morphology than the control at 0 and 18 h, except for Linco-Spectin at 18 h and after freezing-thawing and tetracycline after freezing and thawing the sample which were the same as the control. Sperm viability was not affected by antibiotics before and after freezing. It was concluded that oxytetracycline and penicillin G in both D and D×2 were effective in controlling seminal bacterial contaminations and because of the adverse effect of oxytetracycline on the sperm motility and morphology, it proved not to be suitable for this purpose but penicillin G could be recommended as an additive to the semen extenders.

Key words: Buffalo, semen, antibiotics, bacterial contaminations

INTRODUCTION

Most AI (Artificial Insemination) programs require at least some short-term storage of spermatozoa. Because the media used for storage of spermatozoa are particularly supportive of microbial growth, it is necessary to include antimicrobial agents to prevent massive proliferation of the microorganisms present in ejaculated semen. The inclusion of antimicrobial agents is necessary whether the spermatozoa are stored short term in a liquid medium or cryopreserved. Certain precautions, such as clipping preputial hair and allowing only one intromission into an AV (artificial vagina), can minimize the microbial contamination (Garner, 1991).

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Antibiotics are added to most semen diluents as a prophylactic measure against transmission of pathogenic bacteria and to reduce the load of non-pathogenic organisms that contaminate the semen. In cattle AI, benzylpenicillin and streptomycin are the most widely used antibiotics, for these are efficacious against *C. fetus*. Most other antibiotics either fail to control this organism or are directly detrimental to sperm. Recently, concern over the potential transmission of *Mycoplasma* and *Ureaplassma* species in bovine semen has led to the incorporation of lincomycin and spectinomycin into semen diluents in an effort to control these orgamisms. There is evidence that the efficiency of antibiotics may be reduced in the presence of some components of the diluents, notably egg yolk, hence the practice in some bovine AI centers is to pre-incubate the raw semen with antibiotic cocktails before main dilution occurs (Parkinson, 2001).

Some problems with resistant organisms have been noted. A new combination of antibiotics comprised of gentamycin, tylosin and Linco-Spectin has been used successfully to control certain resistant microorganisms in cryopreserved bull semen (Garner, 1991).

Penicillin (1000 IU mL^{-1}) and Streptomycin sulphate (1.0 mg mL^{-1}) alone or in combination are commonly added to freezing diluents. The microorgamisms present in buffalo semen have been examined and their sensitivity to available antibiotics has been reported. The combination of penicillin and neomycin has been reported to be more effective than the combination of penicillin and streptomycin currently used (Sansone, 2000).

This study was conducted to investigate: (1) effects of 8 different antibiotics in controlling growth of the semen contaminating bacteria, (2) whether duration of the pre-incubation exposure of the semen to the antibiotics could affect the antibiotic potentials and (3) if these antibiotics have any adverse effect on the spermatozoa.

MATERIALS AND METHODS

Semen sample was collected from a 4 year buffalo bull with a history of good fertility in the Buffalo Breeding Center, Northwest of Iran, in Urmia (37° 33'N, 45° 4'E) by using a bovine model artificial vagina. Semen quality was evaluated by determination of the percentage of sperm progressive motility, viability, abnormal morphology and sperm concentration as well as volume, color and viscosity evaluation of the semen according to the procedures recommended by Ax *et al.* (2000) and Barth (1997) by using Olympus BX41 light microscope with a pre-warmed stage (37°C) at x400 magnification. The sample, then, was diluted by tris extender (tris 2.66 g, citric acid 1.4 g, glucose 0.63 g, cystein 0.13 g, double distilled water 80 mL and egg yolk 20 mL); semen quality was re-evaluated to ensure that the dilution has not affected the semen quality. The diluted semen cooled to room temperature, transported as quickly as possible to the microbiology Lab. and divided into 5 mL tubes. A total volume of 120 mL diluted semen was used at this stage. One group of 8 tubes was used as control, one group as recommended dose (D) (according to Prescott and Baggot, 1993) and another group as D×2 (double dose). To the D group of tubes 20 μg mL^{-1} Tiamulin (Damloran Co., Khoram-abad, Iran), 200 mg mL^{-1} Neomycin (Razak Co. Tehran, Iran), 100 mg mL^{-1} Tetracycline (Livestock Drug Manufacturing Co. Tehran, Iran), 100 μg mL^{-1} Fluorophenicol (Erfan Co., Tehran, Iran), 1000 IU mL^{-1} Penicillin G (Jaber-Ebne-Haian Co., Teheran, Iran), 0.15 mg mL^{-1} lincomycin + 0.3 mg mL^{-1} spectinomycin marketed as Linco-Spectin [a commercially available preparation of one part (33.3 g) of lincomycin combined with two parts (66.6 g) of spectinomycin in 50 g packets] (Razak Co., Tehran, Iran), 0.05 mg mL^{-1} Erythromycin (Razak Co. Tehran, Iran) and 50 μg mL^{-1} Oxytetracycline (Damloran Co. Khoram-Abad, Iran) and to the D×2 group of tubes twice this dose was added, respectively. A 100 μL culture on Muller-Hinton agar medium (Merck kGaA, 64271 Darmstadt, Germany) that repeated 4 times for each tube was made at 0 (immediately after adding antibiotics), 2, 6, 12 and 24 h after adding antibiotics to the semen and the results were recorded after 18 h incubation at 37°C. Colonies grown on the control culture were re-cultured on Blood agar (Merck D61, 64271 Darmstadt, Germany) and MacConkey agar medium (Antec. Mo45, Antec. Diagnosis, England) and biochemical tests revealed that the contaminating bacteria belonged to *Arcanobacter* (*Corynebacterium*) species. Colony growth was regarded as an ineffective antibiotic and in cases in which colonies were grown the number of colonies was counted. Because in this study the number of colonies were either too many for counting or none, the results were recorded as + or – signs.

In the next step, semen sample was collected from the same bull, its quality evaluated as mentioned above and diluted with the same extender but this time 7% glycerol was added as cryoprotectant to the extender. Diluted semen was re-evaluated, divided into 5 mL tubes and the antibiotics were added to each tube in twice the recommended dose (D×2). Semen quality evaluation was repeated at 0 (immediately after adding antibiotic), 18 h after keeping the samples at 4°C for equilibration and after being packed in 0.5 mL French straws, frozen in liquid

nitrogen (to -196°C) and later thawed in 37°C water bath for 30 sec. The results were compared with those of the controls which underwent the same process along with the test group without adding antibiotics.

Statistical analysis of the data was performed by using Sigmastat (Sigmastat version 2.03 for windows, Jandel Corporation San Rafael California, USA) and SPSS (SPSS version 11.5 for windows, SPSS Inc., Chicago, IL.USA) software. Chi square and Fisher's exact test were used to compare the results of the antibiotic cultures and time laps effects and the results of semen quality evaluations were tested by repeated measure ANOVA and Bonferroni test was used for the comparison of semen quality results after transforming the data by a procedure recommended by Armitage and Berry (1987).

RESULTS

Cultures of 100 μL samples which repeated four times for each tube were resulted in too many colonies that were uncountable (+) or no colony growth (-). No cultures with a small or countable number of colonies were observed. So the results are depicted as + or –. Table 1 summarizes the results of cultures.

Comparing with the control, of the antibiotics examined, oxytetracycline and penicillin in D and D×2 ($p<0.001$ for both) were effective and erythromycin was effective only in double dose (D×2) ($p<0.001$). Other antibiotics proved to be significantly ineffective. The difference between penicillin and oxytetracycline in D and these antibiotics in D×2 with erythromycin was not significant. Duration of time laps only affected Linco-Spectin at 2 h significantly ($p<0.014$). The results of semen quality evaluations are depicted in Table 2.

Addition of oxytetracycline to the diluted semen (0, $p<0.05$ and 18 h, $p<0.05$) and fluorophenicol at 18 h reduced the sperm motility significantly ($p<0.05$) all the antibiotics had a significantly lower percentage of abnormal sperm morphology ($p<0.05$) at 0 and 18 h than that of the control except for Linco-Spectin at 18 h, which was the same as the control and tiamulin had a lower percentage of abnormal sperm count after freezing-thawing. The sperm livability was not significantly different from controls in antibiotic added samples, but freezing and thawing process apparently had lowered the semen quality (as measured by motility, abnormal morphology and livability) more effectively than the antibiotics.

Table 1: The results of quadruple culture the diluted buffalo bull semen

	Time (h)									
	0		2		6		12		24	
Drugs	D	D×2	D	D×2	D	D×2	D	D×2	D	D×2
Control	++++[a]	++++[a]	++++	++++	++++					
Tiamulin	++++	++ - -	++++	++++	++++	++++	++++	++++	++++	++++
Neomycin	++++	+ -++	++++	+- + -	++ -+	++++	++++	+- ++	++++	++++
Tetracycline	++++	+- +-	++++	+++ -	++++	+++ -	++++	++++	++++	++++
Fluorophenicol	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Penicillin	+ - - -[b]	- - - -[b]	+ -++	- - -+	+- - -	+ - -+	+ - -+	- - - -	++++	++++
Linco-Spectin	++++	- - - -	- - - -	++++	++++	++++	++++	++++	++++	++++
Erythromycin	++++	- - - -[b]	++++	- - - -	++ -+	- - - +	++++	++ -+	+ - -+	+ - - -
Oxytetracycline	- - - -[b]	- - - -[b]	- - - -	- - - -	++++	++ - -	++++	++++	+ - - -	+ - -+

+ = Colony growth (non-effective antibiotic), - = No colony growth (effective antibiotic), D = Recommended dose, D×2 = Twice the recommended dose (double dose), The superscript letter(s) denote a significant ($p = 0.001$) difference with the control

Table 2: Effects of double dose antibiotics on the semen characteristics

	Progressive motility (%)			Abnormal morphology (%)			Live sperm count (%)		
Hours*	0	18	F-T	0	18	F-T	0	18	F-T
Control	85±1.384[a]	85±1.050[a]	60±0.756	8±0.580[a]	8±0.058[a]	12±0.057[a]	92±0.00	92±0.00	70±0.809
Tiamulin	85±1.384[a]	80±0.931[b]	60±0.756	4±0.065[b]	4±0.065[b]	8±0.070[b]	92±0.00	92±0.00	72±0.934
Neomycin	80±1.384[b]	75±0.931[c]	60±0.756	4±0.075[b]	4±0.075[b]	10±0.077[c]	92±0.00	92±0.00	70±0.809
Tetracycline	85±1.384[a]	80±0.931[b]	65±0.749	5±0.089[e]	5±0.089[e]	12±0.047[a]	92±0.00	92±0.00	70±0.809
Fluorophenicol	80±1.384[b]	75±0.858[c]	60±0.756	2±0.094[d]	2±0.094[d]	10±0.077[c]	92±0.00	92±0.00	68±0.622
Erythromycin	80±1.384[b]	85±0.931[a]	65±0.777	2±0.094[d]	5±0.089[c]	10±0.085[c]	92±0.00	92±0.00	70±0.809
Penicillin	85±1.384[a]	85±1.050[a]	65±0.777	5±0.089[e]	5±0.089[c]	10±0.077[c]	92±0.00	92±0.00	70±0.809
Linco-Spectin	80±1.384[b]	80±0.931[b]	60±0.756	2±0.094[d]	8±0.058[a]	12±0.058[a]	92±0.00	92±0.00	70±0.809
Oxytetracycline	75±1.384[c]	75±0.858[c]	60±0.756	2±0.094[d]	5±0.089[c]	10±0.085[c]	92±0.00	92±0.00	70±0.779

*Hours after adding antibiotics and extender to the semen, F-T = Frozen - Thawed semen. The mean values assigned by superscript letter(s) denote a significant difference ($p<0.05$) between groups in each column

DISCUSSION

Contamination of semen during its collection for use in artificial insemination is an unavoidable event in AI programs. Different antimicrobial agents have been added to the semen extenders to overcome this problem. Although some antibiotics, such as penicillin (and streptomycin sulphate), have been used routinely for this purpose in buffalo and other animal species semen extenders for many years, their effectiveness should be checked from time to time to discover any possible bacterial resistance. In this study we tried to find a possible substitute for penicillin among commercially available antibiotics while evaluating its effectiveness. For this purpose 8 different antibiotics, including penicillin, were tested in recommended dose (D) and double dose (D×2) to evaluate their effectiveness and safety for spermatozoa and left for different times in contact with the semen to see whether duration of the time laps after being added to the extender might have any effect, as suggested by Parkinson (2001) for the bull and Douglas-Hamilton *et al.* (1984) for the stallion semen.

Present culture results showed that only oxytetracycline and penicillin in D and D×2 and erythromycin in D×2 were effective ($p<0.001$). Of these three antibiotics, oxytetracycline reduced semen quality as indicated by reduced percentage of motile spermatozoa and elevated percentage of sperms with abnormal morphology, so, not suitable for being recommended and erythromycine was effective only in double dose. Thus, the only antibiotic remaining to be recommended for controlling bacterial contamination of the buffalo semen is penicillin. Excluding Linco-Spectin in D×2, duration of exposing semen to the antibiotics before culture had no significant effect on their anti-bacterial potential.

Holzmann *et al.* (1984) have used tiamulin for eliminating *Mycoplasma* from bovine semen and concluded that addition of up to 70 mg/100 mL (700 μg mL^{-1}) is safe for the spermatozoa when motility and proportion of non-viable spermatozoa were used as criteria of the drug effect. Here, we tested 20 and 40 μg mL^{-1} (D×2) tiamulin which had no effect.

Shin *et al.* (1988) standardized bacteria to 10^5 to 10^6 CFU and inoculated into each mL of raw bovine semen and tested the effectiveness of a combination of gentamycin (500 μg mL^{-1}), tylosin (100 μg mL^{-1}) and Linco-Spectin (300/600 μg mL^{-1}) in controlling *Mycoplasma*, *Ureaplasma*, *Campylobater fetus* subsp. *Venerealis* and *Haemophilus somnus* contaminations and found them more effective for the control of *Mycoplasma* and *Ureaplasma* and equally effective for the control of *C. fetus* and *H. somnus* than standard combination of penicillin and dihydrostreptomycin and polymixin B sulphate. In present study Linco-Spectin in doses of 150/300 and 300/600 μg mL^{-1} (D×2) was not significantly effective.

Lorton *et al.* (1988) added Linco-Spectin at various concentrations to bovine neat semen and to egg yolk citrate, egg yolk tris and heated milk extenders prior to final processing for freezing to -196°C. After thawing, they measured seminal quality by progressive and acrosomal integrity evaluation and reported that Linco-Spectin (300/600 μg mL^{-1}) was not detrimental to seminal quality. Our results indicated that Linco-Spectin in double dose (300/600 μg mL^{-1}) had a significant effect ($p<0.05$) on the abnormal morphology of the buffalo semen.

CONCLUSION

It can be concluded from the results that penicillin G is still a suitable antibiotic for controlling bacterial contaminations of the semen used in AI.

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