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ANTIBIOTIC CONTROL OF CAMPYLOBACTER FETUS SUBSPECIES VENEREALIS IN DILUTED BUFFALO SEMEN STORED AT 5°C

BY

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SUMMARY

Two groups of antibiotic (Pencillin -G- sodium and Streptomycin or Gentamycin, Lincomycin, Spectinomycin and Tylosin) were evaluated for their ability to eliminate C . fetus subsp. Venerealis from diluted buffalo semen stored for 3 days at 5°C as well as their effect on sperm motility and percentage of spermatozoa with intact acrosomes. Semen was experimentally infected with three strains of C. fetus subsp. Venerealis before extended in two different diluents (egg yolk citrate and triladyl diluents). Our result revealed that group 1 and group 2 antibiotics had has the ability to eliminate C. fetus subsp. Venerealis from diluted semen without any deleterious effect on sperm motility or the percentage of spermatozoa with intact acrosomes.

INTRODUCTION

Campylobacter fetus infection is an important cause of infertility and abortion in buffalo. The disease is a venereal one, transmitted either by natural service or by artificial insemination. The disease caused mainly by C. fetus subsp. Venerealis. The infected bulls appear healthy and their semen appears normal (Garcia, Eaglesome and Rigby, 1983). Bulls may remain carrier for years, and they are regarded as causes of spreading of infection (Lander, 1988). The artificial insemination centres effectively has been eliminating C. fetus in extended frozen semen by treating raw semen with antibiotics and by having antibiotics in the extender as Polymyxin-B, Pencillin-G, and Streptomycin sulfate (Elliot, Murphy, Bartlett and Kubista, 1962, Seger, Lank and Levy, 1966, Howard, Vasquez and Amann, 1982, Shin, Kaproth, Lein, Arlitsch and Howe 1985). Combination of Pencillin and Neomycin or Lincomycin and Spectinomycin were added to semen diluent to eliminate C. fe-

tus (Almquist and Zaugg 1974). Incubation of C. fetus infected semen with Pencillin, Streptomycin Lincomycin and Spectinomycin reduced the numbers of C. fetus in diluted semen to nondetectable level (Chen, Redwood and Ellis, 1990). Recently combination of Gentamycin, Tylosin and Lincospectin antibiotics was more effective for control C. fetus subsp. venerealis (Shin, Lein, Patten and Ruhnke 1988; Guerin and Thibier 1993).

This study aimed to compare between the effect of addition Penicillin. G. sodium and Streptomycin (antibiotics combination used in A.I. centres in Egypt) and new antibiotic combination, Gentamycin, Lincospectin and Tylosin to eliminat C. fetus subsp. Venerealis on the sperm motility and the percentage of spermatozoa with intact acrosomes in diluted buffalo semen experimently infected with C. fetus subsp. Venerealis and stored at 5°C.

MATERIALS AND METHODS

I. Bacterial strains:

Three laboratory strains of Campylobacter fetus subsp. Venerealis obtained from Anim, Reprod. Res. Inst., Campylobacter lab., were used in this experiment. Two isolates were from preputial wash of bulls and one isolate was from semen. Each of the three strains was inoculated into 100 ml thiol broth in Rolex tubes and incubated at 37°C for 3 days in a microaerophilic condition (5 % O_2, 10 %, Co_2, 85 % N_2). The growth cultures were centrifuged at 6,000 r.p.m. for 15 min. and the sedment was resuspended in PBs (pH 7.2). The cell concentration was determined by the standard plate count procedure adjusted at $1x10^6$ organisms/ml. (chen, Redwood and Ellism 1990).

II. Semen processing:

Freshly ejaculated semen was kindly supplied by Abassia Centre for frozen buffalo semen belonging to General Organization for Veterinary Services. Semen was collected by using A.V. and examined for individual motility, sperm cell concentration and the percentage of spermatozoa with intact acrosomes (PIA) using unstained wet mounts examined under dark field microscope. The strains suspension in PBs were added to fresh semen ($1x10^6$ organisms/ml semen) and let to stand for 15 min. at 37°C.

Diluents preparation: For preservation of buffalo bull semen at 5°C,semen was diluted with each of two diluents. The first, was egg yolk-sodium citrate (EYC) composed of 20 ml egg yolk and 80 ml sodium citrate 2.9 % . The second diluent was. triladyl (tris based patent diluent, Mini-Tube Comp. Germany) which composed of tris, Citric acid, fructose and glycerol. Triladyl diluent was prepared as one part triladyl, one part egg yolk and 3 parts bi-distilled water with 7 % final glycerol concentration. Each diluent was divided into 2 portions and, 0.5 mg Streptomycin and 500 I.U. Pencillin (Group 1 of antibiotics combination) were added to each ml diluent in the first portion of both diluents. 500 µg Gentamycin, 300 µg Lincomycin, 600 µg Spectinomycin and 100 µg Tylosin (0.3 ml) (Group 2 of antibiotic

combimation) were added to each ml diluent of the second portion of both diluents.

Dilution process: "Campylobacter experimently infected semen was diluted 1:25 at temperature 35°C in the first portions of two diluents had group 1 antibiotics. One ml of infected semen was incubated with 1 ml diluent containing group 2 antibiotics for 15 min at 35°C before finally diluted to the prementioned ratio. (treated semen sample). Campylobacter infected semen was diluted without antibiotics in the two diluents to serve as a positive control. At the same time semen was diluted in both antibiotics groups-treated diluent prior to addition of campylobacter fetus subsp. Venerealis to serve as control negative. Immediately after dilution, diluted semen samples were coold and preserved at temperature 5°C. Diluted semen samples, negative control, antibiotics treated and positive control were examined for sperm motility and the PIA, immediately after dilution as well as 6 , 24, 48 and 72 h later.

Ten dilution trials were made to eliminate C. fetus and determine the effect of antibiotics and diluents on sperm motility and PIA in diluted buffalo semen.

III. Detection of C. fetus subsp. Venerealis in diluted semen sample: Samples from freshly collected semen prior to addition of C. fetus and the diluted semen after addition of antibiotics, negative control and control positive were cultured for detection of C. fetus organisms. After one hours, 6, 24, 48 and 72 h, all samples were examined under phase contrast microscope to detect C. fetus motility. Cultures were taken from all samples by inoculating 0.1 ml into semisolid thiol media and also blood agar plates, for detection of campylobacter organisms. All cultures and plates were incubated at 37°C for 3 days under microaerophilic conditions.
The suspected colonies were confirmed by morphology and biochemical test (Eogelsome and Garcia, 1992).

Estimates of motility and PIA were subjected to appropriate analysis of variance according to Snedcor and Cochran (1980). Whne significant effects were revealed, completed analysis was mde by examining the differences between means using Student's t-Test comparisons.

RESULTS

The effect of two antibiotic combinations with 2 different diluents on sperm motility and the percentage of spermatozoa with intact acrosomes of experimentaly infected semen with C.fetus are shown in table 1 and 2. Analysis of variance and Student's (t) test revealed that addition of 500 I.U. Pencillin G. sodium and 0.5 mg Streptomycin/ml in EYC diluent resulted in a significantly higher sperm motility than addition of 500 µg Gentamycin, 300 µg Lincomycin, 600 µg Spectinomycin and 100 µg Tylosin /ml of the same diluent. This significantly high sperm motility appeared in both treated and negative control semen samples diluted in EYC diluent with group 1 of anti-

Table 1: Effect of different antibiotics treatment and diluents on sperm motility at different time of preservation at 5°C (mean ± S.E.)

Diluent			0 h.	6 h.	24 h.	48 h.	72 h.
Egg yolk citrate	Treated	G. 1	76 ± 1.3	73 ± 1.1	68 ± 0.8 ^{aA}	59.5 ± 1.3 ^{Aa}	52 ± 1.1 ^{Aa}
		G. 2	75 ± 1.3	74 ± 1.3	64.5 ± 1.2 ^{b}	55 ± 1.3 ^{b}	48.5 ± 1.1 ^{Ab}
	Control -ve	G. 1	78 ± 1.3*	72.5 ± 0.8	67 ± 0.8 ^{aA}	60.5 ± 1.4 ^{Aa}	54 ± 1.2 ^{Aa}
		G. 2	78 ± 1.1*	74.5 ± 1.2	64 ± 1.00 ^{b}	56 ± 1.2 ^{b}	50.5 ± 1.1 ^{b}
Triladyl	Treated	G. 1	73 ± 1.1	71 ± 1.8	62 ± 1.5 ^{Ba}	53.5 ± 1.1 ^{Baa}	45 ± 1.5 ^{Ba}
		G. 2	74.5 ± 0.9	72.5 ± 0.8	66.5 ± 1.3 ^{b}	58 ± 1.7 ^{b}	52 ± 1.1 ^{Bb}
	Control -ve	G. 1	75 ± 1.1	71.5 ± 1.5	62.5 ± 1.1 ^{Ba}	54.5 ± 1.2 ^{Ba}	46.5 ± 1.3 ^{Ba}
		G. 2	76.5 ± 0.8	72.5 ± 0.8	66.5 ± 1.1 ^{b}	58.5 ± 1.5 ^{b}	53.5 ± 1.1 ^{b}
Control +ve	Egg yolk citrate		74.5 ± 1.00 **	67 ± 1.9 *	51 ± 1.00 *	33.5 ± 1.3*	13 ± 1.3*
	Triladyl		74 ± 1.00 **	67 ± 1.5 *	52.5 ± 1.1 *	35 ± 1.7*	15.2 ± 1.2*

G.1 = Diluted semen treated with 500 I.U. Pencillin-G-sodium and 0.5 mg Streptomycin/ml diluent.

G.2 = Diluted semen treated with 500 µg Gentamycin, 300 µg Lincomycin, 600 µ g Spectinomycin and 100 µg Tylosine/ml diluent.

Means with different alphapetical superscripts a, b for columns for effect of treatment,

A, B, for rows for diluent in both treated and control -ve and stars for effect of control +ve on the sperm motility are significantly different at least at $P < 0.05$

Table 2: Effect of different antibiotics treatment and diluents on the percentage of spermatozoa with intact acrosomes at different time of preservation at 5°C (mean ± S.E.)

Diluent			0 h.	6 h.	24 h.	48 h.	72 h.
Egg yolk citrate	Treated	G. 1	72.5 ± 0.8^{A}	70.4 ± 1.100^{A}	66.7 ± 0.9 ^{A}	56.3 ± 0.9^{a}	51 ± 0.7^{a}
		G. 2	72 ± 0.6 ^{A}	70.7 ± 0.8 ^{A}	66.3 ± 0.9 ^{A}	54.5 ± 1.2 ^{a}	50.3 ± 0.8^{a}
	Control -ve	G. 1	75.7 ± 0.7 ^{*B}	73.2 ± 0.5 ^{B}	70.1 ± 0.9 ^{*B}	57.7 ± 0.9 ^{*a}	52.6 ± 0.8^{a}
		G. 2	76.6 ± 0.6 ^{*B}	74.3 ± 0.9 ^{B}	70 ± 1.4 ^{*B}	56.8 ± 0.8 ^{*a}	51.5 ± 0.6^{a}
Triladyl	Treated	G. 1	73.3 ± 0.6 ^{A}	71.8 ± 0.8 ^{A}	68.7 ± 1.5 ^{A}	64.8 ± 1.3 ^{b}	59.8 ± 1.8^{b}
		G. 2	72.1 ± 0.5 ^{A}	69.5 ± 0.9 ^{A}	65.4 ± 0.9 ^{A}	62.1 ± 1.00 ^{b}	58.1 ± 1.8 ^{b}
	Control ve	G. 1	77.2 ± 0.6 ^{*B}	75.7 ± 0.7 ^{B}	73.4 ± 1.4 ^{B}	66.6 ± 1.3 ^{a}	60.5 + 1.7 ^{b}
		G. 2	76.4 ± 0.5 ^{*B}	74.5 ± 0.9 ^{B}	69.7 ± 0.8 ^{B}	63.9 ± 0.9 ^{b}	60 ± 1.5^{b}
Control +ve	Egg yolk citrate		72.2 ± 0.6 **	66.2 ± 0.8 *	57 ± 0.8 ^{**A}	51.2 ± 0.7 **	43.6±1.00^{**A}
	Triladyl		73 ± 0.6 **	66.7 ± 0.8 *	59.5 ± 0.8^{**B}	35 ± 0.6 **	47.4 ± 0.7^{**B}

G.1 = Diluted semen treated with 500 I.U. Pencillin-G-Sodium and 0.5 mg Streptomycin/ml diluent.

G.2 = Diluted semen treated with 500 µg Gentamycin, 300 µg Lincomycin, 600 µg Spectinomycin and 100 µg Tylosine/ml diluent.

Means with different alphapetical super scripts A, B for effect of treatment, compared with control negative a, b, for effect of diluent in both treated and control -ve and stars for effect of control +ve on PIA are significantly different at least at $P < 0.05$

biotic combination after 24 h of storage at 5°C and remained for 2 days later. On the contrary, the significantly high sperm motility resulted in treated and control negative semen sampels diluted in triladyl with group 2 of antibiotic combination after 24 h from dilution and storage at 5°C.

Semen experimentlally infected with campylobacter fetus subsp. Venerealis and diluted in both EYC and triladyl diluents without antibiotics (control positive) showed lower sperm motility than control negative or antibiotics treated semen samples immediately after dilution as well as during three days of preservation at 5°C.

Table 2 shows no significant differences between the effect of two groups of antibiotic combination on the PIA in treates semen samples in both diluent. Diluted semen samples treated with antibiotics in EYC and triladyl without campylobacter infection (control negative) has significantly higher PIA than antibiotics treated semen pre-infected with campylobacter immediately after dilution and for 24 h, but this difference disappeared after two days of storage at 5°C. The effect of diluent used for preservation of buffalo semen experimentally infected or free from campylobacter on the PIA appeared after two days storage at 5°C. Semen samples diluted in triladyl resulted in a significantly higher PIA in treated semen samples either by group 1 or 2 antibiotic combination and control negative than semen diluted in EYC 48 and 72 h after dilution. The significantly low PIA resulted in control positive semen samples diluted either in EYC or triladyl.

Diluted semen samples artificially infected with C. fetus subsp. Venerealis strains and treated with either group 1 or group 2 of antibiotic combination were negative for survival of C. fetus subsp. Venerealis strains after 1 h from treatment. C. fetus organism were not detected microscopically or isolated from semen samples experimently infected and treated with group 1 or 2 antibiotic combination as well as control negative semen samples. On the other hand control positive semen samples were positive for survival of C. fetus subsp. Venerealis and the microorganism could be detected microscopically and isolated from them.

During one trial for dilution and detecting the effect of antibiotics on semen parameters, motile Gram +ve bacilli were noticed and isolated from diluted semen samples treated with group 2 of antibiotics combination 24 h after dilution but campylobacter fetus subsp. Venerealis were not detected or isolated in the 10 trials. On the other hand, the addition of Pencillin G Sodium and streptomycin prohibited any growth of any microorganisms as well as campylobacter fetus subsp. Venerealis in the diluted semen stored for 3 days at 5°C.

DISCUSSION

The two combinations of antibiotics, Pencillin G Sodium and Streptomycin or Gentamycin, Lincomycin, Spectinomycin and Tylosin used

in our study for control campylobacter fetus subsp. Venerealis in diluted semen samples were equally effective. All strains of C. fetus were not viable within one hour of antibiotics exposure. All strains of C. fetus in the non antibiotic treated (control positive) samples were viable after dilution and during different days of storage at 5°C. On the other hand, antibiotics-treated and negative control diluted semen in EYC or triladyl maintained a high percent of sperm motility and good PIA during different days of storage at 5°C. Almquist and Zaugg (1974) reported that addition of 1000 I.U. Pencillin and 1000 µg Neomycin, 150 µg Lincomycin and 300 µg Spectinomycin or 1000 I.U. Pencillin and 1000 µg Streptomycin/ml milk diluent used for freezing bull semen in glass ampules results a good non-return rate (68.8, 67.6 and 66.6 % respectively) when treated semen was used in artificial insemination. They suggested that a combination of Pencillin and Neomycin or Lincomycin and Spectinomycin are satisfactory substitutes for Pencillin and Streptomycin in diluted bull semen without impairing fertility. Shin, et al. (1985) used 500 I.U. Polymyxin, 500 I.U. Pencillin-G and 1000 µg Streptomycin Sulphate to be incubated with one ml raw semen at 35°C for 10 min., before diluted in whole milk with the same concentration of antibiotics/ml . They found that combination of the pervious antibiotics had bactericidal effect rather than bacteriostatic activity against C. fetus.

In another trial, Shin, et al. (1988) claimed that combination of Gentamycin 500 µg, Tylosin 100 µg and linco-spectin 300-600 µg/ml diluent was more effective for the control of C. fetus subsp. Venerealis than the standard combination of Pencillin, Dihydrostreptomycin and Polymyxin-B-sulfate

Incubation of fresh diluted semen containing C. fetus subsp. venerealis with 500 I.U. Pencillin, 500 µg Streptomycin, 160 µg Lincomycin and 300 µg of Spectinomycin/ml diluted semen at 35°C for 0, 1, 2, 5, 10, 20 or 40 minutes reduced the number of C. fetus in semen to non-detectable level without significant reduction in sperm motlity (Chen, et al. 1990). Guerin and Thibier (1993), found that the combination of 500 µg Gentamycin, 100 µg Tylosin and 300-600 µg Linco-spectin/ml diluent was the most effective on the strain of C. fetus.

Krause, Weitze and Waberski, (1989) conducted a susceptibility test with tris-egg yolk freezing medium to demonstrate the effect of lincospectin on the diluted semen samples. They demonstrated that lincospectine in concentration 450 µg/ml diluted semen had no detrimental effect on sperm function.

Contrary to our results, Howard, et al. (1982) reported that treatment of raw semen or extender alone with antibiotics was not consistently effective in controlling C. fetus. The dual treatment of raw semen with 1000 µg Streptomycin sulfate and 1000 I.U. Polymycin B-sulfate/ml semen and incorporation of antibiotics, 500 I.U. Polymyin B. sulfate, 1000 mg Streptomycin sul-

fate and 500 I.U. Potassium Pencillin G/ml diluent were effective with egg yolk citrate and egg yolk -tris diluent. However, C. fetus survived in semen that had been treated with antibiotics and then extended with egg yolk tris containing antibiotics. Also . Eaglesome and Garcia (1992) observed that the addition of 500 µg Gentamycin, 300 µg Lincomycin, 600 µg Spectinomycin and 100 µg Tylosin did not a reduce the three strains of C. fetus subsp. Venerealis to non-detectable levels in semen stored in liquid nitrogen.

It can be concluded that addition of 500 I.U. Pencillin G. sodium and 0.5 mg Streptomycin/ml diluent was effective and inexpensive combination of antibiotics for control of C. fetus subsp. Venerealis in bullals semen.

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