Vet. Med. J., Giza 40, NO. 1, 1-9 (1992)

LISTERIA MONOCYTOGENS ISOLATED FROM FROZEN BULL SEMEN.

BY

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(Received: 11.12.1991)

INTRODUCTION

It became evident already in the early days of Artificial Insemiantion (A.I.) that certain bacterial and protozoal venereal diseases such as genital campylobacteriosis and trichomoniasis could spread by A.I. when semen from infected bulls was used. It has been later clearly documented that semen may serve as a vehicle for spread of many infected diseases in cattle as foot and mouth disease, infectious Pustular vulvo vaginitis, Blue Tongue and mycoplasmosis (Bartlett et al., 1976). It has been accepted that micro-organisms will survive during collection processing, freezing and storage of semen at least as do sperm cells. Macpherson and Fish (1954) demonstrated by laboratory methods that *L. monocytogenes*, *Brucella abortus*, *Corynebacterium pyogenes* and *Campylobacter foetus* can survive semen freezing process in a typical egg yolk-citrate extender without antibiotics. Blood and Henderson (1974) stated that the visceral infection of pregnant cattle from injection of infected materials with *L. monocytogenes*, as well as venereal transmission of *L. monocytogenes* may lead to abortion, expermintal inoculation of *L.monocytogenes* into rams had resulted in localization of the organism in testicles and epididymis and suggested the possibility of veneral transission

of *L. monocytogenes* (Smith et al., 1968). *Listeria monocytogenes* had been isolated from human semen (Toaff et al., 1962) who reported the possiblity of sexual transmission.

MATERIAL AND METHODS

A total of 96 straws of frozen semen were derived from lots of imported frozen semen received from different localities at different times during the last 4 years (1988-1991) as illustrated in (Table, 1). The lots of imported frozen semen were sent to the Pyramid Research Institute Labroatory in liquid nitrogen containers under the supervison of Vet. Organization. Each container contained several canisters, every canister contained a group of straws of semen which represent single bull. One straw from each canister was taken out and thawed in a water bath at 37°C for about 30 seconds. The outer surface of each straw was swabed with alcohol (70%), then both ends were cut under complete aseptic conditions and the content of each straw was delivered into a sterile screw capped tube. A measured part of each sample was spread onto Muller Hinton plates for Colony Forming Unit (CFU) count using the standard plate count procedure for determination of the concentration of extracellular bacteria in semen. The second part was directly inoculated onto Columbia CNA (Biomereux), blood agar and brucella agar media and incubated at 4°C and 37°C under 5-10% Co^2 for 24 - 48 hrs. While the third part was inoculated into trypticase soya broth (Difco) and incubated in refrigerator at 4°C for at least 4 weeks with periodical subculture onto Colombia CNA, blood agar and brucella agar media in duplicate then the plates were incubated at 4°C and 37°C under 5-10% Co^2 for 24 - 48 hrs, Suspected colonies were picked up for further identification according to Sneath et al.(1984).*Listeria* was identified by Gram stain,

cellular morphology, catalase test, motility test, haemolysis and other biochemical tests **(Table II)**. Pathogenicity to mice was studied according to Gray and Killenger (1966). The isolates were serotyped using the specific polyvalent and monovalent antiserum type 1 and 4 (Difco code No. 2300-50 & 2301-50). Broth for carbohydrate fermentation were incubated at 34°C and all tubes were held for 14 days. Plates for CAMP phenomenon (Christie et al., 1944) were incubated for 24 to 48 hrs at 34°C in candle jars. A positive CAMP reaction was recognized by the production of rectangular zone of complete haemolysis surrounding the listeria streaks within the weak secondary lytic zone produced by staphylococcal data toxin (Groves and Welshimer, 1977).

Table I: Number of frozen bull semen straws imported from different localities during the last four years:

Groups	Locality	No. of bull straws	Import-date
G1	France	12	1/88
G2	U.S.A.	6	2/88
G3	France	6	4/88
G4	U.S.A.	8	3/89
G5	Germany	3	6/89
G6	Denmark	4	6/89
G7	U.S.A.	9	7/89
G8	U.S.A.	7	12/89
G9	Germany	7	4/91
G10	U.S.A.	10	10/19
G11	U.S.A.	24	10/91

Table II: Biochemical, biological and serological tests performed on L. monocytogenes strains isolated from imported frozen semen.

Different test	Strain No.													
	G1a	G1b	G1c	G1d	G1e	G1f	G1g	G1h	G1i	G1j	GIIa	GIVa	GIVb	GVIIa	GIXa
Cytochrome oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Christensen's urea agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatine liquefication	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simmons citrate agar	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TSI agar for H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in 6.5% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bile tolerance 10%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid production from															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-
Lactose	-	+	-	+	-	+	-	-	+	+	+	-	+	-	+
Xylose	-	-	-	-	+	+	+	-	-	-	-	+	-	-	-
Rhamnose	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+
CAMP test	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+
Pathogenicity for mice	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+
Serological typing	I	I	I	4b	Non	Non	Non	I	Non	4b	Non	Non	!	4b	

Latin number denote the group No.
Arabic No. denote the isolate No.
- = Negative reaction.
+ = Positive reaction.
G = Group.

RESULTS

Out of 96 frozen bull semen samples examined, 14 samples were found to harbour *L. monocytogenes* using direct as well as cold enrichment procedures. The results of the bacteriological examination were summarized in(**Table II**). Eleven isolates of *L. monocytogenes* were pathogenic for mice and 3 isolates were non pathogenic. Nine isoaltes were positive biochemically and serologically for listeria polyvalent antisera, six of which were found belonged to *L. monocytogenes* serotype 1 and the others (3) belonged to serotype 4 b. Five isolates could not be confirmed serologically but only identified biochemically. The highest count of bacteria in semen straws occurred in G4 (9500/straw) **(Table III)**. all CAMP-positive isolates were pathogenic to mice, **(Table II)**.

Table III: Number of frozen semen straws positive for *Listeria moncytogenes* in relation to the total count of extra cellualr *L. monocytogenes* cells/straw

Group	No.of straws examined	Total positive sample	positive samples by direct isolation	positive samples by cold cold enrich-ment	Average bacterial cell/straws	
					Patho-genic	Non patho-genic
GI	12	8	5	3	6	2
GII	6	2	1	1	2	0
GIII	6	0	0	0	0	0
GIV	8	2	1	1	1	1
GV	3	0	0	0	0	0
GVI	4	0	0	0	0	0
GVII	9	1	1	0	1	0
GVIII	7	0	0	0	0	0
GIX	7	1	0	1	1	0
GX	10	0	0	0	0	0
GXI	24	0	0	0	0	0
11 G	96	14	9	5	11	3

DISCUSSION

The significance of contamiantion with potentially pathogenic non specific bacteria in frozen semen has not been settled. The previous trials were based on certain types and limited numbers of non specific bacteria in frozen semen Ostaszko (1976) counted 500 micro-organisms for 1 ml frozen semen as a limit for satisfactory conception rate of 66% and this was approved to propositions made to ISO (1976) but not accepted up till now. Also Debrughe (1961) added that the fertility rates dropped after the use of highly contaminated semen. In fact, the mentioned acceptable number of seminal flora in frozen semen is still a matter of controversy for many regards, Viz; whether or not there is a permissible number of flora that would have not been incriminated when subsequent infections or reproductive disorders take place, and on the other hand, if that number is only for an individual organism or it is a conclusive number for all types of microorganisms that might exist in frozen semen. The present work, which offers the results of the present work in this area of investigation since 1988, presents a powerful explanation for the failure of detection of *L. monocytogenes* by direct isolation from frozen semen straws, since this bacterium is a facultative intracellular parasite (Mackness, 1971), it needs cold enrichment incubation prior to isoaltion. Based on this fact, from the 14 samples which were found to harbour the organism, only 9 isolates were detected by direct isolation which represent the extracellular existance, while the other 5 isolates obtained by the cold enrichment procedures denote the intracellular existance of the bacterim.

The average of the total count of pathogenic bacteria ranged from 4500 to 9500 cells/straw. Since there is no justification for introducing requirements as the type and number of non specific

micro-organisms in frozen semen which might be acceptable for international trade (FAO, 1981), however, the count obtained during the present work strongly drives the attention to the role played by frozen semen as a good vehicle for venereal transmission of *L. monocytogenes*.

From the available literatures, there is a single report of the recovery of *L. monocytogenes* from the semen of rams and goats (Laing, 1970). An analysis of a possible association between repeated abortion in 50 women and L. monocytogenes infections in their husbands revealed the presence of *L. monocytogenes* in the semen of 3 of the husbands (Toaff et al., 1962).

The available literatures failed to reveal any record of infected frozen semen with *L. monocytogenes* therefore, the isolation of this genus from imported frozen semen was considered of interest. Also there is lack of evidence of a causal relationship between the presence of potentially pathogenic *L. monocytogenes* in semen and its fertilizing capacity.

SUMMARY

A total of 96 imported frozen semen straws represent 11 lots (groups) from different localities at different times during the last four years, revealed the isolation of *Listeria monocytogenes* from 14 samples by direct and cold enrichment procedures. Eleven isolates which were CAMP positive proved to be pathogenic to mice. Nine isoaltes could be identified biochemically and serologically where 5 isolates were identified only biochemically. The bacteriological count ranged from 4500-9500 cells/straw and the highest count was in group 4.

REFERENCES

1. Bartlett, D.E.; Larson, L.L.; Parker, W.G. and Haward, T.H. (1976): Specific pathogenic free frozen bovine semen. Proc. 6th tech. Conf. on AI. and reproduction NAAB. pp. 11-22.

2. Blood, D.C. and Henderson, J.A. (1974): Veterinary Medicine. 4th Ed; PP. 307-310.

3. Christie, R. Atkins, N.E. and Munch-Petersen, E. (1944): A note on a lytic phenomenon shown by grands B. streptococci. Aust. J. Exp. Biol. Med. Sci. 22: 197-200.

4. Debruyne, R. (1961): Het opvangen van sperma bi j stieren door massage der zaadampullae en Tijdschrift, XXX. Nr 9- (0: 221-237 in Flemish.

5. FAO (1981): Animal production and Health paper. Disease control in semen and embryos consultation Rome 23-27 February 1981. Annexes. I. VII.

6. Gray, M.L. and Killinger, A.H. (1966): *Listeria monocytogenes* and literic infections. Bacteriol. Rev. 30: 309-382.

7. Groves, R.D. and Welshimer, H.J. (1977): Separation of pathogenic from apathogenic *L. monocytogenes* by three in vitro reactions. Clin. microbiol., 5 (6): 559-563.

8. Laing J.A. (1970): Fertility and infertility in the domestic animals. Balliere, Tindall and Cox., London.

9. Mackaness, G.B. (1971): Resistence to intracellular infection. J. Infec. Dis. 123 : 439-445.

10. Macpherson, J.W. and Fish, N.A. (1954): The survival of pathogenic bacteria in bovine semen preserved by freezing. Am. J. Vet. Res. 15:548.

11. Ostazko, F.J. (1976): In: Variable fertility results in AI. centres and associated factors. Proc. 8th Int. Congress on Animal Reprod. and AI. Vol. 5: 1235-1237.

12. Smith, R.E. et al. (1968): Cornel Vet., 58:389.

13. Sneath, P.H.A.; Nicholas, S.; Elisabek S. and Holt, J.G. (1984): Bergey's manual of systemic bacteriology.

14. Toaff, R.; Kochik, N. and Rabinovilz, M. (1962): Genital listeriosis in the male. Lancet 2 : 482-483.