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Comparative study of physical properties and bacterial contamination between local and imported straws frozen semen

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Abstract
Objective of study to display the differences between the some semen characteristics of imported and frozen of artificial insemination center-Iraqi straws and evaluate the bacterial contamination in these are straws. This study was carried out on two types of frozen semen straws samples storage in liquid nitrogen: Frozen bull semen straws samples imported from, Holland, Germany and U.S.A. (from local market in Iraq) and Frozen bull semen straws samples production from three Holstein bulls used in artificial insemination center of Abou-Ghareeb western of Baghdad. After 48 hours of storage, thawing is achieved by placing the straw into a water bath at 37 ˚C for 30 seconds, then straw was cut from both sides and the first drop of semen was excluded while the second drop was evaluated for the percentages of motility, dead, abnormalities and concentration of sperms in each straws. In addition to bacteriological evaluation whether counting, isolation. Results revealed that the average individual motility percentage of sperm was significantly different between all straws which production from AI.center or imported. Also individual motility percentage of sperm for A.I.center straws more significantly $P<0.05$ than straws imported from Holland, Germany and U.S.A, and individual motility percentage of sperm in straws imported from for Holland increase significantly $P<0.05$ than Germany and U.S.A. The percentage of dead sperms for U.S.A straws and Germany straws different significantly $P<0.05$ compared with Holland straws and all straws which production from all bulls for A.I.center. Present study arise that percentage of abnormalities sperms for U.S.A straws different significantly $P<0.05$ compared with Germany, Holland straws. In addition to individual variation $P<0.05$ in abnormality sperms for straws which production from A.I.center, in spite of recorded lowest percentage of abnormal sperms for A.I.center straws compared with all imported straws. Concentration of sperms for A.I.center straws for all bulls more significantly $P<0.05$ than imported straws**.** Present study revealed that no isolated any bacteria from straws production locally whereas found in imported straws specimens were *Streptococcus faecalis* (16.6%) and *Staphylococcus aureus* (33.3%) and some gram negative bacteria *proteus spp.* (8.3%). Also there were mixed isolates (*Klebsiella spp.* and *Escherichia coli*) 33.3%, (*Pseudomonas aeruginosa* and *Escherichia coli*) 8.3%.

Keywords: Physical, Bacterial, Local, Imported, Frozen semen, Bull, Holestin

Introduction
Hallap., (2005). was indicated that the aim of the breeding industry is to identify genetically superior bulls and maximize the number of offspring conceived with their semen through artificial insemination (AI), thus increasing the dissemination of their genes. Artificial insemination (AI) has been a successful technique that is used for the breeding of cattle and other domestic animal species around the world [1]. The method is a valuable tool that benefits breeders to gain high quality genetic potential from proven bulls [2, 3]. Routinely, semen is packaged in straws approximately 0.25 ml or 0.5 ml, pellets and flattend plastic bags for freezing and storage. The frozen straws and flattened plastic bags are transported in liquid nitrogen for the artificial insemination [4, 5]. However, there is a risk of contamination of semen from pathogens during the packaging and storage that can adversely affect the fertility and reproductive efficiency [6]. In general, fresh semen or every ejaculate contains some nonpathogenic microbial contaminants that are not detrimental for the artificial insemination. However, the excessive load of microbial agents may result in infertile mating [7]. The semen may get contaminated with pathogenic and non-pathogenic microbial agents during processing and storage. These microbial agents gain access to the semen and can transfer serious diseases

in recipient farm animals. This may lead to bacteraemia, viraemia and local infections in different parts of genital tract [8]. According to literature data, the individual microorganisms can successfully survive the low-temperature storage of semen in liquid nitrogen (-196 ° C), both in semen straws, as well as in liquid nitrogen and ice sediment in the containers for storage of bull frozen semen [9]. Several studies evaluated the contamination of bacterial pathogens such as *Acinectobacter cacloaceticus, coxiella burnetti, Escherichia coli, Flavobacterium* species *Pantoeau agglomerans*, *Corynebacterium spp, Staphylococcus aureus, Micrococcus, Leptospira spp. Histophilus somni, Enterobacter cloacae*, *Brucellasuis, Ureaplasma diversum*, *Stenotrophomonas maltophilia*, *Enterobactercoccus, Staphylococcus sciuri*, *Chlamydophila abortus* and *Pseudomonas aeruginosa*, in the frozen semen of farm animals [8, 10-16]. Another's study isolated samples of *Candida albicans* in 9 and *Citrobacter Freundi* in 5 of 351 samples of deep frozen bull semen after thawing [17]. Abro *et al*., 2009. examined 100 samples of frozen bull semen and isolated seven different bacterial species (*Acinetobacter, Actinobacillus lignieresii, Citrobacter, Microccus luteus, Pseudomonas auruginosa, Staphylococcus epidermidis* and *Staphylococcus intermedius*). Bielanski *et al*., 2003. in their work indicated that they isolated 13 different bacterial species from deep-frozen bull semen which were stored in liquid nitrogen from 6 to 35 years. [19] in total of 35 samples of liquid nitrogen confirmed the presence of microorganisms in 21 samples, and identified *Citrobacter freundi, Klebsiella oxitoca, Acinetobacter braumannii, Acinetobacter Iwoffi, Pseudomonas stutzeri, Citrobacter diversus, Citrobacter koseri, Proteus mirabillis* and *Aspergillus sp*. The pathogenic bacteria in the ejaculates can induce a defect in semen parameters, such as reduce sperm count, poor morphology and motility [20]. The presence of a variety of microorganisms in semen leads in reduction of survival rate and fertility of cells; as a consequence, results in nonviable offspring. Therefore the suppression of undesirable contaminant microbial activity in breeder semen is a mandatory condition for artificial insemination of the pedigree stock [21]. Presence of bacteria, fungi and viruses has been detected in semen samples that deteriorate semen quality, as well as, transmit the pathogen to next generation [22]. The role of specific microbes in semen leading to reproductive disorder among dairy animals is well established, Despite sanitary precautions, several ubiquitous and opportunistic microbes find their ways into semen during harvesting, processing and storage of semen [23]. A correlation was shown to exist between bacterial load and semen quality. Increase bacterial load can lead to deterioration sperm motility and viability [24]. Moreover it has been reported that the frozen semen of the bulls has a higher microbial load [24, 25] and there is a highly signifycant negative (P<0.01) correlation of standard plate count with progressive sperm motility [24], live sperm [24, 26] and HOS % [24] both in neat as well as cryopreserved semen. Microbes can also have an indirect effect on motility of sperms by producing toxins [27, 28]. The presence of bacteria in extended semen creates competition for nutrients [29], and also results in the production of metabolic byproducts that may harm the spermatozoa. In addition, some bacteria contain lipopolysaccharides (LPS) in their cell walls which are released when they die; these LPS also cause damage to spermatozoa. The presence of contaminant bacteria in extended boar semen is associated with a decrease in sperm motility and viability [29, 30]. Moreover, bacteria may cause inflammation or disease in inseminated females [31]. Bacteria have a negative effect on sperm quality, either by directly competing with spermatozoa for nutrients supplied by the semen extender or by the production of toxic metabolic byproducts and endotoxins [32]. Therefore, the present study was designed to evaluate the differences between imported and frozen semen with respect to effect of microbial contaminations.

Materials and Methods

This study was carried out on two types of frozen semen straws samples storage in liquid nitrogen:

Type one: Frozen bull semen straws samples imported from, Holland, Germany and U.S.A. (from local market in Iraq)

Type two: Frozen bull semen straws samples production from three Holstein bulls used in artificial insemination center of Abou-Ghareeb western of Baghdad.

1-Estimation of physical properties of semen: Semen was routinely collected from all bulls weekly with the aid of an artificial vagina. As a routine work in the AI center, after collection of semen, the sample was immediately brought to the laboratory, and placed in a water bath at (37-38 ˚C) for evaluation of mass activity individual motility [33], and sperm concentration in straws [34], after evaluation ejaculates which individual motility more 50% diluted with tris-yolk-fructose-glycerol diluter, after 30 minutes, the diluted semen was evaluated for individual motility [33], dead and abnormalities percentages percentage using Eosin-Nigrosin stain [35]. Frozen diluents semen according to routing procedures used by AI Center.

2-Estimation of Microbial load : Blood agar were used for growth and isolation of bacteria from semen frozen straws production from three Holstein bulls used in artificial insemination center of Abou-Ghareeb western of Baghdad. Microscopic evaluation [36], then Gram stain were done to differentiate the bacteria is either gram positive cultured on Manitol Salt Agar [37].

Results and Discussion

1-Physical properties of semen

The average individual motility percentage of sperm was significantly (P<0.05) different between all straws production from AI. center or imported (Table 1). Average individual motility percentage of sperm for A.I. center straws of bulls No 2, No 3 were (60.34, 65.65),respectively; were significantly (P<0.05) superior over bull No1 (55.25), straws from Holland (40.66), Germany (35.34) and U.S.A (33.22); while as bull No 1 were more significantly (P<0.05) than straws from Holland, Germany and U.S.A, and individual motility percentage of sperm in straws for Holland increase significantly(P<0.05) than Germany and U.S.A, but no differences significantly between individual motility percentage of sperm in straws for bull No 2,3 and between Germany and U.S.A. In general Table (1) shows that overall mean of individual motility percentage of sperm for A.I. center straws (60.32) superior significantly (P<0.05) imported straws (37.34).

Table 1: Comparative between individual motility and dead percentage sperms for A.I center and imported straws. (Mean ±SE).

Straws	Bulls	Individual motility %	Overall mean	Dead%	Overall mean
A.I center Straws	Bull No 1	55.25± 6.34 b	60.32±8.21 a	32.15± 4.11 b	22.25 ±4.12 b
	Bull No 2	60.34± 8.23 a		19.34±5.19 c	
	Bull No 3	65.65 ± 4.32 a		10.25±4.12 d	
Imported Straws	Holland	40.66 ± 6.55 c	37.34±6.12 b	39.00 ± 3.33 b	50.12±10.32 a
	Germany	35.34 ± 8.45 d		55.34 ± 6.15 **a**	
	U.S.A	33.22 ± 5.22 d		59.62 ± 3.22 **a**	

Mean values in the same columns with different superscripts differ significantly ($P< 0.05$).

Wilmintje *et al*., 2016 show that Post thawing motility were varies among bull. Post-thaw sperm motility is the most commonly used parameter for evaluation of frozen thawed semen. There is a considerable variation among breeds and individual bulls in retaining fertilizing capacity after freezing and thawing [39]. Results in Table (1) revealed that percentage of dead sperms for U.S.A straws (59.6 2) and Germany straws (55.34) different significantly ($P<0.05$) compared with Holland straws (39.00), A.I. center straws bull No 1(32.15),bull No 2 (19.34) and bull No 3 (10.25),,and the differences significantly ($P<0.05$) between bulls No 1,2,3, and between Holland straws compare with Germany and U.S.A. straws,but the differed no significant between bull No 1 compare with Holland straws and between Germany and U.S.A, and the overall mean (Table 1) recorded lowest percentage of dead sperms for A.I. center straws(22.25) compared with imported straws (50.12).The present study Table (2) arise that percentage of abnormalities sperms for U.S.A straws (33.00) different significantly ($P<0.05$) compared with Germany (25.14), Holland straws (22.66), A.I. center straws for bull 1(19.25),bull 2(16.26) and bull No 3 (13.25),and bull No 3 straws different significantly($P<0.05$) compare with bull No 1,Holland and Germany straws and Germany straws differences significantly ($P<0.05$) compare with bull No 2, but non- significant difference between Holland and Germany straws and between Holland and bull1straws also between bull2 and bull3 straws,in spite of recorded lowest percentage of abnormal sperms for A.I. center straws(17.65) compared with all imported straws (26.38). [40] reported. Wide variations exist in properties of semen among different bulls as well as different ejaculates of the same bull. Present study showed in Table (2) that concentration of sperms(x106) for A.I. center straws for bull No 1 (15.24), bull No 2(20.00) and bull No 3 (20.12), more significantly ($P<0.05$) than

Table 2: Comparative between abnormality percentage and concentration sperm of A.I center and imported straws. (Mean ±SE).

Straws	Bulls	Abnormality %	Overall mean	Concentration x106	Overall mean
A.I center Straws	Bull No 1	19.25 ±2.00 dc	17.56±2.34 b	18.24 ± 0.045 c	19.12±0.031 a
	Bull No 2	16.26±1.23 de		20.00±0.033 b	
	Bull No 3	13.25±1.12 e		20.12±0.021 a	
Imported Straws	Holland	22.66±2.12 bc	26.38±4.12 a	9.20±0.001 d	7.67±0.003 b
	Germany	25.14±1.66 b		5.75±0.0023 d	
	U.S.A	33.00±3.13 a		7.78±0.006 d	

Mean values in the same columns with different superscripts differ significantly ($P<0.05$).

Holland **(**9.20**),** Germany **(**5.75**)** and U.S.A straws (7.78), (16.6%),in addition to the differences significantly ($P<0.05$) between concentration sperms in straws for bull No 1,bull2 and bull3 but the differences no significantly between all imported straws (Table 1), thus concerning overall mean of A.I. center straws (19.12) superior significantly ($P<0.05$) imported straws (7.67) [41]. show that significant differences in sperm concentration have been shown in semen from different bulls.

Bacterial contamination of semen

a. Biochemical tests results: The diagnostic and differential biochemical tests for gram-positive cocci and gram-negative bacilli isolated from semen of bull as in Table (3, 4, and 5).

b. Cultural results

All isolated bacteria found in imported straws specimens were *Streptococcus faecalis* and *Staphylococcus aureus* (33.3%) and some gram negative bacteria *proteus spp.* (8.3%). Also there were mixed isolates (*Klebsiella spp.* and *Escherichia coli*) 33.3%, (*Pseudomonas aeruginosa* and *Escherichia coli*) 8.3%.This finding is enclose agreement to [36, 42] in imported bulls. Abro *et al*., 2009 isolated and characterized 7different pathogenic bacteria from 100 frozen semen samples of cattle spp. [17, 22] isolated samples of *Candida albicans* in 9 and *Citrobacter Freundi* in 5 of 351 samples of deep frozen bull semen after thawing. Bielanski *et al*., 2013. in their work indicated that they isolated 13 different bacterial species from deep-frozen bull semen which were stored in liquid nitrogen from 6 to 35 years. [19] in total of 35 samples of liquid nitrogen confirmed the presence of microorganisms in 21 samples, and identified *Citrobacter freundi, Klebsiella oxitoca, Acinetobacter braumannii, Acinetobacter Iwoffi, Pseudomonas stutzeri, Citrobacter diversus,Citrobacter koseri, Proteus mirabillis* and *Aspergillus spp* [26, 36].

Table 3: Biochemical tests results for gram positive cocci.

Isolated bacteria	Biochemical tests					
	Motility on SIM	Catalase	Growth on MSA	Gelatin liquefaction	Coagulates	Citrate utilization
Staphylococcus aureus	–	+	+	Nt	+	Nt
Streptococcus faecalis	–	+	–	Nt	–	Nt

Nt = not test **+** = positive **–**= negative

Table 4: Results of sugar fermentation test

Isolated bacteria	sugar fermentation				
	Mannitol	Glucose	Sucrose	Lactose	Raffinose
Staphylococcus aureus	+	+	+	+	–
Streptococcus faecalis	–	+	+	+	–

+ = positive – = negative

Table 5: Biochemical tests results for Gram negative bacilli

Isolated bacteria	Biochemical tests								
	Lactose fermentation on MacConkey agar	Oxidase	Triple sugar iron test	Motility on SIM	Gelatin	Phenyl alanine test	Urease	Citrate	Indol
Escherichia coli	LF	–	A/A	V		–	–	–	+
Klebsiella pneumonia	LF	–	A/A	–	–	–	+	+	–
Proteus mirabilis	NLF	–	K/A	+	+	+	+	+	–
Pseudomonas aeruginosa	NLF	+	K/A	+	+	–	V	+	–

V= variable reaction between species, NLF= no lactose fermentation, – = negative, + = positive, A = acid reaction, K = alkaline reaction

Among the identified bacteria, *Bacillus cereus* [44], *B. licheniformis*, Micrococci [45], *Pseudomonas* [46].

In conclusion: Physical properties of the frozen semen produced locally by the AI center were better than the imported straws. In addition, there was no bacterial contamination in the locally produced straws, whereas bacterial contamination was found in the imported straws where the following types of bacteria were found (*Staphylococcus aureus proteus spp. Klebsiella spp.* and *Escherichia coli, Pseudomonas aeruginosa*)

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Authors' Contribution
All authors contributed equally in all the efforts for these articles

References

1. Hallap T. Assessment of Sperm Attributes of Frozen-thawed AI Doses from Swedish and Estonian Dairy Bull Sires, Doctoral thesis Swedish University of Agricultural Sciences, 2005.
2. Verkerk G. Pasture-based dairying: challenges and rewards for New Zealand producers, Theriogenology. 2003; 59(2):553-561.
3. Funk DA. Major advances in globalization and consolidation of the artificial insemination industry, J. Dairy Sci. 2006; 89(4):1362-1368.
4. Bwanga CO, Einarsson S, Rodriguez-Martinez H. Cryopreservation of boar semen. II: Effect of cooling rate and duration of freezing point plateau on boar semen frozen in mini- and maxi-straws and plastic bags. Acta Vet. Scand. 1991; 32(4):455-461.
5. Weitze KF, Stampa E, Richter L, Willmen T, Waberski D. Fertility of frozen boar semen: influence of packaging, number of inseminations and seminal plasma. Reprod. Domest. Animal, 1991; Suppl1:139-142.
6. Russell PH, Lyaruu VH, Millar JD, Curry MR, Watson PF. The potential transmission of infectious agents by semen packaging during storage for artificial insemination. Animal Reprod, Sci. 1997; 47(4):337-342.
7. Thacker BJ, Larsen RE, Joo HS, Leman AD. Swine diseases transmissible with artificial insemination, J Animal Vet. Med. Assoc. 1984; 185(5):511-517
8. Thibier M, Guerin B. Hygienic aspects of storage and use of semen for artificial insemination. Animal Reprod, Sci. 2000; 62:233-251.
9. Vakanjac S, Pavlovi M, Nedi S, Pavlovi V, Masgaš V, Maleti M, *et al*. Isolation of *Candida albicans* and *Citrobacter freundi* from deep frozen semen of bulls, XIII Middle Europian Buiatrics Congress, Belgrade, Serbia, 2013, 182.
10. Ramaswamy V, Latha N, Gnanasubramanian T, Manickam R. Aerobic bacteria in buffalo semen and their antibiogram, Indian J. Animal Reprod. 2002; 23:117-119.
11. Bielanski A, Bergeron H, Lau PC, Devenish J. Microbial contamination of embryons and semen during long term bankng in liquid nitrogen. Cryobiol, 2003; 46:146-152.
12. D'Angelo M, Pavło DL, Melo GM, Rojas N, Souza RJC. thayde. Acceptable microorganisms concentration in a semen sample for *in vitro* embryo production, Braz. J. Microbiol. 2006; 37:571-572.
13. Schlafer DH, Miller RB. Female Genital System. *In:* Grant MaxieM (ed.), Pathology of Domestic Animals, 5th edn. Saunders Elsevier, Philadelphia. 2007; 3:429-564.
14. Vinodh R, Raj GD, Govindarajan R, Thiagarajan V. Detection of Leptospira and Brucella genomes in bovine semen using polymerase chain reaction. Trop Animal Health Prod. 2008; 40(5):323-329.
15. Corona A, Cherchi R. Microbial quality of equine frozen semen. Animal Reprod. Sci., 2009; 115(1-4):103-109.
16. Hobson N, Chousalkar KK, Chenoweth PJ. Ureaplasma diversumin bull semen in Australia: its detection and potential effects, Aust. Vet. J., 2013; 91(11):469-473.
17. Vakanjac S, Pavlovi M, Nedi S, Magaš V, uri M, Maleti M *et al*. The possibility of contamination of deep frozen bull semen during long period of storage in containers with liquid nitrogen, 2014. Proceedings of the International Symposium on Animal Science 2014, Belgrade-Zemun, 2014, 343-346.
18. Abro SH, Wagam R, Tunio MT, Kamboh AA, Munir M. Biochemical activities of bacterial species isolated from the frozen semen of cattle, Agri Social Sci, 2009;

5(4):109-113.
19. Nedic S, Vakanjac S, Duric M, Maletic M. Mikrobiološka analizatesnog azota iz kontejnera za skladištenje dubokog zamrznutog semena, Reprodukcija domacihživotinja, Divzibare, Srbija, 2013, 181.
20. Khalili MA, Pourshafie MR, Saifi M, Khalili MB. Bacterial infection of the reproductive tract of infertile men in Iran, MEFS J. 2000; 5:126-131.
21. Amangeldy R, Nurlygul ZE, Kulzada ML, Lazzat MMM, Indira AM, Gulmira K. Development of Antibacterial complex for Sanitation of Boar and Bull Semen, (2013). Life Science Journal. 2013; 10(12s) http://www.lifesciencesite.com.
22. Joyjit Mitra, Sumit Chowdhury, Santanu Panda, Mrityunjay Chakraborty, Anup Singha. Microbiological evalution of bovine frozen semen samples in west bengal, India, 2016. Explor Anim Med Res, 2016; 6(2):185-191.
23. Sannat C, Nair A, Sahu SB, Sahasrabudhe SA, Kumar A, Gupta AK *et al*. Effectof species, breed, and age on bacterial load in bovine and bubaline semen, Vet World 2015; 8(4):461-466.
24. Shukla MK. Correlation of microbial load of cryopreserved semen with quality of neat and cryopreserved murrah buffalo bull semen, Buffalo Bull. 2005; 24: 84-87.
25. Jaisal S, Katoch RC, Chachara D, Mahajan A. Evaluation of bacterial load in fresh ejaculates of bovine and buffalo bull semen in Himachal Pradesh, Indian J. Anim. Sci., 2000; 70(5):465-467.
26. Ahmed K, Mohan G. Effect of antibiotics on the bacterial load and quality of semen of Murrah buffalo bulls during preservation, Indian J. Anim. Reprod. 2001; 22(1):79-80.
27. Morrell JM, Geraghty RM. Effective removal of equine arteritis virus from stallion semen, Equine Vet. J 2006; 38:224-9.
28. Fraczek M, Szumala-Kakol A, Jedrzejczak P, Kamieniczna M, Kurpisz M. Bacteria trigger oxygen radical release and sperm lipid peroxidation *in vitro* model of semen inflammatory, Fertil. Steril. 2007; 88:1076-85.
29. Althouse GC, Pierdon MS, Lu KG. Thermotemporal dynamics of contaminant bacteria and antimicrobials in extended porcine semen, Theriogenology. 2008; 70:1317-1323.
30. Bussalleu E, Yeste M, Sepúlveda L, Torner E, Pinart E. Effects of different concentrations of enterotoxigenic and verotoxigenic E. coli on boar sperm quality, Anim. Reprod. Sci. 2011; 127:176-182.
31. Jane M Morrell, Margareta Wallgren. Alternatives to Antibiotics in Semen Extenders: A Review, 2014. *Pathogens*. 2014; 3:934-946.
32. Morrell JM, Geraghty RM. Effective removal of equine arteritis virus from stallion semen, Equine Vet. J., 2016; 38:224-9.
33. Chemineau P, Cognine Y, Guerin Y, Orgeure P, Valtet JC. Training Manual on Artificial Insemination in Sheep and Goats, Food and Agriculture Organization of the United Nations, Rome, FAO, Animal Production and Health Paper, 1991, 83.
34. Kreem Iwaid, ALBadry, Sadeq jaafer Zalzala, Faris Faisel Ibrahim, Kamil Fayyad Hurish. Monthly and Seasonality Variation in Freezability of Semen and Associations Between Freezability of Semen With Age and Testicular Dimension of Holstein Bulls Born in Iraq, Paripex - Indian Journal of Research. 2016; 5:P35-38.
35. Bearden HJ. Fuquay, J.W. and Willard,S.T. Applied Animal Reproduction, 6th Ed. New Jersy. Pearson Prentice Hall, 2004.
36. Schulze J, Schiemann M, Sonnenborn U. 120 years of E. coli Its importance in research and medicine, 6th ed. Alfred-Nissle- Gesellschaft, Germany, 2006, 11-13.
37. Quinn PJ, Markey BK, Carter ME, Donnelly WJ, Leonard FC. Veterinary Microbiology and Microbial Diseases Textbook, Printed and bound in Great Britain by International Ltd. Mosby, London, Padstow-Cornwall. 2004, 118-126.
38. Wilmintje MNN, Iis A, Eros S. Semen characteristics and freezing capability of Madura's cattle. Tropentag Vienna, Austria September 18-21, 2016.Confconference on International Research on Food Security, Natural Resource Management and Rural Development organized by the University of Natural Resources and Life Sciences (BOKU) Vienna. 2016,
39. Mathur AK, Kumars S, Prabhkar JH, Mandal DK, Ingalemer, James J. Motility of frozen thawed semen after thermal resistance test, Indian Journal of Animal Reproduction. 2014; 35(1):18-20.
40. Hossain, Khatun MM, Islam MM, OF Miazi. Semen characteristics of breeding bulls at the Central Cattle Breeding and Dairy Farm of Bangladesh, *Bang. J. Anim. Sci.* 2012; 41(1):1-5.
41. Shelke VB, Dhami AJ. Comparative evaluation of physico-morphological attributes and freezability of semen of Gir cattle (Bos indicus) and Jafarabadi buffalo (Bubalus bubalis) bulls, Ind. J Anim. Sci. 2001; 71:319-324.
42. Bhatt CP, Mishra S, Bhatt AD, Lakhey M. Bacterial pathogens in semen culture and their antibiotic susceptibility pattern *in vitro*, International Journal of Biomedical Research 2015; 6(11):909-914. Cabell E Bovine abortion: A etiology and investigations. In Practice 2007; 29:455-463.
43. Bielanski A, Bergeron H, Lau PC, Devenish J. Microbial contamination of embryons and semen during long term bankng in liquid nitrogen, Cryobiol, 2013; 46:146-152.
44. Andersson MA, Jääskeläinen EL, Shaheen R, Pirhonen T, Wijnands LM, Salkinoja-Salonen MS. Sperm bioassay for rapid detection of cereulide-producing Bacillus cereus in food and related environments, Int J Food Microbiol. 2004; 94(2):175-183.
45. Abro SH, Abro R, Tunio M, Rind R, Bughio S. Evidence of bacterial contamination in the frozen bovine semen, Pak J Agri Agril Engg Vet Sci. 2015; 31(1):102-108.
46. Smole I, Thomann A, Frey J, Perreten V. Repression of common bull sperm flora and *in vitro* impairment of sperm motility with Pseudomonas aeruginosa introduced by contaminated lubricant. Reprod Domest Anim 2010; 45:737-742.