# PREVALENT BACTERIA ISOLATED FROM BOAR SEMEN EXTENDED WITH THE INCLUSION OF MORINGA LEAF EXTRACT (MLE) IN BELTSVILLE THAWING SOLUTION.

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## ABSTRACT:
Antimicrobial agents are added to semen extenders to control the growth of microbes contaminating semen. However, non-therapeutic use of antibiotics comes with its challenges as it may contribute to antibiotic resistance. This study compared the efficacy of Natural antibiotic source: MLE as an alternative to Penicillin and streptomycin. Forty ejaculate each of four exotic boar breeds (Large White, Landrace, Duroc and Gene Converter 750 boars) were collected using gloved hand technique and extended with MLE incorporated at 0, 0.25, 0.50, 0.75 and 1.00g/250ml of BTS although 0g inclusion had penicillin and streptomycin as control. Semen samples were analysed for microbiological contamination and isolate identity was deduced from the Bergey's manual of Systematic Bacteriology, results revealed absence of bacteria in 0g MLE for all breeds except Gene Converter 750 where *Alcaligenes spp* was found. Gram –ve bacteria were isolated in all the ejaculates with MLE (0.25-1.00g). *Pseudomonas spp, Aeromonas spp, Enterococcus spp, Alcaligenes spp, Serratia marcescens, Escheria coli* were isolated in all breeds. *Acinetobacter baumanii, Buchholdera mallei, citrobacter spp and Pseudomonas fluorescens* were also isolated. We inferred that the inhibitory activity of the MLE as an antimicrobial agent for swine extender may be limited or inadequate because of the high presence of gram –ve bacteria in MLE inclusions. The presence of bacteria would in turn lead to reduction in the quality of stored semen, the utilization of a higher dosage of MLE is recommended and procedures with high standards of hygiene and microbiological control.
**Key words**: Ejaculate, antimicrobial, antibiotic, extenders and bacterial contamination.

## INTRODUCTION
Bacteriospermia remains a challenge in processing boar semen; it leads to decreased shelf life, motility, sperm agglutination as well as reproductive problems in inseminated females (Althouse *et al*., 2000). The boar itself is a source of bacterial contamination in the ejaculate, likely due to the preputial diverticulum (Althouse and Evans, 1994). Appropriate hygiene during semen collection can decrease contamination (Althouse *et al*., 2005). Sources of contamination could also include human handlers, water used for semen extension, collection and laboratory equipment (Althouse *et al*., 2005). To determine the source of contamination, raw and corresponding extended ejaculates should be cultured, as well as the water source and laboratory equipment. Even if there is no known bacterial problem on site, semen should be cultured regularly to monitor for contamination (Reicks, 2003). Most boar semen extender contains at least one antibiotic with the aim of inhibiting the growth of bacteria; however, there are disadvantages to this practise both for sperm quality and for the environment. The antimicrobials may have detrimental effect on sperm survival, thus limiting the choice of agents that can be added to semen extenders. In a bid to reduce the toxicity caused by bacteria various highly potent broad spectrum antibacterial agents are used. However, use of antibiotics can contribute to the development of antibiotic resistance and this can in turn be transferred to other bacteria in other host species (Johansson *et al*., 2004) one of such extender with the inclusion of broad spectrum antimicrobials is the Beltsville Thawing Solution (BTS). The BTS was initially developed for frozen semen and later adapted to condition fresh swine semen at 15-18°C. Due to challenges of antibiotics resistance, unavailability, expensive price and the ban on some antibiotics the use of natural plant sources to boost and preserve semen is on the increase. *Moringa oleifera* as a plant has an impressive range of medicinal uses with high nutritional value throughout the world. *Moringa* contains pterygospermin which has powerful antibacterial effects (Rao *et al*., 1946). The leaves of *Moringa oleifera* have been reported to be a valuable source of macro and micronutrients, rich source of β-carotene, protein, vitamin C, calcium, potassium and act as a good source of natural antioxidants (Siddhuraju and Becker, 2003). A number of medicinal properties attributed to different parts of Moringa have been recognized by both Ayurvedic and Unani systems of medicines (Mughal

*et al*., 1999). Components of *Moringa* preparations have been reported to have antibacterial activity which includes 4–(4'-O–acetyl-α-L rhamnopyranosyloxy) benzyl isiothiocyanate, niazimicin, pterygospermin, and 4– (α-L-rhamnopyranosyloxy) benzyl glucosinolate (Fahey *et al.*, 2005). The benefit for the treatment or prevention of disease or infection that may accrue from either dietary or topical administration of *Moringa* preparations (e.g. extracts, deconcoctions, poultices, creams, oils, emollient, powders) are not quite well known (Palada, 1996) hence, culturing semen extended in BTS with MLE replacing antibiotics and identifying prevalent bacteria isolated in semen of four exotic breeds of boar is encouraging**.**

## MATERIALS AND METHODS

### Experimental site

The experiment was carried out at the Artificial Insemination Centre of the Trypano-tolerant Livestock Improvement Program of the Institute of Agricultural Research and Training, Ibadan (I.A.R & T) in Ikenne, Ogun state, West Africa (Latitude $6^0$55' N, longitude $30^0$35' E).

### Experimental animals and management

A total of 16 exotic boars of different breeds and of similar age (2year) and weight (150-200kg) namely 4 Large White, 4 Landrace, 4 Duroc and 4 Gene Converter 750 (GC 750) were used for the experiment. The boars were housed intensively and fed balanced ration according to NRC (2005) maintenance requirement for breeding boars.

### Methanolic extraction of *Moringa oleifera* leaf

Fresh *Moringa Oleifera* leaves were obtained from Bora Farm of the Institute of Agricultural Research and Training (I. A. R & T.) Ibadan. The leaves were air dried for two weeks and 2000 g of the powdered leaves was extracted with 500 ml of 70% methanol for 72 hours. The mixture was filtered and extracted on a water bath for evaporation and solidification of the extract (Afolabi *et al*., 2013).

### Preparation of the BTS extender

The Beltsville thawing solution (BTS) was constituted from: Glucose 39.1g, Sodium bicarbonate 1.32g, Tri-sodium citrate 1.32g, EDTA 1.32g, Potassium chloride 0.79g, Streptomycin 1.1g, Penicillin (IU/ml) 1.1g and 1000 ml Distilled water. The Moringa leaf extract were included at T1 (BTS+ antibiotics), T2 (BTS+0.25gMLE), T3 (BTS+0.50gMLE), T4 (BTS+0.75gMLE) and T5 (BTS+1.00gMLE)

### Semen Collection

Semen was collected from boars weekly using gloved-hand technique as described (King and Macpherson, 1973). Forty (40) ejaculates were collected from each breed between 8.00 and 10.00am over a 10 week period. The semen was extended with the constituted Beltsville thawing solution at a ratio 1:10. The extended semen were transferred into labelled tubes with the inclusion levels and then transferred in a climate box to the laboratory.

### Bacteriological Evaluation of Extended Semen

All semen samples were analysed for the presence of microbiological contamination by plating on the following media: Nutrient Agar, MacConkey agar (Oxoid), Blood agar, Sabouraud agar (Oxoid, UK), Manittol Salt Agar and nitrate reduction medium. The media were prepared according to the manufacturer's instructions and the plates were incubated in aerobic conditions at $37^oC$. The phenotypic properties of isolated bacteria were investigated using different methods, depending on type; the identification process was based on the use of the Gram staining and Analytical Profile Index systems (API 10S, API Staph and API 20NE; bioMe´rieux, France). Motility test, Catalase test, Oxidase test, Indole test, Starch hydrolysis, Methyl red test, Voges-Proskauer test, Citrate utilization test, Nitrate reduction test and Sugar Fermentation were all carried out as described (Bergeys manual of Systematic Bacteriology, 2000).

### Biochemical characterization of isolates

Pure cultures of bacteria isolates were subjected to various biochemical tests to determine the probable identity of the bacteria isolates. The result of each test was recorded and the probable identity of the isolates was deduced with reference to the Bergeys manual of Systematic Bacteriology (2000). Bacteriological characteristics were determined as described (Bezuidenhout *et al*., 2002).

## RESULTS AND DISCUSSION

The results of bacteria isolation obtained in this study showed that T1(0g MLE + antibiotics) inhibited the growth of bacteria in all boar semen except the Gene Converter 750, which had *Alcaligenes spp*. Inhibition of bacteria growth observed in T1 attest to the potency of synthetic antibiotics as reported by Vyt *et al*. (2007). The presence of gram –ve bacteria *Alcaligenes spp* in Gene Converter (GC) 750 could be breed dependent as reported by Kommisrud *et al.* (2002). Duroc semen with varying inclusions of MLE had *Alcaligenes spp and Acinetobacter iwoffi* at 0.25g, *Serratia marcescen* and *Alcaligenes spp* at 0.50g, *Citrobacter spp and E coli* at 0.75g inclusion while 1.00g MLE had *Pseudomonas auregenosa and Enterococcus spp*.

The semen samples of Large White had *Aeromonas spp* at T2, T3 and T4 while *Serrattia marcescens and E coli* were isolated at T5. The Landrace boar semen had no growth at T1, while T2 had *Buckholdera mallei;* T3 had *Shewanella putrifaciens and Pseudomonas flourescens,* T4 *had Shewanella spp and Shawanella putrifaciens* while T5 had *Pseudomonas Flourescens and Escherichia coli*. GC 750at T1 had *Alcaligenes spp,* T2 and T3 had *Acinetobacter baumanii* while *Alcaligenes spp* was isolated from T4. In T5, *Alcaligenes spp* and *Enterococcus spp* were the most prevalent. Enterococcus *spp, Pseudomonas flourescens, Serrattia marcescens, Citrobacter spp* were similar to bacteria reported by Althouse *et al*. (2000). Althouse *et al*. (2000) reported that potential sources of contamination include Laboratory equipment, disposable and non-disposable supplies, powdered/reconstituted extenders, boars and the boar stud environment. *Alcaligenes spp, Acinetobacter iwoffi, Aeromonas spp*, *Buckholdera mallei*,*Shawanella putrifaciens*, *Acinetobacter baumanii* and *Alcaligenes spp* were different from bacteria reported byAlthouse *et al.* (2000) and this could be as a result of ecological differences. The results from this study are in line with the works of Althouse and Lu 2005, Okazaki *et al*. 2010, U´ beda *et al*. 2013 and Kuster and Althouse (2016) that largely confirms the general view that Gram-negative bacteria, especially from the *Enterobacteriaceae* family are most prevalent in boar semen. These results are consistent with those of other authors, compiled by Maroto Martı´n *et al*. (2010), who concluded that, of at least 25 different bacteria genera identified in boar semen, the species *Pseudomonas*, *Staphylococcus*, *Proteus*, and *E. coli*, occur most frequently. The isolates in these results suggest the low efficacy of the MLE inclusions. Results are nevertheless consistent with the reports of Althouse *et al*. (2000) and Baracaldo and Ward (2008).

## CONCLUSION

The synthetic antibiotic (penicillin and streptomycin) remains more effective against bacteria in semen when compared to natural antibiotic source (MLE). This study discourages the utilization of all inclusion of MLE (0.25-1.00g) in semen extender BTS as this could result in reproductive challenges in inseminated gilt/sows. A higher dose of MLE is recommended for further research to examine if a higher dose could reduce or further inhibit bacteria population.

**Table 1: The prevalent bacteria isolated from semen samples extended with varying inclusions of MLE in BTS.**

| Breeds | MLE | Prevalent Isolates from sample with MLE |
|---|---|---|
| **Duroc** | T1 (0g) | No growth |
| | T2 (0.25g) | *Alcaligenes spp, Acinetobacter iwoffi* |
| | T3 (0.50g) | *Serratia marcescens, Alcaligenes spp* |
| | T4 (0.75g) | *Citrobacter spp, E coli* |
| | T5 (1.00g) | *Pseudomonas auregenosa, Enterococcus spp* |
| **LargeWhite** | T1 (0g) | No growth |
| | T2 (0.25g) | *Aeromonas spp* |
| | T3 (0.50g) | *Aeromonas spp* |
| | T4 (0.75g) | *Aeromonas spp* |
| | T5 (1.00g) | *Serrattia marcescens, E coli* |
| **Landrace** | T1 (0g) | No growth |
| | T2 (0.25g) | *Buckholdera mallei* |
| | T3 (0.50g) | *Shewanella putrifaciens, Pseudomonas flourescens* |
| | T4 (0.75g) | *Shewnell spp, Shawanella putrifaciens* |
| | T5 (1.00g) | *Pseudomonas Flourescens, Escherichia coli* |
| **GC 750** | T1 (0g) | *Alcaligenes spp* |
| | T2 (0.25g) | *Acinetobacter baumanii* |
| | T3 (0.50g) | *Acinetobacter baumanii* |
| | T4 (0.75g) | *Alcaligenes spp* |
| | T5 (1.00g) | *Alcaligenes spp, Enterococcus spp* |

*All the bacteria isolated are gram –ve

**REFERENCES**

Afolabi, A. O., Hameed, A. A. and Alagbonsi I. A. 2013. Effects of Methanolic Extract of *Moringa oleifera* Leaves on Semen and Biochemical Parameters in Cryptorchid Rats. *African Journal of Traditional Complement Alternative Medicine*.10(5):230-235

Althouse, G. and Hopkins, S., 1995. Assessment of boar sperm viability using a combination of two fluorophores. *Theriogenology*, 43: 595-603.

Althouse, G. C and Kuster, C. E., 2000. Field investigations of bacterial contaminants and their effects on extended porcine semen. *Theriogenology* 53(5): 1167-1176.

Althouse, G. C. and Lu, K. G. 2005. Bacteriospermia in extended porcine semen. *Theriogenology* 63(2): 573-584.

Althouse, G. C. and Evans, L. E., 1994. Closed resection of the preputial diverticulum in the boar. *Agricultural Practice* 15(9): 18-21.

Althouse, G., Kuster, C., Clark, S. and Weisiger, R., 2000. Field investigations of bacterial contaminants and their effects on extended porcine semen. *Theriogenology*, 53, pp. 1167-1176.

Althouse, G., Wilson, M., Kuster, C. and Parsley, M., 1998. Characterisation of lower temperature storage limitations of fresh-extended porcine semen. *Theriogenology*, 50: 535-543.

Fahey, J. W., 2005. *Moringa oleifera*: a Review of the medical evidence for its nutritional, therapeutic, and prophylactic properties. Part 1, *Trees for Life Journal*, 1: 5-7.

Kommisrud, E., Paulenz, H., Sehested, E., and Grevle, I., 2002. Influence of boar and semen parameters on motility and acrosome integrity in liquid boar semen stored for five days. *Acta Veterinaria Scandinavica*, 43: 49–55.

Mughal, M. H. S., Ali, G., Srivastava, P. S. and Iqbal, M., 1999. Improvement of drumstick (Moringa pterygosperma Gaertn.) A unique source of food and medicine through tissue culture, *Hamdard Medicus*, 42: 37–42.

Okazaki, T., Mihara, T., Fujita, Y., Yoshida, S., Teshima, H., and Shimada, M. 2010. Polymixin B neutralizes bacteria-released endotoxin and improves the quality of boar sperm during liquid storage and cryopreservation. *Theriogenology* 74: 1691-1700.

Palada, M. C., 1996. *Moringa oleifera*: A versatile true crop with horticultural potential in the subtropical United States. Hortscience 794-797.

Reicks, D. L., 2003. Bacterial contamination and semen quality. Proceedings of the Allen D. Leman Swine Conference: 169-170.

Rao, R. R., George, M. and Pandalai, K. M., (1946). Pterygospermin: the Antibacterial Principle of *Moringa pterygosperma*, Gaertn. *Nature* 158:745-746.

Saadabi, A. M., and Abu, Z. A. I., 2011. An *in vit*ro antimicrobial activity of *Moringa oleifera* L. seed extracts against different groups of microorganisms. *Asian Journal of Basic and Applied Science*. 5:129-134.

Siddhuraju, P., and Becker, K., 2003. "Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam.) leaves," *Journal of Agricultural and Food Chemistry*, vol. 51, no. 8: 2144–2155.

Vyt, P., Maes, D., Sys, S.U., Rijsselaere, T., Van Soom, A. 2007. Air contact influences the pH of extended porcine semen. *Reproduction of Domestic Animals* 42: 218-220.