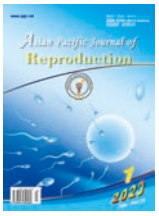

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Bacteriospermia among smallholder artificial insemination boars in the Philippines and potential associated factors

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ABSTRACT

Objective: To determine the prevalence of bacteriospermia, the bacterial load, and the potential factors associated with bacterial contamination in boar semen collected by local smallholder artificial insemination operators.

Methods: Fifteen individual raw semen samples were collected from locally available artificial insemination boars owned by different smallholder boar operators within the 5th district of Leyte, Philippines and were subjected to standard bacteriological culture and identification, including a survey of potentially associated factors. Prevalence and bacterial count were determined accordingly, while boar characteristics and collection practices were clustered following agglomerative hierarchical clustering technique.

Results: One hundred percent contamination with a bacterial count of $(2.01\pm0.38)\times10^3$ CFU/mL was observed. At least 73.33% of the samples were positive for *Bacillus* spp., while other identified isolates included *Enterobacter* spp., *Staphylococcus* spp., *E. coli*, *Pseudomonas* spp., *Citrobacter* spp., and *Klebsiella* spp.

Conclusions: Despite the high prevalence of bacteriospermia, the bacterial count is low. Nevertheless, on-farm practices on boar health and management, semen collection, and sanitation as well as the enhancement of basic protocols to control contamination should be conscientiously considered in smallholder artificial insemination operation.

KEYWORDS: Bacteriospermia; Local artificial insemination boars; Boar sperm; Artificial insemination; Semen quality; Smallholder pigs

1. Introduction

There are obvious reasons why artificial insemination (AI) continues to engage pig farmers with several countries around the world having adopted AI in the last 20 years[1]. Many in the developing countries have also realized the benefits of AI which include among others an increase in production efficiency and profit[2] thus making it more attractive to pig farmers[3]. AI has been instrumental in the control of venereal diseases and the promotion/adoption of new assisted reproductive biotechnologies such as sperm freezing, sperm sexing, and cryobanking among others[2,4–6]. Unlike the natural breeding, AI in pigs makes it possible to inseminate about 1 000-2 000 sows per year[4,7] from a single boar producing about 40 liquid-stored semen doses/week stored for about 5 days[8] with a particularly attractive farrowing rate[9]. In the Philippines where backyard pig farming comprises about 72.1% of the country's pig production[10], processing of boar semen by local AI boar operators for use in distance AI has become a profitable business (about $10.00/dose) and plays a pivotal role for sustained pig production in the face of African swine fever (ASF) and other pig epidemics.

Significance

Bacteriospermia may compromise fertility, requiring treatment and additional cost. However, appropriate regular testing in smallholder artificial insemination operations may be prohibitive and challenging. This study confirms the high prevalence of bacteriospermia and reports potentially associated factors needing attention by artificial insemination operators. Such findings may also call for more support from the industry including government and private sectors as more smallholder pig farmers turn to artificial insemination in the face of pig epidemics.

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The benefits of AI however can only be sustained by ensuring that only high-quality semen doses are used for insemination particularly when semen doses come from locally processed boar semen. Among others, semen quality may be directly or indirectly affected by housing conditions, feeding and nutrition, boar management[2,11–14], and the environment[15–17]. One important yet uncommonly checked aspect of semen quality is bacteriospermia or bacterial contamination of the seminal fluid. Several studies have reported that bacteriospermia could result in poor motility, agglutination, and acrosomal damage[18–20]. Other studies also suggest relevant effects on seminal pH, sperm survival during storage, capacitation, and litter size[20–23].

Different bacterial isolates have already been reported in boar semen[20]. However, there are inherent challenges to testing for bacteriospermia as conventional semen laboratory may not be equipped to conduct routine microbiological tests. Moreover, when semen processing by local AI operators is to be considered, it is hypothesized that bacterial contamination is highly possible and may originate from multiple sources either of animal or non-animal origin[24]. Our study focused on the profiling of bacterial contamination in raw boar semen used to prepare AI doses by local boar operators and sold to smallholder/backyard pig producers. Our aim was to determine the prevalence of bacteriospermia and potentially associated factors that could be useful in designing practical protocols that could help our local AI boar operators ensure high standards of hygiene and sanitation in processing boar semen.

2. Materials and methods

2.1. Boars and preparation of semen samples

Fifteen individual raw semen samples from sexually mature locally available AI boars (*Sus scrofa domesticus*) located within 10 km-60 km of the 5th district of Leyte, Philippines were used in the study. Laboratory experiments were conducted at the PCAARRD-funded boar semen laboratory of the College of Veterinary Medicine, Visayas State University, Baybay City, Leyte, Philippines (10°44'44.5"N, 124°47'48.5"E). These boars aged on average 2-3 years old have been sources of semen for AI doses in at least a year and were collected regularly for insemination of sows within their respective locality. Given that the available number of AI boars was limited due to ASF, raw ejaculates were collected only from selected boars known in the locality either per recommendations from the local agriculture office and only where the boar operators allowed. Boars were not subjected to any experimental manipulation to constitute ethical considerations and semen collection was done by individual boar operators according to their normal AI management practices.

A portion of the raw ejaculate was acquired each time and while at the boar operator's farm, the raw semen was initially filtered and collected into a standard plastic semen collection bag (US Bag® with filter and sprout, Minitube, Germany), placed inside an insulated semen flask, and transferred into a sterile 50-mL conical tube while ensuring as minimal contamination as possible. The semen sample was then transported to the laboratory on a motorcycle using an improvised portable boar semen shipper. Briefly, this portable semen shipper or cool box was made of a polystyrene medicine box (about 21.30 cm × 18.50 cm × 17.20 cm; side thickness: 1.5 cm; top-bottom thickness: 3.0 cm) painted with a commercial waterproofer/sealer on the inner and outer sides of the box as added insulation. Four 50-mL conical tubes may be accommodated inside the box individually inserted into a 4-hole plastic tube holder (with 29 mm wells). A single 400-mL frozen ice block (16.51 cm × 8.99 cm × 3.50 cm) was used to tightly support the plastic holder in place while providing an appropriate temperature inside (about 15 ℃-18 ℃). Further insulation and protection was provided by placing the box inside a thermal bag during the open-air transport on a motorcycle. The semen transport box was regularly sprayed with alcohol each time before use as standard practice to reduce potential contamination.

2.2. Bacterial culture and characterization

Standard microbiological procedure to grow and characterize bacterial contaminants in individual semen samples was conducted about 2-3 sessions/week depending on the availability of the boars and the capacity of the laboratory to carry out the work required. Briefly, upon arrival, about 1-2 mL of pure semen was aliquoted from the original raw semen sample and carefully brought next door to the microbiology laboratory of the College of Veterinary Medicine, Visayas State University for processing and culture.

Initially, appropriate serial dilutions were conducted in three separate tubes of buffered peptone water and 1 mL from each dilution was seeded onto the culture agar plates in three replicates. The sample dilution and agar medium were then mixed thoroughly and allowed to solidify. These inoculated plate count agar plates were then incubated at 37 ℃ for 24-48 h. Thereafter, using a manual colony counter, respective dilution dishes containing 30-300 colonies were counted and multiplied by the appropriate dilution to generate the colony-forming units per milliliter [colony forming unit (CFU)/mL].

Selected colonies from plate count agar were used for culture and identification of specific bacterial genus using different selective growth media and following standard plate streak technique. Bacterial isolates were identified based on their morphological and biochemical characteristics following IMViC tests (Indole test, Methyl red test, Voges-Proskauer test, and Citrate utilization test) as well as Triple Sugar Iron (TSI) tests, Urease test, and gas production among others. Selected cultures were examined under the microscope at 1 000× using an oil-immersion objective (Leica DM500) and photographed directly through the eyepiece (12 MP, f/1.8, 28mm iPhone 8).

2.3. Assessment of boar characteristics and collection practices

A simple survey was conducted upon sample collection through a one-on-one interview with the AI boar operator using a prepared questionnaire to collect information related to boar characteristics and collection/management practices. Relevant questions included boar characteristics (*i.e.*, age and breed) and collection/management practices (*i.e.*, collection frequency, use of gloves, sterilization of materials, cutting of preputial hears, among others).

2.4. Data management and statistical analysis

All data were entered into a spreadsheet using Google Sheet and a Comma Separated Values (CSV) file was downloaded as required by JASP (Version 0.16.2), a free and open-source statistical computer software[25] for appropriate descriptive statistical analyses. Results were expressed in percent contamination and the bacterial load in CFU/mL (mean±SEM). Different clusters showing boar characteristics and management practices by local boar operators were generated by agglomerative hierarchical clustering technique following multiple correspondence analysis using XLSTAT Version 2022.3.1[26] as previously described[27,28]. Thereafter, parallel coordinates plots were produced to better visualize the boar characteristics and management practices between each clusters.

2.5. Ethics statement

Boars were not subjected to any experimental manipulation to constitute ethical considerations. Participation in the interview survey was voluntary. A consent form was part of the interview questionnaire where boar operators were informed and asked to sign before proceeding with the questionnaire.

3. Results

3.1. Prevalence of bacteriospermia and level of contaminations in local AI boars

Fifteen different individual raw ejaculates were brought to the laboratory and successfully cultured for possible bacteriospermia. Following standard procedures on bacterial culture, the level of bacterial contamination was found at an average (mean±SEM) of $(2.01\pm0.38)\times10^3$ CFU/mL. Specific bacterial isolates include *Bacillus* spp. as the most prevalent (73.33%) followed by *Enterobacter* spp. and *Staphylococcus* spp. (60%). Other isolates of particular interest also because of their zoonotic potential include *E. coli* and *Pseudomonas* spp. (33.33%), *Citrobacter* spp. (20.00%), and *Klebsiella* spp. (13.33%). The remaining two contaminants included a possible *Pantoea* spp. (20.00%) and *Micrococcus* spp. (6.67%). The first six of these isolates were documented accordingly in Figure 1.

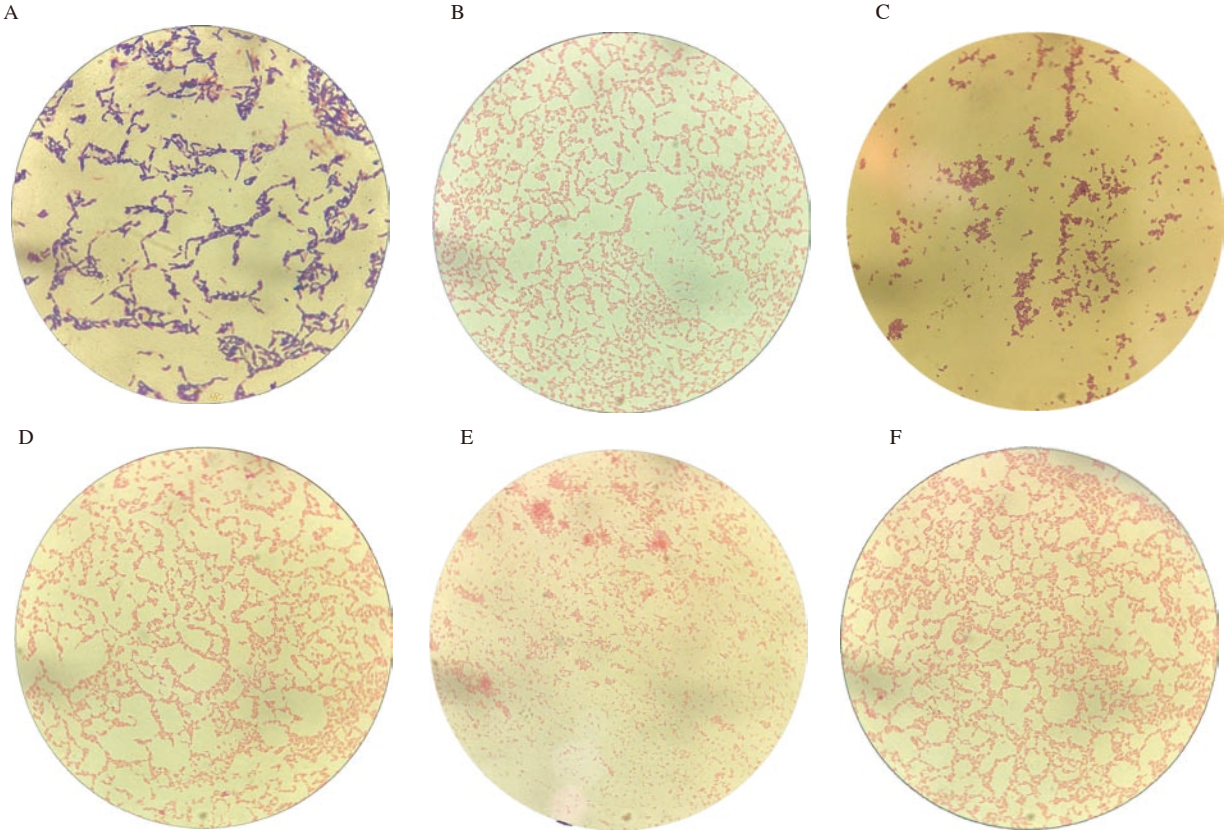

Figure 1. Bacterial contaminants in boar semen collected from local artificial insemination boars in the 5th District of Leyte, Philippines. *Bacillus* spp. (A), *Enterobacter* spp. (B), *Staphylococcus* spp. (C), *E. coli* (D), *Pseudomonas* spp. (E), and *Citrobacter* spp. (F). Magnification at 1 000× using an oil-immersion objective.

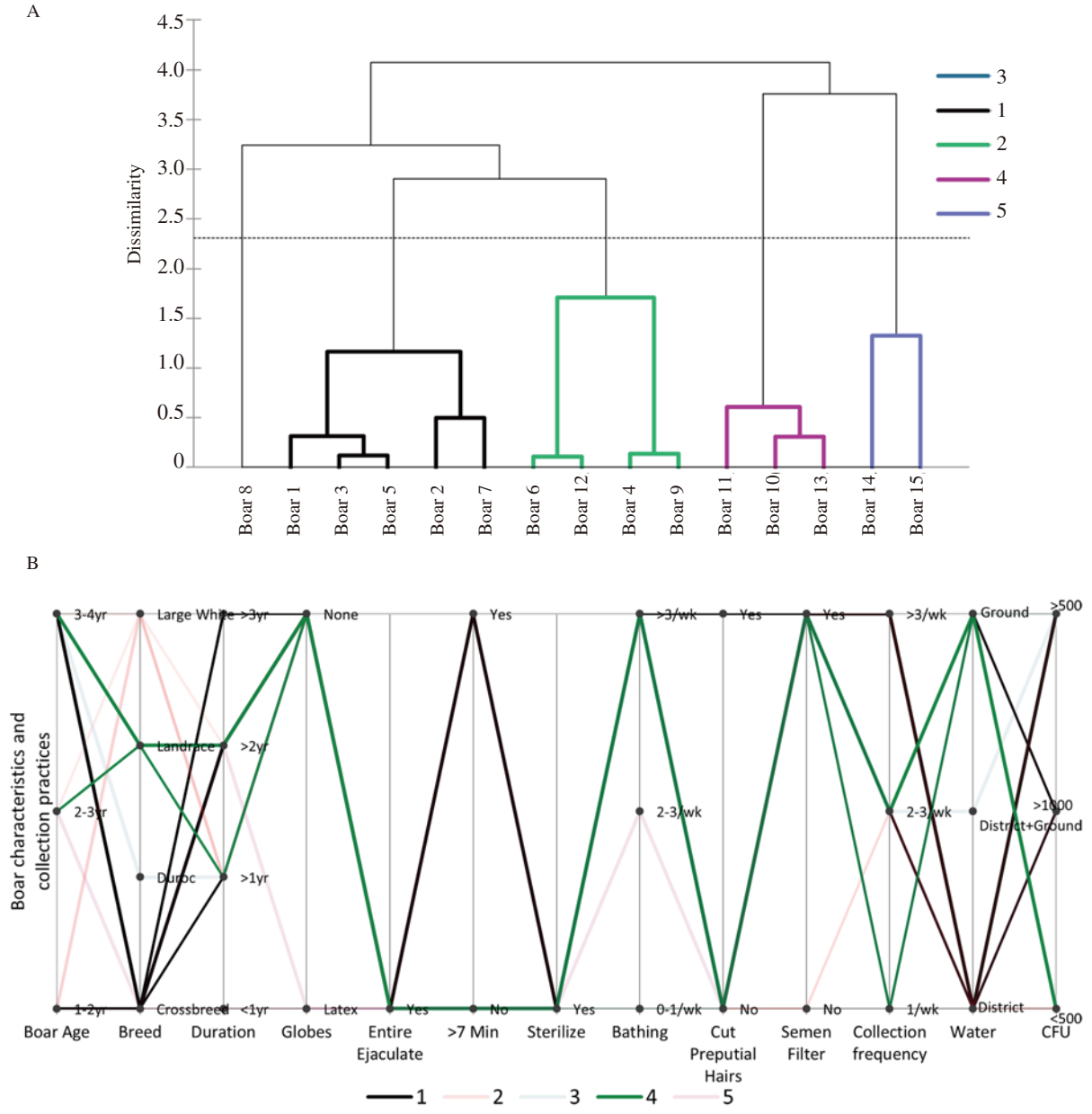

Figure 2. Dendrogram generated by agglomerative hierarchical clustering (A) and parallel coordinate plots (B) highlighting the boar characteristics and artificial insemination practices of local boar operators in cluster 1 (black) and cluster 2 (green) of five different clusters. Cluster 1 boars are characterized by boar age of >3 years (yr), crossbreed, no gloves used during collection, collection of the entire ejaculate, >7 min ejaculation time, no cutting of preputial hairs, and collection frequency of >3 times/week (wk), among others. Cluster 2 boars share almost similar characteristics except for a lesser collection frequency (once/week), purebreed (Landrace), and a lower CFU/mL.

3.2. Boar characteristics and collection practices

Majority of the ejaculates were collected from crossbreed boars (47%) while others came from Large White (27%), Landrace (20%), and Duroc (6.67%) breeds. Notably, the local AI operators who owned these boars had been into AI business for an average of 9.9 years with a minimum and a maximum of 1.5 years and 37 years, respectively. Figure 2 summarizes in five different clusters (Figure 2A) the basic characteristics of boars included in the study such as boar age and breed and the management practices by local boar operators (Figure 2B). Of the clusters generated by agglomerative hierarchical clustering, clusters 1 (boars 1, 2, 3, 5, and 7) and 2 (boars 4, 6, 9, and 12) comprised the majority of the boars at 33.33% and 26.27%, respectively. Cluster 1 boars were characterized by boar age of >3 years, crossbreed, no gloves used during collection, collection of the entire ejaculate, >7 min ejaculation time, cutting of preputial hairs, and collection frequency of >3 times/week, among others. Cluster 2 boars share almost similar characteristics except for a lesser collection frequency (once/week), purebreed (Landrace), <7 min ejaculation time, and a lower CFU/ml, among others. Interestingly, two boars belonged to the same cluster (cluster 5) for having one of the highest CFU/mL.

4. Discussion

Selling AI doses serves as additional source of income making it an attractive small business operation among local boar operators. This is especially true in light of government restrictions due to ASF and COVID-19 where boar-for-hire service boars are prohibited from entering other pig farms. It is important therefore to ensure that the quality of locally processed AI doses is not compromised both in terms of basic semen quality parameters and potential disease contaminants that could affect the overall reproductive performance of the pig farms served. On this study, we have successfully demonstrated the level of bacterial contamination in semen samples collected from AI boars owned and operated by smallholder boar operators, including the identification of bacterial isolates following standard laboratory culture techniques.

Bacterial contamination in boar semen may come from various sources either from animal or non-animal origin[24]. Several of these sources include the faeces, water sources, preputial fluid, feed and bedding materials, ventilation systems, and the personnel involved, among others[18]. Moreover, despite the popular use of the gloved-hand technique in boar semen collection[29], there is still the high possibility for contaminants to end up with the raw semen. While bacteriospermia may not always lead to infertility[30] or infection requiring treatment, there appears to be an extensive evidence showing its negative impact on sperm quality[31] including the possibility of transmitting diseases during AI[32]. Furthermore, bacteriospermia should not be treated lightly, as severe cases could be a sign of severe disruption in the normal microflora of the urogenital tract signifying disease status leading to unnecessary operational costs associated with treatment. It is also possible to suggest that such infections may lead to further complications in the reproductive tract of the male causing potential disturbances in the development and/or maturation of spermatozoa at those respective sections of the reproductive tract.

Reports of bacterial contamination in boar semen both in raw and extended AI doses are not new[24]. Our results show that *Bacillus* spp., *Enterobacter* spp., and *Staphylococcus* spp. were the most prevalent including *E. coli* and *Pseudomonas* spp. to some degree. Moreover, other bacterial isolates reported in this study have also been confirmed by other authors in earlier studies[18,20,21,31,33]. Fortunately, the level of bacterial load we have found may not be considered critical enough to cause major problems, as boar ejaculates were reported to contain on average 10^4-10^5 bacteria/mL[18]. Moreover, as much as 3×10^7 to 3×10^9 spermicidal bacteria have been considered to cause detrimental effects in a standard dose with three billion sperm[34], although this cutoff may have significantly changed in today's standards.

The literature provides an extensive evidence of the detrimental effects of bacteriospermia and may include reduction in sperm motility, sperm concentration, and incidence of spermatozoa with morphologically normal acrosomes[33]. Other studies have reported significant impact on sperm viability and progressive motility at high infective concentrations[35,36], sperm agglutination and acrosome damage[37], and high DNA fragmentation and significant negative effect on fertilization[38]. Based on these detrimental effects and the results of our study, there is a need to improve the existing practices in smallholder AI operations.

Since the steps involved with semen collection are most crucial to potential bacterial contamination[39,40], hygienic recommendations during the process of collection should be devised to minimize microbiological risks. This is particularly true in local AI operations where access to appropriate equipment and materials used for processing semen is limited. In particular, the use of appropriate gloves during collection should be strictly followed to minimize the risk of contamination. In fact, an over-glove may be used to manually evacuate first the preputial fluid to prevent it from trickling through the ejaculate[39]. Using latex-based gloves should also be avoided as this type of gloves are toxic to sperm[41].

Moreover, the current collection duration of >7 min may also be improved as such collection time may predispose to increased bacteriospermia[39]. Additionally, regular cutting of the preputial hairs may also be employed to reduce the risk of bacterial contamination in boar semen[13,20]. Preputial hairs may harbor different microorganisms from the environment and could be a good place for bacterial growth and multiplication. Finally, the practice of collecting the entire ejaculate which is true for all the local boar operators involved in the study may also be revisited. The pre-sperm fraction which is the first part to be emitted is normally absent of sperm[42] and is associated with high bacterial count[39] thus may not be collected after all to reduce bacterial contamination.

Overall, this study could provide baseline information on the level of bacteriospermia in backyard pig production. Some limitations include the identification of bacterial contaminants at the species level and a parallel examination on its impact on semen quality.

Since semen quality involves a host of complex factors allowing the sperm to undergo a series of physiological events including capacitation, acrosome reaction, and successful zona pellucida penetration, it is essential that sanitary procedures in the production of AI doses should be in place[40]. This is particularly important so as not to compromise the fertilizing capacity of spermatozoa associated with bacteriospermia that could result in reduced litter size[21]. Thus, ensuring quality control from semen collection to preparation, and extension to transport to minimize if not fully prevent bacterial contamination should be conscientiously considered.

In conclusion, this study confirms the high prevalence of bacteriospermia among smallholder AI boars while the bacterial load was relatively low. Nevertheless, on-farm practices on boar health and management, semen collection, and sanitation as well as the enhancement of basic protocols to control contamination should be conscientiously considered in smallholder AI operation.

Conflict of interest statement

The authors declare no conflict of interest.

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Authors' contributions

Santiago T. Peña, Jr. contributed to project administration, funding acquisition, study conceptualization, original manuscript writing, review, and editing, and data consolidation and analysis. Ma. Delia A. Pagente, Bianca Therese P. Ymas, and Mark Edd B. Janier equitably contributed to study conceptualization, methodology, sampling and laboratory works, data management and analysis, and review of the manuscript.

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