PREVALENCE OF MICROBIAL CONTAMINATION OF BULL SEMEN FROM YEAR 2018 TO 2021 IN NATIONAL INSTITUTE OF VETERINARY BIODIVERSITY

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ABSTRACT. Breeders in developing countries, particularly Malaysia, are embracing the advantages of using artificial insemination (AI) to produce crossbred progeny with better beef and milk production. This genetic composition of superior bulls helps in improving the product quality of the cattle population in Malaysia. National Institute of Veterinary Biodiversity (IBVK) is an institute that is responsible for supplying high quality bull semen for breeders. This paper provides an overview of bacteriological control in bull semen practiced by IBVK including the process of semen collection, handling, and storage of semen. The implementation of microbiological control is important because it represents the hygiene status of semen. The transmission of infectious disease can cause clinical effects on recipients, decrease productivity, and influence fertility. Furthermore, bacterial contamination in bull semen can also deteriorate sperm quality. Preliminary data were collected from the Department of Veterinary Services (DVS) system, Sistem Maklumat Makmal (SIMMAK) data. The result of this study found that only 0.5 % bacterial contamination was detected from 2018 until 2021 indicating that good hygiene procedures were used during semen collection, which correlate with sperm function and parameters. This study serves as a baseline for more extensive research to better control bacterial contamination in bull semen in future and contributes to the updated information on bull semen processing conducted in IBVK.

Keywords: semen quality, bacteriological control, artificial insemination, ruminant, cattle

INTRODUCTION

The livestock industry is experiencing a high demand for beef and milk production due to the growing population. This economic pressure drives industry to the application of artificial insemination among cattle breeders. This technique allows the use of semen from various bulls around the world without the requirement to transport the bulls, thereby opening up the possibilities of genetic diversity within a breed. Frozen semen has been widely used among cattle breeders in artificial insemination activities in Malaysia. Since 1990, frozen semen has been supplied by IBVK, an institute under the Department of Veterinary Services (DVS) for both department internal usage and commercial use. The source of genetic materials used for artificial insemination are from frozen semen of imported and native breeds such as Belgian Blue, Jersey, Sahiwal, Limousine, Holstein-Friesian, Mafriwal, Kedah-Kelantan, Charolais, and Brahman which are known for having good genetic qualities in terms of beef and milk production. These various bull breeds are maintained at IBVK. Semen collection processes including semen evaluation, dilution with extender, and packaging are routinely performed by IBVK.

To ensure the supplied semen is of good quality, IBVK cooperates with Eastern Zone Veterinary Laboratory (MVZT) Pahang to perform bacterial screening as part of the

quality control. The bull semen straws were delivered to MVZT after an assessment of quality frozen semen was conducted. There, the hygienic quality was measured through routine culture (Abadi *et al*., 2020). Routine culture is a test performed to detect the presence of microorganisms in the sample. The screening of bacterial contamination is crucial due to the fact that bacteria can negatively impact the sperm function (Martínez *et al*., 2014). Bacterial contamination in semen may lead to the spreading of infectious disease to recipient animals and transmission of bacteria can lead to long-term reproductive difficulties and low pregnancy outcomes including infection to the fetus (Aiden, 2012). Generally, standard practices and procedures are practiced by the technicians during the collection, processing, and storage of semen especially ensuring the laboratory to be in sterile and controlled condition.

To date, there have not been any recent studies conducted to evaluate the sperm microbiota associated with bull semen in Malaysia. Therefore, this study aims to determine the apparent prevalence of microbial contamination of bull semen collected in IBVK from 2018 to 2021.

MATERIALS AND METHOD

Semen collection and processing

Freshly ejaculated semen samples (Figure 2) were collected using an artificial vagina (Figure 1) by specially trained personnel. Semen concentration and initial percentage of motile spermatozoa were immediately analyzed after collection using digital photometer and computer assisted semen analysis (CASA), respectively (Figure 3). The semen samples were diluted 1:1 with commercial semen extender containing antibiotics. Semen was packed into polyvinyl chloride (PVC) straws (Figure 4) and balanced for 1 hour at 4°C. After equilibration, the straws were frozen in liquid nitrogen vapor using a computer controlled automatic freezer from 4°C to -15°C at the rate of -3 °C/min and -15°C to -80°C at the rate of -10 °C/min (Figure 5). Prior to cryopreservation in bank semen, sperm survival tests and bacteriological screening were carried out to evaluate the progressive character of the frozen-thawed sperm and hygiene monitoring, respectively (Barszcz *et al*., 2012).

Figure 1. Artificial vagina

Figure 2. Fresh semen of the bull

Figure 3. Semen analysis - the progression of spermatozoon

Figure 4. Semen packaging into 0.25 ml straw

Figure 5. Container with the frozen straws

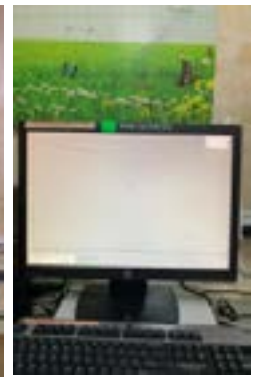

Figure 6. The programmed curve of freezing

Microbiological screening and data analysis

Between 2018 to 2021, a total of 1119 samples from various bulls breeds from IBVK were sent to MVZT (Pahang) for microbial screening. Information on the details about each bull such as breed, age, and sex were available based on the records at IBVK. All data of the samples were subjected to descriptive analysis and presented in graphical forms. For microbiological screening, three samples from each batch were randomly taken from the canister. Microbiological screening was conducted by the bacteriology section following standard procedures established by DVS (2011). Bacterial isolation for the semen samples were conducted in the biosafety cabinet. The semen samples were gently streaked onto the entire surface of blood agar and MacConkey agar using a sterile disposable loop (Figure 7). The inoculated plates were incubated for 24 hours at 37 °C (Figure 8). Following incubation, plates were observed for any bacterial growth and characteristics of each bacterial colony were recorded including the shape, size, color, surface texture, smell, and haemolytic activity. Interpretation of the result was made and reported as "negative" if no colonies formed (no growth of bacteria) on agar and "positive" if there was presence of colonies on agar. For the positive plate, a colony sample from primary culture was picked and streaked into new blood and MacConkey agar to obtain a pure culture of single type bacteria and incubated at 37°C prior bacterial growth and morphological observation after 24 hours. Smear preparation and gram staining process were subsequently done to determine gram negative or gram-positive bacteria. Gram negative bacteria results proceeded to the oxidase test while gram negative bacteria underwent catalase test of biochemical tests which were done by using Microgen® test kit. All the results obtained were then interpreted by the Microgen system where bacterial species were generated. The positive results were sent to the repeated microbiological screening of semen sample from the same batch of the initial batch submitted to MVZT for further confirmation of microbial contamination.

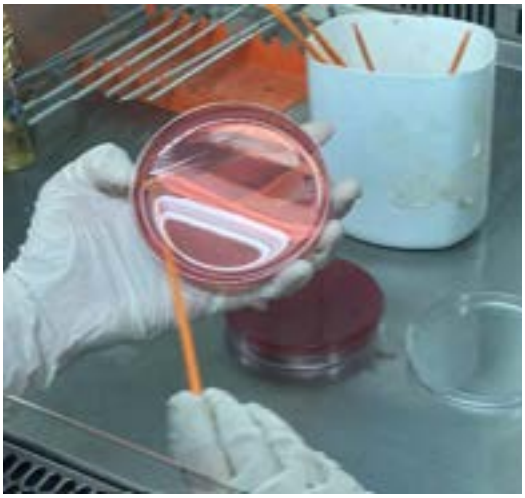

Figure 7. Bacterial isolation of semen sample on blood and MacConkey agar

Figure 8. Incubation for 24 hours at 37°C

RESULTS

Table 1 shows the prevalence of bacterial contamination observed based on the year of study from 2018 to 2021. The number of samples in 2020 decreased by 50 % due to Coronavirus Disease 2019 (COVID-19) lockdown restriction. Out of 1119 samples, six (6) samples were found to be positive, making the overall prevalence for 4 consecutive years low (0.5 %). The occurrence of bacterial contamination was calculated as the proportion of positive samples out of total samples received each year. Two bacteria species were identified throughout the period of study which were *Staphylococcus sp* and *Enterobacter* cloacae. *Staphylococcus sp.* was isolated from Jersey bull semen in 2019, while *Enterobacter* cloacae was isolated from Mafriwal bull semen in 2021 (Table1).

Table 1. Prevalence of positive samples for the year 2018 - 2021

Year	Total No. of Samples (N)	Positive Samples (N)	Bacteria Diagnosed	Prevalence
2018	354	0	Not detected	No bacteria isolated
2019	306	3	Staphylococcus sp.	0.9 % (2019)
2020	120	0	Not detected	No bacteria isolated
2021	339	3	Enterobacter cloacae	0.8 % (2021)

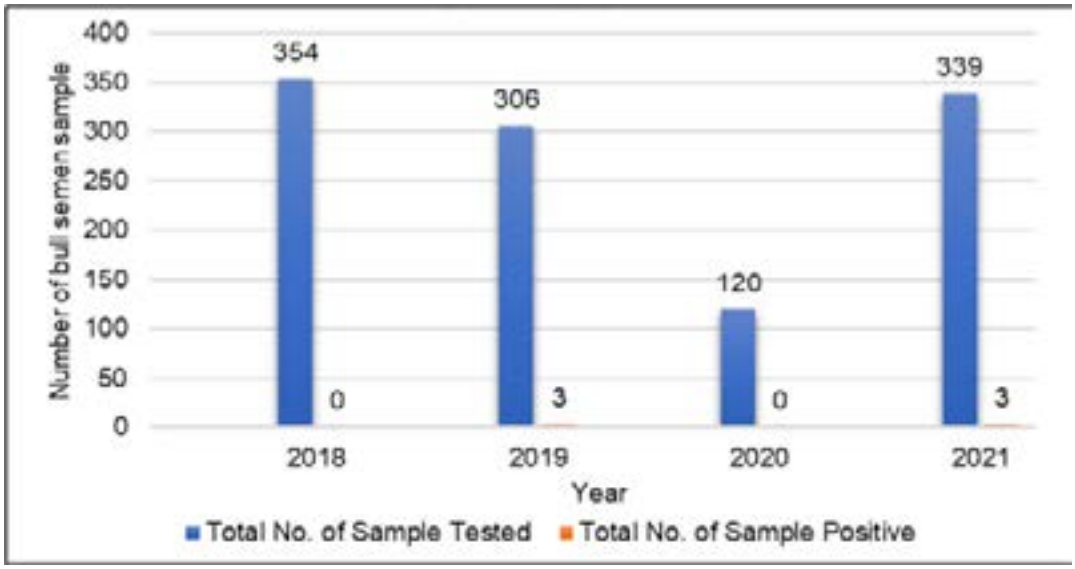

Figure 9. Number of bacterial contamination incidents detected by Eastern Zone Veterinary Laboratory, Pahang, Malaysia (2018- 2021)

From the in-house SIMMAK data, 0.8% (3/339) samples were detected with *Enterobacter cloacae* and 0.9% (3/306) samples were detected with *Staphylococcus sp*, respectively (Table 1 and Figure 9). However, data revealed that the repetition of the test showed negative results of contamination, and this was probably caused by non-animal origin. Another possibility might also be due to the airborne germs from the environment.

Staphylococcus sp and *Enterobacter cloacae*, the two microbes that were found are gram-positive and gram-negative bacteria, respectively (Foster, 2012; Mezzatesta *et al.*, 2012). On blood agar, the colonies of E. cloacae can be identified as whitish, round in shape, smooth, and flat colonies, whereas *Staphylococcus* sp appeared as golden-yellow colonies and often have complete hemolysis (beta hemolysis). According to Shi *et al*. (2016), *Staphylococcus* is one of the most common bacteria found in mammalian and human reproductive systems, and it is closely linked to infertility. Other studies found that Staphylococcal infection has a detrimental effect on sperm activity, including deterioration of sperm motility, morphology, and vitality as well as decrease in semen volume and concentration. Aside from that, *E. cloacae* is a normal microflora in both animal and human digestive systems. This species is a major bacteremia that also causes lower respiratory and urinary tract infections (Mezzatesta *et al.*, 2012). Previously, Prieto-Martinez *et al*. (2014) found that the presence of *E. cloacae* in boar semen impaired sperm quality and semen preservation.

In terms of artificial insemination, contamination with these species; *Staphylococcus sp* and *E. cloacae* causes disruption on the sperm function which consequently reduces the efficiency of artificial insemination and lowering the likelihood of success (Noelia *et al.,* 2014). Althouse (2008) reported that contaminated semen used in an artificial insemination activity has resulted in post-insemination vulvar discharges and reduced herd reproductive performance.

Moreover, the relationship between bacterial contamination and sperm function has been widely investigated in other organisms including humans and other livestock. Previous study by Abadi *et al*. (2020) stated that at least 25 different genera of bacteria have been detected in contaminated semen including the species of *Corynebacterium, Staphylococcus, Micrococcus, Bacillus, Escherichia, Proteus, Pseudomonas, Klebsiella, Streptococcus, Citrobacter, Enterobacter*, and *Stenotrophomonas*, the most commonly occurring ones being *E. coli* and *Staphylococcus aureus*. According to a study by Martin *et al*. (2010), the presence of *E. coli* in boar semen causes sperm agglutination and has a negative impact on the size of the offspring born from sows inseminated with such semen. Eventually, this is in line with the finding of a report from Moretti *et al*. (2009), who worked on human semen and found that *E. coli* infections in semen resulted in reduced sperm motility as well as causing disrupted acrosome function. These microorganisms can contaminate semen by producing harmful metabolic byproducts and competing with spermatozoa for nutrients provided by the semen extender (Althouse, 2008).

According to Zalzala *et al*. (2017), non-pathogenic microbial contaminants that are present in fresh semen are typically not dangerous for artificial insemination and are not a major cause of infertility. However, earlier studies showed a significant reduction of sperm motility and detrimental effect on the sperm quality due to high amounts of microorganism presence in semen of boar which resulted in mating sterile as bacteria can negatively affect the sperm function (Goldberg *et al*., 2013). In addition, a study by Sanocka *et al*. (2005) reported that extended semen with high levels of bacterial contamination has a shorter shelf life and higher occurrence of sperm agglutination. Sperm agglutination is a condition in which motile sperms stick to each other with various orientation, resulting in damaged acrosomes and reduced sperm motility (Barszcz *et al*., 2012). As a result, it is critical to avoid factors that could lead to contamination because it brings lots of negative impact on the animal reproductive system and production.

DISCUSSION

In Malaysia, the prevalence of microbial contamination in bull semen has been poorly studied until now. The use of frozen semen for artificial insemination is becoming extremely prevalent in commercial beef and dairy cattle due to the fact that this technique can increase economic income and has high output (Manafi, 2011). The broad use of artificial insemination in the cattle industry requires a high standard hygienic control of bull semen as the presence of bacteria can negatively impact the sperm quality, which further results in economic losses for breeders and industry players. A more thorough study pertaining to microbial contamination loads in semen should be conducted in the future in order to ensure the hygiene status of quality semen.

Previous studies suggested that improving sanitary procedures, keeping bulls clean, regular rinsing of preputial cavity, and addition of antibiotics are all effective ways to improve semen quality (Mitra *et al.,* 2016). Other than that, it is also important to ensure that the bull is not stressed as study by Sannat *et al*. (2015) mentioned that environmental conditions can cause stress to bulls and could also contribute to the proliferation of the bacterial population as some exotic breeds are unable to withstand Malaysia's climate. Some studies also discovered several aspects that may contribute to bacterial loads in semen and potentially impacting the

operation efficiency. The factors can be classified either from animal or those of non-animal sources such as hygiene parameters pertaining to the male (feces, age, preputial fluid, and length of preputial hair), proper pre-collection hygiene (unsterilized equipment, dust, dirty benchtop, human contamination, and poor hygienic environments), cleanliness, ambient temperature, and humidity (Goldberg *et al.*, 2013; Althouse, 2008). Study by Abadi *et al*. (2020) reported that microbial contamination of bull semen is associated with unhygienic conditions during semen collection, transportation, and processing. Additionally, it has been discovered that bacterial contamination is also found to be associated with types of bull breeds. The type of breeds has been found to yield the same or different bacterial strain (Sannat *et al.*, 2015). Table 2 lists the common sources of bacterial contamination in semen and preventative measures.

CONCLUSION

Overall, this finding showed that good hygiene procedures were practiced during semen collection, processing, and storage in IBVK. However, there is still room for improvement in order to constantly produce high-quality semen, including a focus on proper and safe semen handling as well as maintaining a hygienic environment during semen processing. The safe storage of germplasm and prevention of disease transmission to recipients are required as those factors have a big impact on the country's livestock economy.

Table 2. Common sources of bacterial contamination in semen and precautions to minimize bacterial contamination

Sources of Bacterial Contamination	*Precautions*
Fecal	Cleaning and drying of the ventral abdomen are required before semen collection
Skin/hair	Trimming any hair surrounding the preputial orifice
Preputial cavity fluids	Clean the preputial opening and the surrounding area if the area is wet
Personnel/ technicians	Avoid any contact with materials using bare hands and wash hand regularly
Water	Checking on purified water should be done at least once every three months
Fume hood ventilation system	Regular calibration is necessary to ensure effective operation
Laboratory cleanliness	Thorough cleaning, disinfecting contaminated lab equipment, and countertop
Reusable laboratory materials	Clean using a laboratory grade detergent with water, then rinse with distilled water and finish with a 70 % alcohol rinse
Semen extender	Divide bulk products into smaller portions for daily use

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