Bacterial contamination and antibiotic susceptibility profile of *Sus scrofa domesticus* semen

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Abstract

The fastest-growing industry among all livestock is piggery, with traditional pig farmers from the lowest socio-economic strata in charge of the pork sector and pig raising. Artificial insemination (AI) is the most widely used method for reproduction, aiming to boost fertility rates and increase supply. However, sperm quality can be negatively impacted by various factors, such as varicocele, accessory gland infection, immunological factors, and endocrine causes. Antimicrobial control in semen dosages is essential due to high storage temperatures. Ten semen ejaculates were collected from Intensive Cattle Development Project (ICDP), Upper Shillong weekly using gloved hand method from boars, mounted on dummies, and placed in pre-warmed bags and laboratory analysis was conducted in North-Eastern Hill University, Shillong and identification was confirmed at Pasteur Institute, Shillong. *Pseudomonas aerugionosa* is the most predominant bacterial species found in this study. Antibiotic resistance rates show 100% for Tetracycline, Ciprofloxacin, Imipenem, 90% for Gentamicin, 80% for Kanamycin, and Chloramphenicol. Understanding microorganisms and using advanced techniques like artificial insemination (AI) is crucial for improving the pig business. Breeders need a high-quality and high-quantity semen supply to improve production. Analyzing microorganisms in semen is essential for understanding the effects of collecting and storing swine semen in extenders. Monitoring diet, intake, and surroundings is crucial for boar semen utilization. Researching and boosting reproductive rates is essential for maintaining a balanced pig business. Implementing semen microbiological procedures is simple and affordable.

Keywords: boar semen; sperm; infection; bacteria; artificial insemination.

Introduction

In emerging nations like India, agriculture is significant. Besides from supplying the nation with food, agriculture also frees up labour, generates savings, supports the market for industrial goods, and generates foreign exchange. The development of agriculture is essential to the growth of the economy as a whole (Kumar et al., 2015). The fastest-growing industry among all livestock is piggery, which is thought to be the most productive. In India, traditional pig farmers from the lowest socio-economic strata are in charge of the pork sector and pig raising. The pig rearing is a source of livelihood and sustainable income generation of farmers in the rural areas of Terai region and hilly tribal terrains of North East India (Das et al., 2021; Muhindro Singh 2023). A well-managed pig production system may significantly reduce hunger, provide nutritional security, and stop environmental deterioration (Monteiro et al., 2021). When artificial insemination is used to produce animals, male fertility is of the utmost importance. Hence for the pig business, tracking and evaluating the quality of boar semen has significant economic implications (Tripathi et al., 2010). Sperm quality can be negatively impacted by a variety of circumstances, such as varicocele, accessory gland infection, immunological factors, and endocrine causes (Birthal et al., 2012). To achieve a greater rate of success in the process of fertilization, most widely used process is the Artificial Insemination.

The fertility rate has been significantly dropping around the globe in recent decades. To feed the expanding population in all of the nations, this dropping rate poses a serious challenge. Techniques like artificial insemination can be used to boost fertility rates and increase supply in order to close the supply-demand imbalance. In most intensive pig production systems around the world, artificial insemination (AI) is the favoured method for reproduction (Lopez et al., 2017). In order to provide disease-free bull studs with semen free of possible pathogens, national and international organizations have established rules. Cattle are most frequently used in the global distribution of frozen semen. Following the eradication of the main epizootic diseases, emphasis has been directed on illnesses including IBR, BVD, and blue tongue, where clinical symptoms are seldom noticeable but viral detection in semen is crucial (Philpott 1993). To do this, fresh extended ready-to-use semen doses are either produced on-site by boars or obtained from AI centres. To produce semen profitably, it is vital to get the most high-quality semen possible from each boar (Kashiha et al., 2013). Due to the relatively high storage temperature (17 °C), antimicrobial control in semen dosages for artificial insemination of pigs is essential (Luther et al., 2021). Oxidative stress in the testicular micro-environment can lead to aberrant sperm morphology, reduced spermatogenesis, and sperm DNA damage (Omu 2013). Pathological bacterial strains that directly affect gametogenic cells and indirectly by encouraging the formation of antibodies that may be directed against the sperm glycocalyx complex are two possible ways that microorganisms might cause defects in spermatozoa function. According to studies, bacterial contamination may impair the capacity of pig sperm to perform the acrosome response , cause spermatozoa to agglutinate or move slowly, or change the shape of the cells. (Pastorelli et al., 2016). Distinguishing between benign and harmful bacterial strains would be made easier by identifying the bacterial strains in boar semen and researching their association with sperm characteristics. The purpose of this study is to ascertain the harmful impacts of various microorganism species in Meghalaya.

Materials & Methodology

The work was carried out at the Microbiology Laboratory, Department of Biotechnology and Bio-informatics, North Eastern Hill University, Shillong (793022), Meghalaya. Ethical clearance was provided by the Assistant Director, Intensive Cattle development Project (ICDP), Upper Shillong letter no. ICD/NPCBB/TRG-2/2022-23/981 dated 17/08/2022.

Culture media and chemicals

Nutrient agar, Plate count agar, Mannitol salt agar, Mueller–Hinton agar, MacConkey agar (HiMedia, India), Ethanol, crystal violet,sodium chloride, Gram's iodine ,decolorizer, Peptone, MR-VP, Simmons Citrate agar hydrogen peroxide, TSI agar, (HiMedia, India), antibiotic susceptibility disc - Penicillin-G (10mcg), kanamycin (30 mcg), ceftizoxime(30 mcg), tetracycline (30 mcg), erythromycin (10 mcg), vancomycin (30 mcg), ciprofloxacin (5 mcg), imipenem (10 mcg), piperacillin (10 mcg), gentamicin (10 mcg) and Chloramphenicol (30mcg) (Hi Media, India).

Semen Collection

Semen samples were collected from Hampshire pig (*Sus scrofa domesticus*), which were raised and taken care by Intensive Cattle Development Project (ICDP), Upper Shillong. Total of ten semen ejaculates were collected from five pigs, two from each pig. The semen was collected by gloved hand method thrice weekly from each boar. The boar was allowed to mount on the dummy. After successful mounting and the boar started expelling the penis from the prepuce, the semen collector would grasp the corkscrew tip of the penis applying gentle pressure. The semen was then collected inside pre-warmed (38°C) collection bags fitted with filters and placed inside insulated collection cups (Minitube, Germany).

Collection of samples

The Intensive Cattle Development Project (ICDP), Upper Shillong, was the location where raw semen samples were gathered. A thermoflask that had been set to 37°C using a thermometer was used to collect and transport 50mL of the material to the microbiology lab at North Eastern Hill University in Shillong. It was stored in a water bath at 37°C in the laboratory after arrival.

Sperm motility Test

Samples were placed on slides above the heating block before the experiment began and their motility was checked by looking at them under the microscope at 10X and 20X magnification. Further testing was carried out when motility was discovered to be more than 70%.

Isolation and characterization of bacterial culture

100 µL of the sample were taken and put on plate count agar plates (PCA) using a micropipette. Using sterile spreaders, semen samples were evenly distributed on the plates, and they were then allowed to grow for 24 hours at 37°C in an incubator. Then, using the quadrant streaking technique, it was subcultured on Nutrient agar plates, after which it was grown on Mannitol salt agar plates and MacConkey agar plates to obtain the pure culture.

Gram staining of bacteria

Gram's staining was used to distinguish between Gram positive and Gram negative microorganisms. A loop full of bacteria was used to spread a thin layer of culture onto glass slides. Following air drying and heat fixing, the smear was stained for 60 seconds with crystal violet (Hi media), 60 seconds with Gram's iodine (Himedia), 30 seconds with Gram's decolourizer (Hi media), and 30 seconds with safranin (Hi media). Following a distilled water wash, the smear was air dried. The slide was then examined at 40X and 100X magnifications using the microscope (Aneja et al., 2003).

Biochemical identification and VITEX MS

To identify the unknown isolates a number of biochemical tests were performed which included oxidase test, catalase test, indole test, methyl red test, voges-proskauer test, citrate test was performed. Confirmation of identification of isolates was done using VITEX MS at Pasteur Institute, Shillong.

Antibiotic susceptibility test using disc diffusion method

The antibiotic susceptibility test for the bacterial isolates was determined against twelve antibiotics penicillin-G (10mcg), and chloramphenicol (30 mcg), kanamycin (30 mcg), ceftizoxime (30 mcg), tetracycline (30 mcg), erythromycin (10 mcg), vancomycin (30 mcg), ciprofloxacin (5 mcg), imipenem (10 mcg), piperacillin (10 mcg), gentamicin (10 mcg), ampicillin (10 mcg) using standard antibiotic discs (Hi Media, India). The test isolates were diluted in 0.5% saline water and were inoculated uniformly onto Mueller–Hinton agar (MHA) plates using a cotton swab and the twelve antibiotic discs were antibiotics were placed equidistantly on the agar plates. Results were recorded after 24 hours of incubation at 37°C. For each antibiotic disc the zone of inhibition for each antibiotic was measured in millimeters.

Results

Sperm Motility

Motility was examined before being subjected to experimentation. The material was pipetted into a glass slide that was lukewarm and covered with a cover slip. It was then inspected under a microscope at 10x, 20x, and 40x for motility and morphology. All samples showing motility >70% were taken into consideration for further experiments. Samples showing motility <70% were discarded due to improper collection or extended sexual abstinence.

Spread plate method

100µL of the samples were poured in the centre of plate count agar plates and using a sterilized spreader it was spreaded uniformly. After 24 hours of incubation growth was observed and colonies were counted.

Streak plate method

To produce pure colonies, 10 isolates (S1, S2, S3, S4, S5, S6, S7, S8, S9, S10) were streaked in different nutrient agar plates after being separated from the spread plate. Pure colonies were streaked, and the development of these colonies was observed.

Culture characteristics of bacterial isolates

The morphological characteristics such as size, shape, color and nature of colonies were observed. In addition, all the isolates were tested by Gram's staining to check whether they belong to Gram positive or negative. The details of the morphological characteristics and Gram staining are shown in Table 1. All samples showed cocci shape and shape of colony varies from smooth to mucoid. Microscopic examination was done at 100x oil immersion lens to determine gram positive or gram negative bacteria.

Table 1: Morphological characters of bacterial isolates

Isolate No.	Whole shape of colony	Size of colony	Edge/ margin	Opacity	Elevation	Gram Nature	Shape
S1	Smooth	Small	Entire	Opaque	Raised	Positive	Coccoid
S2	Smooth	Small	Entire	Opaque	Raised	Positive	Coccoid
S3	Smooth	Small	Entire	Opaque	Raised	Positive	Coccoid
S4	Mucoid	Large	Undulate	Opaque	Raised	Negative	Coccoid
S5	Smooth	Small	Entire	Opaque	Raised	Negative	Rod
S6	Mucoid	Large	Undulate	Opaque	Raised	Negative	Rod
S7	Mucoid	Large	Undulate	Opaque	Raised	Negative	Rod
S8	Smooth	Small	Entire	Opaque	Raised	Negative	Rod
S9	Mucoid	Large	Undulate	Opaque	Raised	Negative	Rod
S10	Smooth	Small	Entire	Opaque	Raised	Negative	Rod

Table 2: Antibiotic showing its average diffusion zone, number (%) of resistant isolates

Antibiotics	Average diffusion zone (mm)	Number (%) of resistant isolates (n=10)
Kanamycin (30 mcg)	9.1	8 (80)
Ceftizoxime (30 mcg)	9.5	5 (50)
Penicillin-G (10 mcg)	2.6	2 (20)
Chloramphenicol (30 mcg)	12.3	8 (80)
Tetracycline (30 mcg)	15.2	10 (100)
Vancomycin (30 mcg)	4.6	3 (30)
Ciprofloxacin (5 mcg)	32.9	10 (100)
Imipenem (10 mcg)	28.1	10 (100)
Piperacillin (10 mcg)	15.3	8 (80)
Gentamicin (10 mcg)	16.8	9 (90)
Erythromycin (10 mcg)	3.4	3 (30)

Table 3: Identification of isolates through VITEX MS

Sample no.	Bacterial Species
S1	Kluyvera intermedia
S2	Kocuria kristinae
S3	Kocuria kristinae
S4	Pseudomonas fluorescens
S5	Pseudomonas aerugionosa
S6	Pseudomonas aerugionosa
S7	Pseudomonas aerugionosa
S8	Pseudomonas aerugionosa
S9	Pseudomonas aerugionosa
S10	Pseudomonas aerugionosa

Biochemical characterization of the isolates

The different biochemical tests were performed for the ten isolates to know their biochemical characteristics. The results of biochemical tests of the bacteria isolated from semen sample were all positive for Catalase and Oxidase test, sample 1-4 negative and sample 5-10 positive for Indole test, sample 9 negative and rest of the samples were positive for Methyl Red test, sample 1,2,7 negative and were positive for Voges Proskauer test, sample 1,2,4 negative and rest were positive for Citrate test. In TSI tests sample 1,2,6,7 had yellow butt, red slant, sample 3,4,8,10 had red butt, red slant and sample 5,9 had yellow butt, red slant.

Antibiotic Sensitivity test

The zone of inhibition of the isolates were different in each plate. Antibiotic resistance was seen in 100% for Tetracycline, Ciprofloxacin, Imipenem, 90% for Gentamicin, 80% for Kanamycin, Chloramphenicol (Table 2). This would suggest that older generation of antibiotic should be discontinued and newer generation should be included to combact antimicrobial resistance (AMR).

Identifications by VITEK MS

Identifying bacteria was done using VITEK MS (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) at Pasteur Institute, Shillong involves comparing the mass spectral profiles of unknown bacterial isolates with an extensive reference database of known spectra. The following bacteria (Table 3) were observed after the identification:

Discussion

It is usual for bacterial contamination to occur during routine boar sperm collection and processing. Similarly, our findings show that all of the raw semen samples taken in various BS included one or more microorganisms. The presence of more than 10^2 CFU mL^{-1} of aerobic mesophiles in the semen, according to Goldberg et al., (2013), suggests a highly polluted sample, which may influence the quality of the semen.

The presence of S. aureus may result in a decrease in the number of spermatozoa, suppression of motility, morphological alterations, and decreased fertilizing capacity. A large number of spoilage and harmful bacteria can participate in both the adhesion and biofilm development processes (Tremblay et al., 2014), The collection of a bacteria-free ejaculate is a significant challenge. Thus, our findings indicated that all samples had ejaculated with microbes belonging to the gram-positive cocci family. Additionally, these microorganisms may contribute to inflammatory processes in inseminated sows' endometrium (Mazurova et al., 2015).

While sperm motility, sperm morphology, and concentration of the semen doses generated are the metrics frequently used to evaluate semen quality, a microbiological analysis is not a usual practise in the evaluation of swine semen and may reveal additional potential markers of poor semen quality. Since poor semen quality is one of the main reasons why female animals, including swine, are unable to reproduce, microbiological examination should be used, especially since it is a simple and affordable approach.

On the other hand, our findings indicated a significant resistance to Ciprofloxacin (100%) and Imipenem (100%). It has been demonstrated that using beta-lactams in a mixed bacterial population of susceptible and resistant cells causes the beta-lactam to be rapidly degraded by resistant cells (in as little as 2.5 h), removing whatever selection pressure the antimicrobial offered (Korpimäki et al., 2003). We think this process of degradation may have contributed to the high amount of piperacillin resistance seen in our results, given that mixed isolation was the main finding. Although penicillin or piperacillin is an antibiotic that should only be used against Gram positive bacteria, it was utilised since it is an ingredient in the majority of commercial semen extenders because it is impossible to determine where the contamination originated. Since the majority of the bacteria we isolated belonged to Gram negative bacteria, the high level of resistance was predicted based on the fact that piperacillin has a broad spectrum of activity against Gram negative bacteria.

We concluded from the results that one or more genera of bacteria were present in all of the raw semen samples collected in various boar semen. A good efficacy (in vitro) of Vancomycin on the primary contaminants detected in boar semen has also been demonstrated by our results on the examination of susceptibility to antimicrobial agents. However, there was a significant resistance to Ciprofloxacin (100%) and piperacillin (80%). Therefore, for a proper quality control and decreased risk of contamination of semen doses in boar studs, understanding of bacterial dynamics on contamination processes, as well as identification of major bacterial contaminants genera, and their source and profile of antimicrobial susceptibility, are mandatory.

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