

Research Article

Identification, Molecular Confirmation, and Antibiotic Sensitivity of Bacteria Isolated from Repeat Breeder Cows in Rangpur Division

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Abstract

Aims: The study was conducted from January to June 2023 to observe the prevalence of repeat breeding syndrome in cows, to isolate and identify the bacteria with their molecular confirmation, and antibiotic sensitivity in Bangladesh.

Study design & Place and duration of study: The study was conducted under the supervision of the Department of Medicine, Surgery, and Obstetrics, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200. Some laboratory work was performed at the Microbiology Department of Pathology and Parasitology laboratory, HSTU, from January to June 2023.

Methodology: A questionnaire was used to identify repeat breeding syndrome. Bacteria were isolated and identified from the cervical mucus of affected cows. For confirmation of the detected bacteria, PCR was used. The agar disc diffusion method was utilized to investigate the antibiotic sensitivity of the detected isolates against widely used antibiotics in vitro.

Results: The prevalence of Repeat Breeding (RB) was 41.33%. The prevalence of *E. coli* was 40%, *Staphylococcus aureus* 40% and *Klebsiella spp.* 10%. Molecular detection of *E. coli* and *Staphylococcus aureus* was confirmed by using Eco 223 (F), Eco 455 (R), Sau 234(F), and Sau 1501(R) primers. The target genes were 16S and 23S rRNA, and the size of the product amplified at 232 bp and 1267 bp, respectively. According to the antibiogram profile, *E. coli* was resistant to ciprofloxacin, penicillin, tetracycline, amoxicillin, and erythromycin, but sensitive to ceftioxaone and gentamicin. While *Staphylococcus aureus* was resistant to ampicillin and erythromycin, it was sensitive to ciprofloxacin, ceftioxaone, and levofloxacin. It was also intermediately resistant to vancomycin and amoxicillin. *Klebsiella spp.* Showed resistance to ceftioxaone, penicillin, and amoxicillin, while remaining sensitive to ciprofloxacin, tetracycline, and intermediate resistance to erythromycin and gentamicin.

Conclusion: This study concluded that *E. coli*, *Staphylococcus aureus*, and *Klebsiella spp.* are the most common bacteria causing repeat breeding syndrome in cows.

More Information

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Keywords: Antibiotic sensitivity; Bacteria; Molecular confirmation; PCR; Repeat breeding



Introduction

The Contribution of the cattle industry to the nation's GDP is 1.85%, 20% of jobs are directly and 50% are indirectly provided by the livestock industry [1]. Pregnancy rates are used to estimate the economic status of dairy farms [2]. Repeat breeding (RB) has been defined as failure to conceive from 3 or more successive services in the absence of detectable abnormalities [3]. Repeat breeding has significant effects on cattle breeding, leading to huge economic loss for the dairy breeders due to more inseminations, increased calving interval, and culling rate [4]. Several studies on Repeat Breeding (RB) have been conducted over the years [5,6]. Various factors, such as infectious agents affecting the sperm or embryo, hormonal deficiency, reproductive immunology, oocyte quality, nutrition, feeding, and season, may be responsible for repeat breeding syndrome [7,8]. Many specific and non-specific uterine infections are associated with either fertilization failure or early embryonic mortality in repeat breeder cattle [9]. Several bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus* spp., *Proteus* spp., *Enterobacter* spp., *Corynebacterium* spp., and *Pseudomonas aeruginosa*, were isolated from the cervical mucus of repeat breeder cattle in different studies [10,11]. The incidence of fertilization failure and early embryonic death following fertilization may also lead to repeat breeding in cows [9,12]. Recently, higher progesterone levels during oestrus in Repeat Breeding (RB) heifers compared to healthy animals have been indicated as one possible cause of Repeat Breeding (RB) [13]. There are only a few studies that have been conducted to detect and molecularly characterize of causal bacteria of Repeat Breeding (RB). In Bangladesh, some experiments have also been conducted to investigate the causes of repeat breeding [9]. Several antibiotics have been used for the treatment of repeat breeder cows suffering from uterine infections. Proper selection of antibiotics is needed to prevent antimicrobial resistance [14]. However, there are very few investigations on the identification and molecular characterization of infectious agents isolated from repeat breeder cows and their antibiotic resistance in our country. Therefore, considering the above points, the major objectives of the research were to observe the prevalence of repeat breeding syndrome in the Rangpur division of Bangladesh, to isolate and identify the bacteria with their molecular confirmation causing repeat breeding syndrome, and to determine the antimicrobial sensitivity of the bacteria isolated from repeat breeder cows.

Materials and methods

Study area

The study was conducted under the supervision of the Department of Medicine, Surgery, and Obstetrics, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, from July 2022 to December 2023. The research was ethically approved by the Institute of Research

and Training (IRT) of Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, approval number: HSTU/IRT/4356(1).

Data collection for prevalence study

A structured questionnaire was prepared to acquire farm and cow-level management, demographic, health, production, and reproduction data. The questionnaire was designed to comprise mostly closed and open-ended (categorical) questions to ease data processing, minimize variation, and improve the precision of responses.

Selection of cows for sample collection

This study was conducted in different dairy farms located in the Rangpur division of Bangladesh. A total of 300 cows were selected for the study. The cows were between 3 to 10 years old, with body condition scores (BCS) ranging from 1 to 3.5 and body weight (325-480 kg). Repeat breeding (n = 124) cows were selected based on owner complaint, history of repeated conception failure, breeding records, nature of estrous cycle, and per rectal examination of reproductive tract.

Clinical examination

To confirm the absence of clinical abnormalities, the uterine horns and ovaries of the cows were examined by rectal palpation. Both horns were then examined from the base to the tip for their size and symmetry, tone, and thickness of the wall. In case of uterine asymmetry, the bigger horn was palpated to confirm pregnancy or pyometra. Acyclicity, possibly associated with chronic uterine infection, was assessed by palpating the thick uterine wall with or without intraluminal content, along with the presence of palpable corpus luteum. The vaginal discharges of the cows were evaluated by a gloved hand, and only the animals with clear mucus were chosen for this study.

Collection of samples

The cervical mucus samples were collected by using a sterile cotton stick when animals were in estrus. After restraining of animal in Travis, the vulvar and perineum region was washed with a mild antiseptic, ethyl alcohol (95%) solution, and wiped properly with absorbable sterile cotton. The recovered cervical mucus samples were transferred into tubes containing Phosphate-Buffered Saline (PBS) and placed in an ice box, and then transported to the laboratory as soon as possible after collection.

Procedure of isolation and identification of bacteria

Laboratory preparation: Glassware, such as test tubes, micropipettes, cylinders, conical flasks, petri dishes, glass plates, slides, and vials, should be immersed in a dishwashing detergent solution for at least an entire night before use. After brushing, thorough washing, and autoclaving the glassware

for 15 minutes at 121 °C under 15 lbs. of pressure per square inch, the glassware was properly cleaned and sterilized. Items that had been autoclaved were dried at 50 °C in a hot air oven. Autoclaving was used to sanitize plastic disposables (micropipette tips).

Instruments and appliances: All items of glassware and appliances used during the course of the experiment were test tubes, conical flasks, petri dishes, pipettes, slides, cover slips, glass rod spreader, water measuring cylinder, dropper, stop watch, distilled water, forceps, scissors, tray, experimental test tube, stopper, vortex mixture, labeling tape, micropipette, test tube racks, water bath, bacteriological incubator, freeze (-20 °C), refrigerator (4 °C), sterilizing instruments, hot air oven, centrifuge tubes and machine, ice boxes, electronic balance, syringe and needle, immersion oil, cotton, slide, compound microscope, spirit lamps, match lighter, bacteriological loop, inoculum loop, autoclave machine, laminar air flow casting tray, well combs, voltage source, gel box, uv light source, microwave, gel- electrophoresis, PCR machine, spinner, genetic analyzer etc.

Media for culture: The isolation, characterization, and growth of bacteria from cervical mucus were accomplished using various bacteriological culture media and reagents. The culture media and materials used in this experiment are Nutrient broth (HIMEDIA, India), Methyl Red-Voges Proskauer (MR-VP) broth (HIMEDIA, India), Buffered Peptone Water (HIMEDIA, India), Indole broth (HIMEDIA, India), Nutrient agar base (Scharlau, Spain), MacConkey Agar medium (HIMEDIA, India), Eosin Methylene Blue (EMB) Agar (HIMEDIA, India), Mannitol salt agar (Scharlau, Spain), Triple Sugar Iron (TSI) Agar slant (HIMEDIA, India), Simmons citrate agar (HIMEDIA, India), Blood agar (HIMEDIA, India), Mueller-Hinton agar (MHA) (HIMEDIA, India).

Chemicals, reagents, and solutions: The reagents used during the study were crystal violet, iodine solution, gram's iodine, acetone alcohol, normal saline, distilled water, ethidium bromide, safranin, methyl red solution, catalase test reagent hydrogen peroxide, (3% solution), kovac's reagent, ethyl alcohol (70% and 95%), gel-loading dye, tae buffer, wash buffer, elusion buffer, pcr master mix and other common laboratory chemicals and reagents.

Antibiotic discs: To identify the antibiotic sensitivity profile of several bacterial isolates with various classes of antibiotics. The antibiotic discs used were readily accessible commercially. By measuring the size of the zone of inhibition that forms when different amounts of the agent diffuse into the medium around the disc, this method made it possible to figure out how sensitive different antibiotics are. The antibiotics were examined in tests against the chosen bacterium, are listed below, along with their disc concentrations in Table 1.

Isolation and identification of bacteria

The samples were collected for the isolation and

Table 1: Antibiotics with their disc concentration.

Sl. No.	Name of the Antibiotics	Concentrations (µg/disc)	Company Name
01	Ampicillin (AM)	10	Bioanalyse® Turkey
02	Vancomycin (VA)	30	
03	Erythromycin (E)	15	
04	Amoxicillin (AX)	30	
05	Levofloxacin (LEV)	5	
06	Ciprofloxacin (CIP)	5	
07	Ceftriaxone (CRO)	30	
08	Penicillin G (P)	10	
09	Tetracycline (TE)	10	
10	Gentamicin (GN)	10	

identification of bacteria by morphology, cultural characteristics, and staining characteristics. The characterization of bacteria was done by cultural and biochemical reactions. The isolated bacteria were tested for the sensitivity patterns of different antibiotics, and finally performed a molecular test was finally performed by PCR.

Culture of bacteria: Samples (uterine pus) were collected in a sterile cotton swab stick containing Phosphate-Buffered Saline (PBS) in a sterile tube from different farms. Samples were brought to the medicine, surgery, and obstetrics laboratory to maintain an aseptic condition. Samples were cultured on primary culture (nutrient agar) and incubated at 37 °C for 24 hours. Then secondary culture was done on MacConkey blood agar from nutrient agar and incubated at 37 °C for 24 hours. Again, subculture on selective media (MSA, EMB, and Blood agar) and incubation at 37 °C for 24 hours was done. The isolation of the pure cultures was done. Gram's staining was performed to identify gram-positive and gram-negative bacteria, their morphology, and staining characteristics. Biochemical characterization of isolates by catalase, TSI, Indole, Simmons citrate, MR, and VP tests was done. After that, the determination of antibiotic sensitivity patterns was done by the disc diffusion method. Finally, molecular characterization by DNA extraction, reaction mixing, PCR amplification, agarose gel preparation, and electrophoresis was performed.

Antibiotic sensitivity tests

The standard Kirby-Bauer disk diffusion method was used to determine the antimicrobial susceptibility profile of the isolates [15] according to the recommendations of the National Committee for Clinical Laboratory Standards (CLSI, 2022). Sensitivity to antibiotics was studied on a Muller-Hinton Agar plate using the different types of commercial antibiotic discs. The antibacterials used are Ampicillin (AM) -10, Vancomycin (VA) -30, Erythromycin (E) -15, Amoxicillin (AX) -30, Levofloxacin (LEV) 5, Ciprofloxacin (CIP) -5, Ceftriaxone (CRO) -30, Penicillin G (P) -10, Tetracycline (TE) -10, Gentamicin (GN) -10. Antibiotic disks were applied using some sterile forceps. Agar plates were incubated at 37 °C for 24 hours. After overnight incubation at 37 °C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant

or sensitive in accordance with company recommendations. All isolates were tested for sensitivities to 10 routine and practical antibiotics. Antimicrobial agents with their disc concentration and diameter of zone of inhibition are shown in Table 2.

DNA extraction

At first, 200 µl of Phosphate-Buffered Saline (PBS) was taken in an Eppendorf tube with the help of a micropipette. Then, one bacterial colony was taken from pure culture and added to the Eppendorf tube containing Phosphate-Buffered Saline (PBS) with the help of an inoculum. Then 130 µl of Buffer ATL was added to each tube and mixed gently. Then 15 µl of proteinase K was added to the tube, vortexed, and incubated at 56 °C for three hours, invert mixed occasionally. Then 200 µl of Buffer AL was added to the sample, pulsed for 15 seconds, and incubated at 70 °C for 10 minutes. Then 200 µl of ethanol (100%) was added, pulse-mixed, and touch-spun. Then samples were transferred to a Qiagen mini spin column, spun at maximum speed for 60 seconds, and the filtrate was discarded. Then 500 µl of Buffer AW1 was added to each tube, spun at maximum speed for 60 seconds, and the filtrate was discarded. Then the transfer of the column to a fresh collection tube was done. Then 500 µl of Buffer AW2 was added to each tube; spin max for 60 secs; discard the filtrate. The transfer of the spin column to a fresh 1.5-ml labeled Eppendorf tube was performed. Then 100 µl of Buffer AE was added to the membrane. Then the tube was incubated at room temperature for a minimum of 10–15 minutes. Spin at 6000 rpm for 60 seconds was done. Store the collected DNA at -20 °C until PCR amplification is done.

PCR

Selection of primer for detecting *E. coli* and *Staphylococcus aureus*: There were two types of primer used in this study. One was for *E. coli*, and another was for *Staphylococcus aureus* (Table 3).

- Eco 223 (F) and Eco 455 (R) primer:** The band size was 232 bp to confirm the presence of *E. coli*.
- Sau 234 (F) and Sau 1501 (R) primer:** The band size was 1267 bp to confirm the presence of *Staphylococcus aureus*.

Table 2: Antimicrobial agents with their disc concentration and diameter of the zone of inhibition.

Antimicrobial Agents	Sensitive or more	Intermediate	Resistant or less
Ampicillin (AM) -10	≥17	15-16	≤14
Vancomycin (VA) -30	≥17	15-16	≤14
Erythromycin (E) -15	≥23	14-22	≤13
Amoxicillin (AX) -30	≥18	14-17	≤13
Levofloxacin (LEV) - 5	≥17	14-16	≤13
Ciprofloxacin (CIP) -5	≥21	16-20	≤15
Ceftriaxone (CRO) -30	≥23	20-22	≤19
Penicillin G (P) -10	≥29	-	≤28
Tetracycline (TE) -10	≥15	12-14	≤11
Gentamicin (GN) -10	≥15	13-14	≤12

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper.

Amplification of extracted DNA for confirmation of *E. coli* and *Staphylococcus aureus*: PCR product was formed by mixing all the essential reagents (master mix, forward primer –Eco 223, Sau 234||, reverse nuclease-free 455, Sau 1501||, nuclease-free water, and extracted DNA) in a PCR tube (Table 4). Then the tubes were placed in the well of the PCR machine and optimum conditions for PCR were commanded (Tables 5, 6). The overall procedure was followed according to Riffon, et al. [16].

Gel and Gel-electrophoresis: 0.75 g of agarose powder was measured. Agarose powder was mixed with 50 mL 1xTAE in a microwavable flask. Microwave for 2 min was performed until the agarose was completely dissolved. The agarose solution was cooled down to about 50 °C for about 5 minutes. Ethidium bromide (EtBr) (2.5 µl) was added to a final concentration of approximately 0.2-0.5 µg/ml. The agarose was poured into a gel tray with the well comb in place. Newly poured gel was placed at room temperature for 30-45 minutes until it had completely solidified. The sample was applied to the gel. Adjustment of voltage or current (gel-electrophoresis about

Table 3: Properties of the primer used in this study.

Specificity	Primer name	Primer sequence (5'-3')	Target gene	Annealing temp (°C)	Size of product amplified (bp)	Ref
<i>E. coli</i>	Eco 223 (F)	ATC AAC CGA GAT TCC CCC AGT	16S & 23S rRNA	55	232	[16]
	Eco 455 (R)	TCA CTA TCG GTC AGT CAG GAG				
<i>S. aureus</i>	Sau 234 (F)	CGA TTC CCT TAG TAG CCG CG	16S & 23S rRNA	58	1267	
	Sau 501 (R)	CCA ATC GCA CGC TTC GCC TA				

Table 4: Preparation of PCR product for Eco 223, Eco 455, Sau 234, Sau 1501 primer.

Sl. No.	Name of the Products	Volume (µl)
01	Master mix	12.5
02	Extracted DNA	5
03	Primer F (Eco 223, Sau 234)	1
04	Primer R (Eco 455, Sau 1501)	1
05	Water	5.5
	Total	25

Table 5: Condition of PCR for *Staphylococcus aureus*,

Step name	Temperature	Duration time	Number of cycles
Preheat	94 °C	2 minutes	1
Denaturation	94 °C	45 seconds	35
Annealing	58 °C	45 seconds	
Extension	72 °C	2 minutes	
Final extension	72 °C	6 minutes	1
Holding	4 °C	overnight	1

Table 6: Condition of PCR for *E. coli*.

Step name	Temperature	Duration time	Number of cycles
Preheat	94 °C	3 minutes	1
Denaturation	94 °C	30 seconds	35
Annealing	55 °C	30 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	3 minutes	1
Holding	4 °C	overnight	1

70-100 volts) was performed. The setup of the run time about 30-45 minutes, was done. When DNA migrated sufficiently, as judged from the migration of bromophenol blue in loading buffer, the power supply was switched off. The gel was placed on the imaging system in the dark chamber of the image documentation. The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired, and saved in a USB flash drive.

Statistical analysis

The generated data was compiled in an MS Excel sheet and analyzed using the statistical analytical software SPSS (version 29.0.1.0). Prevalence was analyzed by one-way ANOVA. All significant differences were considered at $p < 0.05$.

Results

The research was aimed at the diagnosis, isolation, and identification of causal agents of repeat breeding in dairy cows.

Prevalence of major reproductive disorders in dairy cattle in Rangpur division

The prevalence of major reproductive disorders of dairy cattle is presented in Table 7.

Prevalence of different types of bacteria found in repeat breeding

Prevalence of different types of bacteria isolated from Repeat Breeder cows is shown in Table 8. The prevalence of *E. coli* (40%), *Staphylococcus aureus* (40%), and *Klebsiella spp.* (10%) was most common in all repeat breeder cows.

Cultural characteristics of bacteria

The cultural characteristics of *E. coli*, *Staphylococcus aureus*, and *Klebsiella spp.* on various culture media are presented in Table 9.

Table 7: Prevalence of major reproductive disorders in dairy cattle in Rangpur Division.

Name of reproductive disorders (n = 300)	Frequency	Percent of total affected animals (%)
Anestrus	94	^b 31.33
Abortion	16	^c 5.33
Endometritis	8	^d 2.66
Dystocia	21	^c 7.00
Repeat breeding syndrome	124	^a 41.33
Retained placenta	29	^c 9.66
Uterine prolapse	4	^d 1.33
Vaginal Prolapse	4	^d 1.33

^{abcd}Different superscript letters in the same column differ significantly (at least $p < 0.05$).

Table 8: Prevalence of Different Types of Bacteria Found in Repeat Breeding.

Types of Bacteria	Total sample (n)	Positive (n)	Prevalence (%) (n/n)
<i>E. coli</i>	10	4	^a 40
<i>Staphylococcus aureus</i>	10	4	^a 40
<i>Klebsiella spp.</i>	10	1	^b 10

^{ab}Different superscript letters in the same column differ significantly (at least $p < 0.05$).

The bacteria were isolated from cervical mucous samples by using spread plate methods on NA, MAC, MSA, EMB, and BA. All the isolates produced different kinds of colonies on the different agar plates.

E. coli produced a large, mucoid, white colony on nutrient agar, a large, mucoid, rose-pink colony on MacConkey agar, and a green metallic sheen with reflected light on Eosin methylene blue agar.

Staphylococcus aureus produced a white, glistening colony on nutrient agar, a small white colony on blood agar, and yellowish colonies yellow zone on mannitol salt agar.

Klebsiella spp. Produced creamy white, circular, raised, fluidal colony on nutrient agar, Sticky, wet appearing, mucoid colony on MacConkey agar, Large, Mucoid, Pink to purple colonies with no metallic sheen on EMB agar.

Characterization of bacteria by using different biochemical tests:

- The identified isolates were characterized by using different biochemical tests (MR, VP, TSI, Indole, Catalase test, Citrate test, etc.) This observation also revealed that isolated organisms were *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella spp.*
- Catalase test was performed by placing a drop of hydrogen peroxide on a slide and thoroughly mixing the colony of the bacteria to be tested. The catalase test was positive (presence of bubbles) for *Staphylococcus aureus*, *Klebsiella spp.*, and *E. coli*.
- MR reactions were positive (presence of a red colored colony on the surface of the media) for *Staphylococcus aureus*, *Escherichia coli*, and MR reactions were negative (absence of a red colored colony on the surface of the media) for *Klebsiella spp.*
- Indole tests were negative (absence of a cherry red colored ring on the surface of the media) for *Staphylococcus aureus* and *Klebsiella spp.*, on the other hand, positive (presence of a cherry red colored ring on the surface of the media) for *Escherichia coli*.
- VP tests were positive (presence of a red colored colony on the surface of the media) for *Staphylococcus aureus*, *Klebsiella spp.*, and negative (absence of a red colored colony on the surface of the media) for *Escherichia coli*.
- Simon's citrate agar was positive (formation of Prussian blue color on the slant) for *Staphylococcus aureus*, *Klebsiella spp.*, and negative (no colored change of the medium) for *E. coli*.
- TSI was positive (yellow butt, yellow slant, presence of gas, and absence of H_2S) for *E. coli* and *Klebsiella spp.*

Table 9: The cultural characteristics of *E. coli*, *Staphylococcus aureus*, and *Klebsiella spp.* on various culture media.

Types of media	Cultural properties	Morphological properties	Remarks
Nutrient agar	White, glistening colonies	Purple/violet colored and small, round, and coccus-shaped organism. Arranged in singles, pairs, and clusters	<i>Staphylococcus aureus</i>
Mannitol salt agar	Small whitish or yellowish colony		
Blood agar	Produce a small white colony.		
Nutrient agar	Large mucoid, white colony	Pink colored, small, rod-shaped organism. Arranged in single, pair, or short chain.	<i>E. coli</i>
MacConkey agar	Produce a large mucoid, rose-pink colony.		
Eosin Methylene Blue agar	Green metallic sheen with reflected light.		
Nutrient agar	Creamy white, circular, raised, fluidal colony	Pink color, small, rod-shaped, arranged in a single row.	<i>Klebsiella spp.</i>
MacConkey agar	Sticky, wet appearing, mucoid colony		
Eosin Methylene Blue agar	Large, mucoid, pink to purple colonies with no metallic sheen		

Results of morphological properties by Gram's staining technique

The obtained results revealed that the gram-positive violet color, cocci-shaped, arranged in clusters indicated *Staphylococcus aureus* (Figure 1), the Pink color, small, rod-shaped, arranged in single, indicated gram-negative *Klebsiella spp.* (Figure 2). It also observed that gram's Gram-negative pink color, small, rod-shaped organisms arranged in single, pair, or short chains indicated *E. coli* (Figure 3).

Result of the antibiotic sensitivity test of isolated bacteria

To ascertain the sensitivity and resistance pattern against frequently used antibiotic discs, the isolated *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella spp.* were treated with antibiotics. The antibiogram profile revealed that *E. coli* was sensitive to Ceftriaxone, Gentamicin, and resistant to Ciprofloxacin, Penicillin, Tetracycline, Amoxicillin, and Erythromycin. *Staphylococcus aureus* was sensitive to Ciprofloxacin, Ceftriaxone, and Levofloxacin, intermediately resistant to Vancomycin and Amoxicillin, and resistant to Ampicillin, Erythromycin. *Klebsiella spp.* was sensitive to Ciprofloxacin, Tetracycline, and intermediately resistant to Erythromycin, Gentamicin, and resistant to Ceftriaxone, Penicillin, and Amoxicillin. The results of antibiotic sensitivity tests are shown in Tables 10-12. Antibiotic sensitivity patterns are shown in Figures 4-6.

Molecular confirmation of *E. coli* and *Staphylococcus aureus* by the PCR technique

Molecular confirmation of *E. coli* and *Staphylococcus aureus* was confirmed by using Eco 223, Eco 455, Sau 234, and Sau 1501 primers. The target genes were 16S and 23S rRNA, and the size of the product amplified at 232 bp and 1267 bp, respectively. The results of the PCR for *E. coli* and *Staphylococcus aureus* are shown in Figures 7,8, respectively.

Discussion

The experiment was carried out to isolate and identify the bacteria with molecular confirmation causing repeat breeding syndrome in cows. Three different bacterial species (*Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella spp.*) were identified and isolated in this research. Based on the

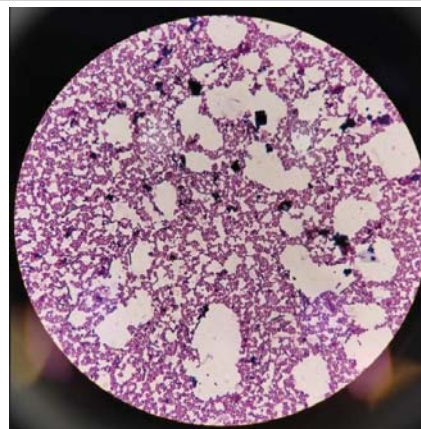
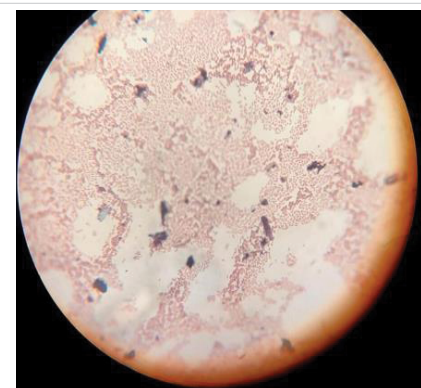
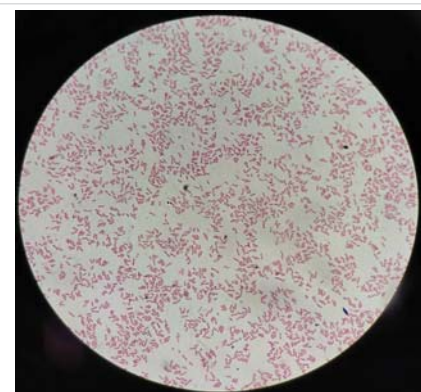

Figure 1: Violet color, cocci-shaped, arranged in clusters, indicated Gram-positive *Staphylococcus aureus*.

Figure 2: Pink color, small, rod-shaped, arranged in single, indicated gram-negative *Klebsiella spp.*

Figure 3: Pink color, rod-shaped, arranged in a single, indicated gram-negative *Escherichia coli*.

Table 10: Zone diameter interpretative standards for *E. coli*.

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Ciprofloxacin (CIP)-5	0	R
Ceftriaxone (CRO)-30	28	S
Penicillin G (P)-10	0	R
Tetracycline (TE)-10	0	R
Amoxicillin (AX)-30	0	R
Gentamicin (GN)-10	16	S
Erythromycin (E)-15	6	R

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper.

Table 11: Zone diameter interpretative standards for *Staphylococcus aureus*.

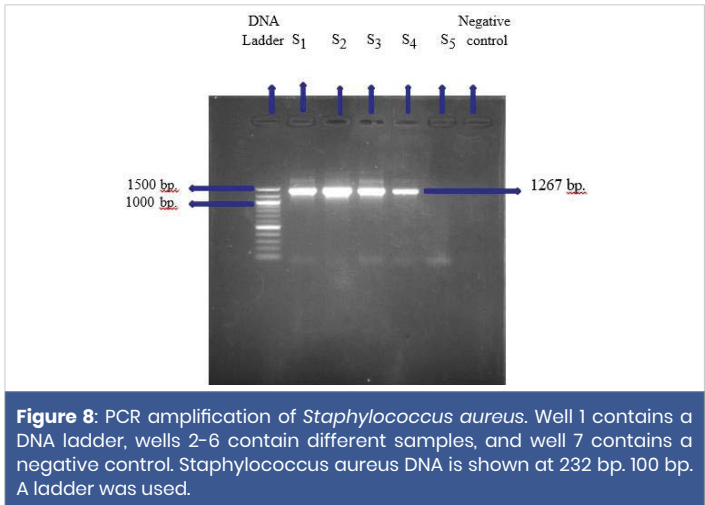
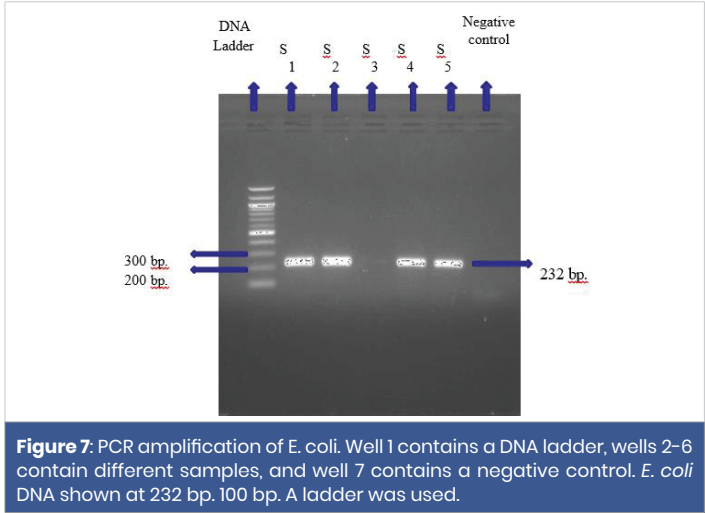
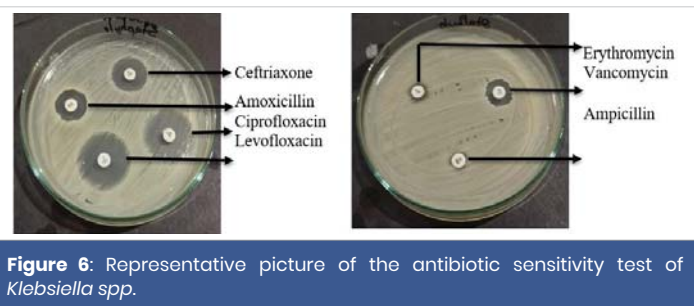
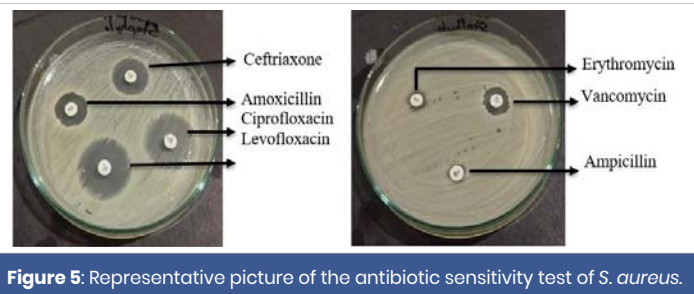
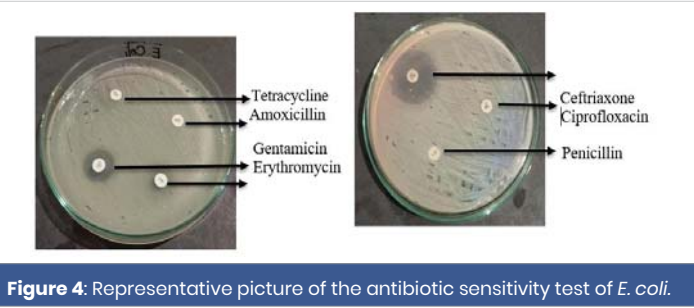
Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Ciprofloxacin (CIP)-5	26	S
Ceftriaxone (CRO)-30	20	S
Levofloxacin (LEV)-5	26	S
Vancomycin (VA)-30	15	I
Amoxicillin (AX)-30	17	I
Ampicillin (AM)-10	6	R
Erythromycin (E)-15	8	R

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper.

Table 12: Zone diameter interpretative standards for *Klebsiella spp.*

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Ciprofloxacin (CIP)-5	26	S
Ceftriaxone (CRO)-30	15	R
Penicillin G (P)-10	0	R
Tetracycline (TE)-10	19	S
Amoxicillin (AX)-30	0	R
Gentamicin (GN)-10	14	I
Erythromycin (E)-15	20	I

Source: The Clinical and Laboratory Standards Institute (CLSI-2022), revised paper.



morphology of the colony, Gram reaction, microscopic features, biochemical properties, and molecular confirmation was done to identify the bacteria were identified from the isolates. The selected isolates were further tested for antibiotic sensitivity using commercially available antibiotics and the conventional Kirby-Bauer disk diffusion method [17].

The results of the present research revealed that the major reproductive problems were anestrus, repeat breeding syndrome, dystocia, endometritis, retained fetal membrane, abortion, and uterine and vaginal prolapse. Among all the reproductive disorders found in this study, repeat breeding syndrome was the highest (41.33%), and anestrus was the second (31.33%) common reproductive disorder. The prevalence of retained placenta, abortion, dystocia, uterine prolapse, endometritis, and vaginal prolapse was 9.66%, 5.33%, 7.00%, 1.33%, 2.66%, and 1.33%, respectively. The prevalence of repeat breeding syndrome was higher in this study than the findings of others [6,18-23]. The causes of the highest prevalence found in this research might be due to improper management of farms, lack of hygienic conditions of farms, improper insemination, failure to detect ovulation and poor semen quality, age, nutrition, and lack of skilled artificial inseminators in the Rangpur division.

The prevalence of *E. coli* was 40%, *Staphylococcus aureus* 40% and *Klebsiella spp.* 10% found in the present study. Compared to our findings, similar results were reported in the other studies [24].

In the present study, cultural characterization of isolated *Staphylococcus aureus* on nutrient agar produced white, glistening colonies; on mannitol salt agar, exhibited yellow colonies with yellow zones; and on blood agar produced small white colonies (β -hemolysis). The cultural characterization of *Escherichia coli* on nutrient agar produced large, mucoid, white colonies; on EMB agar produced a green metallic sheen with reflected light, and on MacConkey agar produced large, mucoid, rose-pink colonies. Cultural characterization of *Klebsiella spp.* on nutrient agar produced creamy white, circular, raised, fluid colonies, and on MacConkey agar produced sticky, wet appearing, mucoid colonies, and on EMB agar produced large, mucoid pink to purple colonies with no metallic sheen. These characteristics of different bacteria observed in this research were consistent with previous studies [24,25].

The biochemical tests showed that *Staphylococcus aureus* made catalase (+ve), methyl red (MR) (+ve), Voges-Proskauer (VP) (+ve), Simon's Citrate Utilization (+ve), and indole (-ve). Catalase (+ve), methyl red (+ve), indole (+ve), Triple sugar iron (+ve), Voges-Proskauer (VP) (-ve), and Simon's Citrate Utilization (-ve) were all made by *Escherichia coli*. *Klebsiella spp.* Produced catalase (+ve), Voges-Proskauer (VP) (+ve), Simon's Citrate Utilization (+ve), indole (-ve), and Methyl Red (MR) (-ve), Triple sugar iron (+ve). These results showed similarity with previous studies [24,25].

In Gram's staining, the obtained results revealed that the gram-positive violet color, cocci- arranged in clusters, indicated *Staphylococcus aureus*. Gram-negative, pink-colored, small rod-shaped, arranged in single rods indicated *Klebsiella spp.* It also revealed that the gram's Gram-negative pink color and small, rod-shaped organisms arranged in single, pair, and short chains indicated *E. coli*, which is agreed by others [24,25].

In recent years, there has been a surge in antibiotic resistance (ABR) throughout the world [26,27]. ABR poses a significant risk in terms of mortality and economic burden worldwide. However, the developing countries are more affected because of the widespread misuse of antibiotics, non-human antibiotic use, poor quality of drugs, inadequate surveillance, and factors associated with individual and national poverty (poor healthcare standards, malnutrition, chronic and repeated infections, unaffordability of more effective and costly drugs) [28,29]. Bangladesh, a developing country of Southeast Asia with a high degree of ABR, poses a regional and global threat. To ascertain the sensitivity and resistance pattern against frequently used antibiotic discs, the isolated *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella*

spp. were treated with antibiotics. The antibiogram profile revealed that *E. coli* was sensitive to Ceftriaxone, Gentamicin, and resistant to Ciprofloxacin, Penicillin, Tetracycline, Amoxicillin, and Erythromycin. *Staphylococcus aureus* was sensitive to Ciprofloxacin, Ceftriaxone, Levofloxacin, intermediately resistant to Vancomycin and Amoxicillin, and resistant to Ampicillin, Erythromycin. *Klebsiella spp.* was sensitive to Ciprofloxacin, Tetracycline, and intermediately resistant to Erythromycin, Gentamicin, and resistant to Ceftriaxone, Penicillin, and Amoxicillin. These results are similar to previous studies [24,30]. In a human study performed in Chittagong in 2003, typhoid patients were found to be unresponsive to second-line therapy (ciprofloxacin). First-line therapy was not even attempted because of existing resistance [31]. Therapeutic failures like this are not rare at all. Furthermore, in relation to this, multiple studies have demonstrated irrational antibiotic prescribing by physicians, a habit of self-medication among patients, and the indiscriminate use of antibiotics in agriculture and farming in different parts of the country [32,33]. The susceptibility pattern of *E. coli* showed resistance to sulphamethaxazole (40%), polymyxin (100%), tetracycline (100%), oxacillin (40%), gentamycin (40%), and cefoxitin (100%) [34]. The isolates of *A. pyogenes* showed resistance to polymyxin (66.66%), tetracycline (66.66%), oxacillin (16.66%), gentamycin (50%), and cefoxitin (16.66%). *Klebsiella spp.* Showed resistance to only cefoxitin (100%) and was susceptible to all the tested antimicrobials [34]. The isolates of *Streptococcus spp.* and *C. fetus* were susceptible to all the tested antimicrobials [34].

Molecular detection of *E. coli* was confirmed by using Eco 223 (F) primer and primer sequence (5'-3') ATC AAC CGA GAT TCC CCC AGT, Eco 455 (R) primer and primer sequence (5'-3') TCA CTA TCG GTC AGT CAG GAG. The target genes were 16S and 23S rRNA, and the size of the product amplified at 232 bp. *Staphylococcus aureus* was confirmed by using Sau 234 (F) primer and primer sequence (5'-3') CGA TTC CCT TAG TAG CGG CG and Sau 1501 (R) primer and primer sequence (5'-3') CCA ATC GCA CGC TTC GCC TA. The target genes were 16S and 23S rRNA, and the size of the product amplified at 1267 bp. Other studies had reported similar results [16,35].

Molecular approaches are highly effective tools in the advancement of novel diagnostic tests. PCR-based approaches utilizing the 16S or 23S rDNA region sequences have been effectively employed in the identification of numerous bacterial species, particularly in cases where labor-intensive techniques like ribotyping prove to be time-consuming [36]. One of the primary benefits of polymerase chain reaction (PCR) is its capacity to utilize minute quantities of nucleic acid materials, hence obviating the need for culture, while also offering advantages in terms of speed and ease of analysis. Hence, we opted for polymerase chain reaction (PCR) amplification of DNA regions that encode ribosomal RNA (rRNA) due to the existence of hypervariable areas, which aid

in the creation of oligonucleotide probes with high specificity, as well as common regions that enable the design of universal probes. Furthermore, the presence of several copies of rDNA allows for signal augmentation [37].

Conclusion

The prevalence of repeat breeding syndrome was 41.33%. *E. coli*, *Staphylococcus aureus*, and *Klebsiella spp.* are the most common bacteria isolated from repeat breeder cows. PCR bands of *Escherichia coli* and *Staphylococcus aureus* were shown at 232 bp and 1267 bp, respectively. *E. coli* was sensitive to Ceftriaxone, Gentamicin, and resistant to Ciprofloxacin, Penicillin, Tetracycline, Amoxicillin, and Erythromycin. *Staphylococcus aureus* was sensitive to Ciprofloxacin, Ceftriaxone, Levofloxacin, and intermediately resistant to Vancomycin and Amoxicillin, but resistant to Ampicillin, Erythromycin. *Klebsiella spp.* was sensitive to Ciprofloxacin, Tetracycline, and intermediately resistant to Erythromycin, Gentamicin, and resistant to Ceftriaxone, Penicillin, and Amoxicillin. Treatment for repeat breeding syndrome should be done after performing an antibiotic sensitivity test. The results should be useful for farmers and veterinary practitioners to assess bacterial load in the genital tract, causing repeat breeding syndrome and uterine infections.

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Disclaimer (Artificial Intelligence)

The author (s) hereby declare that no generative AI technologies such as large language models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during the writing or editing of this manuscript.

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