



## Antimicrobial Susceptibility of Bacterial Causes of Abortions and Metritis in Buffaloes (*Bubalus bubalis*) in Bareilly Region, Uttar Pradesh, India

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### Abstract

Infectious reproductive disorders including abortions and metritis are important hurdles towards economic dairy farming. This study was undertaken to determine infectious bacterial causes of metritis and abortions in buffaloes and determination of their antimicrobial susceptibility. Samples of stomach contents of 23 aborted foeti and uterine aspirates of 20 buffaloes suffering from metritis were received for bacteriological analysis from Referral Veterinary Polyclinic and Centre for Animal Disease Research and Diagnosis. To identify the causal bacteria standard bacteriological method for isolation and identification of bacteria and antimicrobial susceptibility assay of isolates were followed. From 13 cases of abortion only single type of bacteria (*Aeromonas media* 1, *Alcaligenes faecalis* 2, *Brucella abortus* 4, *Enterobacter agglomerans* 1, *Escherichia coli* 3, *Klebsiella pneumoniae* ssp. *pneumoniae* 1, *K. varians* 1) was detected while in 10 samples more than one type of bacteria were isolated. Antibodies in high titres ( $\geq 40$ ) were detected in serum samples of 17 aborting buffaloes showing possibility of systemic infection before abortion. Antibodies were detected against *A. media* (1), *A. faecalis* (1), *B. abortus* (4), *E. agglomerans* (2), *Enterobacter amnigenus* (1), *E. coli* (3), *K. pneumoniae* ssp. *pneumoniae* (2), *Kocuria varians* (1), *Salmonella enterica* ssp. *enterica* ser Dublin (1) and *Xenorhabdus poinarii* (1). From uterine aspirates of six animals with metritis single type of bacteria (*Acinetobacter lwoffii* 3, *Aeromonas salmonicida* ssp. *salmonicida* 1, *E. coli* 1, *K. varians* 1) was detected while 14 samples had more than one type of bacteria. Irrespective of source, >90% bacterial isolates were susceptible to ajowan oil, cinnamon oil, cinnamaldehyde, carvacrol and thyme oil. Tigecycline was one of the most effective antibiotics but for other antibiotics susceptibility varied with source and type of bacteria. A total of 31.58% bacteria associated with metritis were resistant to carbapenems and were more often resistant to other antibiotics (MRI=0.45) than isolates from aborted fetuses (MRI=0.33). Though *B. abortus* and *E. coli* were main causes of abortions, there was no fixed set of bacteria causing metritis and abortions in buffaloes. Multiple drug-resistant (MDR) and carbapenem drug-resistant (CR) bacteria are more often associated with metritis than with abortions.

**Keywords:** *Aeromonas media*, *Alcaligenes faecalis*, *Enterobacter agglomerans*, *Kocuria varians*, *Micrococcus varians*, *Salmonella Dublin*, *Xenorhabdus poinarii*.

### Abbreviations

As per the 2019 livestock census, India holds 109.85 million buffaloes (*Bubalus bubalis*) [1] and milch buffaloes contribute about 55% of total milk production in the country [2]. Buffalo (*Bubalus bubalis*) meat constitutes about 30% of the total meat production of India [3] and buffalo meat exports account for

89.08% of total animal products export from India [4]. Though India holds >58% of world buffalo population, health and production problems of buffaloes are not taken as seriously as of cattle in India [5]. For economically rearing livestock, productive and reproductive health is an important determinant. The buffalo breeding efficiency indicated by healthy reproductive system depends on many

genetic, anatomical, physiological, nutritional, managerial and infectious factors. Frequent reproductive problems are considered as one of the most important reasons branding buffaloes as poor breeders [6]. Abortions in buffaloes are common and the incidence may vary from 1.51% to 7.1% in different breeds [7]. Galiero [8] and Satish and co-workers [9] enlisted *Brucella* spp., *Arcanobacterium pyogenes*, *Chlamydophila* spp., *Coxiella burnetii*, *Bacillus licheniformis*, *Campylobacter fetus venerealis*, *E.coli*, *Leptospira* spp., *Listeria monocytogenes* as important bacterial causes of abortions in Mediterranean buffaloes along with viral, fungal and protozoal causes. A few studies in different parts of buffalo rearing regions indicated that uterine infections are common in buffaloes and are major cause of repeat breeding [10]. In Iraq, common bacteria causing metritis include *E. coli*, *A. pyogenes*, *Bacteroides fragilis*, *Fusobacterium necrophorum*, *Streptococcus* spp., *Staphylococcus* spp., and *Pseudomonas* spp. [10,11]. In India, 9.66% incidence of metritis has been reported [12]. The present study reports bacterial profile of 23 abortion samples and 20 uterine aspirates of buffaloes diagnosed clinically having metritis to understand the common bacterial causes associated with the two important ailments of buffaloes and to reveal antimicrobial susceptibility of the isolates so that proper therapeutic intervention can be suggested.

### Materials and Methods

- Samples:** The stomach contents of aborted foeti during post-mortem examination were aseptically aspirated with sterile needle and syringe, transferred to sterile screw capped tubes and brought to the laboratory on ice within an hour of collection. In laboratory, samples were stored on ice till processing within 0-2 h of arrival.
  - The uterine aspirates collected aseptically by the clinicians in Referral Veterinary Polyclinic, ICAR-Indian Veterinary Research Institute, Izatnagar, were received in sterile sample collection vials in the Clinical Epidemiology Laboratory and kept on ice till tested within 0-2 h of receipt.
  - From all cases of abortions, dam blood samples were also collected for serum in vacutainer tubes (BD, Difco) and submitted to the laboratory for testing the serum primarily for antibodies to *Brucella*. The serum samples were stored at -20°C till tested.
  - Processing of samples for isolation of bacteria:** The stomach contents and uterine aspirates were directly inoculated onto blood agar (BA) plates and McConkey agar (MA) plates in duplicate and streaked for isolated colonies. One mL of each sample was also inoculated into 10 mL thioglycollate medium (BD, Difco) in screw capped tubes and incubated for 24 h at 37°C. Thereafter, from each tube of thioglycollate medium growth was streaked on to BA and MA plates in duplicate. One set of the plates was incubated aerobically at 37°C and other in microaerobic environment with 5% CO<sub>2</sub> at 37°C for 24-48 h. Three to five colonies of each type observed on plates were picked up for further characterisation using growth, morphological, staining and biochemical characteristics as described earlier [13-15]. Bacterial strains tentatively identified as *Brucella* spp. and *Salmonella* spp. were submitted to National *Brucella* Centre (Izatnagar) and National *Salmonella* Centre (Vet), respectively for confirmation and serotyping.
  - Detection of antibodies in dam serum for bacteria isolated from aborted foetuses:** For detecting *Brucella* antibodies by standard tube agglutination test OIE's standard guidelines [16] were followed. *Brucella* plain antigen was procured from Division of Biological Products, ICAR-Indian Veterinary Research Institute, Izatnagar, India.
  - Besides, pure cultures of the bacteria isolated from stomach contents of foetus were grown in tryptic soy broth (TSB, BBL Difco) medium for 18 h at 37°C and then equal amount of 1% formaldehyde saline [13] was added and tubes were kept overnight after shaking frequently. Then the broth cultures were centrifuged at 5000 rpm for 20 min and pellet was washed through suspending it in 10 mL sterile normal saline solution. Centrifuged again, supernatant discarded and pellet was resuspended in 0.5% formal saline to the 0.5 OD at 600 nm to use it as antigen for micro-agglutination test [13] with serially diluted respective dam serum.
  - Antimicrobial susceptibility assay:** All isolates were tested for antimicrobial sensitivity using disc diffusion assay on Mueller Hinton agar (MHA) or 5% serum added (for fastidious bacteria) MHA. After 24 h of incubation, diameter of zone of growth inhibition around each disc was measured in mm. Isolates were classified either sensitive or resistant following CLSI [17,18] guidelines. All media and antimicrobial discs (amoxicillin, amoxicillin + clavulanic acid, ampicillin, ampicillin + sulbactam, azithromycin, cefepime, cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, cotrimoxazole, enrofloxacin, erythromycin, gentamicin, imipenem, meropenem, moxalactam, nitrofurantoin, penicillin, piperacillin, piperacillin tazobactam, tetracycline and tigecycline) were purchased from BBL Difco, USA. Gram positive isolates were also tested for susceptibility to novobiocin and vancomycin, while Gram negative isolates were also tested for colistin and aztreonam susceptibility.
- All bacterial isolates were tested using disc diffusion assay for susceptibility to 13 herbal antimicrobials using discs containing 1 mg of >99% pure active herbal contents in each discs namely, ajo-

wan (*Tachyspermum ammi*) oil (Subh Flavor and Fragrance Pvt. Ltd., New Delhi), holy basil (*Ocimum sanctum*) oil (Subh Flavor and Fragrance Pvt. Ltd., New Delhi), cinnamaldehyde (Sigma Aldrich, USA), carvacrol (Sigma Aldrich, USA), lemongrass (*Cymbopogon citratus*) oil (Sigma Aldrich, USA), thyme (*Thymus vulgaris*) oil (Sigma Aldrich, USA), citral (Sigma Aldrich, USA), cinnamon (*Cinnamomum verum*) oil (Sigma Aldrich, USA), agarwood (*Aquilaria malaccensis*) oil (Naga Fragrance Ltd., Dimapur, India), patchouli (*Pogostemon cablin*) essential oil (Subh Flavor and Fragrance Pvt. Ltd., New Delhi), betel (*Piper betle*) leaf essential oil (Subh Flavor and Fragrance Pvt. Ltd., New Delhi), guggul (*Commiphora wightii*) oil and sandal (*Santalum album*) wood oil (Government Sandalwood Oil factory, Mysore, India). Testing was done in the way similar to antibiotics and any measurable zone of inhibition around disc was measured in mm. The test strain showing measurable growth inhibition was classified as sensitive else resistant as described earlier [19].

- **Multiple drug resistance indices (MRI) and multiple drug resistance (MDR):** Bacteria resistant to three or more therapeutically important classes of antibiotics were classified as MDR. The ratio between number of antimicrobials resisted and number of antimicrobials tested for each isolate was expressed as MRI. Similarly multiple herbal drug resistance indices (MHRI) and multiple herbal drug resistance (MHDR) were also determined.
- **Metallo- $\beta$ -lactamase (MBL) detection:** It was phenotypically determined using ETEST<sup>®</sup> MBL, (Bio-Merieux) for all carbapenem resistant strains (CLSI, 2014) as described by the manufacturer of the E-test strips. For determining minimum inhibitory concentration of imipenem and meropenem, E-test was performed as per BioMerieux E-strips and CLSI [18] guidelines.
- **Extended spectrum  $\beta$ -lactamase (ESBL) production assay:** All cephalosporin resistant isolates were tested using ETEST<sup>®</sup> ESBL (Bio-Merieux) as described by the producer of the E-test strips.
- **Statistical analysis:** The antimicrobial susceptibility and growth inhibition data for different types of bacteria against different antimicrobials was compared using Chi-square tests, odds ratio and correlation coefficient analysis.

## Results

### Bacteria isolated

From aborted foeti stomach contents of a total of 23 cases, 55 bacterial isolates belonging to 21 genera and 30 species were detected. Of the 23 samples, 13 samples had only single type of bacteria (*Aeromonas media* 1, *Alcaligenes faecalis* 2, *Brucella abortus* 4, *Enterobacter agglomerans* 1, *Escherichia coli* 3, *Klebsiella pneumoniae* ssp. *pneumoniae* 1, *Kocuria varians* 1), while 10 samples yielded more than one type of bacterial isolates (Table 1).

Of the 20 uterine aspirates, six samples had only single type of bacteria (*Acinetobacter lwoffii* 3, *Aeromonas salmonicida* ssp. *salmonicida* 1, *E. coli* 1, *K. varians* 1), while remaining 14 samples had more than one type of bacteria (Tab. 1). In total 38 isolates of 13 genera belonging to 16 species were detected in 20 samples.

### Antibodies in serum of aborted buffaloes

Of the 23 aborted dam serum samples, 17 samples contained antibodies in high titres ( $\geq 40$ ) against bacteria isolated from respective aborted foeti. Antibodies were detected against *A. media* (1), *A. faecalis* (1), *B. abortus* (4), *E. agglomerans* (2), *Enterobacter amnigenus* (1), *E. coli* (3), *K. pneumoniae* ssp. *pneumoniae* (2), *K. varians* (1), *Salmonella enterica* ssp. *enterica* ser Dublin (1) and *Xenorhabdus poinarii* (1). Six serum samples had no detectable agglutinins against bacterial isolates from respective foeti. *Brucella* antibodies could be detected in all samples of the dam buffaloes where *B. abortus* was isolated from stomach contents of their aborted foeti.

### Herbal antimicrobial susceptibility of bacterial isolates from aborted foeti

Ajowan oil discs inhibited growth of all 55 bacteria isolated from aborted foeti while guggul oil was the least effective antimicrobial. The susceptibility for herbal antimicrobials varied for Gram positive (G+ve) and Gram negative (G-ve) bacterial isolates. None of the G+ve isolate was susceptible to betel leaf oil (BLO) and guggul oil (GO), while agarwood oil (AWO) was the least effective on G-ve bacteria. Gram positive bacteria were significantly more often resistant to holy basil oil (OR. 9.52; CI<sub>95%</sub>, 1.10-82.02) than G-ve bacterial isolates but the susceptibility to citral was in reverse order i.e., G-ve bacteria had 16.82 times higher odds (CI<sub>95%</sub>, 1.01-280.08) to be resistant to citral than G+ve bacteria. However, there was no significant difference in multiple herbal drug resistance (MHDR) and multiple herbal drug resistance indices (MHRI) of G+ve (average MHDR, 5.33; average MHRI, 0.41) and G-ve (average MHDR, 5.95; average MHRI, 0.46) bacteria.

### Herbal antimicrobial susceptibility of bacterial isolates from metritis samples:

The most effective herbal antimicrobials killing about 90% or more isolates included ajowan oil (AO), cinnamon oil (CO) and carvacrol (Tab. 2). The guggul oil was the least effective against G+ve bacteria and agarwood oil against G-ve bacteria isolates. Though in general, G+ve bacteria were more often sensitive than G-ve bacteria for herbal antimicrobials, the difference was only significant for thyme oil (TO) and patchouli essential oil (PEO) susceptibility. Gram positive bacteria had 9.75 times higher odds to be resistant (CI<sub>95%</sub>, 1.30-79.95) to thyme oil than G-ve bacteria while for PEO G-ve bacteria had 4.79 times higher odds (CI<sub>95%</sub>, 1.26-18.24)

to be more resistant than G+ve bacteria. There was no significant difference in multiple herbal drug resistance (MHDR) and multiple herbal drug resistance indices (MHRI) of G+ve (average MHDR, 5.00; average MHRI, 0.38) and G-ve (average MHDR, 5.44; average MHRI, 0.42) bacteria.

On comparison of susceptibility of G-ve bacteria from two different origins to herbal antimicrobials, no significant difference was evident for most of the herbal antimicrobials except for holy basil oil (HBO) and lemongrass oil (LGO). Bacteria from metritis cases were more often resistant to HBO (OR, 7.84; CI<sub>95</sub>, 1.23-50.15) than those from aborted foeti while the order was reverse for susceptibility to LGO (OR, 3.23; CI<sub>95</sub>, 1.18-8.87). Similarly, on comparing G+ve bacteria from aborted foeti and metritis aspirates, no significant difference was detected in their susceptibility to any of the 13 herbal antimicrobials tested.

#### Antimicrobial susceptibility of bacterial isolates from aborted foeti

The antimicrobial susceptibility assay on 55 bacterial isolates revealed that tigecycline was the most effective antibiotic followed by ceftriaxone, imipenem, meropenem, colistin, chloramphenicol and gentamicin inhibiting >80% of the isolates while erythromycin was the least effective inhibiting <19% of the isolates. The antimicrobial resistance patterns differed for G+ve and G-ve bacteria (Tab. 3). All 12 isolates of G+ve bacteria were sensitive to tigecycline, cefotaxime, ceftriaxone, penicillin and tigecycline while none of the antibiotic could restrain growth of all the 43 G-ve bacterial isolates from aborted foeti. Gram positive bacteria were significantly more often resistant to ceftazidime (OR, 5.75; CI<sub>95</sub>, 1.12-29.41), gentamicin (OR, 5.42; CI<sub>95</sub>, 1.24-23.82) and moxalactam (OR, 6.17; CI<sub>95</sub>, 1.49-25.99) than G-ve bacteria while the later were more often resistant to amoxicillin (OR, 11.33; CI<sub>99</sub>, 1.58-81.53), ampicillin + sulbactam (OR, 25; CI<sub>95</sub>, 1.33-43.26), and erythromycin (OR, 126; CI<sub>99</sub>, 11.72-1354.58) than G+ve bacteria. Of the 12 G+ve bacteria only one isolate of *Staphylococcus warneri* was resistant to meropenem and imipenem (imipenem MIC 4 µg/ mL). However, of the 43 G-ve bacterial isolates five (one isolate each of *Acinetobacter calcoaceticus*, *A. bestiarum*, *Enterobacter amnigenus*, *Hafnia alvei* and *Moellerella wisconsensis*) produced MBL (imipenem MIC >32 µg/ mL). Seven of the 12 G+ve bacteria were resistant to one or more cephalosporins and 9 were classified as MDR. Of the 43 G-ve bacteria, 23 produced ESBL and 33 had MDR. There was no significant difference in MDR and MRI of G+ve and G-ve isolates from aborted foeti.

#### Antimicrobial susceptibility of bacterial isolates from metritis samples

Of the 38 isolates, 11 were G+ve and 27 were G-ve bacteria and their antimicrobial susceptibility patterns varied significantly. However, there was no significant difference in multiple drug resistance among G+ve (10/11) and G-ve bacteria (23/27). Ampicillin + sulbactam, chloramphenicol and penicillin inhibited growth of all 11 G+ve isolates while penicillin failed to inhibit growth of all the G-ve isolates (Tab. 3). For ampicillin, G-ve bacteria were significantly more often (OR, 1.61; CI<sub>95</sub>, 1.12-22.41) resistant than G+ve bacteria but susceptibility was in reverse order for ceftazidime (OR, 9.29; CI<sub>95</sub>, 1.04-82.96), gentamicin (OR, 5.28; CI<sub>95</sub>, 1.14-24.47) and moxalactam (OR, 19.8; CI<sub>99</sub>, 1.82-215.61). One strain each of *Enterococcus faecium* and *K. varians* were resistant to carbapenems (imipenem MIC >32 µg/ mL), while of the 10 isolates of carbapenem resistant (CR) G-ve bacteria, an *E. coli* isolate was resistant to only meropenem (MIC 4 µg/ mL) but sensitive to imipenem (MIC 0.19 µg/ mL). Other nine isolates of G-ve CR strains (*Acinetobacter lwof-fii* 5, *Alcaligenes denitrificans* 1, *Aeromonas salmonicida* ssp. *salmonicida* 1 and *E. coli* 2) produced MBL. For all MBL positive G-ve bacteria, imipenem MIC was >32 µg/ mL) except for *A. salmonicida* ssp. *salmonicida* (imipenem MIC 4 µg/ mL).

On comparing antibiotic susceptibility of G+ve bacteria of foetal calf and uterine aspirate origin, no significant difference was evident for most of the antibiotics, all isolates from aborted foeti were susceptible to azithromycin, cefotaxime and ceftriaxone while 6, 7 and 7 of the 11 isolates from metritis cases were resistant to these antibiotics, respectively. On comparing susceptibility of G-ve bacterial isolates of the two sources, difference in their susceptibility to most of the antibiotics was significant but isolates of metritis samples were many more time resistant to colistin (OR, 7.06; CI<sub>99</sub>, 1.45-34.24), cotrimoxazole (OR, 4.82; CI<sub>99</sub>, 1.19-19.46), erythromycin (OR, 56.06; CI<sub>99</sub>, 8.01-392.61), imipenem (OR, 3.8; CI<sub>95</sub>, 1.11-12.98) and piperacillin (OR, 3.36; CI<sub>95</sub>, 1.23-9.18).

#### Comparison between antimicrobial susceptibility of bacteria of different species and bacteria of same species of different origin

Only those groups were compared where numbers of isolates were ≥6. *Escherichia coli* isolates from aborted foeti (8) and metritis cases (9) had almost similar pattern of resistance for herbal and conventional antimicrobials barring a few exception. One of the eight *E. coli* isolates from aborted foeti was resistant to HBO while 7 of the 9 isolates from metritis cases were resistant (OR, 24.5; CI<sub>95</sub>, 1.79-336.24). Nitrofurantoin and carbapenems inhibited all *E. coli*

from aborted foeti but 4 and 2 *E. coli* isolates from metritis cases were resistant, respectively. Similarly, ceftazidime and piperacillin + tazobactam were significantly more often (OR, 13.33; CI<sub>95</sub>, 1.07-166.38) effective on *E. coli* isolates from aborted foeti than those for metritis cases. *Alcaligenes faecalis* (6) and *E. coli* (8) from aborted foeti had no significant difference in their susceptibility to herbal antimicrobials but all *A. faecalis* isolates and only four isolates of *E. coli* were resistant to nitrofurantoin. All *E. coli* from aborted foeti were susceptible to ceftriaxone, chloramphenicol, colistin, cotrimoxazole and piperacillin tazobactam but 3, 2, 3, 4 and 3 *A. faecalis* isolates were resistant to these antimicrobials, respectively. For tetracycline, *A. faecalis* isolates had 35 times higher odds (CI<sub>95</sub>, 1.74-703.03) to be resistant than *E. coli* Isolates.

On comparing antimicrobial susceptibility of *A. lwoffii* (6) and *E. coli* (9) isolates from cases of metritis revealed that all *A. lwoffii* were susceptible to BLO, citral, HBO, LGO, azithromycin and tetracycline while among *E. coli* from metritis 7, 6, 7, 7, 6 and 5 isolates, respectively were resistant to these antimicrobials. However, 4 of 6 *A. lwoffii* isolates were resistant but all 9 *E. coli* isolates were susceptible to moxalactam.

**Correlation among antimicrobial activity of herbal antimicrobials and conventional antimicrobials**

On analyzing correlation between zones of inhibitions (ZIs) around herbal and conventional antimicrobials for different bac-

teria, for majority of the antimicrobials either there was no significant correlation or was positive correlation, negative correlation was detected only for a few pairs (Tab. 4). Most of the herbal antimicrobials tested had significant correlation among ZIs (r, ≥ 0.21; p, ≤ 0.05) except a few viz., ZIs around guggul oil discs had positive but insignificant correlation (r, ≤ 0.21; p, ≥ 0.05) with ZIs around carvacrol, cinnamaldehyde, HBO and SWO discs; ZIs of SWO discs were also not in significant correlation with ZIs induced by BLO and thyme oil discs. Similar positive correlation was evident among ZIs of different antibiotics. Although aztreonam had negative correlation with several antibiotics including azithromycin, colistin, erythromycin, gentamicin, imipenem, penicillin, tetracycline and tigecycline, it was significant only with colistin (r, -0.28; p, 0.01). Similarly, moxalactam ZIs also had insignificant negative correlation with ZI of several antibiotics including, amoxicillin, ampicillin, ampicillin + sulbactam, azithromycin, colistin, penicillin, tetracycline and tigecycline but it turned significant with erythromycin (r, -0.26; p, 0.05). Besides, significant negative correlation with aztreonam ZIs (r, -0.28; p, 0.01), colistin had insignificant negative correlation with ZIs of ampicillin + sulbactam, cefepime, cefotaxime, ceftazidime, meropenem, moxalactam and penicillin. Vancomycin was another important antibiotic having significant negative correlation with antimicrobial activity of chloramphenicol (r, -0.24; p, 0.05) but insignificant negative correlation with azithromycin and tigecycline.

Case Number	Bacteria detected in foetal calf stomach contents	Antibodies in dam serum detected (agglutination titre) for	Bacteria detected in uterine aspirate of buffaloes suffering from metritis
1	<i>Edwarsiella tarda</i> , <i>Enterococcus avium</i> , <i>Kocuria varians</i> , <i>Streptococcus bovis</i> , <i>Aerococcus</i> spp.	No	<i>Bacillus pantothenicus</i> , <i>Vibrio alginolyticus</i>
2	<i>Salmonella enterica</i> ssp. <i>enterica</i> ser Dublin, <i>Enterococcus durans</i> , <i>Staphylococcus aureus</i> , <i>Aeromonas bestiarum</i>	<i>S. Dublin</i> (40)	<i>E. coli</i> , <i>H. alvei</i>
3	<i>Brucella abortus</i>	<i>B. abortus</i> (>320)	<i>E. agglomerans</i> , <i>Proteus vulgaris</i>
4	<i>B. abortus</i>	<i>B. abortus</i> (>320)	<i>Acinetobacter lwoffii</i> , <i>E. agglomerans</i> , <i>P. vulgaris</i>
5	<i>Alcaligenes faecalis</i>	<i>A. faecalis</i> (>320)	<i>A. lwoffii</i>
6	<i>Escherichia coli</i> , <i>Eanterobacter agglomerans</i>	<i>E. coli</i> (320)	<i>A. lwoffii</i> , <i>P. vulgaris</i>
7	<i>E. coli</i>	<i>E. coli</i> (320)	<i>B. pantothenicus</i> , <i>E. coli</i>
8	<i>E. coli</i>	<i>E. coli</i> (320)	<i>E. coli</i> , <i>E. faecalis</i>
9	<i>Klebsiella pneumoniae</i>	<i>K. pneumoniae</i> (80)	<i>K. varians</i> , <i>Streptococcus morbillorum</i>
10	<i>E. agglomerans</i>	<i>E. agglomerans</i> (40)	<i>K. varians</i> , <i>Streptococcus pneumoniae</i>
11	<i>E. coli</i> , <i>E. agglomerans</i>	<i>E. agglomerans</i> (>320)	<i>K. varians</i>
12	<i>K. pneumoniae</i> , <i>Xenorhabdus bovienii</i>	<i>K. pneumoniae</i> (40)	<i>Aeromonas salmonicida</i> ssp. <i>salmonicida</i>
13	<i>K. pneumoniae</i> , <i>Alacligenes faecalis</i> , <i>A. denitrificans</i> , <i>Pseudomonas testosteronii</i> , <i>Erwinia cyperipedii</i>	No	<i>E. coli</i> , <i>Staphylococcus intermedius</i>

14	<i>Enterobacter amnigenus, E. agglomerans</i>	<i>E. amnigenus</i> (80)	<i>A. lwoffii</i>
15	<i>Xenorhabdus poinarii, X. bovienii, Staphylococcus warnerii</i>	<i>X. poinarii</i> (>320)	<i>A. denitrificans, E. coli</i>
16	<i>Acinetobacter calcoaceticus, E. coli, Hafnia alvei, Bacillus subtilis, Moellerella wisconsensis</i>	No	<i>A. denitrificans, A. bestiarum</i>
17	<i>Aeromonas media</i>	<i>A. media</i> (160)	<i>A. lwoffii</i>
18	<i>B. abortus</i>	<i>B. abortus</i> (>320)	<i>E. coli, Enterococcus faecium</i>
19	<i>B. abortus</i>	<i>B. abortus</i> (>320)	<i>E. coli</i>
20	<i>A. faecalis</i>	No	<i>E. coli, E. faecium</i>
21	<i>Achromobacter xyloxidans, Aeromonas schindleri, Streptococcus agalactiae, Streptococcus uberis</i>	No	
22	<i>M. vairans</i>	<i>M. vairans</i> (320)	
23	<i>E. coli</i>	No	

**Table 1:** Bacteria detected in aborted foetus stomach contents, antibodies in aborted dam buffalo serum and uterine aspirate samples of buffaloes suffering from metritis.

The most effective to the least effective herbal antimicrobials (% resistant isolates) on bacterial isolated from			
Aborted foeti stomach contents		Uterine aspirates of buffaloes with metritis	
Gram positive isolates, n = 12	Gram negative isolates, n = 43	Gram positive isolates, n = 11	Gram negative isolates, n = 27
Ajowan oil (0.00)	Ajowan oil (0.00)	Ajowan oil (0.00)	Cinnamledehyde (0.00)
Carvacrol (0.00)	Thyme oil (0.00)	Carvacrol (0.00)	Carvacrol (3.70)
Cinnamledehyde (0.00)	Carvacrol (2.33)	Cinnamledehyde (9.09)	Thyme oil (3.70)
Cinnamon oil (8.33)	Cinnamledehyde (4.65)	Carvacrol (27.27)	Ajowan oil (11.11)
Citral (8.33)	Holy basil oil (6.98)	Lemongrass oil (27.27)	Carvacrol (11.11)
Thyme oil (8.33)	Carvacrol (9.30)	Thyme oil (27.27)	Holy basil oil (37.04)
Lemongrass oil (33.33)	Citral (60.47)	Citral (36.36)	Citral (44.44)
Holy basil oil (41.67)	Lemongrass oil (72.09)	Holy basil oil (36.36)	Lemongrass oil (44.44)
Sandalwood oil (66.67)	Betel leaf oil (74.42)	Patchouli essential oil (54.55)	Betel leaf oil (51.85)
Agarwood oil (83.33)	Sandalwood oil (81.40)	Agarwood oil (63.64)	Guggul oil (81.48)
Patchouli essential oil (83.33)	Guggul oil (93.02)	Sandalwood oil (63.64)	Sandalwood oil (81.48)
Betel leaf oil (100.00)	Patchouli essential oil (93.02)	Betel leaf oil (72.73)	Patchouli essential oil (85.19)
Guggul oil (100.00)	Agarwood oil (97.67)	Guggul oil (81.82)	Agarwood oil (88.89)

**Table 2:** Herbal antimicrobial susceptibility among bacterial isolates from stomach contents of aborted foeti and uterine aspirates from buffaloes suffering from metritis.

The most effective to the least effective antimicrobials (number of resistant isolates) on bacteria isolated from			
Aborted foeti stomach contents (resistant strains)		Uterine aspirates of buffaloes with metritis (resistant strains)	
Gram positive isolates, n = 12	Gram negative isolates, n = 43	Gram positive isolates, n = 11	Gram negative isolates, n = 27
AS (0)	TGC (1)	AS (0)	TGC (1)
AZM (0)	Cl (5)	C (0)	C (3)
CTX (0)	G (5)	P (0)	CPM (4)
CTR (0)	I (5)	TGC (1)	G (5)
P (0)	MR (5)	I (2)	Mox (5)
TGC (0)	CTR (6)	MR (2)	CTX (8)
I (1)	Mox (6)	Nf (3)	MR (8)
MR (1)	C (7)	A (4)	T (8)

Nf (1)	CPM (8)	CPM (4)	CTR (9)
C (2)	Ef (10)	Pi (4)	I (9)
Co (2)	PIT (10)	PIT (4)	Ef (11)
Pi (2)	AZM (11)	Ef (5)	AZM (12)
PIT (2)	CTX (11)	AZM (6)	PIT (12)
AMX (3)	T (12)	Co (6)	AC (13)
AC (3)	Pi (13)	E (6)	Cl (13)
Ef (3)	Nf (15)	G (6)	AT (14)
E (3)	Co (16)	T (6)	CA (14)
T (3)	AT (20)	AC (7)	Nf (14)
CPM (4)	CA (20)	CTX (7)	AS (16)
Va (4)	AS (22)	CTR (7)	Pi (16)
G (5)	AC (24)	Va (7)	A (20)
A (6)	A (33)	AMX (8)	Co (20)
Mox (6)	AMX (34)	Mox (9)	AMX (23)
NB (8)	P (41)	NB (9)	E (23)
CA (10)	E (42)	CA (10)	P (27)

**Table 3:** Antimicrobial susceptibility among bacterial isolates from stomach contents of aborted foeti and uterine aspirates from buffaloes suffering from metritis.

AMX: Amoxicillin; AC: Amoxicillin + Clavulanic Acid; A: Ampicillin; AS: Ampicillin + Sulbactam; AZM: Azithromycin; AT: Aztreonam; CPM: Cefepime; CTX: Cefotaxime; Ca: Aeftazidime; CTR: Ceftriaxone; C: Chloramphenicol; Cl: Colistin; Co: Cotrimoxazole; Ef: Enrofloxacin; E: Erythromycin; G: Gentamicin; I: Imipenem; MR: Meropenem; Mox: Moxalactam; Nf: Nitrofurantoin; NB: Novobiocin; P: Penicillin; PiL: Piperacillin; PIT: Piperacillin + Tazobactam; T: Tetracycline; TGC: tigecycline; Va: Vancomycin

Antimicrobial	Significant negative correlation ( $r, \leq -0.21$ ; $p, \leq 0.05$ ). With zone of inhibition by
Betel leaf oil	Ceftriaxone, imipenem
Cinnamon oil	Ampicillin + sulbactam
Lemongrass oil	Moxalactam
Patchouli essential oil	Aztreonam
Ampicillin + sulbactam	Cinnamon oil
Aztreonam	Patchouli essential oil, colistin
Ceftriaxone	Betel leaf oil
Chloramphenicol	Vancomycin
Colistin	Aztreonam
Erythromycin	Moxalactam
Imipenem	Betel leaf oil
Moxalactam	Lemongrass oil, erythromycin
Vancomycin	Chloramphenicol

**Table 4:** Negative correlation among zone of inhibition of bacteria isolated from stomach contents of buffalo aborted foeti and uterine aspirates of buffaloes with metritis against antimicrobial discs.

## Discussion

Abortions [6,7] and metritis [12,20] are two important reproduction problems of buffaloes affecting their productivity and profitability. The study has not determined the real occurrence of abortion and metritis in buffaloes but concentrated on types of bacteria present in samples received in the laboratory for bacterial identification. Of the 55 bacteria isolated from stomach contents of aborted foeti, a few including *Aeromonas media*, *Alcaligenes faecalis*, *Brucella abortus*, *Enterobacter agglomerans*, *Escherichia coli*, *Klebsiella pneumoniae* ssp. *pneumoniae*, and *K. varians* were isolated as pure culture indicating that they might be the main or important cause of abortion. Besides, many of those bacteria which were present in mixed culture in aborted foeti had antibodies in respective aborted animals (*E. agglomerans*, *E. amnigenus*, *E. coli*, *K. pneumoniae* ssp. *pneumoniae*, *Salmonella enterica* ssp. *enterica* ser Dublin and *Xenorhabdus poinarii*) indicating that at one or other stage of abortion these bacteria might have also invaded the dam body to induce antibody production and might be an important cause of abortion. *Brucella abortus* has often been reported to cause abortion in bovines [21] and is an endemic problem in India and isolation of the pathogen has often been reported in Bareilly region in aborted foeti [22]. Except *E. coli* and *Brucella abortus*, many of the bacteria identified in the present study having a causal association with abortion in buffaloes are not listed among important causes of abortion in earlier studies [8,9] and it might be due to difference in geographical distribution of pathogens and also the environmental and husbandry factors might be important. However, many of the similar types of bacteria as identified in this study are reported to cause of abortions, as *Salmonella enterica* serovars including *S. Dublin* in cattle [23-25], *Aeromonas* species in equids [26] and bovines [27], *Enterobacter agglomerans* in equids [28,29], *Moellerella wisconsensis* in bovines [30,31], *Xenorhabdus bovienii* and *X. poinarii* in bovines [30], *K. pneumoniae* in cattle [27]. However, *K. varians* identified as cause of abortion and or metritis is rarely reported earlier. Though *K. varians* strains are often considered as non-pathogenic commensally present and colonizing on the skin, mucosa and oropharynx have also been reported to cause opportunistic infections involving almost all systems of the body [32,33].

A total of 38 bacterial isolates belonging to 16 species (*Acinetobacter lowffii*, *Aeromonas bestiarum*, *A. salmonicida*, *Alcaligenes denitrificans*, *Bacillus pantothenicus*, *E. agglomerans*, *E. faecalis*, *E. faecium*, *E. coli*, *Hafnia alvei*, *K. varians*, *Proteus vulgaris*, *Staphylococcus intermedius*, *Streptococcus morbilorum*, *S. pneumoniae* and *Vibrio alginolyticus*) of 13 genera detected in uterine aspirates of buffaloes suffering with metritis indicates about diversity in microbiota associated with uterine infection in buffaloes. Many of the bacteria detected in the study have also been identified as part of

reproductive tract microbiota and cause of abortion and metritis in bovines and other animals [11,12,27,30,31,34,35] but some like *K. varians* and *V. alginolyticus* have rarely been associated with bovine infections but reported in human as opportunistic pathogens. Similar types of bacteria have been reported in vaginal tract of apparently healthy sows [35] and a closely related animal, mithun (*Bos frontalis*), to buffaloes [34] indicating that the bacteria identified in the present study might be opportunistic pathogens of reproductive tract. *Acinetobacter lowffii* and aeromonads often causes uterine infection in humans [36]. Isolation of *B. pantothenicus*, a known probiotic bacteria is a naturally occurring bacteria in synbiotics and may be affecting reproductive tract functionality [37], from uterus might either be an accidental contamination or due to therapeutic use of the bacteria with some intrauterine antibiotics.

Comparatively higher level of antimicrobial resistance observed in bacteria isolated from cases of metritis than those from abortion cases indicated the commensality of the bacteria in reproductive tract or their acquisition through frequent manoeuvring of reproductive tract for therapeutic purposes i.e., nosocomial infection. The nosocomially acquired and commensally occurring bacteria are known to possess higher level of resistance than community acquired infections [38,39].

Though carbapenems are not permitted for use in livestock, identification of carbapenem resistant and MBL producing bacteria from abortion and metritis cases of buffaloes is an alarming health hazard but not novel as many of such bacteria have been reported world over due to communal spread of MDR strains and spread of resistance factors among different bacteria associated with abortions and metritis [40,41]. Emergence of carbapenem resistant bacteria is not only reported in domestic animals [41,42] but in captivated pets and wild animals too [43]. However, it certainly emphasizes the need for an early intervention to curb spread of AMR in uncontrolled manner in livestock.

In the present study some of herbal antimicrobials including ajowan oil, thyme oil, cinnamon oil, carvacrol and cinnamaldehyde proved better than many of the antibiotics on metritis and abortion causing bacteria. Herbal antimicrobials have been advocated as an alternative to antibiotics especially in management of MDR pathogens [44,45]. In treatment of bacterial metritis, antimicrobial potential of herbs can be exploited easily through incorporating herbal preparation either in liposomal or other nano-encapsulation delivery vehicles as suggested earlier for gastrointestinal tract infections [46].

The study concluded that there is no fixed set of bacteria causing metritis and abortions in buffaloes and it may vary depending

on geography and the target host. Similarly, antimicrobial susceptibility of bacteria not only depends on type of bacteria but with the problem they are associated with. Although a few of the antibiotics were comparatively more effective, are not recommended for animal use. Thus the antimicrobial susceptibility testing of the isolates to determine the therapeutic usefulness may be valuable in handling reproductive tract infections. Uterine tract infections are usually chronic in nature and the treatment can be deferred for a few days to get the antimicrobial susceptibility report rather than wasting valuable antimicrobials and precious breeding time of buffaloes. Although herbal antimicrobials may be tried as antibiotic alternatives, studies on suitable formulations are needful for conformity in the therapeutic outcome.

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### Conflict of Interest

The author declares that there is no conflict of interest.

### Contribution of Authorship

BRS, KPS, AMP conceived, designed, analysed the study, KPS, AMP collected samples, BRS did bacteriological analysis and BRS, KPS, AMP, DKS prepared and reviewed manuscript.

### Details of Ethical Approval

Study involved clinical samples collected as routine activity in veterinary polyclinic it not required any ethical approval.

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