STUDIES ON AEROBIC BACTERIA ISOLATED FROM BOVINE SUBCLINICAL AND CLINICAL ENDOMETRITIS

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CERTIFICATE

This is to certify that the thesis entitled "***STUDIES ON AEROBIC BACTERIA ISOLATED FROM BOVINE SUBCLINICAL AND CLINICAL ENDOMETRITIS***" submitted by **Mrs. PAVITHRA, B. S.,** I. D. No. **MVSK 1804** in partial fulfillment of the requirements for the award of **MASTER OF VETERINARY SCIENCE** in **VETERINARY MICROBIOLOGY** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by her during the period of her study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, association ship, fellowship or other similar titles.

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Affectionately dedicated to:

My father Late Shivanna
who taught me about dreams
and how to catch them.

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LIST OF ABBREVIATIONS/ ACRONYMS

%	:	Per centa
&	:	And
/	:	Per
<	:	Less than
>	:	More than
µg	:	Microgram
µL	:	Microlitre
agn43	:	Antigen 43
AH & VS	:	Animal husbandry and veterinary services
AMR	:	Anti microbial resistance
BHI	:	Brain heart infusion
C. bovis	:	*Corynebacterium bovis*
CE	:	Clinical endometritis
CLSI	:	Clinical Laboratory Standards Institute
CoNS	:	Coagulase Negative *Staphylococcus*
CoPS	:	Coagulase Positive *Staphylococcus*
csgA	:	Curli fimbriae
DNA	:	Deoxyribonucleic acid
DW	:	Distilled water
E. coli	:	*Escherichia coli*
e.g	:	Example
EDTA	:	Ethylene Diamine Tetra acetic acid
et. al	:	*Et.*

alili	:	and others
F	:	Forward
Fig.	:	Figure
fimH	:	Type 1 fimbriae
fyuA	:	Yersiniabactin
gm(s)	:	Gram/s
hr(s)	:	Hour(s)
i.e.	:	*id est* or that is
K. pneumoniae	:	*Klebsiella pneumonia*
kpsMT II	:	Protectin
M	:	Molar
mA	:	Milli ampere
MDR	:	Multi drug resistant
mecA	:	Gene encoding PBP2A (penicillin-binding protein 2A)
Min	:	Minutes
mL	:	Millilitre
mM	:	Milli molar
m-PCR	:	Multiplex PCR
MRSA	:	Methicillin resistant *Staphylococcus aureus*
MRSS	:	Methicillin sensitive *Staphylococcus aureus*
Nacl	:	Sodium Chloride
NCBI	:	National Center for Biotechnology Information
NFW	:	Nuclease Free Water
ng	:	Nano gram
nuc	:	Thermonuclease

°C	:	Degree Celsius
OD	:	Optical Density
P. aeruginosa	:	*Pseudomonas aeruginosa*
P. mirabilis	:	*Proteus* mirabilis
PBS	:	Phosphate Buffer Saline
PCR	:	Polymerase Chain Reaction
PMN	:	Polymorphonuclear neutrophils
Pmol	:	picomole (s)
Psi	:	Pounds per square inch
pvt	:	Private
R	:	Reverse
R. equi	:	*Rhodococcus equi*
rpm	:	Revolutions per minute
S. aureus	:	*Staphylococcus aureus*
S. uberis	:	*Streptococcus uberis*
SE	:	Subclinical endometritis
Sec	:	Seconds
soda	:	Super oxide dismutase-A
spa	:	Gene encoding *Staphylococcus aureus* protein A
Taq	:	*Thermus aquaticus*
T. pyogenes	:	*Trueperella pyogenes*
TBE	:	Tris Borate EDTA
TE	:	Tris EDTA
U	:	Unit
uidA	:	Gene encoding beta-glucuronidase

uspA	:	Gene encoding Universal stress protein
UV	:	Ultra Violet
V	:	Volts
VF	:	Virulence Factor
v/v	:	volume / volume
viz..	:	Videlicet (namely)
w/v	:	weight / volume

Introduction

I. INTRODUCTION

The uterine diseases such as endometritis, metritis and pyometra are a major concern for dairy farmers and are highly prevalent in high yielding dairy cows. The causes of uterine diseases are multifactorial in nature including infectious etiological agents (Lewis, 1997). They are a result of the contamination of the uterus that occur during and after calving (Azawi, 2008; Sheldon and Dobson, 2004). Diagnosis of the uterine diseases is done by evaluating the uterine discharge, the clinical signs of the illness, and by investigating the cytology and by taking into account the number of days since parturition (Sheldon *et al*., 2006).

Endometritis may appear as subclinical form often the untreated leading to clinical form. Clinical endometritis is characterized by the presence of purulent (>50% pus) or mucopurulent (approximately 50% pus, 50% mucus) vaginal discharge at 21 or more days postpartum, accompanied by a prominent leukocyte infiltration into the uterine lumen and is not accompanied by systemic signs. This form of endometritis is easily detectable by clinical examination of the animal (LeBlanc *et al*., 2002b; Sheldon *et al*., 2006; Ingale *et al.,* 2016).

Subclinical endometritis is characterized by an increased proportion of polymorphonuclear neutrophils (PMN) in the endometrium, >18% neutrophils in uterine cytology samples collected 21-33 days post-partum or >10% neutrophils at 34–47 days, in the absence of clinical endometritis (Kasimanickam *et al*., 2004). Therefore, detection of subclinical form mainly relies on the cytology of the uterine discharge to determine the neutrophils count (Dubuc, 2011; LeBlanc, 2014).

During the first two weeks of post-partum, around 80–100% of dairy cows can harbor a broad diversity of bacteria, which includes potential pathogens. Depending on the balance between the immune response and pathogenicity of bacteria causing uterine infection (Sheldon *et al*., 2009), 15–20% of cows develop clinical endometritis and 30% develop subclinical endometritis beyond 3 weeks postpartum (Cheong *et al*., 2011). Postpartum endometritis has a negative effect on reproductive performance as it delays resumption of ovarian cycles, prolongs postpartum luteal phases, increases days to first service and days open, and decreases the conception rate (Kasimanickam *et al*., 2004; Wang *et al*., 2018).

After calving, most of the bacteria, isolated from the uterus of normal cow are responsible for different types and degrees of infection depending on the health status of the animals (Abere and Belete, 2016).

Either a single isolate or mixed isolates are obtained from subclinical and clinical endometritis. The most common single aerobic bacterial isolates cultured from cows with subclinical endometritis are *Escherichia coli*, *Staphylococcus aureus*, proteus species, *Trueperella pyogenes*, *Enterobacter* species, *Corynebacterium* spp., *Klebsiella* spp., *Citrobacter* spp. and *Rhodococcus equi* (Takamtha *et al*., 2013; Abreham *et al*., 2017; Sahadev *et al*., 2017).

The bacteria isolated from the post-partum endometritis are classified as an obligate uterine pathogens which includes *T. pyogenes, E. coli* potential uterine pathogens such as non hemolytic streptococci and opportunistic bacteria like *Klebsiella* spp*., Proteus* spp. and coagulase-negative staphylococci (Sheldon *et al*., 2004a; Williams *et al.,* 2005; Nibret *et al*., 2013).

The bacterial infections of uterus are treated with wide range of antibiotics. There is a need to evaluate the efficacy of such therapeutic agents from time to time due to continuous emergence of drug resistant bacterial strains (Barman *et al*., 2013) and thereby preventing the further usage of antibiotics and to help the farmers in reducing extra burden in terms of money for treatment of affected animals.

Antibiotic resistance being recognized as a top public health and veterinary medicine challenge of the 21st century (Thomson *et al*., 2004). Improper use of antimicrobial agents is the paramount factor for the emergence and dissemination of these antimicrobial resistant bacteria (Sayah *et al*., 2005). Hence, veterinarians should support the judicious use of antimicrobials to reduce the emergence and spreading of resistances. Culture and sensitivity test is considered as one of the best methods to evaluate the efficacy of these agents and to detect the resistance if any at the early stages.

Various research works have been carried out on the bovine reproductive tract with endometritis and have recognized a wide array of bacteria and their corresponding sensitivity to different antibacterial agents (Nibret *et al.,* 2013; Brodzki *et al.,* 2014; Sharma *et al.,* 2017). As different types of infectious agents are involved in uterine infections and hence cannot conclude a set recommendation of drugs as ideal. Identification and *in vitro* culture sensitivity against antimicrobial agent should be carried out in principle. Subsequently following the test reports in treatment regimen with an approved drug is the most practical, efficient and economical approach (Sharma *et al.,* 2017; Kumar *et al.,* 2018).

It is required to know the minimum inhibitory concentrations (MICs) of the antimicrobial agents available before choosing an appropriate antibiotic to treat a postpartum uterine infection caused by primary pathogens in a particular geographic region (Sheldon *et al*., 2004b).

The routine zone diffusion assays can only categorize laboratory outcomes as susceptible, intermediate susceptible or resistant, and will not quantify resistance by actual concentration of the antibacterial agents. The advantage of determining the minimum inhibitory concentration (MIC) is that it quantifies the resistance more accurately and infers required antimicrobial concentration can be modified to achieve target therapeutic serum/tissue concentration where pharmacokinetic /pharmacodynamic data are available (Balouiri *et al*., 2016).

The varied sensitivity and even resistance to antibacterial agents by bacterial isolates obtained from endometritis cases can be attributed to indiscriminate use of commonly used antibiotics and subsequently, development of resistant strains due to bacterial mutation (Kumar *et al*., 2018)

In addition to the conventional detection methods, molecular techniques of polymerase chain reaction (PCR), multiplex PCR and others are found to be efficacious in expeditious and confirmatory diagnosis of reproductive infections in farm animals caused by various infectious agents (Vidal *et al.,* 2004; Sheldon *et al.,* 2010; Bicalho *et al.,* 2012).

The study of virulence factors represents a new way of understanding the bacterial pathogenicity and intensifying the knowledge obtained from this will explain the mechanism behind the establishment of postpartum uterine infections in cows (Luana *et al*., 2019).

Keeping these points in view, the present study was undertaken with the following objectives:

1. Isolation and phenotypic identification of aerobic bacteria obtained from subclinical and clinical endometritis in dairy cattle.
2. Study of antibiogram profile of the bacterial isolates.
3. Detection of major virulence genes in the predominant bacterial species by PCR.

Review of literature

II. REVIEW OF LITERATURE

Reproductive disorders like subclinical endometritis, clinical endometritis, metritis and pyometra are a curse to farming community (Sheldon *et al.,* 2008). A large array of microflora causes uterine infection and endometrial inflammation leading to conception failure which is considered to be the chief cause of endometritis (Singh *et al.,* 1996).

Economic viability of dairy-farming depends on the fertility of its dairy cows. Reproductive performance of dairy cows deteriorates to maximum extent when uterine disorders occur in postpartum period. The knowledge of the pathophysiology behind uterine disorders like subclinical endometritis, endometritis and metritis and its sequelae over a period of time (short-term and long-term), has significantly increased due to their major impact on viability of dairy sector in past few decades (Huszenicza *et al*., 1999; Gilbert *et al*., 2005; Williams *et al*., 2007; Sheldon *et al*., 2009).

Repeat breeding due to SE in the postpartum period is one of the most important reproductive disorders in cattle whose average prevalence rate ranges from 10% to 14% in dairy cows (Båge *et al*., 2002; Yusuf *et al*., 2010). Cheong *et al*. (2011) reported that beyond 3 weeks postpartum the incidence of CE was 15-20% and that of SE was 30%.

Subclinical endometritis is not associated with the visible turbidity of uterine discharge but it does affect the reproductive performance of the animal by increasing the number of inseminations per conception, increased calving interval and thereby reduces the overall reproductive performance of the cow even ending up in culling of animals (Kasimanickam *et al.,* 2005; Sheldon *et al.,* 2008; Le Blanc 2008; Chapwanya *et al.,* 2010).

Using of endometrial cytology in post-partum repeat breeding animals can minimize the productivity loss and lead to timely diagnosis of SE, and can aid in improvising the reproductive performance (Sharma *et al*., 2017).

2.1 Diagnosis of subclinical endometritis in cows by endometrial cytology

Neutrophils are the first cells to arrive on the scene when any body tissue encounters an extraneous bacterial infection. They form first line of defence even in post-partum period to check the invasion of pathogenic organisms. Hence large numbers of neutrophils are recruited in the uterine lumen as first sign of inflammation. In the uterus, the establishment and propagation of infection is controlled by phagocytic activity of neutrophils (Butt *et al.,* 1993). This uterine inflammation due to bacterial invasion in the postpartum uterus with hand in hand involution of uterus by endometrial repair occur normally (Ahmadi *et al.,* 2006). But in some cases, this phase of inflammation can extend more than the normal threshold leading to SE (Le Blanc, 2014).

Endometrial cytology being preferred technique to diagnose SE in both field and research setups. It is the simple, cost effective and more reliable conclusive method to diagnose SE (Dubuc *et al*., 2010; De Boer *et al*., 2014).

The conclusive indicator in SE is the proportion of polymorphonuclear neutrophils (PMN) in the total number of endometrial cells recovered from the uterus during diagnosis. There are different thresholds of PMN cells for concluding as SE being mentioned by various authors, ranging from 5% (Barlund *et al*., 2008; Gilbert *et al*., 2005; Plöntzke *et al*., 2010; Madoz *et al*., 2013; Melcher *et al*., 2014) to more than 18% (Kasimanickam *et al*., 2004).

SE occurring beyond 8 weeks postpartum will hamper the fertility of dairy cows. There are various methods by which one can collect the sample for analyzing uterine cytology as it is conclusive indicator of uterine health (Sheldon and Dobson, 2004; Kasimanickam *et al.,* 2004). The approaches for collecting sample are vaginoscopy, ultrasonographic assessment of uterine fluid volume, ultrasonographic assessment of endometrial thickness, uterine biopsy, uterine lavage and cytobrush method, which can be used to evaluate the relationship between PMN cells and conception rate in SE cases (Kasimanickam *et al.,* 2005; Barlund *et al.,* 2008).Among them the relative sensitivity of cytobrush method was the highest followed by the lavage cytology which were, 93.9% and 92.3% respectively (Barlund *et al.,* 2008). The sensitivity and specificity of lavage cytology was high in similar studies (Drillich *et al*., 2004; Dubuc *et al*., 2010; Kasimanickam *et al*., 2005a; Oral *et al*., 2009).

The active inflammatory process is indicated by presence of neutrophils in the uterine lumen (Butt *et al.,* 1993).

Identification of SE and the precise evaluation of uterine condition in cows with no uterine/vaginal discharge could be done by obtaining cytological samples by flushing the uterine lumen (Barlund *et al*., 2008; Pleticha *et al*., 2009).

Cytobrush method is also termed as low-volume lavage (Dini *et al*., 2015). The sampling using cytobrush is easier and capitulate an *in-situ* sample with less altered cells (Kasimanickam *et al*., 2005) but it evaluates only small part of endometrium (Brook,1984; LeBlanc *et al*., 2010: De Boer *et al*., 2014). In contrast the uterine lavage method is economical which needs more technical expertise and the sample obtained will be more representative of major portion of the uterus. It ensures high harvest of

PMN cell as it collects the larger surface of endometrium (Ball *et al*., 1988; Roszel and Freeman, 1988; Bonnett *et al*., 1991; Bourke *et al*., 1997; Bohn *et al*., 2014).

The threshold of PMN cells for concluding as SE by endometrial cytology as a diagnostic technique corresponded to >5% Neutrophils at 40–60 days postpartum (Kasimanickam *et al.,* 2004).

In another study it was concluded that SE is indicated if PMNs exceed 18% (21-33 d) and 4% (48-62 d) after parturition (Vinita *et al*., 2018).

Endometrial Cytology Interpretation

Days in milk (DIM)	**PMN (%)**	**References**
27±6 40±6	>18 >10	LeBlanc *et al.* (2002a); Kasimanickam *et al.* (2004); Sheldon *et al.* (2006); Brodzki *et al*. (2014)
35±7	>8	Barlund *et al*. (2008); Salah *et al.* (2017)
28±10	>5	Plontzke *et al*. (2010)
35±3 56±3	>6 >4	Singh *et al*. (1998); Dubuc *et al*. (2010)
37±7	>9	McDougall *et al*. (2007)
27±6 40±6 55±7	>8 >6 >4	Madoz *et al.* (2013)
>60	≥1	Pascottini *et al*. (2016)

2.2 Diagnosis of clinical endometritis in cows

Clinical endometritis is characterized by mucopurulent or purulent discharge after 26 days in milking (LeBlanc, 2008). Diagnosis of CE can be made using simple method by manual clinical examination of vagina and expulsion of the mucus from uterus for inspection. This technique is inexpensive and quick which provides additional sensory evaluation by visualizing the turbidity, colour and odour of the discharge and facilitate to grade the same (Sheldon *et al.,* 2002; Williams *et al.,* 2005).

Transrectal palpation method is also used in diagnosis of CE but when compared to uterine culture, it is only 22% sensitive (Miller *et al.,* 1980). The confirmatory diagnosis of CE can be ensured by the microbiological examination of uterine swabs but the samples thus obtained may not represent the entire uterine environment which is the striking drawback in this approach (Purohit *et al*., 2013). Determining the bacterial load which are causing pathological changes in the uterus by obtaining samples through swab method is considered most accurate in 21-28 day post calving animals (Olson *et al.,* 1986; Bretzlaff, 1987; Noakes *et al.,* 1991; Bonnett *et al.,* 1993; Lewis, 1997; Bondurant, 1999; LeBlanc *et al.,* 2002b; Sheldon *et al.,* 2004).

Vaginal mucus scoring is one of the methods to determine the severity of CE. It will be done on a scale of ‘0 to 3’. In which ‘0’ being clear/translucent; ‘1’ being mucus containing few flecks of white purulent material; ‘2’ being 50% white or off-white purulent material and ‘3’ on the scale represents more than 50% white or off-white purulent material or even blood mixed (Williams *et al*., 2005; Sheldon *et al*., 2006; Senosy *et al*., 2009).

Based on the visual inspection by the farmer, mainly the purulent vaginal discharge or discharge adhered to vulva/tail/perenium and further confirmation of the

same by transrectal examination by veterinarian, it was found that incidence of CE was 20% in 50 days postpartum cows (Hendricks *et al.,* 2006). Similar record was published by Somasekara (2003), where they found that the incidence of CE based on the history of purulent discharge was 20.3%.

2.3 Aerobic bacterial aetiology of subclinical endometritis

Many specific and nonspecific pathogens of the genital tract affect the fertility of cow thereby causing economic loss to the farmers. Uterine environment is favourable for the growth of anaerobic organisms which synergistically promotes the growth of various pathogenic aerobes ending up in bacterial infection of uterus (Huszenicza *et al.,* 1999). Around 80 to 100% of the cows do have bacterial contamination of the uterus in the first 2 weeks postpartum which can be attributed to various factors during and after calving (Markusfeld, 1987; Hussain *et al.,* 1990; Kim and Kang, 2003; Foldi *et al.,* 2006). Larger part of these bacterial contaminations gets eliminated without any external intervention during the first 5 weeks after parturition, but to a small but considerable portion of these animals end up in the uterine infections. The pathogenic bacteria cause inflammation of uterus, termed histologically as of endometritis (Noakes and England*,* 2009; Sheldon *et al.,* 2006)

Among the cervical mucus samples of SE collected in repeat breeding cows, 80 to 100% of the samples were positive for aerobic bacteria (Chandrakar *et al*., 2002; Ahmed and Bhattacharyya, 2005; Mane *et al*., 2009)

Many literature reports suggest that the reason behind mixed infections detected from vaginal discharges of the affected cows with SE is the synergism among those organisms and uterine environment (Das *et al.,* 1996; Huszenicza *et al.,* 1999; Abd El-Hafeez *et al*., 2001; Chandrakar *et al.,* 2002; Zaman *et al.,* 2015).

In SE, the recovered bacterial isolates can be either a single or mixed with an extent of 42.16 per cent and 57.83 per cent respectively. Among which *Escherichia coli* were found in 45.71 per cent being most common single bacterial cause of SE followed by *Staphylococcus* spp. detected in 42.86 per cent, *Proteus* spp. and *Enterobacter* spp. Each detected in 5.71 per cent cases (Sahadev *et al*., 2017).

The bacteria frequently isolated from uterine swabs of cows with SE were *E. coli, S. aureus* and *Proteus* spp. Whereas, the *Streptococcus* spp., *Klebsiella* spp., *Pseudomonas* spp., *A. pyogenes* and *Bacillus* spp. were less frequently isolated (Singla *et al.,* 2004).

In cows with SE the most common isolates reported were non lactose fermenting organisms of *Enterobacteriaceae* family. *E. coli*, *Streptococcus* spp., *Staphylococcus* spp., *Pseudomonas* spp. and *Bacillus* spp. were other common bacteria isolated (Barman *et al.,* 2013).

Out of 72 samples collected from repeat breeding cows with SE, aerobic bacteria were detected in 61 samples (84.72%), among which *Staphylococcus aureus* (38.88 %) was predominant followed by *E. coli* (36.11 %), *Streptococcus* spp. (33.33 %), *Enterobacter* spp. (22.22 %), *Proteus* spp. (18.05 %) and *Pseudomonas* spp. (16.67 %) (Behera *et al*., 2015).

Staphylococcus spp., *Bacillus* spp., *Streptococcus* spp. and *E coli* are the predominant bacteria isolated from SE and among them *Bacillus* spp. was found in higher percentage (43.5 %). It was also reported that these bacteria are common opportunistic bacteria in normal cows that can take upper hand any time and turn into pathogenic bacteria and also synergistically cause mixed infections (Ahuja *et al*., 2017).

Out of 23 samples collected in SE post-partum dairy cows, 13 bacterial isolates were gram-positive and 10 bacterial isolates were gram-negative. Among them 7 samples showed mixed bacterial infections. *E. coli* (32.26%) was found to be predominant followed by *Bacillus cereus* (22.58%), *Staphylococcus aureus* (16.13%). Mixed cultures were *Bacillus cereus* + *Staphylococcus aureus (9.68%)* and *Escherichia coli* + *Proteus vulgaris* (3.23%) (Sharma *et al*., 2017).

2.4 Aerobic bacterial aetiology for clinical endometritis

The bacteria isolated from the post-partum endometritis was classified as obligate uterine pathogens which included *T. pyogenes* and *E. coli,* potential uterine pathogens like non hemolytic Streptococci and opportunistic bacteria like *Klebsiella* spp*., Proteus* spp. and coagulase-negative *Staphylococci* spp. (Sheldon *et al*., 2004; Williams *et al.,* 2005)

Similarly, *E. coli, Klebisella* spp., *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Proteus* spp., and *Streptococcus* spp., are more commonly isolated from endometritis cases in cows (Bhat and Bhattacharyya 2012; Shweta 2003; Bonnett *et al*., 1991).

In the study conducted by Ngarmkum *et al.,* (1993) it was found that *Staphylococcus aureus* (32.14%) was predominant bacteria followed by *Acinetobacter anitratus* (25.0%), *E. coli* (21.43%) and *Corynebacterium pyogene*s (14.29%).

Virakul *et al*. (1995) reported that in 30 days post-partum dairy cow the isolates obtained were *Staphylococcus aureus* (28.2%), *Corynebacterium pyogene*s (23.1%) and *E. coli* (17.9%).

Nibret *et al*. (2013) published that the study performed by them resulted in 22 (91.66%) bacteriologically positive swabs from which *A. pyogenes* (25%) was frequently isolated bacteria followed by *Streptococcus* spp., and *E. coli* (20.8% each), *S. aureus* (12.5%)*, Klebsiella* Spp. (8.3%) and *C. fetus* (4.2%).

In another study, the results obtained after bacterial cultivation of endometritis were *E. coli* (24.07%), *Bacillus* spp., (20.37%) *Streptococcus* spp., (14.81%), and *Staphylococcus* spp. (11%) (Takamtha *et al.,* 2013).

In a study conducted with cervico-vaginal mucus samples collected from cows with CE, it was concluded that non-lactose fermenters belonging to the family *Enterobacteriaceae* were the most common isolates (25%), followed by *Escherichia coli* (20%), *Corynebacterium* spp., (15%), *Streptococcus* spp., (15%), *Staphylococcus* spp., (10%), *Pseudomonas* spp., (10%) and *Bacillus* spp., (5%) (Barman *et al*., 2013).

Among 30 uterine samples collected, 5 were sterile and rest 25 were detected with bacterial growth, of which *E. coli* was found in 36.66%, *Klebsiella* spp., in 30% of the positive samples, followed by *Proteus* spp., being in 13.33%, *Pseudomonas aeruginosa* in 6.66% and *Clostridium* spp., in 3.33% of 25 samples. As the samples were collected from confirmed CE cases, the authors also concluded that 5% of the samples which were sterile might be due to unfavorable media conditions that did not permit the growth of those undetected micro-organisms **(**Udhayavel *et al*., 2013).

Patel *et al*. (2019) reported that *Staphylococcus* spp., as predominant causative agent recovered from CE affected cows followed by *Bacillus* spp., *E. coli*, *Streptococcus* spp., *Salmonella* spp. and *Corynebacterium* spp.

2.5 Phenotypic characterization of aerobic bacteria isolated from SE and CE

Clinical endometritis samples collected from postpartum cows were cultured for aerobic bacteria on blood agar (sheep blood) and brain heart infusion medium (enriched media) at 37°C for 24h. phenotypic identification was done by cultural characteristics, Gram's staining, morphology, hemolytic characteristics and biochemical profile with TSI agar and motility-indole-lysine (MIL) medium along with other biochemical tests such as catalase, citrate, urease and growth on MacConkey agar (Takamtha *et al*., 2013).

Uterine aspirates obtained from cows affected with endometritis was transported to laboratory in sterile Stuart media as transport media which upon cultural isolation on Blood agar and MacConkey agar, and phenotypic identification done using Gram staining characteristics, hemolysis pattern and biochemical tests. *Staphylococcus aureus* was identified based on distinctive colonial morphology like white colour and lemon-yellow colour in blood agar. *Streptococcus* spp*.,* was identified as the colony was white, small, hard drew drop like colonies with hemolysis in blood agar. *Escherichia coli* was detected as Red colonies surrounded by a precipitation zone around the colony. *Trueperella pyogenes* was identified as tiny colonies surrounded by a narrow zone of complete hemolysis in blood agar and staining characters showed gram positive pleomorphic rods. *Klebsiella* spp., was identified based on typical pink to red-coloured colonies on MacConkey agar and *Campylobacter fetus* was identified as round, small, slightly raised, smooth, translucent colonies with a typical "dewdrop" appearance (Nibret *et al*., 2013).

Putative identification of staphylococcal strains causing CE in cows was done based on colony morphology, staining characteristics in Gram staining and catalase

activity which were further confirmed as staphylococci by the tube coagulase method (Zhao *et al*., 2014).

Abreham *et al*. (2017) incubated the endometritis sample obtained from the uterus of slaughtered cows on BHI broth and later inoculated on 7% sheep blood agar. The phenotypic identification of bacteria was performed based on cultural characteristics, Gram staining, morphology, hemolytic characteristics and biochemical tests such as catalase test, oxidase test, motility test by sulfide indole motility medium and oxidation-fermentation test followed by secondary biochemical tests like MR test, VP test, citrate utilization test, indole test, urease test gas production test and profile with inoculating on TSI agar, EMB agar, Edward's media and MSA. The predominant bacteria isolated and identified was *E. coli* followed by *Bacillus* spp., *Trueperella pyogenes, Staphylococcus* spp., *Streptococcus* spp., *Proteus* spp., *Corynebacterium* spp., *Klebsiella* spp., *Citrobacter* spp., and *Rhodococcus equi.*

Staphylococcus spp., *Bacillus* spp., *Streptococcus* spp., *E. coli*, *Salmonella* spp., and *Corynebacterium* spp., were recovered from cows affected with endometritis. The samples were streaked onto Blood agar and MacConkey agar, and phenotypic identification was done using Gram staining characteristics and biochemical tests such as oxidase, catalase tests and KOH (Patel *et al*., 2019).

2.6 Antimicrobial susceptibility

2.6.1 Antimicrobial susceptibility by disc diffusion

Antibiogram is useful data to decide the most efficient antibacterial agents. It may not exactly mimic the *in vivo* conditions as the susceptibility reported *in vitro* may be due to instability of the organisms and non-favorable culture environment (Udhayavel *et al*., 2013). But still, it gives an overview of susceptibility of the

organisms to a particular antibacterial agent and thereby helping the clinicians to choose best possible agent in their treatment protocol. Indiscriminate use of drugs leads to change in susceptibility of an organism to a given antibiotic (Sharma *et al.,* 2009).

Antibiogram not only helps in deciding the sensitive antibiotic for treatment of endometritis but also helps in constant monitoring of the resistance patterns of etiological agents causing bovine endometritis and avoiding unnecessary resistance to antimicrobial agents (Malinowski *et al.,* 2011).

Endometritis literature review suggests that the bacteria showing sensitivity to one antibacterial agent in one study will show resistace in other, for instance the *E. coli* recovered from the CE samples were sensitive to chloramphenicol, gentamycin, enrofloxacin, polymyxin B, cefquinome and ceftiofur (Sheldon *et al*., 2004; Drillich *et al*., 2001) and resistant to oxytetracycline, enrofloxacin, lincomycin and amikacin. The authors also inferred that using resistant antibiotics along with EDTA Tris according to their study plan increased the efficacy to approximately 92% (Farca *et al*., 1997).

In an antibiogram study of bacteria isolated from repeat breeder cattle, sensitivity was reported to gentamicin (80.6%), tetracycline (67.8%), chloramphenicol (61.3%) and resistance was reported to ampicillin (96.8%) nitrofurantoin (87.1%) co-trimoxazole (77.4%), streptomycin (74.2%), penicillin (71%) (Singh *et al*., 1998).

In another study by El-Kader and Shehata (2001), for the isolates obtained from healthy heifers and clinical endometritis affected cows, highest sensitivity was recorded to ciprofloxacin (92%) and gentamicin (90%).

Gram negative bacteria showed high sensitivity to antimicrobial agents like ampicillin and gentamicin (Sadig, 2010). Gentamicin (93%) and cephalexin (77%)

were highly sensitive to gram negative aerobic bacteria (Takamtha *et al.,* 2013). Sharma *et al*. (2019) reported that ciprofloxacin and enrofloxacin (63.50% and 60.32% respectively) were highly sensitive in isolates obtained from cows suffering from endometritis. While the resistant antibacterial agents were penicillin (83.64%), oxytetracycline (42%), amoxicillin (26%) and streptomycin (25%) (Takamtha *et al.,* 2013; Sharma *et al*., 2019).

Barman *et al*. (2013) reported in their study that one coagulase negative *Staphylococcus* spp., was resistant to most of the antibiotics but most of the organisms were found to be highly sensitive to enrofloxacin followed by moderately sensitive to ampicillin, penicillin, gentamicin, oxytetracycline, furazolidone and nitrofurantoin. Other organisms obtained in the study were *Corynebacterium* spp., *Streptococcus* spp., *Staphylococcus* spp., *Bacillus* spp. NLF Gram negative organisms, *E. coli, and P. aeruginosa.* Among these two NLF Gram negative organisms were resistant to ampicillin and *P. aeruginosa* was resistant to nitrofurantoin and furazolidone.

Antibiogram study conducted by Nibret *et al.,* 2013 confirmed that norfloxacin was 100% efficacious against *Escherichia coli* followed by marbofloxacin (100%), rifaximin (97%), gentamycin (96%) and amoxicillin and clavulanic acid (95.5%). Whereas Gram-negative bacteria isolated in the study were sensitive to neomycin (100%), norfloxacin (100%), and cefaperazone (95%). Relatively less sensitivity was shown to rifaximin (86.7%) and oxytetracycline (80.9%). It was also reported that *E. coli* showed 100% resistance to polymyxin, tetracycline and cefoxitin followed by 40% resistance determined to sulphamethaxazole and oxacillin. *S. aureus* showed resistance to ampicillin, vancomycin and oxacillin. Isolates of *A. pyogenes* showed 66.66% resistance to polymyxin and tetracycline, gentamycin (50%), oxacillin (16.66%) and cefoxitin (16.66%). 100% resistance to cefoxitin was recorded in *Klebsiella* spp.

Isolates obtained in two different studies by Rao and Seshagiri (1997) and Barman *et al*. (2013), antibiotic sensitivity pattern of endometritis in cows were 96.87% and 100% resistant to penicillin respectively.

Sharma *et al*. (2017) reported that levofloxacin was most sensitive in both gram-positive and gram-negative pathogens and penicillin was the most resistant for both gram-positive and gram-negative pathogens. The antibiogram data obtained in their study revealed that pathogens were sensitive for levofloxacin, enrofloxacin and ciprofloxacin against both *E. coli* and Gram-positive aerobes like *B. cereus, S. aureus* and *Streptococcus* spp. *E. coli* was resistant to cloxacillin, penicillin and ampicillin in decreasing order of resistance whereas Gram positive aerobes were resistant to penicillin, cloxacillin and amoxycillin respectively.

Sensitivity recorded in bacteria causing uterine infection with gentamicin and ofloxacin was 98.41% respectively which was highest followed by ciprofloxacin 63.50%. Resistance was noted to penicillin (83.64%), ceftriaxone (10.17 %) and tetracycline (7.94%). The more sensitivity to ciprofloxacin was probably due to less use of the same at field level (Sharma *et al.,* 2017). In another similar study it was reported that highest sensitivity was observed to ciprofloxacin (96%) and enrofloxacin (92%) (Mshelia *et al*., 2014).

Bacterial isolates obtained from endometritis cases showed highest sensitivity for ciprofloxacin (71%), followed by enrofloxacin (65%) and ofloxacin (52%) and resistance was highest for penicillin followed by metronidazole and tetracycline (Kumar *et al.,* 2018). Sadig (2010) reported that the aerobic bacteria from vagina of cross-bred dairy cows during early postpartum showed high sensitivity to penicillin

(90%). Hence judicious and cautious use of antibacterial agents in treatment is essential to avoid resistance (Barman *et al.,* 2013; Sharma *et al.,* 2017; Kumar *et al.,* 2018).

The reason for less efficacy of antibacterial agents can be due to presence of necrotic debris and purulent materials especially for sulfonamides and aminoglycosides (Smith and Risco, 2002) and that for β-lactam antibiotics and cephalosporins can be due to ß-lactamase enzyme producing organisms in post-partum uterus (Deori and Phookan, 2015). The resistance to oxytetracycline may be due to its indiscriminate use soon after parturition both intrauterine mode and parenteral route of administration (Risco *et al.,* 2007).

Antibiogram pattern of bacteria causing endometritis in cows showed that chloramphenicol was very less sensitive (8%) whereas highest sensitivity was shown by ceftriaxone (64%), followed by gentamicin, enrofloxacin and chlortetracycline (Udhayavel *et al*., 2013).

In 43 cross bred repeat breeding cows, cervico - vaginal discharge was collected and antibiogram was studied which revealed sensitivity to ciprofloxacin (100%) tetracycline (90.25%), ceftriaxone (56.5%), amoxyclav (49%) and resistance to ampicillin (85%), cefuroxime (75%) and cefotaxime (74%) (Ahuja *et al*., 2017).

In a recent study cervico-vaginal discharge was collected and assessed for antibiogram in 78 abandoned cows diagnosed with CE which revealed that isolates were sensitive for ciprofloxacin (63.50%) and enrofloxacin (60.32%) and resistant for penicillin (83.64%) (Singh *et al*., 2019).

2.6.2 Minimum Inhibitory Concentration

MICs obtained by use of MIC strip technique have previously been shown to correlate closely with the standard broth dilution method (Gavan *et al*., 1980).

Watts and Rossbach (2000) reported that for *C. bovis* MIC50 and MIC90 values for ceftiofur was 0.125 µg/mL and 0.5 µg/mL with dilution range ≤ 0.06–64.0 µg/mL in broth microdilution method. The data on *in vitro* activity of antimicrobial agents commonly used to treat bovine SE and CE caused by *C. bovis* strains is not available. The lack of *in vitro* antimicrobial susceptibility data may be due to hurdles in cultivating *C. bovis*, as it fails to grow without lipid supplementation of the basal medium (Funke *et al*., 1997; Watts and Rossbach, 2000).

Jacks *et al*. (2003) reported that *R. equi* MIC50 and MIC90 values for ceftiofur were ≤0.5 µg/mL and 1.0 µg/mL with microtitration strip method having a range of 0.06–64.0 µg/mL.

In agar incorporation method of detection of minimum inhibitory concentration, lowest MIC90 and MIC50 values (<0.06 µg/mL) was reported in cefquinome and enrofloxacin for *E. coli* isolates, whereas oxytetracycline and cephapirin showed highest values. Similarly, *A. pyogenes* showed lowest MIC values for all the cephalosporins and again highest MIC50 value was reported in oxytetracycline (16 µg/ml). MIC50 values for anaerobic bacteria were again lowest in cephalosporins and highest in enrofloxacin and oxytetracycline (Sheldon *et al*., 2004).

Pohl *et al*., (2018) reported that *T. pyogenes* MIC50 and MIC90 values for ceftiofur were 0.25 µg/mL and 0.5 µg/mL respectively and for *E. coli* they were 0.5 µg/mL and 1 µg/mL respectively. It was also concluded that *E. coli* showed emerging

resistance to ceftiofur which was indicated by non-inhibition even at highest concentration of ceftiofur.

Jong *et al*. (2018) reported that MIC value of *Escherichia coli* for ceftiofur ranged between 0.12–2.0 µg/mL in broth dilution method and that for *Staphylococcus aureus* was 0.25–4.0 µg/mL. The MIC values for *Streptococcus uberis* was 0.06-2 µg/mL and for *Klebsiella* spp. was 0.12-1 µg/mL.

2.7 Molecular characterization of bacterial isolates associated with bovine endometritis

Pathogenicity and virulence are often used synonymously as there is no clear demarcation between the two terms. The inherent ability of a microbe to cause disease in a host is termed virulence and pathogenicity indicates the potential to transmit and speed of transmitting along with virulence (Thomas and Elkinton 2004). Pathogenesis is also explained as the mechanism by which disease is caused by a pathogen in the host and virulence factor is a gene that contributes to virulence when expressed in such organism (Mokady *et al*., 2005). One more explanation is that virulence is concerned with bacteria whereas pathogenesis is from the host point of view (Weiss, 2002).

2.7.1 *Eschericha coli*

Luque *et al*. (2017) reported in their study that *E. coli* isolates from reproductive tract of cows and sows could be confirmed by targeting species specific *uidA* gene.

The *fimH* gene was used for confirmation of *E. coli* by PCR in endometritis affected buffaloes. *E. coli* was isolated from 25 cases, out of 103 (24.27%). 17 cases out of 25 (68%) showed positive for *E. coli fimH* gene (Ingale *et al.,* 2016).

In a study that evaluated virulence factor genes of *E. coli* isolates recovered from endometritis such as *papEF* (P-fimbriae), *afaBC* (a fimbrial adhesion 1 or *Afa1*), *sfaDE* (Sfimbriae), *cnf1* (cytotoxic necrotizing factor 1, *CNF1*), *iucD* (aerobactin), *hlyA* (α-hemolysin), *hlyE* (hemolysin E), *eaeA* (intimin), *stx1* and *stx2* (shiga toxins 1 and 2), *Sta* (heat-stable enterotoxin a), *F5* (K99), *F41* fimbriae, *F17* fimbriae, and *csgA* (curli subunit protein), a recognizable association of virulence factor genes with endometritis was not found . Virulence factor gene *fimH* was not detected in this particular study (Silva *et al*., 2009).

Sheldon *et al.* (2010) reported that *E. coli* isolated from uterine diseases have properties like increased adhesion and invasion to bovine endothelial cells in comparison to other types like diarrhoeagenic and extraintestinal pathogenic *E. coli* isolated from cows without uterine disease. It was also reported that the uterine isolates lacked a number of virulence factors normally associated with virulence in the extraintestinal and diarrhoeagenic pathogenic *E. coli* which inferred that these isolates of *E. coli* might be specific for uterine diseases of bovine.

Bicalho *et al*. (2010) revealed in their study that there were 32 different virulence factors in relation to postpartum uterine disease caused by *E. coli* and also detected eleven virulence genes, among them six were found to have a significant association with endometritis viz., *fimH* (type 1 pili), *kpsMII* (gene coding for capsule protein), *astA* (gene coding for endotoxin), *cdt* (cytolethal distending toxin), *hlyA* (gene coding for the toxin) and *ibeA* (gene coding for a factor that facilitates invasion). They also found that there was an 87% prevalence of *fimH* gene was seen as a virulence factor associated with endometritis. Pilus along with the *fimH* adhesin present in uropathogenic *E. coli* facilitates adhesion to the epithelial cells and it is also responsible for invasion (Martinez *et al*., 2000).

In another study, the ferric yersiniabactin uptake receptor *fyuA* gene was associated with endometritis which is involved in iron uptake (Sheldon *et al*., 2010).

Bicalho *et al*. (2011) concluded that type 1 pilus was the virulence factor associated with the uterine disease, and the same was coded by gene *fimH* which coded for an adhesive subunit of the protein. Six virulence genes were detected from the *E. coli* isolated from cows with postpartum uterine disease and showed that there was a negative correlation between *fimH* and reproduction. The results also sustained the notion that *F. necrophorum* being facilitated by *E. coli* based on the observations made in the study that the presence of *fimH* in 1-3 postpartum days had almost 16 times increased chance of finding *F. necrophorum* at 8-10 days postpartum.

Among six virulence factor genes (*fimH*, *astA*, *cdt*, *kpsMII*, *ibeA* and *hlyA*.) associated with uterine infection in cows *fimH* was most prevalent and significantly associated with metritis and increased risk of about 4.6 times to result in endometritis when it was compared with the cows which had a uterine infection but samples found negative for *E. coli* (Bicalho *et al.,* 2010, Bicalho *et al*., 2011; Yang *et al*., 2016).

Ingale *et al*. (2016) conducted a study to isolate *Escherichia coli* from the uterus of 25 buffaloes affected with endometritis, *E. coli fimH* gene was detected in 68% of cases (17 cases) and inferred that *the fimH* gene was associated with endometritis.

In a study randomly collected uterine samples (40 cows), *E. coli* was isolated from 45% (18 cows) of the samples. Among 18 samples, 27.7%, 50% and 22.2% were diagnosed as metritis, CE and SE, respectively. Biofilm production gene *Agn43a*, a virulence factor was detected in 33.3% of the isolates. *Agn43b* VF gene was found in 11 of the 18 *E. coli* isolates (61.1%). Six isolates showed both *Agn43a* and *Agn43b* VF genes. Summary of the study disclosed that fact that 61.1% (11 of 18) of the samples

had either of the biofilm VFs and of these 27.27% were diagnosed as metritis and 72.72% were CE (Kasimanickam *et al.*, 2016).

The virulence factor genes of *E. coli* obtained from extra-intestinal infections such as *fimH*, *iucD/aer*, *hlyA*, *kps*, *usp*, *vt1*, and *vt2* were incidentally found to be associated with postpartum endometritis (Luana *et al*., 2019).

Among 15 genes evaluated for virulence factor, seven were found in the study. Among them *fimH* (87%), *agn43* (41%) and *csgA* (35%) were the most prevalent and genes *traT* (27%), *fyuA* (11%), *hlyA* (5%) and *kpsMT II* (5%) were in low proportion. *FyuA* was significantly higher ($p < 0.05$) in endometritis affected cows. *FimH* (fimbriae type 1) was in higher prevalence, whose expression was essential for adhesion of pathogenic *E*. *coli* to endometrial cells (Gonzalez *et al*., 2020).

It was also concluded that no samples were proved positive for the typical diarrheagenic *E. coli* genes (Silva *et al*., 2009; Gonzalez *et al*., 2020).

2.7.2 ***Staphylococcus aureus***

Sequencing of the polymorphic X region of the protein A gene (*spa*) is the base for *spa* typing and it will be present in all strains of *Staphylococcus aureus*. The X region is constituted of a variable number of 24-bp repeats flanked by well-conserved regions (Hallin *et al.,* 2009).

In a study carried out intending to screen milk samples for subclinical mastitis, 186 out of 246 samples were found with *S. aureus* detected by PCR. Among them, 95 *S. aureus* isolates revealed the presence of *nuc* gene and *sodA* gene was detected in 87 isolates. *E. coli* was found in 48 isolates which was confirmed by *alr* gene-based simplex and by a multiplex PCR (Hegde, 2011).

In a study 250 samples, including 200 milk samples from animals and 50 nasal swabs from animal workers were collected and subjected to bacterial isolation and molecular detection confirmed the presence of *S. aureus* isolates in 184 samples by detection of 270bp sized *nuc* gene by PCR (Sarkar *et al*., 2014).

Among 167 mastitis milk samples, *S. aureus* strains were isolated from 39 samples. Antibiogram results revealed that 61.5% (24 of 39) were multidrug-resistant and 28.2% were resistant to vancomycin (MICs = 2μg/mL). All isolates were sensitive to oxacillin but 48.7% were detected with *mecA* gene, which concluded the prevalence of oxacillin susceptible *mecA* positive strains in India (Mistry *et al*., 2016).

Yadav *et al*. (2018) carried out a study intending to assess the prevalence of *S. aureus*, including methicillin-resistant *S. aureus* (MRSA) from the clinical pyogenic samples of domestic animals in India. Results showed that there was a 40% prevalence of *S. aureus* among clinical pyogenic samples of domestic animals. 57.5% (23isolates) of *S. aureus* isolates were positive for *mecA* gene detected by PCR-based amplification.

Dan *et al*. (2019) investigated antimicrobial resistance, virulence gene profiles, and genotypes of *S. aureus* from clinical mastitis and endometritis in dairy cows which included 151 endometritis swab samples and 186 milk samples from clinical mastitis. 155 *S. aureus* strains were isolated of which 14.2% (22) were detected as MRSA. MSSA was significantly lower than MRSA. It was also found that the majority of *S. aureus* isolates isolated from dairy cows were multidrug-resistant which was confirmed by the presence of multiple virulence genes and the results of the study were posing a potential public-health risk.

Staphylococcus aureus isolates obtained from clinical samples of mastitis and endometritis from cattle also revealed 4 subtypes (t779, t2883, t13751 and t1939) of *spa* gene upon molecular typing detected as four characteristic bands in PCR analysis (Dan *et al*., 2019).

Out of 450 milk samples from all agro-climatic zones of West Bengal,94 isolates were of *S. aureus*. After biochemical confirmation, the isolates obtained were further subjected to PCR for detection of *nuc* gene (thermonuclease) which was species specific to *Staphylococcus aureus*. It was found that Nine (9.6%) *S. aureus* isolates were confirmed both phenotypically and by PCR as MRSA possessing *mecA* gene. As an adjunct to phenotypic confirmation, a 14mm or less diameter zone for oxacillin (1 µg) and 21mm or less diameter zone for cefoxitin (10 µg) in the disc diffusion test serves as a breakpoint for confirming the isolate as MRSA. MRSA isolates obtained are classified as t304 and t6297 types based on *spa* typing, and the global prevalence of 0.53% was reported in European countries for t304 type. Only one clone of MRSA with *spa* type t6297 being reported to date from India from bovine milk samples (Achintya *et al.,* 2019).

Identification of *S. aureus* using PCR amplification of the *nuc* gene (~270 bp) which codes for extracellular thermostable nuclease protein of *S. aureus* is considered as a gold standard method (David *et al.,* 2010). Compared to the 16S rRNA gene which is common to *Staphylococcus* genus, the *nuc* gene is more specific in detecting isolates specific to the species *S. aureus* (Sahebnasagh *et al*., 2014). The *nuc* gene of *S. aureus,* which is species-specific molecular target for confirmation of *S. aureus* isolates was amplified in 24 samples from subclinical and clinical mastitis (Rocha *et al*., 2019).

In a study of clinical and subclinical mastitis that affected indigenous and cross-bred cattle from Kolkata, a total of 118 milk samples were analyzed and 38 samples were found positive for *Staphylococcus aureus* isolates of which 15 isolates were MRSA phenotypically. This was further confirmed by the detection of the *mecA* gene. Further *nuc* PCR was performed for genotypic confirmation, but only 10 isolates found positive inferring that there was mixed infection with other Staphylococcus organisms which was also resistant to methicillin along with *S. aureus*. The *S. aureus*-specific staphylococcal protein A(*spa*) is analyzed for *spa* typing which is a unique repeat code that is assigned for each new base composition of the polymorphic repeat found in a strain. The repeat succession for a given strain determines its *spa* type. Three samples were positive for *spa* of which only one was having a typeable sequence and the *spa* type was t267 (Singh *et al*., 2019).

In a study with the objective of molecular characterization of *Staphylococcus aureus* to be isolated from the milk samples, it was disclosed that 42 *S. aureus* isolates were detected among which 10 were multidrug-resistant strains. All the MDR strains were having *nuc* gene and were found positive for *16S rRNA.* It was also revealed that antibiotic-resistant genes like *mecA* (40%), *ermC* (70%)*,* and *aacA-aphD* (30%) were detected in the *S. aureus* isolates (Kumari *et al*., 2020).

The extracellular thermostable nuclease protein of *S. aureus* is coded by *nuc* gene which is also species-specific molecular target for detection of *S. aureus* isolates, have been detected in almost 100% of MDR *S. aureus* strains (Kabir *et al.,* 2017; Javid *et al*., 2018). Likewise, presence of *mecA* gene is considered as an authenticated method to detect methicillin resistance in *S. aureus* isolates. Antimicrobial resistance is an emerging threat to human and animal health. It is also found that MRSA strains are resistant to other routinely used antimicrobial class such

as beta lactams, aminoglycosides, fluoroquinolones and macrolides. In one of the studies which detected 10 MDR *S. aureus* isolates *mecA* (40%) gene was detected (Kumari *et al*., 2020). Keyvan *et al.* (2020) reported MRSA with *mecA* gene with higher prevalence of 75.4%.

Material and methods

III. MATERIAL AND METHODS

The study was conducted in the Department of Veterinary Microbiology, Veterinary College, Shivamogga, a constituent institute under Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar, Karnataka State.

3.1 General considerations

The glassware used in this study were of neutral glass of Corning or Borosil India Ltd. make. The culture media, buffers and other biochemical reagents were prepared in MilliQ (millipore) water. The chemicals of analar, excellar or molecular biology grade were used for the preparation of various solutions and reagents. The culture media, buffers, reagents, kits and other requirements were obtained from M/s. Biosciences, Merck specialties Pvt. Ltd., Mumbai; BD, BBL, and DIFCO, USA; E-Merck (India) Ltd; Hi-media, Mumbai; Sigma Aldrich, USA; Sisco Research Laboratories Pvt. Ltd., Mumbai and Synergy Scientific Services, Chennai. Plasticware including microcentrifuge tubes, cryovials, micropipette tips, petri dishes, and autoclave bags were procured from M/s. Tarson Products Pvt. Ltd., Kolkata.

3.1.1 Preparation of glassware and plasticware

The glassware used in the study were prepared by soaking them in detergent (Teepol) solution overnight. The following day, washed thoroughly in running tap water, followed by a rinse in deionized/ distilled water (DW). The oven-dried glassware was then packed and sterilized in a hot air oven for one hour at 160 $^{\circ}$C. The new plasticware including microcentrifuge tubes and micropipette tips used in the study were sterilized by autoclaving at 121 $^{\circ}$C for 15 min at 15 psi (Collee *et al*., 1989).

3.1.2 Preparation of media and reagents

Brain heart infusion broth and agar, mannitol salt agar, blood agar base, Edward's media, esculin agar, egg yolk agar, EMB agar, MacConkey agar, Muller Hinton Agar (MHA), nutrient agar and broth, Voges-Proskauer's test medium, peptone water, glycerol, hydrogen peroxide, methanol, Gram staining kit and Giemsa staining kit were obtained from M/s Hi media Laboratories Ltd., Mumbai. The media and reagents were prepared as recommended by the manufacturer or as per the standard procedure and autoclaving was done for sterilization (Collee *et al*., 1989).

Dehydrated media bases of the desired medium were added to the recommended quantity of distilled water and the media were dissolved by boiling in the microwave oven and poured into the conical flasks. The flasks were cotton plugged and autoclaved at 121 °C 15 lbs for 15 minutes and allowed to cool. The cooling media were poured into the sterilized petri plates in the laminar airflow and allowed to solidify. The plates were checked for sterility before using for inoculation. For the preparation of slants, the media were poured into the test tubes (10ml-15ml), cotton plugged and autoclaved at 121 °C 15 lbs for 15 minutes. After autoclaving, the tubes were kept at the slanting position until solidify. The media were stored at 4 °C until further use (Collee *et al*., 1989).

Blood agar was prepared using a blood agar base (Hi media Laboratories Ltd., Mumbai) to which 5% of sheep blood was added as per standard procedure. Edward's medium is prepared using a dehydrated media base to which 5% sheep blood was added as per standard procedure (Collee *et al*., 1989).

Egg yolk agar was prepared using a dehydrated media base to which sterile egg yolk emulsion was added as per standard procedure (Collee *et al*., 1989).

Preparation of Nutrient-glycerol broth

Nutrient broth (sterile) - 85 ml

Glycerol (sterile) - 15 ml

Glycerol was sterilized in hot air oven at 160°C for one hr and added to the nutrient broth which was sterilized by autoclaving, both were mixed well and aliquots of 1ml in sterile tubes was stored at 4 °C for further use.

3.1.3 Equipment, reagents and chemicals

The following equipment/instruments which were made available in the Department of Veterinary Microbiology, Veterinary College, Shivamogga were used in the study.

- Incubator (Lab line instruments)
- Hot air oven (Lab line instruments)
- Autoclave (Apollo Scientific Surgicals Co., Bangalore)
- Deep freezer (Lab line instruments)
- Microscope (Olympus India Pvt LTD)
- Laminar air flow (Kadavil Electro Mechanical Industries., Kerala)
- Water bath (Lab line instruments)
- Refrigerated eppendorf centrifuge (Hermle Labor Technik)
- Electronic balance (Shimadzu Corporation., Japan)
- 0.5 Mc Farland scale (M/s Hi media Laboratories Ltd., Mumbai)
- Antibiotic zone scale
- Sterile swab with wooden applicator

The following reagents, stains and chemicals were used in the study.

- Gram staining kit (Himedia)
- Giemsa staining kit (Himedia)
- Rabbit plasma (Himedia)
- Normal saline
- Hydrogen peroxide (3%)
- Proteinase K (Himedia)
- Phenol chloroform (1:1) (Himedia)
- Ethanol (Himedia)
- Magnesium chloride (Genie)

3.2 Collection of samples

The samples for the present study were collected from dairy cows located in and around Shivamogga district during infertility camps organised by local veterinarians. Samples were also received from veterinary dispensaries/hospitals. Collected samples were subjected for cytology, bacterial culture and antimicrobial susceptibility assay and molecular studies.

3.2.1 Collection of samples from repeat breeding cows by uterine lavage method

Procedure

The perineal area of selected cattle was washed with 0.01 percent potassium permanganate solution before sample collection, which was followed by swabbing with 70 % ethyl alcohol. Using a sterile catheter, 20 mL of sterile normal saline solution (20 mL) was infused into the uterus. The uterus was gently massaged by per rectal palpation, for a few seconds to ensure the proper distribution of infused normal saline and the fluid was aspirated back with the same catheter. The aspirate was then

transferred immediately to a 10 mL sterile modified polystyrene centrifuge tube (Corning, New York, USA) without any preservatives and stored in a cool box with ice. The sample thus collected under sterile conditions was brought to the laboratory within 2 hrs and few drops of sample was used to inoculate BHI broth for bacterial isolation and the remaining fluid was centrifuged at 7000 rpm for 5 min. A drop of sediment was smeared on to a clean microscopic slide, air dried and utilized for cytological examination (Gilbert *et al.*, 2005).

A total of 82 uterine lavages were collected from all the suspected animals irrespective of age, body condition score and parity. These samples were subjected to cytology for SE confirmation.

3.2.2 Collection of samples from clinical endometritis cases

The cows diagnosed to be suffering from endometritis by gynaecological examination were considered as clinical cases for sample collection. The perineal area of selected cattle was washed with 0.01 percent potassium permanganate solution before sample collection, which was followed by swabbing with 70 % ethyl alcohol. Endometrial discharge was collected using artificial insemination (AI) gun equipped with AI sheath. The discharge collected was transferred to a sterile screw capped vials equipped with sterile swabs without any preservatives and then transported in a cool box with ice in upright position. The sample was then brought to the laboratory within 2 hrs. Few drops of sample was used to inoculate BHI broth in laminar air flow for bacterial isolation (Sharma *et al.*, 2019).

A total of 51 samples were collected from the cows diagnosed to be suffering from CE. The samples were collected from all the suspected animals irrespective of

age, body condition score and parity. These samples were subjected to aerobic bacterial isolation and identification.

The detailed information of the cases with regard to the history, breed, age of cattle and parity including the treatment history were recorded for all the samples collected.

3.3 Cytological assessment of uterine lavage samples

Uterine lavage fluid collected from cows with repeat breeding history were subjected to microscopic examination. The uterine lavage sample was centrifuged at 7000 rpm for 5 min. The pellet obtained was smeared on to a clean microscopic slide and air dried. The slides were then fixed and stained using Giemsa stain (Hi-Media, Mumbai) as per the instructions mentioned in the kit. The slides were evaluated microscopically at 400x magnification, for endometrial cells and other associated cells like PMN cells, neutrophils etc., (Gilbert *et al*., 2005; Moges and Ethiopia 2015; Salah *et al*., 2017; Sharma *et al*., 2019).

3.4 Isolation of bacterial agents from uterine lavage / endometrial discharge samples

Both uterine lavage samples (n=82) and endometrial discharge samples (n=51) were processed for bacterial isolation using standard bacteriological methods.

3.4.1 Culturing of samples for bacterial isolation

The uterine lavage fluid and uterine discharge samples collected was initially inoculated in BHI broth and incubated for 12-24 hr at 37 °C. Based on colony morphology and Gram staining, presumptive identification of bacterial agents was done. A loopful of inoculum was then streaked onto already prepared petri plates of

brain heart infusion agar, mannitol salt agar, MacConkey agar, blood agar and incubated for 24 hrs at 37 $^{\circ}$C. The examination was done as per the standard procedure (Cruickshank *et al*., 1975; Brooks *et al*., 2001).

3.4.2 Identification of bacterial agents by phenotypic methods

3.4.2.1 Gram staining and cultural characters

Gram staining kit, which contained Crystal violet, Gram's iodine, decolourizer and Safranin was procured from M/s Hi-Media, Mumbai. Staining was carried out as per the instructions mentioned in the kit for all the culture isolates and categorized as Gram -positive and Gram-negative bacteria. In addition, colony characteristics on the agar plates and lactose fermenting ability on MacConkey were recorded. Then the selective plating of isolates was carried out for further confirmation. The relevant biochemical tests were performed for Gram-positive and Gram-negative isolates so obtained as per the standard procedure and the results were documented (Collee *et al*., 1989).

3.4.2.2 Biochemical tests

The recovered isolates confirmed by Gram staining technique were further subjected to biochemical tests such as coagulase, esculin hydrolysis test, catalase tests, oxidase test, IMVC tests, urease test and citrate utilization test etc (Barrow and Feltham 1993; Collee *et al*., 1989).

3.4.2.2.1 Coagulase test

All the staphylococcal isolates primarily confirmed by Grams staining were first subjected to tube coagulase test. Following the standard procedure, about 0.3 ml of 18 hr old culture prepared in BHI broth was mixed with 0.5ml of diluted rabbit plasma (1:4 in PBS) and incubated overnight at 37 $^{\circ}$C. The result was recorded at 1 hr, 4 hr and

after overnight incubation. Confirmation was made based on formation of clot/ stiff gel which remained in place when tube was tilted through 90º angle or inverted was considered as positive for coagulase production.

3.4.2.2.2 Catalase test

Ebullition of gas bubbles upon addition of 24 hr culture to 3% hydrogen peroxide on a clean glass slide indicated the positive reaction. Absence of the same indicated the negative reaction.

3.4.2.2.3 Oxidase test

Oxidase reaction is carried out by spreading a well isolated colony on the oxidase disc which was already placed on clean glass slide by sterile forceps. The reaction was observed within 5-10 seconds at 25-30 °C. If purple colour develops, then the reaction was considered positive (Collee *et al*., 1989).

3.4.2.2.4 Indole production test

The test organism was inoculated into peptone water and incubated at 37 °C for 24 hr. Then, Kovac's reagent (1ml) was run down along sides of the test tube. Appearance of pink colour in the reagent layer within a minute of test indicated positive reaction.

3.4.2.2.5 Methyl red (MR) test

The test organism was inoculated into glucose phosphate medium (MR – VP medium) and incubated at 37 °C for 24 hr. Few drops (about two drops) of Methyl red reagent were added, the mixture was shaken well and examined. Appearance of red colour indicated positive reaction, whereas orange or yellow colour indicated negative reaction.

3.4.2.2.6 Voges –Proskauer test

The test organism was inoculated into glucose phosphate medium (MR–VP medium) and incubated at 37 °C for 24 hr. Then, three mL of 5% alpha-naphthol solution of and one mL of 40% potassium hydroxide were added to inoculated MR – VP medium. Development of bright pink color within 30 minutes indicated positive reaction.

3.4.2.2.7 Urease test

The test organism was inoculated to a slope of urea agar slant medium and incubated at 37 ºC for 24 hr. Change in color to red indicated positive reaction.

3.4.2.2.8 Citrate utilization test

Simmon´s citrate medium was inoculated with test organism and incubated at 37 ºC for 24 hr and then examined for growth and change in colour. Streak of growth and change in colour to blue was considered positive for citrate utilization.

3.4.2.2.9 Motility test

Young broth culture was observed under direct microscopy by placing a drop of the broth culture at the center of the coverslip and then inverting the same on the pre-fixed ring glass slide forming a hanging drop preparation. Initially, it was viewed under low power and later observed with high power dry objective under reduced illumination. The bacteria were considered motile if the movement of organisms were noticed (moving away or towards the cells) (Markey *et al*., 2013).

3.4.2.2.10 Sugar fermentation tests

5 mL of sterile peptone water (pH 7.2) along with Andrad's indicator solution was transferred to several sterile test tubes depending on the number of sugars to be tested. Mannitol, glucose, sorbitol, rhamnose, mannose, trehalose, xylose, galactose,

maltose, arabinose and sucrose sugars discs were aseptically added to the broths containing loopful recommended culture (Markey *et al.*, 2013) and incubated at 37 °C for 24-48 hours. Change in the colour of the broth from yellow to pink indicated acid production.

3.4.2.3 Identification of bacteria

The purified isolates obtained were identified according to the criteria described by Barrow and Feltham (1993), which included staining reaction, organism morphology, growth condition, colony characteristics on different media, hemolysis pattern on blood agar, motility and biochemical characteristics.

3.4.3 Preservation of pure culture

After phenotypic identification by biochemical tests, pure cultures of bacterial isolates were streaked onto slants of nutrient agar and preserved at 4 ºC for further study. Alternatively, cultures were also preserved in sterile nutrient glycerol (15%) broth vials at -20 °C until further use.

3.5 Molecular identification of major isolates

3.5.1 Identification of *E. coli* and *S. aureus* by molecular methods

3.5.1.1 DNA extraction

The DNA was extracted as per the procedure described by Arakere *et al*. (2005).

3.5.1.1.1 Materials

Uniflex™ DNA isolation kit (Genei, Bangalore), spectrophotometer, sterile DNase free micropipette tips and micro centrifuge tubes (Genei, Bangalore), micropipettes (Eppendorf, Germany), Ethanol (96 -100%).

3.5.1.1.2 Procedure

The isolates in pure culture were inoculated to BHI broth and incubated overnight at 37 °C. Then the culture broth was centrifuged at 6,000 rpm for 10 minutes to collect cells which were further suspended in lysis buffer (phosphate buffered saline containing 0.5% sodium dodecyl sulphate and 100µg/ml proteinase K). The cell suspension was incubated at 37 ºC for one hour, and an equal volume of phenol: chloroform (1:1) mixture was added to the cell suspension and vortexed. Centrifugation of the samples was done at 12,000 rpm for 20 minutes and the supernatant aqueous phase was transferred to a fresh sterile DNase free tube. Precipitation of DNA was done by centrifugation at 12,000 rpm for 20 minutes at room temperature after adding 0.1 suspension volume of 3 M sodium acetate (pH 5.2) and 2.0-3.0 suspension volumes of 99% cold ethanol. Cold ethanol (70%) was used to wash the DNA pellet twice, then air dried, and suspended in 500µL of TE buffer [10 Mm Tris-HCl (pH 8.0) 1 mM EDTA (pH 8)].

3.5.1.2 Determination of purity and yield of the DNA samples

UV spectrophotometry was used to estimate the purity and concentration of the extracted genomic DNA. An aliquot of 20µL of DNA sample was dissolved in 0.98 ml of sterile DW in a 1 mL microcuvette and the optical density (OD) was read at 260nm and 280nm using UV spectrophotometer. Sterile DW was used as blank in the procedure (Boesenberg *et al.*, 2012). Then the ratio of 260/280 OD was calculated and the DNA samples with the 260/280 OD value of 1.7 to 1.9 was considered as pure.

3.5.1.3 Agarose gel electrophoresis for confirmation of DNA

The DNA was confirmed by agarose gel electrophoresis following the procedures explained by Lee *et al.* (2012). The 0.5 µl of DNA was used on 0.8 % agarose gel to check the purity by electrophoresis method.

3.5.1.3.1 Equipment

a. Horizontal electrophoresis apparatus with power back (Bangalore Genei, India)

b. Weighing balance (Shimazdu)

c. Microwave oven (M/s. BPL Pvt. Ltd.)

d. Gel documentation unit (Bio-Rad, USA)

3.5.1.3.2 Reagents

a. Agarose (Synergy Scientific Services, Chennai)

b. Tris - Borate EDTA buffer (TBE buffer) (10X, pH 8.2)

Tris base	108.0 g
Boric acid	55.0 g
EDTA disodium salt	8.3 g
Double D.W.	1000.0 ml

The stock solution was sterilized by autoclaving and made to 1X before use.

c. **Gel loading dye (6X)** was procured from Genei Bangalore and stored at 4°C. Gel loading dye was routinely made at 6X concentration with 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol.

d. **Ethidium bromide (**10 mg**)** was procured from Biosciences, Merck specialties Pvt. Ltd., Mumbai. To 10 mg of ethidium bromide, one mL of DW was added and the

suspension was properly mixed to dissolve the dye. The stock solution was stored at RT and protected from direct light.

e. **100 bp DNA ladder** (Genei Bangalore)

3.5.1.3.3 Preparation of the gel

Agarose (0.8 %) was prepared in Erlenmeyer flask by adding 0.8g of agarose to 100 mL of running buffer (TAE buffer: 40 mM Tris-acetate, 1 mM EDTA) and then mixed well by swirling. Agarose was melted in a microwave oven, at 30 s intervals, the flask was removed and swirled to mix well. This was repeated until the agarose has completely dissolved. Already diluted ethidium bromide dye was added to a concentration of 0.4 μg/ml. Agarose was allowed to cool on the bench top. Gel receptacle (tray) is placed in casting apparatus and appropriate comb was placed into the gel mould to create wells. Molten agarose was transferred to the gel mould and then it was allowed to set at room temperature. Comb was removed and gel was placed in the gel box (Viljoen *et al*., 1993).

3.5.1.3.4 Setting up of gel equipment and separation of DNA fragments

Power supply to apparatus was programmed to desired voltage (1-5 V/cm between electrodes). Running buffer (TAE) with sufficient quantity was added to cover the surface of the gel and power supply to the apparatus was ensured by connecting the gel box through leads. Lid was removed and DNA samples were loaded into already created wells in the gel carefully and slowly. DNA size marker was also loaded along with the samples. Lid was replaced and gel was placed in such a way that cathode (black leads) was closer the wells than the anode (red leads) and power was turned on. Gel running was carried out until the dye has migrated to an appropriate distance (Lee *et al*., 2012).

3.5.1.3.5 Visualization of separated DNA fragments

After completion of electrophoresis, power supply was turned off and gel box lid was removed. Gel removed from the gel box using gel pan. Excess buffer was removed from the surface of the gel by draining. Gel tray was placed on tissue paper to absorb and ensured removal of any extra running buffer. Gel was exposed to UV light after removing from the gel tray and DNA bands which elicited orange fluorescent bands were recorded. Subsequently the gel was placed in gel documentation unit for observation and photography. At the end, gel was properly disposed off as per standard recommendations.

3.5.1.3.6 Storage of DNA

After confirmation of purity and yield, the DNA samples were stored at -20 °C for further use.

3.5.2.1 PCR-based detection of *E. coli*

The duplex PCR was carried out by targeting *uidA* and *uspA* genes following the procedure described by Godambe *et al*. (2017).

Table 1. Primers used in detection of *E. coli* (Bej *et al*., 1991; Chen and Griffiths, 1998)

Sl No.	Gene targeted	Name of the primer	Oligonucleotide Sequence (5′ - 3′)	Product size (bp)
1.	*uidA*	*uidA*-F	TGGTAATTACCGACGAAAACGGC	162
		uidA-R	ACGCGTGGTTACAGTCTTGCG	
2.	*uspA*	*uspA*-F	CCGATACGCTGCCAATCAGT	884
		uspA-R	ACGCAGACCGTAGGCCAGAT	

The reaction mixture of 25 µl each was prepared in 0.2 ml thin-walled PCR tubes placed in mini cooler as shown below (Table 2).

Table 2. Reagents used for *uidA* and *uspA* genes-based duplex PCR

Reagents (concentration)	Volume
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 µL
Primer *uidA*-F (12.5 µL/mL)	1 µL
Primer *uidA*-R (12.5 µL/mL)	1 µL
Primer *uspA*-F (12.5 µL/mL)	1 µL
Primer *uspA*-R (12.5 µL/mL)	1 µL
Template (*E. coli* DNA)	3 µL
Nuclease free water	5.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Godambe *et al*., 2017).

<table>
<tr><th>Initial denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final extension</th></tr>
<tr><td rowspan="2">94 °C, 60 sec</td><td>94 ° C, 60 sec</td><td>64 ° C, 30 sec</td><td>72 ° C, 30 sec</td><td rowspan="2">72 °C, 7 min followed by hold at 4 °C</td></tr>
<tr><td colspan="3">Repeated for 35 cycles</td></tr>
</table>

An *E. coli* strain which was earlier confirmed by species-specific PCR targeting *uidA* and *uspA* genes followed by sequencing of amplicons was used as positive control in this study. *S. aureus* isolates were used as negative control.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded onto a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and ‘no template control’ were also loaded into one

well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR (Bio-Rad., U.S.A) for observation and photography. At the end, the gel was properly disposed off as per standard recommendations.

3.5.2.2 Detection of virulence genes in *E. coli* using PCR

All the isolates of *E. coli* were subjected to PCR for detection of five major virulence genes *fimH, fyuA, kpsMTII, csgA* and *agn43* as shown below.

3.5.2.2.1 Detection of *fimH* gene

All the isolates of *E. coli* were subjected to PCR for detection of *fimH* gene using published primers (Table 3) and procedure described by Gonzalez *et al*., 2020.

Table 3. Primer sequences used for detection of *fimH* gene in *E. coli*

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
fimH	*fimH* -F	TGCAGAACGGATAAGCCGTGG	508
	fimH -R	GCAGTCACCTGCCCTCCGGTA	

The reaction mixture of 25 µL each was prepared in 0.2 mL thin-walled PCR tubes placed in mini cooler (Table 4).

Table 4. Reagents used for *fimH* gene-based PCR

Reagents (concentration)	**Volume**
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 µL
Primer *fimH*-F (12.5 µL/ml)	1 µL
Primer *fimH*-R (12.5 µL/ml)	1 µL
Template (*E. coli* DNA)	3 µL
Nuclease free water	7.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Gonzalez *et al.*, 2020).

Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**
95 °C 12 min	94 °C, 30 Sec	63 °C, 30 Sec	68 °C, 3min	72 °C, 10 min followed by hold at 4 °C
	Repeated for 25 cycles			

An *E. coli* strain ENDO4e which was earlier confirmed for *fimH* gene by PCR followed by sequencing of amplicons was used as positive control throughout the study. *S. aureus* isolates were used as negative control.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded into a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and 'no template control' were also loaded into one well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength

TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR (Bio-Rad., U.S.A) for observation and photography. At the end, the gel was properly disposed off as per standard recommendations.

3.5.2.2.1.1 Sequencing of the purified PCR products

The purified PCR amplicons were commercially sequenced at Eurofiins Genomics India Pvt. Ltd., Bengaluru on an ABI – PRISM dye terminator DNA sequencing apparatus using T_3 sequencing. The sequence data generated were received as colored electropherograms, AB1 and FASTA files.

3.5.2.2.1.2 Analysis of nucleotide sequence

The sequence quality and the chromatogram were analyzed in MEGA software. The sequence reads obtained from both forward and reverse primers were analyzed and edited using MEGA version X software.The sequence data was further analyzed by BLAST and Clustal method with Weighted residue weight table software program. MegAlign of DNA STAR was used for further analysis of nucleotide sequences.

3.5.2.2.2 Detection of *fyuA* gene in *E. coli*

The *fyuA* gene-based PCR was carried out using published primers (Table 5) and the procedure described by Gonzalez *et al*. (2020).

Table 5. Primer sequences used for detection of *fyuA* gene in *E. coli*

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
fyuA	*fyuA* -F	TGATTAACCCCGCGACGGGAA	880
	fyuA -R	CGCAGTAGGCACGATGTTGTA	

The reaction mixture of 25 µL each was prepared in 0.2 mL thin-walled PCR tubes placed in mini cooler (Table 6).

Table 6. Reagents used for *fyuA* gene-based PCR

Reagents (concentration)	Volume
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 µL
Primer *fyuA*-F (12.5 µL/mL)	1 µL
Primer *fyuA*-R (12.5 µL/mL)	1 µL
Template (*E. coli* DNA)	3 µL
Magnesium chloride	1 µL
Nuclease free water	6.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Gonzalez *et al*., 2020).

Initial denaturation	Denaturation	Annealing	Extension	Final extension
95 °C 12 min	94 °C, 30 sec	63 °C, 30 sec	68 °C, 3min	72 °C, 10 min followed by hold at 4 °C
	Repeated for 25 cycles			

An *E. coli* strain ENDO9e, which was earlier confirmed to possess *fyuA* gene by PCR followed by sequencing, was used as positive control throughout the study.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded onto a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and 'no template control' were also loaded into one well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR (Bio-Rad., U.S.A.) for observation and photography. At the end, the gel was properly disposed off as per standard recommendations.

Sequencing of the purified PCR product and analysis of nucleotide sequence was performed as described in section 3.5.2.2.1.2 and 3.5.2.2.1.1.

3.5.2.2.3 Detection of *kpsMTII* gene in *E. coli*

The *kpsMTII* gene-based PCR was carried out using published primers (Table 7) and the procedure described by Gonzalez *et al*. (2020).

Table 7. Primer sequences used for detection of *kpsMTII* gene in *E. coli*

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
kpsMTII	*kpsMT II*-F	GCGCATTTGCTGATACTGTTG	272
	kpsMT II-R	CATCCAGACGATAAGCATGAGCA	

The reaction mixture of 25 µL each was prepared in 0.2 ml thin-walled PCR tubes placed in mini cooler as shown below (Table 8).

Table 8. Reagents used for *kpsMTII* gene-based PCR

Reagents (concentration)	Volume
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 µL
Primer *kpsMTII*-F (12.5 µL/mL)	1 µL
Primer *kpsMTII*-R (12.5 µL/mL)	1 µL
Magnesium chloride	1 µL
Template (*E. coli* DNA)	3 µL
Nuclease free water	6.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Gonzalez *et al.*, 2020).

Initial denaturation	Denaturation	Annealing	Extension	Final extension
95 ° C 12 min	94 °C, 30 sec	63 °C, 30 sec	68 °C, 3min	72 °C, 10 min followed by hold at 4 °C
	Repeated for 25 cycles			

An *E. coli* strain ENDO2e, which was earlier confirmed to possess *kpsMTII* gene by PCR followed by sequencing, was used as positive control throughout the study.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded into a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µl/10ml. Marker (DNA ladder), positive control, negative control and 'no template control' were also loaded into one well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength

TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR (Bio-Rad., U.S.A) for observation and photography. At the end gel was properly disposed off as per standard recommendations.

Sequencing of the purified PCR product and analysis of nucleotide sequence was performed as described in section 3.5.2.2.1.2 and 3.5.2.2.1.1.

3.5.2.2.4 Detection of *csgA* gene in *E. coli*

The PCR was carried out for *csgA* gene responsible for biofilm formation using published primers (Table 9) and the procedure described by Gonzalez *et al*. (2020).

Table 9. Primer sequences used for detection of *csgA* gene in *E. coli*

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
csgA	*csgA* -F	ACTCTGACTTGACTATTACC	200
	csgA -R	AGATGCAGTCTGGTCAAC	

The reaction mixture of 25 µL each was prepared in 0.2 ml thin-walled PCR tubes placed in mini cooler as shown below (Table 10).

Table 10: Reagents used for *csgA* gene-based PCR

Reagents (concentration)	**Volume**
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 µL
Primer *csgA* -F (12.5 µL/mL)	1 µL
Primer *csgA* -R (12.5 µL/mL)	1 µL
Template (*E. coli* DNA)	3 µL
Nuclease free water	7.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Gonzalez *et al*., 2020).

<table>
<tr><th>Initial Denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final Extension</th></tr>
<tr><td rowspan="2">94 °C, 5 min</td><td>94 °C, 30 sec</td><td>55 °C, 30 sec</td><td>72 °C, 15 sec</td><td rowspan="2">72 °C, 2 min followed by hold at 4 °C</td></tr>
<tr><td colspan="3">Repeated for 30 cycles</td></tr>
</table>

An *E. coli* isolate ENDO7e, which was earlier confirmed to possess *csgA* gene by PCR followed by sequencing was used as positive control throughout the study.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded into a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and ‘no template control’ were also loaded onto one well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR

(Bio-Rad., U.S.A) for observation and photography. At the end, the gel was properly disposed off as per standard recommendations.

Sequencing of the purified PCR product and analysis of nucleotide sequence was performed as described in section 3.5.2.2.1.2 and 3.5.2.2.1.1.

3.5.2.2.5 Detection of *agn43* gene in *E. coli*

The *agn43* gene-based PCR was carried out using published primers (Table 11) and the procedure described by Gonzalez *et al*. (2020).

Table 11. Primer sequences used for detection of *agn43* gene in *E. coli*

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
agn43	***agn43*-F**	CTGGAAACCGGTCTGCCCTT	433
	***agn43*-R**	CCTGAACGCCCAGGGTGATA	

The reaction mixture of 25 µL each was prepared in 0.2 mL thin-walled PCR tubes placed in mini cooler as shown below (Table 12).

Table 12. Reagents used for *agn43* gene-based PCR

Reagents (concentration)	Volume
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 µL
Primer *agn43*-F (12.5 µL/ml)	1 µL
Primer *agn43*-R (12.5 µL/ml)	1 µL
Template (*E. coli* DNA)	3 µL
Nuclease free water	7.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Gonzalez *et al*., 2020).

<table>
<tr><th>Initial denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final extension</th></tr>
<tr><td rowspan="2">94 °C, 1 min</td><td>94 °C, 1 min</td><td>60 °C, 1 min</td><td>72 °C, 1 min 20 sec</td><td rowspan="2">72 °C, 10 min followed by hold at 4 °C</td></tr>
<tr><td colspan="3">Repeated for 30 cycles</td></tr>
</table>

An *E. coli* strain ENDO12e, which was earlier confirmed to possess *agn43* gene by PCR followed by sequencing, was used as positive control throughout the study.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded into a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and 'no template control' were also loaded into one well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR (Bio-Rad., U.S.A) for observation and photography. At the end gel was properly disposed off as per standard recommendations.

Sequencing of the purified PCR product and analysis of nucleotide sequence was performed as described in section 3.5.2.2.1.2 and 3.5.2.2.1.1.

3.5.3 *Nuc* gene-based PCR for confirmation of *Staphylococcus aureus*

The PCR was carried out by targeting *nuc* gene as per the procedure described by Hegde (2011). The PCR was carried out using published primers (Table 13) and the procedure described by Hegde (2011).

Table 13. Primers used for detection of *S. aureus* by PCR

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
nuc	*nuc*-F	AAACACCGAGTAATACGCCG	180
	nuc-R	TTTAGCGTTCCCAAATGTTCAG	

The reaction mixture of 25 μL each was prepared in 0.2 mL thin-walled PCR tubes placed in mini cooler as shown below (Table 14).

Table 14. Reagents used for *nuc* gene-based PCR

Reagents (concentration)	Volume
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 μL
Primer *nuc*-F (12.5 μL/mL)	1 μL
Primer *nuc*-R (12.5 μL/mL)	1 μL
Template (staphylococcal DNA)	3 μL
Nuclease free water	7.5 μL
Total	**25 μL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Hegde, 2011).

<table>
<tr><th>Initial denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final extension</th></tr>
<tr><td rowspan="2">94 °C, 3 min</td><td>94 °C, 30 sec</td><td>60 °C, 30 sec</td><td>72 ° C, 1 min 30 sec</td><td rowspan="2">72 °C, 5 min followed by hold at 4 °C</td></tr>
<tr><td colspan="3">Repeated for 30 cycles</td></tr>
</table>

A *S. aureus* strain which was earlier confirmed by species-specific PCR targeting *nuc* genes followed by sequencing of amplicons was used as positive control in this study. *E. coli* isolates were used as negative control.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded into a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and 'no template' control were also loaded into one well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR (Bio-Rad., U.S.A) for observation and photography. At the end, the gel was properly disposed off as per standard recommendations.

3.5.3.2 Detection of *spa* gene in *S. aureus* using PCR

All the isolates of *S. aureus* were subjected to PCR for detection of *spa* gene responsible for its virulence. The PCR was carried out using published primers (Table 15) and the procedure described by Aires *et al*. (2006).

Table 15. Primers used for detection of *spa* gene by PCR

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
spa	*spa* -F	AAGTAGACATTTTTGGCGTTCC	287
	spa -R	AGAACCATCAAACTCGTATAGC	

The reaction mixture of 25 µL each was prepared in 0.2 mL thin-walled PCR tubes placed in mini cooler as shown below (Table 16).

Table 16. Reagents used for *spa* gene-based PCR

Reagents (concentration)	Volume
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2x)	12.5 µL
Primer *spa*-F (12.5 µL/ml)	1 µL
Primer *spa*-R (12.5 µL/ml)	1 µL
Template (Staphylococcal DNA)	3 µL
Nuclease free water	7.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Aires *et al*., 2006).

Initial denaturation	Denaturation	Annealing	Extension	Final extension
94 $^{\circ}$C, 5 min	94 $^{\circ}$C, 30 sec	56 $^{\circ}$C, 30 sec	72 $^{\circ}$C, 45 sec	72 $^{\circ}$C, 5 min followed by hold at 4 $^{\circ}$C
	Repeated for 30 cycles			

S. aureus strain SA6 which was earlier confirmed by species-specific PCR targeting *spa* genes followed by sequencing of amplicons, was used as positive control in this study. *E. coli* isolates were used as negative control.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded into a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and ‘no template’ control were also loaded into one well each and gel was made to run in a gel electrophoresis apparatus using a 1X strength TAE buffer. Subsequently the gel was placed in gel documentation unit Gel Doc XR

(Bio-Rad., U.S.A) for observation and photography. At the end, the gel was properly disposed off as per standard recommendations.

3.5.3.3 Assignment of *spa* type

The edited files using procedure as described in section 3.5.2.2.1.2 and 3.5.2.2.1.1. were trimmed and for the assignment of *spa* type, the sequences which are present immediately after the signature sequence at 5′ end (RCA MCA AAA) and upto 18 or 19 sequences prior to signature sequences at the 3′ end (TAY ATG TCG T) were taken into account to arrive at the size of the amplicon and to deduce the *spa* repeat. *Spa* type was deduced using the database (http://spatyper.fortinbras.us/) available online. Furthermore, it was compared using the RidomSpaServer (http:// wwwspaserver.ridom.de/) for the spa repeats succession (Rup察ttsch *et al*., 2006).

3.6 Antimicrobial susceptibility testing

The antibiotics which were commonly used by practicing veterinarians for the treatment of endometritis in the study area and the antibiotics which have been suggested for treatment of endometritis (Noakes and England, 2009) were selected for the antibiogram assay (Table 17).

Materials: BHI broth, Muller Hinton Agar (MHA) plates, PBS, 0.5 Mc Farland scale, sterile swab with wooden applicator, antibiotic zone scale (Himedia Laboratories, Mumbai).

Table 17. List of antimicrobial discs used in the study for the antibiogram assay

Sl. No.	Chemotherapeutic agents	Symbol	Concentration
1	Amikacin	AK	30 µg
2	Amoxicillin/Sulbactum	AMS	30/15 µg
3	Amoxyclav	AMC	50/10 µg
4	Ampicillin	EX	10 µg
5	Cefaperazone	CPZ	75 µg
6	Cefazolin	CZ	30 µg
7	Cefotaxime	CTX	30 µg
8	Cefoxitin	CX	30 µg
9	Ceftrixone	CTR	30 µg
10	Chloromphenicol	C	10 µg
11	Ciprofloxacin	CIP	5 µg
12	Co-trimaxazole	COT	25 µg
13	Enrofloxacin	EX	30 µg
14	Gentamicin	GEN	10 µg
15	Nitrofurantoin	NIT	300 µg
16	Oxytetracyclin	O	30 µg
17	Penicillin	P	2 units
18	Polymixin B	PB	100 units
19	Streptomycin	S	10 µg
20	Tylosin	TL	15 µg

Procedure

Antibiogram assay was performed following the standard disc diffusion method. The bacterial inoculum was adjusted to 0.5 on Mc Farland scale and were mat cultured on Muller Hinton agar petriplates using sterile non-toxic swab. The antibiotic discs were deposited aseptically onto the agar surface with centres at least 30 mm apart and examined after incubation for 12-24 hours at 37 $^{\circ}$C as per the standard procedure

for disc diffusion method as described by Bauer *et al*. (1966). The zone of inhibition was then measured using antibiotic zone scale and expressed in millimetres. Sensitivity/resistance was assessed by comparing the values of the standard chart provided with the discs. The interpretation was done in accordance to performance standards for antimicrobial disks susceptibility tests, Clinical Laboratory Standard Institute (CLSI, 2019). The antimicrobials used in this study are listed in Table 17.

3.6.1 Detection of methicillin resistance in *S. aureus*

Methicillin resistance in *S. aureus* was detected phenotypically using cefoxitin 30 mcg disc as per the procedure mentioned in (3.6). The isolates were considered resistant, if the zone of inhibition was ≤21 mm and susceptible if it was ≥22 mm (CLSI, 2019).

3.6.2.1 Detection of *mec*A gene in *S. aureus* by PCR

The PCR procedure described by Chitra *et al*. (2015) was followed for detection of *mec*A gene (Table 18).

Table 18. Primers used for detection of *mecA* gene in *S. aureus*

Gene targeted	Name of the primer	Oligonucleotide sequence (5′ - 3′)	Product size (bp)
mec A	*mec A* -F	CAAACTACGGTAACATTGATCGC	210
	mec A -R	GCCTATCTCATATGCTGTTCCT	

The reaction mixture of 25 µL each was prepared in 0.2 ml thin-walled PCR tubes placed in mini cooler as shown below (Table 19).

Table 19. Reagents used for *mec*A gene-based PCR

Reagents (concentration)	Volume
Master mix (Ampliqon Taq DNA polymerase master mix RED, 2X)	12.5 µL
Primer *mec A* -F (12.5 µL/mL)	1 µL
Primer *mec A* -R (12.5 µL/mL)	1 µL
Template (staphylococcal DNA)	3 µL
Nuclease free water	7.5 µL
Total	**25 µL**

Subsequent to addition of above reagents, the contents were centrifuged briefly, to facilitate proper mixing. PCR reaction was carried out using thermal cycler (Biorad T 300) with the thermal conditions set as detailed below (Chitra *et al*., 2015).

<table>
<tr><th>Initial denaturation</th><th>Denaturation</th><th>Annealing</th><th>Extension</th><th>Final extension</th></tr>
<tr><td rowspan="2">94 °C, 3 min</td><td>94 °C, 30 sec</td><td>60 °C, 30 sec</td><td>72 °C, 30 sec</td><td rowspan="2">72 °C, 5 min followed by hold at 4 °C</td></tr>
<tr><td colspan="3">Repeated for 30 cycles</td></tr>
</table>

S. aureus strain SA15, which was earlier confirmed by species-specific PCR targeting *mecA* genes followed by sequencing of amplicons, was used as positive control in this study. *E. coli* isolates were used as negative control.

After successfully achieving PCR reaction, the amplified product (3µL) was loaded into a pre-prepared 1.5-2 % agarose gel (prepared in 1X strength TAE buffer) containing ethidium bromide at the concentration of 1µL/10mL. Marker (DNA ladder), positive control, negative control and ‘no template control’ were also loaded into one well each and the gel was made to run in a gel electrophoresis apparatus using a 1X strength TAE buffer. Subsequently the gel was placed in gel documentation unit Gel

Doc XR (Bio-Rad., U.S.A) for observation and photography. At the end, the gel was properly disposed off as per standard recommendations.

Sequencing of the purified PCR product and analysis of nucleotide sequence was performed as described in section 3.5.2.2.1.2 and 3.5.2.2.1.1.

3.8 MIC determination

The MIC was determined and interpreted based on the recommendations of Clinical and Laboratory Standards Institute guidelines (CLSI, 2019).

Materials: BHI broth, Muller Hinton Agar (MHA) plates, PBS, 0.5 Mc Farland scale, sterile swab with wooden applicator, Ezy MIC strips with MIC scale (Himedia Laboratories, Mumbai).

3.8.1 EZy strip method

The bacterial inoculum was adjusted to 0.5 on Mc Farland scale and mat cultured on Muller Hinton agar petriplates using sterile non-toxic swab. The Ezy MIC strips with MIC scale were positioned aseptically with sterile forceps onto the agar surface with MIC scale facing upwards. The plates were incubated at 37 °C for 12 – 24 hrs. The zone of inhibition in the form of an ellipse was then examined and MIC value was read as the value at which the zone convenes the comb-like projections of the strips and not the handle. The MIC was interpreted as greater than the highest concentration of the strip when no zone of inhibition was observed. When the zone of inhibition was below the concentration, then the MIC was recorded as less than the highest concentration of the strip. The interpretative chart (supplied with the MIC strips) was used to compare MIC values, which was in accordance with the performance standards for antimicrobial disk susceptibility tests, Clinical Laboratory Standards Institute (CLSI, 2019).

3.8.2 Broth dilution (macro dilution) method

3.8.2.1 Materials: Nutrient broth, PBS, 0.5 Mc Farland scale, sterile 5 mL tubes, antibiotic to be tested.

3.8.2.2 Method

The test was performed by preparing two-fold dilutions of an antibacterial in a series of tubes (5 mL tubes) containing a nutrient broth. Then, 1948.8 μL of nutrient broth was added in the first tube and then 1 mL (1000 μL) in the remaining tubes. Now, 51.2 μL of antibacterial solution dissolved in appropriate solvent was added to the first tube in each row and then after mixing the content, 1 mL was serially transferred from this tube to the second tube in each of the rows (serial two-fold dilution). Then the contents of the second tube of each of the rows were mixed and 1 mL was transferred to the third tube in each of the rows. This serial dilution was repeated up to sixteenth tube. This provided antibacterial concentrations of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03, 0.015, 0.008 μg/mL in the first to sixteenth tube respectively. Each tube was inoculated with actively growing pure culture suspension of the test bacterium that contained between 10^4 and 10^5 bacteria/mL. The required concentration of bacteria was achieved by adjusting the bacterial cell suspension turbidity matching to 0.5 on McFarland Scale by adding 1X PBS. Drug (antibacterial) and control broth controls were maintained following the same procedure but without adding bacterial suspension. Culture (inoculums) control was also maintained but without adding the antibacterial. The inoculated tubes of broth were incubated at 35– 37 °C for 24 hours and then observed for visible growth in the form of turbidity in the tubes. The highest dilution of the antibacterial that inhibited growth (showing no visible growth/ no turbidity in the tube) was considered to be representing the minimal inhibitory concentration (MIC) of that particular drug (CLSI, 2019).

Results

IV. RESULTS

The present study was carried out with an objective to isolate aerobic bacteria from subclinical and clinical endometritis in dairy cattle, identification of bacterial agents by phenotypic methods, to study antibiogram profile of the bacterial isolates using disc diffusion method and to determine minimum inhibitory concentration assay. In addition, the detection of major virulence genes in the predominant bacterial species by PCR was also carried out. The results obtained during the period of study are documented under the following subheadings.

4.1 Collection of samples

A total of 82 uterine lavage samples with history of repeat breeding and 51 samples from clinical endometritis cases were collected in the study.

4.2 Cytological evaluation of uterine lavages for detection of SE

Lavage samples that showed 1 - 5% of PMN cells were considered as cytologically positive indicating SE status. Out of 82 samples, 63 were cytological positive (SE) and 19 were cytological negative (Plate 1).

All the samples viz., cytologically positive samples (n=63), cytologically negative samples (n=19) and samples collected from CE cases (n=51) were processed further for isolation of aerobic bacteria.

4.3 Isolation and identification of aerobic bacteria

4.3.1 Isolation of aerobic bacteria

Among 63 lavage samples from SE processed for isolation, aerobic bacterial growth was observed in 62 samples and one sample did not show any growth. Four

samples showed heavy contamination and hence they were not considered for further processing. The remaining 58 samples were subjected for isolation of aerobic bacteria.

Among 19 cytology negative samples processed, aerobic bacterial growth was observed in four samples and the remaining 15 samples were negative for aerobic bacteria and hence 04 samples were subjected for further isolation of aerobic bacteria (Table 20).

All the endometrial discharge samples (n=51) collected from CE cases showed bacterial growth (Table 20).

Pure culture was obtained from all the culturally positive samples and the isolates obtained were subjected for identification by phenotypic methods.

4.3.1 Identification of bacterial isolates by phenotypic methods

All the isolates were characterized by morphological, cultural and biochemical methods. The phenotypic properties considered for genus/species detection included staining morphology, cultural characteristics and biochemical profile of the isolates as mentioned in Tables 21 and 22. The genus/species was assigned to the isolate based on the phenotypic properties as shown in the Table 23(Plate 2- 6).

Gram negative bacterial isolates recovered from SE, cytology negative samples and CE samples were 31 (49.20%) and 30 (44.11%) respectively and similarly, gram positive isolates were 32 (50.79%) and 38 (55.88%) respectively.

The number of single bacterial isolates recovered from SE, cytology negative samples and CE samples were 41 (70.68%), 04 (100%) and 27 (52.94%) respectively and that of mixed isolates were 17 (29.31%), 0 and 24 (47.06%) respectively (Tables 24- 26).

Table 20. Aerobic bacteria recovered from lavage samples/uterine discharge

Type of sample	Number of samples	Negative for bacteria	Discarded	Processed
SE	63	01	04	58
CE	51	0	0	51
Cytology negative	19	15	0	4

Plate 1. **Cytology of uterine lavage showing PMN cells in Giemsa-stained smear (100x)**

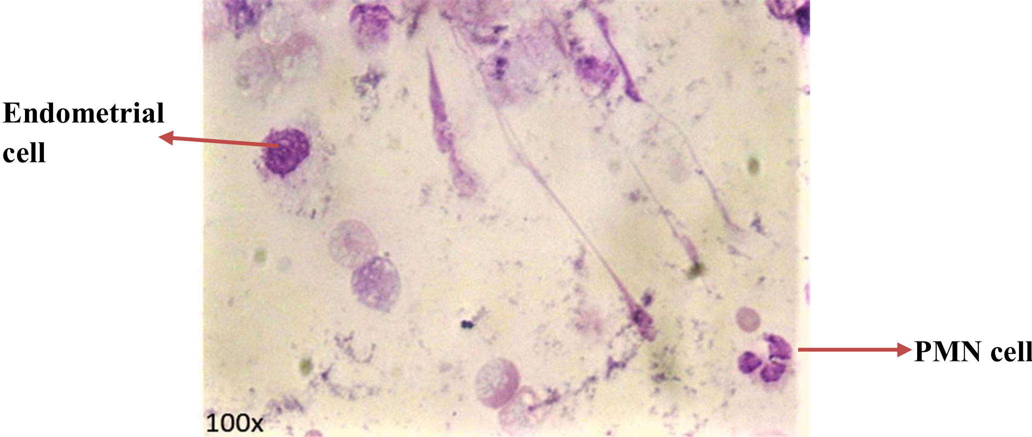


Table 21. Phenotypic properties of Gram-negative bacteria that were studied for assigning species

	Escherichia coli	***Klebsiella pneumoniae***	***Proteus mirabilis***	***Pseudomonas aeruginosa***
Gram stain	Negative	Negative	Negative	Negative
Shape	Rods	Rods	Rods	Rods
Motility	Motile	Non motile	Motile	Motile
MR	+	-	+	-
VP	-	+	-	-
Indole	+	-	-	-
Catalase	+	+	+	+
Oxidase	-	-	-	+
Urease	-	+	+	-
Citrate	-	+	+	+
TSI	Y/Y	Y/Y with gas	Y/Y with gas	R/R
Growth on MacConkey	LF	LF	NLF	NLF
Growth on EMB	Metallic sheen			
Growth on BHI agar	+	+	Swarming	+
Pigment production				+
Mannitol	+	+	-	+
Mannose	+			-
Trehalose	+	+	+	
Xylose	+	+	+	
Mannose	+			
Rhamnose	+			
Sorbitol		+	-	-
Nitrate reduction		+	+	+
Maltose		+	-	-
Sucrose			-	-
Arabinose		+		-
Esculin hydrolysis		+		

+ Positive; - Negative; Y-Yellow; LF- Lactose fermenter; NLF- Non-Lactose fermenter, R- Red

Table 22. Phenotypic properties of Gram-positive bacteria that were studied for assigning species

	S. aureus	*S. uberis*	*B. cereus*	*R. equi*	*C. bovis*
Gram stain	Positive	Positive	Positive	Positive	Positive
Shape	Cocci in bunches	Cocci in chain	Rods	Pleomorphic	Pleomorphic
Motility	Non motile	Non motile	Motile	Non motile	Non motile
Spores	-	-	+	+	-
Capsule staining				+	
Catalase	+	-	+	+	+
Oxidase	-	-	-	-	+
Coagulase	+				
Urea hydrolysis	+		-	+	-
MR	+				
VP	+	+	+		
Indole	-		-		
Esculin hydrolysis		+		-	-
Hemolysis on blood agar	Hemolytic	Non-hemolytic	Hemolytic	Non-hemolytic	Non-hemolytic
CAMP test		-		+	
Growth on macConkey agar	-	-	-		
Growth on egg yolk agar			Opaque zone		
Growth on potassium tellurite agar					Black centered colony
Sodium hippurate		+			
Sorbitol		+			
Mannitol fermentation	+	+			
Nitrate utilization	+		+	+	+
Citrate utilization	+		-		
Mannose	+		-		
Trehalose	+	+			
Xylose	-		-		
Galactose			-		
Glucose			+	-	+
Maltose				-	-
Sucrose				-	-
Colony morphology on BHI agar	Round, smooth, raised, glistening and grey to golden yellow	Pin point, white, round	Grey-white, granular colonies with wavy edge	Orange colour	

+ Positive; - Negative;

Table 23. Bacterial isolates recovered in the study

Subclinical endometritis	Clinical endometritis	Cytology negative samples
E. coli	*E. coli*	*E. coli*
Staphylococcus aureus	*Staphylococcus aureus*	
Streptococcus uberis	*Streptococcus uberis*	
Pseudomonas aeruginosa	*Bacillus cereus*	
Proteus mirabilis	*Corynebacterium bovis*	
Klebsiella pneumoniae	*Rhodococcus equi*	
Bacillus cereus	*Klebsiella pneumoniae*	
	Proteus mirabilis	

Table 24. Bacterial isolates recovered from SE cases

Bacteria isolated	Isolates			Percent
	Single	Mixed	Total	
E. coli	18	5	23	36.51
Staphylococcus aureus	13	5	18	28.57
Streptococcus uberis	6	3	9	14.28
Bacillus cereus	2	3	5	7.94
Klebsiella pneumoniae	2	2	4	6.35
Proteus mirabilis	0	2	2	3.17
Pseudomonas aeruginosa	0	2	2	3.17
Total	41	22	63	

Table 25. Bacterial isolates recovered from cytology negative cases

Bacteria isolated	Isolates		
	Single	Mixed	Total
E. coli	**04**	**0**	**04**

Table 26. Bacterial isolates recovered from CE cases

Bacteria isolated	Isolates			Percent (%)
	Single	Mixed	Total	
Escherichia coli	10	16	26	38.24
Staphylococcus aureus	7	11	18	26.47
Corynebacterium bovis	2	4	6	8.82
Streptococcus uberis	2	3	5	7.35
Bacillus cereus	2	3	5	7.35
Rhodococcus equi	2	2	4	5.88
Klebsiella pneumoniae	0	3	3	4.41
Proteus mirabilis	0	1	1	1.47
Total	27	41	68	

Among the bacterial isolates obtained, *Escherichia coli* and *Staphylococcus aureus* were found predominant in both SE and CE cases. Hence, the isolates belonging to these two species were subjected for detection of virulence genes using PCR.

4.4.1 Identification of *E. coli* by molecular method

Phenotypically confirmed *E. coli* isolates (n=53) were further subjected to PCR for molecular identification by targeting the *uspA* and *uidA* genes for genotypic confirmation. All the isolates (n=53) yielded 884 bp and 162 bp amplicons respectively, specific for *E. coli* (Plate 7A).

4.4.2 Detection of virulence genes of *E. coli* by PCR

Uniplex PCRs targeting the *fimH*, *fyuA*, *KPSMTII*, *csgA* abd *agn43* genes were carried out for the *E. coli* isolates (n=53) which were confirmed upto species level by PCR as mentioned in 4.4.1

4.4.2.1 Detection of *fimH* gene

Of the 53 isolates subjected for *fimH* gene-based PCR, 50 isolates were found to be positive for the presence of *fimH* gene which yielded amplicons of 508 bp size (Plate 8 and Table 27). PCR product of the isolate ENDO4e was sent for commercial sequencing (Eurofins Genomics India Pvt Ltd) and the sequence showed 100% sequence similarity with the corresponding partial *fimH* gene sequence of *E. coli* (Fig.1).

4.4.2.2 Detection of *fyuA* gene

Thirty of 53 isolates were found to be positive for the presence of *fyuA* gene which yielded amplicons of the 880 bp size (Plate 9 and Table 27). PCR product of the isolate ENDO9e was sent for commercial sequencing (Eurofins Genomics India Pvt Ltd) and the sequence showed 99.72% sequence similarity with the corresponding partial *fyuA* gene sequence of *E. coli* (Fig.2).

4.4.2.3 Detection of *kpsMTII* gene

Fourteen of the 53 isolates were found to be positive for the presence of *kpsMTII* gene which yielded amplicons of the size corresponding to 272 bp in the DNA ladder (Plate 10 and Table 27). PCR product of the isolate ENDO2e was sent for commercial sequencing (Eurofins Genomics India Pvt Ltd) and the sequence showed 98.89% sequence similarity with the corresponding partial *kpsMTII* gene sequence of *E. coli* (Fig.3).

4.4.2.4 Detection of *csgA* gene

Of the 53 isolates subjected for PCR, 15 isolates were found to be positive for the presence of *csgA* gene yielding amplicons of the 200 bp size (Plate 11 and

Table 27). PCR product of the isolate ENDO7e was sent for commercial sequencing (Eurofins Genomics India Pvt Ltd) and the sequence showed 100% sequence similarity with the corresponding partial *csgA* gene sequence of *E. coli* (Fig.4).

4.4.2.5 Detection of *agn43* gene

Eleven of the 52 isolates were found to be positive for the presence of *agn43* gene which yielded amplicons of the size corresponding to 433 bp in the DNA ladder (Plate 12 and Table 27). PCR product of the isolate ENDO12e was sent for commercial sequencing (Eurofins Genomics India Pvt Ltd) and the sequence showed 100% sequence similarity with the corresponding partial *agn43* gene sequence of *E. coli* (Fig.5).

Table 27. PCR based detection of major virulence genes in *E. coli* isolates (n=53)

Gene targeted	*E. coli* isolates obtained from		
	SE cases (n= 23)	CE cases (n= 26)	Cytology negative cases (n=4)
fimH	23 (100) *	26 (100)	01(25)
fyuA	11 (47.82)	19 (73.07)	0
kpsMTII	4 (17.39)	10 (38.46)	0
csgA	6 (26.08)	9 (34.61)	0
agn43	0	11 (42.30)	0

***Data in the parenthesis indicates percentage**

4.4.3 Identification of *S. aureus* by molecular method

Phenotypically confirmed staphylococcal isolates were subjected to species-specific PCR by targeting the *nuc* gene for genotypic confirmation. All the isolates (n=36) subjected to *nuc* gene-based PCR yielded 181bp amplicon specific for *S. aureus* (Plate 7B).

4.4.4 Detection of *spa* gene in *S. aureus*

PCR targeting the *spa* gene was carried out for all the 36 *S. aureus* isolates. Amplicon size was determined by sequencing of the amplified PCR product of the isolate SA6 (Plate13) and sent for commercial sequencing (Eurofins Genomics India Pvt Ltd). Comparison of edited nucleotide sequence of the isolate with deposited sequences of NCBI showed 99.73% similarity in the sequence corresponding to partial *spa* gene sequence of *S. aureus* (Fig.6). *Spa* type of the sequenced *S. aureus* isolate (SA6) was determined to be t359.

All the isolates of *S. aureus* (n=36) were positive for *spa* gene and yielded amplicons of size ranging from ~280 bp to ~350 bp.

4.5 Antibiogram profile of the bacterial isolates

4.5.1 Disc diffusion method

Disc diffusion method of antimicrobial susceptibility test was performed for all the 135 bacterial isolates obtained from SE and CE and cytological negative samples as mentioned in the section 3.6.1. The isolates were further categorized either sensitive or as resistant based on the CLSI guidelines (CLSI, 2019) (Plate 15B and Table 28).

E. coli isolates showed highest sensitivity to ciprofloxacin (91.83%) followed by ceftriaxone (87.75%) and chloramphenicol (81.63%) whereas showed resistance to penicillin (93.87%), polymyxin B (79.59%) ampicillin (77.55%) and nitrofurantoin (75.51%) respectively (Fig.8).

S. aureus isolates were found sensitive to tylosin (97.22 %), followed by nitrofurantoin (94.44 %), ciprofloxacin (91.66%), co-trimoxazole (88.88%) and

resistant to penicillin (83.33%), gentamicin (83.33%) and chloramphenicol (75%) respectively (Fig.9).

R. equi isolates exhibited highest sensitivity to gentamicin (100%) and ciprofloxacin (100%) followed by enrofloxacin (75%) and co-trimoxazole (75%), whereas showed resistance to penicillin (100%), polymyxin B (100%), nitrofurantoin (100%) and AMS30/15(100%).

All the isolates of *P. aeruginosa* were found sensitive (100%) to ciprofloxacin, cefotaxime, cefaperazone and similarly, all were resistant (100%) to penicillin, nitrofurantoin, streptomycin and polymyxin B.

Proteus mirabilis isolates were found sensitive to ciprofloxacin (100%), co-trimoxazole (66.66%) and enrofloxacin (66.66%). Maximum resistance of 100% was recorded for most of the antibiotics used.

K. pneumoniae isolates exhibited sensitivity to ciprofloxacin (85.71%), gentamicin (85.71%) and ceftriaxone (71.42%) whereas resistance to penicillin (71.42%), nitrofurantoin (85.71%), ampicillin, (85.71%) and AMS30/15 (85.71%).

S. uberis isolates exhibited highest sensitivity to nitrofurantoin (85.7%) and highest resistance to oxytetracycline (100%). As interpretative criteria with regard to break point value specific for bacterial species was not available for some of the antibacterial agents used in the study, zone of inhibition could not be interpreted for such antibaterials viz., ciprofloxacin, gentamicin, streptomycin, amikacin, enrofloxacin, ampicillin, polymyxin B, cefotaxime, cefaperazone, amox-clav 50/10, cefazolin, tylosin.

All the bacterial isolates recovered in the study were tested for antimicrobial susceptibility using agents listed in Table 17. Although the zone of inhibitions was recorded, the result of *Corynebacterium bovis* and *Bacillus cereus* isolates could not be interpreted as most sensitive or most resistant owing to unavailability of interpretation criteria during the period of study.

4.5.2 Detection of phenotypic methicillin resistance

Of the 18 *S. aureus* isolates obtained from SE cases, none of the isolates were resistant to cefoxitin and of the 18 *S. aureus* isolates obtained from CE cases, two isolates were resistant to cefoxitin (Plate 15A).

4.5.3 Detection of *mecA* gene in *S. aureus*

All the isolates of *S. aureus* were further subjected to PCR targeting the *mecA* gene. Of these, one isolate (SA15) was found to be positive for the presence of the *mecA* gene which yielded amplicon of the size corresponding to 210 bp in the DNA ladder (Plate 14A). PCR product of this isolate (SA15) was sent for commercial sequencing (Eurofins Genomics India Pvt Ltd). Comparison of edited nucleotide sequence with deposited sequences of NCBI using BLAST tool showed 100% sequence similarity with the corresponding partial *mecA* gene nucleotide sequence of *S. aureus* (Fig.7).

Table 28 a. Antimicrobial susceptibility pattern of the bacterial isolates

Bacteria		Ciprofloxacin	Co-trimoxazole	Ceftriaxone	Gentamicin	Penicillin	Streptomycin	Amikacin	Enrofloxacin	Ampicillin	Polymyxin B	Cefotaxime	Chloramphenicol	Cefaperazone	AmoxClav 50/10	Oxytetracycline	Cefazolin	AMS30/15	Nitrofurantoin	Tylosin
Escherichia coli	S	91.8	77.5	87.7	71.4	6.1	22.4	26.5	79.5	22.4	20.4	24.4	81.6	69.3	26.5	36.7	79.5	30.6	24.4	-
	R	8.1	22.4	12.2	28.5	93.8	77.5	73.4	20.4	77.5	79.5	75.5	18.3	30.6	73.4	63.2	20.4	69.3	75.5	-
Staphylococcus aureus	S	91.6	88.8	72.2	55.5	16.6	47.2	44.4	83.3	30.5	33.3	33.3	25	63.8	47.2	50	52.7	41.6	94.4	97.2
	R	8.3	11.1	27.7	44.4	83.3	52.7	55.5	16.6	69.4	66.6	66.6	75	36.1	52.7	50	47.2	58.3	5.5	2.7
Klebsiella pneumoniae	S	85.7	42.8	71.4	85.7	28.5	42.8	42.8	71.4	14.2	28.5	57.1	71.4	42.8	42.8	28.5	71.4	14.2	14.2	-
	R	14.2	57.1	28.5	14.2	71.4	57.1	57.1	28.5	85.7	71.4	42.8	28.5	57.1	57.1	71.4	28.5	85.7	85.7	-
Rhodococcus equi	S	100	75	50	100	0	25	50	75	25	0	50	25	75	50	25	75	0	0	100
	R	0	25	50	0	100	75	50	25	75	100	50	75	25	50	75	25	100	100	0

Data in the table are in percentage; S- Sensitive; R- Resistant; Grey cells- Interpretation criteria unavailable

Table 28 b. Antimicrobial susceptibility pattern of the bacterial isolates

Bacteria		Ciprofloxacin	Co-trimoxazole	Ceftriaxone	Gentamicin	Penicillin	Streptomycin	Amikacin	Enrofloxacin	Ampicillin	Polymyxin B	Cefotaxime	Chloramphenicol	Cefaperazone	AmoxClav 50/10	Oxytetracycline	Cefazolin	AMS30/15	Nitrofurantoin	Tylosin
Proteus mirabilis	S	100	66.6	33.3	33.3	0	33.3	0	66.6	33.3	0	33.3	66.6	66.6	33.3	0	66.6	0	0	-
	R	0	33.3	66.6	66.6	100	66.6	100	33.3	66.6	100	66.6	33.3	33.3	66.6	100	33.3	100	100	-
Pseudomonas aeruginosa	S	100	50	100	50	0	0	100	50	50	0	100	0	100	50	100	50	50	0	-
	R	0	50	0	50	100	100	0	50	50	100	0	100	0	50	0	50	50	100	-
Streptococcus uberis	S	-	57.1	35.7	-	28.5	-	-	-	-	-	-	14.2	-	-	0	-	50	85.7	-
	R	-	42.8	64.2	-	71.4	-	-	-	-	-	-	85.7	-	-	100	-	50	14.2	-

Data in the table are in percentage; S- Sensitive; R- Resistant; Grey cells- Interpretation criteria unavailable

4.6 Determination of minimum inhibitory concentration

4.6.1 Determination of MIC of ceftiofur

Determination of MIC of ceftiofur for bacterial isolates was done by following broth dilution method (Plate 15C and Table 29). Minimum inhibitory concentration of *E. coli, S. aureus*, *S. uberis* isolates ranged between 0.25-1, 0.25-2, 0.125-0.25 µg/mL respectively. MIC values of ceftiofur in *R. equi*, *C. bovis*, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae,* B. *cereus* was 0.25, 8, 16, 16, 2 and 16 µg/mL respectively and there was no inhibition found for 6.6% of the *E. coli* isolates and 50% of *P. aeruginosa* isolates even at 256 µg/mL.

4.6.2 Determination of MIC of ciprofloxacin

The results of the antimicrobial susceptibility assay by disc diffusion method indicated the highest sensitivity to ciprofloxacin and hence, MIC was determined to ciprofloxacin by following Ezy MIC strip method. Minimum inhibitory concentration of ciprofloxacin in *E. coli* was 0.032 µg/mL, 0.125 µg/mL. MIC of *S. aureus* were 0.064 µg/mL, 0.125 µg/mL. MIC of *S. uberis* isolates were 0.38 µg/mL. MIC values for *Rhodococcus equi, Corynebcterium bovis, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Bacillus cereus* were 0.032 µg/mL, 0.75 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 0.5 µg/mL and 0.19 µg/mL respectively (Table 30 and Plate 15D).

4.6.3 Determination of MIC of gentamicin

As gentamicin was being most commonly used drug for treatment of endometritis in repeat breeding cases, determination of MIC was done to gentamicin by following Ezy MIC strip method.

Minimum inhibitory concentration range of gentamicin in *E. coli* was 0.01 µg/mL to 1 µg/mL. MIC of *S. aureus* isolates were 0.001 µg/mL and 1 µg/mL. MIC of *S. uberis* isolates were 0.01 µg/mL. MIC values for *Rhodococcus equi, Corynebcterium bovis, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae* and *Bacillus cereus* were 0.1 µg/mL, 2 µg/mL, 0.1 µg/mL, 0.1 µg/mL, 10 µg/mL and 0.1 µg/mL respectively. Results of gentamicin MIC are shown in Table 31 (Plate 15D).

Table 29. MIC values of ceftiofur

Bacteria (n=number of isolates)	Antimicrobial concentration(µg/ml)																
	0.008	0.015	0.03	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	>256
E. coli (30)						9 (30*)	11 (36.66)	8 (26.66)									2 (6.6)
S. aureus (30)						11 (36.66)	8 (26.66)	9 (30)	2 (6.6)								
S. uberis (5)					2 (40)	3 (60)											
R. equi (2)						2 (100)											
C. bovis (2)					1 (50)						1 (50)						
P. aeruginosa (2)												1 (50)					1 (50)
P. mirabilis (2)												1 (50)					1 (50)
K. pneumoniae (2)									2 (100)								
Bacillus cereus (2)												1 (50)					1 (50)

	Sensitive		Resistant		Interpretation criteria unavailable	*	Data in the parenthesis indicates percentage

Table 30. MIC values of ciprofloxacin

Bacteria (n=number of isolates)	Antimicrobial concentration (µg/ml)															
	0.016	**0.023**	**0.032**	**0.047**	**0.064**	**0.125**	**0.19**	**0.25**	**0.38**	**0.50**	**0.75**	**1**	**1.5**	**2**	**3**	**4**
E. coli **(12)**			04 (33.33*)			06 (50)								02 (16.66)		
Staphylococcus aureus (12)					02 (16.66)	06 (50)								02 (16.66)		
Streptococcus uberis (4)									02 (50)							02 (50)
Rhodococcus equi (2)			02 (100)													
Corynebcterium bovis (2)											01 (50)			01 (50)		
Pseudomonas aeruginosa (2)								02 (100)								
Proteus mirabilis (2)										**02 (100)**						
Klebsiella pneumoniae (2)										**01 (50)**			**01 (50)**			
Bacillus cereus (2)							01 (50)							01 (50)		

	Sensitive		Resistant		Interpretation criteria unavailable	*	Data in the parenthesis indicates percentage

Table 31. MIC values of gentamicin

Bacteria (n=number of isolates)	Antimicrobial concentration (µg/mL)												
	0.001	**0.01**	**0.10**	**0.25**	**0.5**	**1**	**2**	**5**	**10**	**30**	**60**	**120**	**240**
E. coli (10)		04 (40*)	03 (30)			02 (20)		01 (10)					
Staphylococcus aureus (10)	03 (30)	04 (40)				01 (10)	02 (20)						
Streptococcus uberis (03)		02 (66.66)					01 (33.33)						
Rhodococcus equi (01)			01										
Corynebcterium bovis (01)							01						
Pseudomonas aeroginosa (01)			01										
Proteus mirabilis (01)			01										
Klebsiella pneumoniae (01)									01				
Bacillus cereus (01)			01										

	Sensitive		Resistant		Interpretation criteria unavailable	*	Data in the parenthesis indicates percentage

PLATE 2. Phenotypic identification of *E. coli* and *K. pneumoniae*

A. *E. coli* growth on MacConkey agar showing characteristic lactose fermentation

B. *E. coli* growth on EMB agar showing metallic sheen

C. IMVC, urease and TSI test of *E. coli*

D.*K. pneumoniae* growth on MacConkey agar showing characteristic lactose fermentation and mucoid colony

E. IMVC, urease and TSI test of *K. pneumoniae*

F. Esculin and sugar fermentation tests of *K. pneumoniae*

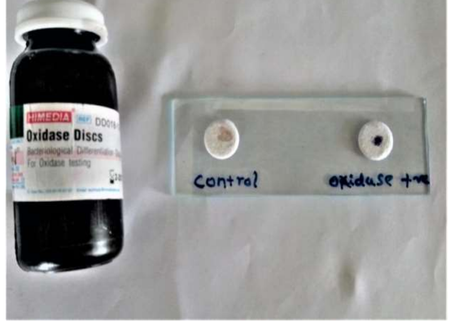
PLATE 3. Phenotypic identification of *Proteus mirabilis* and *Pseudomonas aeruginosa*

A. *Proteus mirabilis* growth on MacConkey agar showing no lactose fermentation

B. *Proteus mirabilis* showing swarming growth on BHI

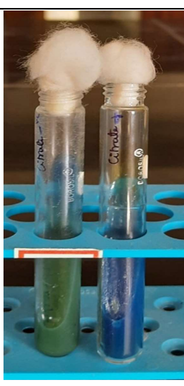
C. IMVC, urease and TSI test of *Proteus mirabilis*

D. *Pseudomonas aeruginosa* growth on MacConkey agar showing no lactose fermentation



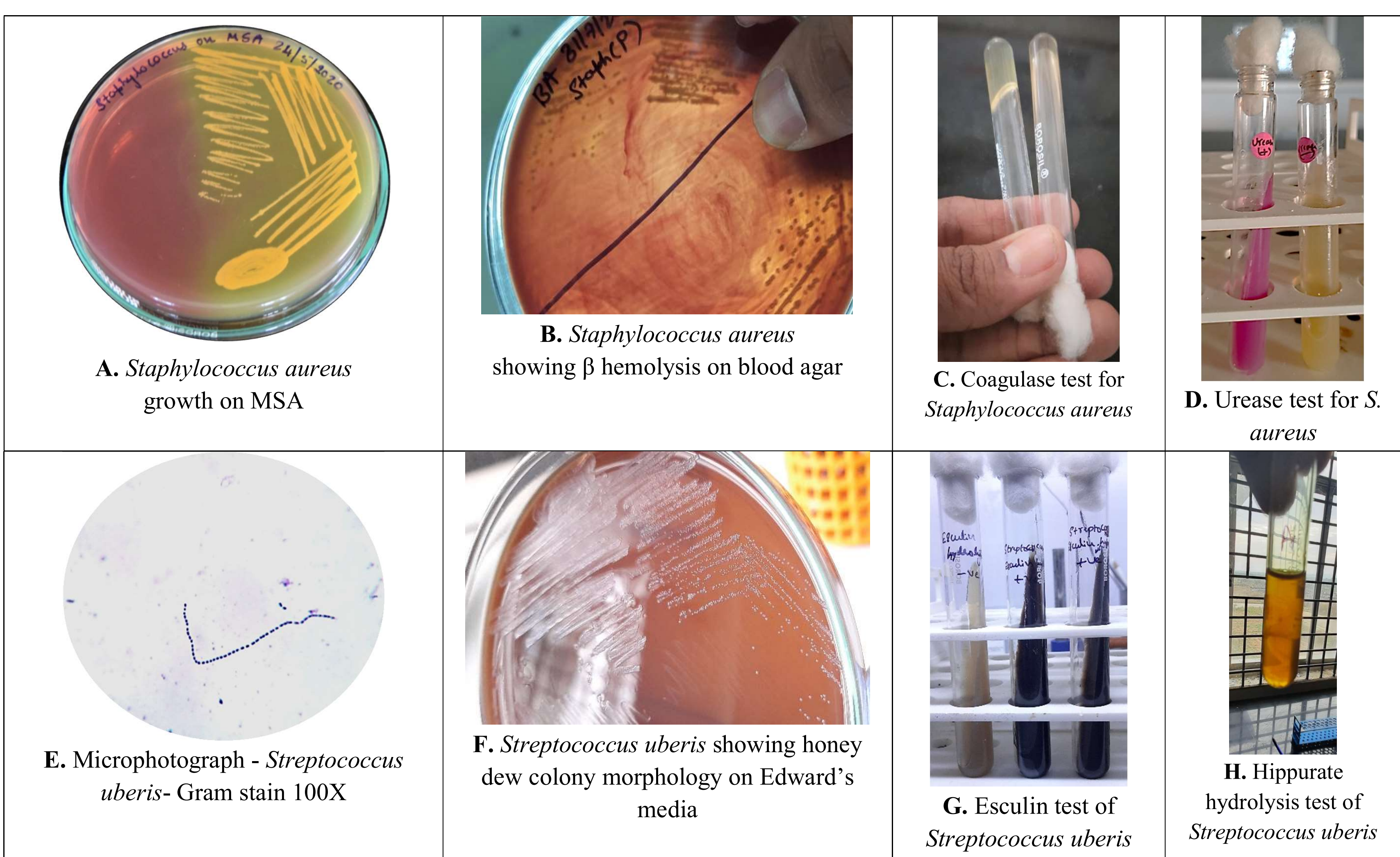
E. Oxidase test in *Pseudomonas aeruginosa*

F. Pyocyanin Pigment production in *Pseudomonas aeruginosa*



G. Citrate test in *Pseudomonas aeruginosa*

PLATE 4. Phenotypic identification of *Staphylococcus aureus* and *Streptococcus uberis*



A. *Staphylococcus aureus* growth on MSA

B. *Staphylococcus aureus* showing β hemolysis on blood agar

C. Coagulase test for *Staphylococcus aureus*

D. Urease test for *S. aureus*

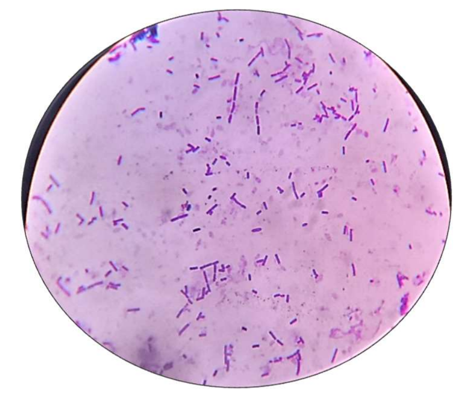
E. Microphotograph - *Streptococcus uberis*- Gram stain 100X

F. *Streptococcus uberis* showing honey dew colony morphology on Edward's media

G. Esculin test of *Streptococcus uberis*

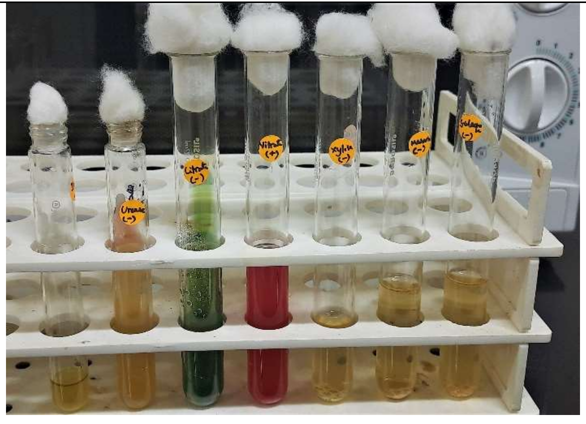
H. Hippurate hydrolysis test of *Streptococcus uberis*

PLATE 5. Phenotypic identification of *Bacillus cereus* and *Corynebacterium bovis*

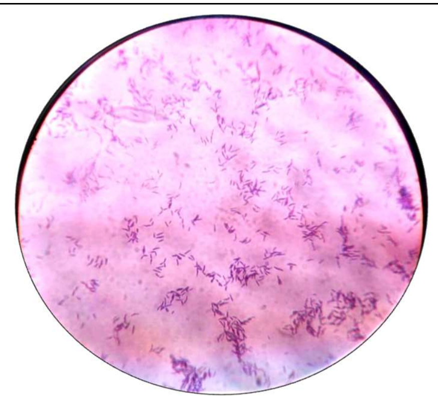


A. Microphotograph- *Bacillus cereus* Gram stain 100X

B. *Bacillus cereus* growth on egg yolk agar



C. Indole, urease, citrate and sugar fermentation tests of *Bacillus cereus*

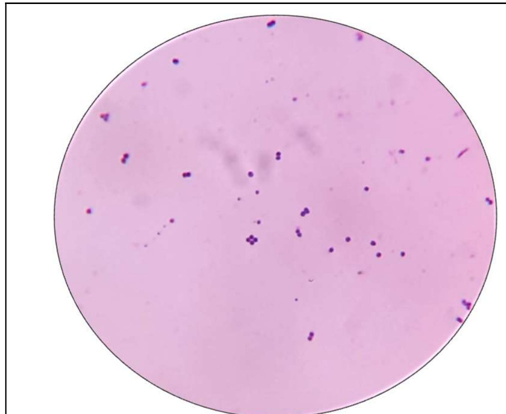


D. Microphotograph- *Corynebacterium bovis* Gram stain 100X

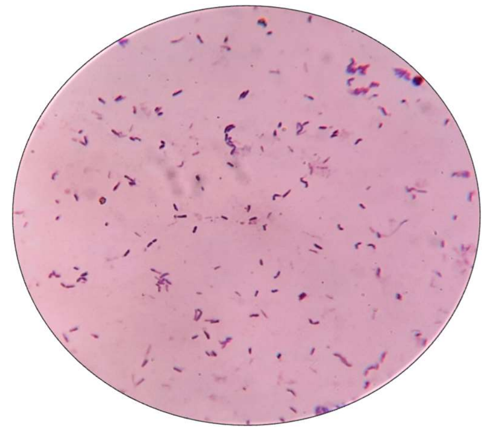
E. *Corynebacterium bovis* Growth on potassium tellurite agar

F. Urease, esculin, nitrate and sugar fermentation test of *C.bovis*

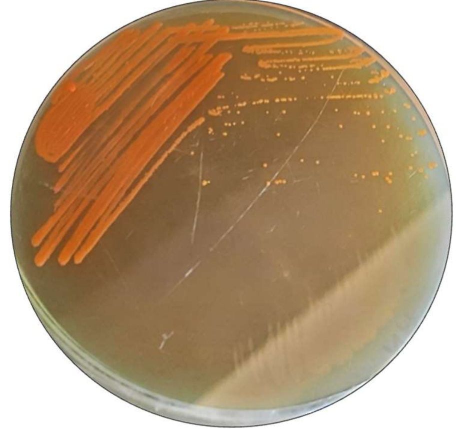
PLATE 6. Phenotypic identification of *Rhodococcus equi*



A. Microphotograph- *Rhodococcus equi* Gram stain 100X (broth culture-cocci)

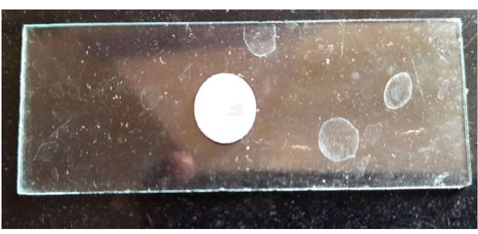


B. Microphotograph- *Rhodococcus equi* Gram stain 100X (agar culture-pleomorphic)



C. *Rhodococcus equi* growth on BHI

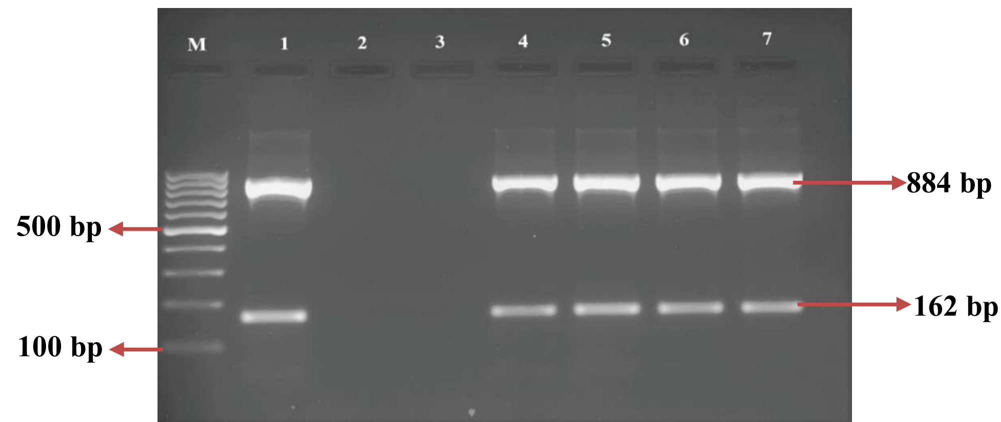
D. Catalase test for *Rhodococcus equi*



E. Oxidase test for *Rhodococcus equi*

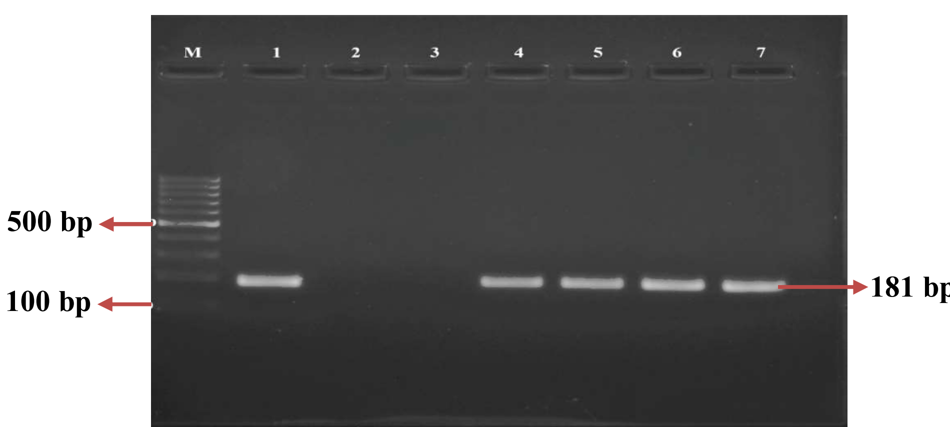
F. Urease, esculin, nitrate and sugar fermentation test for *Rhodococcus equi*

PLATE 7A. Partial amplification of *uid*A and *usp*A genes of *E. coli* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*E. coli*)
Lane 2: Negative control (*S. aureus*)
Lane 3: No template control
Lane 4 – 7: Test samples showing partially amplified product of *uid*A (162 bp) *and usp*A (884 bp) gene of *E. coli* isolates

PLATE 7B. Partial amplification of *nuc* gene of *S. aureus* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*S. aureus*)
Lane 2: Negative control (*E. coli*)
Lane 3: No template control
Lane 4 – 7: Test samples showing partially amplified product of *nuc* (181bp) of *S. aureus* isolates

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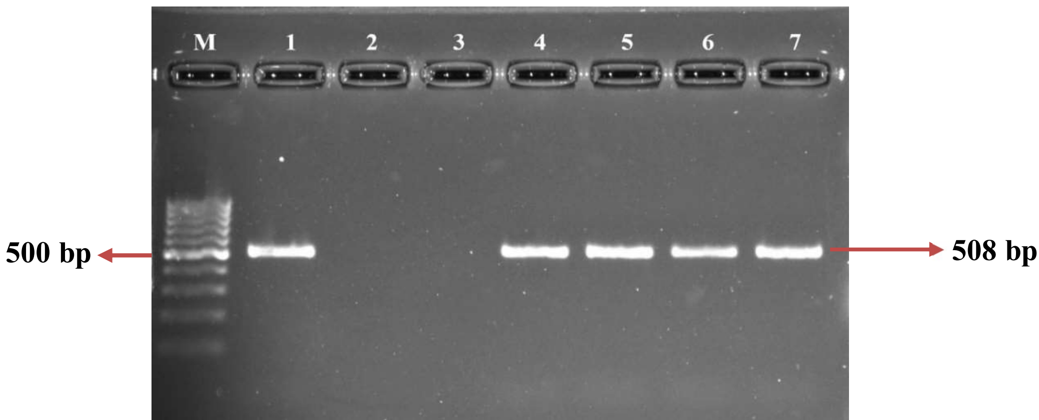
PLATE 8. Partial amplification of *fimH* gene of *E. coli* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*E. coli*)
Lane 2: Negative control (*S. aureus*)
Lane 3: No template control
Lane 4 – 7: Test samples showing partially amplified product of *fimH* (508 bp) gene of *E. coli* isolates

Fig 1. Chromatogram of *fimH* gene obtained by sequencing with forward primer

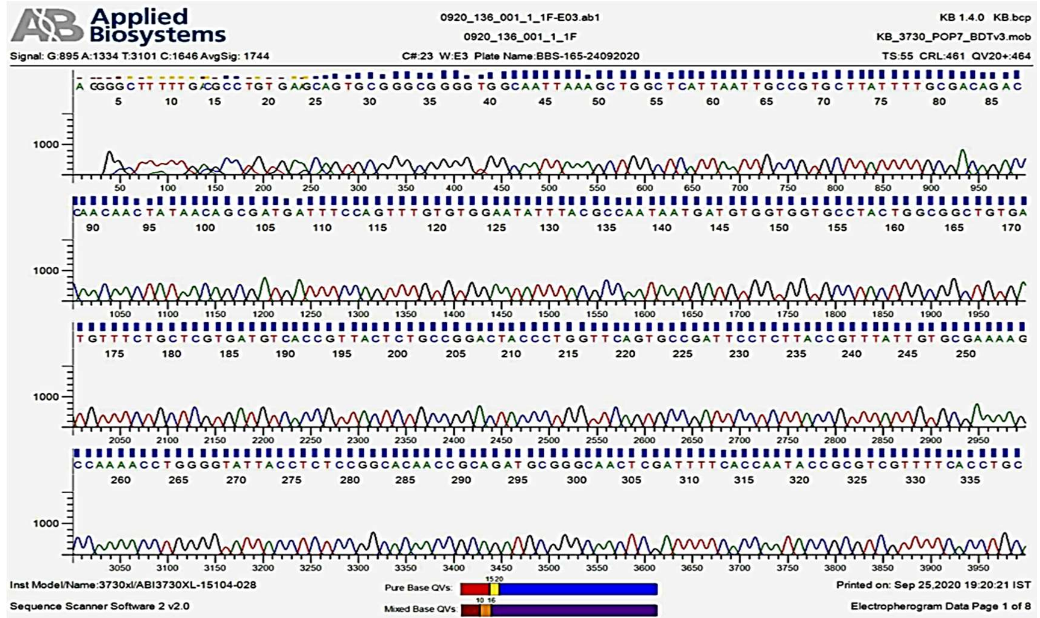
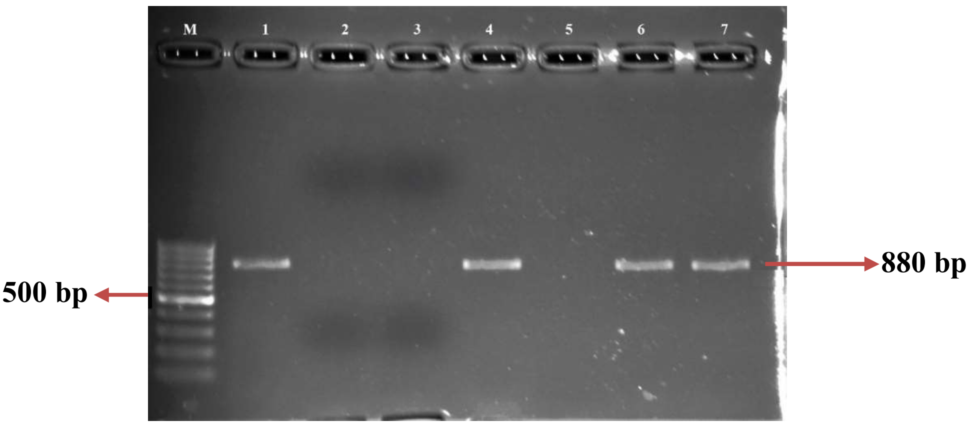


PLATE 9. Partial amplification of *fyuA* gene of *E. coli* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*E. coli*)
Lane 2: Negative control (*S. aureus*)
Lane 3: No template control
Lane 4, 6 &7: Test samples showing partially amplified product of *fyu*A (880 bp) gene of *E. coli* isolates
Lane 5: Test sample negative for *fyu*A (880 bp) gene

Fig 2. Chromatogram of *fyuA* gene obtained by sequencing with forward primer

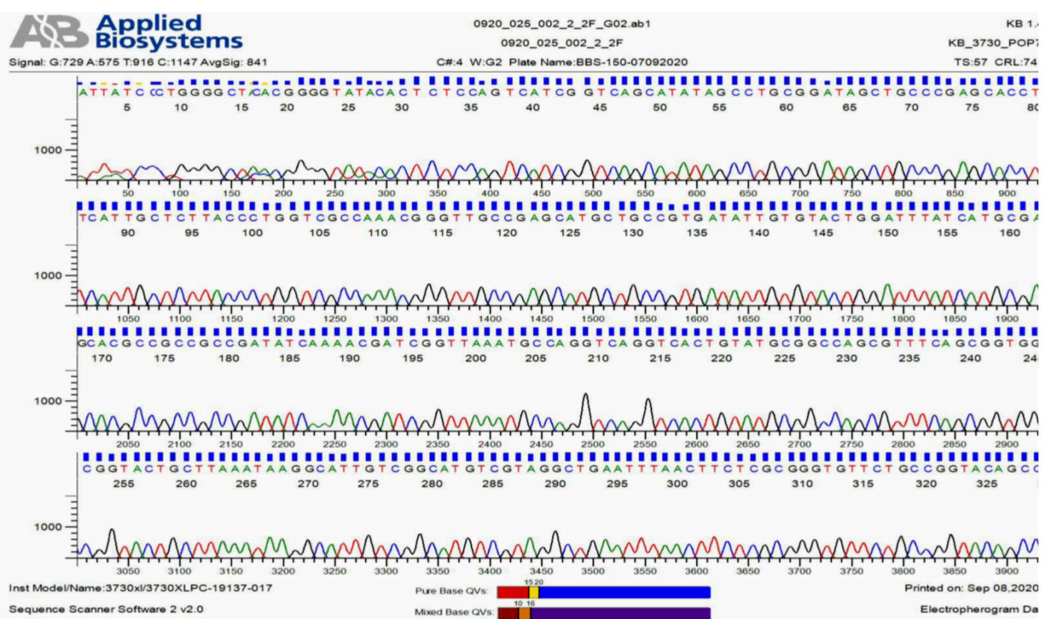
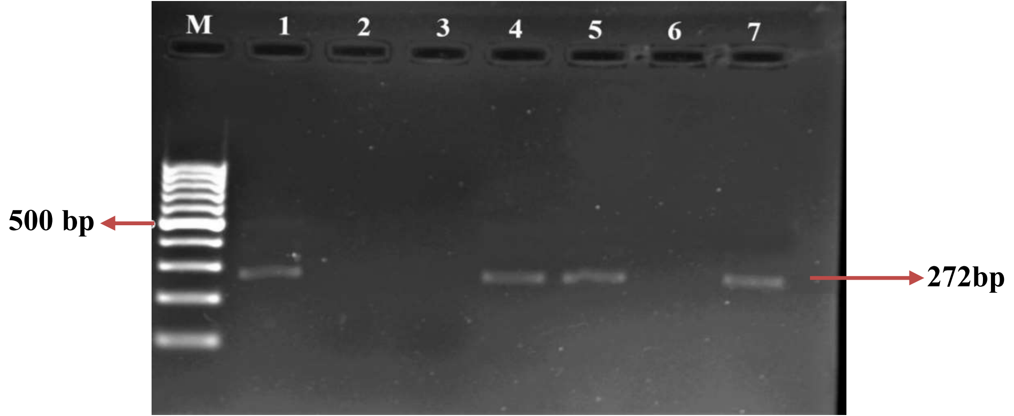


PLATE 10. Partial amplification of *kpsMTII* gene of *E. coli* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*E. coli*)
Lane 2: Negative control (*S. aureus*)
Lane 3: No template control
Lane 4, 5 & 7: Test samples showing partially amplified product of *kpsMT* II (272bp) gene of *E. coli* isolates
Lane 6: Test sample negative for *kpsMT* II gene

Fig 3. Chromatogram of *kpsMT II* gene obtained by sequencing with forward primer

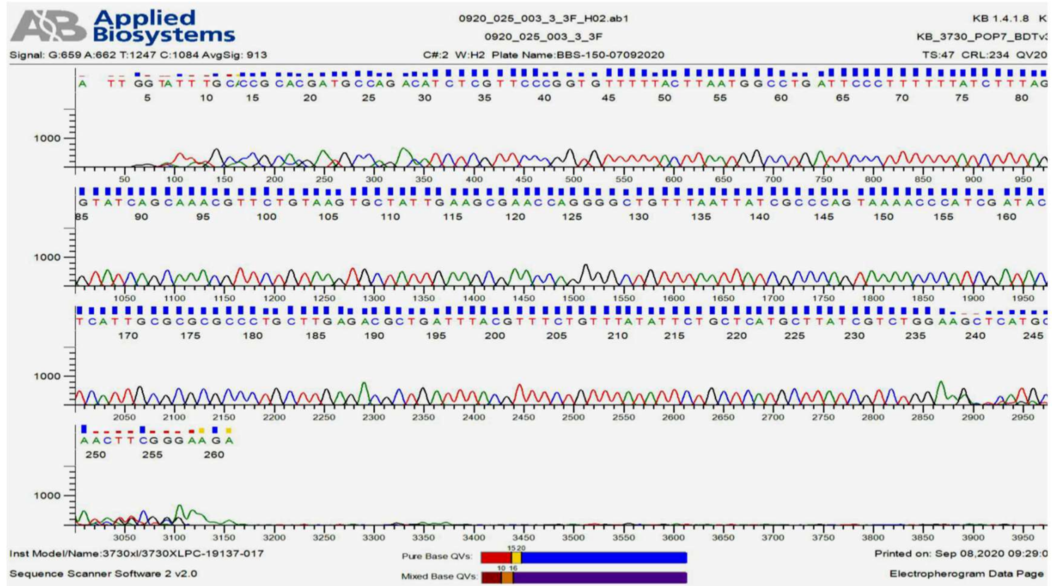
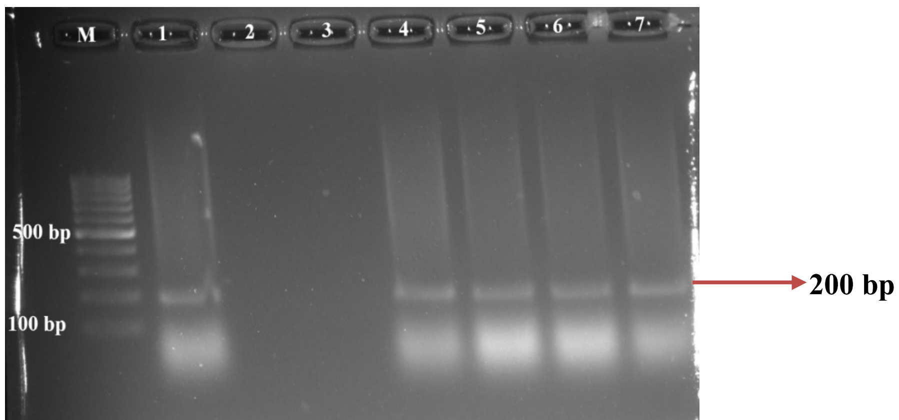


PLATE 11. Partial amplification of *csgA* gene of *E. coli* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*E. coli*)
Lane 2: Negative control (*S. aureus*)
Lane 3: No template control
Lane 4- 7: Test samples showing partially amplified product of *csgA* (200 bp) gene of *E. coli* isolates

Fig 4. Chromatogram of *csgA* gene obtained by sequencing with forward primer

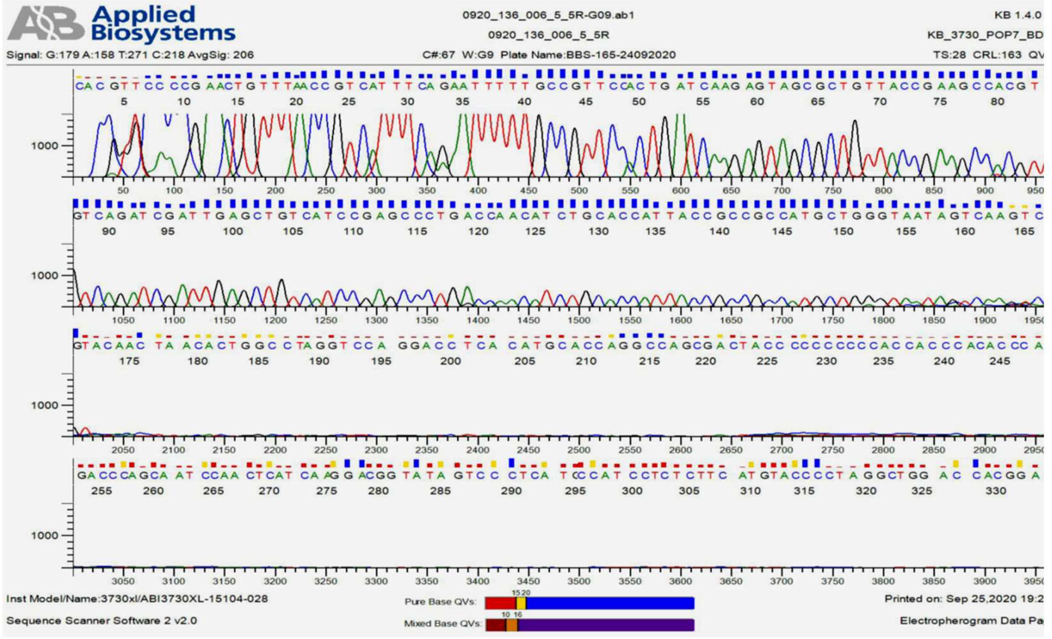
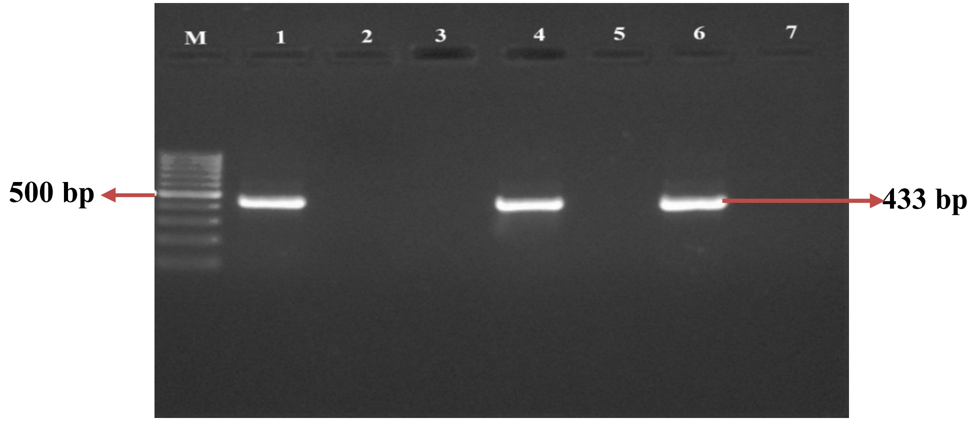


PLATE 12. Partial amplification of *agn43* gene of *E. coli* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*E. coli*)
Lane 2: Negative control (*S. aureus*)
Lane 3: No template control
Lane 4 and 6: Test samples showing partially amplified product of *agn43* (433 bp) gene of *E. coli* isolates
Lane 5 and 7: Test sample negative for *agn43* gene

Fig.5. Chromatogram of *agn43* gene obtained by sequencing with forward primer

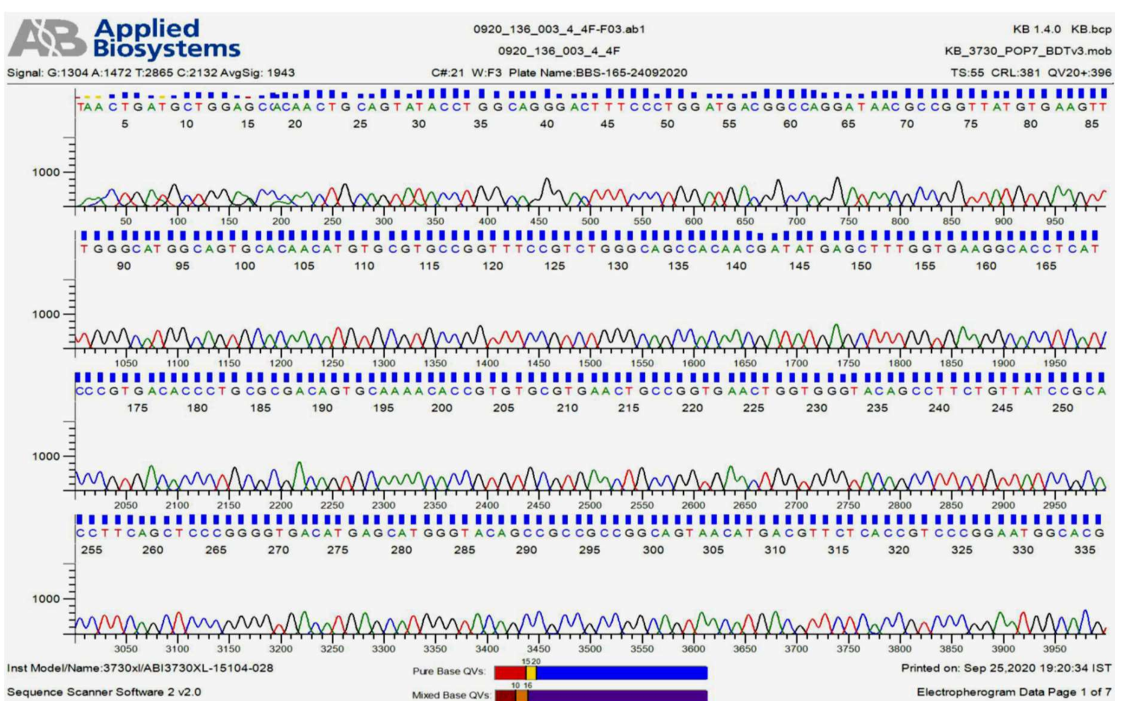
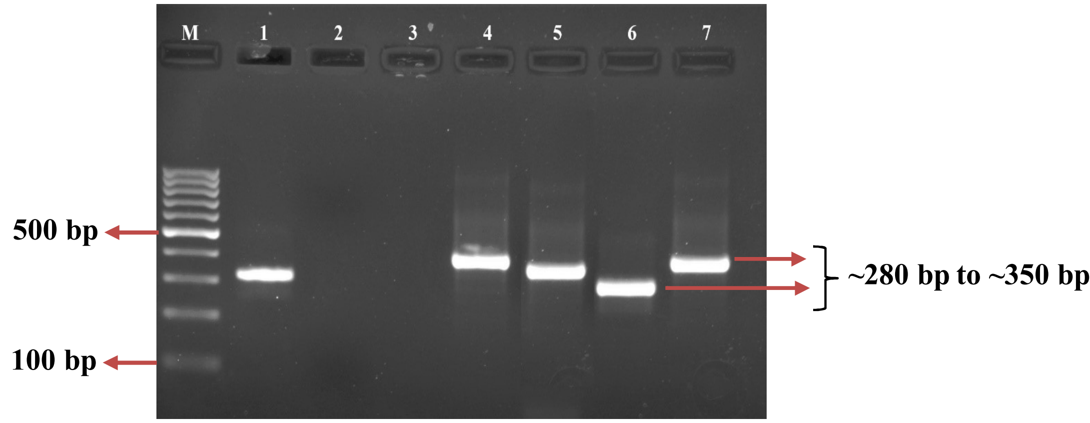


PLATE 13. PCR based detection of *spa* gene (virulence gene) of *S. aureus*



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*S. aureus*)
Lane 2: Negative control (*E. coli*)
Lane 3: No template control
Lane 4 – 7: Test samples showing partially amplified product of ***spa***
(size ranging from ~280 bp to ~350 bp) of *S. aureus* isolates

Fig 6. Chromatogram of *spa* gene obtained by sequencing with forward primer

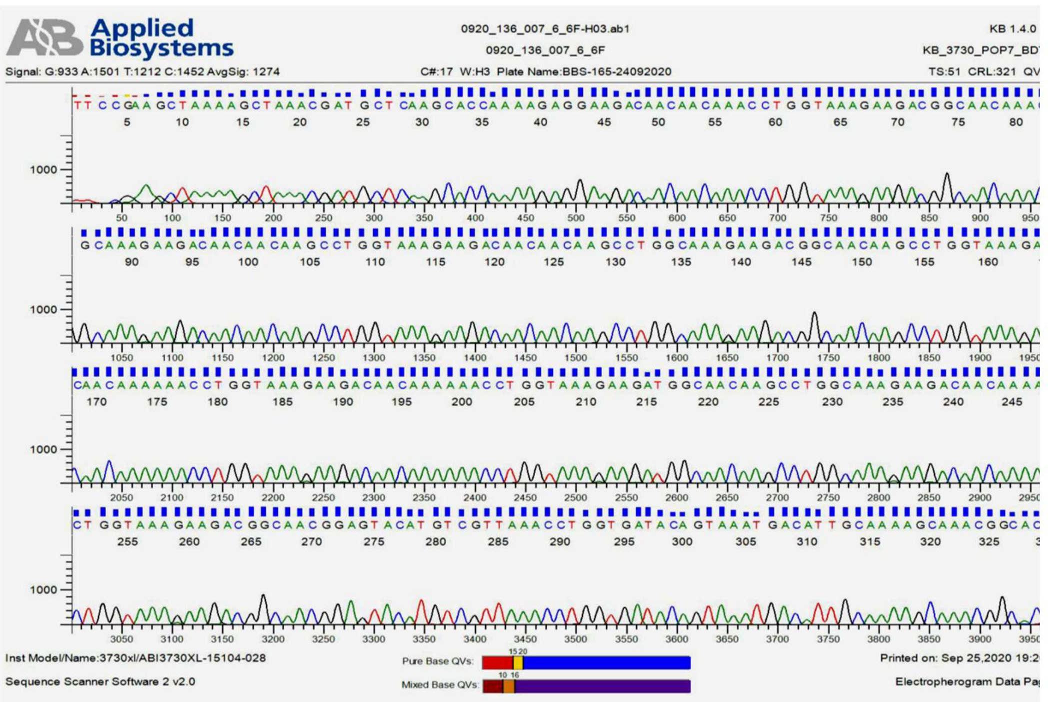
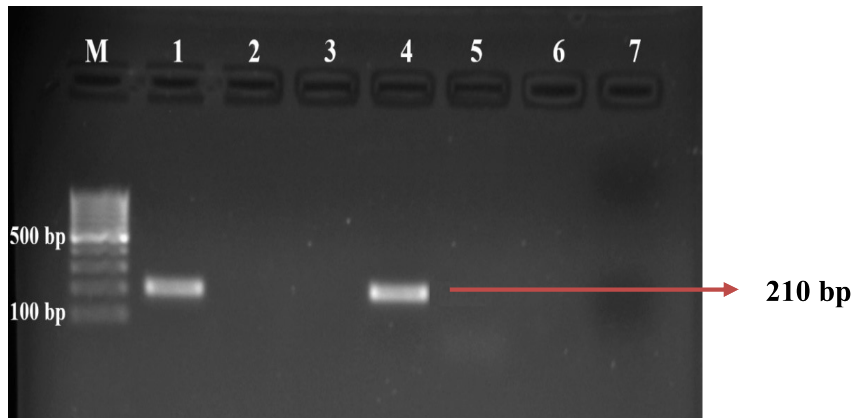


PLATE 14. Partial amplification of *mecA* gene of *S. aureus* by PCR



Lane M: 100 bp DNA ladder
Lane 1: Positive control (*S. aureus*)
Lane 2: Negative control (*E. coli*)
Lane 3: No template control
Lane 4: Test sample showing partially amplified product of *mecA* (210 bp) gene of *S. aureus* isolates
Lane 5 to 7: Test sample negative for *mecA* gene

Fig.7. Chromatogram of *mecA* gene obtained by sequencing with reverse primer

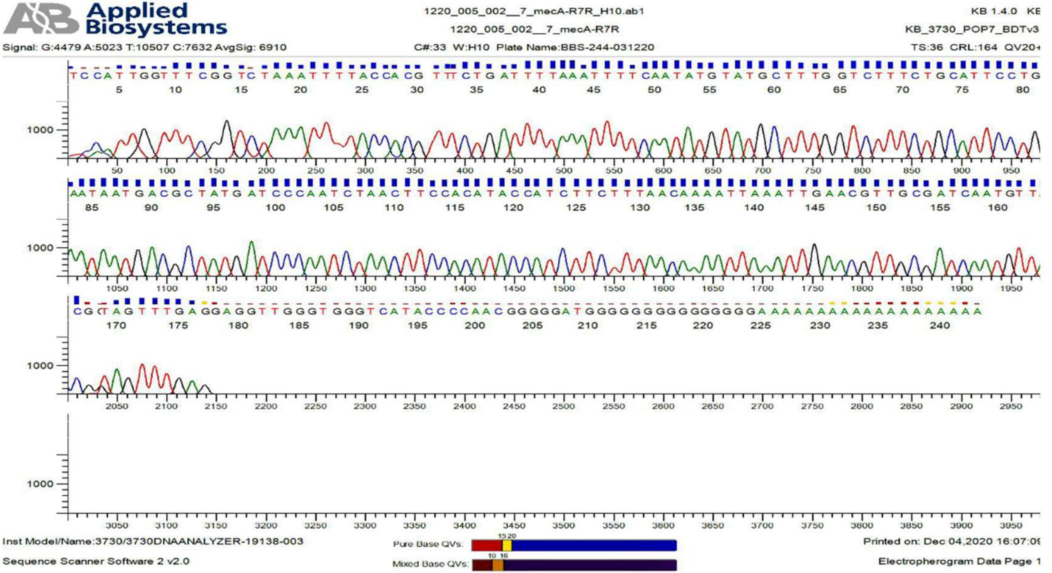
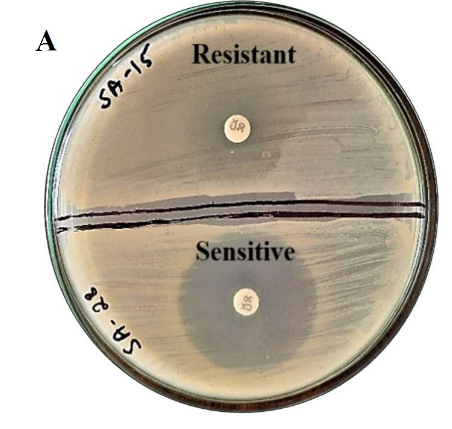
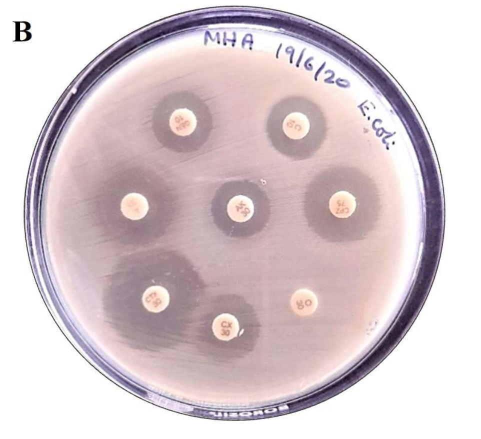


PLATE 15. Antibiogram Assay

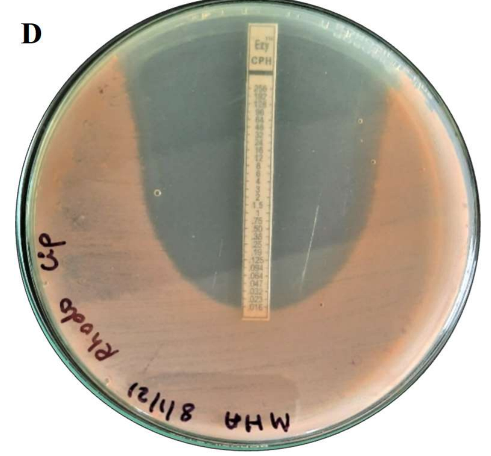


Phenotypic methicillin resistance and susceptibility of *S. aureus* isolates

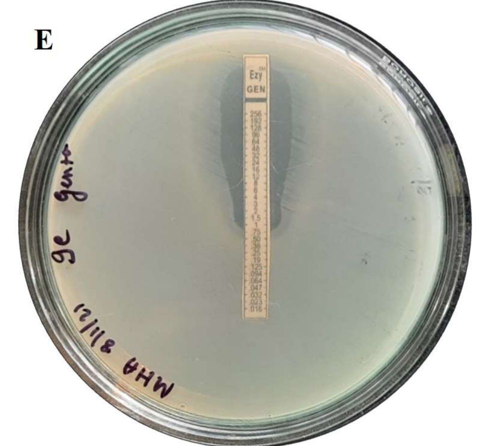


Representative ABST plate of *E. coli* (ENDO32e) showing sensitivity and resistant pattern to various antibacterial agents

C. Representative MIC of ceftiofur against *Corynebacterium bovis* (Cy-2) by broth dilution method with controls



Representative Ezy strip plate of *R. equi* (R-4) showing MIC pattern to ciprofloxacin strip



Representative Ezy strip plate of *E. coli* (9e) showing MIC pattern to gentamicin strip

Fig. 8. Antimicrobial susceptibility pattern of *E. coli* isolates

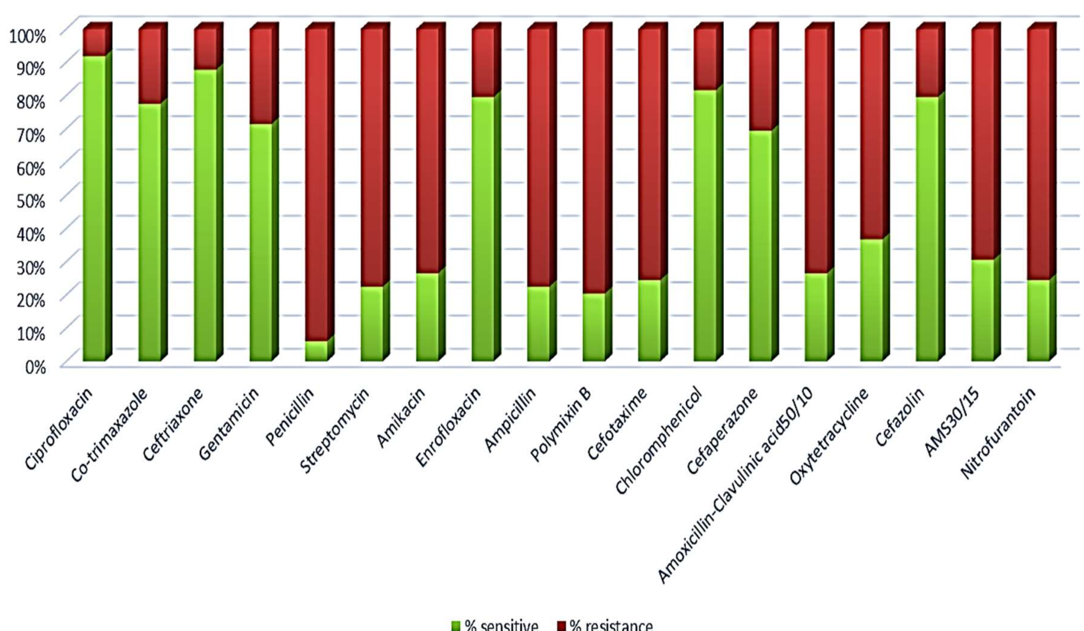
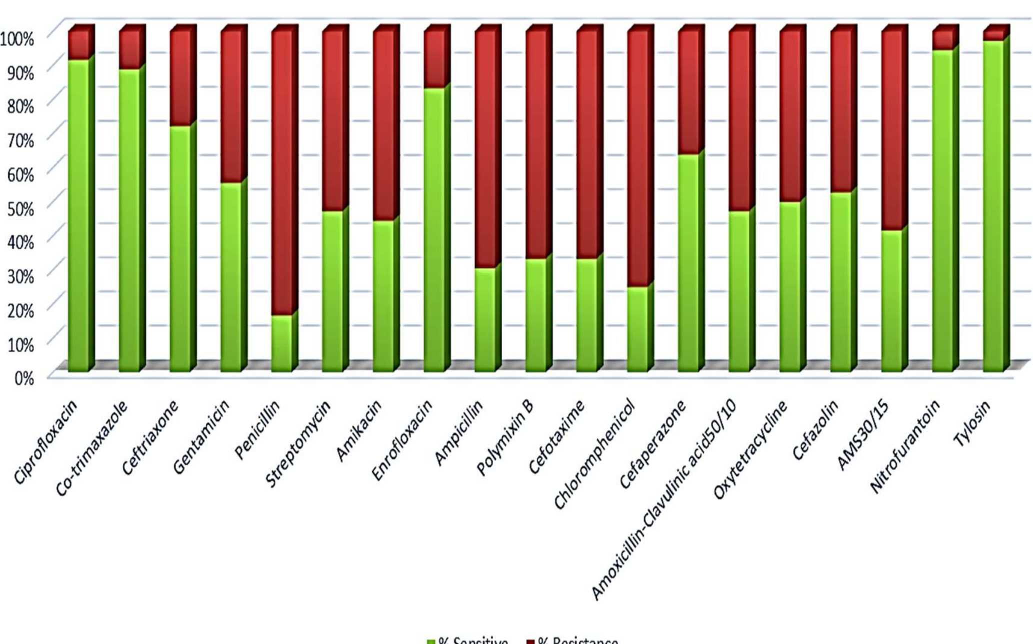


Fig. 9. Antimicrobial susceptibility pattern of *S. aureus* isolates



Discussion

V. DISCUSSION

Uterine inflammatory processes in dairy cows may persist for enduring time periods, triggering a detrimental effect on further reproductive capacity (Gilbert *et al*., 2005; Salasel *et al*., 2010; Gilbert, 2011). Subclinical endometritis refers to cows with no clinical signs of endometritis but having an increased percentage of polymorphonuclear cells (PMNs) in endometrial cytology not only affects milk yield but also decreases reproductive performance of animal (Kasimanickam *et al*., 2004). Mere presence of a mucopurulent discharge in the vagina concludes CE (Purohit *et al*., 2013). Endometritis also has detrimental effect not only on economy of milk production in dairy animals but also in terms of increased culling rate and to a lesser extent, the cost of treatment (Galvão, 2012).

5.1 Cytological evaluation of uterine lavages for detection of SE

The endometrial cytology was reported to be more reliable method than uterine biopsy or bacteriology for diagnosis of sub-clinical endometritis as the uterine biopsy outputs are influenced by the estrous cycle whereas there is no significant change in estrous cycle in superficial endometrial layer (Madoz *et al*., 2013). Hence measuring the proportion of PMNs in endometrial cytology is the hallmark of SE diagnosis. The influx of neutrophils from the peripheral circulation into the endometrium and uterine lumen is due to compromise in host immune system and microbial population (Vinita *et al*., 2018).

The proportion of PMN cells in the total number of endometrial cells in uterine lavage is indicative for SE. Different thresholds of PMN for defining SE have been used, ranging from 5% to 18% (Kasimanickam *et al*., 2004). Salasel *et al*. (2010) have used a threshold of 3%. In the present study a threshold value of 1-5% range of PMN cells was considered to indicate SE by cytology in repeat breeding cows. As suggested

by Pothmann *et al*. (2015). Of 82 samples processed for cytology in the present study 63 samples revealed proportions of PMN cells in the range of 1-5% in the uterine lavage which indicated 76.83% positivity for SE. Pothmann *et al*., (2015) reported 48.2% of the total samples to be positive for SE in their study. The fact should be noted that the animals were not with similar days open, from which the samples were collected in the present study.

5.2 Bacterial isolation from endometritis

Bacterial isolation has been reported from uterine secretions of cows suffering from endometritis by Barman *et al*. (2013).

In present study, 70.68% of the SE samples yielded as single etiological agent and 29.31% of the samples showed mixed infection. Whereas in CE, 52.94% of the samples yielded single isolate and 47.05% samples yielded more than one isolate.

The findings were in close accordance with the findings of Bhat *et al*. (2013), in which single organism was isolated in 76.67% samples, whereas mixed infections were observed in 23.33% samples (n=60) of repeat breeding cows with SE.

In another study conducted by Chandrakar *et al*. (2002), where 70% single isolates and 30% mixed isolates were recovered. Similarly, Mane *et al*. (2009) reported 80% single and 20% mixed isolates in their bacterial spectrum and antibiogram study in repeat breeding cows.

Contrary to the results of the present study, Behera *et al*. (2015) where reported the recovery of single organism only 31.15 % of the samples, whereas mixed infections were observed in 68.85 % samples (n=61) of repeat breeding cows.

In another study on CE Raval *et al*. (2018) recovered 63.3% mixed isolates among 30 CE affected crossbred cows.

As suggested by several research workers, the reason behind mixed infections detected from vaginal discharges of the affected cows with endometrits is the synergism among those organisms and uterine environment (Dholakia *et al.,* 1987; Das *et al.,* 1996; Huszenicza *et al.,* 1999; Abd El-Hafeez *et al*., 2001; Chandrakar *et al.,* 2002; Zaman *et al.,* 2015).

In contrast to our study, Sahadev *et al*. (2017) reported lesser percentage (42.16 %) of single isolates and higher percentage (57.83 %) of mixed isolates in their study conducted in Karnataka state. Considerably, a very high percentage of single isolates (95%) was recovered by Manjhi *et al*., (2019) with least mixed isolates (5%).

In present study, a variety of aerobic bacteria such as *E. coli, S. aureus, S. uberis, P. aeroginosa, P. mirabilis, K. pneumoniae* and *B. cereus* were recovered from SE cases. Additionally, *R. equi, C. bovis* were also recovered in CE cases along with *E. coli, S. aureus, S. uberis, P. mirabilis, K. pneumoniae* and *B. cereus.*

Similar bacteriological profile was reported by Patel *et al*. (2019) in which the vaginal discharges of endometritis cows were positive for aerobic organisms such as *E. coli, Staphylococcus* spp*., Bacillus* spp*., Streptococcus* spp*., Salmonella* spp*., Klebsiella* spp*., Corynebacterium* spp*.,* and *Proteus* spp*.*

Aerobic bacteria such as *E. coli, Klebsiella* spp., *Staphylococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Proteus* spp., and *Streptococcus* spp., were frequently isolated from SE cases in cows (Bonnett *et al*., 1991; Shweta, 2003; Bhat and Bhattacharyya, 2012).

Similar to the bacteria obtained in the present study, Manjhi *et al*. (2019) also obtained *E. coli, Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp. and *Pseudomonas* spp., isolates both in SE and CE cases.

5.2.1 Phenotypic identification

In the present study, the bacteria isolates obtained from bovine endometritis cases were identified phenotypically based on colony characteristics, growth on selective media cultural and biochemical tests, sugar fermentation tests (Table 21 and 22) and accordingly the bacterial species was assigned (Table 23).

Similar criteria was adopted by Abreham *et al*. (2017) wherein the phenotypic identification of bacteria obtained from endometritis sample was performed based on cultural characteristics, Gram staining, morphology, hemolytic characteristics and biochemical tests such as catalase test, oxidase test, motility test by sulfide indole motility medium and oxidation-fermentation test followed by secondary biochemical tests such as MR test, VP test, citrate utilization test, indole test, urease test gas production test including cultural characteristics on TSI agar, EMB agar, Edward's media and MSA. The bacteria isolated and identified were *E. coli* predominantly followed by *Bacillus* spp., *Trueperella pyogenes, Staphylococcus* spp. *Streptococcus* spp., *Proteus* spp., *Corynebacterium* spp., *Klebsiella* spp., *Citrobacter* spp. and *Rhodococcus equi.*

Similarly, Behera *et al*. (2015) also identified the bacterial isolates of endometritis obtained by cytobrush method based on colony characteristics, Grams staining and various biochemical tests.

5.2.2 Bacterial isolates obtained from endometritis cases

Among the bacterial isolates obtained in the present study, *E. coli* and *S. aureus* were found predominant. *E. coli* found in 36.50% and 38.24% and *S. aureus* found in 28.57% and 26.47% of SE and CE samples respectively. This is in accordance with findings of Manjhi *et al*. (2019) who reported *E. coli* as predominant species (41.67%,

n=35) followed by *Staphylococcus* species (40.48%, n=34), *Bacillus* species (10.71%, n=9), *Streptococcus* species (7.14%, n=6) in SE. In contrast to our study *Staphylococcus* species was the most prevalent species (33.33%, n=39) followed by *E. coli* (27.35%, n=32), *Streptococcus* species (15.39%, n=18), *Bacillus* species (15.39%, n=18) and *Pseudomonas* species (8.54%, 10) in CE.

E. coli was the most predominant isolate obtained in several studies; Sharma *et al*. (2017) obtained *E. coli* as most predominant (32.26%) followed by *Bacillus cereus* (22.58%), *Staphylococcus aureus* (16.13%) in 23 subclinical endometritis affected cows. Similarly, *E. coli* was frequently (45%, 8 out of 40 samples) in the studies conducted by Kasimanickam *et al*. (2016) and in the study conducted by Raheel *et al*. (2020) (48.3%, 58 out of 120 samples) on SE.

In Karnataka state, Sahadev *et al*. (2017) also reported *E. coli* as predominant aerobic bacterial species (45.72%) followed by *Staphylococcus* species (42.86%) in cows with SE. The other pathogenic aerobic bacteria isolated less frequently were *Proteus* spp. and *Enterobacter* spp.

Contrasting results were reported by Behera *et al*. (2015) where aerobic bacteria were detected in 61 (84.72%) of 72 samples collected from repeat breeding cows. *Staphylococcus aureus* (38.88 %) was found to be predominant, followed by *E. coli* (36.11 %), *Streptococcus* spp. (33.33 %), *Enterobacter* spp. (22.22 %), *Proteus* spp. (18.05 %) and *Pseudomonas* spp. (16.67 %).

The findings of Barman *et al*. (2013) were contradictory to the present study findings, as non-lactose fermenters of *Enterobacteriaceae* family were the predominant isolates (25%), followed by *Escherichia coli* (20%), *Corynebacterium* spp. (15%)

Streptococcus spp. (15%), *Staphylococcus* spp. (10%), *Pseudomonas* spp. (10%), and *Bacillus* spp. (5%) in CE affected cows.

The presence of *E. coli* in postpartum uterus has been associated with the persistence of uterine infection (Mateus *et al*., 2002). Establishment of the infection is certainly dependent on the immunity status of the host, type and virulence of the bacteria involved and environmental conditions such as contaminated animal shed and bedding materials, season, and cow perineal hygiene score (Silva *et al*., 2009). Sheldon *et al*. (2002) found that endometrial pathogenic *E. coli* recovered from postpartum cows was distinct from enteric or extraintestinal pathogenic *E. coli*.

5.3 Species detection by PCR

5.3.1 Species detection of *E. coli* by PCR

Although nine different species of bacteria were detected in the present study, *E. coli* and *S. aureus* isolates constituted majority of the isolates and hence these isolates were further subjected to species specific PCR.

In the present study all the isolates (n=53) were genotypically confirmed as belonging to *E. coli* by targeting *uspA* and *uidA* genes by duplex PCR.

Earlier, either *uidA* coding for β-D-glucuronidase enzyme alone (Luque *et al*., 2017; Gonzalez *et al*., 2020) or along with *uspA* coding for universal stress protein (Godambe *et al*., 2017; Montso *et al.,* 2019) were targeted for confirmation of *E. coli* by PCR. However, Godambe *et al*. (2017) could detect only 77% of *E. coli* isolates by targeting both the genes.

Alternatively, the *fimH* gene was also used for confirmation of *E. coli* by PCR in endometritis affected buffaloes. *E. coli* was isolated from 25 of 103 cases (24.27%)

and of 25 (68%) *E. coli* isolates were detected by targeting *fimH* gene (Ingale *et al.,* 2016).

5.3.2 Species detection of *S. aureus* by PCR

In present study, the total number of *S. aureus* isolates subjected for species detection by *nuc* gene was 36 and all the isolates gave positive results with the expected amplicon of 181bp size.

Identification of *S. aureus* using PCR amplification of the *nuc* gene (~270 bp) which codes for extracellular thermostable nuclease protein of *S. aureus* is considered as a gold standard method (David *et al*., 2010). Compared to the 16S rRNA gene which is common to *Staphylococcus* genus, the *nuc* gene is more specific in detecting isolates specific to the species *S. aureus* (Sahebnasagh *et al*., 2014). However, 16S rRNA gene could also be a potential target for species identification of *S. aureus* isolates (Yehui *et al*., 2019).

In a study, which included 200 milk samples from animals and 50 nasal swabs from animal workers subjecting to bacterial isolation and molecular detection confirmed the presence of *S. aureus* in 184 samples by detection *nuc* gene (270bp size) by PCR (Sarkar *et al*., 2014).

5.4 Detection of virulence genes in major isolates

5.4.1 Detection of virulence genes in *E. coli*

E. coli and *S. aureus* were predominant isolates in the current study constituting 64.88% of all the isolates and hence only these two species were subjected for detection of virulence genes.

Identification of *E. coli* virulence factor genes associated with metritis and endometritis is of prime importance to conclude *E. coli* as primary cause for uterine infection.

Virulence factor distribution varies between herds and regions (Kasimanickam *et al*., 2016) because many published literatures used smaller number of herds as study population (Silva *et al*., 2009; Bicalho *et al*., 2010; Sheldon *et al*., 2010) as in the present study and this may lead to inconsistent association of VF with SE and CE in general. But at the same time the data so obtained can serve as a guideline to improve managemental perspective of the sample population.

In the present study, in SE *fimH*, *fyuA*, *kpsMT*II, *csgA* were detected in 100%, 47.82%, 17.39%, 26.08% respectively of the *E. coli* isolates whereas, in CE they were detected 100%, 73.07%, 38.46%, 34.61% of the isolates respectively.

FimH adhesin of type 1 fimbriae is an important adhesion and invasion factor present in uropathogenic *E. coli*, which is a major cause of increasing bacterial persistence and stimulating mucosal inflammation (Langermann *et al*., 1997). It is significantly associated with CE of human, dogs, mice and bovines (Ofek *et al*., 1977; Aronson *et al.,* 1979; Sheldon *et al.,* 2010; Krekeler *et al.,* 2012). In present study, *fimH* was detected in all the *E. coli* isolates recovered from both SE and CE which signified its presence as an important VF in endometrial pathogenic *E. coli* isolates.

Bicalho *et al*. (2010) reported *fim*H (type 1 pili), *kpsMII* (gene coding for capsule protein) as predominant genes among 11 different virulence factor genes targeted viz *astA* (gene coding for endotoxin), *cdt* (cytolethal distending toxin), *hlyA* (gene coding for the toxin) and *ibeA* (gene coding for a factor that facilitates invasion). The percentage of prevalence of *fimH* gene being an important VF associated with

endometritis varied from 68% (Ingale *et al*., 2016) to 87% (Bicalho *et al*., 2010; Gonzalez *et al*., 2020).

They also found that there was an 87% prevalence of *fimH* gene was seen as a virulence factor associated with endometritis.

Virulence factor *kpsMTII* is known to be associated with cellulitis in chickens and urinary tract infections in women and encodes for the capsular proteins K1 or K5, allowing bacteria to escape phagocytosis (de Brito *et al.,* 2003; Moreno *et al*., 2005, 2009). In present study it was detected in 17.39% of *E. coli* isolates in SE and to an extent of 38.46% in CE *E. coli* isolates indicating more expression in CE possibly indicating increased virulence.

Similar set of genes were targeted by Gonzalez *et al*. (2020) who reported that among 15 genes evaluated for virulence factor, 7 were found and prevalence was *fimH* (87%), *agn43* (41%) and *csgA* (35%). The genes *traT* (27%), *fyuA* (11%), *hlyA* (5%) and *kpsMT II* (5%) were in low proportion and concluded that *fyuA* was significantly higher ($p < 0.05$) in endometritis affected cows and *fimH* (fimbriae type 1) was in higher prevalence.

Kasse *et al*. (2016) reported that only *hra1* and *kpsMTII* were associated with uterine infections by *E. coli* out of 40 VF genes targeted in the study. *KpsMTII*, a VF gene was also detected in the present study in *E. coli* from the samples collected from both SE and CE cases in cows.

Biofilm is produced by bacteria by secreting extracellular polymeric substances upon attaching to a surface. Many genes are activated which in turn produce polysaccharides and other molecules are used for biofilm formation. Up to 800 genes can be activated when a bacterium joins a biofilm community and it protects the bacteria

from potential threats including antibiotics. The antibiotic resistance that biofilms create is a challenge that requires novel therapeutic approaches (Kasimanickam *et al*., 2016).

Antigen 43 (Ag43), a prominent surface protein of *E. coli* is the product of the *agn43* gene. This represents an entire family of closely related autotransporter proteins and a self-recognizing adhesin, characterized by both receptor recognition and receptor target. They promote bacterial biofilm formation by induction of microcolony formation (Kjaergaard *et al*., 2000) even in infection of bladder cells by uropathogenic *E. coli* infection (Anderson *et al*., 2003). It also confers aggregation and fluffing of cells and promote biofilm formation, uptake and survival in macrophages, and long-term persistence of *E. coli* in the infected tissue (Klemm *et al*., 2004). Uropathogenic strain of *E. coli* was reported to be associated with uterine infection for the first time by Kasimanickam *et al*. (2015). In present study *agn43* gene was found in 42.30% of *E. coli* isolates in CE cases which supports the conclusion of Kasimanickam *et al*. (2015) that uropathogenic *E. coli* is establishing as endometrial pathogenic strain causing uterine infection in dairy cows and can transmit antibiotic-resistant gene to endometrial pathogenic *E. coli* strain. They also found that biofilm gene *agn43* was detectable only in CE and metritis but not in SE. *Agn43* was detected in 61.1% (11 of 18) of the samples of which 27.27% were found in metritis and 72.72% in CE strains. In the present study biofilm gene *agn43* was not found in SE isolates.

The detection of VF genes in *E. coli* could vary depending on the methodology adopted in the study. In addition, the distribution of VF varies between farms and regions (Sheldon *et al*., 2010; Bicalho *et al*., 2010; Silva *et al*., 2009; Kasse *et al*., 2016). In the present study also, samples were taken from limited geographical region (in and around Shivamogga district of Karnataka) which could explain variation in distribution

of VFs in *E. coli*. However, in India literature are scanty with regard to detection of VF genes in *E. coli* isolated from endometrial discharge and hence data was not available for analyzing distribution of VFs in *E. coli.*

5.4.2 Detection of *spa* gene in *Staphylococcus aureus*

The most widely used first line typing method for epidemiological studies of *S. aureus* is *spa* typing which allows rapid characterization of isolates based on phylogenetic relationships (Hasman *et al*., 2010; Porrero *et al.,* 2012).

PCR targeting the *spa* gene as VF was carried out for all the 38 *S. aureus* isolates. *Spa* gene was detected in all the isolates subjected for PCR and the *spa* type obtained by sequencing one of the PCR products was t359.

There is a solitary report on *spa* typing of endometrial *S. aureus* strains which revealed 4 subtypes (t779, t2883, t13751 and t1939) of *spa* gene upon molecular typing by PCR analysis. The study also included the *S. aureus* strains obtained from mastitis in addition to endometrial strains obtained from cattle (n=155) (Dan *et al*., 2019).

Reports are plenty on spa types of *S. aureus* isolates associated with mastitis. Mitra *et al*. (2013) reported t359 a similar *spa* type as predominant *spa* types along with other two *spa* types viz t267 and t6877. They observed that *spa* types t267 and t359 were found among the isolates from different herds indicating the predominant nature of the clones across the dairy herds in their sample population.

Spa type t359 obtained in the present study was also reported in bovine milk *S. aureus* isolates from Brazil, Canada and Japan, (Aires *et al*., 2006; Hata *et al*., 2010).

In a recent study on *spa* typing of mastitic *S. aureus* isolates, t359, t7867 and t33841 were found to be the predominant *spa* types associated with bovine mastitis (Sheela *et al*., 2019).

5.5 Antimicrobial susceptibility

The antibiotics which were commonly used by veterinarians for the treatment of endometritis in the study area and the antibiotics which have been suggested for treatment of endometritis were selected for the antibiogram assay.

As a routine veterinary practice, infections of uterus caused by bacteria are treated with antibiotics. However, the efficacy of such therapeutic agents needs to be evaluated from time to time due to continuous emergence of drug resistant bacterial strains which plays a significant role in the failure of conception as a result of inflammation, denudation of uterine mucosa and change in the pH of the uterine and vaginal secretions (Sheldon and Dobson, 2004; Singh *et al.,* 2019).

Various researchers have carried out studies on the bovine genital tract with endometritis and identified a wide array of bacteria and their sensitivity pattern to different antibiotics (Nibret *et al.,* 2013; Brodzki *et al.,* 2014; Sharma *et al.,* 2017). Ideally, identification and *in vitro* sensitivity against antimicrobial agent and subsequent treatment with an approved drug is the most practical, economical and an efficient approach to give a cognitive prognosis of repeat breeding and CE (Sharma *et al.,* 2017; Kumar *et al.,* 2018).

In the present study, antimicrobial susceptibility patterns were varied among different bacterial species. Species wise susceptibility pattern are shown in Table 28. *E. coli*, the most predominant species, showed high susceptibility to ciprofloxacin (91.8%), followed by ceftriaxone (87.7%), enrofloxacin (79.5%), gentamicin (71.4%) etc. *S. aureus*, another predominant strain, was found susceptible to tylosin (97.2%), nitrofurantoin (94.4%), ciprofloxacin (91.6%) and co-trimoxazole (88.8%). Both the species were considerably found resistant to penicillin and polymyxin B.

Researchers from diverse geographical locations have reported varying pattern of susceptibility to antibiotics with regard to bacterial strains associated with endometritis.

Takamtha *et al.* (2013) and Sharma *et al*. (2019) found that the isolates obtained from cows suffering from endometritis were highly sensitive to ciprofloxacin (63.50%) and enrofloxacin (60.32%). While the resistance was noticed to antibacterial agents viz., penicillin (83.64%), oxytetracycline (42%), amoxicillin (26%) and streptomycin (25%).

Sensitivity recorded in bacteria causing uterine infection to various antibiotics were ciprofloxacin (71%), enrofloxacin (65%) and ofloxacin (52%). Moderate sensitivity was recorded in oxytetracycline (44 %) and gentamicin (45%) (Kumar *et al.,* 2018).

The antibiogram study conducted by Sharma *et al*. (2017) revealed that both *E. coli* and Gram-positive aerobes like *B. cereus*, *S. aureus* and *Streptococcus* spp., were sensitive to levofloxacin, enrofloxacin and ciprofloxacin in decreasing order of sensitivity. *E. coli* was resistant to cloxacillin, penicillin and ampicillin in decreasing order of resistance whereas Gram positive aerobes were resistant to penicillin, cloxacillin and amoxycillin.

In contrast to the present study, the antibiotic sensitivity of isolates was found to be maximum for ceftriaxone and sulbactam combination 91.67% in a study conducted by Manjhi *et al*. (2019) and for levofloxacin was 89.07%, but the sensitivity pattern to ciprofloxacin (79.69%), ceftriaxone (73.43%), enrofloxacin (61.45%) and gentamicin (56.78%) was in close agreement with current study.

Bajaj *et al*. (2018) in their study recorded antibiogram in uterine lavage of normal, sub-clinical and clinical endometritic postpartum buffaloes and found maximum sensitivity to ceftriaxone + sulbactam (93.54%) followed by levofloxacin (87.08%), ceftriaxone (80.64%), ciprofloxacin (74.19%), enrofloxacin (58.06%) and gentamicin (54.83%), respectively.

The in vitro antibiotic sensitivity test indicated that the highest number of isolates (64%) were sensitive to ceftriaxone, followed by gentamicin, enrofloxacin and chlortetracycline (32%), but chloramphenicol showed sensitivity in minimum number (8%) of isolates (Udhayavel, 2013).

5.5.1 Detection of methicillin resistance

Staphylococcus aureus strains that are oxacillin and methicillin resistant are considered resistant to β- lactam agents, including cephalosporins and carbapenems. The mechanism of resistance to penicillinase-stable β-lactams including methicillin, isoxazolyl penicillins and cephalosporins in *S. aureus* (MRSA) is an altered target site due to an acquired penicillin-binding protein encoded by the gene *mecA* (Catry *et al*., 2010). Resistance to these agents drastically limits therapeutic options and is a worldwide emerging problem in both animals and humans.

An increased number of reports on methicillin resistant *Staphylococcus aureus* (MRSA) in livestock have been recorded in recent years. MRSA has also been found in companion animals, cows and horses, and transmission between humans and animals are also reported (Leonard and Markey, 2008; Weese and Duijkeren, 2010). Extension of the host range of MRSA to different animal species and its zoonotic concerns related to the persons in direct contact with MRSA-positive animals demands a critical review on the factors associated with this emerging zoonotic bacterium from both the

veterinary and public health point of view. The irrational extensive use of antimicrobials against diseases, appears to be an important risk factor for the spread of MRSA although data are still sparse in this context (Catry *et al*., 2010).

Reports on study of methicillin resistance in endometrial *S. aureus* are limited in Indian as well as global context.

In this present study, phenotypic resistance to cefoxitin was detected in 2 of 36 *S. aureus* isolates tested (5.55%) and *mec*A gene was detected in one isolate (2.78%).

Dan *et al*. (2019) in his study stated that out of the 155 *S. aureus* strains (186 milk samples from clinical mastitis cases and 151 endometritis swab samples), 133 (85.8%) were methicillin-susceptible *S. aureus* (MSSA) and 22 (14.2%) were MRSA by phenotypic method. *Mec*A gene was detected in 14.2% of the MRSA strains.

Ten MDR *S. aureus* isolates were reported in a study on mastitis (Kumari *et al*., 2020) and *mecA* gene was detected in only 40% of them. Very high prevalence (75.4%) of *mecA* gene was detected in another study on bovine mastitis (Keyvan *et al.,* 2020).

In a study which was designed to account for potential variation in AMR prevalence in common mastitis pathogens due to geographical and epidemiological differences by Canadian researchers Saini *et al*. (2012), a very low prevalence rate (0.05%) of MRSA (1 out of 1802 *S. aureus* isolates) was reported, similar to the findings of the current study.

5.6 Determination of minimum inhibitory concentration

5.6.1 MIC of ceftiofur

Optimal dosing of antimicrobial agents is essential for any therapeutic success, and designing dosing regimens requires an integration of both pharmacokinetic and

pharmacodynamic principles along with the invitro MIC of any antimicrobial agent against microbes of interest.

Ceftiofur is a third-generation cephalosporin which has become the gold-standard therapeutic agent for cattle with reproductive tract infections (Credille and Giguere, 2015). Ceftiofur administration by systemic route in dairy cows affected with dystocia, retained fetal membrane, or both have reduced the incidence of metritis by 70% compared with cows treated with estradiol cypionate (Risco and Hernandez, 2003).

In the present study, MIC of ceftiofur for *E. coli*, *S. aureus*, *S. uberis* isolates ranged between 0.25-1, 0.25-2, 0.125-0.25 µg/mL respectively. MIC of *R. equi*, *C. bovis*, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae*, *B. cereus* was 0.25, 8, 16, 16, 2, 16 µg/mL respectively.

Subcutaneous administration of ceftiofur at the dose of 1 mg/kg body weight after parturition resulted in concentrations of ceftiofur derivatives in uterine tissues exceeding the reported minimal inhibitory concentrations (MICs) for the common pathogens like *Escherichia coli*, *Fusobacterium necrophorum*, *Trueperella pyogenes* and *Bacteroides* spp. (Okker *et al*., 2002).

MIC of *E. coli* isolates(n=20) from bovine endometritis cases ranged between 0.25 (18) and 0.5 (2) µg/mL and the isolates showed 100% sensitivity (Henriques *et al*., 2014).

In a study conducted by Sheldon *et al*. (2014), MIC of ceftiofur against *E. coli* strain (n=20) isolated from the uterus of postpartum cows with pyrexia and/or metritis and/or endometritis was 0.25µg/mL (8 isolates) and 0.5 µg/mL (12 isolates) respectively.

MIC of *E. coli* isolates (n=85) were found to be 0.25 (29.4%), 0.5 (60%) and 1(4.7%) µg/mL and 5.9% of the isolates were not inhibited by the even at the highest concentration of the drug 125 µg/mL. Dilution range used was 0.0 3 to 125 µg/mL (Pohl *et al*., 2018).

Riesenberg *et al*. (2014) determined the MICs of 32 antimicrobial agents for 200 isolates of *Rhodococcus equi* of animal origin by applying a recently described broth microdilution protocol. MIC values of ceftiofur were 0.25 µg /ml (1), 0.5 µg /ml (3), 1 µg /ml (8), 2 µg /ml (16), 4 µg /ml (14), 8 µg /ml (128), 16 µg /ml (30) and dilution range were 0.03 to 64 µg /ml.

In another study (Jong *et al*., 2018) *Escherichia coli* MIC values for ceftiofur ranged between 0.12–2.0 µg/mL in broth dilution method and for *Staphylococcus aureus*, it was 0.25–4.0 µg/mL. The MIC values for *Streptococcus uberis* was 0.06-2 µg/mL and for *Klebsiella* spp., was 0.12-1 µg/mL.

MIC values for ceftiofur against *S. aureus* and *R. equi* as reported by Carlson *et al.* (2010) was 0.5-1 µg/mL and 0.125 µg/mL respectively.

5.6.2 MIC of ciprofloxacin

Although MIC values of ciprofloxacin varied with very wide range among different bacterial species, the MIC values for most of the isolates were low (Table 30).

MIC values for ciprofloxacin in *E. coli* isolates was in the range of 0.008-0.25µg/ml in the study conducted by Pohl *et al*. (2018).

González *et al*. (2010) studied the MIC values for enrofloxacin (along with its biologically active metabolite, ciprofloxacin) against Gram-negative strains (*E. coli* and *P. aeruginosa*) and Gram-positive (β-haemolytic) strains and its values ranged from 0.25–2.0 and 1.5–3.0µg/mL respectively. Endometrial ciprofloxacin concentrations

were also detected to be above the MIC for the Gram-negative and positive isolates following i.v. treatment at 5mg/kg in mares and plasma concentrations were also maintained in the study.

S. uberis showed 100% sensitivity to enrofloxacin and MIC value ranged between 0.25-1 µg/mL (Kaczorek *et al*., 2017).

A study on antimicrobial susceptibility of *S. aureus* and *S. pseudointermedius* isolated from various animal species revealed that *S. aureus* was found the second most predominant species of bacteria obtained and the MIC of the ciprofloxacin was 0.064 - 0.125 µg/mL and of gentamicin was 0.001 -1 µg/mL (Rubin *et al*., 2011).

Effect of ciprofloxacin therapy for the treatment of endometritis in cows was studied by Purohit and Sharma (2007) and reported 87.5 % efficacy in mild endometritis cases, 66.7% efficacy in moderately affected cows and lesser efficacy (26.6%) in severe endometritis cases. Based on therapeutic efficacy they concluded that ciprofloxacin might be useful in the therapy of endometritis. Similar invitro results were obtained in the present study and hence MIC determination of the same was performed by method described in 3.8.1 and results are as depicted in Table 30.

5.6.3 MIC of gentamicin

In a study to determine the PK-PD integration of selected antibacterial agents used to treat mastitis against local bacterial isolates (Pavithra *et al*., 2018) the gentamicin MIC value for *E. coli, S. aureus* and *P. aeruginosa* isolates were found to be 2 µg/mL, 0.25 µg/mL and 0.5 µg/mL respectively. Whereas considerably low MIC values of gentamicin were noticed for *E. coli, S. aureus* and *P. aeruginosa* in the present study and they were 0.01- 1 µg/mL, 0.001-1 µg/mL and 0.10 µg/mL respectively.

Pohl *et al*. (2018) in their study of minimum inhibitory concentrations of frequently used antibiotics against *Escherichia coli* and *Trueperella pyogenes* isolated from uteri of postpartum dairy cows reported that MIC values for gentamicin for *E. coli* isolates was in the range 0.25-2µg/mL.

S. uberis showed 96% resistance in a study of phenotypic and genotypic antimicrobial susceptibility patterns of *Streptococcus* spp. isolated from cases of clinical mastitis in dairy cattle in Poland by Kaczorek *et al*. (2017) to gentamicin. Moderate sensitivity with MIC range of 32-128 µg/mL was observed in 4% isolates with the least susceptibility towards gentamicin. On the contrary least MIC value (0.01 µg/mL) was recorded for gentamicin against *S. uberis* in the present study (Kaczorek *et al*., 2017).

MIC values for gentamicin against *S. aureus* and *R. equi* as reported by Carlson *et al.* (2010) was 0.5 µg/mL and 0.25 µg/mL respectively in an *in vitro* study to determine antimicrobial activity of tulathromycin and other antimicrobial agents against *R. equi*. In present study, MIC value of gentamicin against *S. aureus* and *R. equi* was 0.001-1 µg/mL, 0.10 µg/mL respectively and majority of the isolates were in the lower range of MIC (Table 31).

Against *B. cereus*, gentamicin MIC values were in the range of 0.094–0.75 µg/mL and that for ciprofloxacin was 0.047–0.5 µg/mL in a study of MICs for *Bacillus cereus* along with other *Bacillus* spp., from isolates of clinical and environmental sources by Turnbull *et al*. (2004). The MIC values recorded in the present study was 0.10 µg/mL.

There are several reasons for variability of an MIC measurement viz., inter-strain differences, intra-laboratory variability and inter-laboratory variability.

To summarize, it was concluded that existence of biological variation and assay variation in steps like inoculum preparation, media, incubation temperature and incubation time. This variation is also well recognized in the accepted MIC ranges of quality control strains, which often span over two to three dilutions and even four dilutions in some cases (Mouton *et al*., 2018).

Optimal success with antimicrobial agents is essential for any therapeutic regimen and hence integration of both pharmacokinetic and pharmacodynamic principles along with the invitro MIC of any antimicrobial agent against microbes of interest becomes vital for the successful therapeutic management of SE and CE in field scenario.

Summary

VI. SUMMARY

The present study was taken with the objective of isolation and phenotypic identification of aerobic bacteria associated with subclinical and clinical endometritis cases in dairy cattle. Further, antimicrobial susceptibility patterns were also evaluated with the bacterial isolates obtained, including methicillin resistance in *S. aureus* isolates. In addition, presence of major virulence genes in the predominant bacterial species was investigated using PCR.

A total of 133 samples were collected from bovines which included 82 uterine lavage samples from SE and 51 endometrial discharge samples from CE cases. Subclinical cases of endometritis were determined by cytology of endometrial lavage. Out of 82 uterine lavages collected from RB cases, 63 were diagnosed as sub clinical endometritis by endometrial cytology and remaining 19 samples were cytologically negative. All the samples from SE cases (n=63) and CE cases (n=51) were processed for bacterial isolation. A total of 63 isolates from SE and 51 isolates from CE were recovered from the samples which were identified by morphological, cultural, and biochemical methods to species level.

The aerobic bacteria recovered from SE were *E. coli, S. aureus, S. uberis, P. aeruginosa, P. mirabilis, K. pneumoniae* and *B. cereus*. Whereas, in CE *R. equi* and *C. bovis* were also recovered along with *E. coli, S. aureus, S. uberis, P. mirabilis, K. pneumoniae* and *B. cereus.*

In subclinical endometritis, 70.68% of the samples yielded single isolate and mixed infection was observed in 29.31% of the samples. Whereas in CE,single bacterial agent was isolated in 52.94% of samples and more than one agent in 47.05% of the samples.

Among the bacterial isolates obtained, *Escherichia coli* and *Staphylococcus aureus* were found predominantly in both SE and CE cases. *E. coli* was found in 36.50% and 38.24% of the samples whereas, *S. aureus* was found in 28.57% and 26.47% of the samples in SE and CE respectively, indicating that *Escherichia coli* and *Staphylococcus aureus* were frequent cause of endometritis in bovines. Further, major virulence genes were detected using PCR in these two predominant bacterial isolates to elucidate the virulence pattern in the causative agents.

The PCR was employed as molecular method in this study for the detection of species of *E. coli* and *S. aureus*. For *E. coli uidA* and *uspA* was targeted by duplex PCR and for *Staphylococcus aureus, nuc* gene was targeted. PCR method was also used for the detection of virulence genes in both the bacterial species. For all *E. coli* isolates virulence genes viz., *fimH*, *fyuA*, *kpsMT*II, *csgA* and *agn43* were targeted. In *Staphylococcus aureus*, *spa* gene was detected and additionally, antibiotic resistance gene *mec*A was also detected in *S. aureus* isolates using published primers.

In subclinical endometrits, *fimH*, *fyuA*, *kpsMT*II and *csgA* were detected in 100%, 47.82%, 17.39% and 26.08% of the isolates respectively, whereas, in CE detection rate was 100%, 73.07%, 38.46% and 34.61% of the isolates respectively. *Agn43*, a biofilm gene, was not found in any of the SE isolates whereas it was found in 42.30% of the CE isolates indicating the presence of the potential virulence factors more in *E. coli* isolates of CE than SE.

The emergence of MRSA as a cause for concern because of close human-animal association and its zoonotic potential was considered to be studied in the present work. Therefore, in present investigation, phenotypic (using cefoxitin disc) and genotypic

(targeting *mec*A) methicillin resistance in *S. aureus* (n=36) isolates was determined and it was detected in two (5.55%) and one isolates (2.78%) respectively.

Polymerase chain reaction products obtained from all the targeted virulence genes of *E. coli* and *S. aureus* along with antibiotic resistance gene *mecA* one each were sent for commercial sequencing. The alignment of partial sequences of *fimH*, *fyuA*, *kpsMT*II, *csgA, agn43* of *E. coli* revealed 100 *per cent,* 99.72 *per cent*, 98.89 *per cent*, 100 *per cent* and 100 *per cent* similarities respectively, with the corresponding deposited sequences of *E. coli* in database. The sequence alignment of *S. aureus spa* and *mecA* genes revealed 99.73% and 100% similarities with the corresponding deposited sequences of *S. aureus* in database respectively.

Antimicrobial susceptibility assay by disc diffusion method was performed for all the 135 bacterial isolates obtained in the study and interpreted based on CLSI guidelines. But interpretation criteria for *S.uberis*, *C. bovis* and *B. cereus* were not available for most of the antibacterial agents used in the study. Over all in the study, ciprofloxacin was found highly sensitive and penicillin was found highly resistant. Since ceftiofur disc was unavailable, MIC was determined by broth dilution method and the MIC was 0.25-1 µg/mL in *E. coli,* 0.25-2 µg/mL in *S. aureus* and 0.125-0.25 µg/mL in *S. uberis*. 0.25 µg/mL in *R. equi*, 8 µg/mL in *C. bovis*, 16 µg/mL in *P. aeruginosa*, 16 µg/mL in *P. mirabilis*, 2 µg/mL in *K. pneumoniae,* 16 µg/mL in B. *cereus* and there was no inhibition in 6.6% of the *E. coli* isolates and in 50% of *P. aeruginosa* isolates even at 256 µg/mL.

Ezy MIC strip method was employed for determination of minimum inhibitory concentration of ciprofloxacin and gentamicin. The MIC for ciprofloxacin ranged between 0.032 µg/mL to 0.125 µg/mL for *E. coli* and 0.064 µg/mL to 0.125 µg/mL for

S. aureus. Similarly, MIC values for *S. uberis*, *Rhodococcus equi, Corynebcterium bovis, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneummoniae, Bacillus cereus* were found to be 0.38 µg/mL, 0.032 µg/mL, 0.75 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 0.5 µg/mL and 0.19 µg/mL respectively.

The MIC for gentamicin against *E. coli* was ranged between 0.01 µg/mL to 1 µg/mL and that of *S. aureus* were 0.001 µg/mL to 1 µg/mL. For *S. uberis* isolates, it was 0.01 µg/mL. Similarly, MIC values for *Rhodococcus equi, Corynebcterium bovis, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneummoniae, Bacillus cereus* were 0.1 µg/mL, 2 µg/mL, 0.1 µg/mL, 0.1 µg/mL, 10 µg/mL and 0.1 µg/mL respectively.

The outcome of the present study gives an overview of aerobic bacteria associated with SE and CE, their antibiogram and the prevalence of virulence factors in the respective predominant bacteria in bovines.

Conclusions

This study evaluated bacterial agents associated with subclinical and clinical endometritis cases in bovines with regard to their antimicrobial susceptibility and profile of major virulence genes involved.

- *E. coli* was the most frequently isolated bacteria from endometrial samples followed by *S. aureus* in both SE and CE cases.
- *C. bovis* and *R. equi* were isolated only in CE cases.
- Isolates obtained from CE cases possessed a higher number of virulence genes than from SE cases.
- Ciprofloxacin was found more efficacious antimicrobial agent invitro than other antimicrobial agents and had a wider range of MIC value.

- MIC value of gentamicin across different species of bacteria was less compared to other antibacterial agents.
- Ceftiofur showed a promising outcome with MIC value being low and less variable.
- Detection of methicillin resistance in *S. aureus*, although in very few isolates, signifies the need for continuous monitoring and demands for judicious use of antimicrobial agents in routine treatment.
- The findings contribute to the understanding of bacterial etiological profile in bovine endometritis, there is a need to explore their pathogenic potential role in causing uterine diseases using experimental animal models.

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Abstract

VIII. ABSTRACT

Bacterial endometritis results in lower and delayed conception rates in affected cows. The present study was conducted to isolate and characterize aerobic bacteria from subclinical (SE) (n=82) and clinical endometritis (CE) (n=51) cases in cows. In addition, studies on antibiogram profile, MIC and major virulence genes were also carried out. Fifty eight of 82 uterine lavage samples showing 1-5% of PMN cells by endometrial cytology were considered as subclinical endometritis cases. Bacterial isolation and phenotypic characterization of the isolates revealed *E. coli, S. aureus, S. uberis, P. aeroginosa, P. mirabilis, K. pneumoniae* and *B. cereus* from SE and in addition to these, *R. equi* and *C. bovis* were also recovered in CE cases. PCR was used to confirm the bacterial species targeting *uspA* and *uidA* genes in *E. coli* and *nuc* gene in *S. aureus*. Major virulence genes in *E. coli* viz., *fimH, fyuA, kpsMTII, csgA* were detected by PCR in 100%, 47.82%, 17.39%, 26.08% of the SE isolates, and in100%, 73.07%, 38.46%, 34.61% of CE isolates respectively. *Spa* gene was detected in all *S. aureus* isolates and the *spa* type was t359. The antibiogram study by disc diffusion method using most commonly used 19 antibacterials revealed that most of the isolates were susceptible to ciprofloxacin, enrofloxacin, gentamicin and ceftriaxone, and resistant to penicillin and polymyxin B. MIC values for ceftiofur, ciprofloxacin and gentamicin were within the susceptibility range for most of the isolates. Out of 36 *S. aureus* isolates, phenotypic methicillin resistance was detected in two whereas *mecA* gene was detected only in one isolate by PCR. The study gives an insight into the etiological profile, antibiogram, virulence potential of the bacterial agents associated with bovine endometritis.

Key words: Endometritis, aerobic bacteria, virulence genes, antimicrobial susceptibility, minimum inhibitory concentration