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Determination of antibiotic sensitivity and biofilm concentration against mastitis causing bacterial isolates

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Abstract
Mastitis is one of the most commonly occurring diseases of dairy animals. It is the most important cause of economic losses to the dairy industry in India and throughout the world. Hence, the present study designed to conduct with the main purpose to study the antibiotic sensitivity of fluroquinolones and tetracyclines class antibiotics and biofilm formation for major bacterial isolates known to cause mastitis in dairy herds. Bacterial isolates causing mastitis namely *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Staphylococcus capitis, Staphylococcus schleiferi, Enterobacter aerogenes* were procured from Department of Veterinary microbiology, Department of Veterinary public health Pookode, Kerala and Veterinary College, Shivamogga, Karnataka. The antibiotic susceptibility testing and biofilm formation tests were conducted. Antibiotic sensitivity test revealed that *E. coli* isolates revealed highest sensitivity to Tetracycline class antibiotics (oxytetracyclines, tetracyclines, doxycycline) and Fluroquinolones class antibiotics (Enrofloxacin, Norfloxacin, Ciprofloxacin and Ofloxacin) followed by extended spectrum beta-lactamase (ESBL) *E. coli.* In conclusion, tetracyclines class antibiotics were found to be more sensitive against mastitis causing microorganism as compared to fluroquinolones class antibiotics.

Keywords: Mastitis, Fluroquinolones, Tetracyclines, Antibiotic, *E. coli*

1. Introduction
Globally, mastitis is one of the most important infectious diseases in dairy herds. The majority of clinical cases are caused by a limited number of specific pathogens, but in fact, a very large array of bacterial species may infect the udder. India ranks number one in the world in terms of total milk production with 146.3 million tons of milk produced annually (Jamali H *et al.* 2018) [1]. Mastitis is one of the most commonly occurring diseases of dairy animals causing huge economic losses to the dairy industry in India and throughout the world (Redogorelse, 2019) [2].

Mastitis is the inflammation of the udder tissue parenchyma characterized by pathological changes in the mammary gland tissues such as edema, redness, and increase in the gland temperature as well as several changes in the physical and chemical properties of the milk (Acar and Rostel, 2001) [3]. The panorama can also vary between countries and regions within each country. In order to implement suitable strategies against mastitis, it is important to understand the panorama of udder pathogens and to monitor trends over time. The consequence of mastitis is restricted not only to the dairy farmers but is also a concern to the consumers because of increasing antimicrobial resistance due to the extensive and indiscriminate use of antimicrobials for the management of mastitis (Mader *et al*. 2021) [4].

It has been reported that more than 150 bacterial species implicated with the mastitis and they have been categorized into three main categories *viz.* environmental, contagious and opportunistic (Perrosn *et al.* 2016) [5]. Hence, sustaining the efficacy of antibiotics is very important for dairy cow welfare and herd economics. Acquired antibiotic resistance in bacteria is, however, an increasing threat, and surveillance of antibiotic susceptibility of bacteria, including mastitis-causing pathogens is recommended by the World Organisation for Animal Health (OIE) and several other organisations (Acar and Rostel, 2001; Mader *et al*. 2021) [3, 4]. Results of such monitoring will guide therapeutic decisions and show possible trends, indicating a possible need for interventions regarding antibiotic use (Mader *et al*. 2021) [3].

Hence, the current research investigation was undertaken to study the antibiotic sensitivity of fluroquinolones and tetracyclines class antibiotics and biofilm formation for major bacterial isolates known to cause mastitis in dairy herds.

2. Materials and Methods

The present study was carried out in the department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode.

2.1 Collection of samples

Bacterial isolates causing mastitis namely *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Staphylococcus capitis*, *Staphylococcus schleiferi*, *Enterobacter aerogenes* were procured from Department of Veterinary microbiology, Department of Veterinary public health Pookode, Kerala and Veterinary College, Shivamogga, Karnataka.

2.2 Preparation of culture media

Dehydrated media manufactured by m/s HiMedia were used for the study. Culture media were reconstituted with double glass distilled water as per the manufacturer's direction and sterilized by autoclaving at 121 °C and 15 psi pressure for 15 minutes. They were then cooled to about 45 °C and poured to sterile petri plates and test tubes and incubated at 37 °C for 24 hr. to check sterility.

2.3 Antibiotic discs

Antibiotic discs used in the study were enrofloxacin (10µg), ciprofloxacin (10µg), norfloxacin (30µg), ofloxacin (10µg), tetracycline (30µg), oxytetracycline (30µg), doxycycline (10 µg) The zone of inhibition of bacterial growth around each disc including the diameter of the disc was measured and interpreted as sensitive, moderately sensitive or resistant by comparing the ranges given by the manufacturer.

2.4 Antibiotic susceptibility testing (ABST)

The antibiotic susceptibility testing was carried out for bacterial isolates of *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Staphylococcus capitis*, *Staphylococcus schleiferi*, and *Enterobacter aerogenes*. The bacterial isolates were subjected to disc diffusion as per the method described by Bauer, 1966.

2.5 Congo red agar method for Biofilm study

Autoclaved Congo red agar is poured to sterilized petri-plates and incubated for 24hr. After sterility checking the bacterial strains were streaked on the plates and further streaked plates were incubated for 24-48hrs at 37 °C. Biofilm production was assayed after incubation period.

3. Results

Mastitis causing microorganisms namely *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Staphylococcus capitus*, *Staphylococcus schleiferi*, *Enterobacter aerogenes* formed the subjects of the study.

3.1 Antibiogram

3.1.1 E Coli isolate 1

The isolate showed zone of inhibition of 26mm around ciprofloxacin followed by 24mm around enrofloxacin, norfloxacin and ofloxacin. Whereas, sensitivity of oxytetracyclines was 5mm, and tetracyclines & doxycycline was 3mm (Plate. 1 and Figure 1).

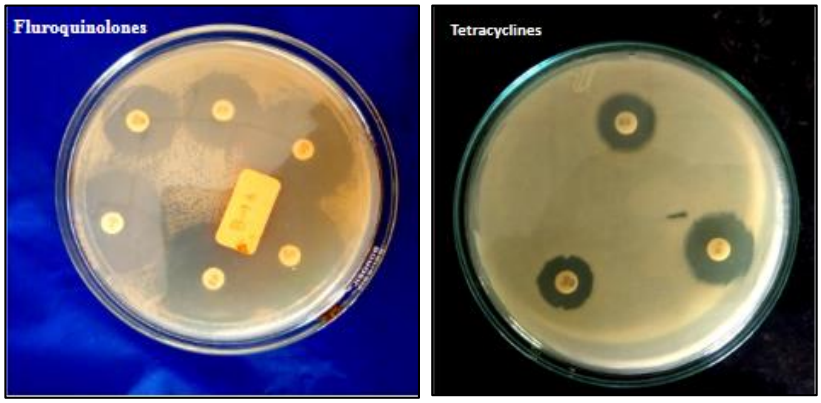

Plate 1: Antibiotic sensitivity test of *E. coli*

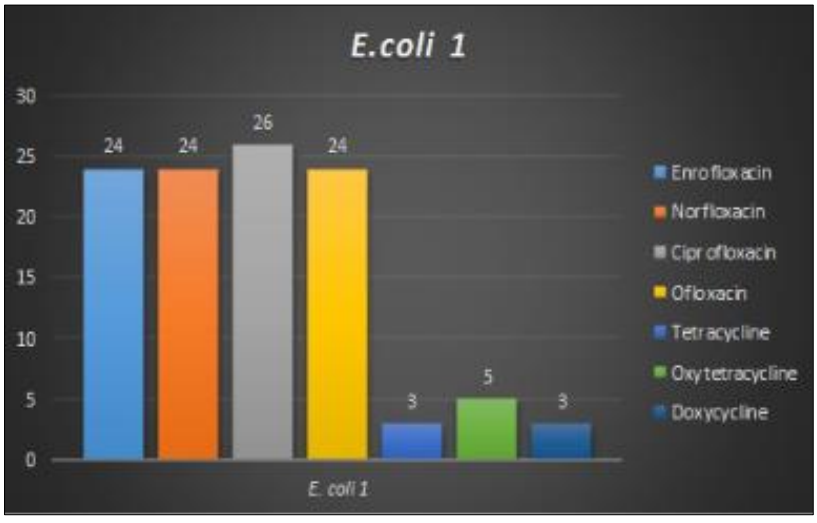

Fig 1: Zone of inhibition (mm) of *E Coli 1*

3.1.2 E. Coli isolate -2

E Coli isolate-2 showed zone of inhibition of 26mm for Norfloxacin and Ofloxacin followed by 24mm for Ciprofloxacin, and 23mm for Enrofloxacin. Whereas, in case of tetracycline group antibiotics *E Coli* isolate-2 showed zone of inhibition 8mm for Oxytetracyclines followed by 5mm and 4 mm for tetracycline and doxycycline respectively (Fig. 2).

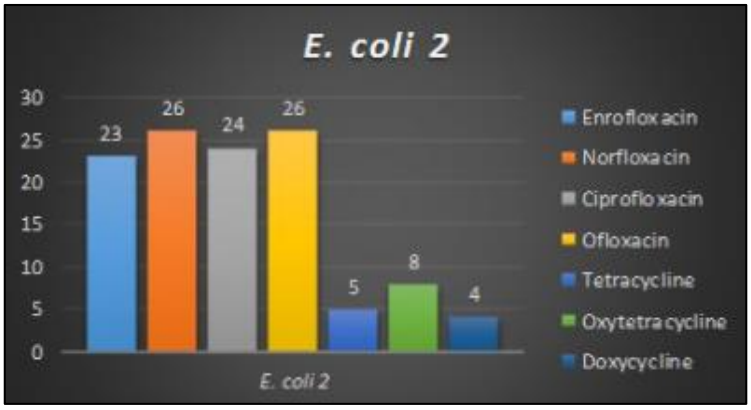

Fig 2: Zone of inhibition (mm) of *E. Coli* isolate-2

3.1.3 E. Coli isolate-3

E. coli isolate-3 showed zone of inhibition of 26mm for enrofloxacin followed 24mm for norfloxacin, ofloxacin, ciprofloxacin. While *E. coli* isolate-3 showed zone of inhibition 13mm for tetracycline followed by 12mm for oxytetracycline, and 10 mm for doxycycline (Figure 3).

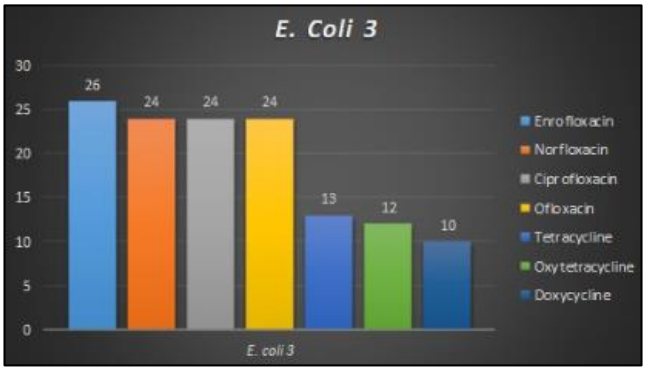

Fig 3: Zone of inhibition (mm) of *E. coli* isolate-3

3.1.4 Extended spectrum beta-lactamase *(ESBL) E. coli*

ESBL E. Coli. Showed zone of inhibition of 30mm for enrofloxacin followed by 28mm for ciprofloxacin & ofloxacin and 26mm for norfloxacin. In case of tetracycline groups *ESBL E. Coli.* Showed zone of inhibition of 5mm for oxytetracycline and Doxycycline discs and 3mm for tetracycline (Figure 4).

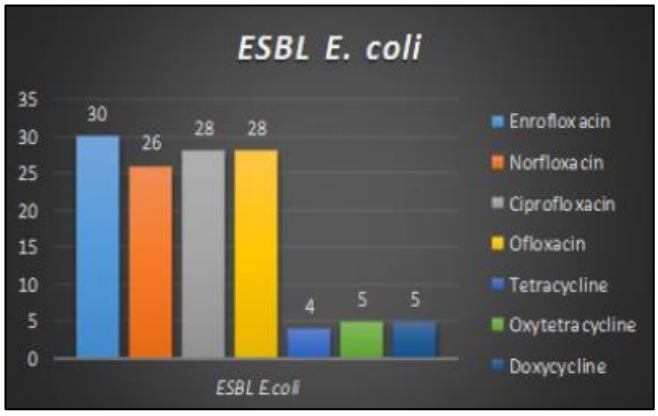

Fig 4: Zone of inhibition (mm) of ESBL *E. coli*

3.1.5 Staphylococcus aureus

Zone of inhibition for bacterial isolate of *S. aureus* was found to be 20mm for ciprofloxacin followed by 18mm for norfloxacin, 17mm for enrofloxacin and 16mm for ofloxacin. Whereas, in case of tetracycline groups *S. aureus* exhibited zone of inhibition of 14mm, 13mm, and 12mm for oxytetracycline, doxycycline, and tetracycline (Plate 2 and Figure 5).

Plate 2: Antibiotic sensitivity test of *S. aureus*

Fig 5: Zone of inhibition (mm) of *S. aureus*

3.1.6 Klebsiella pneumoniae

K. Pneumoniae was sensitive for enrofloxacin and ciprofloxacin with zone of inhibition 20mm followed by 19mm for norfloxacin and ofloxacin. However, in case of

tetracyclines group *K. Pneumoniae* showed zone of inhibition 13mm for oxytetracycline followed by 12mm for doxycycline (Plate 3 and Figure 6).

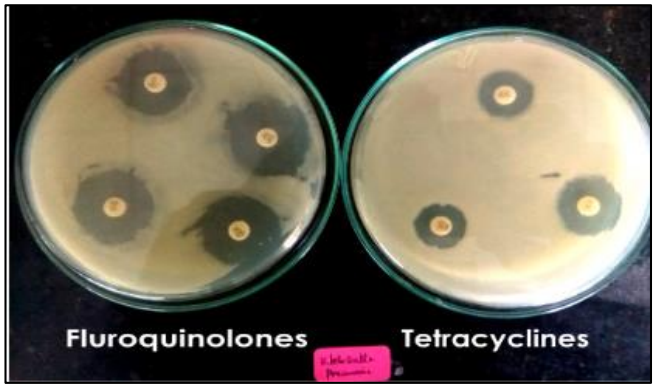

Plate 3: Antibiotic sensitivity test of *K. pneumonia*

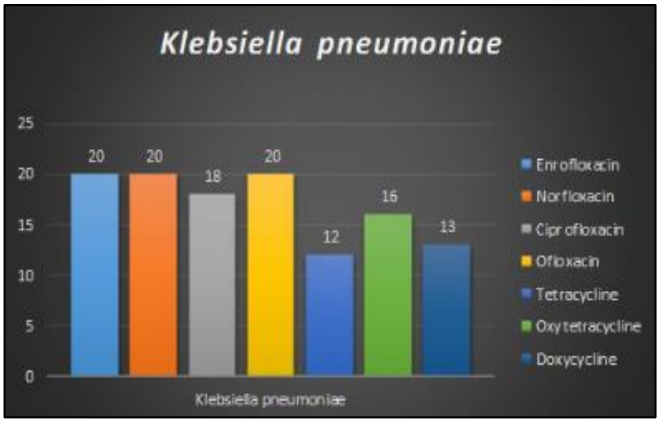

Fig 6: Zone of inhibition of *K. pneumoniae*

3.1.7 Staphylococcus capitus

S. capitus exhibited zone of inhibition of 26mm for norfloxacin followed by 25mm for enrofloxacin, 23mm for ofloxacin, 22mm for doxycycline, and 20mm for ciprofloxacin, tetracycline and oxytetracycline (Plate 4 and Figure 7).

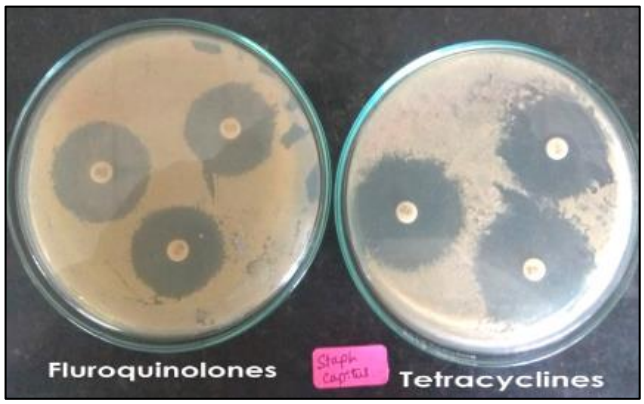

Plate 4: Antibiotic sensitivity test of *S. capitis*

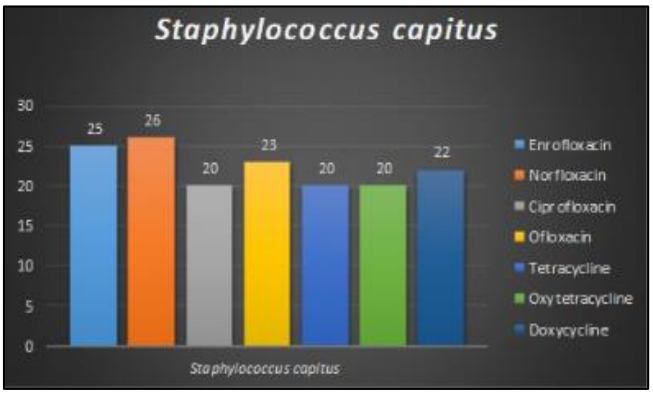

Fig 7: Zone of inhibition (mm) of *S. capitis*

3.1.8 Staphylococcus schleiferi

Both fluroquinolones and tetracyclines class antibiotics were highly sensitive for *S. schleiferi* organism. *S. schleiferi* exhibited zone of inhibition of 19mm for Norfloxacin and doxycycline followed by norfloxacin (18mm), ciprofloxacin (15mm), ofloxacin & tetracycline (14mm) (Plate 5 and Figure 8).

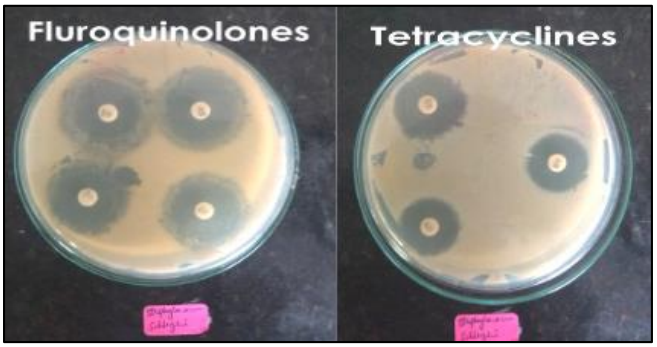

Plate 5: Antibiotic sensitivity test of *S. schleiferi*

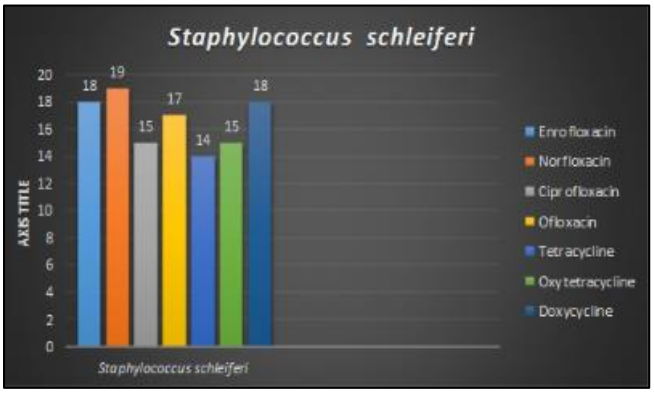

Fig 8: Zone of inhibition of (mm) of *S. schleiferi*

3.1.9 Enterobacter aerogenes

Enrofloxacin and Ciprofloxacin was found to be the most sensitive antibiotics against *E. aerogenes* with zone of inhibition 18mm followed by norfloxacin (17mm) and ofloxacin (16mm). With regards to tetracycline class antibiotics *i.e.*, doxycycline, tetracyclines, and oxytetracycline showed zone of inhibition 8mm, 7mm, and 4mm respectively (Plate 7 and Figure 10).

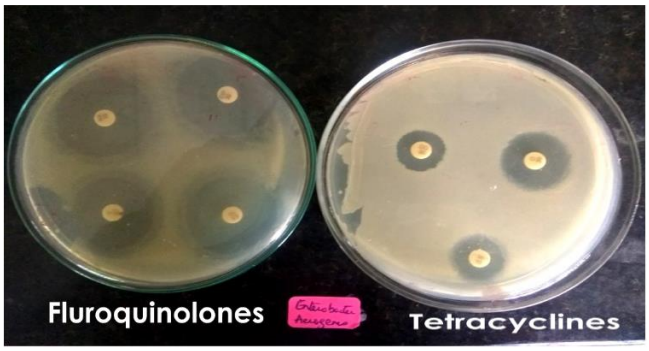

Plate 7: Antibiotic sensitivity test of *E. aerogenes*

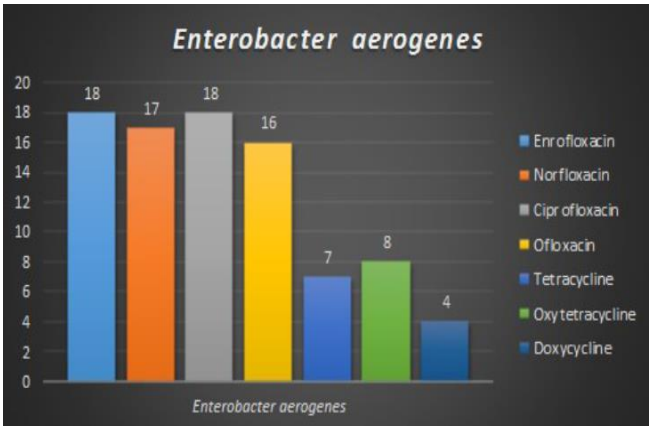

Fig 10: Zone of inhibition (mm) of *E. aerogenes*

3.2 Biofilm formation

With absence of intense black pigment in isolates and presence of red colonies it was interpreted as negative biofilm for the all the organisms tested *viz*. *E. coli, S. aureus, K. pneumoniae, S. capitis*, *S. schleiferi*, *E. aerogenes* (Plate 8-13). These findings depicted that all the bacterial isolates tested was sensitive for the selected fluroquinolones and tetracycline class antibiotics *viz*. norfloxacin, ciprofloxacin, ofloxacin, doxycycline, tetracycline, and oxytetracycline; the biofilm concentration was found to be resistant in all the cases (Plate 8-12).

4. Discussion

Mastitis is characterized by an increase in somatic cells, especially leukocytes, in the milk and by pathological changes in the mammary tissue (Ranjan *et al.* 2010) [7]. The occurrence of mastitis is an outcome of interplay between the infectious agents and the management practices (Sudhan and Sharma, 2010) [8] and the overall loss caused by mastitis to the national economy has been estimated to be Rs 16,072 million (Singh, 1994) [9]. Various microorganisms implicated to because mastitis includes *Staphylococcus*, *Streptococcus* and members of *Enterobacteriaceae etc*. Bacteriological culturing is considered most suitable, accurate and reliable method to confirm the presence of the causative organisms and many investigations have assured it as the gold standard for identifying intramammary infections and for developing a specific mastitis control program for a dairy herd. In the present study we found the presence of major bacterial pathogens causing mastitis namely of *E. coli*, ESBL *E. coli*, *S. aureus, K. pneumoniae*, *S. capitis S. schleiferi*, and *E. aerogenes*. The presence of Staphylococcus and Streptococcus organisms in mastitic milk is a common finding which has been observed by various workers. Fujikura and Shibata on bacteriological examination revealed that 72.4% of samples showed the presence of *S. aureus*, coagulase negative *Staphylococcus (CoNS), S. agalactiae, S. dysgalactiae, S. uberis, Corynebacterium pyogenes, Pseudomonas aeruginosa, Bacillus cereus* and members of *Enterobacteriaceae*. Various research investigators in the literature reported prevalence of *S. aureus* which was found to be 43% from the mastitic animals (Fujikura and Shibata, 1965; Gonzalez *et al*. 1980) [10, 11].

The data from various other studies indicated *Staphylococcus* to be the dominant organism among all the bacteria isolated from subclinical mastitic cases (Swartz, 1984; Schukken *et al.* 1989; Kerro Dego and Tareke, 2003; Singh *et al*. 2018) [12, 13, 14, 15]. High rate of isolation of *Staphylococcus spp.* is mainly attributed due to the fact that the principal reservoirs of *Staphylococcus spp.* are the skin of the udder and milk of the infected gland. Additionally, Staphylococcus has the capacity to penetrate into the tissue producing deep seated foci protected by a tissue barrier (Ranjan *et al.* 2010) [7]. Also, the high frequency of staphylococcal mastitis is considered to be due to the existence of inadequate hygiene in the dairy industry, poor animal health services and lack of proper attention to the health of the mammary gland in general. A combination of all or two or more than two organisms was found in 86 % samples indicating that more than one organism was causing mastitis. The above finding is similar to the findings of many workers in which they reported the presence of mixed infection (Gurleen *et al*. 2017; Saidi *et al*. 2013; Hawari and Al-Dabbas, 2008) [16, 17, 18].

ABST test helps to understand the resistance and susceptibility of bacteria towards a particular drug and thus helping in the choice of drug to be used for treatment. Overuse and misuse of antimicrobial agent led to an increased antimicrobial resistance around the world leading to treatment failures in infectious diseases of human and animal. Thus, seven antibiotics were chosen for the study according to their common use in research, human medicine and veterinary practice. They belonged to the following groups: Quinolones (Enrofloxacin, ciprofloxacin, norfloxacin and ofloxacin) and Tetracyclines (oxytetracycline, tetracycline and doxycycline). Antibiogram study of the individual organisms isolated *viz*., *E. coli, S. aureus, K. pneumoniae, S. capitus, S. schleiferi, E. aerogenes* was done and the *E. coli* isolates and *ESBL E. coli* revealed highest sensitivity to enrofloxacin, norfloxacin, ofloxacin and ciprofloxacin and highest sensitivity to oxytetracycline, doxycycline and tetracyclin followed by *ESBL E. coli*. The results of this study were similar to the earlier findings (Gurleen *et al.* 2017; Mir *et al*. 2014; Moges *et al*. 2011) [16, 19, 20].

Plate 8. Biofilm formation
Escherichia coli (negative)

Plate 9. Biofilm formation
Klebsiella pneumoniae (negative)

Plate 10. Biofilm formation
Staphylococcus capitus (negative)

Plate 11. Biofilm formation
Staphylococcus schleiferi (negative)

Plate 12. Biofilm formation
Enterobacter aerogenes
(negative)

5. Conclusions

In conclusion, based on antibiotic sensitivity test Tetracyclines class antibiotics i.e., oxytetracyclines, tetracyclines, doxycycline was found to be more sensitive antibiotics against mastitis causing microorganism as compared to Fluroquinolones class antibiotics i.e., enrofloxacin, norfloxacin, ciprofloxacin and ofloxacin.

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