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In *Vitro* Biofilm Study in Tetracycline Resistant *Escherichia Coli* from Mastitis Samples

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ABSTRACT

Antimicrobial resistance is a global concern in both human and veterinary medicine as there is therapeutic failure for infectious disease. Every year millions of people dying worldwide because of multiple drug resistance (MDR). Multiple drug resistant bacteria and genes can transfer between humans and animals. Transfer of MDR bacteria and genes form food producing animals to humans has great contribution in spread and occurrence of MDR. Drug resistance in mastitis producing organism is very common due to indiscriminate use of antibiotics in the treatment of mastitis. Among various resistance mechanisms biofilm formation of antibiotic resistance is gaining importance as mastitis causing organism are more prone to biofilm production due to availability of raw materials like lactose for biofilm synthesis and causing therapeutic failure. In this study biofilm is assayed by simplest method called congo red dye assay in which congo red dye is used and sucrose is added as energy source. Six tetracycline resistant *E. coli* strains are analysed for biofilm and all *E coli* isolates from mastitis milk were negative for biofilm production. *E coli* used in this study failed to produce biofilm may be because involvement of other resistant mechanisms like altered binding site, Efflux pump and inactivation of antibiotics by enzymes.

Keywords : Congo Red Dye, Coli Strains, Escherichia Coli, Vitro, MDR, EPS, Staphylococcus

I. INTRODUCTION

Antibacterial resistance is the ability of bacteria to resist the effects of an antibacterial to which they were once sensitive. Antibiotic resistance is one of the biggest threats to global health, food security, and development today (WHO, 2017). Multiple drug resistance is the ability of bacteria to resist the effects of an antibacterial to which they were once sensitive. Broadly, it is the property of bacteria that overcome the bacteriostatic or bactericidal effects of antibiotics, to which they were earlier sensitive, resulting in their survival despite exposure to standard doses of antibiotic(Sharma *et al.*, 2018). MDR occurs by various mechanisms like impermiability of antibiotics, target site mutation or efflux pump,inactivation of antibiotics by active enzymes and biofilm formation (Poole, 2001). Impermiability of antibiotics into the cell occurs due to difference in the cell wall structure of gram positive and gram negative organisms whereas target mutations occurs due to decreased influx or increased efflux of antibiotics. Inactivation of antibiotics by enzyme produced by bacteria is another mechanism of resistance seen in penicillins which are inactivated by penicillinase enzymes. Efflux systems are the proteins or glycoproteins located inside the cell membrane responsible for the removal of toxic materials from the cell. Along with other toxic materials they also cause efflux of antibacterial. Efflux pumps are the major contributor to the MDR and they causes resistance to various class of antibacterial (Biochimie, 2005). It was also reported that efflux pumps contribute to biofilm formation by supply of raw materials to produce extracellular matrix.

Biofilm is defined as microbial communities adhered to a biotic or abiotic surface and enclosed within an extracellular polymeric substance (EPS) produced by the bacteria (Percival *et al.*, 2011) Biofilm contribute approximately 61% of human zoonotic disease and 99% of bacteria in nature exists inn biofilm.it is also estimated that cells in biofilm can be up to 10,000 times more resistant than normal cells(Chakraborty *et al.*, 2018)..Biofilm contributes to 65% of nosocomial infections in humans. It is difficult to eradicate biofilm as they are resistant to host defence mechanism and antibiotics fails to act on bacteria inside the biofilm due to their impermiability. Biofilm contribute to resistance by various mechanism also it is the main reason for therapeutic failure in many clinical conditions due to failure of entry of antibiotics through biofilm extracellular matrix to produce their desirable effect on host(Of *et al.*, 2015). Biofilm increases the pathogenicity of bacteria and decrease the susceptibility of bacteria which is used for preventive and therapeutic approach in cases of infectious disease (Gupta *et al.*, 2016).

Among various infectious disease of bovines like mastitis, foot and mouth disease, haemorrhagic septicaemia (HS) mastitis is a major economic disease because reduced milk production, loss due to veterinary service, loss due to treatment, loss due to milk discarded , loss due to culling etc. (Nabi *et al.*, 2014).

Etiology of bovine mastitis was classified as bacterial mastitis; mycotic/fungal/algal mastitis; mycoplasmal and nocardial mastitis. Viral mastitis is least clinical significance. Among these bacterial mastitis is pathologically and thus economically the most significant Saheen *et al.* (2016). Among various etiological agent mastitis due to *staphylococci* are the most common mastitis in all animals like cows, buffaloes, ewes, does, llamas, dromedaries, rabbits, dolphins and women. They are closely followed by *streptococci* and *E. coli* which also contributed to the major prevalence of mastitis. Less commonly, other Gram-positive (*Actinomyces* spp., *Corynebacterium* spp., *Bacillus* spp., *Mycobacterium* spp., *Enterococcus* spp., *Clostridium* spp.) and Gram-negative (*Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp., *Serratia* spp., *Proteus* spp., *Pasteurella* spp., *Mannheimia haemolytica*) Contreras and Rodríguez (2011) Among the various mastitis causing bacteria *E coli* contributes to highest percentage. It was reported that 56.1% of the bacterial isolates showed resistance to one or more antibiotics. Among them (50.64%) of *E. coli* bacteria shown resistance to antibacterial agents which was the major pathogen showing predominant resistance to antimicrobial agents followed by *Staphylococcus* species (44.25%) Chandrasekaran *et al* . (2014[a]). Mastitis due to *E coli* can be a transient infection and persistent infection. Favouring production of biofilm occurs in the infected mammary gland due to stress condition produced on bacteria. Stress is produced to *E coli* in the infected mammary gland due reduced concentration of glucose and lactose by the fermentation of bacteria(Costa *et al.*, 2014).

II. MATERIALS AND METHODS

Escherichia coli isolates from mastitis are collected from department of Microbiology ,Veterinary college, Bangalore, Karnataka Veterinary and Animal Sciences University. These isolates were previously confirmed as *E coli* and their resistance pattern for tetracycline also confirmed. Collected samples are incubated for 24 hours at 37^0c temperature. Culture was confirmed for *E coli* by gram staining and are subcultured. Sub cultured strains were stored in -20 through glycerol preservation for future use. Antibiogram of *E coli* strains is done by Muller Hinton agar according to CLSI guidelines. Bacterial culture adjusted for 0.5 Mcferland unit and are smeared on Muller Hinton agar plate. Tetracycline and oxytetracyline disc were place on agar plates and incubated for 24 hours at 37^0c temperature. Zone of inhibition for the antibiotics is measured and analysed for resistance pattern.

Resistant strains were selected for further biofilm study to check the resistance mechanism by Congo red dye method (Dias *et al.*, 2013) and (Mariana *et al.*, 2009) Congo red agar is prepared by addition of congo red dye (Himedia) 0.8gram, sucrose 36 gram, brain heart infusion agar at 56 gram per 1000 ml of distilled water which is autoclaved for 21 minutes at at 121 temperature,15 lb pressure and the plates were incubated at 37 for 24 hours. Resistant *E coli* strains were streaked on congo red plates and incubated for 48 hours. Plates are analysed for biofilm formation by colour of colonies on congo red plates after 48 hours of incubation. Biofilm producing strains form black colonies whereas non biofilm producers produce pink colonies.

III. RESULTS AND DISCUSSION

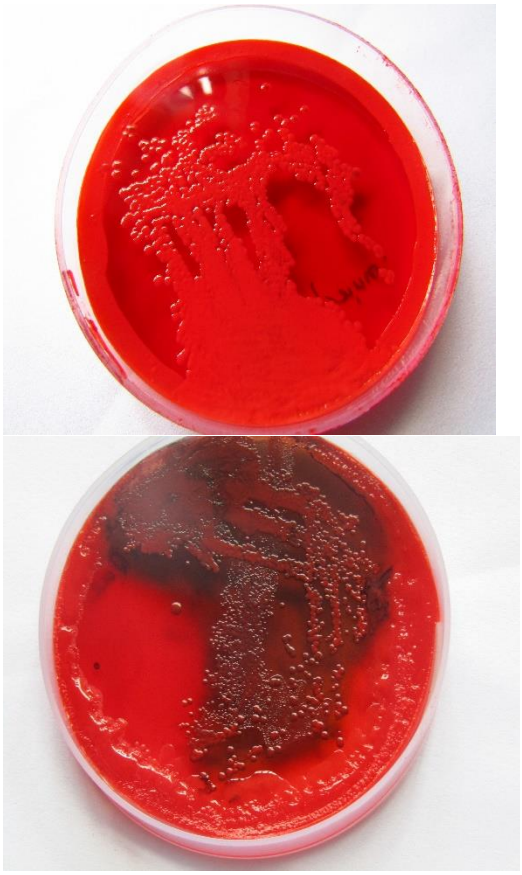

Fig 1 : Congored agar plates with biofilm positive and negative sample:

Pink colony indicates negative biofilm formation, black glistening colony indicates positive biofilm formation. All the twelve strains of tetracycline and oxytetracycline resistant *E coli* isolates from mastitis shown negative for biofilm formation by congored dye method even after treating with antibiotic and piperine. *E coli* used in this study failed to produce biofilm may be because involvement of other resistant mechanisms like altered binding site, Efflux pump, inactivation of antibiotics by enzymes etc (Biochimie, 2005, Sana *et al.*, 2015). Biofilm formation may be hindered by other contributing factors like incubation temperature (Of *et al.*, 2015). In *in vitro* conditions, compared to gram positive bacteria, gram negative shows less biofilm producing phenomenon because of micro anatomy of cell wall of gram positive and gram negative. Small pore size in gram negative bacteria like *E coli* are not much prone for biofilm production due to failure in supply of biochemical molecules for extracellular polysaccharide matrix. Polysaccharide in the gram negative bacteria are neutral or poly ionic, whereas polysaccharide in gram positive bacteria is cationic in nature (Vu *et al.*, 2009). Biofilm production by *E coli* is variable and it depends on strains, different growing conditions and different environmental factors. Remarkable variability also seen with the growth medium used(Naves *et al.*, 2008).

IV. CONCLUSION

Microorganism of capable of growing in free form (plantoic form) or biofilm form (Percival *et al.*, 2011).Infected udder contributes to biofilm production (Costa *et al.*, 2014) but in *in vitro* conditions biofilm production may not fallow the same mechanisms. In the present study none of the strains of tetracycline resistant E *coli* showed biofilm production may be due to involvement of other resistant mechanism.

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