Prevalence of the bovine mastitis causing bacteria and confirmation of the virulence potential of Mammary Pathogenic *E. coli* (MPEC)

By

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(NUST 2017ASAB)

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I certify that this research work titled **"Prevalence of the bovine mastitis causing bacteria and confirmation of the virulence potential of Mammary Pathogenic *E. coli* (MPEC)"** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Asim Zahoor Abbasi

Master of Science in Industrial Biotechnology

Registration No. 00000205105

DEDICATION

Dedicated to the beloved memory of my brother (Zahid Mahmood Abbasi) who departed without saying a goodbye!

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Asim Zahoor Abbasi

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List of Abbreviations

CRA	Congo Red Agar
E. coli	*Escherichia coli*
EDTA	Ethylenediamine Tetraacetic acid
EMB	Eosin Methylene Blue
ExPEC	Extra-intestinal Pathogenic *Escherichia coli*
FAO	Food and Agriculture Organization of United Nations
M. Luteus	*Micrococcus Luteus*
MDR	Multi-Drug Resistant
ME	Mastitis causing *Escherichia Coli*
MHA	Mueller-Hinton Agar
MPCR	Multiplex Polymerase Chain Reaction
MPEC	Mammary Pathogenic *Escherichia Coli*
N- Broth	Nutrient Broth
N-Agar	Nutrient Agar
O.D	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase Chain Reaction
pH	potential of Hydrogen

S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SFMT	Surf Field Mastitis Test
Spp.	*Specie*
STEC	Shiga Toxin producing *Escherichia coli*
STs	Sequence Types
TE	Trisaminomethane Hydrochloride - Ethylenediamine Tetraacetic acid
Tris HCl	Trisaminomethane Hydrochloride
TSB	Trypton Soy Broth
UPEC	Urinary Pathogenic *Escherichia Coli*
UTI	Urinary Tract Infection
VAGs.	Virulence Associated Genes

Abstract

Bovine mastitis is the inflammation of the mammary glands due to physical injury or microbial infection. It is one of the most common and expensive disease of dairy industry resulting huge economic loss due to reduced milk production. Mastitis can be further classified into clinical and sub-clinical types based on the degree of inflammation. In this study, prevalence of bovine mastitis in the Rawalpindi district along with prevalence of mastitis causing bacteria in mastitis positive milk samples was investigated. Moreover, antibiotic susceptibility pattern, ESBL production, *in vitro* virulence properties, phylogenetic classification, prevalence of virulence associated genes (VAGs) and toxin genes were also analyzed for Mammary Pathogenic *E. coli* (MPEC). The prevalence of mastitis found was 13.98% (10.6% subclinical, 2.11% clinical) and 22% (20.58% subclinical, 1.47% clinical) for buffalos and cattle respectively. The most prevalent isolated bacteria included *S. aureus*, *Streptococcus spp.* and *E. coli* occurring in 80%, 30% and 26.78% respectively of total mastitis positive milk samples. Except for one, all MPEC isolates (95.45%) were Multi-drug resistant (MDR) and ESBL producers. All MPEC isolates were α-hemolytic while most isolates showed positive Congo red binding activity, different motility activities, low to medium biofilm formation and high growth in human urine. Most MPEC isolates belonged to D group (50%) followed by B1, B2 and A group *E. coli* at 27.27%, 18.18% and 9.09% respectively. Among VAGs, *iss* and *fimH* gene occurred in around 95.45% samples while *papC* and *cvaC* occurred in less than half number of samples. Prevalence of Shiga toxins also was high (59.09% for *stx1*and 4.54% for *stx2)* while *hlyA* and *eaeA* were present in 9.09% and 13.63% isolates respectively. The study reports high prevalence of bovine mastitis with very high resistance pattern of MPEC isolates and different virulence associated properties.

1. Introduction

Ever since the dawn of human civilization and earlier, humans have been domesticating animals particularly for milk, meat and other products. Cattle and Buffalos are mostly raised for milk and provide most of the world's milk demand. Milk is a wholesome food replete with macro and micronutrients. Milk and milk products constitute not only an important part of human diet but also contribute to the livelihood of people by providing employment in dairy sector. Increasing standards of living in developing world has increased consumption of dairy products. In 2018, worldwide production of milk increased by 2.2 percent to 843 million tones than the previous year. Number of factors caused increase in global production including improvements in collection of milk, increase in milk yield per cattle and consumer demand. Pakistan is one of the largest producers of Milk and production of Milk in Pakistan has been increasing by over 3 percent in the last few years (FAO, 2019). Thus, any disease affecting health and productivity of bovines not only affect the general wellbeing of the animal but could also devastate livelihood of communities depending on bovines. Besides, diseases having zoonotic potential can spread to human population and with increasing interconnectedness of communities due to increasing trade and travel, the likelihood is higher than ever before. So, it is important to consider wellbeing of not only humans but also the animals and environment.

Increase in use of antibiotics around the world has increased the resistance of microbes to an unprecedented level presenting great challenge to tackle in dairy industry. Resistant microbes can defend themselves against antibiotics by acquiring certain properties which render those antibiotics weak or ineffective. Increasing resistance of mastitis causing microbes not only makes antibiotic therapy ineffective in the future use, but also could lead to transfer of resistant bacteria to humans

(Ungemach, 1999). Since organisms adopt and survive in relation to their environment, resistance patterns differ in different parts of the world. So, it is pertinent to design treatment strategies by investigating resistance patterns and virulence associated properties of pathogens (Aarestrup, 2005)

Bovine Mastitis is one of the most common dairy cattle diseases, and in Pakistan, the disease is classified as primary dairy cattle malady (Hortet P. *et al.*, 1998, khan *et al.,* 1991). The disease causes inflammation of the mammary gland and the degree of inflammation is determined by causative agent and host immune response (Burvenich *et al.,* 2003). Mastitis is associated with increased treatment costs, decreased milk production, and is one of the most common cause of death of dairy cattle (Kossaibati, M. A *et al.,* 1997). Moreover, due to decrease in quality, the milk cannot be processed further into value added products because of decrease in lipids, casein and lactose content while simultaneously increasing ions and enzymes concentration (Girma 2001; Shitandi 2004). Apart from economic losses and compromised animal health, the disease also poses threat to human health because of its zoonotic and food toxin contamination potential (Blum *et al*., 2008, Fernandes *et al*. 2011). Sub-clinical form of Mastitis which usually precedes clinical mastitis, is harder to detect as the cattle shows no visible signs of disease in the milk or on body. The sub-clinical form is also predominant being 15-40 times more widespread than clinical Mastitis (Shearer *et al.,* 2003). In Sub-clinical mastitis, the number of somatic cells exceed 200000 per milliliter of milk (Hinthong *et al*., 2017). Increased numbers of somatic cells are an indicator of infection of the mammary glands because these cells are produced in higher than normal levels in response to infection of the udder. Generally, neutrophils compose 75 percent while epithelial cells constitute 25 percent of total somatic cell count (Sharma *et al.,* 2011)

Mastitis is primarily caused by Bacterial pathogens. The onset of Mastitis occurs when infectious microbial agents cross the animal's teat into the udder, followed by multiplication of pathogenic microbes and production of toxins that are detrimental to the health of the udder. Moreover, due to tissue damage of the mammary glands and elevated vascular permeability, undesirable changes occur in milk quality including presence of blood and blood components, enzymes, salts and proteins. While milk components which determine milk quality like caseins, lactose and fats get decreased (Østerås, 2000; Harmon, 1994). Mastitis causing bacterial pathogens can either be environmental borne or contagious microorganisms. Contagious microorganisms spread from cattle to cattle using cattle udder as main repository site and include bacteria such as *Styphlococcus aureus* and *Strepococcus Agalactiae*. Environment borne microorganisms include *E. coli, Klebsiella species* and *Streptococcus uberis*, and are found primarily in farm settings. In majority of cases, contagious microorganisms cause subclinical mastitis while environmental bacteria cause clinical mastitis (Harmon, 1994). Up to 137 different pathogens have been recognized as causative agents of bacterial bovine mastitis, and among them streptococcus, staphylococcus and gram-negative coliforms such as *E. coli* and *Klebsiella spp*. have been implicated as major causative organisms. Ever since the implementation of mastitis control programs in different parts of the world, the prevalence of mastitis causing microbes has been changing most importantly a decrease in contagious mastitis causing microbes has been seen and an increase of intramammary infections causing environment borne gram negative coliforms (Barkema *et al.,* 1998).

E. coli is a gram negative, rod shaped coliform belonging to Enterobacteriacae family. Coliforms generally reside in the intestine of mammals where they play an important role in maintaining a healthy microbiota by fighting pathogens. *E. coli* particularly resides in the large intestine in a mutually beneficial relationship, and so, are called commensal bacteria. However, pathogenic

strains of *E. coli* also exist and cause severe and fatal infections. Because of the highly adaptive nature of *E. coli*, some strains have acquired virulence properties which make them fit for colonization of new niches where they cause disease. These virulence encoding genes are located on mobile genetic elements which allows transfer of the virulence genes to other bacteria. The virulence associated genes can also be immobilized and made a permanent part of genome of a pathogenic *E. coli* by getting assimilated. The most successful combination of genes responsible for survival and Pathogenesis ensures the survival of pathogenic *E. coli* in the long run to make a "Patthovar". Other than acquisition of mobile genetic elements and assimilation of these sequences, *E. coli* can also have genetic rearrangement, point mutations and deletions to make strains that were once commensal pathogenic (capable of causing various fatal diseases). *E. coli* possess certain adhesins which allow them to colonize areas not in their niche. These adhesins often form particular structures called fimbriae which then are classified into various types based on structure and function. Once bound to a host cell, the cell surface structures of the bacteria can bind to particular receptors on the surface of host cells triggering responses like inflammation, septic shock and death of the host cell and organism (Kaper *et al.*, 2004). Pathogenic *E. coli* have been classified into 8 classes or Pathovars owing to differences in attachment to host cells and mechanism of infection. These pathovars are further divided into two broader categories namely diarheagenic and Extraintestinal pathogenic *E. coli* (ExPEC) (Croxen *et al.,* 2010). Apart from few enteric or intra-intestinal pathogenic *E. coli* (being able to invade and replicate inside epithelial cells and macrophages), most of the pathogenic strains of *E. coli* are Extra-intestinal. The pathogenic strains of *E. coli* that can colonize body organs outside the intestine are named as Extra-intestinal Pathogenic *E. coli* or ExPEC. The ability of Extra-intestinal infection causing *E. coli* seems to relate with the entry of ExPEC into organs other than the intestines (Russo *et al.*, 2000)

E. coli have been divided into four groups on the basis of phylogenetic data. The phylogenetic classification includes four groups (A, B1, B2, and D). Traditionally, the phylogenetic grouping was done by multilocus enzyme electrophoresis and ribotyoping. However, the complexity involved in the usage of both techniques resulted in development of simple and better approach by Clermont *et al.*, 2000 using amplification of three genes i.e., *yjaA*, *chuA* and TSPE4.C2 by multiplex PCR technique. Generally, commensal strains belong to group A of this phylogentic classification while most ExPEC strains belong to group B2 and to some extent to group D.

Intestinal microbiota is subjected to enormous antibiotic selection pressure due to the increase in use of antibiotics in dairy industry. *E. coli* being a resident of lower intestines encounters such pressure which could explain the diversity and resilience of these microbes. Unprecedented use of antibiotics is driving evolution of microbes towards resistance to commonly available antibiotics used in the dairy industry particularly against Amoxicilin, Streptomycin, Oxatetracyclin and Sulfonamides. 3rd and 4th generation cephalosporins and fluoroquinolones are an important class of antibiotics which can be used against *E. coli* causing mastitis. But resistance against these two important classes is also reported around the world due to the production of Extended spectrum beta-lactamase (ESBL) enzymes by *E. coli* albeit at different levels (Suojala *et al*.,2013). Generally antibiotic resistant genes are found in plasmids and therefore can be transferred to other bacteria. The wide scale use of antibiotics helps in selection and transfer of bacterial virulence properties (Soujala *et al*.,2010). The resilience of *E. coli* strains against antibiotics differ among members of different phylogenetic groups expounding role of genetic background. Generally, *E. coli* belonging to A and D group are more likely to be resistant to third generation cephalosporins than other groups while B group *E. coli* are most susceptible among the phylogenetic groups. This explains

the fact that a smaller number of B group *E. coli* are found among other groups of *E. coli* in domestic animals due to extensive antibiotics use (Tenaillon *et al*., 2010).

Being an environmental bacterium, *E. coli* is able to enter the udder and cause disease only if it is not already killed by Host's immune defense system suggesting presence of particular virulence associated properties. These properties include ability to invade and adhere to the epithelial cells of mammary gland followed by establishment of disease. However, there is no definitive set of virulence associated genes to characterize mastitis causing *E. coli*. The most important virulence associated genes investigated in mastitis causing and other pathogenic *E. coli* include adhesion and invasion factors like P-fimriae, *fimH* etc., and other genes responsible for pathogenesis of *E.coli* like shiga toxin, intimin and hemagglutinin transporter and various other genes describing how pathogenic *E.coli* infect various organs and animals (Zhang *et al*., 2018).

2. Review of Literature

2.1 Pathogenicity of *E. coli*

As expected, there is a greater tendency for virulent clone-related *E. coli* groups to express virulence factor, compared to other members of *E. coli* (Johnson *et al*., 1991). Also, the severity of *E. coli* infection is directly impacted by the total number of virulence factors expressed by bacteria. However, despite the established association of virulence factors in wild-type strains with disease development, it is believed that they alone are by themselves not usually enough for pathogenesis, thus underscoring the need to explore other yet unknown virulence-related properties of *E. coli*. Although of both scientific and epidemiological interest, defined clinical role is not apparent for virulence factors. Meanwhile, therapeutic interventions targeting the UTI virulence factor have shown great promise in animal models and thus may provide a basis to improve the prevention of this bacterial disease in humans.

2.2 Virulence factors of Pathogenic *E. coli*

Pathogenic *E. coli* normally contain several virulence factors, including adhesins, toxins, invasins, and capsule (Fernandes *et al*., 2011). Further, this microorganism has developed the means to counter serum factors, iron scavenging etc. Mastitis in cows somewhat is like urinary infection in that the disease develops over time and is caused by environmental bacteria. Virulence factors are important in these diseases as they handle host selection pressure and enable bacterial colonization, persistence and survival (Kaipainen *et al*., 2002). Biofilm formation may also pose additional problems but research in this regard is not yet conclusive, thus underscoring the need to conduct further studies (Melchior *et al*., 2006).

2.3 Extended Spectrum Beta-Lactamase producing *E. coli*

Ever since the year 2010, the increased spread of Extended Spectrum Beta-Lactamase-producing bacteria like *E. coli* has created a state of alarm in healthcare settings (Chong *et al*., 2018). This trend has been partly responsible for bacterial colonization and subsequent development of infection. Along with that, the increased occurrence of ESBL-producing bacteria has led to difficulties in distinguishing between hospital-acquired infections and community-acquired infections (healthy people with no epidemiological link with healthcare settings). This observation that accumulating amount of evidence points to the fact that ESBL-producing bacterial strains are increasing with the passage of time make scientists wonder about the underlying reasons behind this phenomenon. Research is ongoing and although some links are established, it may take some time for scientists and researchers working in modern medicine to answer these fundamental questions related to bacterial diseases.

2.4 Bovine mastitis

Mastitis is a complex multifactorial disease and remains the costliest disease affecting dairy animals. As expected, the incidence of this disease is directly related to the level of exposure to pathogens, as well as other factors such as performance of host udder immune mechanisms, along with environmental factors, as well as interlinkage of all these factors (Manasa *et al*., 2019). Mastitis in clinical setting is established by the appearance of abnormal milk containing visible clots, or milk color changes, swelling of glands etc. This form of mastitis can be per acute, acute, subacute, and chronic. The causative agents of this disease are Klebsiella species such as *Klebsiella pneumoniae*, *Klebsiella oxytoca* or *Escherichia coli* (more common). The milk reduction attributed to clinical mastitis could result in 30% of the total losses. *E. coli* is reportedly amongst the most common pathogens in environmental mastitis and may lead to sudden fever, loss of appetite,

diarrhea, toxaemia and show pain, swelling, with discharge of bloody or watery milk. Necrosis of the mammary epithelium happens due to the severe, naturally occurring clinical *E. coli* mastitis, as well as during severe experimental *E. coli* mastitis (Bradley *et al*., 2001). Early identification of the prevalence and distribution of causative pathogens is a prerequisite to better prevent diseases and guide treatment options.

2.5 Mastitis causing *E. coli*

Other studies have been conducted to assess the genetic relationships of Mammary pathogenic *E. coli* (MPEC) isolated from bovine mastitis-associated milk. Notably, a study analyzed a total of eighty-two *E. coli* samples from bovine mastitis-infected milk samples (Nüesch-Inderbinen *et al*., 2019). They also examined the presence of virulence factors in *E. coli* samples using PCR and performed antibiotic susceptibility testing using disk diffusion method. Their analyses projected the two most prevalent phylogenetic groups: Group B1 accounting for about 41.5% of the isolates and Group A accounting for 30.5% of the isolates. This study identified a total of 35 different sequence types (STs), with STs 1125, 58, 10, and 88 having prevalence of 11, 9.8, 8.5, and 7.3 percent, respectively. Although the virulence factors of the *E. coli* isolates varied somewhat, their study successfully identified the three most common virulence factors *traT*, *fyuA* and *iutA*, with most isolates (72%) containing *iutA* thus underlying the potential biomarker candidate gene. The authors of the study concluded that *E. coli* strains of the sample population displayed a high level of diversity, but noted the occurrence of multiple strains with same STs thus indicating the close relationship between these same STs containing isolates. Nevertheless, their analyses confirmed the high susceptibility of the bacterial isolates to clinically relevant compounds.

2.6 Antibiotic Susceptibility of MPEC

Yet another group similarly conducted a study on the prevalence of *E. coli* in bovine mastitis-associated milk samples (Zhang *et al*., 2018). They performed antimicrobial susceptibility testing and virulence gene profiling. Their analysis also suggested that the *E. coli* isolates majorly were categorized into group A (47/90) and group B1 (22/79). However, in contrast, they found that *ompC* was detected in all the isolates, followed by *fimH* (89.9%), *ECs3703* (88.6%) and *ompF* (73.4%), and they noted the absence of most virulence genes in the bacterial isolates. The isolates showed susceptibility to aminoglycosides and fluoroquinolones as per the antibiotic susceptibility test. Another thing they noted was the inverse function between *ompF* expression level and antimicrobial resistance. Like the previous study, the authors indicated the emergence of antibiotic resistance in *E. coli* from bovine mastitis and recommended the counter control measures as well as further surveillance of bovine mastitis samples to consider a larger sample population for conclusive judgement.

2.7 MPEC Pathotypes

Bovine mastitis associated with *E. coli* or *E. coli* mastitis maybe caused by either environmental, commensal *E. coli* strains or by other pathotypes such as Enteropathogenic *E. coli*, Extraintestinal *E.* coli and Shiga Toxin-Producing *E. coli*. Quite unfortunately, not many studies have been conducted to determine the prevalence of STEC in *E. coli* mastitis and as such this query has not been resolved yet and may remain undiagnosed and underreported and thus not noticed (Murinda *et al*., 2019). This aspect of research is also crucial in order to establish the virulence potential of *E. coli* mastitis and encourage routine surveillance. Hence, public health policy must be updated to enable the detection of STEC-containing *E. coli* mastitis samples. This may inform mitigation

efforts designed specifically for prevention or control of bovine mastitis, the costliest diseases in dairy cows.

2.8 Shiga Toxin-Producing *E. coli* (STEC)

According to multiple studies, STEC strains have a greater tendency to cause milk-poisoning (Solomakos *et al*., 2009, Stephan *et al*., 2008). Especially, mastitis-associated milk and particularly subclinical mastitis contains STEC strains. Non-standard practices during milk collection and processing, as well as contamination of milking machine, along with low quality milk, necessitate the need to take proper care of milk. To make matters worse, the pathogenesis of bovine mastitis is still not understood well, despite being the focus of research. E. coli is a common environmental bacterium known to survive in a variety of environmental conditions. During parturition and after lactation onset in cows, the immune system is not strong enough to face challenges posed by the presence of bacteria. This enables microorganisms like *E. coli* to cause mastitis. Metabolic and hormonal influences may also downregulate immune responses. Also, due to the unique nutritional requirements of the cow during late pregnancy and lactation, ketosis may occur, and the circulating neutrophils antibacterial activity may get affected, thus increasing the risk of cows to develop mastitis (Suriyasathaporn *et al*., 2000). All these factors in unison lead to the greater bacterial presence in the udder to cause problems in cows (Diez-Fraile *et al*., 2003).

3. Materials

3.1 Bacteriological Media Used in the Study

Following media were used for growth, isolation and in vitro pathogenicity Analysis of Bacteria. Recipe of all the media used is described below. All medias were dissolved in distilled water and autoclaved

Table 3.1.1: Nutrient Agar (N-Agar)

Sr. No	Ingredients	Quantity in g/L
1	Nutrient Broth	13
2	Agar	15

Table 3.1.2: Nutrient Broth (N- Broth)

Sr. No	Ingredients	Quantity in g/L
1	Nutrient Broth	13

Table 3.1.3: Trypton Soy Broth (TSB)

Sr. No	Ingredients	Quantity in g/L
1	Trypton Soy Broth	13

Table 3.1.4: MacConkey Agar

Sr. No	Ingredients	Quantity in g/L
1	MacConkey Agar	51.5

Table 3.1.5: Eosin Methylene Blue (EMB) Agar

Sr. No	Ingredients	Quantity in g/L
1	EMB Agar	37.5

Table 3.1.6: Mueller-Hinton Agar (MHA)

Sr. No	Ingredients	Quantity in g/L
1	MHA	38

Table 3.1.7: Blood Agar

Sr. No	Ingredients	Quantity in g/L (w/V) or (V/V)
1	Blood Agar Base	37
2	Sheep Blood*	50ml

Blood Agar media is prepared by autoclaving blood agar base dissolved in distilled water. Blood is added to lukewarm media bottle before pouring of the media into plates.

Table 3.1.8: Cetrimide Agar

Sr. No	Ingredients	Quantity in g/L (w/V) or (V/V)
1	Cetrimide Agar	46.7
2	Glycerol*	10ml

For 1000ml media, 46.7g Cetrimide agar was dissolved in 990ml distilled water and 10ml Glycerol was added to make 1L media.

Table 3.1.9: Congo Red Agar (Berkhoff *et al*., 1986)

Sr. No	Ingredients	Quantity in g/L
1	Congo Red	0.3
2	Bile Salts	1.5
3	Trypticase Soy Agar	30g Tryptone Soy Broth + 15g Agar

3.2 Motility Assay Media

Table 3.2.1: Swimming motility media (Qiao *et al*., 2012)

Sr. No	Ingredients	Quantity in g/L
1	Tryptone	10
2	NaCl	5
3	Agar	3

Table 3.2.2: Swarming motility media (Rashid *et al*., 2000)

Sr. No	Ingredients	Percentage
1	Tryptone	8
2	NaCl	20
3	Agar	5

Table 3.2.3: Twitching Motility media (Meng *et al*., 2005)

Sr. No	Ingredients	Percentage
1	Tryptone	10
2	NaCl	5
3	Yeast Extract	3
4	Agar	10

3.3 Solutions used in 96 well Microtiter dish biofilm assay (Mitchell *et* al., 2015)

Table 3.3.1: Crystal Violet solution (0.1% w/v)

Sr. No	Ingredients	Quantity in g/100 ml
1	Crystal Violet	0.1

Table 3.3.2: Saline solution (0.85% w/v)

Sr. No	Ingredients	Quantity in g/100 ml
1	Sodium Chloride	0.85

Table 3.3.3: Glacial Acetic acid (33% v/v)

Sr. No	Ingredients	Quantity in ml/100 ml
1	Glacial Acetic acid*	33ml

For 33% Glacial Acetic acid solution, 33ml Glacial acid was added and distilled water was added upto 100ml distilled water.

3.4 Solutions for genomic DNA extraction method by Heat Boiling method (Junior *et al*., 2016)

Table 3.4.1: TE Buffer for storage of DNA

Sr. No	Ingredients	Molar
1	Tris HCl	10mM
2	EDTA (pH: 8)	1mM

The pH of TE Buffer was adjusted at 7.5.

3.5 Antibiotics used in the study

The table below describes the antibiotics used in the study including the categories and zone sizes for interpretation of susceptibility or resistance of bacterial isolates (CLSI 2018).

Table 3.5: Antibiotics used in the study

S. No.	Antibiotic	Abbr.	Category	Conc. (µg)	S	I	R
1.	Amoxicillin	AML	Penicillin	10	≥ 17	14-16	≤ 13
2.	Ampicillin	AMP	Penicillin	10	≥ 17	14-16	≤ 13
3.	Amoxicillin-clavulanate	AMC	β-lactam combination agent	20/10	≥ 18	14-17	≤ 13
4.	Doxycycline	DO	Tetracycline	30	≥ 14	11-13	≤ 10
5.	Tetracycline	TE	Tetracycline	30	≥ 15	12-14	≤ 11
6.	Piperacillin-tazobactam	TZP	Penicillin- β-lactamase inhibitor	100/10	≥ 21	18-20	≤ 17
7.	Eratapenem	ETP	Carbapenem	10	≥ 22	19-21	≤ 18
8.	Meropenem	MEM	Carbapenem	10	≥ 23	20-22	≤ 19
9.	Imipenem	IPM	Carbapenem	10	≥ 23	20-22	≤ 19
10.	Gentamicin	CN	Aminoglycoside	10	≥ 15	13-14	≤ 12
11.	Tobramycin	TOB	Aminoglycoside	10	≥ 15	13-14	≤ 12
12.	Streptomycin	S	Aminoglycoside	10	≥ 15	12-14	≤ 11
13.	Amikacin	AK	Aminoglycoside	30	≥ 17	15-16	≤ 14
14.	Ciprofloxacin	CIP	Fluoroquinolone	05	≥ 21	16-20	≤ 15
15.	Norfloxacin	NOR	Fluoroquinolone	10	≥ 17	13-16	≤ 12
16.	Levofloxacin	LEV	Fluoroquinolone	05	≥ 17	14-16	≤ 13
17.	Trimethoprim-sulfamethoxazole	SXT	Folate Pathway Antagonist	25	≥ 16	11-15	≤ 10
18.	Nitrofurantoin	F	Nitrofuran	300	≥ 17	15-16	≤ 14
19.	Erythromycin	E	Macrolide	15	≥22	17-21	≤ 18
20.	Ceftriaxone	CRO	Cephalosporin	30	≥ 23	20-22	≤ 19
21.	Cefotaxime	CTX	Cephalosporin	30	≥ 26	23-25	≤ 22
22.	Ceftazidime	CAZ	Cephalosporin	30	≥ 21	18-20	≤ 17

* Abbr. = Abbreviation Conc.= Concentration

S = Sensitive I = Intermediate sensitivity R = Resistant

3. Materials and Methods

3.6 Study Area

On site testing for prevalence of Bovine mastitis sample collection was done in different dairy farms in Rawalpindi district. The district is situated in Potohar plateau, on the northernmost side of the province of Punjab with city of Rawalpindi as district capital. The district has a total area of 5,285 km^2 and is further divided into six tehsils (Pakistan Bureau of Statistics, 1998). Total population of Cows and Buffalos is 252,298 and 131,212 respectively while population of Lactating cows and buffalos is 104,950 and 69,920 respectively.

3.7 Determination of Sample size

To determine sample size, it is important to know expected prevalence. The expected prevalence of Mastitis in Cows is 23% while in Buffalos is 19% in Rawalpindi district. On the basis of the expected prevalence, total sample size for cows and buffalos was calculated according to the formula described by Thrusfield (Thrusfield, 2005).

$n= 1.96^2 P_{exp} (1- P_{exp})/d^2$

In above formula, n is the sample size, d is desired absolute precision which was taken as 5%, 1.96^2 is the value of standard deviation taken at 95% confidence level, and P_{exp} is the expected prevalence of the disease.

Based on the formula, total number of lactating cows and buffalos required were 272 and 236 respectively.

3.8 Population included in the study

Cross sectional study plan was designed to assess the prevalence of Mastitis in dairy bovines and cattle in Rawalpindi district, report prevalence of major mastitis causing microbes isolated from mastitis milk samples, further proceed E. coli to find its virulence associated properties.

The sampling started in April 2019 and continued until October 2019. The farms included in the study were selected randomly. Lactating cows and buffalos were included in the study from small, medium and large-scale farms. Management system of farms visited was noted in a questionnaire type paper which included measures like sanitation, floor type, management practices, milking method, and physical observation of the animal under study. The animals were either of local or cross breed. Before milking, each teat of the animal was washed with clean water to clean any manure or dirt from teats.

3.9 Examination of the Farms and Animals

Management system of farms visited was noted in a questionnaire type paper which included measures like sanitation, floor type, management practices, milking method, and physical observation of the animal under study. Physical characteristics included hygiene and any physical disabilities if present. The udder was observed for presence of any tick infection, leakage of blood, and blind teats.

The animals were either of local or cross breed. Before milking, each teat of the animal was washed with clean water to clean any manure or dirt from teats.

3.10 Collection of Milk Samples

All the cows and buffaloes in a dairy farm were tested for Mastitis by Surf mastitis test (SFMT) using 3% surf solution in distilled water. Presence of Mastitis in each lactating cow and buffalo from every teat was observed on clean mastitis testing tray for presence of any blood spills, milk colour and coagulation before putting surf solution on each quadrant. After pouing surf solution, the mastitis testing tray containing the milk samples was swirled. Formation of coagulation indicated the presence of Mastitis in the teat. Degree of coagulation was also assessed from high to low indicated by + for low level, ++ for medium, +++ for high, and ++++

for very high level of coagulation (Muhammad *et al*., 1995). The samples were collected in a sterile falcon tube and put in icebox and transported to lab for isolation of microbes.

Mastitis positive milk samples were put into different scoring levels based on degree of coagulation and milk's physical features and were either labelled as Sub-clinical or clinical mastitis samples.

3.11 Bacterial Culturing

After transfer of milk samples to the lab, falcons containing milk samples was vortexed to homogenise the milk. 10µL homogenised milk sample was aseptically inoculated on respective solid media plates and left for incubation for 24 to 48 hours maximum. If present, bacterial colonies appeared on solid media plates after incubation and were further processed.

3.12 Isolation of Bacteria

After transfer of milk samples to the lab, falcons containing milk samples was vortexed to homogenise the milk. 10µL homogenised milk sample was aseptically inoculated on respective solid media plates and left for incubation for 24 to 48 hours maximum at 37 °C. Bacteria were differentiated on the basis of colony characteristics, haemolytic activity, gram staining and biochemical identification tests (Quinn *et al*., 1994). For *S. aureus* and *S. epidermidis* identification, coagulase and catalase tests were performed.

3.12.1 Coagulase Test

For the Coagulase test, blood plasma was used. Bacterial culture taken with sterile inoculating loop was mixed in blood plasma. The bacterial sample was identified as coagulase negative if it was completely dissolved and as positive if bacterial sample formed clots.

3.12.2 Catalase Test

Three percent hydrogen peroxide was used for identification of catalase positive and negative microorganisms (Cheesbrough 1985), 4.2 ml of 35% hydrogen peroxide stock was mixed with 45.8 ml distilled water to make 3% Hydrogen peroxide dilution. Bacterial culture taken with sterile inoculating loop was mixed in 3% hydrogen peroxide and observed for effervescence. Those bacterial samples which produced bubbles were identified as catalase positive and those which did not were identified as catalase negative.

3.13 Extraction of genomic DNA

Genomic DNA of MPEC isolates was extracted to the method defined by Junior *et al*., 2016 with little modification. Overnight incubated bacterial cultured were centrifuged at 14000 rpm for 5 minutes to obtain cell pellet which was dissolved in 200 µL TE buffer and then boiled for 15 minutes. After boiling, each sample was put on ice and centrifuged for 5 minutes at 14000 rpm. The supernatant obtained contained DNA which was then stored at -20°C. The genomic DNA extracted was then used for detection of *uidA* gene (housekeeping gene in *E. coli*), phylogenetic grouping, and detection of virulence associated genes (VAGs) and toxin genes.

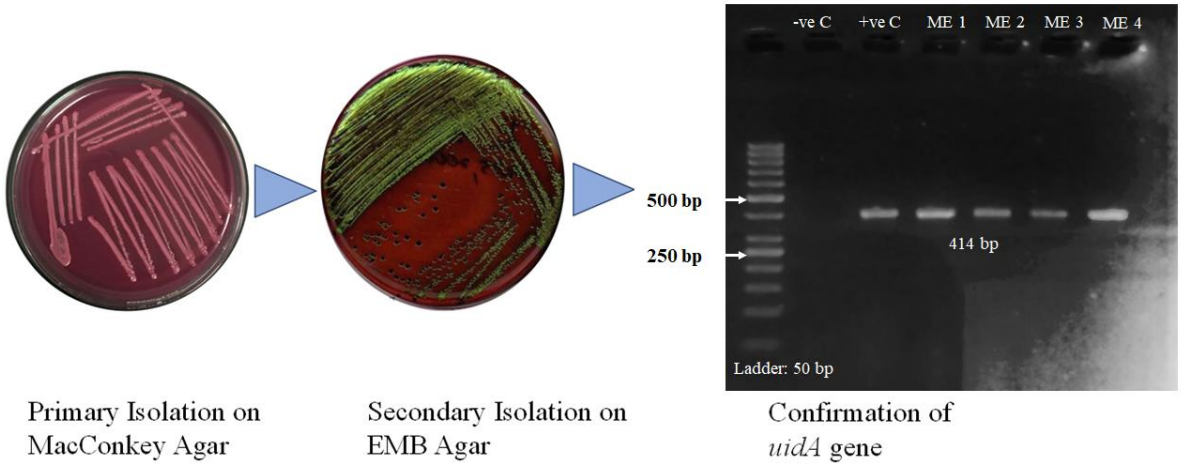


Fig 3.13: Isolation strategy for *E. coli: E. coli* give dark pink colonies obtained on MacConkey and green metallic sheen on EMB. *E. coli* were further confirmed by the amplification of *uidA* gene

3.14 Antibiotic susceptibility tests

Each isolate of *E. coli* obtained was then evaluated for Antibiotic susceptibility according to the guidelines of European Committee on Antimicrobial Susceptibility Testing (EUCAST). The antibiotics used in the study are mentioned in the table:

E. coli isolates grown overnight on Nutrient agar were used to make suspension in saline and vortexed to thoroughly mix *E. coli* colonies in saline medium and adjusted to the turbidity comparable to 0.5 McFarland standard solution. In order to use constant suspension of inoculum for all antibiotic sensitivity tests, 100 µL inoculum was used to make bacterial lawn on MH Agar plates followed by putting antibiotic discs on top of bacterial lawn and incubated for at least 16 hours at 37°C after which zone of inhibition of antibiotics was noted.

3.15 Phenotypic Detection and Confirmation of Extended spectrum β-lactamases (ESBLs)

Bacteria can resist third generation cephalosporins and monobactams by the production of Extended spectrum β-lactamase (ESBL) enzymes but remain ineffective against cephamycins and carbapenems. All Cephalosporins, aztreonam and penicillins are reported resistant if an ESBL is detected.

Double disk synergy (DDS) method was used for the *in vitro* detection of Extended Spectrum B-Lactamase production by *E. coli* samples according to the Clinical and Laboratory Standards Institute (2012) guidelines. In the test, 30 µg antibiotic disks of Ceftazidime (CAZ) and Cefotaxime (CTX)were used. 100 µL of *E. coli* samples at 0.5 O. D were spread on MHA plate. The antibiotics mentioned were then placed on the *E. coli* spread plate at 30mm distance and then left for overnight incubation at 37 °C. If the zone of inhibition for an isolate was found to be ≤ 22mm for CAZ and ≤ 27mm for CTX then the isolate was considered as potential ESBL positive.

To confirm ESBL positive isolate *in vitro*, AMC (20 µg Amoxicillin + 10 µg Clavulanate) disc was used in combination with CTX and CAZ. The procedure involves placing AMC in the centre with CAZ and CTX at 20mm distance apart on either side from AMC. Any disturbance from the original zone of inhibition is considered an ESBL positive isolate.

3.16 Multiplex Polymerase Chain Reaction for Confirmation of ESBL positive isolates

Double disk synergy results can give false negative results due to varying expression of genes encoding ESBLs. So, in order to confirm *in vitro* results it is imperative to detect ESBL encoding genes. In the study, three most common ESBL genes i.e., ^{bla}*CTX-M*, ^{bla}*TEM and* ^{bla}*SHV* genes were amplified using primer sets and MPCR conditions described by MONSTEIN *et al.*, 2007.

3.17 *In vitro* Pathogenicity tests

In order to determine the disease causing potential, MPEC isolates were subjected to following *in vitro* pathogenicity tests:

1. Blood Haemolysis assay
2. Motility Assays
3. Congo Red Binding assay
4. Growth in Human Urine
5. Biofilm Formation ability assay

3.17.1 Blood Haemolysis Assay

Breaking down of Red blood cells is defined as Blood Haemolysis. Pathogenic bacteria can hydrolyse red blood cells by the production of lysine enzymes, and on basis of presence or absence of haemolysis, bacteria can be classified as α-haemolytic (partial hydrolysis), β-haemolytic (complete hydrolysis) and γ-haemolytic (No haemolysis). For that purpose,

blood agar media was prepared using Tryptic soy agar and 5% defibrinated fresh and aseptic sheep blood. Instructions for preparation and interpretation of results was done according to guidelines mentioned by Buxton, 2005

3.17.2 Motility Testing

Bacteria employ flagella, pilli or other mechanisms to move in natural environments which is influenced by physical, chemical or biological factors (Mattingly *et al.*, 2018).

Three types of motility of tested, namely: swimming, swarming, and twitching of MPEC isolates was evaluated.

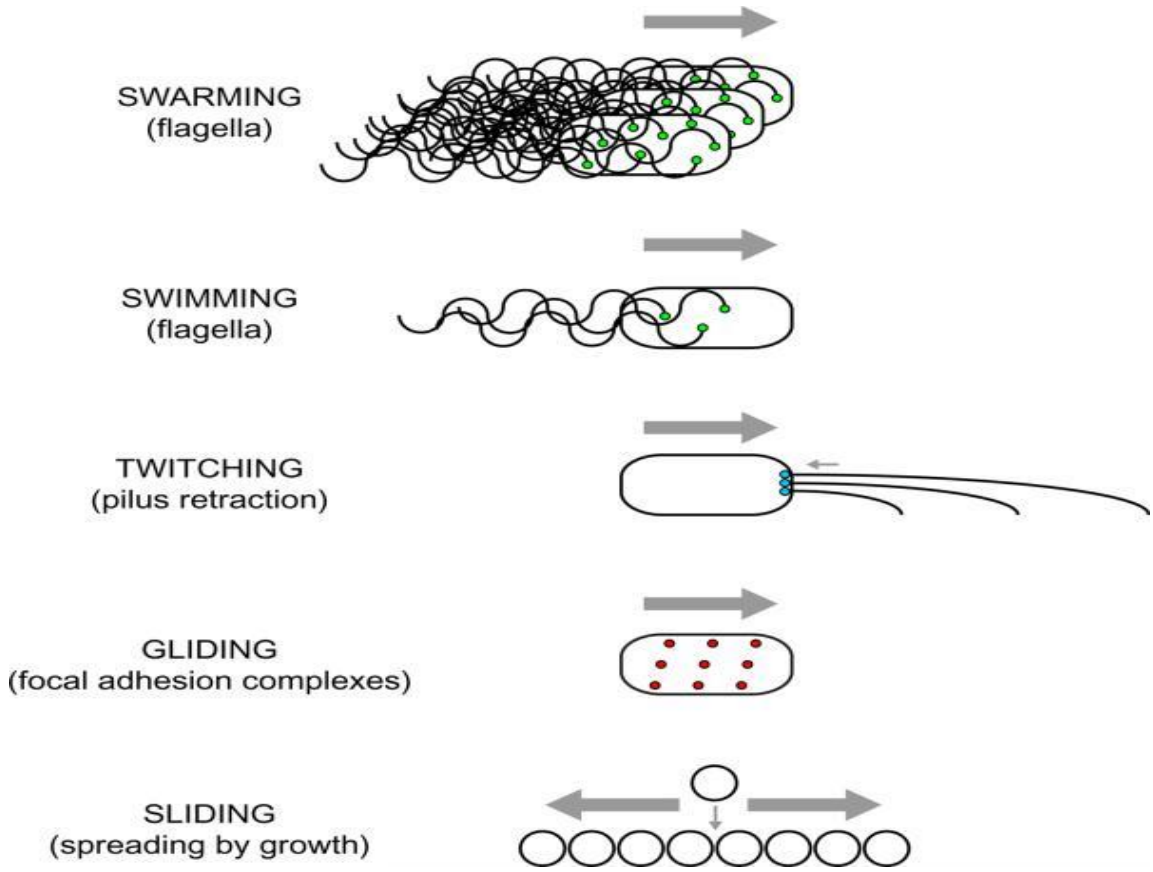


Figure 3.18.2: Mechanisms of Bacterial Motility: Grey arrow represents direction movement (Kearns, 2010)

3.17.2.1 Swimming Motility

It is a movement of single cell in a liquid medium empowered by rotating flagella. Swimming motility was checked according to the method described by (Qiao *et al*., 2012). Swimming tryptone plates were prepared and left at room temperature for 6 hours after which the bacterial samples were stabbed with sterile toothpick in aseptic conditions and left for incubation at 37°C for 18 hours. To analyse the distance covered by bacterial sample after 18 hours incubation, circular turboid zone was measured.

3.17.2.2 Swarming Motility

Multicellular rotating flagellar movement on semi solid surface medium is defined as swarming motility. Mostly in laboratory conditions, more than 0.3% agar concentration is needed to study swarming motility as agar concentration above this percentage inhibits swimming and encourages swarming motility while agar concentration more than 1% inhibits swarming motility (Kearns, 2010). For this purpose, swarming media was prepared according to the recipe described (table)

Swarming plates poured were left at room temperature for 6 hours before being stabbed with bacterial samples with the help of sterile toothpick in aseptic conditions. Incubation was followed for each swarm bacterial plates at 37°C for 18 hours. After intended incubation time, the distanced travelled by each bacterial sample from stabbing point was measured.

3.17.2.3 Twitching Motility

Powered by retracting type IV pili, bacteria are able to move on semisolid or solid medium un-reliant on the presence of flagella. Type IV pili is a hair like projection which extends and retracts thereby facilitating collective movement of bacterial colonies (Turnbull et al., 2014).

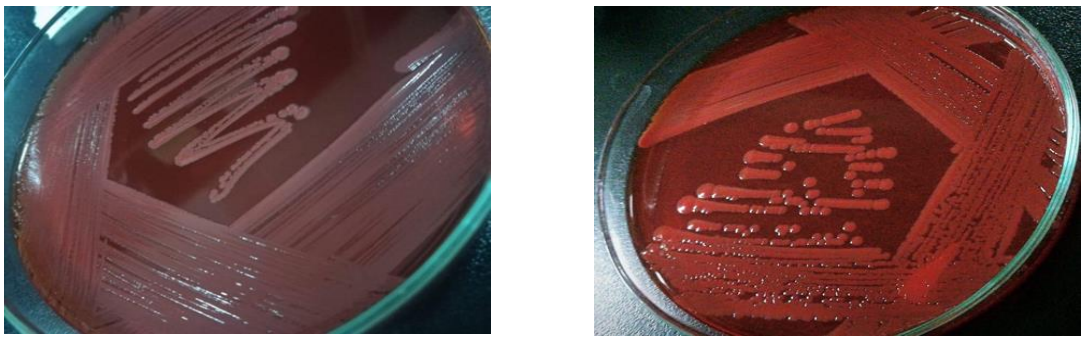
To study twitching motility in-vitro, twitching media was prepared according to the media recipe described by Meng *et al*., 2005 (table). Approximately 3ml twitching media was poured on to the plates and left to dry at room temperature for 6 hours. Each plate was then stabbed

with bacterial samples with the help of sterile toothpick and left for incubation for 48 hours at 37°C. After completion of intended incubation, cultured twitching media was discarded and washed. The attached cells were fixed with methanol and stained with 1% crystal violet solution for 15 minutes. Then plates were washed and remaining stained zone was measured.

3.17.3 Congo Red Binding Assay

Ability of a bacteria to bind with congo red dye is an indication of its pathogenicity. Studies have reported correlation between the ability of *Shigella* and *Yersinia* to invade host cells and their congo red dye binding ability, and this test could also be used to distinguish between pathogenic and non- pathogenic *E. coli* (Berkhoff *et al*., 1986) and could also be used to confirm presence of curli -extracellular fimbriae comprising of amyloid proteins involved in pathogenicity in *E. coli*- (Lloyd *et al*., 2012).

Congo Red (CR) agar was prepared according to procedure defined by Berkhoff *et al*., 1986. For preparation of CR agar media, 0.03% Congo red dye and 0.15% bile salts solution (both autoclaved separately) were added to autoclaved warm Trypticase soy agar medium and poured on petri plates. E. coli isolates were then streaked on CR agar medium and incubated at 37°C for 24 hours. After 24 hours incubation, each streaked CR agar plate was left at room temperature for another 2 days. The isolates which bound with CR dye appeared red while those which did not gave white colonies on CR agar plates.



Unbound Bound

Fig 3.18.3: CR positive and negative isolates based on ability to bind with CR dye: Unbound *E. coli* give pink colony appearance while *E. coli* bound with CR give red appearance

3.17.4 Growth in Human Urine

Ability of bacteria to survive in urine is termed as bacteriuria as urine is antimicrobial in nature and generally retards growth of bacteria. Bacteria with ability to survive, grow and sustain in urine have been implicated in urinary tract infections (UTIs). *E. coli* is recognized as major causative agent of UTI in up to 80% of all UTI cases (Ipe *et al*., 2016). Since *E. coli* are one of the most versatile and adaptable species employing various strategies and environments for survival, we tested whether these MPEC isolates could also survive in urine. MPEC ability to grow in urine was evaluated according to method described by Mitchell *et* al., 2015 with little modifications.

Before the test, Urine samples were taken from 2 healthy males and females, filter sterilized, pooled, poured as aliquots in 2ml Eppendorf tubes and stored at -20°C. To perform the test, each *E. coli* isolate was inoculated in Tryptic soy broth (TSB) and incubated overnight in a shaking incubator at 37°C temperature and 120 rpm speed. Next day, optical density of each isolate was brought to 1. For the test, each isolate adjusted at 1 O.D was inoculated in sterile urine in a 1:100 dilution and 200µL of each urine inoculated sample was pipetted on to 8 contiguous wells (for eight replicate values) in 96 well plate for each sample. For each experiment, UPEC positive control and negative controls (media and non- urine growing bacteria sample used separately) were employed. After inoculation, the 96 well plate was

incubated at 37°C under static condition for 8 hours after which the O.D was measured at 630nm wavelength in Elisa reader. Each experiment was repeated in duplicates.

3.17.5 Biofilm Formation Ability

Bacteria can form one of the most resilient structure in order to survive in harsh conditions. A Biofilm contains many cells under a protective extracellular matrix sheath allowing bacteria to survive in inhospitable conditions and resist survival pressure exerted by antibiotics. Biofilm film formation of MPEC isolates was analysed *in vivo* by 96 well Microtiter dish biofilm assay method. The method is less laborious, effective and has been used to analyse biofilm formation ability of wide range of bacteria like *P. aeuroginosa*, *V. cholera* and *E. coli* etc (O'Toole *et al*.,2011). Biofilm formation ability of MPEC isolates was analysed according to the method described by Mitchell *et* al., 2015. The method can be broadly characterised into two steps.

1. **Growing Biofilm**

   Growing cells in static conditions in a minimal medium for at least 48 hours allows biofilm forming bacterial cells to adhere to the bottom of well in a microtiter dish

I. MPEC isolates were grown overnight in LB broth media in a shaking incubator at 37°C and 120 rpm speed.

II. Next day, O.D of every isolate was brought to 1.

For inoculation in wells of Microtiter plate, 1:100 dilution of each isolate at O.D 1 was prepared in TSB medium

III. 200 µL dilution of each isolate was added to 8 contiguous wells to make 8 replicates for each sample. For each experiment, positive control was a biofilm forming strains from *bacillus spp*. while sterile TSB media was used as a negative control.

IV. Microtiter plate was incubated in static condition for 48 hours at 37°C.

2. Staining and Quantification of Biofilm

Bacterial cells which have adhered to the bottom of the wells are stained with crystal violet and the O.D of the cells is taken.

I. First after 48 hours, microtiter dish is taken out of the incubator and free cells are dumped out by inverting the dish upside down and jerking out excess liquid gently.
II. The wells were then washed with 0.85% saline and dried
III. Next, 200 µL Methanol was added into each well and left for 15 mins after which methanol was discarded and the dish was dried.
IV. After drying, 200 µL of 0.1% crystal violet was added and left for incubation at room temperature for 15 minutes.
V. Crystal violet too was discarded, and the plate was submerged in 0.85% saline to de-stain each well of excessive dye. The dish was blotted on dry paper towels and dried
VI. Onto each well, 200 µL of 33% acetic acid was added and the dish was left for 15 minutes at room temperature.
VII. Absorbance was recorded in Elisa reader at 630 nm UV wavelength

For confirmation of results, each biofilm assay was conducted in duplicates

3.18 Multiplex Polymerase Chain Reaction for Determination of *E. coli* Phylogenetic Group

Multiplex PCR was performed in order to find the phylogenetic group of all the isolated *E. coli* strains according to the protocol defined by Clermont *et al*., 2000 for Phylogenetic classification. This method involves amplification of two virulence genes (*chu A* and *yjA*) and DNA fragment (*TspE4.C2*), and assigns *E. coli* into either one of the four groups i.e., A, B1, B2, and D group based on the presence or absence of certain combination of these genes (fig. 3.18).For this purpose, primers sequence, concentration, and PCR conditions already defined were used (Clermont *et al*., 2000).

After MPCR amplification, gel electrophoresis was done in order to visualise presence or absence of gene amplification using 2% (wt/vol) agarose gel and 0.5 μg/ml ethidium bromide. The gel was visualised in UV transilluminator.

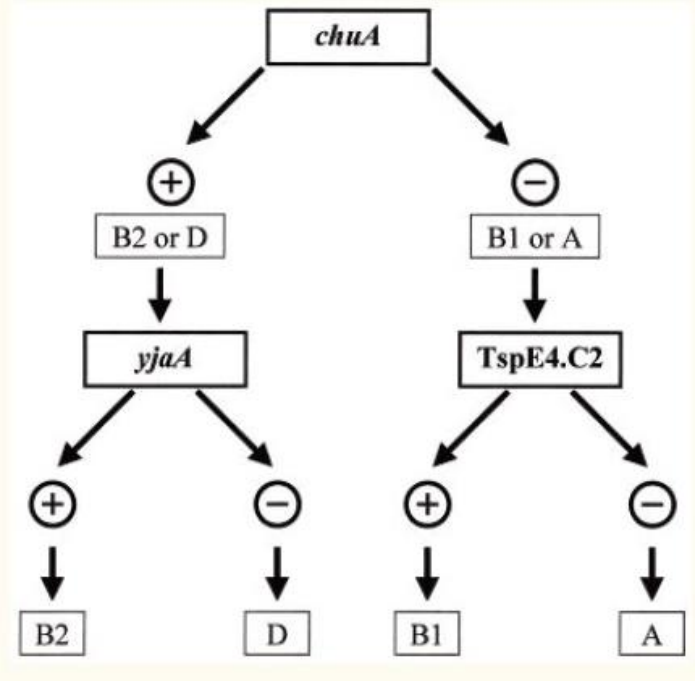


Fig 3.18: Phylogenetic classification based on MPCR amplification of genes (Clermont *et al.,* 2000): B2 *E. coli* contain *chuA* and *yjA* while D lacks *yjA*, and *chuA* is absent in both B1 and A while A group also lack TspE4.C2 gene fragment

3.19 Multiplex Polymerase Chain Reaction for Determination of Virulence associated genes and Toxin Genes

Pathogenic *E. coli* can cause various diseases due to presence of several virulence associated and Toxin genes. The genes responsible for pathogenicity are mostly encoded on Plasmid. These genes enable *E. coli* to resist complement, produce colicin, survive in serum and acquire iron from blood (Trampel *et al.*, 2007). Three MPCR panels were used to amplify different virulence and toxin genes (functions described in the table below) characterised into three combinations for each panel. First two panels consisted of virulence associated genes while third panel consisted of toxin genes.

The first panel contained three genes, namely; *cvaC*, *papC* and *iss* (Primer sequences described in the table 1.11). In a 25 µL PCR reaction tube, 12.5 µL Master mix, a total of 4.5 µL Primers (0.75 µL forward and 0.75 reverse primer of each gene), 2 µL DNA, 0.5 µL Taq Polymerase and 5.5 µL molecular grade Nuclease free were added and amplified using MPCR condition amplified during the study which are as follows: Denaturation at 95°C for 5 mins, followed by 40 cycles of 95°C for 1 min, 55°C for 90s, 7 for 1 min, and final extension of 72°C for 10 mins. The second panel included six genes namely; *fimH*, *papA*, *kpsMT III*, *papEF*, *ibeA*, *ireA* and a pathogenicity island marker (PAI). Protocol for genes amplification including Designed primer sequences for these genes, components of reaction mixture concentrations and MPCR conditions described by Johnson *et al*., 2000 were followed. Lastly, four toxin genes (*stx 1*, *stx 2*, *hlyA* and *eaeA*) were amplified according to the conditions described by Fagan *et al*., 1999 including primer sequences, individual concentrations of PCR components and PCR conditions. To visualise gene amplification, gel electrophoresis was employed using 2% (w/v) agarose gel.

Table 3.19: Primers set used in first panel MPCR

Sr. No.	Gene	Amplicon Size (bp)	Primer Sequence		GC %	Length (bp)
1.	*iss*	227	F	GTTATTTTCTGCCGCTCTGG	50	20
			R	AACCGAGCAATCCATTTACG	45	20
3.	*papC*	440	F	AATAAAAACGTGGCGGACTG	45	20
			R	TATCCTTTCTGCAGGGATGC	50	20
5.	*cvaC*	501	F	CCTCCTACCCTTCACTCTTG	55	20
			R	GGATGGAGACATTGCAGGAT	50	20

4.Results

4.1 Prevalence of Mastitis in Rawalpindi District

Sampling for prevalence of mastitis was done in Rawalpindi district during the period from April to October 2019. Out of the total 236 lactating buffalos and 272 cows tested by SFMT, 13.98 % lactating buffalos were positive for Mastitis out of which 10.6% had subclinical mastitis and 2.11% had clinical mastitis. For dairy cows, mastitis was found in 22% (20.58% and 1.47% for subclinical and clinical mastitis respectively). The prevalence of Mastitis in bovines is different from the expected prevalence (19% for buffaloes and 23% for cows respectively).

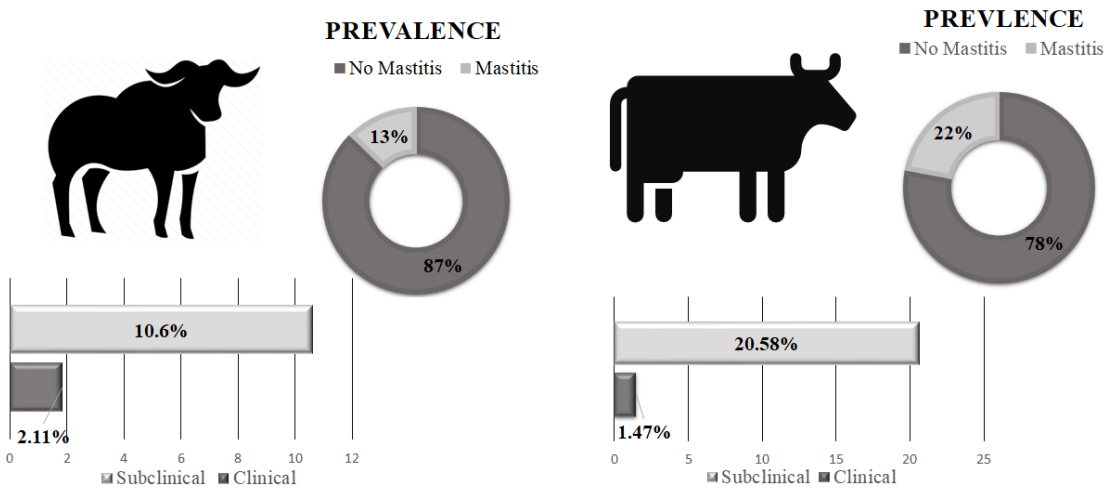


Fig 4.1: Prevalence of Bovine mastitis in Rawalpindi district: On the left, prevalence for mastitis in buffalos is shown while the right side of the diagram shows prevalence of mastitis in cows. In both parts of the diagram bar graph represents clinical and sub-clinical mastitis prevalence

4.2 Prevalence of Bacteria in Mastitis positive Milk samples

Mastitis positive milk samples (subclinical and clinical) were processed to isolate mastitis causing bacteria culturable in our laboratory to find their prevalence. The most prevalent bacteria found was *S. aureus* (80%) followed by *Streptococcus* and *E. coli* (30% and 26%) respectively (fig. 4.2)

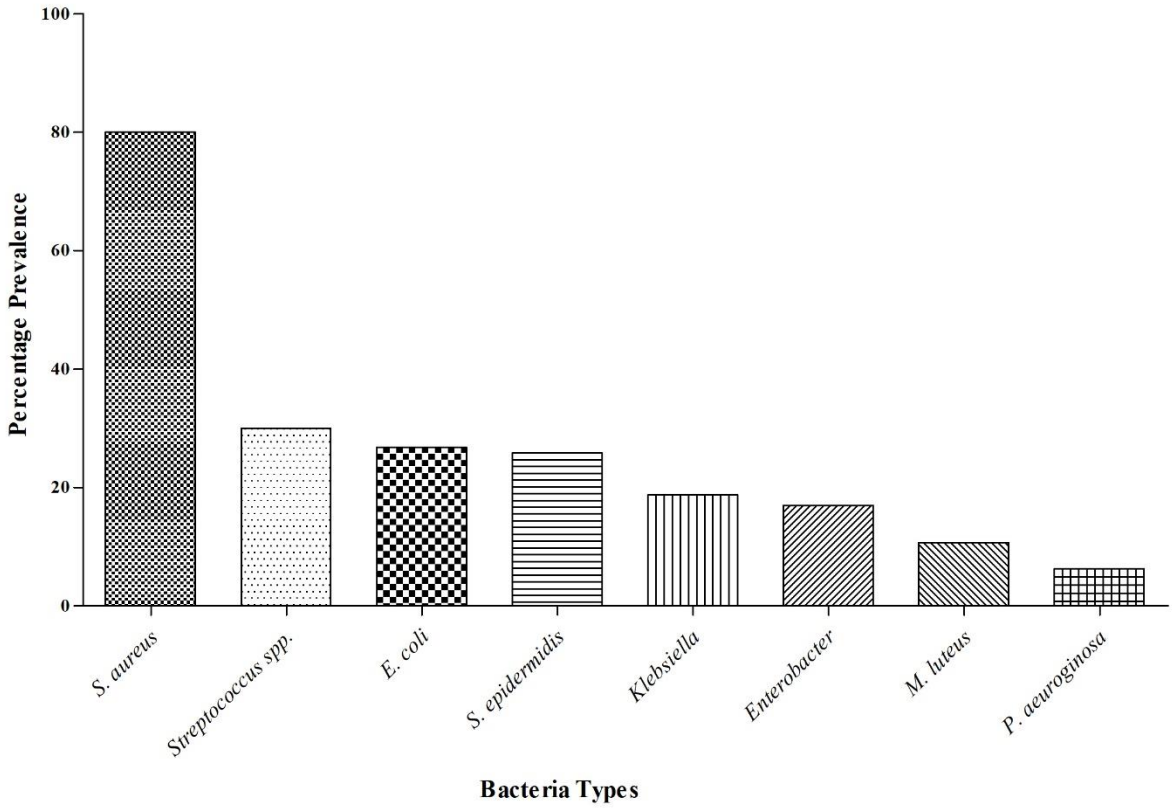


Fig. 4.2: The bar graph showing prevalence of all microbes: The isolates of different bacterial species are represented in different bar graph patterns. X-axis shows bacterial species identified while Y-axis shows percentage of these specie isolates in percentage of total Mastitis positive samples.

This study agrees with earlier reports on *S. aureus* being the major causative agent of bovine mastitis in Pakistan and India (Allore *et al*., 1993). Though being most prevalent in Pakistan, incidences of bovine mastitis caused by *S. aureus* and other contagious bacteria have decreased in many parts of the world due to better milking and farm management practices (Swinkels *et al*., 2013). Other bacteria identified in mastitis positive milk samples were, *S. epidermidis* (25.84%), *Klebsiella spp*. (18.75%), *Enterobacter spp*. (16.96%), *M. Luteus* (10.71%) and *P. aeruginosa* (6.25%).

Among these bacteria, *E. coli* was further investigated for Antibiotic susceptibility pattern and its role in pathogenicity.

4.3 Antibiotic Susceptibility Testing of the *E. coli* isolate

E. coli samples isolated from mastitis positive milk samples were tested for response towards different antibiotics (table 4.3a). All the isolates (n=22) showed complete resistance for AMP (10) and E (5), while 90.90%, 72.72% and 68.18% of isolates showed resistance against AML (10), TE (30) and S (10) respectively. Both AML (10) and AMC (10) are Penicillin antibiotics while E (5), TE (15) and S (10) are Macrolide, Tetracycline, and Aminoglycoside respectively. In the study, 10 antibiotics categories were tested and those isolates which were able to ≥ 1 antibiotic agent in ≥ 3 categories of antibiotics were defined as Multidrug resistant (MDR) according to definition proposed by Magiorakos *et al*., 2012. Except for one all isolate, all (95.45%) were MDR with one isolate being able to show resistance for ≥ 1 antibiotic in all categories (table).

Highest level of sensitivity was shown for LEV (5) and IPM (10) at 95.46% and 77.27% respectively. LEV (5) belongs to Fluoroquinolone class of antibiotic while IPM (10) is a Carbapenem.

Table 4.3 (a): Antibiotic susceptibility pattern of *E. coli* isolates in percentage.

Antibiotics	Sensitive	Intermediate	Resistant
AMP (10)	0	0	100
AML (10)	4.54	4.54	90.9
TE (30)	27.27	0	72.72
DO (30)	31.81	27.27	40.9
AMC (30)	27.27	22.72	50
ETP (10)	40.9	27.27	31.81
TZP (110)	68.18	22.72	9.09
S (10)	13.63	18.18	68.18
CIP (5)	45.45	31.81	22.72
NOR (10)	77.27	18.18	4.54
TOB (10)	54.52	22.72	22.72
CN (10)	72.72	9.09	18.18
IPM (10)	72.72	18.18	9.09
MEM (10)	18.18	31.81	50
E (15)	0	0	100
LEV (5)	95.46	0	4.54
SXT (25)	54.54	9.09	36.36
F (300)	63.63	13.63	22.72
AK (30)	72.72	13.63	13.63
CRO (30)	59.09	0	40.9
CTX (30)	36.36	18.18	45.45
CAZ (30)	72.72	13.63	13.63

Table 4.3 (b): Resistant MPEC isolates against number of antibiotic categories used

Resistance against no. of antibiotic categories	No. of Resistant Isolates	Percentage %
2	1	4.54
3	3	13.63
4	4	18.18
5	1	4.54
6	5	22.72
7	4	18.18
8	1	4.54
9	2	9.09
10	1	4.54

The susceptibility pattern of *E. coli* isolates is also shown in form of bar graph (fig. 4.3 (a)).

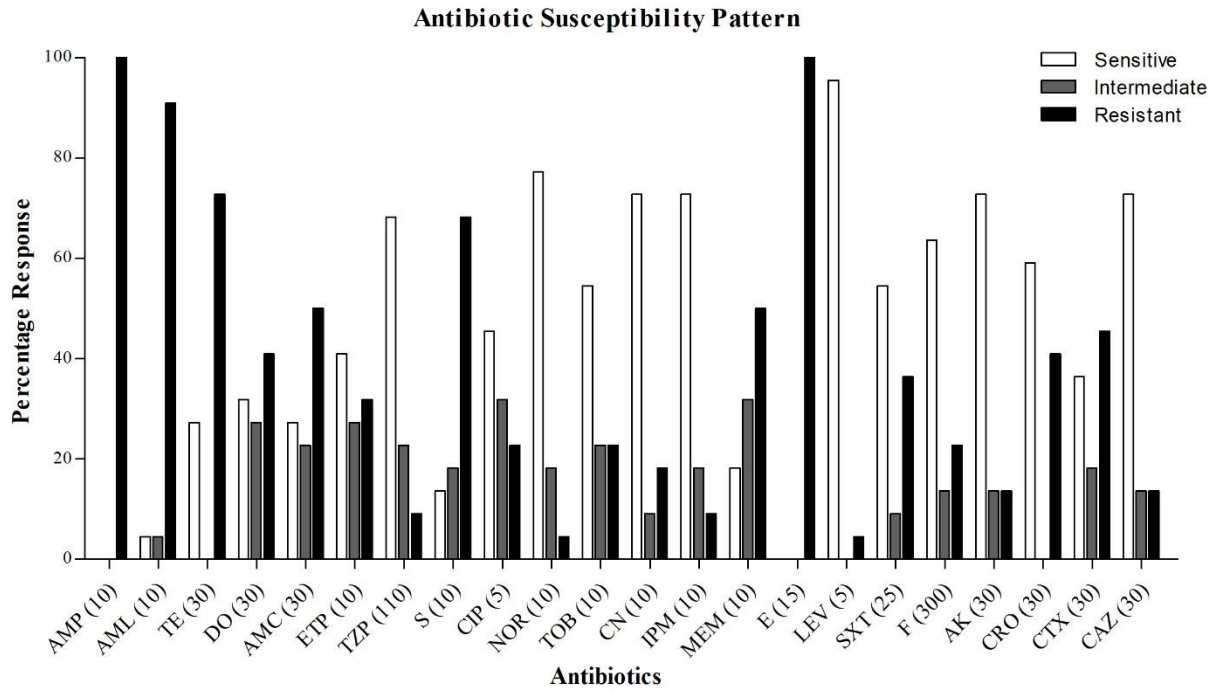


Fig 4.3 (a): Antibiotic Sensitivity Pattern of MPEC isolates: In the X-axis, antibiotics are shown and Y-axis Shows activity against MPEC in Percentage. White bars indicate MPEC sensitive to respective antibiotic while grey and black show intermediate resistance and resistance respectively.

Among the isolates, 16 (73%) showed phenotypic expression of ESBLs checked by Double Disk Diffusion assay (fig 4.3 (b)). But through MPCR, it was found that 21 isolates (95.45%) had at least one of three ESBL genes (*blaCTX-M*, ^{bla}*TEM and* ^{bla}*SHV*) genes. 12 isolates (54.54%) contained a combination of *blaCTX-M* and ^{bla}*TEM* genes, 8 isolates (36.36%) contained only ^{bla}*TEM* gene while only one isolate (4.54%) contained single ^{bla}*CTX-M* gene. No ^{bla}*SHV* gene was found in either isolates. In total, ^{bla}*TEM* gene was present in 20 isolates (90.09%) while ^{bla}*CTX-M* was present in 9 isolates (40.90%). Besides ESBL production, many isolates showed resistance to 3rd generation cephaloaporin antibiotics (CRO (30), CAZ (30) and CTX (30)), and among these, highest resistance was found for CAZ (30) at 45.5% followed by CRO and CTX at 40.9% and 13.63% respectively.

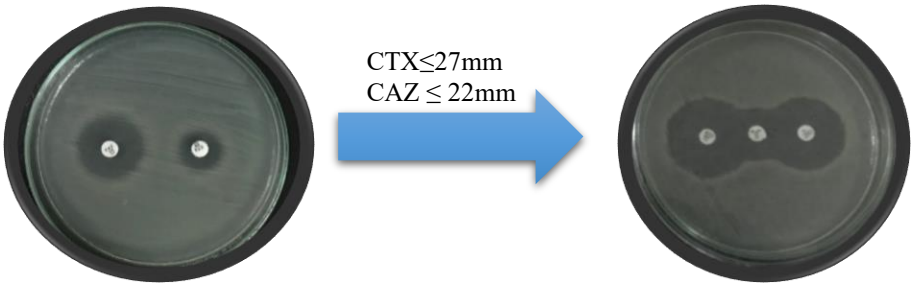


Fig 4.3 (b): DDS method for detection of ESBL positive MPEC isolates: CTX+CAZ were used on the bacterial lawn plate shown on the left while AMC+CAZ++CTX were used on bacterial lawn plate shown in right

4.4 *In vitro* Pathogenicity testing of the *E. coli* isolates

4.4.1 Blood Hemolysis

E. coli that can lyse red blood cells are implicated in variety of intestinal diseases in animals and extra-intestinal diseases in humans (Short *et al.*, 1971). So, in order to find ability of MPEC isolates to lyse red blood cells, blood hemolysis test was performed. All the isolates were alpha hemolytic,

being able to partially degrade red blood cells in blood agar media as indicated by the presence of greenish color around *E. coli* colonies and dark appearance of agar under same cultures.

4.4.2 Motility Testing

Swimming and swarming motility of MPEC isolates analyzed qualitatively as described by Qiao *et* al., 2012. The isolates which showed clear turboid zone away from point of inoculation were considered positive. Accordingly, 19 (86.36%) and 8 isolates (36.36%) were considered positive for swimming and swarming motility respectively. The isolates which showed less than 6mm zone on twitching motility plates were considered non motility for twitching motility (Liaqat *et al*., 2009). So, 17 isolates (77.27%) were found to be positive for twitching motility. No specific association between motility types was found as those positive for one motility were not necessarily positive for other motility types. From the data mentioned above, it is not clear about the quantitative aspects. So, Motility of MPEC is divided into high, medium and low based on zone size measured. Those isolates which showed more than 50mm zone on motility plates were considered highly motile while those that had less than 50mm zone were considered medium motile and bacteria showing less than 20mm zone of motility were low motile. The detailed motility profile of MPEC isolates is shown in the table 4.4.2.

Table 4.4.2: Motility of MPEC isolates

Motility Type	Low (<20mm)	Medium (<50mm)	High (>50mm)
Swimming	8 (36.36%)	3(13.63%)	11(50%)
Swarming	19 (86.36%)	2 (9.09%)	1 (4.54%)
Twitching	22 (100%)	0 (0%)	0 (0%)

Those isolates which showed zone less than 10mm for swimming and swarming motility types were considered negative while for twitching motility it was less than 6mm. It is clear from the

above table that motility decreased as rigidness of media on which MPEC isolates were streaked increased.

4.4.3 Congo Red Binding ability

Ability of MPEC isolates to bind with congo red was checked by congo red binding assay. Total 18 isolates (77.27%) bound to the congo red dye as indicated by red appearance of MPEC colonies on CRA streaked plates, and 5 isolates (22.72%) were congo red dye negative.

4.4.4 Growth in Human Urine

Growth in human urine was performed in 96 well triter plate to analyze the ability of MPEC to grow and survive in human urine as well as to find the zoonotic potential of these isolates. The O.D of MPEC isolates and controls (positive and negative) were compared with O.D of sterile urine after 8 hours. The mean absorbance values obtained from replicate well readings and experiments conducted in duplicates of controls and isolates of each isolate were subtracted from mean absorbance values of negative controls, and a bar graph was made to express all the values using GraphPad PRISM 5.01. Except for one isolate, all other isolates (95.95%) were able to grow beyond 0.5 O.D in 8 hours suggesting their ability to survive in urinary bladder and cause infection. Bacteria with high bacteriuria potential should be able to reach stationary phase (O.D 0.5-0.9) at the allocated incubation time (Roos *et al*., 2006).

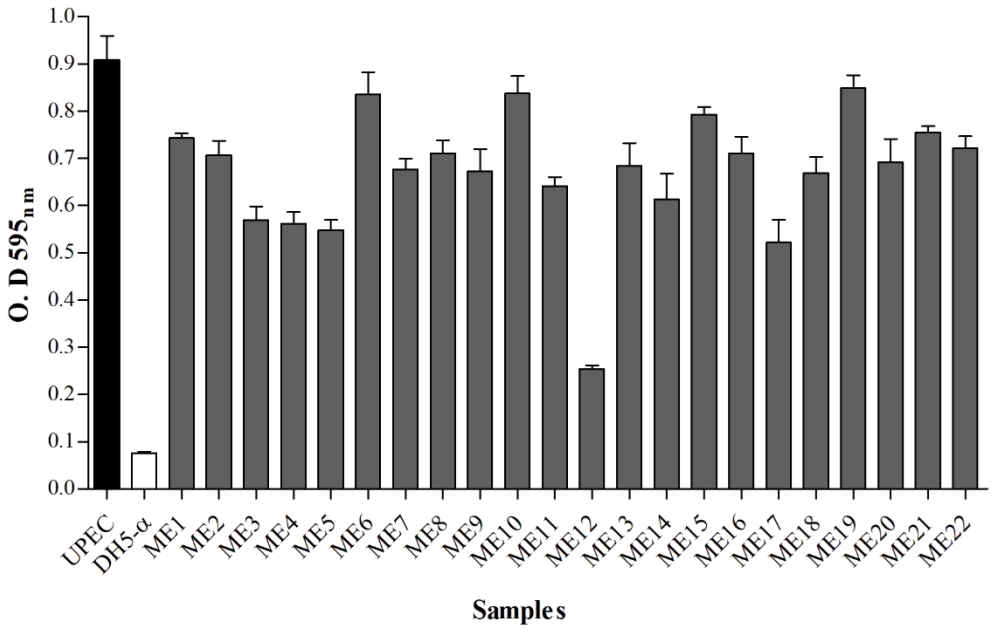


Fig 4.4.4: The bar graph showing O.D of all MPEC isolates after 8 hours incubation in human urine: In the graph, UPEC is a positive UTI causing *E. coli,* DH5-α was used as a negative control. ME represent MPEC isolates and number indicates isolate number. Each reading represents mean of 8 replicate values. Each experiment conducted in duplicates

4.4.5 Biofilm Forming Ability

E. coli is a multifaceted microorganism with biofilm formation ability as one of the characteristics which some *E. coli* possess. Pathogenicity of a bacterium is significantly increased due to their ability to form biofilm. So, biofilm formation ability of MPEC was assessed by 96 well Microtiter dish biofilm assay method. After 48 hours incubation, the microtiter plate was processed for quantification of biofilm formed by MPEC isolates. The mean absorbance values of positive control and MPEC isolates were subtracted from mean absorbance of media. The interpretation of biofilm assay readings was done according to the criteria defined by Qiao *et al*., 2012. Out of 22, 7 (31.81%) isolates showed moderate level biofilm activity while rest of the isolates (68.18%) showed weak biofilm activity. Only two isolates (9.09%) among moderate biofilm formers showed O.D more than 0.2 while rest of the moderate biofilm formers (22.72%) had O.D in range between less than 0.2 and more than 0.14. Isolates having less than 0.14 O.D (O.D less than 2 times O.D of

negative control) were characterized as weak biofilm formers and isolates showing O.D more than 0.14 (O.D more than 2 times O.D of negative control) were classified as moderate biofilm formers.

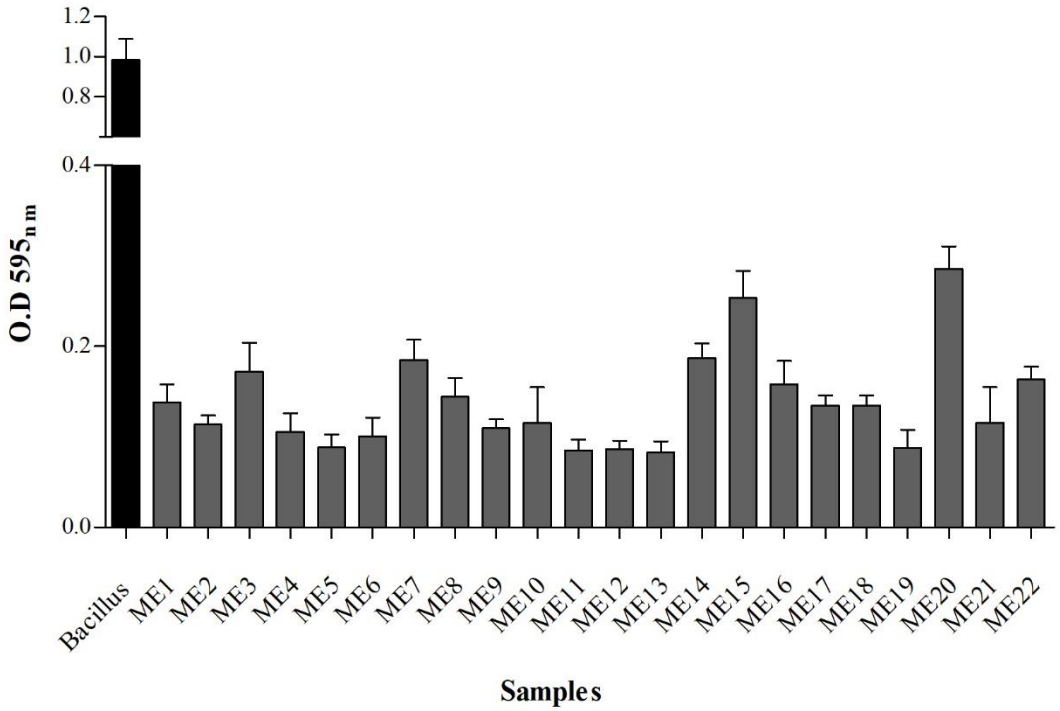


Fig.: 4.4.5: Bar graph showing Biofilm forming ability of MPEC isolates: In the graph, *Bacillus spp.* was taken as positive control while ME along with numerals represent MPEC isolates and the respective isolation number assigned. Readings were taken after 48 hours incubation in TSB. Each reading represents mean of 8 well-replicate values, and each experiment was conducted in duplicate

4.5 Phylogenetic Classification of MPEC isolates

In order to classify the isolated MPEC strains, 22 MPEC isolates were analyzed for phylogenetic grouping. Out of these 22 strains, 11 isolates (50%) belonged to D group, 6 isolates (27.27%) belonged to B1, 4 isolates (18.18%)were B2 group *E. coli* while only 2 isolates (9.09%) belonged to A group. In B2 and D groups, *chuA* is present while it is absent in B1 and A groups. In addition to *chuA*, B2 group *E. coli* also contain *yjA* gene which is absent in D group *E. coli*. B1 and A group *E. coli* both contain *yjA* gene, but the main difference between them is the presence or absence of TspE4.C2 DNA fragment which is present in B1 and absent in A1. The majority of MPEC isolates in this study belonged to D group followed by B1 group isolates.

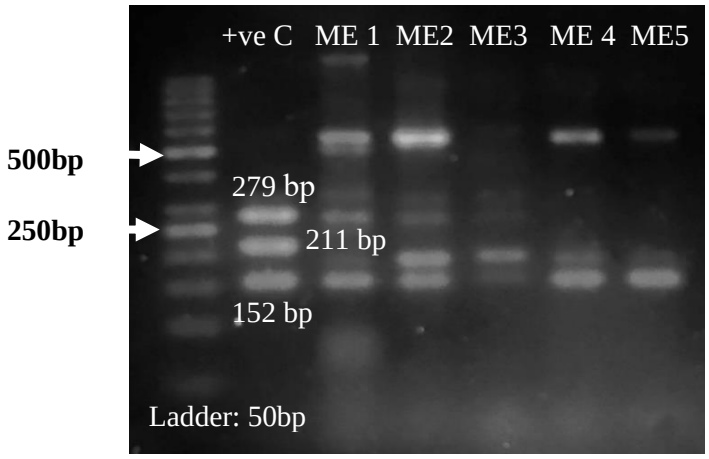


Fig. 4.5: Phylogenetic group determination of MPEC by MPCR: In the gel, +ve C is an APEC strain used as positive control containing all the genes. While ME are Mastitis *E. coli* samples with numbers indicating isolation number

4.6 Virulence associated and Toxin genes Prevalence

Three MPCR panels were done for the detection of different Virulence associated genes and Toxin genes. The overall distribution of these genes is shown in the (table.). The most prevalent virulence associated genes present in *E. coli* were *iss* and *fimH* both present in 21 isolates (95.45%), the two being absent in different isolates. Followed by these, *papC* and *cvaC* were the most common detected genes both being present in 10 isolates (45.45%) though both not being mutually exclusively present or absent in all isolates. Virulence genes that were not detected in any of the isolates (0%) included *papA, papEF, ireA* and *irbA*. Among Toxin genes, stx_1 by far was the most prevalent gene occurring in 13 isolates (59.09%) followed by *hlyA* gene being present in 4 isolates (18.18%).

Table 4.6 (a): Prevalence of Virulence associated genes in MPEC isolates

Virulence associated genes	Present in Isolates	Percentage (%)
iss	21	95.45%
cvaC	10	45%
papA	0	0%
papC	10	45%
papEF	0	0%
kpsMTIII	4	18%
ireA	0	0%
ibeA	0	0%
fimH	21	95.45%
PAI	1	4.54%

Table 4.6 (b): Prevalence of Toxin genes in MPEC isolates

Toxin genes	Present in Isolates	Percentage (%)
stx_1	13	59.09%
stx_2	1	4.54%
hlyA	2	9.09%
eaeA	4	18.18%

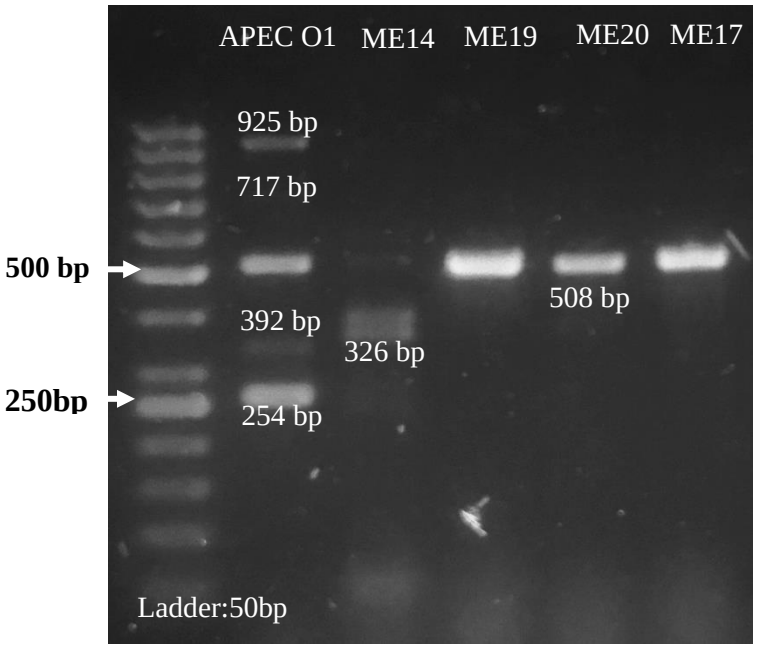


Fig 4.6 (a): MPCR for detection of VAGs: In the gel, APEC O1 is a control while ME are Mastitis *E. coli* Samples

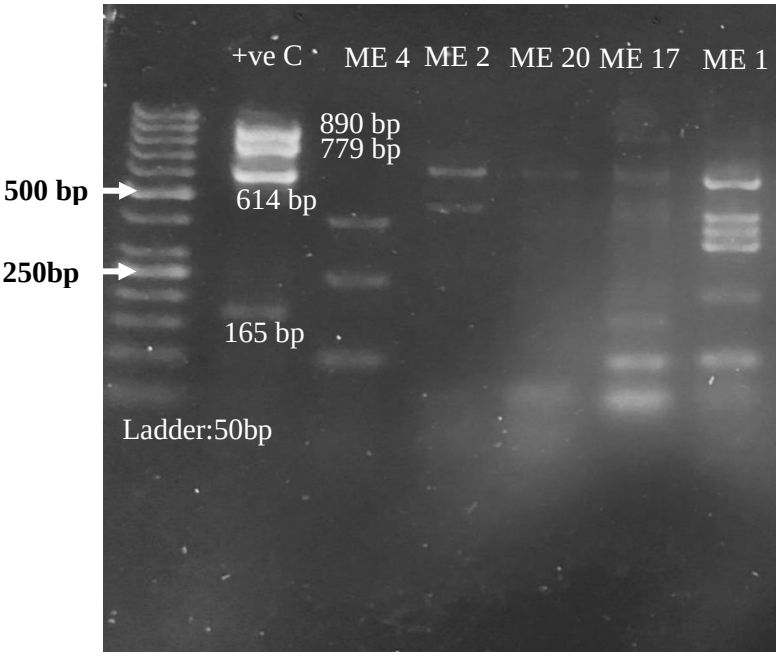


Fig 4.6 (b): MPCR for detection of Toxin genes: In the gel, STEC is a used as a positive control while ME are Mastitis *E. coli* Samples

5. Discussion

The purpose of the current study was to find prevalence of bovine mastitis in Rawalpindi district including prevalence of mastitis causing bacteria. Among these bacteria, virulence associated properties of Mastitis causing *E. coli* was further investigated. The study reports prevalence of Bovine Mastitis in Rawalpindi district, Pakistan from period between April and October 2019. During the study, 236 lactating buffalos and 272 lactating cows were screened for presence of mastitis by SFMT. It was found that 13% buffalos and 22% cows were positive for mastitis. The prevalence found during this study is different from the expected prevalence of 19% and 23% for buffaloes and cows respectively in Rawalpindi district. Scarcity of data exists on the prevalence of bovine mastitis in Pakistan though prevalence for some regions is reported. Study conducted by Bachaya *et al*., 2011 reported 36% prevalence of mastitis in crossbreed cows in Muzaffargarh district while Umar *et al*., 2013 reported 34% prevalence rate in arid areas of the Punjab province. Ali *et al*., 2011 found 44% subclinical mastitis among lactating buffalos in four districts of Punjab including Sialkot, Lahore, Narowal and Okara.

It is noteworthy to mention that previously reported incidence of Subclinical Mastitis among dairy buffaloes in Rawalpindi district including three tehsils i.e., Rawat, Kallar Syedan and Gujjar Khan was 67.3% (Khan *et al*.,2019) conducted between 2017-2019 in 196 lactating buffalos which has a large discrepancy with prevalence found in this study for dairy buffalos. The difference in prevalence could be explained on the number of factors including sampling size, duration and season, management practices and knowledge of the well-being of animals (Khan *et al*., 2006). During the study, we found different levels of husbandry practices among dairy farmers. Most of the farmers including farm managers showed lack of good husbandry practices and animal health

knowledge and therefore practiced none. Overuse of antibiotics and bad sanitary conditions was a common occurrence. However, some farms practiced comparatively better husbandry practices and owners/managers had adequate knowledge of animal health and hygiene.

The very high prevalence of *S. aureus* in mastitis positive samples is an indication of lack of hygiene and good husbandry practices. *S. aureus* is a contagious microbe found naturally in animal's environment such as soil and manure, and spreads to healthy udders from infected udders through contaminated hands used for milking, cleaning towels used on infected udders and then subsequently used on health udders and quite possibly also by flies (Allore *et al*., 1993). Several studies have reported prevalence of *S. aureus* in different areas. In Pakistan, researchers have reported various prevalence rates of *S. aureus*. For instance, 49.53% prevalence of *S. aureus* was reported among mastitis positive buffalos in Samundri tehsil, district Faisalabad (Ali *et* al., 2008) and more than 50% percent in cows and buffalos in tehsil Burewala (Hameed *et* al., 2008). Followed by *S. aureus, Streptococcus spp.* and *E. coli* were the most prevalent causative agent of bovine mastitis found. *Streptococcus spp.* are gram positive spherical bacteria which form a natural microflora of mammals. Like *S. aureus, Streptococcus spp*., *Micrococcus spp*., and *Pseudomonas spp*., also constitute as major mastitis causing bacteria in the bovines (Deb *et al*., 2013). *E. coli* together with *klebsiella spp.* and *Enterobacter spp.* are a member of gram-negative coliform bacteria and are major causative agents of bovine coliform mastitis. While incidences of gram-positive bovine mastitis are decreasing, the incidences of coliform mastitis are on rise and might increase in future as lower somatic cell count (SCC) in bulk milk is a trend among farmers (Schukken *et al*., 2012).

The antibiotic resistance shown by MPEC isolates is very high as most of the strains were MDR (95.45% of all tested MPEC isolates). Moreover, among MDR isolates 18 isolates (85.71%) were

resistant to ≥1 antibiotic in ≥4 antibiotic categories. Increase in resistance to antibiotic categories increases the chances of recurrent infection 1.6 times for each category i.e., isolates showing resistance to four categories of antibiotic have 1.6 times more chances of causing recurrent infection than the isolates resistant to 3 antibiotic categories (Fairbrother *et al*., 2015). Resistance pattern of bacteria depends on the geographical location suggesting common use of antibiotics used to treat the bacterial infections. Here in this study highest resistance is found against Penicillins, Macrolide, and Tetracycline suggesting overuse of these antibiotics in local dairy industry. Therefore, judicious use of antibiotics is necessary since overuse of antibiotics drives bacterial populations towards resistance. Being able to show some level of resistance for every antibiotic used is an indication of diverse resistance pattern of MPEC isolates from local dairy farms. The very high resistance pattern against Carbapenems (50% for MEM and 31.81% for ETP) is of great concern since these antibiotics are considered one of the most potent antimicrobials for treating severe infections. Among these isolates only one showed sensitivity to all three Carbapenem antibiotics. High frequency of ESBL production can drive resistance towards Carbapenems (Murugan *et al*., 2019), and this hypothesis is also corroborated in this study as more than 95% MPEC isolates showed ability to produce ESBLs. The situation seems alarming since these ESBL genes are plasmid borne (Moura *et al*., 2014) and can be transferred to other bacteria, environment and humans who consume such milk. Studies on the dissemination of ESBL positive MPEC are lacking in Pakistan. In a study conducted by ur Rahman *et al*., reported 23.53% ESBL producing *E. coli* samples from a total of 156 *E. coli* samples isolated from milk of mastitis positive dairy cows situated in nine provinces of China. In other parts of the world, ESBL genes were detected in 4.5% of 490 MPEC isolates from Bavaria region in Germany (Eisenberger *et al*., 2018). In a total 316 *E. coli* samples isolated from poultry fecal samples and mastitis milk samples from

cows, 5.9% *E. coli* were confirmed as ESBL positive in study conducted in Odisha, India (Kar *et al*., 2015). The first two studies found ^{bla}*CTX-M* as the most prevalent ESBL gene while Kar *et al* found ^{bla}*SHV* as most prevalent. During this study ^{bla}*TEM* was the most prevalent ESBL gene found occurring in 90.09% of MPEC isolates.

It is evident from the data that the prevalence of ESBL genes in MPEC isolates analyzed during this study is very high as compared with prevalence reported in other studies around the world though smaller *E. coli* sample size in this study is impediment for a full view to compare accurately. Though from the study, sensible use of antibiotics is the need for which it is necessary to devise effective and enforce better farm management practices as poorly managed dairy farms have higher chances of harboring antibiotics resistant bacteria (Murugan *et al*., 2019).

In vitro pathogenicity tests revealed ability of all MPEC isolates to partially hydrolyze the red blood cells and therefore form green zone around bacterial growth due to production of hydrogen peroxide. Hemolysis increases the chance of pathogenic *E. coli* to persist in the udder by increasing iron availability and causing damage to neutrophils (Hogan *et al*., 1990). Most isolates (77.27%) were able to bind with congo red dye indicating pathogenic nature of those isolates. Literature on the association between pathogenicity of *E. coli* and CRA is ambivalent as some authors have found positive correlation while others do not agree on strong association between the two (Lamey *et al*., 2013). So, further research needs to be done in order to find correlation between pathogenicity of *E. coli* and CRA expression. As for ability of bacteria to move, most of the isolates were positive for swimming and Twitching motility however relatively few isolates showed swarming motility. The motility of bacterium can serve as an important measure of bacterial pathogenicity in case of bovine mastitis i.e., motile bacteria have more capacity to cause severe infection than the non motile bacteria (Guerra *et al*., 2019). Biofilm formation also enables bacteria

to resist hostile environment and cause infection persistently. All tested isolates were either weak or moderate biofilm formers with majority being the former (68.18%). In order to find whether MPEC could colonize other organs i.e., urinary tract, growth in human urine test was performed. Except for one isolate, all other isolates had O.D beyond 0.5 after 8 hours of incubation in filtered human urine. This shows that MPEC isolates studied had potential to survive in urinary bladder and might have zoonotic potential as well. High bacteriuria potential is not an indication of actual ability to cause UTI as many *E. coli* can remain asymptomatic even in higher bacterial counts in urinary tract, and some *E. coli* having high bacteriuria ability have been found to show activity against uropathogenic *E. coli* (UPEC) as well. The main difference between uropathogenic and asymptomatic *E. coli* is the presence of type I and P-fimbriae, and both are present in UPEC and absent in non-pathogenic (Roos *et al*., 2006).

Majority of MPEC analyzed during the study belonged to D group phylogenetic classification. Different studies have reported different prevalence rates of phylogenetic groups of bovine mastitis causing *E. coli.* Study conducted by Kempf *et al*., 2016 found A and B1 group as the most prevalent among MPEC isolated from France. Study conducted by Nüesch-Inderbinen *et al*., 2019 found B2 and A group MPEC as the most prevalent *E. coli* isolated from clinical mastitis cases in Switzerland while Zhang *et al*., 2018 also found A and B1 as majority of MPEC phylogroups isolated from bovine mastitis cases in Liaoning, China. Ghanbarpour *et al*., 2010 also found majority of MPEC to be B1, A and D group respectively while no B2 group was found in a study conducted in Kerman province of Iran. Our study presents different demographics of MPEC phylogenetic groups isolated from bovine mastitis cases in Rawalpindi district with majority being D group MPEC but, it would be precipitous to come to conclusion. For better comparison, large sample size of *E. coli* is needed comparable to number of *E. coli* studied in the above-mentioned

studies. All these Phylogenetic groups can be further characterized according to their role in host i.e., A and B1 groups are mainly commensals and diarrheagenic while B2 and D group *E. coli* are ExPEC (Liu *et al*., 2014). Accordingly, our study found most of MPEC to be ExPEC.

The very high prevalence of *iss* and *fimH* genes (95.45% for both) in MPEC isolates is an indication of ability of majority of the MPEC isolates to survive in serum and adhere to or colonize extraintestinal environments outside their natural habitat (gastrointestinal tract) because *iss* gene allows survival in serum while *fimH* is involved in adhesion to epithelial layer of various organs. Though occurring in commensal as well as ExPEC, prevalence of *iss* is much higher in ExPEC where it enables the pathogenic *E. coli* to resist complement mediated killing and studies have shown that the gene confers 100 times more virulence in one day old chicks (Johnson *et al*., 2008). Together with high bacteriuria and high prevalence of *fimH*, these MPEC isolates can be causative agent of UTI and might also have a role in zoonosis since the gene codes for fimH adhesin which mediates binding with α-mannoside found on epithelial lining of various organs including upper urinary tract in humans (Vandemaele *et al*., 2004). Besides these, the genes found in significant numbers (found in 10 isolates) were *papC* and *cvaC* former of which is an outer membrane protein of P pilli (Dodson *et al*., 1993) while the latter is involved in colicin production (Gilson *et al*., 1987).

Among toxin genes, very high prevalence of shiga toxins in MPEC isolates studied is alarming and a public health concern. Shiga toxins are known to be cause of diarrhea in animals and humans, and dairy animals have been long known for harboring Shiga toxin producing *E. coli* (STEC) strains. Studies around the world have reported prevalence of 10-80% prevalence of STEC strains in dairy cows. Accessory virulence factors influence virulence of STEC worth mentioning being *eaeA* and *hlyA*, the former enables attachment with intestinal cells of the host while the former

encodes enterohemolysin (Lira *et al*., 2004). Three of the STEC isolates also contained *eaeA* gene while *hlyA* was found in only one STEC isolate.

The study provides insight into the prevalence of bovine mastitis in Rawalpindi district which is different to the expected prevalence and prevalence reported by Khan *et al*.,2019 for sub-clinical mastitis in dairy buffalos. Insight into the major causative bacterial agents of bovine mastitis found *S. aureus* to be present in majority of bovine mastitis positive milk samples while *E. coli*/ MPEC also occurred in significant number of these samples. Antibiotic resistance pattern and virulence associated properties of the isolated MPEC from bovine mastitis indicate the bacteria to be highly resistant to commonly used antibiotics in dairy industry and highly pathogenic with potential to cause zoonotic diseases albeit, more studies need to be done in order to find role of MPEC in zoonosis.

6. Conclusion

Coming towards the conclusion, our study found high prevalence of bovine mastitis especially among dairy cattle in Rawalpindi district. The most prevalent bacteria found among mastitis positive milk samples was *S. aureus* with *E. coli* being present at significantly high number. The antibiotic susceptibility revealed that most of the MPEC isolates were Multi-drug resistant including medium level resistance for Carbapenems and 3rd generation Cephalosporins with majority being able to produce ESBLs. In vitro pathogenicity results revealed various pathogenic aspects of MPEC such as hemolytic ability of blood cells, congo red binding activity, different motility abilities, biofilm forming ability and growth in human urine. MPEC isolates studied belonged from every group of phylogenetic classification as described by Clairmont *et al*., 2000. Virulence associated genes (VAGs) and toxin genes profiling revealed highest prevalence for *iss* and *fimH* genes while among toxin genes, stx_1 gene was the most prevalent gene found. Mastitis is a disease which renders considerable economic loss to the dairy sector and has a potential of spreading zoonotic diseases. The incidence of the disease can be minimized by adopting sound husbandry practices and stricter controls to ensure minimal antibiotics usage. More data is needed in this respect to analyze pathogenic potential of other major mastitis causing bacteria in order to find suitable therapeutic approaches to control the disease.

7. Future Prospects

The first limitation of the study is that it reports prevalence of bovine mastitis and causative bacterial agents in Rawalpindi district which represents local perspective of the study. A nationwide study is needed to have clear picture of the disease in the country. In the study antibiotic susceptibility pattern and virulence associated properties of *E. coli* were investigated. In order to establish clear cut role of *E. coli* in bovine mastitis and zoonotic diseases, further research needs to be done by conducting *in vivo* trials, whole genome sequence comparisons of commensal as well as pathogenic strains of *E. coli* from different hosts. In the same manner, other major bacterial causative agents of mastitis causing bacteria should be evaluated for their role in pathogenicity. Since antibiotic resistance is becoming a major health concern and the study revealed high antibiotic resistance and virulence potential of MPEC, alternative strategies for treatment of infection should be evaluated. Vaccines should be developed for treating bovine mastitis especially against environmental pathogens like *E. coli* because of their ubiquitous nature. During our study we have found use of indigenous plants such as chilies, garlic and mustard oil by local dairy farmers for treating bovine mastitis. These therapeutic strategies which could be evaluated and possibly exploited as a complete alternative to antibiotics or used in combination with them for synergistic effect.

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