EPIDEMIOLOGICAL INVESTIGATION OF RISK FACTORS OF MASTITIS IN DAIRY BUFFALO AND ANTIBIOTIC RESISTANCE OF *STAPHYLOCOCCUS AUREUS*

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To

My Teachers, Parents and Family
ACKNOWLEDGEMENTS

All praises to “ALLAH”, the Almighty, Most Gracious, the most Merciful and the Sustainer of the worlds, Who created the jinn and humankind only for His worship and raised among the inhabitants of Makkah the Holy Prophet “Muhammad” (peace be upon him.) from among themselves, who recited to them His revelations and purified them, and taught them the Book (Holy Quran) and the Wisdom. Foremost, thanks to Almighty Allah Who blessed me with good health and valued teachers to accomplish my Ph.D studies. I feel great pleasure in expressing my heartiest gratitude and a deep sense of obligation to my distinguished supervisor, Prof. Dr. Mansur ud Din Ahmad, Department of Epidemiology and Public Health, for his able guidance, keen interest, skilled advice, constructive criticism, constant encouragement, valuable suggestions and kind supervision throughout the course of my study and research work. In addition, I owe my thanks to my committee members, Dr. Muhammad Hassan Mushtaq Assistant Professor, Department of Epidemiology and Public Health, and Prof. Dr. Muhammad Sarwar Khan, Department of Clinical Medicine and Surgery, for their time and insights into this study. All these respected teachers have contributed their thoughts, perspectives, insights, and experiences, which have positively impacted the quality of my thesis. Special thanks to Prof. Dr. Michael Philipp Reichel and Dr. Tanveer Hussain who helped me a lot during my research work. Many thanks go to the labs of Epidemiology and Public Health. In the end, I could not envisage the endeavors that my parents, made for my comfortable stay at the campus No acknowledgement could ever adequately express my obligation to my affectionate parents for leading their children into intellectual pursuits. “May Allah give them a long, prosperous and happy life” (Aa’meen). I also extend my heartiest compliments to Dr. Noshaba Fakhar-ud-Din, Fatima Abid, Meerub Abid, brother and sisters whose love and support made it possible to reach this position.

Abid Hussain
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Pakistan is an agricultural country where livestock farming is its integral part and considered to be the backbone of the rural economy. The livestock contribution in national GDP is 11.8% while its share in agricultural value added stands at 55.9%. The agriculture sector involves 43.7% of the labour force in the country. As many as 8.4 million farming families are directly engaged in livestock rearing nationwide that shows the critical dependence of nearly 40 million rural people on the livestock sector for their livelihoods. Milk is by far single most commodity of the livestock sector and the value of milk alone exceeds than the combined value of cash crops such as wheat, rice, maize and sugarcane. Pakistan is third largest milk producing country in the world with an annual production of 50.9 million tons. Milk production in Pakistan is mainly dominated by 34.6 million buffaloes (Anonymous, 2014) having a share 75% of the total milk produced in the country (Jamil et al. 2011; Hussain et al. 2012).

Dairy farming in Pakistan is primarily fragmented into traditional rural, progressive/semi-commercial rural, peri-urban and commercial/corporate farming. However, the traditional rural farming is the largest segment among all. The majority of dairy farmers are smallholders with substantial number of landless farmers. The 61% of the farmers owned only 1-2 animals, 30% of their owned 3-6 animals and the remaining 9% farmers have more than 6 animals herd size. A number of notable livestock diseases, both infectious and non-infectious, are quite common in Pakistan. These diseases are not only life threatening but also have substantial impact on the production of dairy animals towards economic distress for the farmers. Among the infectious diseases mastitis has been ranked on top for economic losses because of decreased milk production.
INTRODUCTION

It results not only in milk losses but also deteriorate its quality.

Mastitis is a complex and multi factorial disease and prevalent around the globe in herds. It is an inflammation of the parenchyma of the udder characterized by physical, chemical and pathological changes in the milk and udder. Mastitis mostly occurs in two forms i.e. clinical and subclinical. The clinical mastitis can easily be identified with abnormal milk, udder swelling and disorder of the affected animal, while subclinical mastitis, mostly gone unnoticed by farmers because of non-apparent milk changes (Radostits et al. 2000). For every case of clinical mastitis, there are 20-40 times as many cases of subclinical mastitis and this subclinical mastitis causes losses in the dairy industry (Erskine et al. 2002; Schultz et al. 1978).

Mastitis can be divided in the environmental and contagious form on the basis of its etiological agents. The environmental mastitis is derived from the environment in which the animal lives. Contagious mastitis can be transmitted from one buffalo to another during milking from infected to healthy animal. The contagious pathogens are *Streptococcus agalactiae*, *Staphylococcus aureus* and mycoplasma spp. Among the contagious mastitis pathogens, *Staphylococcus aureus* is most frequently responsible for this disease. *Staphylococcus aureus* produce toxins that destroy cell membranes and can directly damage milk producing tissue. The leukocytes are attracted to the area of inflammation, where they attempt to fight the infection. Initially, the bacteria damage the tissues lining the teats and gland cisterns within the quarter, which eventually leads to formation of scar tissue. The bacteria then move up into the duct system and establish deep-seated pockets of infection in the milk alveoli. This is followed by the formation of abscesses that wall-off the bacteria to prevent spread but allow the bacteria to avoid detection by the immune system. The abscesses prevent antibiotics from reaching the bacteria and are the
primary reason (Petersson et al. 2010). *S. aureus* is modified to survive in the mammary gland and usually causes a subclinical mastitis and can be shed in the milk, facilitating transmission to healthy animals during the milking process (Radostits et al. 2007). The main reasons for the failure of any of the antibiotic therapy in most cases of Staphylococcal mastitis are inadequate concentration of antibiotics reaching at the site of infection, bacterial antibiotic resistance, L-forms of bacteria (sensitive to beta-lactam antibiotics), bacterial dormancy and tissue barriers, as this organism lies in deep seated foci. It causes pockets in the depth of udder surrounded by fibrous tissue where antibiotics cannot gain access (Sandholm et al. 1991).

In principle, antibiotic sensitivity against the available chemotherapeutic agents in a region or locality should be done from time to time to enable the veterinarians to prescribe the most suitable antibiotics against the prevailing diseases.

The present study was planned with the following aims and objectives:

- To assess the prevalence of mastitis in buffaloes.
- To identify the epidemiological risk factors of mastitis.
- To assess the resistance of *Staphylococcus aureus* against commonly used antibiotics.
- Molecular characterization of virulent gene of indigenous strains of *Staphylococcus aureus*.
REFERENCES


INTRODUCTION

Mammites, desvaches, laitieres. 9: 88-106.

2.1 Buffalo

In South Asia, the water buffalo is reared as a primary dairy animal. The water buffalo (Bubalus bubalis) is also called as the ‘Black Gold’. Water buffalo plays a vital role in Asia and Far-Eastern countries where 96.0% of its total population exists. At present, Pakistan has a buffalo population of 34.6 million (Anonymous 2014). Buffalo is recognized as the world’s second most important milk producing species. In buffalo milk production, it has been estimated that 95.0% of world’s buffalo milk is produced in Asia (FAO, 2010). Pakistan is the fifth largest milk producer in the world (FAO, 2010). In Pakistan, the increasing interest in buffalo breeding during recent years owes to the increased demand of buffalo milk with more butterfat, protein and lactose, which is essential for buffalo mozzarella cheese and makes it more valuable than bovine milk (Moroni et al. 2006).

2.2 Mastitis

Mastitis is a complex and multifactorial disease and it is an important dairy health problem with physical, chemical and pathological alterations in milk and udder. It is an inflammation of the parenchyma of the udder and is characterized by physical, chemical and pathological changes in the milk and udder. Udder inflammation may be caused by any injurious agent, including physical trauma, chemical irritants, infectious agents and their toxins. Mastitis occurs worldwide among dairy animals and it has an extreme zoonotic and economic impact (Radostits et al. 2007).

Mastitis mostly occurs in two forms i.e. clinical and subclinical. The clinical mastitis can be easily identified with abnormal milk, udder swelling and the disorder of the affected animal while in case of subclinical mastitis, there only increases in somatic cell count with no apparent milk changes (Radostits et al. 2000). In subclinical mastitis, milk production may decrease and
bacteria are present in the milk. It has been reported from previous studies that annual losses due to mastitis in dairy industry are approximately $2 billion and $526 million in the USA and India, respectively (Varshney and Naresh 2004; Donovan et al. 2005). For every case of clinical mastitis, there are 20-40 times as many cases of subclinical mastitis and this subclinical mastitis causes the losses in the dairy industry (Erskine, 2002; Schultz et al. 1978). Mastitis can be divided in the contagious and environmental form on the basis of its etiological agents. Among the etiological agents, bacteria are the most common ones, the greatest share of which resides widely distributed in the environment of dairy cows, and hence pose a common threat to the mammary glands (Bradley, 2002). The cost of milk production increases due to the cost of treatment along with the loss of milk due to reduced volumes and its poor quality (Radostits et al. 2007). Mastitis severely affects milk quality and production of infected animals and has a tendency to spread rapidly within the herds and to other animals. Compositional changes in the milk of infected animals with mastitis depend upon the inflammatory response of mammary gland, extent and pathogenicity of infectious agent along with amount of infected tissue, leakage of blood, various enzymes, proteins, salts, decreased concentration of lactose and fat in milk (Osteras, 2000).

2.2.1 Anatomy of Udder

The glands and teats of domestic animals are collectively known as udder. A buffalo udder is composed of two halves, each of which has two teats and each teat drains a separate gland (quarter). The quarters are separated by connective tissue and each has a separate milk collecting system. The two halves of the bovine udder are separated by the median suspensory ligament, which is formed by two lamellae of elastic connective tissue originated from the abdominal tunic. The posterior extremity of its ligament is attached to the prepubic tendon. The lateral suspensory ligaments are composed largely of fibrous, non-elastic strands given rise to numerous lamellae that
penetrate the gland and become continuous with the interstitial tissue of the udder. The lateral suspensory ligaments are attached to the prepubic and subpubic tendons, which in turn are attached to the pelvic symphysis. The lateral and median suspensory ligaments are the primary structure supporting the bovine udder. The bovine teat has a small cistern terminating at its distal extremity in the streak canal, which is the opening to the exterior of the teat. Radiating downward from its internal opening into the streak canal is a structure known as Furstenberg’s rosette, which is composed of about seven or eight loose folds of double layered epithelium and underlying connective tissue; each fold has a number of secondary folds. The primary structure responsible for the retention of milk is a sphincter muscle surrounding the streak canal. Large ducts empty into a gland cistern located above each teat. These ducts branch profusely, ultimately ending in secretory units called alveoli. Alveoli are generally recognized as the basic functional units of the lactating mammary gland (Husveth, 2011).

Milk is formed in the epithelial cells of the alveolus. The alveolus are approximately 100 to 300 µm in diameter. The size of the alveolus is affected by many factors, primarily the amount of milk in the lumen. The alveoli are grouped together in units known as lobules, which are surrounded by more extensive connective septa. The alveoli are surrounded by contractile myoepithelial cells that are involved in the milk-ejection. Myoepithelial cells are also located along the ducts (Husveth, 2011).
Figure: 2.1 Anatomy of the Bovine Udder.
2.3 Mastitis and its Pathogens

The major pathogens responsible for mastitis include *Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Escherichia coli*. *Staphylococcus aureus* and *Streptococcus agalactiae* serve as contagious pathogens while *Escherichia coli*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* act as environmental pathogens (Waller et al. 2009). Among a very large number of pathogens causing mastitis in buffalo and cow, *Staphylococcus aureus* and *Streptococcus agalactiae* are the most frequent etiological agents and induce an extensive and wide variety of pathologies in lactating animals (Marjan et al. 2009; Cheng et al. 2010). Intramammary infections with *Staphylococcus aureus* are associated usually with increased somatic cell count. *Staphylococcus aureus* is contagious and spreads easily within dairy herds (Barkema et al. 2006; Marjan et al. 2009). Prevalence of mastitis pathogens varies from one dairy herd to other dairy herd, but bacterial pathogens are most prominent and act as a first line of infection throughout the world (Bradley, 2002; Unnerstad et al. 2009). Mastitis causing bacteria are divided into two groups contagious and non-contagious, which include gram positive bacteria, whose cell wall consists of peptidoglycan layers that withhold crystal violet stain and gram negative that lack peptidoglycan layers and teichoic acid incorporated within the peptidoglycan. The major gram positive bacteria include *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Streptococcus agalactiae* (Piepers et al. 2009). In gram negative mastitis pathogens, *Escherichia coli* acts as the primary pathogen (Unnerstad et al. 2009). Mastitis is mostly caused by a wide range of bacteria mainly *Staphylococcus aureus* followed by *Streptococcus agalactiae*, *Staphylococcus hyicus*, *Staphylococcus epidermidis*, Bacillus spp., *Staphylococcus hominis*, *Escherichia coli*, *Staphylococcus xylosus*, *Streptococcus dysgalactiae* and Corynebacterial spp. (Ali et al. 2008;
Unnerstad et al. 2009; Waller et al. 2009). In spite of growth inhibitory properties of keratin, bacteria are able to survive in teat canal causing inflammation (Trinidad et al. 1990). Milk is sterile and free from pathogens when it is secreted by the mammary gland and it is infected with different micro-organisms when flows out of the udder. Generally, various pathogenic and non-pathogenic microorganisms excluding mastitis dairy animals cause changes in color, taste, aromas of milk and certain pathogens result in food borne diseases. Pathogenic organisms which are responsible for the deterioration of milk and its product quality, mainly enter into milk through improper milking, handling, storage and finally unhygienic conditions of workers.

*Staphylococcus aureus* produce toxins that destroy cell membranes and can directly damage milk producing tissue. The leukocytes are attracted to the area of inflammation, where they attempt to fight the infection. Initially, the bacteria damage the tissues lining the teats and gland cisterns within the quarter, which eventually leads to formation of scar tissue. The bacteria then move up into the duct system and establish deep-seated pockets of infection in the milk alveoli. This is followed by the formation of abscesses that wall-off the bacteria to prevent spread but allow the bacteria to avoid detection by the immune system. The abscesses prevent antibiotics from reaching the bacteria and are the primary reason (Petersson et al. 2010).

2.4 Economic Importance and Characterization of Mastitis

In Pakistan, economic losses due to mastitis have serious concerns (Farooq et al. 2008). The importance of domestic animals may be realized from the fact that the rural population is generating, 30-40% of their income from livestock and almost 35-40 million populations are involved in the livestock industry (Anonymous 2013). Various expenses like decreasing in milk production, treatment cost, increased labor charges are compound conditions where culling becomes necessary. The improved management practices can reduce the prevalence of mastitis.
While investigating the total expenses that are involved in mastitis prevention and its treatment, scientists have determined some strategies based on the direct impacts of disease. Miller et al. (1993), determined cost of mastitis prevention was $14.50 per animal per year. Economic losses due to mastitis may be divided into: 70.0% lower milk production, 8.0% discarded milk due to veterinary medication, 8.0% treatment and veterinary charges and 14.0% premature culling (Barkema et al. 2006; Halasa et al. 2007). Mastitis results in premature removal of herd, alters reproduction, increases culling thus causing serious economic loss to farmers (Parker et al. 2008). According to Wells et al. (1998), worldwide losses due to mastitis were estimated to be approximately $35 billion annually. In the US, overall costs of mastitis were estimated to be $1.5–2.0 billion per year, while losses from reduced milk production and higher replacement costs associated with high somatic cell counts (SCC), due to subclinical mastitis were estimated at $960 million (Wells et al. 1998). In 2007, the USDA reported that mastitis was considered to be one of the most costly diseases of the dairy industry (USDA, 2008). The average cost of generic clinical mastitis (CM) per cow and year in high-yielding dairy cows, based on collecting data from 5 large herds since 2004 to 2006 in New York State was $71, with an incidence of 39.7 CM cases per 100 cows per year and the average cost of a CM case was $179. This cost comprised of $115 due to milk yield losses, $14 due to increased mortality, and $50 due to treatment-associated costs (Barkema et al. 2006). In Africa, according to Mungube et al. (2005), production losses associated with subclinical mastitis were estimated 5.6% of the Addis Ababa Milk Shed. Losses were highest (9.3%) for urban dairy farms and small-scale farms (6.3%). The estimates of the financial losses ranged from $29.1, for dairy herds in secondary towns, to $66.6 for urban dairy farms. A total loss of $38 was estimated for each cow per lactation (Mungube et al. 2005).
2.5 Prevalence of Mastitis

According to a study conducted by Ali (2009), the overall prevalence of subclinical mastitis was 23.9%. Sharma et al. (2007) reported the overall prevalence of subclinical mastitis (SCM) by quarter was 42.0% in Chhattisgarh State, India by the Modified California Mastitis Test (MCMT). Elbers et al. (1998), conducted a study in Southern part of Netherland and reported the incidence rate was 12.7 quarter/year per. Hameed et al. (2012) conducted a study in Burewala Pakistan, and reported a total of 673 animals (n=291 cattle, n=382 buffaloes) from 300 livestock farmers and tested using the Surf Filed Mastitis Test (SFMT) for the presence of mastitis. A prevalence of clinical and subclinical mastitis was 24.6 and 36.4% in buffaloes, respectively, and subclinical mastitis was more vital in varying from 10–50%, 5–20% in cow and buffaloes respectively than clinical mastitis (Joshi and Gokhale 2006) in India. Anwar et al. (2013) reported that *Staphylococcus aureus* prevalence was highest and the most frequently bacterial species such as in Nili Ravi and Kundhi 40.4% and 34.9%, while in the study by Hussain et al. (2013) the subclinical mastitis prevalence was recorded to be 15.2% in Lahore, Pakistan. According to Megersa et al. (2010), subclinical mastitis and clinical mastitis were 11.2%, 4.3% respectively in Southern Ethiopia, while mastitis overall prevalence was 15.5%. Busato et al. (2000) described the quarter wise subclinical mastitis prevalence was 21.2% and overall prevalence of subclinical mastitis (SCM) was 36.5% in Switzerland while Buchaya et al. (2005) the quarter wise overall prevalence of mastitis as 58.7% and the animal wise was 78.0% in Bangladesh.

2.6 Quarter wise prevalence

According to Bansal et al. (1995), subclinical mastitis was present in 23.9% of buffaloes and 11.3% of buffalo’s udder quarters while Prabhakar et al. 1995 stated that the overall monthly incidence was 4.1 percent and Khan et al. (2004), by using the Surf field mastitis test, recorded
27.0, 4.0 and 10.0 % prevalence for subclinical mastitis, clinical mastitis and blind quarters, respectively. Lalrinthuanga et al. (2003) revealed that 37.5% and 11.6% for mastitis by animal and quarter, respectively while Ahmad et al. (1991) described the subclinical mastitis prevalence was 7.0%. Physical examination of udders revealed (7.2%) blind teats. Islam, et al. (2011) showed the overall clinical mastitis prevalence and subclinical mastitis as 4.5% and 37.2%, respectively while another study by Islam et al. (2012) showed the overall prevalence of subclinical mastitis (SCM) 29.0%. Zeryehun et al. (2013) revealed abnormalities of the udder as evidence of mastitis 74.7% in cows and 9.6% clinical and 55.1% were subclinical mastitis while the incidence of clinical mastitis was 22.8% (Peeler et al. 2000). Sargeant et al. (1998) mentioned a prevalence of 19.8% of cows experiencing one or more cases of clinical mastitis during lactation and the bacteria isolated was *Staphylococcus aureus* (6.7%). Oezenc et al. (2008) investigated the mastitis per quarter and animal 36.5%, 69.1%, respectively. Bachaya et al. (2011) investigated *Staphylococcus aureus* was a predominant bacterial species recovered from mastitis and it shows dominancy 48.6% and following bacterial species were isolated such as *Staphylococcus aureus* (48.6%), *Bacillus cereus* (2.9%), *Escherichia coli* (10.0%), *Micrococcus luteus* (15.7%), *Proteus vulgaris* (4.3%), *Pseudomonas aeruginosa* (1.4%), *Streptococcus dysgalactiae* (11.4%), *Streptococcus uberis* (4.3%) and *Citrobacter* species (1.4%). Ali et al. (2011) stated the overall prevalence of subclinical mastitis was 44.0% and it was the highest (58.0%) in dairy buffaloes. The lowest prevalence was in organized farms with reasonable good management conditions (32.0%). The overall prevalence of SCM was 36.5% of a CMT Test (Islam et al. 2012b). Khan and Muhammad (2005) studied the overall subclinical mastitis prevalence 27.0%, clinical mastitis 4.0% and prevalence in buffaloes was 29.0%. Ahemd et al. (2012) studied the overall prevalence of mastitis (clinical and subclinical) and found it in 60.3% buffaloes. The clinical mastitis
prevalence was higher in peri-urban 25.1% than rural 9.7% and incidence was highest during 4 to 6 months after calving both in peri-urban 45.8% and rural areas 45.1%. Mastitis was maximum during third lactation both in peri-urban 19.0% and rural (23.0%) localities. In the folded thumb milking method, prevalence was higher than suckling calves. Stage of lactation and lactation number, source of milk let down, method of milking and floor condition have their effect on mastitis (Bilal et al. 2004).

The clinical mastitis prevalence of individual quarter in buffaloes was 8.0%, while quarter-based prevalence of subclinical mastitis was 16.0% in buffaloes (Hameed et al. 2012). The overall quarter wise prevalence was 58.7% in buffaloes (Bachaya et al. 2005). According to Memon et al. (2012), the prevalence of subclinical and clinical mastitis at quarters 20.2% and cow level 52.3% was recorded, respectively. Occurrence of subclinical mastitis in the left rear quarter was high 26.7%, while Sharif et al. (2007) investigated that the overall quarter-wise prevalence was 37.7% quarters and mastitic quarters maximum prevalence was found in right rear (30.4%) followed by left front, right front and left rear with values of 24.5, 23.8 and 21.2%, respectively. Bilal et al. (2004) observed the incidence of mastitis was higher in hindquarters 73.3 and 63.1% than in forequarters 26.6 and 36.8% in peri-urban and rural areas, respectively while Sharma et al. (2007) mentioned the overall quarter-wise prevalence of SCM was 39.0% by Modified Wide side Test, 42.0% of Modified California Mastitis Test and 45.0% of SCC, 1.9%. Oezenc et al. (2008) investigated that mastitis was higher per quarter 36.5% and animal 69.1% while (Joshi and Gokhale 2006) the prevalence was increased with lactation number and animals in 4th to 5th month of lactation were more susceptible (59.5%) while hind quarters were more affected (56.5%) than fore quarters (43.5%). The maximum quarter wise prevalence was in Nili Ravi and Kundhi 60.1% and 77.3%, respectively, (Anwar et al. 2013). The incidence of mastitis in cows was higher in the
hind quarters than in the forequarters (Prem and chand et al. 1995) as well as in buffaloes hind quarters the incidence of mastitis was higher than fore quarter, similarly, left quarters prevalence (44.0%) was higher than right quarters (33.3%) (Shukla et al. 1997) while mastitis prevalence was highest in the left fore quarters (29.3%) then left hind (28.0%), right hind (22.0%) and the right fore (20.7%) quarters. Due to the common practice of milkmen milking the prevalence was higher (Ramachandraiah et al. (1998). Lalrintluanga et al. (2003) screened the lactating cows an quarter infections was 63.44%, more frequently than any other combination of quarters and left hind quarters (30.2%) were more frequent and single factor as compared to the other three quarters same as Bilal et al. (2004) mastitis incidence was higher in hind quarters (73.3 and 63.1%) than in forequarters (26.6 and 36.8%).

2.7 Animal related risk factor of mastitis:

2.7.1 Age

Rahman et al. (2009) identified the risk factors about dairy farms and management and age and lactation wise distribution of mastitis cases varied in different age groups, being highest in 6-8 years of age group (40.5%) while according to Bilal (1999) the relative risk of clinical mastitis increased with the age and it was maximum in buffaloes 10-11 years as well as Ahmed et al. (2012) significantly associated (p<0.05) of age with the prevalence of mastitis and the prevalence (33.4%) of mastitis of 5–7 years of age were low, while the animals aged between 14 to 16 mastitis was highest (80.0%). According to Waage at el. (1998), mastitis increased as age of animal increased. Hameed et al. (2012) described that age is considered as a risk factor of mastitis as well as by Zeryehun et al. (2013) age was also considered as major intrinsic risk factors that influenced the prevalence of mastitis resembles with Rady and Sayed (2009) who investigated that age is an intrinsic risk factor of mastitis. According to Sharma et al. (2007) the prevalence was highest at 3
to 9 years of age. While Islam et al. (2012b) the prevalence of subclinical mastitis (SCM) was significantly (p<0.01) higher (47.6%) in age group more than 13 years than others. Bilal (1999) mentioned the relative risk for clinical mastitis increased with the age increase and it was maximum in buffaloes 10-11 years while Lalrintluanga et al. (2003) screened the lactating cows and higher number of cases were in 4-6 years age group (51.1%) while, Feroze (1992) noted that age wise mastitis varied in different age group and mastitis was highest in 6-8 years age group (40.5%).

2.7.2 Breed

The dairy buffalo breeds popular for milk production in Pakistan are Nili Ravi and Kundhi. The breed characteristics of Nili Ravi buffaloes are as massive animals having curled horns with an average weight 350-450 kg. The whole of the body skin is black in colour with five white marks on the body (on forehead and all the four hooves). This striking feature of the breed is locally pronounced as “Panj Kalyan” in native language. Males attain their maturity at the age of 30 months and females at 36 months. The first calving has been recorded at 46 months of age with an average milk yield of 1800-2500 liters per lactation with 6.5% butter fat. (Bilal et al. 2006; Shah et al. 1994).

Kundhi breed buffaloes are also massive animals having jet-black colour with an average weight 300-400 kg. The have broader horns at the base and taper upward and inward, giving them a fishhook shape. The average age of maturity of these animals is 30 months in males and 36 months in females. The milk yield per lactation is 1700-2200 liters with over 6.5% butter fat (Bilal et al. 2006: Shah et al. 1994).

2.7.3 Parity

According to Ahmed et al. (2012) lactation number (parity) is significantly associated (p<0.05) with the mastitis prevalence as well as Bilal et al. (2004) investigated that lactation
number has a significant effect on mastitis and during third lactation both in peri-urban (19.0%) and in rural areas (23.0%) maximum cases of mastitis were seen. Lactation number was considered as an intrinsic risk factor of mastitis (Hameed et al. 2012) same as parity was considered as major intrinsic risk factor of mastitis (Zeryehun et al. 2013). According to Busato et al. (2000) quarter level 21.2% for lactation period 7–100 days and 34.5% for 101 to 305 days post-partum subclinical mastitis prevalence was increased while Oezenc et al. (2008) studied that during the first 4 months of lactation after the 5th lactation prevalence was higher. According to Prem and chand et al. (1995), incidence of mastitis increased as the lactation number increased while according to study in Nepal by Joshi and Shrestha (1995), the prevalence of bovine clinical mastitis was the highest (17.6%) during first lactation, declining in successive lactations. Ramachandraiah et al. (1998) investigated that the subclinical mastitis was increased with the increase in lactation number same as during third lactation mastitis increased (Bilal et al. 2004). Similarly, Thirunvukkarasu and Prabaharan (1998) concluded that, lactation number has significantly associated with mastitis (P<0.05).

2.7.4 Stage of lactation

Stage of lactation was investigated by Ahmed et al. (2012) that has significant association (p<0.05) with the prevalence of mastitis and it was associated with the prevalence highest (54.5%) during the initial stage of lactation (0 to 1 month) than by last 2 months (10–12 months) as 54.17% and mid-stages (1–3 and 3–10 months) of lactation as 28.6% and 37.5%. Hameed et al. (2012) concluded that the stage of lactation is a significant risk factor of mastitis and Sharma, et al. (2007) described that the stage of lactation has an influence on infection rate and it was higher during the late lactation followed by early and mid-stage of lactation. According to Bilal et al. (2004) stage of lactation is a physiological factor and it has its effect on clinical mastitis in buffalo same as
Zadoks et al. (2001) lactation stage has a significant effect on mastitis and rate of *S. aureus* infection. Megersa et al. (2010) investigated that late stage of lactation has an effect on mastitis (OR = 4.3, 1.8, 10.4) of udder have a high risk than early lactation same as Kavitha et al. (2009) who also reported that the first stage of lactation and the last part of dry period were more prone to mastitis in buffaloes. Zeryehun et al. (2013) studied the prevalence of mastitis at the stage of lactation was 87.2%, 65.9% and 73.1% in early, mid and late lactation, respectively, and this variation was statistically significant (P<0.05) as Islam et al. (2012b) described the prevalence of SCM was the highest in late lactation (72.4%) followed by early (40%) and mid lactation (27.6%). Hussain et al. (2013) revealed the significant difference between lactation stage (P<0.001) same as Lalrintluanga et al. (2003), who screened the lactating cows and incidence of mastitis was higher in the early stage of third lactation (30.6%). Stage of lactation was found significantly associated with mastitis (P<0.05) (Thirunvukkarasu and Prabaharan 1998). The mastitis cases were a higher number (53.6%) during early stage of lactation than in middle and late stages and affected animals showed a 20.6 % decrease in milk yield (Pyroala et al. 1991). Morin et al. (1998) examined the cows for clinical parameters including various udder and milk characteristics, lactation number and stage of lactation showed significant effect on clinical mastitis.

### 2.7.5 Udder and Teat injury

Hussain et al. (2013) revealed the significant difference between various groups, including teat/udder pathology, teat (P<0.001) have a significant positive association with mastitis. Ahmed et al. (2012) stated lesion on udder/teat were significantly associated (p<0.05) with mastitis. According to Hameed et al. (2012), udder and teat injury is significant risk factors of mastitis same as Biffa et al. (2005) revealed that the udder/teat injuries by ticks were the main risk factors of mastitis while Zadoks et al. (2001) teat end callosity and infection with was not significant as risk
factors. Udder abnormalities and milking hygiene have significant association with mastitis (P<0.05) (Thirunvukkarasu and Prabaharan 1998) while according to the Oltenacu et al. (1990), the incidence rates of trampled teats, udder injuries and clinical mastitis as well as the inter relationship between the 4 disorders depend upon the stall length, manure system, type of bedding and calving disorders and cows in herds with liquid manure system were at higher risk of udder injuries and mastitis. Lower risk of both udder injuries and mastitis was found for cows in herds with short stall size (180 cm) as compared to herds with large stall size (205- 219 cm) length.

2.7.6 Udder and Teat edema

In a case-control study by Waage et al. (1998), udder edema, teat edema and milk leakage were the significant risk factors and teat edema at calving as risk factors for clinical mastitis caused by Staphylococcus aureus. Hussain et al. (2012b) used the univariate and bivariate logistic regression including teat shape and udder shape with the occurrence of mastitis and determined the association of some morphometric characteristics of the udder with mastitis in dairy cattle same as Hussain et al. (2013) revealed significant difference between various groups, including teat shape, and udder shape (P<0.001). Shukla et al. (1997) investigated the relationship of teat type, teat length and quarters effect on the mastitis and the teat tips were categorized into four types viz., funnel, round, flat and plate shaped. Mastitis prevalence was maximum in case of funnel shaped teat tip.

2.8 Management, Housing and Environmental Risk Factors related to Mastitis

2.8.1 Management and Farm Hygienic

According to investigation by Fadlelmoula et al. (2007), the management and hygienic risk factors of mastitis were associated with mastitis prevalence and the results show that the probability of mastitis was higher, in tie-stall housed cows, and with the use of the pipe milking
In a Swedish study (Ekman et al. 2004), management practices like, use of wood shavings of any kind reduced the risk of finding *Staphylococcus aureus*, but increased the probability of *Klebsiella* spp., four times as compared to straw or peat. The correlation between sawdust and acute cases of clinical mastitis caused by *Klebsiella* spp. was well known (Oz et al. 1985). Neaveet al. (1969) evaluated the value of hygiene systems in the control of mastitis and how this control can be improved by changes in hygiene and milking machines and by better use of therapy and hygiene systems designed to prevent the transfer of pathogens and more particularly to eliminate residual contamination at the completion of milking have been shown to reduce the number of new infections. Furthermore, the combination of such hygiene systems with effective antibiotic therapy, which reduces the duration of infection, generally resulted in a decrease of more than 50.0% in the incidence of infection within a year. It is probable that further reduction in the incidence of infection can be made by improving management techniques and it can be achieved by improving methods of mechanical milking designed to prevent infection occurring during milking and by the use of better teat dips than by the development of more comprehensive hygiene systems. Ahmed et al. (2012) described the hygiene of milking process has significant association (p<0.05) with the mastitis prevalence and it was concluded by Zaki et al. (2010) who described that both environmental and hygienic measures surrounded the animals are major risk factors of mastitis. Zeryehun et al. (2013) described the prevalence of mastitis in cows that were managed in poor hygienic condition was 82.6%, while those managed in good hygienic condition showed an infection rate of 59.6% and this prevalence were significantly different (P<0.05). Erskine et al. (2002) performed milking hygiene and management practices of each herd and milking-machine function was evaluated and it was concluded that management practices have a significant effect on the control of mastitis.
2.8.2 Condition of floor

Hameed et al. (2012) revealed that bricks floor, uneven floor, poor drainage system are risk factors of mastitis while Bilal et al. (2004) investigated the management factors like method of milking and floor condition have their effect on clinical mastitis in buffalo and bricks and cemented floor were more mastitis as compare to Kacha floors. Hameed et al. (2012) analyzed that poor drainage system and low frequency of dung removal are a risk factor of mastitis.

2.8.3 Teat Dipping

An investigation by Kavitha et al. (2009) depicted that teat dipping has a significant effect on mastitis furthermore, farmers’ education regarding the risk factors and about teat dipping is essential as Peeler et al. (2000) described the teat dipping can reduce 50.0% of mastitis occurrence. According to Neave et al. (1969), improved methods of mechanical milking designed to prevent infection occurring during milking and by the use of better teat dips than by the development of more comprehensive hygiene systems. Teat dipping treatments reduced occurrence of mastitis (Galton et al. 2004; Philpot and Pankey 1978). Mastitis control practices like post milking antiseptic teat dipping, prompt detection and treatment of clinical cases and dry therapy can reduced the mastitis (Oliver, 1975).

2.8.4 Milker hands

Larsen et al. (2000) investigated the transmission of infection for bovine mastitis by milkers with *Staphylococcus aureus* and Ali (2009) considered hand milking as a source of risk factor of mastitis used as a lubricant during milking and milker’s hands is often heavily soiled with milk during this process while in developing countries like Pakistan dairy animals are mostly hand milked used as a lubricant during milking and milker’s hands are often soiled with milk during this process.. Gonzalez et al. (1990) investigated that the milkers are the reservoir of source of
infection and its transmission to susceptible cows may be in direct contact with the milkers or by mechanical transfer from cow to cow via the teat cups. Hand-milked dairy animals have higher prevalence of mastitis than machine milked-animals (Lafi et al. 1994; Motie et al. 1985).

2.8.5 Methods for milk letdown

Mastitis prevalence was higher in animals milked with folded thumb pressure as compared to milk let down through suckling calves (Bilal et al. 2004) as Hameed et al. (2012) mentioned that hard milking, calf suckling, folded thumb milking techniques are risk factors of mastitis. In Pakistan, Oxytocin injection is used commonly for the letdown of milk particularly in buffaloes. At the time of milking by hand the calf is used in most of the animals. Newbould (1970) recommended that the stimulus for these phenomena also affects the smooth muscles around the proximal part of the teat duct near Furstenberg’ Rosette resulting in its dilation. Thus, instead of keeping the duct closed, the smooth muscles around the duct under stimulation, open the proximal end, and allow direct access for micro-organisms to the cistern. In theory at least, animals milked by exogenous oxytocin are at a greater risk to develop mastitis than animals not receiving this milk let down (Allen, 1990). On hand-milked animals, the technique of milking is also important in relation to prevalence of mastitis and factors affecting the mastitis prevalence of clinical in buffaloes by Bilal et al. (2004) encountered 4.2 magnitudes higher prevalence (39.2%) in buffaloes milked by folded-thumb method (thumb-knuckle and finger method) than those milked by full-hand method (9.0%). The relatively higher prevalence was ascribed to relatively larger herd size, use of hired labor for milking (who cannot be desirably careful in milking) and more common housing of animals on the brick floor. Kavitha et al. (2009) evaluated the milking method and the incidence of clinical and subclinical mastitis was high when knuckling was practiced as compared
to stripping with the full-hand milking. An association between risk factor and mastitis were higher infection rates when dairy animals are milked by milking machine (Oezenc et al. (2008)).

2.9 Some other Determinants Related to Mastitis

2.9.1 Foot and mouth Disease, Pox and Reproductive disorders

Diseases like foot and mouth disease and pox are particularly known to predispose mastitis in cattle and buffaloes. According to a study in Pakistan by Muhammad et al. (1998), an investigation of an outbreak of pox in 6 small holder dairy farm (buffalo = 185; cattle = 7) a cumulative incidence rate of 55.3% was recorded over a one month period. Milk from nearly 47.0% of the affected teats and 23.0% of the adjacent unaffected ones reacted positive to California mastitis test (CMT). According to Radostits et al. (2000) observation, foot and mouth disease, vesicles may appear on the teat as to the involvement of the teat orifice the severe mastitis. Cows with the retained placenta were three times more likely to develop mastitis during hospitalization than animals without retained placenta. Ali (2009) investigated on the outbreak of pox in 6 small holder dairy farms and a cumulative incidence rate of 55.3% was recorded over a one month period and milk from nearly 47.0% of the affected teats and 23.0% of the adjacent unaffected ones reacted positive to California mastitis test. According to Bilal (1999) dairy buffaloes having retained placenta, metritis, vaginal prolapses and dystocia at calving are risk factors of mastitis and by Barker (1998) clinical mastitis during the first 45 days of lactation shows 2.7 times higher risk of abortion. Esmat and Badr (1996) studied lactation failure and purulent uterine discharge (metritis) in 127 dairy cows from 3 dairy farms in relation to mastitis. Eighty seven cows (68.5%) had acute mastitis and 23 (18.1%) subclinical one and bacteriological examination of the udder and uterine secretion of cows showed mastitis-metritis syndrome.
2.9.2 Previous exposure to mastitis

Vaarst and Enevoldsen (1997) studied previous udder infections, which showed two-thirds of the clinical mastitis cases, severe local inflammations were found in 21.0% of the cases and some indications of generalized signs. Enevoldsen and Sorensen (1992) stated that previous mastitis statuses are considered to be much more important predisposing factors as same as Karimuribo et al. (2006) conducted a cross-sectional study and 4.0% of cows had clinical mastitis during the previous year.

2.10 Diagnosis, Isolation and Identification of *Staphylococcus aureus* Mastitis

2.10.1 Mastitis diagnosis methods

Clinical and subclinical mastitis were diagnosed by using the California Mastitis Test (CMT) and Surf Field Mastitis Test (SFMT) by Islam et al. (2009). According to Ali et al. (2008), the diagnosis of subclinical mastitis was based on the results of the Surf Field Mastitis Test (SFMT). Sharif et al. (2007) screened milk samples by SFMT for the presence of subclinical mastitis as well as Sharif and Muhammad (2009) mentioned the most common mastitis pathogens are contagious and environmental pathogens and most common causes of udder disease include *Staphylococci* (*Staphylococcus aureus* & *S. epidermidis*). Soomro et al. (1997) used three chemical tests viz., Bromothymol blue test, chloride test and Whiteside test on milk for screening of subclinical mastitis and revealed that 19.7%, 15.9% and 13.8% quarters were affected with subclinical mastitis. Ali et al. (2011) identified the bacterial isolates of mastitis and found the highest prevalence was of *Staphylococci* (28.3%). Chavoshi and Husaini (2012) determined the major causes of buffalo’s subclinical mastitis are teat skin opportunistic bacteria.
2.10.2 Physical examination of udder

Clinical and subclinical mastitis prevalence in buffalo in Hyderabad, Pakistan was investigated by Soomro et al. (1997) and the physical examination of the udder of these animals revealed 6.0% clinical (chronic) and 1.0% congenital abnormalities of udder while Milne et al. (2003) correlated the clinical signs with the bacteriological findings and reduced milk yield, swollen or hard udders, watery milk and being systemically sick. Falkenberg et al. (2004) evaluated clinical examination and the appearance of teat lesions and the status of the teat duct especially the existence of hyperceratosis were defined and prevalence of *Staphylococcus aureus* mastitis was high. Klass et al. (2004) conducted a study to evaluate the disease characteristics of *Staphylococcus aureus* mastitis in buffaloes and visual inspection and palpation of udder and teat. Sargeant et al. (1998) described the abnormal milk with a hard or swollen udder and milk plus systemic signs of illness related to mastitis.

2.10.3 Isolation of *Staphylococcus aureus*

Rahman and Rahman (1985) examined the milk samples derived from apparently healthy udders in Bangladesh and 350 representatives isolates showed distribution to be 42.0% *Staphylococci*, 12.6% *Streptococci*, 22.0% *Coliforms*, 20.0% Gram-positive aerobic sporeformers, 1.4% *Pseudomonads* and 2% *Corynebacteria*, while Smith (2000) isolated *Staphylococcus aureus* 13.3% from mastitis cases. In Istanbul, Firat and Uysal (1986) collected milk samples and examined the nature of mastitogens and found that 34.0% were coagulase-positive *Staphylococci*, 16.0% coagulase-negative *Staphylococci*, 51 (13.0%) gram-negative bacteria, 28 *Streptococcus agalactiae*, 22 *Streptococcus uberis*, 27 *Streptococcus faecalis*, and 21 *Streptococcus dysgalactiae*. Trinidad et al. (1990) reported that most common bacterial pathogen were *Staphylococcus aureus* as it was from isolated from teat canal (31.0%) and from quarters (12.3%). Prabhakar et al. (1995)
described that *Staphylococci* were the major causative organisms (34.2% *Staphylococcus aureus* and 13.2% coagulase-negative *Staphylococci*), followed by *Str. agalactiae* (14.7%), *E. coli* (10.5%), *Pseudomonas* spp (7.9%), *Str. pyogenes* (3.9%), *Klebsiella* spp. (3.9%), *Str. dysgalactiae* (2.6%), *Proteus* spp. (2.6%), *Str. uberis*, Diptheroids and mixed infections (1.3% each). No organism could be isolated from 2.6% quarters. Gonalez et al. (1990) isolated *Staphylococcus aureus* (43.0% of samples) *S. epidermidis* (21.0%), *Str. Uberis* (19.0%), *Str. Agalactiae* (13.0%), *Str. Dysgalactiae* (9.0%), *Corynebacterium pyogenes* (1.3%), *Corynebacterium bovis* (7.0%) and coliform (1.7%) from subclinical cases of mastitis in cows. Rady and Sayed (2009) recovered *Staphylococcus aureus* from milk samples and prevalence was 52.5%. Hameed et al. (2008) also reported that prevalence of *Staphylococcus aureus* was the highest 53.9% by a study conducted on lactating buffaloes in Pakistan. According to investigation of Hussain et al. (2012a), the disease characteristics of *Staphylococcus aureus* mastitis in buffaloes and isolates of *Staphylococcus aureus* were obtained on the basis of growth on a Staph-110 medium, colony morphology and hemolytic pattern on 5% sheep blood agar plates and isolates were identified by slide coagulase test, tube coagulase test and Staphytect plus test. Ali et al. (2008) recovered the isolates of *Staphylococcus aureus* and it was the most frequently bacterial species accounting for 49.5% of all the isolates while Ericsson et al. (2009) the most frequently isolated bacterial species were *Staphylococcus aureus* constituting 21.3% of the diagnoses.

### 2.11 Antibiotic Resistance Profiles of *Staphylococcus aureus* strains isolated from Mastitis:

The antibiotic resistance of *Staphylococcus aureus* strains isolated from mastitic milk is well known around the globe. It may vary from country to country and depends on various factors. Some of the resistant reports are as under. In Argentina, the antibiotic resistance of *Staphylococcus aureus* was reported as 40.3%, 11.6% and 3.4% to penicillin, erythromycin and gentamycin, respectively Gentilini et al. (2000). In India, Sharma et al. (2007) also reported the resistance
against ampicillin most. Sharma et al. (2007) revealed the antimicrobial susceptibility test for the bacterial strains isolated from subclinical mastitis milk samples were sensitive to oxytetracycline 17.4% and resistant to streptomycin. In Brazil, Pianta and Fallavena (1980) observed 40.0% resistance of *S. aureus* against oxytetracycline. A study was conducted in China (Sun et al. (2013) and reported *Staphylococcus aureus* resistant to gentamycin, tetracycline and erythromycin.

### 2.12 Emergence of Antimicrobial Resistance

There are several factors responsible for the emergence of antimicrobial resistance. Some of these include misuse of antibiotics, lack of access to appropriate treatment and failure to complete treatment courses etc. In Staphylococci the resistance to multiple antimicrobial agents is driven by the acquisition of distinct hereditary elements comprising plasmids, transposable genetic elements and genomic islands. These elements incorporate resistance genes and are exchanged via horizontal gene transfer between interrelated bacterial strains and even between different species and genera (Chambers, 2001; Schito, 2006).

### 2.13 *Staphylococcus aureus* Genome

The *Staphylococcus aureus* genome consists of a single circular chromosome of about 2.7 to 2.9 Mbp containing about 2600 genes composed of core and auxiliary (accessory) genes. It has a G+C content of 33.0%. In silico analysis, core genome makes up about 75.0% of the *Staphylococcus aureus* genome and it is highly conserved between isolates. These genes are associated with common species functions and are not essential for growth and survival, including virulence genes. It included, binding proteins, exoenzymes, toxins, and capsule biosynthetic cluster (Lindsay and Holden, 2004). The accessory genome accounts for about 25.0% of any *Staphylococcus aureus* genome, and mostly consist of mobile genetic elements that transfer horizontally between strains. These elements include bacteriophages, pathogenicity islands, chromosomal cassettes, genomic islands, plasmids and transposons. Accessory genes typically
have a different G + C content than those in the core genome, often because they are obtained from other species of bacteria. Many of these genetic elements are known to carry genes associated with virulence, drug and metal resistance, with substrate utilization and miscellaneous metabolism. Therefore, the distribution and horizontal spread of these elements can have important clinical implications. The identification and characterization of these elements provide insights into how *Staphylococcus aureus* causes disease, and their diversity (Shittu et al. 2007). Bacteria obtain genetic information from other cells or the surrounding environment in three ways: (1) uptake of free DNA from the environment (transformation), (2) bacteriophage transduction, and (3) direct contact between bacterial cells (conjugation). Bacteriophages or bacterial viruses seem to have the greatest impact on staphylococcal diversity and evolution. They are known to transfer genes such as *lukF-PV* and *lukS-PV* that encode the Panton- Valentine leukocidin (PVL) components which is strongly associated with severe forms of pneumonia (necrotic pneumonia) in human caused by community-acquired *S. aureus* strains; the staphylokinase gene (sak) gene, which is a potent plasminogen activator that could facilitate bacterial spreading through its fibrin-specific blood clotting activities and the enterotoxin genes (Shittu et al. 2007; Malachowa and DeLeo, 2010). *S. aureus* pathogenicity islands (SaPIs) often carry super antigen genes, such as toxic shock syndrome (*tst*) and enterotoxins B and C, implicated in toxic shock and food poisoning. Three families of genomic islands have been discovered in sequenced strains of *Staphylococcus aureus* strains. They carry exotoxin and lipoprotein genes (Shittu et al. 2007). All the sequenced *Staphylococcus aureus* strains are known to carry one or more free or integrated plasmids. All types of *Staphylococcus aureus* plasmids frequently carry antibiotic resistance genes, or resistance to heavy metals or antiseptics. Some virulence genes are also known to be carried on plasmids, such as exfoliative toxin B and some super antigens (Lindsay and Holden, 2004). Other mobile
genetic elements found in the *Staphylococcus aureus* chromosome include Staphylococcal cassette chromosomes (SCCs). They encode antibiotic resistance and/or virulence determinants. SCCs encode the methicillin resistance gene (*mecA*) (Shittu et al. 2007; Malachowa and DeLeo, 2010).

2.14 Molecular optimization of Coagulase and Fibronectin binding protein A of *Staphylococcus aureus*

New molecular techniques have emerged which are very helpful for detection, identification and characterization of milk borne pathogens, e.g. PCR, RT-PCR, Microarray, Multilocus Typing which are rapid, sensitive and specific and give a better differentiation among species, serotypes or strains. Various molecular techniques have been used for identification of *Staphylococcus aureus*. Among them, Polymerase Chain Reaction (PCR) is most currently used for identification of *Staphylococcus aureus*. With the help of PCR, we will be able to detect a single genome of *Staphylococcus aureus* with accuracy and sensitivity. Many infectious agents that are missed by microbiological, serological tests and DNA probe are detected by PCR. Therefore, now-a-days PCR based tests are mostly used for the clinical and epidemiological investigation of pathogenic bacteria (Ahmadi et al. 2010; Firyal et al. 2009; Kalorey et al. 2007). According to Sun et al. 2013, in china Staphylococcus isolates were electrophoresis (PFGE) analysis, spa typing and minimal inhibitory concentration determination while Kumar et al. (2011) studied in India that the *Staphylococcus aureus* recovered from the buffaloes distribution of virulent genes such as Coagulase and Fibronectin binding protein A and genetic form of *Staphylococcus aureus* isolates were further used for segregation or culling of infected animals from harmful strains to reduce the dissemination of pathogenic microorganisms.
2.15 \textit{Staphylococcus aureus} Virulence Determinants

Exoproteins of \textit{Staphylococcus aureus} contribute to its facility to colonize and cause disease in hosts. These virulent factors cause colonization through the damaged mucosa and skin, propagation to the body, and evasion of host defense mechanisms. Approximately, 50 potential virulence factors in \textit{S. aureus} with a wide range of biologic activities have been reported. Staphylococcal infections occur in a stepwise manner, each step involving one or several specific virulence factors (Ferry et al. 2005). Nearly all strains secrete a group of enzymes and cytotoxins include four hemolysins (alpha, beta, gamma, and delta), nuclease, proteases, lipases, hyaluronidase, and collagenase. The main function of these proteins may be to convert local host tissues into nutrients required for bacterial growth. Each of these toxins is known to have potent effects on cells of the immune system, but many of them have other biological effects as well. Their primary function in vivo may be to inhibit host immune responses to \textit{Staphylococcus aureus} (Dinges et al. 2000). The orchestrated expression of multiple virulence factors is the key to the success of staphylococcal pathogenesis. \textit{Staphylococcus aureus} virulence factors are located either on the chromosome or on mobile elements such as plasmids, transposons and bacteriophages. Acquisition of genes mediating antibiotic resistance, such as the \textit{mecA} gene conferring resistance to methicillin, can further favor epidemic spread by promoting the acquisition of additional virulence factors. Expression of most virulence factors in \textit{Staphylococcus aureus} is controlled by the accessory gene regulator (\textit{agr}) locus, which encodes a two-component signaling pathway whose activating ligand is a bacterial-density sensing peptide (auto inducing peptide) also encoded by \textit{agr} (Lina et al. 2003). A polymorphism in the amino acid sequence of the auto inducing peptide and of its corresponding receptor (AgrC) has been described. \textit{Staphylococcus aureus} strains can be divided into four major groups on this basis: within a given group, each strain produces a peptide that can activate the \textit{agr}
response in the other member strains, whereas the auto inducing peptides produced by the different groups are usually mutually inhibitory (Ji et al. 1997). Links between a peculiar agr type and a specific staphylococcal syndrome have been shown for toxic shock syndrome (TSS) and staphylococcal scalded skin syndrome (SSSS). TSST-1- producing isolates belong to agr specificity group III and mostly belong to a single clone, as shown by multilocus enzyme electrophoresis (MLEE) (Musser et al. 1990; Ji et al. 1997). Most exfoliating-producing strains responsible for SSSS belong to agr group IV, but these strains have not been investigated (Jarraud et al. 2002). *Staphylococcus aureus* virulence factors include surface proteins that promote colonization of host tissues, invasion factors that promote bacterial spread in tissues (leukocidin, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule), biochemical properties that enhance their survival in phagocytes (catalase production), and membrane damaging toxins that lyse eukaryotic cell membranes (hemolysins, leukotoxin) (Turkyilmaz and Kaya, 2006).

**2.16 Transmission of *Staphylococcus aureus***

*Staphylococcus aureus* is transmitted from an infected to an uninfected mammary gland during the milking process. Shared equipment, udder cloths, and even milkers’ hands can transmit *Staphylococcus aureus* between cows, if good hygienic practices are not followed. Environmental factors, such as bedding, housing, and foodstuffs can also be contaminated and play a role in spreading *Staphylococcus aureus* infections. Thorne et al. (1960) isolated *Staphylococcus aureus* from the floor of dairy barns. Also, Spencer et al. (1952) were able to maintain *Staphylococcus aureus* in vitro on sterile straw for at least 49 days, which raises the possibility that bedding may be a source of the organisms. However, in low *Staphylococcus aureus* prevalence herds, 0 of 183 bedding samples were positive for *S. aureus*, and 4 of 208 bedding samples from high
Staphylococcus aureus prevalence herds were positive for Staphylococcus aureus (Roberson et al. 1994). These findings suggest that bedding may play a limited role in spreading infection (Roberson et al. 1999). To cause mastitis, Staphylococcus aureus must enter the mammary gland through the teat sphincter. Contaminated wash cloths or milking equipment may bring the bacteria to the teat canal. After bacteria gain access to teat ducts and grows in milk producing tissue, mastitis occurs. After Staphylococcus aureus enters the teat canal, neutrophils respond to the infection. If the leukocytes succeed, there will only be mild signs. If Staphylococcus aureus growth continues and leukocytes are not able to prevent bacterial growth, swelling of the mammary gland and changes in the milk will occur resulting in clinical mastitis. Infections caused by Staphylococcus aureus strains that produce α-toxin can cause gangrenous mastitis, which can lead to death due to toxemia or culling of the infected cow. In addition to the mammary glands, Staphylococcus aureus has been found on other body sites of animals and humans. Haveri et al. (2008) reported that the Staphylococcus aureus strains causing bovine intramammary infection (IMI) were not different from those isolated from extra mammary sites. Teat skin and especially teat canals were important potential reservoirs of Staphylococcus aureus causing IMI. Heifers that had Staphylococcus aureus on their skin were at a higher risk to develop Staphylococcus aureus mastitis than heifers without skin colonization (Sears et al., 2003). Roberson et al. (1994) reported that milk from heifers that have Staphylococcus aureus on their teat skin could be another source of S. aureus. Although some authors (Haveri et al. 2008; Thorne et al. 1960) have reported that Staphylococcus aureus on the udder skin were genetically similar to Staphylococcus aureus isolates from milk samples, it has also been reported that teat skin is not an important reservoir for Staphylococcus aureus mastitis because less than 4% of cows had the same type of Staphylococcus aureus on teat skin as the type that causes Staphylococcus aureus mastitis (Fox et al. 1992).
2.17 Control of *Staphylococcus aureus*

Correct milking procedures play a significant role in reducing new intramammary infections. Washing the udder with water or sanitizing solution and drying with individual towels, preferably disposable towels are recommended for preventing the spread of infection among dairy animals (National Mastitis Council, 2003; Sears et al. 2003). To prevent spread, milkers should wear disposable gloves and sanitize the equipment for each use (Roberson et al. 1999). Germicidal teat dip after each milking is an effective method in preventing spread of infection from cow to cow (National Mastitis Council, 2003). Both pre and post milking teat disinfection have significant impacts on reducing new infections by *Staphylococcus aureus* and Streptococci (Vestweber et al. 1994). Quarters which were dipped before and after milking showed a lower rate of new infections than quarters, which were dipped only post-milking (Vestweber et al. 1994). Back flushing, which is an automated way of washing milking clusters with water and rinsing them with disinfectant after the removal of milking uniform teats, may also decrease the spread of infection. However, back flushing may be less beneficial in herds that follow effective post milking teat dipping.

Intramammary dry cow antibiotic therapy is one component of mastitis control programs. One of the goals of dry cow therapy is to prevent new mastitis infections from occurring during the dry period, as cows are susceptible to infection during this time and it may also cure existing infections. Intramammary antibiotic dry cow therapy cure rates are higher than those achieved during lactation because udders have less volume and antibiotic is present for a greater length of time. In some countries, including the USA, it is recommended that all quarters should be routinely treated at drying off (Robert et al. 2006), while other countries, such as Finland, Norway, and Switzerland typically use selective treatment based on identification of udder infection (Pitkala et al. 2004; Osteras et al. 2006). Cows with *Staphylococcus aureus* should be segregated and milked last to
Culling is considered the most effective way to lower the prevalence of *Staphylococcus aureus* in dairy herds (Radostits et al. 2000). *Staphylococcus aureus* may reside inside the udder and on teat skin, and they provide the most important source of infection to other cows in the herd. With the limited success of dry cow treatment of chronic infections, culling is the most reliable way to remove the organism and reduce exposure to other cows and heifers. However, culling is primarily recommended for chronically infected cases.

### 2.18. Statement of Problem

Mastitis is a complex and multi factorial disease and it is an important dairy health problem with physical, chemical and pathological alterations in milk and udder. Mastitis mostly occurs in two forms i.e. clinical and subclinical. Mastitis can be divided in the contagious and environmental form on the basis of its etiological agents. Various microorganisms are responsible for mastitis, including bacteria, Mycoplasma, yeast and fungi etc. Among all, bacteria are most frequently responsible for this disease. *Staphylococcus aureus* is held accountable for 50.0% of cases of mastitis in buffaloes (Shakoor, 2006). The main reasons for the failure of any of the antibiotic therapy in most cases of *Staphylococcus aureus* mastitis are inadequate concentration of antibiotics reaching at the site of infection, bacterial antibiotic resistance, L-forms of bacteria (sensitive to beta-lactic antibiotics), bacterial dormancy and tissue barrier as this organism lies in deep seated foci. It causes pockets in the depth of udder surrounded by fibrous tissue where antibiotics cannot gain access (Sandholm et al. 1991). In principle, antibiotic sensitivity against the available chemotherapeutic agents in a region or locality should be done from time to time to enable the veterinarians to prescribe the most suitable antibiotics against the prevailing diseases. In Pakistan mastitis losses might be higher, because prevention practices of mastitis are not...
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adopted by dairy farmers. The present study was planned with the following aims and objectives: Assess the prevalence of mastitis in buffaloes and identify the epidemiological risk factors of mastitis. To assess the resistance of *Staphylococcus aureus* against commonly used antibiotics. Molecular characterization of virulent gene of indigenous strains of *Staphylococcus aureus*. 


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CHAPTER 3
EXPERIMENT -1

RISK FACTORS ASSOCIATED WITH MASTITIS IN DAIRY BUFFALOES

ABSTRACT
The cross sectional study was carried out between January to December 2012 to investigate the prevalence of mastitis in dairy buffaloes and risk factors. Total sample size for this survey consists of 1,036 lactating buffaloes. The farms were categorized as small scale 5-10, medium scale 11-30 and large scale having more than 30 dairy animals. The overall prevalence of mastitis was recorded as 49%. Among mastitis cases the prevalence of clinical mastitis was 10.2% as a whole. The overall prevalence of subclinical mastitis was 38.8% as diagnosed with a California mastitis test. In a univariable analysis: area, herd size, age, breed, parity, stage of lactation, reproductive disorders, udder shape, previous exposure to mastitis, ease of milking, udder edema, lesion on udder/teat, swelling on udder/teat, condition of floor, frequency of dung removal, exposure to FMD, udder wash, teat dipping, milking techniques and tick infestation were significantly associated \((P< 0.25)\) with a prevalence of mastitis. These 20 significant variables were further selected for multivariable analysis to estimate the independence of the effect of the variables. The final model identified area, age, breed, parity, stage of lactation, udder shape, ease of milking, previous exposure to mastitis, udder edema, lesion on udder/teat, exposure to FMD, teat dipping and tick infestation were significantly associated with the prevalence of mastitis in buffaloes \((p<0.05)\). Mastitis can be minimized by improving management and animal related potential risk factors.

**Keywords:** Mastitis, Buffaloes, Prevalence, Risk factors, Age, Breed, Parity
Milk produced from dairy animals provides an important dietary source of rural as well as urban population. However, milk production often does not fulfill the country’s requirements due to various factors. Mastitis is reported to be one of the common problems of the dairy industry all over the world. The economic losses due to mastitis occur because discarded milk, reduction in the quality of milk and the cost of treatment (Radostits et al. 2007; Fekadu, 1995). In Pakistan mastitis has also been reported as one of the top ranking diseases responsible for economic losses (Hussain et al. 2005). It has been reported that annual losses due to mastitis in USA are approximately $2 billion while in India $526 million (Donovan et al. 2005; Varshney and Naresh 2004). Mastitis can affect any herd at any time, therefore all herds are potentially susceptible.

Mastitis can be caused by a number of pathogens. Broadly mastitis can be defined as two categories i.e. contagious and environmental. The contagious mastitis is caused by *S. aureus*, *St. agalactiae*, etc. It can spread from the infected quarters to other quarters or animals during milking (milker hands or machines). The environmental mastitis can be caused by many other organisms such as *Str. uberis*, *Str. dysgalactiae*, coliforms, etc. which are commonly present in the environment (bedding, flooring, droppings). It can infect the glands between milking as well as during the dry period (Radostits et al. 2007). Mastitis is a multifactorial disease, closely related to the production system and environment in which the dairy animals are kept (Mekibib et al. 2010). That’s why monitoring udder health performance is impossible without reliable and affordable diagnostic methods (Zadoks and Schukken, 2006). Furthermore, it can be divided into two groups: clinical, subclinical mastitis. Mustafa (2003) reported that only a small proportion of udder infection results in "clinical mastitis" whereby there are changes in udder condition and milk quality. The vast majority of cases exists as subclinical; with estimated 20-40 cases for every...
clinical mastitis case within the herd. Therefore mastitis problems may be present within a herd
despite no visible presence within the animal or the milk. Subclinical mastitis is responsible for
the greatest financial losses associated with mastitis. It is estimated to cause 70% of the total losses.
These losses however, are difficult to demonstrate producers since they are associated with
decreased milk production caused by the effects of chronic inflammation of the mammary gland.

Bovine mastitis can be caused by physical or chemical agents but the majority of cases are
infectious and usually caused by bacteria. The disease has been reported by several authors on the
prevalence and mastitis in different parts of the world (Biffa et al. 2005; Hussain et al. 2013;
Chishty et al. 2007). Several of these studies have shown mastitis is caused by a range of bacteria
and *Staphylococcus aureus* is major pathogenic. Moreover, there are no proper control measures
in order to contain the disease because of its multifactorial nature. Milk contaminated from
affected dairy animals with bacteria may render it inappropriate for human consumption. Zoonotic
diseases potentially transmitted by raw milk include brucellosis, caseous lymphadenitis,
leptospirosis, listeriosis, melioidosis, Q-fever, staphylococcal food poisoning, toxoplasmosis and
tuberculosis (Mungube et al. 2005; Radostits et al. 2007). These conditions are associated with
public health risks brought about by contaminated raw milk and antibiotic residues as a
consequence of drug therapy (Andrew et al. 2009).

Mastitis in dairy herds occurs as a complex interaction between the host, the environment
and agent. The most common risk factors for mastitis in dairy herds can be divided in two groups:
animal related risk factors and environmental and management risk factors. Animal related risk
factors include: breed, age, parity/lactation number, stage of lactation, lesions on udder/teat,
swelling on udder/teat, udder shape Teat shape, udder edema, treat edema, and ease of milking.
Management, environmental and housing related risk factors included: stimulus for milk letdown,
number of animals milked by the same milker, udder washing, milking technique, dairy husbandry system, condition of floor, tick infestation, the source of drinking water, frequency of dung removal per day, farm hygiene and husbandry practices. Other risk factors associated with mastitis included: previous exposure to mastitis, reproductive disorders, History of exposure to foot and mouth disease and pox lesions on udder and teat (Nigo et al. 2013; Sarker et al. 2013; Elbaby et al. 2013). Many studies report risk factors for mastitis are associated with farm management, hygiene management, the breeding environment, milking technology, feeding, the calving, season and preventive health management (Bludau et al. 2014). In an individual herd, animal related factors are breed, parity, period of lactation, udder and teat morphology, age at calving, milk leakage, udder edema, milk production, number of milk somatic cells and reproductive disorders (Nyman et al. 2007). Moreover, the stage of lactation, lactation number, trauma to udder, teat and teat canal, loose teat sphincters, lesions on teat skin, immunological status of each mammary gland, bulk of infection in the environment and managemental conditions are amongst the determinants which dictate the level of mastitis incidence (Radostits et al. 2007).

In developing countries like Pakistan owing to small herd sizes, dairy animals are mostly hand milked. Mastitis in hand milked animals were nearly twice as frequent as in machine-milked ones (25.1 VS 14.6%) Motie et al. (1985). Infectious agents of mastitis may be transmitted from infected to healthy animals through the milker's hand (Oliver, 1975) especially because milk is often used as a lubricant for milking. The infection originates either from the infected udder or the contaminated environments and the major sources of pathogens and means of transmission include infected quarters and soiled udder, contaminated milking machines, teat cups, milker’s hands, washing clothes, flies and surgical instruments. Several studies have investigated various risk factors for mastitis (Barnouin et al. 2005; O’Reilly et al. 2006). The results of these studies differ,
but some risk factors are in concordance. However, these studies were conducted in different
countries under varying conditions, which may explain different results, and no specific studies on
risk factors for mastitis have been performed under the climatic, housing and dairy breed
conditions.

Mastitis prevalence was found to be significantly influenced by stage of lactation, parity,
breed, milk yield, anatomical abnormality of the udder, and some management aspects including
nutrition (Almaw et al. 2008). There are numerous risk factors identified by many researchers that
influence the occurrence of mastitis such as age, parity, lactation stage, milk yield, breed, previous
mastitis record, floor type, disinfection of fingers and teat dipping, etc. (Karimuribo et al. 2006;
Madut et al. 2009). There are some reports on the magnitude of the disease, but information
relating to its risk factors is insufficient (Kahir et al. 2008). Such information is important to
envisage when designing appropriate strategies that would help to reduce its prevalence and effects.
However, virtually all of the published information about the risk factors for mastitis refers to dairy
breeds of cattle, and little information available for water buffaloes. Though a high probability
exists that these identified risk factors may also be observed among these species, the degree of
influence by these factors is still unknown.
MATERIALS AND METHODS

Study Area

The present study was conducted in two districts Lahore and Bhimber of Pakistan. Lahore is a capital of Punjab province and it lies between longitudes 74° 20' 37 east and latitude 31° 32' 59 north and the altitude is 209 meters above the sea level. The Lahore features hot, semi-arid climate with an average of temperature 110°F in summer and 35°F in winter and average annual rainfall is from 470.1 to 738 millimeters. While, Bhimber is a district of Azad Kashmir and lies 74° 4' 0 east and latitude 32° 58' 60 north and average altitude is 314 meters from sea level. Bhimber is semi-hilly and mountainous area with some stretches of plains. The weather of Bhimber is an arid climate with average temperature 102°F in summer and 22°F in winter. It has a very diverse climate; ranging from sub-humid subtropical, to moist temperate, dry cold temperate, very cold temperate. The mean annual rainfall varies from 800 to 1100 millimeters. Buffalo population in Lahore and Bhimber was 403128 and 147539, respectively (Anonymous, 2006).

Study Population and Design

The present cross sectional study was carried out between January to December 2012 to investigate the mastitis prevalence in dairy buffaloes and epidemiological investigation of risk factors. The study area was consist of two district i,e Lahore and Bhimber. The total buffalo population in Pakistan is 34.6 million Buffalo. There is no registration of dairy farmers to kept small dairy herds at their premises. Unfortunately, list of herds was not available due to this reason convenience sampling was adopted. From the study area the population of district Lahore was higher as compared to district Bhimber. But due to limited sources total 50 dairy herds from two district, 25 from each were selected. The total sample size for this study was consisted of 1,036 lactating buffaloes.
Inclusion and exclusion criteria

All the lactating buffaloes were included. Dry buffaloes and heifers were excluded from the study.

Questionnaire and data collection

The questionnaire was designed with the guideline of Nigo et al. (2013), Ali (2009), Biffa et al. (2005), Sarker et al. (2013), Mustafa et al. (2013), Elbaby et al. (2013) and Kiveria et al. (2006). A questionnaire was designed for collection of data farm demography, climate and management practices. It was having some dichotomous, polychotomous questions and farmer’s interviewing and farm records were used for collection of data. The related data were obtained from farm owners’ interviews and farm records. All the risk factors were arranged into categories in three groups, i.e. Animal related risk factors, management (environmental and housing) related risk factors and others associated risk factors of mastitis. Animal related risk factors include: breed, age, parity/lactation number, stage of lactation, lesion on udder/teat, swelling on udder/teat, udder shape Teat shape, udder edema, treat edema, and ease of milking. Management, environmental and housing related risk factors included: stimulus for milk letdown, number of animals milked by the same milker, udder washing, milking technique, condition of floor, tick infestation, the source of drinking water, frequency of dung removal per day. Other risk factors associated with mastitis included: previous exposure to mastitis, reproductive disorders, history of exposure to foot and mouth disease and pox lesions on udder and teat.

Physical Examination of the Udder and Teat in Clinical Mastitis

In case of clinical mastitis udder and teats were examined visually and thoroughly palpated for detecting any possible fibrosis, visible injury, cardinal signs, tick infestation and viscosity. The size and consistency of the udder and quarters were inspected for the presence of any anatomical
malformation, such as disproportional symmetry, swelling, firmness and blindness. Information regarding previous history of mastitis and quarters effected were obtained and recorded on the questionnaire by interviewing farm owners. Information or the presence of clots, flakes, watery, any bloody secretions and physical appearance of milk secretion were examined from each quarter according to guidelines of Mekibib et al. (2010), Kivaria et al. (2007) and Elbably et al. (2013).

**California Mastitis Test**

Screening of subclinical mastitis was performed by the California Mastitis Test (CMT) with the guideline of Iqbal et al. (2006). About 5 ml milk was mixed with equal volume of CMT reagent in mastitis paddle. Mixing was accomplished by gentle circular motion of the paddle in a horizontal plane. The reaction developed almost immediately with milk containing a high concentration of somatic cells. The peak of reaction was obtained within 10 seconds and scored.

**Statistical analysis**

All the collected data through questionnaire was entered into an Excel sheet of Microsoft Office Excel (2003) and transferred for analysis to R software and SPSS version 16.0. Firstly, all the data were labelled into sub heading and analyzed by using descriptive statistics for the frequency and cross tabulation tables. In Univariable analysis \( p \leq 0.25 \) was considered significant while risk factors significant in the univariable analysis were furtherly selected as potential candidates for multivariate analysis using the Binary Logistic Regression with the forward Stepwise method. All the risk factors in the Logistic Regression, having \( p \leq 0.05 \) were considered significantly associated with mastitis and the strength of association was determined by the Odds Ratio (OR) at 95% Confidence interval (CI).
RESULTS

Descriptive Epidemiology

Prevalence of Clinical and Subclinical Mastitis

A total of 1036 buffaloes were examined for mastitis. Of the population studied, the animals examined in district Lahore were 598 buffaloes belonging to 25 herds, while the remaining 438 were from 25 herds maintained in Bhimber district. The overall prevalence of mastitis was recorded 49.0% (508/1036 animals). The prevalence of mastitis recorded in Lahore was 55.5% (332/598 animals) while in district Bhimber it was 40.2% (176/438 animals) (Table 3.1).

Among mastitis cases the prevalence of clinical mastitis was 10.2% (106/1036 animals) as a whole. However, the prevalence of clinical mastitis in district Lahore was 11.9% (71/598 animals) whereas it was 8.0% (35/438 animals) in district Bhimber. The overall prevalence of subclinical mastitis was 38.8% (402/1036) as diagnosed with a California mastitis test. The district wise prevalence was subclinical mastitis was 43.6% (261/598 animals) and 32.2% (141/438 animals) in the districts of Lahore and Bhimber, respectively.

Among the total of 508 cases of mastitis 20.9% (106/508) were of clinical mastitis while the remaining 79.1% (402/508) was subclinical mastitis. The clinical and subclinical mastitis on district wise among the mastitis cases was 21.4% (71/332) and 78.6% (261/332) in Lahore while in Bhimber it was 19.9% (36/176) and 80.1% (141/176), respectively.

Prevalence of Mastitis on the basis of Herd Size

The herds were categorized into three groups i.e. Small comprising of 5-10 animals, medium sized 11-30 animals and large having more than 30 animals. The overall prevalence of mastitis was 40.2%, 46.5% and 52.6% in small, medium and large herds, respectively. In district
Lahore the herd wise mastitis prevalence was 45.7%, 52.5% and 56.8%, while in district Bhimber it was 29.9%, 40.3% and 44.0%, respectively (Table 3.2).

Age wise distribution of mastitis

The dairy animals were categorized into three age groups; G1 (3-5 years), G2 (6-8 years) and G3 (9 and above). The prevalence of mastitis recorded in was 38.2%, 40.8% and 68.9% in G1, G2 and G3, respectively. In district Lahore the prevalence of mastitis in different age groups was 44.8%, 46.7%, 71.0% of G1, G2 and G3, respectively. Similarly, in Bhimber it was 29.4%, 36.1% and 64.5% in G1, G2 and G3, respectively (Table 3.3).

Breed wise distribution of mastitis

The dairy buffaloes were categorized into three groups on the basis of their breed B1 (Nili Ravi), B2 (Kundhi) and B3 (Non-descriptive). The prevalence of mastitis in buffaloes on the basis of the breed was recorded 52.2%, 40.0% and 44.7% in B1, B2 and B3, respectively. In the district Lahore prevalence of mastitis was recorded 58.1%, 50.0%, and 47.4% while in Bhimber it was 44.1%, 28.3% and 39.3%, respectively (Table 3.4).

Parity wise prevalence of mastitis

The dairy buffaloes were categorized into three groups on the basis of their parity P1 (1 parity) P2 (2-5 parity) and P3 (≥6 parity). The overall prevalence of mastitis on the basis of parity was recorded 26.1%, 48.4% and 58.0% in P1, P2 and P3, respectively. In district Lahore prevalence of mastitis was recorded 32.1%, 54.2% and 65.1% in P1, P2 and P3, respectively. Similarly, the prevalence of mastitis on the basis of lactation number was recorded in Bhimber 17.5%, 41.1% and 47.1% in P1, P2 and P3, respectively (Table 3.5).
Stage of lactation wise prevalence of mastitis

The dairy animals were divided into three categories (early, mid and late) on the basis of their lactation stage. The overall prevalence of mastitis was recorded as 43.5%, 55.4% and 43.8%, in early, mid and late stage of lactation, respectively. In the district Lahore prevalence of mastitis on the basis of stage of lactation was recorded 46.7%, 63.0% and 50.8%, while in district Bhimber it was 39.2%, 44.6% and 34.9% in early, mid and late, respectively (Table 3.6).

Quarter level prevalence

The quarter wise prevalence of mastitis among LF, RF, LR, and RR was recorded at 9.4%, 28.0%, 8.2% and 19.4%, respectively. In district Lahore the quarter wise prevalence of mastitis was 11.0%, 30.8%, 10.5% and 22.7%, while in district Bhimber it was 7.1%, 24.2%, 5.0% and 14.8%, respectively (Table 3.7). The one quarter, two quarters, three quarters and four quarters prevalence were recorded 38.5%, 5.8%, 4.1% and 0.7%, respectively. In district Lahore the quarter level prevalence of mastitis was recorded 42.1%, 7.2%, 5.4% and 0.8%, while in district Bhimber it was 33.6%, 3.9%, 2.3% and 0.5%, respectively (Table 3.8).

Udder Shape

In dairy buffaloes the udder shape was categorized in two i.e. pendulous and normal. The prevalence of mastitis was the highest in pendulous udder (51.6%) than normal (43.0%) (Table 3.10). In the district Lahore prevalence of mastitis on the basis of udder shape was 58.0% (243/419) and 49.7% (89/179) in pendulous and normal udder, respectively. In district Bhimber it was recorded as 42.9% (132/308) and 33.8% (44/130) in pendulous and normal udder, respectively.
**Teat Shape**

Teats shape was categorized into three types on the basis of shape, i.e., funnel, round, and flat. The whole prevalence of mastitis in funnel, round and flat teats was recorded 51.1%, 47.2% and 49.3%, respectively (Table 3.10). In the Lahore prevalence of mastitis was recorded 54.9% (73/133), 53.8% (98/182) and 56.9% (161/283) while in Bhimber it was 45.7% (43/94), 38.1% (51/134) and 39.1% (82/210) in funnel, round and flat teats, respectively.

**Ease of milking**

In dairy buffaloes ease of milking was categorized in two (soft milking and hard milking). The prevalence of mastitis in soft milking was highest 44.7% (421/941) and lowest in hard milking 91.6% (87/95) (Table 3.10). In district Lahore the prevalence of mastitis was in soft milking (51.3% (278/542/) and in hard milking it was 96.4% (54/56). In district Bhimber the prevalence of mastitis was in soft milking 35.9% (143/399) and in hard milking it was 84.6% (33/39).

**Milking Techniques**

The milking techniques were categorized such as complete hand milking and knuckling. The prevalence of mastitis in animals milked by knuckling was 51.3% as compared with complete hand milking was 43.3% (Table 3.11). In district Lahore the prevalence of mastitis on the basis of milking techniques was recorded relatively higher in knuckling milking 57.5% (233/405) than complete hand milking 51.3% (99/193). Similarly, in district Bhimber the prevalence of mastitis was higher 43.8% (145/331) in knuckling than complete hand milking 29.0% (31/107).

**Number of animals milked by same milker**

The dairy buffaloes were categorized on the basis of the number of animals milked by the same milker into three groups G1 (1-5), G2 (6-10), G3 (≥11). The whole prevalence of mastitis
was recorded 53.9% and 47.7% and 49.4% in G1, G2 and G3, respectively (Table 3.11). In the Lahore prevalence of mastitis was recorded 57.2% (91/159), 53.7% (182/339) and 59.0% (59/100) and in Bhimber it was 28.6% (6/21), 41.8% (145/347) and 35.7% (25/70) in G1, G2, and G3, respectively.

Frequency of dung removal

In dairy buffaloes the frequency of dung removal was categorized in two i.e. once in a day and twice in a day. The prevalence of mastitis was recorded higher in once in a day (49.8%) than twice in a day (43.0%) (Table 3.11).

Condition of floor

The floor bedding on dairy farms were categorized as concrete and soiled bedding. The prevalence of mastitis was higher in soiled (50.4%) than concrete floor 45.4% (Table 3.11). In district Lahore the prevalence of mastitis was on soiled floor 69.1% (284/411) and on concrete floor 55.2% (48/87). The prevalence of mastitis was on solid floor 39.5% (96/243) and on concrete floor 41.0% (80/195) in district Bhimber.

Source of water

There were three categories of source of water, such as pond, canal and sub-surface water prevalence of mastitis was 50.8%, 46.0%, and 56.3%, respectively (Table 3.11). In district Lahore the prevalence of mastitis was recorded at 55.4% (113/204), 52.5% (155/295) and 64.6% (64/99) in the pond, canal and sub-surface water, respectively. While in district Bhimber it was 43.3% (55/127), 38.6% (100/259) and 40.4% (21/52/) in river, pond and sub-surface water, respectively.
Tick infestation

The prevalence of mastitis on basis of tick present on farm was recorded 55.8% while it was 41.8% where ticks were not present (Table 3.11). In district Lahore the prevalence of mastitis was 64.8% (184/284), while in district Bhimber it was 45.7% (116/254) were ticks were present. The prevalence of mastitis without tick infestation was 47.1% (148/314) and 32.6% (60/184) in district Lahore and Bhimber, respectively.

Reproductive disorders

The prevalence of mastitis on the basis of reproductive disorders was recorded 62.8% while the prevalence of mastitis was 45.0% where reproductive disorders was absent (Table 3.9). In district Lahore the prevalence of mastitis was 50.7% (231/456) with history of reproductive disorder and it was 71.1% (101/142) without reproductive disorder. While in district Bhimber it was 50.0% (46/92) where reproductive disorder was present and it was 37.6% (130/346) where reproductive disorder was absent.

Exposure to FMD

The prevalence of mastitis on the basis of history of exposure to FMD was recorded 58.4% while prevalence of mastitis was 47.2% where FMD history was not reported (Table 3.11). In district Lahore the prevalence of mastitis was 70.4% (69/98) where FMD history was present and it was 52.6% (263/500) where FMD was not reported. In district Bhimber it was 41.2% (28/68) with history of FMD exposure and it was 40% (148/370) without FMD history.

Previous exposure of mastitis

The overall prevalence of mastitis was 74.9% in buffaloes having previous exposure to mastitis while it has 47.3% in buffaloes without previous record of mastitis (Table 3.10). In district Lahore it was recorded as 81.6% (84/103) in previously exposed to mastitis and 50.1% (248/495) not
exposed to mastitis. In district Bhimber it was 65.8% (50/76) in previously exposed to mastitis and it was 34.8% (126/362) in not exposed to previous mastitis.

**Univariable and Multivariable Analysis of Risk Factors**

In total 25 variables were studied and analyzed by univariable analysis. Out of these 25 variants; 23 variables were further selected for multivariable analysis to estimate the independence of the effect of the variables on the basis of selection criteria (Table 3.9, 10, 11). The final model identified 12 variables as potential risk factors of mastitis in buffaloes (Table 3.12).

**Area**

In univariable analysis the prevalence of mastitis was significantly associated ($p<0.001$) in district Lahore as compared to district Bhimber (Table 3.9). Similarly in multivariable analysis area was significantly associated ($p<0.017$) with the prevalence of mastitis (Table 3.12).

**Herd size**

With univariable analysis the prevalence of mastitis was significantly associated ($p<0.02$) with larger herds (OR of 1.65, 95% CI 1.07-2.54) when compared with small herds, while medium herd was not statistically significant with a prevalence of mastitis (Table 3.9). In multivariable analysis herd size was not statistically significant ($p>0.05$).

**Breed**

The univariable analysis showed that the Kundhi breed had less prevalence of mastitis as compared with Nili Ravi breed was associated significantly ($p>0.002$). The odds ratio of Kundhi was 0.61 times less than the odds of Nili Ravi breed when compared with Kundhi breed. Similarly, Kundhi breed was associated significantly ($p>0.193$) with a prevalence of mastitis when compared with Non-descriptive breed (Table 3.9). The multivariable analysis showed that the Nili Ravi breed was highly associated ($p<0.003$) with a prevalence of mastitis.
when compared with Kundhi breed. The odds of Kundhi breed showed that prevalence of mastitis is 0.61 times less than Nili Ravi breed (Table 3.12).

**Age wise distribution of mastitis**

The univariable analysis showed that older age buffaloes (>8 years) were significantly associated (p< 0.001) with the prevalence of mastitis than young (3-5 years) and adult (>5-8 years) groups having OR 3.59 with 95% confidence interval (CI) of 2.63-4.89 (Table 3.9). In old age buffaloes, there is three times risk of mastitis as compared to young buffaloes. The multivariable analysis showed that older age buffaloes were highly significant (p< 0.001) with a prevalence of mastitis having OR 2.22 with 95% confidence interval (CI) of 1.44-3.43 when compared to young buffaloes. Similarly, adult buffaloes also had significance (p< 0.008) associated with prevalence of mastitis when compared to young buffaloes. The odds of adult buffaloes exposure was 0.53 (CI 95%: 0.34-0.85) times less than the exposure in young buffaloes (Table 3.12).

**Parity wise prevalence of mastitis**

The univariable analysis showed the prevalence of mastitis was highly significant (p< 0.001) with the increase in the parity number OR of 16.43 with 95% confidence interval of 5.91-45.68. (Table 3.9). The multivariable analysis showed that prevalence of mastitis was statistically significant (p<0.001) when increase parity number with OR. 9.14 having 95% confidence interval of 2.9-28.82 (Table 3.12).

**Stage of lactation wise prevalence of mastitis**

The univariable analysis showed that mid lactation stage was significant (p< 0.003) associated with prevalence of mastitis when compared to the early lactation stage with OR 1.61 at 95% CI of 1.17-2.21 (Table 3.9). The prevalence of mastitis was non-significant (p>0.942) in late lactation stage when compared to early stage. The odds of late lactation were (OR 1.01) having
less than one times prevalence of mastitis (95% CI of 0.72-1.42). The multivariable analysis showed that mid lactation stage significantly higher (p>0.003) with a prevalence of mastitis (OR 1.85; 95% CI, 1.23-2.79) while late lactation stage was not statistically associated with prevalence of mastitis (p>0.05) (Table 3.12).

Reproductive disorders

The reproductive disorder was associated with the prevalence of mastitis (p<.001) in univariable analysis. Buffaloes with reproductive disorder were two times more prone to mastitis as compared to healthy buffaloes (OR 2.064) (Table 3.9). In multivariable analysis, reproductive disorder was not associated with prevalence of mastitis (p>0.05).

Udder Shape

The univariable analysis showed that pendulous udder was strongly associated with prevalence of mastitis when compared to normal udder (Table 3.10).

Teat Shape

In the univariable analysis flat teats were associated (p< 0.125) with a prevalence of mastitis in funnel teat shape when compared to funnel shaped teat OR (1.06) (Table 3.10). In multivariate analysis teat shape was not associated with prevalence of mastitis (p<0.05).

Previous exposure to Mastitis

The previous exposure of mastitis was highly associated (p<.000) with a prevalence of mastitis having OR. 3.846 in univariable analysis (Table 3.10). In multivariable analysis the buffaloes with previous exposure to mastitis were three times more prone to infection as compared to new exposure of mastitis OR. 3.44 (955 CI; 2.14-5.53). Similarly, previous exposure to mastitis was strongly associated with prevalence of mastitis (p<.001) (Table 3.12).
Ease of milking

The univariable analysis showed that soft teat consistency may be due to loose teat sphincter was highly significant ($p<.000$) with a prevalence of mastitis when compared with hard teat consistency having OR. 13.432 (95% CI; 6.43-28.02) (Table 3.11). In multivariable analysis, soft teat was highly significant ($p<.001$) associated with prevalence of mastitis having OR. 14.48 (95% CI; 6.43-32.58) (Table 3.12).

Udder Odema

The univariable analysis showed that udder edema was strongly associated ($p<.000$) with the prevalence of mastitis having OR.13.747 at 95% CI of 5.92-23.95 (Table 3.10). In multivariable analysis udder edema was significant ($p>0.023$) with prevalence of mastitis having OR. 4.92 (1.25-19.36 at 95% CI) (Table 3.12).

Lesions on udder and teat

The lesions on the udder or teat were statistically associated ($p<.000$) with the prevalence of mastitis while buffaloes having a lesion on udder and teat were 13 times more prone to mastitis as compared to not lesion on the udder or teat in univariable analysis (OR 13.061; 6.25-27.26) (Table 3.10). In multivariable analysis lesions on the udder or teat were highly significant ($p<.001$) with a prevalence of mastitis having OR 6.84 (2.88- 16.21) at 95% confidence interval (CI) (Table 3.12).

Swelling on udder and teat

The swelling on udder and teat was highly associated ($p<.000$) with the prevalence of mastitis having OR. 11.917 (Table 3.10) in univariable analysis while in multivariable analysis, it was not associated with prevalence of mastitis.
Condition of floor

The univariable analysis showed that soiled floor was significant (p<.086) associated with prevalence of mastitis having OR 1.222 (95% CI; 0.929-1.609) (Table 3.11). But in multivariable analysis condition of floor was non-significant.

Frequency of dung removal

The frequency of dung removal has significant (p>0.171) effect on prevalence of mastitis in univariable analysis (Table 3.11) while in multivariable analysis it was not associated with prevalence of mastitis.

Source of water

In univariable and multivariable analysis the source of water has non-significant effect on prevalence of mastitis.

Exposure to FMD

The exposure to FMD was associated with the prevalence of mastitis (p<.008) with having OR 1.57 (Table 3.11) by univariable analysis. The multivariable analysis, exposure to FMD was as that prevalence of mastitis was significant p< 0.020. Buffaloes with a history of FMD exposure were 1.67 more prone to mastitis as compared to healthy buffaloes (OR 1.67; 95% CI; 1.08-2.58) (Table 3.12).

Udder wash

The univariable analysis showed buffaloes without udder washing at milking time have significant effect on prevalence of mastitis (p<.042) (Table 3.11).

Teat dipping

The univariable analysis showed buffaloes without teat dipping were associated with prevalence of mastitis (p<.042) than teat dipped buffaloes. Buffaloes without teat dipping were
1.40 times more susceptible to mastitis (95% CI; 1.01-1.95) (Table 3.11). In the multivariable analysis, prevalence of mastitis had a significant effect (p>0.03). Buffaloes without teat dipping were 1.76 times more susceptible to mastitis (95% CI; 1.04-2.97) (Table 3.12).

**Milking Techniques**

Milking method knuckling was found to be highly associated (p>0.019) with the prevalence of mastitis by univariable analysis as compared with complete hand milking (OR 1.38; 95% CI, 1.05-1.80) (Table 3.11).

**Tick infestation**

The tick infestation was strongly associated with the prevalence of mastitis (p<.000) having OR. 1.757, when compared to non-tick infestation buffaloes (Table 3.11) by univariable analysis. Similarly, in multivariable analysis the prevalence of mastitis was highly significant (p<.001). Buffaloes with tick infestation were two times more prone to mastitis when compared with non-tick infestation (OR. 2.83; 95% CI; 1.99-4.01) (Table 3.12).
Table 3.1: Prevalence of Clinical and Subclinical Mastitis in Lactating Buffaloes

<table>
<thead>
<tr>
<th>Districts</th>
<th>Mastitis</th>
<th>Total examined</th>
<th>Frequency</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lahore</td>
<td>Clinical</td>
<td>598</td>
<td>71</td>
<td>11.8</td>
<td>9.5-14.6</td>
</tr>
<tr>
<td></td>
<td>Subclinical</td>
<td>598</td>
<td>261</td>
<td>43.6</td>
<td>39.7-47.6</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>598</td>
<td>332</td>
<td>55.5</td>
<td>51.5-59.4</td>
</tr>
<tr>
<td>Bhimber</td>
<td>Clinical</td>
<td>438</td>
<td>35</td>
<td>8.0</td>
<td>5.7-10.8</td>
</tr>
<tr>
<td></td>
<td>Subclinical</td>
<td>438</td>
<td>141</td>
<td>32.2</td>
<td>27.9-36.7</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>438</td>
<td>176</td>
<td>40.2</td>
<td>35.5-44.8</td>
</tr>
<tr>
<td>Overall</td>
<td>Clinical</td>
<td>1036</td>
<td>106</td>
<td>10.2</td>
<td>8.4-12.1</td>
</tr>
<tr>
<td></td>
<td>Subclinical</td>
<td>1036</td>
<td>402</td>
<td>38.8</td>
<td>35.8-41.8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1036</td>
<td>508</td>
<td>49.0</td>
<td>46-52</td>
</tr>
</tbody>
</table>
Table 3.2: Prevalence of Mastitis on basis of Herd size in Lactating Buffaloes

<table>
<thead>
<tr>
<th>Districts</th>
<th>Herd Size</th>
<th>Total examined</th>
<th>Mastitic</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lahore</td>
<td>5-10 animals</td>
<td>35</td>
<td>16</td>
<td>45.7</td>
<td>29.2-62.2</td>
</tr>
<tr>
<td></td>
<td>11-30 animals</td>
<td>204</td>
<td>107</td>
<td>52.5</td>
<td>45.6-59.2</td>
</tr>
<tr>
<td></td>
<td>More then 30</td>
<td>359</td>
<td>204</td>
<td>56.8</td>
<td>51.7-61.9</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>598</td>
<td>332</td>
<td>55.5</td>
<td>51.5-59.5</td>
</tr>
<tr>
<td>Bhimber</td>
<td>5-10 animals</td>
<td>67</td>
<td>20</td>
<td>29.9</td>
<td>19.8-41.6</td>
</tr>
<tr>
<td></td>
<td>11-30 animals</td>
<td>196</td>
<td>79</td>
<td>40.3</td>
<td>33.6-47.2</td>
</tr>
<tr>
<td></td>
<td>More then 30</td>
<td>175</td>
<td>77</td>
<td>44.0</td>
<td>36.8-51.4</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>438</td>
<td>176</td>
<td>40.2</td>
<td>36.4-44.1</td>
</tr>
<tr>
<td>Overall</td>
<td>5-10 animals</td>
<td>102</td>
<td>41</td>
<td>40.2</td>
<td>31-49.9</td>
</tr>
<tr>
<td></td>
<td>11-30 animals</td>
<td>400</td>
<td>186</td>
<td>46.5</td>
<td>41.6-51.4</td>
</tr>
<tr>
<td></td>
<td>More then 30</td>
<td>534</td>
<td>281</td>
<td>52.6</td>
<td>48.4-56.8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1036</td>
<td>508</td>
<td>49.0</td>
<td>46-52</td>
</tr>
</tbody>
</table>
Table 3.3: Prevalence of Mastitis on basis of Age in Lactating Buffaloes

<table>
<thead>
<tr>
<th>Districts</th>
<th>Age</th>
<th>Total examined</th>
<th>Mastitic</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lahore</strong></td>
<td>3-5 years</td>
<td>172</td>
<td>77</td>
<td>44.8</td>
<td>37.4-52.2</td>
</tr>
<tr>
<td></td>
<td>6-8 years</td>
<td>195</td>
<td>91</td>
<td>46.7</td>
<td>39.7-53.7</td>
</tr>
<tr>
<td></td>
<td>9 and above</td>
<td>231</td>
<td>164</td>
<td>71.0</td>
<td>64.9-76.6</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>598</td>
<td>332</td>
<td>55.5</td>
<td>51.5-59.5</td>
</tr>
</tbody>
</table>

| Bhimber   | 3-5 years | 187            | 55       | 29.4           | 23.2-36.2   |
|           | 6-8 years | 144            | 52       | 36.1           | 28.6-44.2   |
|           | 9 and above| 107         | 69       | 64.5           | 55.1-73.1   |
|           | Total     | 438            | 176      | 40.2           | 35.7-44.9   |

| Overall   | 3-5 years | 382            | 146      | 38.2           | 36.6-44.8   |
|           | 6-8 years | 316            | 129      | 40.8           | 35.5-46.3   |
|           | 9 and above| 338         | 233      | 68.9           | 63.8-73.7   |
|           | Total     | 1036           | 508      | 49.0           | 46-52       |
Table 3.4: Prevalence of Mastitis on basis of Breed in Lactating Buffaloes

<table>
<thead>
<tr>
<th>Districts</th>
<th>Breed</th>
<th>Total examined</th>
<th>Mastitic</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lahore</td>
<td>Nili</td>
<td>425</td>
<td>247</td>
<td>58.1</td>
<td>53.4-62.7</td>
</tr>
<tr>
<td></td>
<td>Ravi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kundhi</td>
<td>116</td>
<td>58</td>
<td>50.0</td>
<td>41-59</td>
</tr>
<tr>
<td></td>
<td>Non-descriptive</td>
<td>57</td>
<td>27</td>
<td>47.4</td>
<td>34.70-60.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>598</td>
<td>332</td>
<td>55.5</td>
<td>51.5-59.4</td>
</tr>
<tr>
<td>Bhimber</td>
<td>Nili</td>
<td>311</td>
<td>137</td>
<td>44.1</td>
<td>38.6-49.6</td>
</tr>
<tr>
<td></td>
<td>Ravi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kundhi</td>
<td>99</td>
<td>28</td>
<td>28.3</td>
<td>20-37.4</td>
</tr>
<tr>
<td></td>
<td>Non-descriptive</td>
<td>28</td>
<td>11</td>
<td>39.3</td>
<td>22.7-58.1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>438</td>
<td>176</td>
<td>40.2</td>
<td>35.7-44.9</td>
</tr>
<tr>
<td>Overall</td>
<td>Nili</td>
<td>736</td>
<td>384</td>
<td>52.2</td>
<td>48.6-55.8</td>
</tr>
<tr>
<td></td>
<td>Ravi</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kundhi</td>
<td>215</td>
<td>86</td>
<td>40.0</td>
<td>33.6-46.7</td>
</tr>
<tr>
<td></td>
<td>Non-descriptive</td>
<td>85</td>
<td>38</td>
<td>44.7</td>
<td>34.4-55.3</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1036</td>
<td>508</td>
<td>49.0</td>
<td>46-52</td>
</tr>
</tbody>
</table>
Table 3.5: Prevalence of Mastitis on basis of Parity in Lactating Buffaloes

<table>
<thead>
<tr>
<th>Districts</th>
<th>Parity</th>
<th>Total examined</th>
<th>Mastitic</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lahore</td>
<td>1 Parity</td>
<td>81</td>
<td>26</td>
<td>32.1</td>
<td>24.6-41.7</td>
</tr>
<tr>
<td></td>
<td>2-5 Parity</td>
<td>282</td>
<td>153</td>
<td>54.2</td>
<td>49.3-59.1</td>
</tr>
<tr>
<td></td>
<td>6≥ Parity</td>
<td>235</td>
<td>153</td>
<td>65.1</td>
<td>59.8-70.1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>598</td>
<td>332</td>
<td>55.5</td>
<td>52.2-58.8</td>
</tr>
<tr>
<td>Bhimber</td>
<td>1 Parity</td>
<td>57</td>
<td>10</td>
<td>17.5</td>
<td>10.4-27.1</td>
</tr>
<tr>
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<td>2-5 Parity</td>
<td>226</td>
<td>93</td>
<td>41.1</td>
<td>35.8-46.6</td>
</tr>
<tr>
<td></td>
<td>6≥ Parity</td>
<td>155</td>
<td>73</td>
<td>47.1</td>
<td>40.6-53.7</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>438</td>
<td>176</td>
<td>40.2</td>
<td>36.4-44.1</td>
</tr>
<tr>
<td>Overall</td>
<td>1 Parity</td>
<td>138</td>
<td>36</td>
<td>26.1</td>
<td>19.3-33.9</td>
</tr>
<tr>
<td></td>
<td>2-5 Parity</td>
<td>508</td>
<td>246</td>
<td>48.4</td>
<td>44.1-52.8</td>
</tr>
<tr>
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<td>508</td>
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Table 3.6: Prevalence of Mastitis on basis of Stage of Lactation in Lactating Buffaloes

<table>
<thead>
<tr>
<th>Districts</th>
<th>Lactation stage</th>
<th>Total examined</th>
<th>Mastitic</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lahore</td>
<td>Early</td>
<td>135</td>
<td>63</td>
<td>46.7</td>
<td>38.1-55.4</td>
</tr>
<tr>
<td></td>
<td>Mid</td>
<td>276</td>
<td>174</td>
<td>63.0</td>
<td>57.2-68.6</td>
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<tr>
<td></td>
<td>Late</td>
<td>187</td>
<td>95</td>
<td>50.8</td>
<td>43.6-57.9</td>
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<td>598</td>
<td>332</td>
<td>55.5</td>
<td>51.5-59.4</td>
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<tr>
<td>Bhimber</td>
<td>Early</td>
<td>97</td>
<td>38</td>
<td>39.2</td>
<td>29.8-49.1</td>
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<td>Mid</td>
<td>195</td>
<td>87</td>
<td>44.6</td>
<td>37.7-51.6</td>
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<tr>
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<td>Late</td>
<td>146</td>
<td>51</td>
<td>34.9</td>
<td>27.5-42.9</td>
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<td>438</td>
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<td>35.5-44.8</td>
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<td>Overall</td>
<td>Early</td>
<td>232</td>
<td>101</td>
<td>43.5</td>
<td>37.2-49.9</td>
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<td>Mid</td>
<td>471</td>
<td>261</td>
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<td>508</td>
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Table 3.7: Quarter wise Prevalence of Mastitis in Lactating Buffaloes

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<tr>
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<th>Quarter</th>
<th>Total Quarter examined</th>
<th>Mastitic Quarter</th>
<th>Prevalence (%)</th>
<th>95% CI</th>
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<tr>
<td>Lahore</td>
<td>LF</td>
<td>598</td>
<td>66</td>
<td>11.0</td>
<td>8.7-13.7</td>
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<td>LR</td>
<td>598</td>
<td>184</td>
<td>30.8</td>
<td>27.2-34.6</td>
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<td>RF</td>
<td>598</td>
<td>63</td>
<td>10.5</td>
<td>8.2-13.1</td>
</tr>
<tr>
<td></td>
<td>RR</td>
<td>598</td>
<td>136</td>
<td>22.7</td>
<td>19.5-26.2</td>
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<td>Bhimber</td>
<td>LF</td>
<td>438</td>
<td>31</td>
<td>7.0</td>
<td>4.9-9.8</td>
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<td>LR</td>
<td>438</td>
<td>106</td>
<td>24.2</td>
<td>20.4-28.4</td>
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<td></td>
<td>RF</td>
<td>438</td>
<td>22</td>
<td>5.0</td>
<td>3.2-7.4</td>
</tr>
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<td></td>
<td>RR</td>
<td>438</td>
<td>65</td>
<td>14</td>
<td>11.7-18.4</td>
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<td>Overall</td>
<td>LF</td>
<td>1036</td>
<td>97</td>
<td>9.4</td>
<td>7.7-11.2</td>
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<td>1036</td>
<td>290</td>
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<td>25.3-30.7</td>
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<td>RF</td>
<td>1036</td>
<td>85</td>
<td>8.2</td>
<td>6.6-10.1</td>
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<td>1036</td>
<td>201</td>
<td>19.4</td>
<td>17.1-21.9</td>
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Table 3.8: Prevalence of Mastitis in Lactating Buffaloes having Mastitis in 1, 2, 3 and 4 Quarters

<table>
<thead>
<tr>
<th>Districts</th>
<th>No. of Quarter</th>
<th>Total buffalo examined</th>
<th>Total positive</th>
<th>Prevalence (%)</th>
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<tr>
<td>Lahore</td>
<td>One quarter</td>
<td>598</td>
<td>252</td>
<td>42.1</td>
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<td>Two quarter</td>
<td>598</td>
<td>43</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>Three quarter</td>
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<td>32</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>Four quarter</td>
<td>598</td>
<td>05</td>
<td>0.8</td>
</tr>
<tr>
<td>Bhimber</td>
<td>One quarter</td>
<td>438</td>
<td>147</td>
<td>33.6</td>
</tr>
<tr>
<td></td>
<td>Two quarter</td>
<td>438</td>
<td>17</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Three quarter</td>
<td>438</td>
<td>10</td>
<td>2.3</td>
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<td>Four quarter</td>
<td>438</td>
<td>02</td>
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<tr>
<td>Overall</td>
<td>One quarter</td>
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<td>399</td>
<td>38.5</td>
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<td>Two quarter</td>
<td>1036</td>
<td>60</td>
<td>5.8</td>
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<td></td>
<td>Three quarter</td>
<td>1036</td>
<td>42</td>
<td>4.1</td>
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<td>Four quarter</td>
<td>1036</td>
<td>07</td>
<td>0.7</td>
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Table 3.9: Univariable Analysis for Association of different Risk Factors with Mastitis
*Significant: $P < 0.25$

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Level</th>
<th>Total examined</th>
<th>+Ve No.</th>
<th>-Ve No.</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Area</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lahore</td>
<td>598</td>
<td>332</td>
<td>266</td>
<td>0.54</td>
<td>.42-.69</td>
<td>&lt;0.001*</td>
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<tr>
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<td>Bhimber</td>
<td>438</td>
<td>176</td>
<td>262</td>
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<tr>
<td><strong>Herd Size</strong></td>
<td>Small (5-10)</td>
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<td>41</td>
<td>61</td>
<td>Ref.</td>
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<td>Medium (11-30)</td>
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<td>214</td>
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<td>.83-2.01</td>
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<td>1.07-2.54</td>
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<td>Nili Ravi</td>
<td>736</td>
<td>384</td>
<td>352</td>
<td>Ref.</td>
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<td>-</td>
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<td>Kundhi</td>
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<tr>
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<td>3-5 year</td>
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<td>236</td>
<td>Ref.</td>
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<td>-</td>
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<td>6-8 year</td>
<td>316</td>
<td>129</td>
<td>187</td>
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<td>0.82-1.51</td>
<td>0.48</td>
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<tr>
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<td>≥9</td>
<td>338</td>
<td>233</td>
<td>105</td>
<td>3.59</td>
<td>2.63-4.89</td>
<td>&lt;0.001*</td>
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<td>138</td>
<td>36</td>
<td>102</td>
<td>Ref.</td>
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<td>-</td>
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<td>2 to 5</td>
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<td>262</td>
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<td>1.01-2.65</td>
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<td>226</td>
<td>164</td>
<td>3.96</td>
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<td>146</td>
<td>187</td>
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<td>361</td>
<td>441</td>
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<td>1.53-2.78</td>
<td>0.000*</td>
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<td>234</td>
<td>147</td>
<td>87</td>
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**Table 3.10: Univariable Analysis for Association of different Risk Factors with Mastitis**

<table>
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<tr>
<th>Risk Factors</th>
<th>Level</th>
<th>Total examined</th>
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<th>-Ve No.</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
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<tr>
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<td>Funnel</td>
<td>227</td>
<td>116</td>
<td>111</td>
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<td>0.61-1.2</td>
<td>0.364</td>
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<td>Round</td>
<td>316</td>
<td>149</td>
<td>167</td>
<td>1.06</td>
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<td>0.125*</td>
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<td>Flate</td>
<td>493</td>
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<td>0.709</td>
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<td>Pendolous</td>
<td>727</td>
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<td>352</td>
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<td>Normal</td>
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<td>133</td>
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<td>Pendolous</td>
<td>727</td>
<td>375</td>
<td>352</td>
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<td>374</td>
<td>483</td>
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<td>941</td>
<td>421</td>
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<td>Hard</td>
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<td>471</td>
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<td>423</td>
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<td>09</td>
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*Significant: $P<0.25$
### Table 3.11: Univariable Analysis for Association of different Risk Factors with Mastitis

*Significant: \( P < 0.25 \)

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Level</th>
<th>Total examined</th>
<th>+Ve No.</th>
<th>-Ve No.</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value</th>
</tr>
</thead>
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<td>Condition of floor</td>
<td>Concrete</td>
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<td>128</td>
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<td>Soiled</td>
<td>754</td>
<td>380</td>
<td>374</td>
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<tr>
<td>Frequency of dung removal</td>
<td>Once in a day</td>
<td>922</td>
<td>459</td>
<td>463</td>
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<td>Twice in a day</td>
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<td>49</td>
<td>65</td>
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<td>255</td>
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<td>459</td>
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<td>154</td>
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<td>Teat dipping</td>
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<td>1.01-1.95</td>
<td>0.042*</td>
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<td>865</td>
<td>412</td>
<td>453</td>
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<td>Complete hand</td>
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<td>1.05-1.80</td>
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<td>238</td>
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<td>83</td>
<td>Ref.</td>
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<td>327</td>
<td>359</td>
<td>0.5963</td>
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<td>G3 (≥ 11)</td>
<td>170</td>
<td>84</td>
<td>86</td>
<td>0.7580</td>
<td>-</td>
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Table 3.12: Multivariable Analysis of Potential Risk Factors with Prevalence of Mastitis

<table>
<thead>
<tr>
<th>Potential risk factors</th>
<th>Level</th>
<th>Regression Coefficient</th>
<th>Standard error</th>
<th>OR</th>
<th>95% CI</th>
<th>P-value (Wald)</th>
<th>P-value (LR)</th>
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<tr>
<td>Age</td>
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<tr>
<td></td>
<td>6-8 year</td>
<td>-0.6274</td>
<td>0.2349</td>
<td>0.53</td>
<td>0.34-0.85</td>
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<tr>
<td></td>
<td>≤9</td>
<td>0.7963</td>
<td>0.2214</td>
<td>2.22</td>
<td>1.44-3.42</td>
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<td>Breed</td>
<td>Nili Ravi</td>
<td>Ref.</td>
<td></td>
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<td></td>
<td>Kundhi</td>
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<td>0.36-0.81</td>
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<td>Non-descriptive</td>
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<td>0.2957</td>
<td>0.69</td>
<td>0.39-1.23</td>
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<tr>
<td>Parity</td>
<td>1st</td>
<td>Ref.</td>
<td></td>
<td></td>
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<td>2-5</td>
<td>0.8927</td>
<td>0.3150</td>
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<td>≥6</td>
<td>1.8057</td>
<td>0.3310</td>
<td>6.08</td>
<td>3.18-11.64</td>
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<td>Lactation stage</td>
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<td>Mid</td>
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<td>0.2101</td>
<td>1.85</td>
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<td>Late</td>
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<tr>
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<td>1.2357</td>
<td>0.2418</td>
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<td>Ref.</td>
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<tr>
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<td>1.67</td>
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<td>Ref.</td>
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<tr>
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<td>1.9225</td>
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<tr>
<td>Ease of milking</td>
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<td>Soft</td>
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<td>14.48</td>
<td>6.43-32.58</td>
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<tr>
<td>Teat Dipping</td>
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<td>Ref.</td>
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<td>1.04-2.97</td>
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<td>Bhimber</td>
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<td>0.45-0.93</td>
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</tbody>
</table>

*Significant: P < 0.05
DISCUSSION

Prevalence of Clinical and Subclinical

In the present study the overall prevalence of mastitis was 49.0%. Among these 49.0% clinical mastitis was 10.2%, while subclinical mastitis was 38.8%. The finding of previously study conducted in Khyber Pakhtunkhwa province of Pakistan by Ali et al. (2014), who reported that overall prevalence of clinical and subclinical mastitis was 13.6% and 41.8 %, respectively which is close to the present study. Prevalence of mastitis in present study agreed with a prevalence of mastitis from previous studies carried in Sudan by Nigo et al. (2013), Junaidu et al. (2011) in Nigeria and Hashemi et al. (2011) in Iran. The findings in the present study are in accordance to Ali (2009) who reported prevalence of clinical mastitis was 14.0% to 31.75% in Faisalabad, Pakistan, while, the finding of some previous studies (Mustafa et al. 2014: Bilal et al. 2004) who reported the prevalence of mastitis in buffaloes was more than 40.0% in Lahore and Faisalabad, Pakistan, respectively.

The results of the present study about the prevalence of subclinical mastitis (38.0%) are in accordance with previous studies by Ayano et al. (2013) in Ethiopia, Nigo et al. (2013) in Sudan, Hashmi and Muneer (1981) in Lahore, Pakistan, Anwar and Chaudhry (1983) who reported prevalence of subclinical mastitis was 31.0% to 47.0% in cattle and buffaloes in Lahore, Pakistan. The prevalence of subclinical mastitis was reported by Mustafa et al. (2014) as 59.6%, which was higher than present study. However, the prevalence of subclinical mastitis in this study is relatively higher than Sarkar et al. (2013) and Rahman et al. (2009) in Bangladesh who reported prevalence of subclinical mastitis as 9.0% to 20.0%. In present study significant statistical association of mastitis with locality (p-value =.000), is supported by a previous two studies Nigo et al. (2013) in Sudan and Biffa et al. (2005) in Ethiopia, p-value =.003 and p-value=.001, respectively.
EXPERIMENT -I

Area wise prevalence of mastitis

In the present study, the prevalence of mastitis was different in both localities 55.5% and 40.2% in Lahore and Bhimber, respectively. Our finding is supported by previous studies conducted by Nigo et al. (2013) in Sudan and Biffa et al. (2005) in Ethiopia who reported that the prevalence of mastitis was different in different localities. Basically, mastitis is a complex disease involving interactions of several factors, mainly of management, environment, and other factors relating to animal and causative organisms. Its prevalence is expected to vary from place to place. In this study, the prevalence of mastitis varies from previous studies. It might be due to different geographical locations, animal genetics, milking methods and management practices that applied in farms in different localities. This difference in prevalence of mastitis in area wise may be due to different management practices and environmental contamination.

Various studies has been conducted on prevalence of mastitis and with the passage of time prevalence of mastitis is increases. Previously a study was conducted in 2011 in district Lahore and prevalence of mastitis was recorded in buffalo as 40.7% (Ali et al. 2011). The present study shows, trend of mastitis in increases in Lahore. Increased prevalence is related with increased buffalo population and poor management practices on dairy farms. The prevalence of mastitis was high in district Lahore as compared to district Bhimber. The reason behind could be geographical location. The district Lahore have large population as compared to District Bhimber. District Lahore is an urban area and dairy farms are located close to urban population and industrial area and dairy farms are in form of large size population colonized as compared to district Bhimber.

Quarter wise prevalence

In our study, quarter level prevalence was recorded (16.2%) while district wise it was 18.17% and 12.9%, Lahore and Bhimber, respectively. The quarter wise prevalence of mastitis among LF,
RF, LR, and RR was recorded at 9.4%, 28.0%, 8.2% and 19.4%, respectively. The mastitis was higher in hind quarters as comparable to forequarters. Results of the present study were in line with previous studies of Bilal and Muhammad (2004), Nigo et al. (2013), Gebrekrustos et al. (2012), Shari et al. (2005) and Chishty et al. (2007) who reported that in hindquarters prevalence of mastitis was higher than that of front quarters. Single quarter involvement was seen in maximum number of animals and this is congruent with the finding of Ali (2009) and Hussain et al. (2013) who reported that single quarter involvement was high and very rare involvement of the all quarters. In the present study quarter wise prevalence of mastitis showed that highest prevalence was in single and two quarters, which were in accordance to Iqbal and Siddique (2004), Mahantesh et al. (2014) who reported that mastitis involvement was more in a single quarter (52.7%), Similarly Singh and Shankar have recorded higher incidence of mastitis in a single quarter (17.4%), as compared to two (2.6%), three (0.3%), and four quarters (2.7%). The present study was in line to Ali et al. (2014), Mustafa et al. (2014), Hussain et al. (2013), Bilal et al. (2004) who reported that prevalence of mastitis was higher in rear quarters than in fore quarters in buffaloes. Bilal and Muhammad (2004) reported a higher incidence in hind quarters (63.1%) and forequarters (36.8%) in buffaloes. Results of the present study are in accordance with previous studies (Egan and Meancy1987) who reported that the higher prevalence in left rear quarter (34.0%) followed by the right front (28.0%), right rate (21.8%) and left front (15.3%). Further, among hind quarters, left hind quarters were found to be more susceptible to infection. The prevalence of mastitis in left rear quarters was higher than in other quarters. The possible explanation about the left rear quarters may be due to practices adopted by farmers. In Pakistan, the farmers, mostly milked the buffaloes from the left side. When milker milked the right rear quarter his hand touches the left quarter continuously. If the milker’s hand is contaminated it can transmit the infectious to the left rear
EXPERIMENT -1

quarter. In our view, buffalo hind quarters are large size and have high milk production as compared to front quarters. The hind quarters are more exposed to dirt when they lie down on the floor as well as they are more contaminated with fecal materials. It may be due to reasons that unhygienic conditions of the legs and presence of contaminated dung can help in the occurrence of mastitis. Also, they are in direct touch with the hind limbs at milking time. The difference in quarter wise prevalence of mastitis is probably due to the fact that predisposing factors like teat size, injury, defective sphincters, and so forth could vary from quarter to quarter.

**Previous exposure to Mastitis**

In our study previous exposure of mastitis was statistically significant (p<. 001) with a prevalence of mastitis having OR 3.84 at 95% CI of 2.67-5.53) by univariable analysis. In multivariable analysis, it was highly significant (p<. 001) having OR 3.44 of 2.14-5.53. The present study significant association (p<.000) of previous exposure to mastitis was in accordance with previous studies (Abera et al. 2012; Nigo et al.  2013; Biffa et al. 2005) who reported a significant association (p<.000) of previous exposure to mastitis with a prevalence of mastitis. Elbably et al. (2013) described that previous exposure to mastitis was at greatest risk of being re-infected, and stress factors could put the glands at greater risk of re-infection. In our study, the dairy animals with previous history of mastitis are three times more susceptible to mastitis as described by Biffa et al. (2005) who reported that dairy animals with a history of previous mastitis are two to five times more susceptible to mastitis than those without a history of previous mastitis. Previously affected dairy animals are at greater risk of being re-infected, suggesting that repeated challenges of the mammary tissues with microorganisms coupled with other stress factors could put the glands at greater risks of re-infection. Findings of the present study are in line with previous studies (Erskine et al. 2002; Lents et al. 2002) who reported that treatment of clinical mastitis may
EXPERIMENT -1

suppress the clinical signs, but infective agents are not completely eliminated and infection may remain in subclinical form.

**Herd size wise distribution of mastitis**

In the present study, the prevalence of mastitis was increased as the herd size increased. These findings are in accordance to Ali et al. (2014), Nigo et al. (2013), Kivaria et al. (2013) and Islam et al. (2010) who reported that the prevalence of mastitis was high in large size herds as compared to small size herds. In the univariable analysis, the large herd size was significantly (p<0.02, having OR 1.65 at 95% CI of 1.07-2.54) associated with prevalence of mastitis which is in accordance to Ali et al. (2014), Kivaria et al. (2013), Nigo et al. (2013) and Islam et al. (2010) who reported significant (p< 0.05) association of large herd's size with a prevalence of mastitis.

**Age wise distribution of mastitis**

In the present study, the prevalence of mastitis was in G1 (3-5 years) G2 (6-8 years) D3 (9 and above), 38.2%, 40.8% and 68.9%, respectively. It has been observed from the present results that prevalence of mastitis increased with increase in age. Findings of the present study are in line to previous study conducted by Ali et al. (2014), Ali (2009), Ayano et al. (2013), Kurjogi et al. (2014), Naghmana et al. (1984), Biffa et al. (2005) and Rasool et al. (1985) who reported that rate of mastitis increase with age. Similarly, Bhikane et al. (2002), Kumar and Sharma (2002) and Elbably et al. (2013) reported the prevalence of mastitis in the age of 9 years old was higher than younger animals. The univariable analysis shows that age wise was mastitis is highly significant (p< 0.000). The logistic regression showed in final Step that it age group 6-8 years was highly significant (p< 0.000) with OR. 2. 22 (1.44-3.43). In present results, age was shown significantly (p<0.000) associated with prevalence of mastitis which is close to previous study conducted by Makibib et al. (2010) who reported that age was significant (p < 0.05) associated with prevalence
of mastitis. This study agreed that increase in prevalence rates with the advancing age may be due to gradual suppression of the immune system of the body and structural changes in the udder and teats. This higher prevalence at this age might be due to decreased immunity of dairy animals and resistance of bacteria to antibiotics that were indiscriminately used for the treatment of mastitis during previous infections.

**Parity wise prevalence of mastitis**

In the present study it has been observed that the prevalence of mastitis increases as the parity number increases. The prevalence of mastitis continuously increased with increase in parity number. Our findings are in line with the finding of Biffa et al. (2005), Ali et al. 2014 and Chishty et al. (2007), who reported prevalence of mastitis increased with parity number. The present study reveals parity numbers were highly significant (p< 0.000, OR 16.43 having 95% CI of 5.91-45.68) with a prevalence of mastitis and these findings are similar to finding of previous studies (Elbably et al. 2013; Biffa et al. 2005; Ali, 2009) who reported parity has a significant association with the prevalence of mastitis. This increase in mastitis could be due to increasing ease of penetration of teat duct by a pathogen and accumulation of previous infection as it was confirmed by Tadesse and Chanie (2012) that old dairy animals especially after four calving are more prone to mastitis.

**Stage of lactation wise prevalence of mastitis**

In the present study, it has been observed that the prevalence of mastitis was high in mid lactation as compared to early and late stage of lactation. The present study finding is in line with Breen et al. (2009); Almaw et al. (2007) and Abera et al. (2012) who reported prevalence of mastitis was higher in mid lactation than early and late stage of lactation. In our study, the mid stage of lactation was significantly (p<.003) associated with prevalence of mastitis when compared, having OR 1.61 at 95% CI of 1.17- 2.21. Regarding stage of lactation current results
agreed with the findings of Hussain et al. (2013), Almaw et al. (2007) and Abera et al. (2012) who reported that the stage of lactation was significantly (P-value 0.01) associated with prevalence of mastitis. This may be due to high milk production in mid lactation, incomplete milking, continuously milking for a long time, and putting pressure on a teat, changes in both immune function and nonspecific host defense mechanism.

**Udder Shape**

In the present study the prevalence of mastitis was higher in pendulous udder (51.6%) rather than normal udder (43.0%) respectively. The relationship between udder shape and prevalence of mastitis was significant (P<0.012). The present studies were similar to previous studies conducted by Ali (2009), Compton et al. (2008), Breen et al. (2009) and Hussain et al. (2013) that mastitis was higher in pendulous udder than normal udder. Our univariable analysis is close to Sarkar et al. (2013) and Ali (2009) who reported that pendulous type of udder had significant (p<0.05) association with prevalence of mastitis and pendulous udders because pendulous udder sweep the ground all the time that facilitate ascending entry of mastitic pathogens. Additionally, the pendulous type of udder does not wear well and hanging down due to bigger size, and it is more likely to become injured than an udder held up close to the body. It might be due to the reason that, pendulous udder get injuries and abrasion which may facilitate pathogens to grow.

**Teat Shape**

The overall prevalence of mastitis funnels, round and flat shaped teats was recorded 51.1%, 47.2% and 49.3%, respectively. In the present study, mastitis prevalence was high in flat and round teat than funnel shaped teat. In the present study, flat teats were associated (p<0.125) with a prevalence of mastitis in funnel shaped teats when compared having OR 1.06 at 95% CI of 0.75-
The results of the present study are in accordance to previous studies conducted by Ali (2009) who reported a significant association of teat shape with a prevalence of mastitis. The reasons for flat shaped teat may have more chances of exposure to environmental contamination and injuries.

**Lesion on udder and teat**

The overall prevalence of mastitis in the present study, on the basis of lesion on udder and teat was 18.3%. Similarly, Matios et al. (2009) and Chishty et al. (2007) reported teat injuries and lesions predispose the udder to infection that might be the reason of higher prevalence of mastitis in injured teats. Furthermore, it was reported by Uddin et al. (2009) and Sori et al. (2005) that teat injury provides a medium for the growth of the pathogenic bacteria, which affect the udder and in case of injuries, the risk of an infection increases. Results of the present study are in accordance to Elbably et al. (2013) and Bekele and Molla (2001) who suggested that heavy tick infestation and teat lesions might be responsible for udder infection and also lead to udder abnormalities and deformities of teats. The lesions on udder and teat were highly significant ($p<.000$, OR 13.06 at 95% CI of 6.25-27.26) on the prevalence of mastitis, same as in multivariable analysis, it was highly significant ($p<.001$, OR 6.84 at 95% CI of 2.88-16.21) and these findings were in accordance to Biffa et al. (2005) and Ali (2009) who reported mastitis was significantly associated with udder/teat injuries. In Pakistan, as part of the traditional dairy husbandry practices, calves are kept away from their dams over a long period of time and are only allowed to suckle for a short duration and inadequate milk supply, calves suck vigorously, inducing teat injuries and hence exposing dams to teat injuries.

**Udder edema**

The udder edema was strongly associated ($p<.000$) with the prevalence of mastitis having OR 13.747 while in logistic regression it was significant $p<0.02$ to OR 4.92 (1.25-19.36). Findings
of the present study are in line with Ali (2009) who reported a highly significant association between mastitis status and predisposing factors like udder edema and teat edema.

Milking Techniques

The prevalence of mastitis in animals milked by knuckling was high (51.3%) as compared with complete hand milking (25.6%). Milking method, knuckling was found to be highly associated (p>0.019) with the prevalence of mastitis by univariable analysis as compared with complete hand milking (OR 1.38; 95% CI, 1.05-1.80). The present study is in accordance to Chishty et al. (2007) and Ali, (2009) who reported that there is a significant association between knuckling milking and prevalence of mastitis in buffaloes. This high prevalence of mastitis in buffaloes milked by folding thumb might be due to trauma inflicted by knuckling milking. The use of knuckling technique predisposed the mastitis. Moreover, the use of folded thumb the teat cistern is injured and has often been considered to be conducive to the development of adverse effects of the teat canal.

Ease of milking

The soft milking was highly significant (p<.000) when compared with hard milking having OR 13.43 at 95% CI of 6.43-28.02. In logistic regression it was highly significant (p<.001) with OR 14.48 at 95% CI of 6.43-32.58). Similarly, in previous studies (Chishty et al. 2007 and Ali, 2009) who reported highly significant association between the soft milking and prevalence of mastitis. This high prevalence of mastitis in soft milking might be due to loose sphincter of teat and infectious agents can easily enter and grow in teat canal and udder.
**Teat dipping**

The teat dipping was significantly (p< .042, OR 1.40) associated with prevalence of mastitis. Present study agreed with Hillerton et al. (1993) and Hu et al. (1990) who reported mastitis prevalence can be minimized by using teat dipping before and after milking.

**Number of animals milked by same milker**

In the present study, the prevalence of mastitis was recorded 53.9%, 47.7% and 49.4% in G1, G2 and G3, respectively. It has been observed that the prevalence of mastitis increases with increasing number of animals milked by same milker. In general, mastitis was increased as the number of animals milked by same milker increased, but it had non-significant effect on prevalence of mastitis. The present study findings are accorded with Oliver, (1975) and Ali, (2009) who reported the prevalence of mastitis increases with increasing number of animals milked by same milker. In developing countries like Pakistan, dairy animals are milked mostly by the hand milking method and milk is often used as a lubricant during milking and milker’s hands are often heavily soiled with milk during this process. It is predominantly caused by contagious mastitis pathogens which are usually transmitted at the time of milking. Infectious agents of mastitis may be transmitted from infected to uninfected animals through milkers hands, especially owning to the proclivity of using milk foam in the milking as a lubricant.

**Frequency of dung removal**

The prevalence of mastitis was recorded highest in dung is removed once in a day, 49.8% than twice in a day (43.0%). The univariable analysis showed that a frequency of dung removal has a significant effect on prevalence of mastitis (P<0.171, OR 1.31 at 95% CI of 0.88-1.94). Similar results by Carrol (1977) that with once in a day cleaning of sheds, the incidence of mastitis in buffaloes was 21.1%, whereas when cleaning of sheds was done twice a day, 15.8% of animals
developed mastitis. In the present study, findings have a significant effect on mastitis same as to Nago et al. (2013) and Ali (2009). Environmental mastitis pathogens with reservoirs in dung, floor, bedding etc. Are only occasionally associated with mastitis. Therefore, mastitis control practices directed against environmental mastitis pathogens are not likely to be as potentially rewarding as practices aimed at controlling contagious pathogens.

**Condition of floor**

The prevalence of mastitis was higher on soiled floor, 50.4% than concrete floor 45.4%. The univariable analysis showed that condition of floor has no significant effect (p<.086) with mastitis. Similarly, Ali (2009) and Mukabib et al. (2010) reported non-significant association condition of floor with a prevalence of mastitis in buffaloes. In Pakistan mostly dairy animals are managed on soiled floor. In soiled floor buffaloes udders and legs are not properly clean before milking and infection can easily transmit at milking time from diseased to healthy animal.

**Source of water**

The prevalence of mastitis was 50.8%, 46.0% and 56.3% pond, canal, subsurface water, respectively. In pond and canal the prevalence of mastitis was higher as compare to subsurface water. Similarly, previous studies by Nigo et al. (2013), Kivaria et al. (2004) who reported that river and pond mostly have stagnant water where microorganism can be multiplied and be transmitted from infected animals to non-infected animals. The source of water has no significant effect on prevalence of mastitis.

**Tick infestation**

The prevalence of mastitis on basis of tick infestation was 55.8% in exposed animals. High tick infestation might have contributed to the occurrence of injuries which causes mastitis.
Tick infestation in this study showed a significant association with mastitis (p<.000), similar to Tolosa et al. 2013 and Biffa et al. (2005) who reported a significant association (P <0.01) with a prevalence of mastitis. The present study agreed with previous studies by Almaw et al. (2008), Bekele and Molla (2001) and Elbably et al. (2013) who reported that ticks transmit the mastitis widely throughout the world and ticks left small abrasion and lesion on teat and udder skin which may let the ease of organisms penetration inside udder.

**Reproductive disorders**

The prevalence of mastitis on the basis of reproductive disorder was recorded 62.8% which were highly significant (p<.000) with mastitis. Ali (2009) determined a significant association with the reproductive disorders and mastitis prevalence in buffaloes. The association between prevalence of mastitis and reproductive disorders was in line with the findings reported previously by Esmatand Badr (1996) and Ali, (2009) who reported that reproductive disorders were three times more likely to develop mastitis. Dairy Buffaloes with the retained placenta were likely to develop mastitis. According to Bilal (1999) dairy buffaloes having retained placenta, metritis, vaginal prolapses and dystocia at calving are risk factors of mastitis.

**Exposure to FMD**

In present study exposure to FMD in both univariable and multivariable analysis has significant effect on mastitis. Diseases like FMD and pox are particularly known to predispose mastitis in cattle and buffaloes. According to Radostits et al. (2000) observation, foot and mouth disease, vesicles may appear on the teat as to the involvement of the teat orifice to mastitis.

In conclusion, dairy buffaloes should be regularly screened and monitored for prevention and control of mastitis. The husbandry management practices should be kept in consideration while combating mastitis. Basically mastitis is interaction effects of various management practices,
such as the interaction of teat dipping before and after milking and proper sanitation of the farm have significant effects on mastitis prevention. The control strategies should be based on the improvement of management practices since these factors predispose animals to mastitis. The study showed that various risk factors are significantly associated with the prevalence of mastitis, which need to be considered in the control of the disease. Particularly, special importance should be given to prevention and control of subclinical mastitis. Knowledge of the potential risk factors is vital for the control of bovine mastitis. In general, mastitis can be prevented by implementation of various hygienic and preventive measures.

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ANTIBIOTICS RESISTANCE AND SENSITIVITY OF *STAPHYLOCOCCUS AUREUS* CAUSING MASTITIS IN BUFFALOES

ABSTRACT

The present study was conducted to determine the antibiotic sensitivity of *Staphylococcus aureus* isolated from mastitis buffaloes. Clinical mastitis was diagnosed on the basis of physical examination of the udder, teats and milk. Subclinical mastitis was diagnosed with the help of California Mastitis Tests. Among a total of 1036 buffaloes, 508 were found to have mastitis (49.0%). A total of 673 mastitic milk samples from different animal quarters were collected from different livestock farms having 106 and 567 clinical and subclinical mastitis, respectively. The prevalence of mastitis was 49.0% with 95% Confidence interval of 46.4-51.5, while prevalence per quarter was 16.2% with 95% Confidence interval of 15.3-17.2. From these 673 milk samples, Staphytect plus test confirmed 236 (35.0% at 95% CI 32-38.2) isolates as coagulase positive *Staphylococcus aureus*. Antibiotic sensitivity of all coagulase positive *Staphylococcus aureus* was performed by disk diffusion method. The sensitivity of gentamycin, oxytetracycline, erythromycin, kanamycin, chloramphenicol, tylosin, ciprofloxacin, doxycycline, amoxicillin, streptomycin and trimethoprim to *Staphylococcus aureus* was 80.1%, 75.4%, 74.6%, 73.3%, 72.4%, 71.6%, 69.9%, 69.1%, 63.2%, 56.0% and 54.7%, respectively. While on the other hand, the resistance pattern of gentamycin, oxytetracycline, erythromycin, kanamycin, chloramphenicol, tylosin, ciprofloxacin, doxycycline, amoxicillin, streptomycin and trimethoprim was 11.0%, 19.9%, 15.3%, 20.8%, 20.8%, 28.4%, 22.9%, 17.3%, 36.8%, 34.3% and 45.3%, respectively.
Keywords: Mastitis, Buffaloes, *Staphylococcus aureus*, California mastitis Test, Antibiotics.

INTRODUCTION

Pakistan is rich in livestock, which contributes 11.8% of total GDP with 35 million rural populations engaged in raising livestock (Anonymous, 2014). The buffalo population in this country is 34.7 million heads (Anonymous, 2014). Buffaloes are recognized as the second most important milk producing species around the world (FAO, 2010). Among the various diseases which affect milk production, mastitis is a major one (Shakoor, 2006). Mastitis is basically a tenderness of the mammary gland and is characterized by physical, chemical and pathological changes in the milk and glandular tissue. In the case of subclinical mastitis, milk is apparently normal with an increase in the somatic cell count (Radostits et al. 2000). In Pakistan, mastitis eradication may not be possible, but hygienic management practices can reduce the incidence (Rahman et al. 2009). It has been observed by Mustafa et al. (2011) that the prevalence of mastitis, both clinical and subclinical in buffalo was 40.4% and 59.6%, respectively.

A wide variety of organisms are associated with mastitis, however the most frequent pathogens are bacteria, such as *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus dysagalactiae*, *Streptococcus uberis*, *Streptococcus agalactiae* (Radostits et al. 2000). However, more than 50% of cases of mastitis are caused by *Staphylococcus aureus* (Shakoor, 2006). *Staphylococcus aureus* creates pockets in the inner lining of mammary gland, which is bounded by fibrous tissue and where antibiotics cannot respond. In Pakistan, antibiotic therapy during dry period and teat dipping are not in practice, that’s why losses due to mastitis are higher (Arshad, 1999). The cure rate of *Staphylococcus aureus* mastitis was 52.0% (Sol et al. 2000).

Changes in the nature of proteins expressed by the organism, mutation or horizontal changes in its genome may lead to bacterial resistance. It is common that with the passage of time
bacteria may develop resistance to currently available antibacterial drugs by either new mutations or the exchange of genetic information that is, putting old resistant genes into new hosts (Tenover, 2006). For example, methicillin resistance of *Staphylococcus aureus* is primarily due to changes in the penicillin binding protein that consequently inhibit cell wall synthesis. Expression of the different gene results in an alternative penicillin binding protein that has a low affinity for most β-lactam antibiotics, thereby allowing, these strains replicate in the presence of methicillin and related antibiotics. The objectives of the present study were to determine the prevalence of mastitis in buffalo and the evaluation of commonly used antibiotics in the treatment of mastitic buffaloes.

**MATERIALS AND METHODS**

**Sampling procedure and sampling area**

A cross sectional study was carried out for investigation of the prevalence of mastitis in dairy buffaloes. The present study was conducted in the peri-urban area of district Lahore and district Bhimber.

**Selection Criteria and Diagnosis of Mastitis**

Only lactating buffaloes were incorporated in the present study and those which were pregnant, in the dry period and heifers were excluded. Animals suffering with clinical mastitis were diagnosed upon physical examination by the presence of clinical signs such as swelling of the udder and teat, udder edema, udder and teat injury, abnormal milk secretion etc. Animals with normal udder quarters were screened by California Mastitis Test (CMT) (Iqbal et al. 2006). A total of 4144 quarters of buffaloes was examined by CMT from 1036 lactating buffaloes. Animal and quarter wise prevalence of mastitis was recorded in a questionnaire.
Collection, Transportation and Culturing of Samples

The recommendation of National Mastitis Council (1990) was considered for the collection, transportation, culturing and isolates of *Staphylococcus aureus*. All milk samples were collected in sterilized screw capped vials and samples transported by placing them in the crushed ice to the Microbiology Laboratory in the Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Lahore. Conventional methods were used for the isolation and identification of *Staphylococcus aureus*.

Staph. 110 medium was used as a selective medium for growth of Staphylococcal species. Biochemical tests performed were Gram’s staining, catalase test, coagulase test, Mannitol salt ager and Staphytect plus test (Oxoid UK). After confirmation, pure culture of *Staphylococcus aureus* was preserved in 20% glycerol for further study.

Antibiotic Sensitivity of *Staphylococcus aureus*

According to the guideline of Clinical Laboratory Standard Institute (CLSI. 2005), the disk diffusion method was performed for antibiotic sensitivity. *Staphylococcus aureus* ATCC 25923 was used as the quality control organism and trimethoprim, erythromycin, ciprofloxacin, doxycycline, amoxicillin, streptomycin, tetracycline, tylosin, gentamycin, kanamycin, chloramphenicol were used in antibiotics sensitivity testing, taking into account the previous historical records over one decade that these antibiotics were commonly used in the treatment of buffalo mastitis (Hussain et al. 2013; Hameed et al. 2008).
RESULTS

A total of 673 milk samples from 1036 lactating buffaloes was collected. The animal wise prevalence of mastitis was 49.0% with 95% confidence interval of 46.4-51.5. While the prevalence of clinical and subclinical mastitis was 10.2% and 38.8%, respectively. The table (4.1) indicates that, only 236 confirmed as *Staphylococcus aureus* by coagulase test, mannitol salt agar and staphytect plus test. All these 236 isolates of *Staphylococcus aureus* were subjected to antibiotic sensitivity test by disk diffusion method. The zones of inhibition of antibiotics were recorded with antimicrobial zone gauge. The resistance and sensitivity of *Staphylococcus aureus* to antibiotics has been shown in a number (n=236) and percentage (%) in table 4.2, respectively. The resistant pattern of gentamycin, erythromycin, doxycycline, oxytetracycline, kanamycin, chloramphenicol, ciprofloxacin, tylosin, streptomycin, amoxicillin and trimethoprim was 11.0%, 15.3%, 17.3%, 19.9%, 20.8%, 20.8%, 22.9%, 28.4%, 34.3% 36.8% and 45.3%, respectively (Table 4.2). The sensitivity of gentamycin, oxytetracycline, erythromycin, kanamycin, chloramphenicol, tylosin, ciprofloxacin, doxycycline, amoxicillin, streptomycin and trimethoprim to *Staphylococcus aureus* was 80.1%, 75.4%, 74.6%, 73.3%, 72.4%, 71.6%, 69.9%, 69.1%, 63.2%, 56.0% and 54.7%, respectively. The graphical presentation of resistance and sensitivity of *Staphylococcus aureus* has been shown in figure 4.1 and 4.2.
Table No. 4.1. Isolation and identification of *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Gram’s Staining</th>
<th>Catalase test</th>
<th>Slide coagulase test</th>
<th>Staphytect plus test</th>
<th>Mannitol salt ager test</th>
</tr>
</thead>
<tbody>
<tr>
<td>+Ve</td>
<td>-Ve</td>
<td>+Ve</td>
<td>-Ve</td>
<td>+Ve</td>
</tr>
<tr>
<td>407</td>
<td>-</td>
<td>407</td>
<td>-</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td></td>
<td>171</td>
<td></td>
<td>171</td>
</tr>
</tbody>
</table>

Table No. 4.2. Percentage (%) and numbers of *Staphylococcus aureus* resistant, intermediate and sensitivity to antibiotics (n=236)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Resistant</th>
<th>Intermediate</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamycin</td>
<td>11.0% (26)</td>
<td>9.0% (21)</td>
<td>80.1% (189)</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>19.9% (47)</td>
<td>4.6% (11)</td>
<td>75.4% (178)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15.3% (36)</td>
<td>10.2% (24)</td>
<td>74.6% (176)</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>20.8% (49)</td>
<td>6.0% (14)</td>
<td>73.3% (173)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>20.8% (49)</td>
<td>6.8% (16)</td>
<td>72.4% (171)</td>
</tr>
<tr>
<td>Tylosin</td>
<td>28.4% (67)</td>
<td>0.0% (0)</td>
<td>71.6% (169)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>22.9% (54)</td>
<td>7.2% (17)</td>
<td>69.9% (165)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>17.3% (41)</td>
<td>13.5% (32)</td>
<td>69.1% (163)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>36.8% (87)</td>
<td>0.0% (0)</td>
<td>63.2% (149)</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>34.3% (81)</td>
<td>9.7% (23)</td>
<td>56% (132)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>45.3% (107)</td>
<td>0.0% (0)</td>
<td>54.7% (129)</td>
</tr>
</tbody>
</table>
Figure 4.1. Pattern of resistant of *Staphylococcus aureus* against Antibiotics.
Figure 4.2. Pattern of sensitivity of *Staphylococcus aureus* against Antibiotics.
DISCUSSION

Mastitis is economically unbearable and complicated disease in dairy industry worldwide. *Staphylococcus aureus* is considered as a leading causative organism of mastitis in dairy animals. Due to the complex nature of *Staphylococcus aureus*, the treatment and control of mastitis are failed. The drug resistance is a significant feature of *Staphylococcus aureus* (Wyder et al. 2011). Antibiotic resistance is an emerging problem all over the world. Mastitis in dairy animals is the common reason for antibiotic use in dairy animals. *Staphylococcus aureus* resistance is increased in case of mastitis to several antibiotics. In this study, the resistance to trimethoprim is high as compared to other drugs, it might be due to its constant use in the last decades. In Pakistan, all these tested antibiotics are in practice in case of mastitis treatment, but among them trimethoprim, amoxicillin, streptomycin, ciprofloxacin and oxytetracycline are regularly used in mastitis treatment.

In the present study, trimethoprim sensitivity against *Staphylococcus aureus* was 54.7% and its resistance was 45.3%. Our finding is accordance in with many studies (Wang et al. 2015; Kurjogi et al. 2011; Hussain et al. 2013; Ikiz et al. 2013) who reported the sensitivity of trimethoprim was 50 to 77%. In this study, the resistance to trimethoprim is high as compared to other drugs, it might be due to its constant use in the last decades. Amoxicillin sensitivity in the present study was 63.2%, which is similar to previous studies (Farooq et al. 2008, Idriss et al. 2014; Jeykumar et al. 2013; Khan et al. 2004) who reported amoxicillin as a suitable drug for treatment of mastitis. Tylosin was 71.6% sensitive in the present study, which was not in line with the study of Brinda et al. (2010) who reported its sensitivity 28%. This might be due to frequent use to tylosin in their localities. In the present study, streptomycin sensitivity against *Staphylococcus aureus* mastitis was 56%, same as in previous studies (Idriss et al. 2014; Kurjogi et al. 2011; Farooq et al. 2008) reported their sensitivity from 50 to 80%. *Staphylococcus aureus*
sensitivity against antibiotic may be attributed to geographical variation. In the present study, ciprofloxacin sensitivity against *Staphylococcus aureus* mastitis was 69.9%. Same as in some previous studies (Wang et al. 2015; Jeykumar et al. 2013; Li et al. 2009; Unakal et al. 2010; Khan et al. 2004) reported their sensitivity from 50 to 90%. *Staphylococcus aureus* sensitivity might be attributed to the extent from locality to locality.

In the current study, *Staphylococcus aureus* was 75.4% sensitive and less resistance to oxytetracycline (19.9%). These findings are in accordance with previous studies who reported that *Staphylococcus aureus* was 59 to 88% sensitive to oxytetracycline (Jeykumar et al. 2013; Li et al. 2009; Khan et al. 2004; Zahid et al. 2004; Ikiz et al. 2013). The results of the current study revealed that the isolates were 74.6% sensitive to erythromycin. It is in line with many previous studies (Khan et al. 2004; Alekish et al. 2013; Brinda et al. 2010; Gianneechni et al. 2002; Unakal et al. 2010) who reported erythromycin sensitivity ranging from 78 to 95%. The sensitivity of ciprofloxacin was *Staphylococcus aureus* was 69.9%, which is in compliance with the studies (Jeykumar et al. 2013; Khan et al. 2005; Mustafa et al. 2014; Hussain et al. 2013) who reported ciprofloxacin as the drug of choice. In the present study, doxycycline sensitivity was 69.1%, which is similar to previous studies conducted by Wang et al. (2015) and Alekish et al. (2013) who reported the sensitivity of doxycycline in mastitis was 50 to 70%. In this study *Staphylococcus aureus* was 73.3% sensitive to kanamycin similarity, in previous studies (Idriss et al. 2014; Farooq et al. 2008; Anjum et al. 2010) described kanamycin as the drug of choice in mastitis. Some other studies (Mustafa et al. 2014; Kurjogi et al. 2011; Brinda et al. 2010; Anjum et al. 2010) who also reported their sensitivity less than 40%. *Staphylococcus aureus* isolated from buffalo mastitis was 72.4% sensitive to chloramphenicol, which is close to the previous finding by Wang et al. (2015), Li et al. (2009) and Khan et al. (2004) who reported 52 to 91% respectively. The present study reveals, the sensitivity of gentamycin to Staphylococcus was 80.1%. Gentamycin has been shown
as the drug of choice against *Staphylococcus aureus* similarly, many studies (Akram et al. 2013; Jeykumar et al. 2013; Anjum et al. 2010; Hameed et al. 2008; Iqbal et al. 2004) who reported gentamycin as a drug of choice in case of *Staphylococcus aureus* and its sensitivity was 80%.

In conclusion, for successful treatment of mastitis, antibiotic susceptibility testing should be done to regulate the effectiveness of antibiotics. Proper isolation and identification of the causative organisms and proper selection of antibiotics play important role in minimizing and control of the mastitis. In our study the resistant pattern of trimethoprim, amoxicillin, streptomycin and ciprofloxacin was high as compared to other used antibiotics. Thus, select the appropriate antibiotic and mastitis treatment should be started after antibiotic sensitivity testing. Furthermore, there is a need to routinely investigate and record the resistance and sensitivity of *Staphylococcus aureus* mastitis in dairy buffaloes.

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MOLECULAR CHARACTERIZATION OF COAGULASE AND FIBRONECTIN BINDING PROTEIN A GENES OF STAPHYLOCOCCUS AUREUS ISOLATED FROM MASTITIS IN RIVER BUFFALOES

ABSTRACT

Mastitis is a major dairy herd problem mainly caused by Staphylococcus aureus. Virulent coagulase and fibronectin binding protein A genes of S. aureus from mastitic buffalo milk samples, collected from 50 dairy herds located in Lahore and Bhimber districts. A total of 1036 buffaloes and 4144 quarters were screened for clinical and subclinical mastitis. Among total of 673 milk samples, S. aureus was isolated from 236 samples. The S. aureus isolates were subjected to molecular characterization of coagulase and fibronectin binding protein A genes and phylogenetic tree (using MEGA6.1 software package) was constructed. It revealed that the coa gene of S. aureus isolated from mastitic buffalo milk samples, could be grouped in two clades which were closely related to S. aureus isolates from Japan, India and Taiwan, while S. aureus isolates from Germany, UK and USA were distantly related. Phylogenetic tree based on fnbA gene sequences indicated that all Pakistani isolates were clustered together in one clade in closeness with isolates from Switzerland and Ireland. While isolates from Thailand and USA were placed distantly. These results indicated the genetic relatedness of Pakistani isolates with other reported isolates from different parts of the world. This particular knowledge may support in effective diagnosis, treatment and control of mastitis in river buffaloes in Pakistan. These findings will also be helpful in future for designing suitable mastitis control strategies in the country.

Keywords: Buffalo, Mastitis, Staphylococcus aureus, Coagulase, Fibronectin binding protein A.
Mastitis is an inflammation of the parenchyma of the udder which also results in physical, chemical and pathological changes in the udder. Inflammation of the udder may be caused by any kind of injuries such as physical trauma, chemical irritants, toxins or infectious agents. The bovine mastitis is one of the major disease responsible for reduction in milk production (Hussain et al. 2012). Mastitis is manifested in two forms, i.e. clinical and subclinical. The clinical mastitis is manifested by abnormal milk with or without udder swelling, while in subclinical mastitis milk production may decrease and bacterial counts increase in milk with no physical abnormality (Radostits et al. 2000). However, subclinical mastitis is diagnosed by somatic cell count by various methods such as White Side Test, California Mastitis Test and Surf Filed Mastitis Test. The prevalence of subclinical mastitis is 20-40 times more as compared to clinical mastitis (Erskine, 2002).

*S. aureus* is considered the most common causative agent of mastitis in dairy animals and it possesses property of genetic heterogeneity. Its genetic variability has contributed to the emergence of distinct epidemiologic profiles of strains prevalent in a herd (Fitzgerald et al. 2003; Karimuribo et al. 2005; Tollersrud et al. 2000). *S. aureus* does not have a host preference among animal species (Aires-de-Sousa et al. 2007; Mork et al. 2005). *Staphylococcus aureus* is responsible for a variety of infections in human and animals (Bartlett and Hulten, 2010; Gu et al. 2013; Khan et al. 2013) and treatments become more difficult due to its emerging strains. *S. aureus* can be divided into a number of subtypes, among them only a few are responsible for mastitis in different geographical locations of the world (Aarestrup et al. 1994; Hookey et al. 1999). Rapid and accurate identification of *S. aureus* is important for the control and prevention of the infectious mastitis (Hookey et al. 1998).
Pathogenesis of mastitis may be caused by extracellular toxins, enzymes and surface antigens (Luong et al. 2003; O’Riordan and Lee, 2004). Coagulase gene of *S. aureus* is considered an important virulence factor. Amplification of *S. aureus* coagulase gene (coa) has been recommended as an accurate method for identification of virulent strains of *S. aureus* (Morandi et al. 2010; Sabat et al. 2006; Goh et al. 1992). Sequencing of the coagulase gene shows great diversity in *S. aureus* population (Costa et al. 2012). Information regarding the genetic diversity of *Staphylococcus aureus* isolated from mastitis in cow is available but such information regarding *S. aureus* from buffalo mastitis is limited (Faryal et al. 2009). Various studies described that bovine mastitis is caused by a wide variety of *Staphylococcus aureus* genotypes (Smith et al. 2005). *Staphylococcus aureus* from mastitis represents a genetic heterogeneity (Fagiolo and Lai, 2007; Fournier et al. 2008). It has more than 50 virulence factors, which enable it to attach to a variety of hosts, and cause a multitude of diverse infections and the fibronectin binding protein A (*fnbA*) is one of them. Pathogenicity of *Staphylococcus aureus* is due to the expression of cell surface protein that can bind to host proteins (Broughan et al. 2011; Burke et al. 2010; Nashev et al. 2004; Novick, 2000). Among different virulent genes, either *fnbA* or *fnbB* gene of *Staphylococcus aureus* is considered a major virulence factor causing bovine mastitis. In pathogenesis, the first step of infection starts with the adherence of the pathogen to the teat canal of the udder (Ahmed et al. 2001; Arrecubieta et al. 2008). Effective entrance of bacteria into host occurs by possible when there is the formation of a bridge between bacterial fibronectin binding proteins (*fnbps*) and host fibronectin (Fowler et al. 2000). The present study was designed to find the genetic diversity of *Staphylococcus aureus* isolated from buffaloes with mastitis on the basis of *fnbA* gene. So the present study was designed to find the genetic diversity of *S. aureus* isolated from buffaloes with mastitis on the basis of coagulase gene.
MATERIALS AND METHODS

Collection of milk samples

The present study was carried out in District Lahore (Punjab) and Bhimber (Azad Jammu and Kashmir), Pakistan. Lactating buffalo disregards of the parity were selected while buffaloes in the dry period and heifer were excluded. A total of 4144 quarters from 1036 buffaloes was screened for the presence of clinical and subclinical mastitis. Fifty dairy herds were screened (25 from each district). The data were collected and categorized into three groups (small, medium and large having 5 to 10, 11 to 30, and more than 31 dairy buffaloes, respectively) during the study period. Clinical mastitis was diagnosed on the basis of signs and symptoms while subclinical mastitis was diagnosed California Mastitis Test (CMT) as discussed by Iqbal et al. (2006). All the milk samples positive for clinical or subclinical mastitis were collected in sterilized screw capped vials and transported to the Microbiology Laboratory, Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Lahore in a cooler to maintain temperature below 4°C. The samples were processed within 12 h after collection from the field.

Isolation and identification of S. aureus

Each of the collected samples was streaked on Staph-110 and sheep blood agar plates. Gram’s staining followed by a biochemical test, e.g., Catalase test, Coagulase test, Staphytect plus test and Mannitol fermentation were performed for the characterization of the S. aureus isolates. Pure cultures of S. aureus were preserved in 20% glycerol and kept at -80°C till further use.

DNA Extraction

After microbiological examination of Staphylococcus aureus, pure cultures were preserved in 20% glycerol in eppendrof tubes and freeze down at -80 °C freezer for further
investigations. Before DNA extraction freeze down samples were streaked on Sheep blood agar and after 24 hour incubation 4 to 5 fresh colonies of *Staphylococcus aureus* were dissolved in normal saline. DNA extraction was performed as prescribed by Genomic DNA Purification Kit (Thermo Scientific USA). For DNA extraction mixed 200 μl of sample with 400 μl of lysis solution and incubates at 65°C for 5 min. Then instantly add 600 μl of chloroform, gently emulsified by inversion and centrifuge the sample at 10,000 rpm for 2 min. Precipitation solution was prepared by mixing 720 μl of sterile deionized water with 80 μl of supply 10X concentrated precipitation solution. Transfer the upper aqueous DNA to a new tube and add 800 μl of freshly prepared precipitation solution, mixed gently by several inversions for 1-2 min and centrifuge at 10,000 rpm (~9400 x g) for 2 min. Supernatant discarded completely and dissolved DNA pellet in 100 μl of NaCl solution by vortexing gently. Add 300 μl of cold ethanol, let the DNA precipitate (10 min at -20°C) and spin down (10,000 rpm for 3-4 min). Removed ethanol and wash pellet once with 70% cold ethanol and dissolve DNA in 100 μl of sterile deionized water by vortexing.

**DNA Quantification and storage**

DNA quantification of the every sample was done with the help of Gel electrophoresis using 0.8 % Agarose gel and DNA ladder 100 bp (Fermentas USA). All extracted DNA samples were adjusted at same concentration level i.e.50 ng/μL. DNA quantification was also done with Nanodrop Thermo (Fermentas USA) for further confirmation. Extracted DNA was dissolved in nucleus free water and working aliquots were made while stock DNA samples were being stored at -20 ºC freezer.
Selection of Primers

Primers for gene coagulase and fibronectin binding protein A were synthesized from GeneWorks Australia as reported previously by Kumar et al. (2011).

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Primer Name</th>
<th>Nucl. No</th>
<th>Sequence (5'--------3')</th>
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<tbody>
<tr>
<td>1</td>
<td>Staph_Coag-F</td>
<td>22</td>
<td>AACAAAGCGGCCCATCATTAAG</td>
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<tr>
<td>2</td>
<td>Staph_Coag-R</td>
<td>23</td>
<td>TAAGAAATATGCTCCGATTGTCG</td>
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<tr>
<td>3</td>
<td>FBNA-F</td>
<td>17</td>
<td>GCGGAGATCAAAGACAA</td>
</tr>
<tr>
<td>4</td>
<td>FBNA-R</td>
<td>18</td>
<td>CCATCTATAGCTGTGTGG</td>
</tr>
</tbody>
</table>

Primers Optimization and PCR Amplification

PCR reactions were carried out in 25 μL reaction mixtures, using 50 ng of DNA, 10 mM of Tris HCl (pH 8.8 at 25 °C), 50 mM of KCl, 0.08%, MgCl2 (2 mM), 250 μM of each dNTPs, 0.2 μM of forward and reverse primers and 0.5 U of Taq DNA polymerase (Thermo Fisher Scientific Inc. USA). DNA isolated from pure culture of Staphylococcus aureus ATCC 25923 was taken as positive control while nuclease free water was taken as negative control. The PCR mixture without template was taken as PCR control to check for the possibility of any contamination.

DNA amplification was carried in thermocycler (Kyratec SC 200) with following cyclic conditions: Initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C and 57 °C for 30 sec for Coagulase and Fibronectin binding protein A gene, respectively. Then extension at 72 °C for 30 sec. A final extension at 72 °C was carried out for 5 min. All the PCR products were run on 1.2 % agarose gel stained with ethidium bromide and visualized under UV light in Gel Documentation System (BioRad USA) and gel photographs were saved for future reference.
Sequencing and Phylogenetic Analysis

Positive PCR products were precipitated with 70 % ethanol (80µL) and finally dissolved in 20 µL of double distilled deionized water. The representative samples of coa and fnbA (coa =11 and fnbA =07) gene were sent to Australian Genome Research Facility Ltd (AGRF), University of Adelaide for Sanger’s sequencing. Sequences were edited using Codon Code Aligner and highest similarities of coa gene with that of other strains were searched using the BLAST tool of GenBank, NCBI. (Woo et al. 2001). Phylogenetic trees of all sequences were constructed using MEGA6.1 by Neighbor Joining method (Sudhir and Sudhindra, 2000) with 1000 bootstrap values (Tamura et al. 2011). Alignments, pairwise distance estimations, and similarities/variations at nucleotides levels of all Pakistani sequences and those reported from NCBI GeneBank were also performed by using MEGA6.1 software.
RESULTS

Identification of *S. aureus* isolates from mastitic milk samples

A total of 4144 quarters from 1036 buffaloes was screened and 673 (106 clinical and 567 subclinical) milk samples from 508 buffaloes were examined for mastitis. Out of 106 clinical mastitis milk samples examined, 71 belonged to Lahore and 35 to Bhimber; whilst, out of 567 subclinical mastitis milk samples that were examined, 365 belonged to Lahore and 202 to Bhimber. Among these 673 milk samples, 437 isolates of Staphylococci were recovered. All these 437 isolates of Staphylococci were Gram positive, catalase positive and showed hemolytic property on 5% sheep blood agar. Out of these isolates, only 236 were confirmed coagulase positive *S. aureus*.

Amplification of *coa* and *FnbA* gene PCR Products

Among the total 236 *S. aureus* isolated, PCR was performed on 11 isolates. These 11 representative isolates were selected on from different location/sampling pockets. The results of amplification of *coa* gene has been shown in figure 5.1. The PCR products were categorized into five classes on the basis of their molecular size on electrophoresis of agarose gel. The approximately the product size was from 430 to 840 bp. But the majority of isolates having product size 575 bp. In case of *FnbA* gene all the strains having same product size 1150 bp on agarose gel.
Figure 5.1. Agarose gel electrophoresis of *coa* gene PCR amplification products. 100 bp DNA ladder, lane 1-2: 540 bp, lane 3,6: 550 bp, lane 4-5,10: 525 bp, lane 7: 430 bp and lane 9: 840.
Figure 5.2 Agarose gel electrophoresis of \( FnbA \) gene PCR amplification products. 100 bp plus DNA ladder, lane 1-10: 1150 bp.
Phylogenetic tree of coa gene of *S. aureus* isolates/strains from Pakistan and different geographical locations

Coagulase gene from *S. aureus* strains were amplified and PCR products of 430 to 840 bp were confirmed on agarose gel. PCR products were sequenced and nucleotide sequences were compared with other coagulase gene sequences in the GenBank. Phylogenetic tree was constructed by aligning coagulase gene sequences of the present study isolates with selected coagulase gene sequences in the GenBank. Phylogenetic tree of all Pakistani (n=11) sequences was constructed separately (Figure 5.3) and then combined with all other sequences (n=9) in the NCBI GenBank database. Phylogenetic tree showed that all Pakistani isolates were grouped together in two clades with high bootstrap values showing high genetic relatedness with some isolates from Japan (GenBank accession numbers AB373752 and AB373753), while some isolates from India (JX240355) and Taiwan (CP003603 and CP003166) were relatively distantly placed as compared to above mentioned Japanize isolates. Isolates from Germany (AJ306908), UK (FN433596 and BX571856) and USA (CP006044) were placed distantly as compared to isolates from Japan, India and Taiwan (Figure 5.4).

Phylogenetic tree of fnbA gene of *S. aureus* isolates/strains from Pakistan and different geographical locations

The isolated sequence was compared with other *fnbA* gene sequences found in NCBI GenBank. PCR product size was approximately 1150bp and phylogenetic tree was constructed by aligning *fnbA* gene sequences with *fnbA* gene sequences found in GenBank. Phylogenetic tree of all Pakistani (n= 7) sequences was constructed separately (Figure. 5.5) and combined with all other sequences founded from NCBI GenBank (n=10). Phylogenetic tree showed that all Pakistani isolates were grouped together in one clades with high bootstrap values showing closeness with
isolates from Switzerland (AM07599 and AM076025) and Ireland (AM749008 and AM749014) while isolates from Thailand (JN848748 and JN848745) and USA (JF809640 and JF809661) were placed distantly (Figure 5.6).

**Pairwise similarities and divergence among substitutions of Coagulase gene**

Table 5.3 shown the similarities and divergence among Pakistani and NCBI reported strains from the world. All the Pakistani strains of coagulase gene were 99.98-100% similar while their divergence was 0.02%. The Japanizes strains were close to Pakistan strains and their similarity and divergence was 99.97% and 0.03%, respectively. The Pakistani strains were 99.93% similar and 0.07% divergent from Indian and German strains. Similarly, the Pakistani strains were 99.94% similar and 0.06% were divergent from the UK and Taiwan reported strains.

**Pairwise similarities and divergence among substitutions of FnbA**

In table 5.4 the similarities and divergence among Pakistani and NCBI reported strains has been shown. All the Pakistani strains of FnbA were 99.96-100% similar while their divergence was 0.04%. The Switzerland strains were close to Pakistan strains and their similarity and divergence was 97.1% and 2.9%, respectively. The Pakistani strains were 96-97.2% similar and 2.8-3.2% divergent from strains reported from Ireland. The Pakistani strains were 91.3-91.8% similar and 8.2-8.7% were divergent from Thailand and USA reported strains.
Figure 5.3. *Coa* Phylogenetic tree (MEGA6.1, NJ method with 1000 bootstrap value) showing evolutionary relationship among all Pakistani strains.
Figure 5.4. Phylogenetic tree (MEGA6.1, NJ method with 1000 bootstrap value), based on coagulase gene sequences, showing evolutionary relationship of Pakistani strains/isolates with selected reference strains from different geographical locations. Pakistani strains/isolates have been indicated with closed triangles.
Figure 5.5. Fibronectin Binding Protein A, Phylogenetic tree (MEGA6.1, NJ method with 1000 bootstrap value) showing evolutionary relationship among all Pakistani strains.
Figure 5.6. Phylogenetic tree (MEGA6.1, NJ method with 1000 bootstrap value), based on \textit{Fnba} gene sequences, showing evolutionary relationship of Pakistani strains/isolates with selected reference strains from different geographical locations. Pakistani strains/isolates have been indicated with closed triangles.
Table 5.1. Coa Pairwise genetic distance among all Pakistani strains.

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### Table 5.2. *Fnba* Pairwise genetic distance among all Pakistani strains

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Table 5.3. Pairwise genetic distance relationship between isolated strains and some reference strains based on coagulase gene sequence.

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The number of base substitutions per site from averaging over all sequence pairs within each group is shown. The diagonal bold faced numbers show the mean inter populational evolutionary diversity estimates.
Table 5.4. Pairwise genetic distance relationship between isolated strains and some reference strains based on fibronectin binding protein A.

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The number of base substitutions per site from averaging over all sequence pairs within each group is shown. The diagonal bold faced numbers show the mean inter populational evolutionary diversity estimates.
DISCUSSION

Mastitis is common in dairy herds around the globe. It leads to economic losses and is a serious threat to animal and public health. Udder infection with *Staphylococcus aureus* considered to be the leading cause. Mastitis can be minimized by investigation of particular pathogens and their virulence factors involved in pathogenesis. The molecular characterization of pathogens is an essential part to track the spread of contagious infections from one region to others or among dairy herds (Khan et al. 2013). With the development of molecular techniques and their application, now it is possible to differentiate between pathogenic and nonpathogenic strains of *S. aureus*. Staphylococcus is a normal inhabitant of the skin and nails. The isolation of *Staphylococcus aureus* from milk alone is unequivocal to determine its role in the pathogenesis. *S. aureus* virulent gene surveillance could be helpful in detecting genetic diversity among these major mastitis causing pathogens. In the present study the identification of virulent strains coagulase and fibronectin binding protein A were targeted. Coagulase enzyme activity is the principle criteria for diagnosis of *S. aureus* from other coagulase negative *Staphylococcus* because coagulase can convert fibrinogen to fibrin.

In *S. aureus* identification, coagulase production (coagulase test) has been considered as an important criteria for phenotypic identification (Upadhyay et al. 2012; Gharib et al. 2013). In molecular investigation coagulase gene amplification has been considered as a more accurate method for *Staphylococcus aureus* typing. But in case of molecular identification, the presence of coagulase gene is highly polymorphic and does not give similar amplicons in amplification. This amplicon difference in coagulase gene size is due to that 3’ end contains a series of 81bp tandem repeats and number of which may differ between strains (Upadhyay et al. 2012; Goh et al. 1992). Coagulase gene consists of three distinct regions: (i) the N-terminus containing the prothrombin-
binding site, (ii) a central region which is highly conserved, and (iii) a C-terminal region, each encoding 27-amino acid residues (Janwithayanuchit et al. 2006).

The present study showed that prevalence of coagulase positive *S. aureus* was quite common in both localities studied (i.e. Lahore and Bhimber) and molecular characterization of *S. aureus coa* gene showed genetic variation. Similarly, prevalence of *S. aureus* mastitis in buffaloes is of great concern in Asian countries and these animals are major contributor to the milk industry (Aires-de-Sousa et al. 2007; El-Jakee et al. 2010). Present study agreed with previous study that PCR amplifications of *coa* gene, revealing extensive polymorphism with predominance of one or more of *coa* gene amplified products responsible for mastitis in buffaloes (Sindhu et al. 2010). The phenotypic and genotypic variation was observed in some of the cases. These observations are supported by previous reports as a mutation in the coding sequence or a plasmid number (Kumar et al. 2010). Virulence factor diversity and their various combinations might cause changes in the level of pathogenicity, and spreading of infections within and between animals (Fournier et al. 2008; Kumar et al. 2010). The end region of *coa* gene differs among *S. aureus* isolates, both in their number and in location (Goh et al. 1992), so the coagulase is an important feature used worldwide for epidemiological investigations of *S. aureus*. It is also the easiest gene to analyze polymorphism and generates multiple distinct polymorphism patterns.

The present study results showed heterogeneity in the *coa* gene of *S. aureus* strains in Pakistan and it was in accordance with the heterogeneity reported elsewhere in other strains of *S. aureus* (Aarestrup et al. 1995; Khan et al. 2013; Momtaz et al. 2011; Su et al. 2000). Less variation in *coa* gene of *S. aureus* was found in the present study, which agrees with Mork et al. (2005) that small number of closely related genotypes of *S. aureus* are responsible for mastitis in a locality. The coagulase gene is polymorphic and genetically variable and this polymorphism is due to multi
allelic forms at the 3' end of the gene, which differs in their sequence and restriction sites (Wisal et al. 2005). The PCR amplicons variation in size of coagulase gene could be due to polymorphism among different isolates obtained from different herds and previous studies have also confirmed PCR product variation using molecular analysis of the coagulase gene (Kalory et al. 2007; Reinoso et al. 2008; Khan et al. 2013); however, the reason of this polymorphism is still unknown (Saei et al. 2009). Present study agreed with the previous study conducted by Khan et al. (2013) in Pakistan that this polymorphism may be due to mutations by which different nucleotides are inserted or deleted particularly in the 3´ end the coagulase gene. The different coa gene products and their polymorphism for Staphylococcus aureus and it showed great genotypic variability among the organisms (Sanjiv et al. 2008).

In the present study, coagulase strains produced five types of amplicon (430, 525, 540, 550 and 840 bp) and only one was dominant (525 bp). These five types of amplicons are in accordance with findings of Sanjiv et al. (2008) reported that a single coa genotype was dominant from 5 genotypes and PCR amplification produced 400, 500, 600, 900 and 1000-bp amlicons with 600-bp being the most common in a geographical region. In the present study, PCR product size ranged from 430 to 840 bp which is close in size from PCR product variation (500 to 580 bp) reported by Hookey (1998), confirming the polymorphic nature of coagulase gene found in different strains of S. aureus. In 2004, similarly finding were observed by Salasia et al. in Germany and amplicons of 510, 600, 680, 740, or 850 bp size, but only one was dominant.

Previous studies suggested that mastitis is caused by S. aureus strains harboring more than one coa genotype and that only 1 or 2 genotypes were dominated (Aslantas et al. 2007; da Silva and da Silva, 2005; Nashwa et al. 2011). Momtaz et al. (2011) suggested that mastitis in cattle is
caused by certain strains of *S. aureus*, mostly carrying a coagulase gene, and this information might be helpful for control and management of mastitis caused by *S. aureus*.

**Fibronectin Binding Protein A**

In the present study, the molecular characterization of *Staphylococcus aureus fnbA* gene shows genetic variation and can be helpful in the reduction of mastitis and control of this disease. Some virulent factors are accountable for the adhesion and studies showed that the rate of infection by *fnbA* genes is 100% (Nashev et al. 2004). Fibronectin binding proteins play an important role in virulence action of *Staphylococcus aureus* infection (Foster and Hook, 1998; Aricola et al. 2005). *FnbA* genes 99% were detected by Mishaan et al. (2005) from orthopedic infections while in another study rate of *fnbA* gene infection was 76.1% (Zmantar et al. 2008). The genetic component, namely *fnbA*, is responsible for both clinical and subclinical mastitis. Moreover, the distribution of pathogenic characteristics is equally responsible for maintaining mastitis and this information provides clues for control of such harmful bacterial strains. (Kumar et al. 2011). The variability in phenotypic and genotypic patterns may be due to the different sites in which the *Staphylococcus aureus* is isolated such as clinical and subclinical mastitis milk.

In the present study, PCR product size was 1150bp which is close to 1279bp (Yapar and Avkan, 2011) and 1280bp Kumer et al. 2011 and it was different in size (191bp) Kouidhi et al. 2010 and 128bp (Atshan et al. 2012) that confirming the polymorphic nature of *fnbA* gene found in different strains of *Staphylococcus aureus*. The present study agreed with previous study conducted by Kumar et al. (2010) that animals infected with harmful strains could be helpful in the culling and control of mastitis. It could be recommended from this studies that healthy buffaloes must be milked first and those having mastitis milked at last or with a separate milking unit as it is suggested by Voelk et al. (2014). Similarly, other additional management practices
may be beneficial, such as treating or culling, biosecurity measures, wearing gloves, fore stripping, teat cleaning, and post milking teat disinfection.

The present study results showed heterogeneity in *Staphylococcus aureus* strains of Pakistan based on *fnbA* gene sequence and is an important factor in controlling human and bovine *Staphylococcus aureus* infections. This heterogeneity could cause mutations in gene of *S. aureus*. Similarly, heterologous expression of *fnbA* gene of *Staphylococcus aureus* has been reported by Que et al. (2001). Almost all strains of *Staphylococcus aureus* have fibronectin-binding proteins A encoded by *fnbA* gene (Jonsson et al. 1991). The evidence for the virulent nature of *fnbA* gene had been demonstrated by Kuypers and Proctor (1989). *Staphylococcus aureus fnbA* gene is the key factor for pathogenesis and because it can form biofilm and adhesion to host (Kouidhi et al. 2010; Palmqvist et al. 2005). Adhesion formation is considered as a major virulence factor (Vaudaux et al. 1990).

It is concluded from present study that sequence analysis of virulent genes of *S. aureus* is very important for epidemiological investigation of outbreak (mastitis). *S. aureus* isolates showed genetic diversity in their virulent genes, but only a few gene variants were predominant. The present study also revealed that the strain variation of *S. aureus* could be occurring within and between herds and also between different locations.
REFERENCES


Luong TT, Dunman PM, Murphy E, Projan SJ, Lee CY. 2006. Transcription profiling of the mgrA regulon in *Staphylococcus aureus*. J bacteriology. 188: 1899-1910.


(RAPD) and multilocus variable tandem repeat analysis (MLVA) for typing 


EXPERIMENT - 3


Mastitis is one of the commonest problem in dairy herds; responsible for toll of economic losses. The etiology is complex and considered to be the outcome of the interaction of various factors associated with the host, pathogens and the environment. The present study was carried out in two districts with different ecology i.e. Lahore and Bhimber. In the present study, prevalence of mastitis, its risk factors, *S. aureus* (most common pathogen of mastitis), antibiotic sensitivity and virulent factor coagulase and Fibronectin binding protein A were focused.

A cross sectional study was carried out between January to December 2012 to estimate the prevalence of mastitis and to investigate various epidemiological risk factors. A total of 1036 buffaloes was examined for mastitis. Of the population studied, the animals examined in district Lahore were 598 buffaloes belonging to 25 herds, while the remaining 438 were from 25 herds maintained in Bhimber district. The overall prevalence of mastitis was recorded 49%. The prevalence of mastitis in Lahore was 55.5%, while in district Bhimber it was 40.2%. Among mastitis cases the prevalence of clinical mastitis was 10.2% as a whole. However, the prevalence of clinical mastitis in district Lahore was 11.8%, whereas it was 8% in district Bhimber. The overall prevalence of subclinical mastitis was 38.8%. The district wise prevalence of subclinical mastitis was 43.6% and 32.2% in districts of Lahore and Bhimber, respectively. The overall prevalence of mastitis was 40.2%, 46.5% and 52.6% in small, medium and large herds, respectively. The quarter level prevalence was recorded as 16.20% (673/4144 quarters from 1036 animals). The district wise prevalence of mastitis at quarter level was recorded 18.17% in district Lahore while it was 12.87% in district Bhimber. The buffaloes having mastitis in one quarter, two quarters, three quarters and four quarters were 78.5%, 12%, 8% and 1.5%, respectively. The
prevalence of mastitis was increased as the age of buffaloes increased. The prevalence of mastitis was highest in Nili Ravi 52.2% as compared to Kundhi and non-descriptive 40% and 44.7%, respectively.

In Univariable analysis: area, herd size, age, breed, parity, stage of lactation, reproductive disorders, udder shape, previous exposure to mastitis, ease of milking, udder edema, lesion on udder/teat, swelling on udder/teat, condition of floor, frequency of dung removal, exposure to FMD, udder wash, teat dipping, milking techniques and tick infestation were significantly associated ($P < 0.25$) with a prevalence of mastitis. These 20 significant variables were further selected for multivariable analysis to estimate the independence of the effect of the variables. The final models identified were area, age, breed, parity, stage of lactation, udder shape, ease of milking, previous exposure to mastitis, udder edema, lesions on udder/teat, exposure to FMD, teat dipping and tick infestation were significantly associated ($p< 0.05$) with a prevalence of mastitis.

A total of 673 mastitic quarter milk samples from different buffalo quarters were collected from different livestock farms having 106 and 567 clinical and subclinical mastitis, respectively. These milk samples were subjected to microbiological examination. From these milk samples, 236 isolates were confirmed as coagulase positive $S.\text{ aureus}$. The sensitivity of gentamycin, oxytetracycline, erythromycin, kanamycin, chloramphenicol, tylosin, ciprofloxacin, doxycycline, amoxicillin, streptomycin and trimethoprim to $S.\text{ aureus}$ was 80.1%, 75.4%, 74.6%, 73.3%, 72.4%, 71.6%, 69.9%, 69.1%, 63.2%, 56% and 54.7%, respectively. While on the other hand the resistant pattern of gentamycin, oxytetracycline, erythromycin, kanamycin, chloramphenicol, tylosin, ciprofloxacin, doxycycline, amoxicillin, streptomycin and trimethoprim was 11%, 19.9%, 15.3%, 20.8%, 20.8%, 28.4%, 22.9%, 17.3%, 36.8%, 34.3% and 45.3%, respectively.
The identification of virulence factors such as the *coa* and *fnbA* is necessary for minimizing and control strategies of mastitis. The virulent genes were amplified by using gene specific primers. In case of *coa* gene the PCR amplicon products were categorized into five classes on the basis of their molecular size on electrophoresis of agarose gel. Approximately the product size was from 430 to 840 bp. But, the majority of isolates were having product size 575 bp. In case of *fnbA* gene all the strains having same product size 1150 bp on agarose gel. The PCR products were sent to Australian Genome Research Facility Ltd, University of Adelaide for sequencing. In phylogenetic analysis it revealed that the *coa* gene of *S. aureus* strains, isolated were grouped in two clades which were closely related to *S. aureus* isolates from Japan, India and Taiwan, while *S. aureus* isolates from Germany, UK and Canada were distantly related. These findings will be helpful in future for mastitis control strategies. In case of *fnbA* gene sequences showed, that all Pakistani isolates were grouped in one clades with high boot strap values showing closeness with isolates from Switzerland and Ireland. While isolates from Thailand and USA were placed distantly.

The pairwise distance of *coa* showed that similarities and divergence showed that all the Pakistani strains of coagulase gene were 99.98-100% similar while their divergence was 0.02%. The Japanizes strains were close to Pakistan strains and their similarity and divergence was 99.97% and 0.03%, respectively. The Pakistani strains were 99.93% similar and 0.07% divergent from Indian and German strains. Similarly, Pakistani strains were 99.94% similar and 0.06% were divergent from the UK and Taiwan reported strains. The pairwise distance of *fnbA* showed that similarities and divergence showed that all the Pakistani strains of *fnbA* were 99.96-100% similar while their divergence was 0.04%. The Switzerland strains were close to Pakistan strains and their similarity and divergence was 97.1% and 2.9%, respectively. The Pakistani strains were 96-97.2%
similar and 2.8-3.2% divergent from strains reported from Ireland. The Pakistani strains were 91.3-91.8% similar and 8.2-8.7% were divergent from Thailand and USA reported strains.

Mastitis can be minimized by improving the management and animal related these potential risk factors. Screening of lactating buffaloes should be done on a regular basis. Pre and post teat dipping is recommended. Wear the gloves or hand wash before and after milking. The mastitis treatment should be started after antibiotic sensitivity and select the appropriate antibiotic. These molecular information of virulent genes of *S. aureus* could be helpful in the control of mastitis and development of vaccines.