CHARACTERIZATION AND ANTIBIOGRAM OF SELECTIVE PATHOGENS IN PNEUMO-ENTERITIC GOATS

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Roll No. 0214/03

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DEDICATED TO MY BELOVED FAMILY AND TEACHERS

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The Author

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List of Abbreviation

Abbreviation	Elaboration
%	Percentage
<	Less than
>	Greater than
°C	Degree Celsius
≥	Greater than equal to
≤	Greater than equal to
µl	Microlitre
BaCl2	Barium Choloride
Bp	Base pair
BPW	Buffered Peptone water
BBA	Blood base agar
BGA	Brilliant green agar
CLSI	Clinical laboratory and Standard Institute
Ctg	Chittagong
CI	Confidence interval
CVASU	Chittagong Veterinary and Animal Sciences University
DNA	Deoxyribo Nucleic Acid
e.g.	Example
EDTA	Ethylene Di amine tetra acetic acid
EMB	Eosin methylene blue
et al.	And his associates
etc.	Et cetera
Gm	Gram
H_2SO_4	Sulphuric acid
i.e.	That is
Kb	Kilo base
LPS	Lipopolysaccharide
mL	Milli Litre
Mm	Milli miter

MS	Master of Science
MSA	Mannitol salt agar
MR	Methyl red
MRSA	Methicillin Resistant *Staphylococcus aureus*
Mbp	Mega base pairs
NPM	Naso Pharyngeal mucosae
PRTC	Poultry Research and Training Centre
RAJ	Recto-Anal Junction
Temp.	Temperature
TSI	Triple sugar iron
XLD	Xylose lysine deoxycholate agar

Abstract

Pneumonia and enteritis are still the most common and costly diseases affecting small ruminants. Indiscriminate use of antibiotics in animals against bacterial infections has led to emergence of multidrug-resistant strains. The sensitivity and resistance of antimicrobials to different drug with associate risk factors in pneumoenteritic goats is crucial to investigate to ensure effective treatment as well as taking necessary steps for minimizing the antimicrobial resistance. Hence, the cross sectional study was conducted to characterize the selective pathogens of pneumo-enteritis in goat along with their antibiogram during the period of July- December 2015. A pre-tested questionnaire was used for farm level risk factor analysis. One hundred and fifty goats (n=150) were sampled having pneumonia and/or enteritis. Both nasal and fecal swabs were collected from each goat and the samples were subjected to culturing using a series of agar and broth. Biochemical tests were done for *Staphylococcus aureus* and *Salmonella sp;* and finally PCR was done for *Salmonella sp* targeting TetA and blaTEM gene. The prevalence of *Staphylococccus spp* in pneumoenteritic goats was 28%. Among the *Staphylococcus spp*, the coagulase positive Staphylococcus was 43% whereas the coagulase negative *Staphylococcus aureus* was 57%. The prevalence of *Salmonella sp* was 16.7% in pneumoenteritic goats. The isolated *Salmonella sp* containing TetA gene were 50% of the total isolates whereas the *Salmonella sp* containing blaTEM gene were 72% of the isolates. However, both the Salmonella and Staphylococcus were highly prevalent in pneumoenteritic goats and were resistant to most of the antibiotics used except cefotaxime and potentiated sulphonamides (Sulfamethoxazole and Trimethoprim).

Keywords: *Isolation, Staphylococcus sp, Salmonella sp, Pneumoenteritis, goat, Antibiogram*

Chapter-1: Introduction

Chapter-1: Introduction

Goat is an economically important livestock in many parts of the world, where it is kept as a source of meat, milk and fiber. Among the notable diseases of goat, pneumonia and enteritis are considered as common respiratory and gut associated illness throughout the world (Daniel et al., 2006).The pneumonia related diseases of goat have long been recognized as one of the most important health problems with zoonotic importance associated with goat and sheep-raising throughout the world (Afolabi et al., 2012) and it is noted as most common goat diseases in the world. One of the diseases affecting goat production in Bangladesh is pneumoenteritis being catarrhal enteritis associated with pneumonia. It is caused primarily by virus with predisposing factors like cold, worms and stress although secondary bacterial infection is likely to be involved. Amongst all the respiratory diseases, pneumonia is one of the major problems in goat characterized by fever, anorexia, painful coughing, dyspnea, mucopurulent nasal discharge and depression (Daniel et al., 2006). The bacteria involved in enteritis is yet to discover and unknown. However, some associated bacterial agents are likely to be involved with goat rearing and management system which is not published yet in any part of the world even in Bangladesh.

Disease outbreaks resulting from food borne microbial pathogens are a major public health concern and result in economic loss for the food industry (Ellingson et al. 2004; Hein et al. 2006). Salmonellosis and both coagulase positive and negative staphylococci in human is one of the leading causes of food borne bacterial enteritis in many countries (Tirado and Schmidt 2001; Côté et al., 2004; Klerks et al., 2004). *Salmonella spp* are widely distributed in nature, gaining entry to almost all aspects of the human food chain.

In recent years, many attentions have been focused on goat pneumonia in Bangladesh. Large number of goat population die each year due to pneumonia at the early stage of their lives (Asaduzzaman et al., 2013). Though it caused death of juvenile goat, it has also great influence on adult. Some study revealed that small kids are more prone to be affected by the pneumonia causing pathogen though it likely to depends on seasonal variation and rearing management. There are several, well recognized

infectious species of the organisms in goats causing high morbidity and mortality of adult animals and their young offspring (Momin et al., 2011).

Many bacterial agents colonizes in the mucosa of the upper respiratory tract and nasopharynx, breach the innate mucosal defense, including the mucociliary apparatus and antimicrobial factors through releasing different types of toxic substances that establish infection in lung (Ackermann and Brogden, 2000).

Respiratory tract pathogens include *Staphylococcus sp, Escherichia coli, Bacillus sp* etc. Beside these there are several other organisms reported for the occurrence of pneumonia in goats such as *Actinomyces pyogenes, Arcanobacterium pyogenes, Neisseria catarrhalis* and *Proteus vulgaris, Pasteurella multocida* (Obasi et al., 2001; Hutt and Goossens, 2001; Ozbey and Muz, 2004; Shafarin et al., 2007), *Streptococcus pyogenes* (Obasi et al., 2001); *Mycoplasma capricolum* (Ostrowski et al., 2011). Bacterial enteritis is also considered as an inflammation of the intestines caused mainly by pathogenic strains of *staphylococcus sp and Salmonella sp* (Meshram et al., 2009). Enteric disease, often presenting as a bloody or profuse watery diarrhea with pyrexia, is the commonest clinical manifestation, but a wide range of clinical signs, which include acute septicemia, abortion, arthritis, necrosis of extremities and respiratory disease may also be seen (Meshram et al., 2009).

Two types of *staphylococcus sp* coagulase negative and positive are likely to be associated in the mucosal infection and diarrhea. It's directly correlated with the meat production, growth performance, reproduction and public health. *Salmonella enteritidis* produces enterotoxins which are invasive to cause inflammatory change within the intestine leading to diarrhea. Bacterial enteritis remains the most common clinical problem in the Goats (Meshram et al., 2009). Despite improvement in management practices, and prevention and treatment strategies, pneumonia and enteritis are still the most common and costly diseases affecting small ruminants. Indiscriminate use and misuse of antibiotics in animals against bacterial infections has led to emergence of multidrug-resistant strains.

It is of utmost important to know the sensitivity and resistance of antimicrobials to different drug with associate risk factors in pneumoenteritic goats to ensure effective

treatment as well as taking necessary steps for minimizing the antimicrobial resistance.

The most causative agents of pneumoenteritis in goats are quite necessary to explore in order to the proper control and identifying associated risk factors for its prevention. Among the most common agents *Staphylococcus sp* is the prime important to causing pneumo-enteric infections. As far I know, the characterization of this important pathogen from pneumo-enteritis has not yet been done.

In this context, the present study was conducted to characterize the selective pathogens of pneumo-enteritis in goat along with their antibiogram.

Objectives:

1. To isolate and identify the presence of selective bacterial pathogens in pneumoenteritic goats.
2. To determine antibiogram pattern of *Staphylococcus spp*
3. To determine the presence of genes implicated in the resistance to β-lactams (blaTEM), Tetracycline (tetA) of *Salmonella*.

Chapter-2: Review of literature

Chapter-2: Review of literature

Literature related to antimicrobial sensitivity and resistant to selective pathogens and their characterization and possible public health risk (s) are reviewed in this chapter. The key purpose of this chapter is to provide latest information regarding the research work which is addressed here.

2.1. Pneumoenteritis

Pneumoenteritis is one of the diseases mostly affecting goat production in Bangladesh is being catarrhal enteritis associated with pneumonia. It is caused primarily by virus with predisposing factors like cold, worms and stress although secondary bacterial infection is likely to be involved. Amongst all the respiratory diseases, pneumonia is one of the major problems in goat characterized by fever, anorexia, painful coughing, dyspnea, mucopurulent nasal discharge and depression.

Viral, bacterial, fungal and parasitic infections break down tissue defense barriers of the digestive and respiratory tracts resulting in loss of natural defense mechanism of the host animals. All these increase the production cost associated with expensive treatments. Pneumonia occurs when the lungs of goats become inflamed by infectious and non-infectious agents. The most frequent causes of respiratory infection have been associated with the invasion of various bacterial species (Gonçalves et al., 2010). Most of the infectious agents causing respiratory disease are ubiquitous in nature and commonly nasopharynx of different animals is their normal habitation. However, when the local resistance of respiratory mucus is lowered, bacteria growing in the nose and throat extend down wards, producing multiple bacterial infections including *Staphylococcus sp, Escherichia coli, Bacillus sp, Mannheimia haemolytica, Actinomyces pyogenes and several Mycoplasma species* lead to enzootic or primary pneumonia in small ruminants, although their pathogenic effects are prominent when they are infected with viruses (Radostits et al., 2007; Omer et al., 2012).

Bacterial enteritis is the most important cause of diarrhea in lambs and goat kids. The strategies followed by these bacteria to cause infection and disease generally include an interactive group of virulence determinants, sometimes co-regulated, which are suited for the interaction of a particular bacterium with a specific host. Bacterial

pathogens have evolved numerous strategies to exploit their host's cellular processes so that they can survive and persist in the host animal (Sausa, 2006). Despite the fact that most of the bacteria usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, even normal “nonpathogenic” strains can cause debilitating and sometimes fatal diseases in animals, birds as well as in human (Jafari et al., 2012). Many bacterial pathogens have been implicated in goat enteritis. The most common bacterial causes of enteritis in goats include *Escherichia coli, Salmonella sp, Clostridium sp, Bacillus sp* (Meshram et al., 2009). Systemic infection caused by various enteric bacteria characterized by fever, anorexia, and weakness, followed by coma and death. Shiga-like toxins produced by *E. coli* damage the endothelial cells in the kidneys, pancreas, brain, and other organs, thus inhibiting those organs ability to function (Abdullah et al., 2010).

2.2. Effects of pneumoenteritis

The pneumonia related diseases of goat have long been recognized as one of the most important health problems with zoonotic importance associated with goat and sheep-raising throughout the world. Disease outbreaks resulting from food borne microbial pathogens are a major public health concern and result in economic loss for the food industry (Ellingson et al., 2004; Hein et al., 2006). Salmonellosis and staphylococci in human is one of the leading causes of foodborne bacterial enteritis in many countries (Tirado and Schmidt, 2001; Côté et al., 2004; Klerks et al., 2004). Klerks *Salmonellas* are widely distributed in nature, gaining entry to almost all aspects of the human food chain.

For this reason, the real-time PCR has become a powerful tool for the detection of food borne pathogens. PCR is a rapid, sensitive and specific assay for the detection of food borne pathogens such as *Salmonella spp.* (Bohaychuk et al., 2007; Eyigor et al., 2010; Kurowski et al., 2002; Malorney et al., 2004).

This technique would be a highly valuable tool for the rapid identification of *Salmonella* reservoirs in goat herds, especially when questions such as the *Salmonella* freedom of animal groups or the efficacy of cleaning and disinfection procedures need to be tested in a very short period of time. However, all PCR test for *Salmonella spp.*

have been developed for food (very little other bacterial contamination) and most of the studies are based on artificial contaminated samples (Ellingson et al., 2004; Fey et al., 2004; Hein et al., 2006; Klerks et al., 2004; Malorny et al., 2004).

2.3. *Staphylococcus sp*

2.3.1. *Staphylococcus aureus*

Staphylococcus aureus was discovered in Aberdeen, Scotland in 1880 by the Surgeon Sir Alexander Ogston in pus from surgical abscesses (Ogoston, 1984), abbreviated to *S. aureus* or *Staph aureus* in medical literature. *Staphylococcus aureus* is a Gram-positive spherical bacterium approximately 1 μm in diameter. Its cells form grape-like clusters, since cell division takes place in more than one plane. On a rich medium, *S. aureus* forms medium sized "golden" colonies. On sheep blood agar plates, colonies of *S. aureus* often cause β-hemolysis (Ryan and Ray, 2004). The golden pigmentation of *S. aureus* colonies is caused by the presence of carotenoids and has been reported to be a virulence factor protecting the pathogen against oxidants produced by the immune system (Liu et al., 2005). Staphyloccoci are facultative anaerobes capable of generating energy by aerobic respiration, and by fermentation which yields mainly lactic acid. *Staphylococcus sp.* is catalase-positive, a feature differentiating them from *Streptococcus sp.*,and they are oxidase-negative and require complex nutrients, e.g., many amino acids and vitamins B, for growth. *S. aureus* is very tolerant of high concentrations of sodium chloride, up to 1.7 molar. Another feature of the Staphylococcus genus is the cell wall peptidoglycan structure that contains multiple glycine residues in the crossbridge which causes susceptibility to lysostaphin (Crossley and Archer, 1997). *S. aureus* produces coagulase which interacts with prothrombin in the blood causing plasma to coagulate by converting fibrinogen into fibrin. Blood coagulation is used to distinguish *S. aureus* from others members of the genus, which are collectively designated as coagulase-negative staphylococci (Ryan and Ray, 2004). Since it is a frequent etiological agent of human diseases and exhibits resistance to a growing number of therapeutic agents, *S. aureus* is also one of the most intensively studied bacterial species (Williams et al., 1959).

2.3.2. Cell wall structure

The staphylococcal cell wall is a dynamic, semi-rigid structure. It is composed of three components: peptidoglycan, teichoic acids and surface proteins. Of these three

constituents, peptidoglycan is the major component which builds the murein sacculus. Structurally, peptidoglycan forms a macromolecular net in which glycan strands are cross-linked by short peptides. The glycan strands are composed of repeating disaccharide units of β-1-4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid (Labischinski, 1992). The majority of glycan chains have a length of 3–10 disaccharide units. Extending from the carboxyl residue of the β-1-4 linked acetylglucos amine moiety is the stem peptide with the sequence: l-alanyl-d-isoglutaminyl-l-lysyl-d-alanyl-d-alanine (Scheffers and Pinho, 2005). A series of five l-glycine residues are attached to the l-lysine of the stem peptide which is a characteristic feature of the *S. aureus* cell wall. The pentaglycine cross-bridge is synthesized in a sequential manner by a family of FemABX non-ribosomal peptide transferases (Rohrer et al., 2003).

Another component of the *S. aureus* cell wall is teichoic acids, which are polymers of ribitol residues or polymers of glycerol phosphate. Teichoic acids contribute to the negative charge present on the cell surface that plays a role in acquisition of ions, also and have been reported to be a component of *S.aureus* phage receptor (Chatterjee, 1969).

The last component of the *S. aureus* cell wall is surface proteins, including microbial surface components recognizing adhesive molecules (MSCRAMMs). These proteins contain a signal sequence directing their secretion and the LPXTG motif. The LPXTG motif is cleaved by sortase and then the proteins are covalently attached to the peptidoglycan. Protein A, fibronectin binding protein, collagen binding protein and clumping factor A, among many others, are components of the cell wall and are attached in this manner (Foster and Hook, 1998).

2.3.3. *Staphylococcal* surface protein

The ability of *Staphylococcus sp* to cause infection is due to cell surface-associated proteins that mediate attachment to the host extracellular matrix termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Clumping factors A and B (ClfA and ClfB), protein A and the fibronectin binding proteins A and B (FnBPA and FnBPB) are characterized by an N-terminal signal sequence for Sec dependent secretion and a C-terminal cell wall-anchoring domain for covalent linkage to the cell wall. The ability of Staphylococcus sp to colonize the nasal

epithelium is in part due to the MSCRAMMs ClfB (O'Brien et al., 2002) and IsdA (Clarke et al., 2006), which promote adhesion to desquamated epithelial cells.

2.3.4. Pathogenesis

Staphylococcus sp is a ubiquitous commensal bacterium on skins and anterior nares, but frequently causes severe infections. Rapid and direct identification of *Staphylococcus sp* is crucial for proper management of patients with skin infections, abscesses, septicemia/bacteremia, gastroenteritis, endocarditis, toxic shock syndrome and certain food intoxications (Kateete et al., 2010). *Staphylococcal* food poisoning includes symptoms such as sudden onset of nausea, vomiting, abdominal cramps and diarrhea (Balaban and Rasooly, 2000). On heating at normal cooking temperature, the bacteria may be killed but the toxins remains active (Presscott et al., 2002). *Staphylococcal* enterotoxins are highly heat resistant and are thought to be more heat resistant in foodstuffs than in a laboratory culture medium (Bergdoll, 1983). Besides these, enterotoxins producing *Staphylococcus sp* are the most dangerous and harmful for the human health. About 50 % strain of this organism are able to produce enterotoxins associated with food poisoning (Payne and Wood, 1974). Illness through *Staphylococcus sp* range from minor skin infection such as pimples, boils, cellulites, toxic shock syndrome, impetigo, and abscesses to life threatening disease such as pneumonia, meningitis, endocarditis, and septicaemia (Soomro et al., 2003).

2.3.5. Virulence factors of *Staphylococcus sp*

2.3.5.1. Virulence factors

S. aureus is equipped with a great variety of virulence factors, which include both structural and secreted products participating in the pathogenesis of infection. The virulence factors of *S. aureus* with their function are presented in the following table.

Table 2.1: The virulence factors of *S. aureus* with their function

Factors	Function
Attachment improving agents	
• **"Microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs)**	• Attachment to host tissues and initiate colonization leading to an infection (Gordon & Lowy, 2008).
• **Fibronectin binding proteins A and B (FnbpA and FnbpB)**	• Attachment of bacterial cells to an extra-cellular matrix component, fibronectin, and to plasma clot (Switalski et al., 1993)
• **Collagen binding protein, Cna**	• Adherence of *S. aureus* to collagenous tissues and cartilage (Switalski *et al.*, 1993)
• **Clumping factor A and B (ClfA and ClfB)**	• Adherence of bacterial cells to fibrinogen, wound and foreign body infections (Foster & Hook, 1998).
• **Plasma-sensitive surface protein (Pls)**	• Binding to both fibrinogen and fibronectin (Hauck & Ohlsen, 2006)
• **Protein A**	• Binds IgG in "wrong orientation" on the surface of *S. aureus* cells disrupt opsonization and phagocytosis (Switalski et al., 1993)
Exotoxins	
• **Alpha-hemolysin**	• Osmotic cytolysis Cytolysis of human platelets and monocytes (Menestrina et al., 2001)
• **Panton-Valentine leukocidin (PVL)**	• Associated with (CA-MRSA) (Foster, 2005).
• **Gammahemolysin (Hlg)**	• Cytotoxic to erythrocytes (Kaneko & Kamio, 2004)
• **Leukocidin (Luk)**	• Cytotoxic to leucocytes (Kaneko & Kamio, 2004)
Superantigen toxin	
• **Enterotoxins A, B, C, D, E, G, Q**	• Gastroenteritis (Baker & Acharya, 2004)
• **TSST-1**	• Toxic shock syndrome (Baker & Acharya , 2004)
Regulators of virulence	
• **(*agr*) (Novick & Geisinger, 2008)**	• Affects lipase and protease production
• ***icaADBC* genes (Rohde *et al.*, 2001)**	• Affects biofilm formation
• ***icaR***	• Enhance regulatory effect of *ica* gene
• ***seb, tsst, ear* (Gill *et al.*, 2005)**	• Expression of enterotoxins
• ***tsst* (Gill *et al.*, 2005)**	• Expression of TSST-1

2.3.6. Diseases in humans associated with *S. aureus*

S. aureus is a commensal and a pathogen. The anterior nares are the major site of colonization in humans. About 20–30% of individuals are persistent carriers of *S. aureus*, which means they are always colonized by this bacterium, and 30% are intermittent carriers (colonized transiently) (Wertheim et al., 2005). Colonization significantly increases the risk of infections since it provides a reservoir of the pathogen from which bacteria are introduced when host defense is compromised (Kluytmans et al., 1997). Patients with *S. aureus* infections are usually infected with the same strain that they carry as a commensal (Williams et al., 1959). *S. aureus* is one of the main causes of hospital-and community-acquired infections which can result in serious consequences (Diekema et al., 2001). Nosocomial *S. aureus* infections affect the bloodstream, skin, soft tissues and lower respiratory tracts. *S. aureus* can be a cause of centralvenouscatheter-associated bacteremia and ventilator-assisted pneumonia. It also causes serious deep-seated infections, such as endocarditis and osteomyelitis. Hospitalized patients are particularly exposed to *S. aureus* infections due to their compromised immune system and frequent catheter insertions and injections (Lindsay & Holden, 2004). The SENTRY Surveillance Program investigating worldwide *S. aureus* infections during a two-year period has revealed that this pathogen is the leading cause of bloodstream, lower respiratory tract and skin/soft tissues infections in all regions surveyed (Diekema et al., 2001).

2.3.7. Animal infections by *Staphylococcus sp*

Infections due to *Staphylococcus sp* have been reported in many mammal species as well as in wild and domestic birds and in some reptiles. Some animals are asymptomatic while others suffer from respiratory, gastrointestinal, or skin and soft tissue infections. *Staphylococcus sp* is a significant cause of mastitis in cows and small ruminants (Vanderhaeghen et al., 2010). Whether animals can be persistent carriers of *Staphylococcus sp*in a manner similar to humans has yet to be determined. However, animals can intermittently harbor *Staphylococcus sp*. A recent study found that 10% of healthy dogs visiting a clinic for regular vaccinations harbored *Staphylococcus sp* (Rubin and Chirino-Trejo, 2010). Molecular analyses of isolates from different animals have revealed that there are some strains that appear to be host-adapted to a particular animal species (horses, cattle, pigs, sheep, chickens, or humans) and other strains can colonize multiple species of animals (Cuny et al.,

2010). *Staphylococcus sp* can be transferred between humans and animals, and frequently infections in companion animals can be traced back to their human caretakers (Rutland, 2009).

2.3.8. Prevalence of *Staphylococcus spp*

Although it is widely accepted that small ruminants can carry numerous zoonotic agents, it is unknown which of these agents actually occur in sheep and goats. One study conducted in Germany and sampled feces and nasal liquid of 48 animals (28 goats, 20 sheep). The presence of *Staphylococcus sp*in 75% of both sheep and goats could be demonstrated (Anna-Katarina Schillinga et al., 2012). Another study was conducted among animals in Qassim region, a total of 400 samples were collected from camels, sheep, cows, and goats. From 334 Staphylococci recovered 158 (47.3%) were coagulase positive *Staphylococcus sp*, among them 90 (57%) were MRSA and 68 (43%) were methicillin-sensitive *Staphylococcus aureus* (MSSA). The reaming strains 176 (52.7%) were coagulase negative *Staphylococcus sp*, including 32 (18.2%) were methicillin resistant coagulase negative Staphylococcus and 144 (81.8%) were methicillin-sensitive coagulase negative *Staphylococcus sp* (Alzohairy, 2011). The nasal swab from each goat was analyzed using standard methods in West Africa. Three hundred and twenty-eight isolates were obtained. The most frequently isolated species was *Streptococcus sp*, while *Escherichia coli* and *Staphylococcus sp* were the second dominant bacteria. Other species were isolated at relatively lower rates (Emikpe et al., 2009).

2.3.9. Antibiotic resistant genes in *Staphylococcus sp*

The resistance to antimicrobial agents is an increasingly global problem worldwide, especially among nosocomial pathogens. Staphylococci have become one of the most common causes of nosocomial infections. Multidrug-resistant staphylococci pose a growing problem for human health. The rise of drug-resistant virulent strains of *Staphylococcus sp*, particularly methicillin-resistant *Staphylococcus sp*(MRSA) is a serious problem in the treatment and control of staphylococcal infections (Duran et al., 2012). Methicillin resistance is defined as the strains of *Staphylococcus sp* that are resistant to the isoxazoylpenicillins such as methicillin, oxacillin and flucloxacillin. MRSA are cross-resistant to all currently licensed β-lactam antibiotics (Loomba et al., 2010).

2.3.10. Screening of *Staphylococcus spp* by various cultural and biochemical tests

Phenotypic tests are the mainstay in the diagnosis of staphylococcal infections, in which coagulase tests are usually confirmatory for Staphylococcus sp (Mugalu et al., 2006). Coagulase testing is performed using the slide coagulase (SCT) or the tube coagulase (TCT) methods (Koneman et al., 1997). Although these tests efficiently identify *Staphylococcus sp*, their performances vary from setting to setting and need improvement (Bello et al., 2006). Several laboratories in developing countries screen for presumptive *Staphylococcus sp* based on growth on Mannitol salt agar (MSA) and confirmation is done with the TCT. In many settings, the use of the TCT is curtailed by reliance on human plasma, since the recommended plasmas (from rabbit, horse) are either expensive or if locally available, are of poor quality. Although coagulase tests are invaluable for identification of *Staphylococcus sp*, few studies have evaluated their use in routine practice (Kateete et al., 2010). Catalase test was performed following Cowan (1985) and was used to differentiate bacteria, which produce the enzyme catalase, such as Staphylococci, from that non-catalase one such as, Streptococci. This test was done for evaluation of gas bubbles of Hydrogen peroxide. In addition, diagnostic laboratories are occasionally faced with organisms with biochemical characteristics that do not fit into the patterns of a known genus and species (Woo et al., 2001). Mannitol salt agar was developed for the presumptive isolation of *Staphylococcus sp* in a single step, which is convenient for diagnostic laboratories (Kateete et al., 2010). Furthermore, Mannitol negative MRSA (Methicilin Resistant Staphylococcus sp) was reported from clinical specimens in Kwazulu Natal province, South Africa (Shittu et al., 2007). El-Jakee et al., (2008) characterized and identified 78 *S. aureus* isolates secured from different animals and human origins using the most important conventional biochemical tests as anaerobic glucose fermentation, catalase, coagulase, acetone production, novobiocin sensitivity and mannitol fermentation. In view of the above, the common identification methods for *Staphylococcus sp* were evaluated, aiming at improving the diagnosis of *Staphylococcus sp* through a combination of available phenotypic methods.

2.3.11. Antibiotic susceptibility testing

The disk diffusion test, which is also known as the Kirby-Bauer test, is the most commonly used method for susceptibility testing (Reese et al., 1996). The entire surface of agar media, usually Mueller Hinton agar, is inoculated with a standardized

amount of bacteria. Next, paper disks impregnated with standardized quantities of antimicrobial agents are placed on the agar surface and the plates are incubated for 24 hours at 35°C. During the incubation time, the antimicrobial agents diffuse from the disk into the agar. If the organism is susceptible (killed or growth inhibited) to the antimicrobial agent, a clear zone (inhibited growth of tested bacteria) around the antimicrobial disk will appear. The diameters of the zones of inhibition are measured and compared to interpretive break point values published by CLSI which assign a value of "susceptible", "intermediate" or "resistant" to the isolate. (Adamu et al., 2010) conducted an study where he found that the isolates were highly susceptible to Ciproxin (91.1%), Norfloxacin (90.2%), Rifampicin (73.2%), Streptomycin (72.3%), Erythromycin (71.4%), Norbactin (64.3%) and moderately susceptible to Peflacin (57.4%), Gentamycin (51.8%), Lincocin (50.9%), Chloramphenicol (42.0%) but showed resistance to Ceftazimide (7.1%), Cefotaxime (14.3%) and Ampiclox (31.3%). In another study staphylococcal isolates showed moderate resistance to amoxicillin-clavulanic acid (28.9%), and trimethoprim-sulphamethoxazole (32.2%) and susceptible to vancomycin (Duran et al., 2012).

2.3.12. Coagulase positive versus coagulase negative *Staphylococcus spp*

Coagulase positive staphylococci, other than *S. aureus*, can cause infections in humans and animals. Some veterinary isolates of coagulase positive staphylococci are classified in the *S. intermedius* group (SIG). Recent molecular analyses demonstrated that bisolates of *S. intermedius* detected in a large number of different animals and geographic locations have some significant differences and the species can best be reclassified into three clusters: *S intermedius, S. pseud**o**intermedius*, and *S. delphini* A and B. These three species constitute the *S. intermedius* group (SIG) (Sasaki et al., 2007).

S. intermedius group pathogens produce a number of virulence factors (coagulase, hemolysins, exfoliative toxin and others) similar to those associated with *S.aureus* (Fitzgerald 2009; Iyori et al., 2010).When animals are injured, sick, or otherwise weakened, these bacteria may cause skin, ear, and wound infections (Weese and Duijkeren, 2010). Some SIG isolates also produce enterotoxins and could potentially cause food borne intoxication. One foodborne outbreak in southwestern U.S. in 1991 affecting over 265 people was traced to *S. intermedius* producing type-A enterotoxin in a butter blend (Khambaty et al., 1994).

Compared to coagulase-positive staphylococci, coagulase-negative staphylococci are rarely pathogenic and are often considered to be opportunistic pathogens, such as *S. epidermidis* is for humans (Cheung and Otto, 2010). However, occasionally coagulase-negative staphylococci produce enterotoxins and have been associated with food borne outbreaks (Veras et al., 2008). Certain coagulase-negative staphylococci are important components of meat starter cultures (Fadda et al., 2010). Recent investigations found that genes coding for staphylococcal virulence factors were rare in coagulase- negative staphylococci isolated from sausage and cheese. Of 129 strains tested, only one contained a gene coding for an enterotoxin and none were capable of producing toxic shock syndrome toxin. Some strains did have genetic information coding for hemolysins and some were capable of producing biogenic amines. Of somewhat greater potential concern was the presence of antibiotic resistance genes in 71% of isolates, with nearly half the strains resistant to more than one antibiotic (Even et al., 2010).

Currently, medical attention is paid mainly to coagulase negative staphylococci because they represent a serious therapeutic problem. Coagulase-negative staphylococci are mostly normal skin commensals and are much less pathogenic than *S. aureus* (Livermore, 2000). Although they are generally considered as almost non-pathogenic microorganisms, they play major role activators of nosocomial infections. Moreover, they often become multi-resistant to antibiotics (Eiff et al., 2002; Blahova et al., 2003; Blahova et al., 2004), constituting a continuously evolving reservoir of resistance genes which can be transferred to *S. aureus* (Archer, 1988). In recent years, there has been muchwritten about the emergence of methicillin-resistant *S.aureus* and methicillin-resistant coagulase-negative staphylococci (Tiemersma et al., 2004; Normanno et al., 2007).

2.3.13. Public health significance

The possibility that dogs and cats could act as the source for zoonotic staphylococcal infections in humans was suggested many years ago. Recent reports suggest that pig farmers are at increased risk of nasal Staphylococcus sp colonization including MRSA colonization (Voss et al., 2005). Several reports have presented information suggesting that animals may serve as reservoirs for MRSA infection of humans. In one case, a dog was implicated as a reservoir for the re-infection of two nurses after

their treatment to eliminate carriage of MRSA while in another a cat was implicated as the source of MRSA for nurses in a geriatric nursing facility (Scott et al., 1988).

2.3.14. *Salmonella sp*

2.3.14.1. History

The genus *Salmonella* was named after Daniel Elmer Salmon, an American veterinary pathologist. Salmon, along with Theobald Smith (1886), discovered the organism that causes hog cholera, *Salmonella enterica* var. *Choleraesuis* (Durecko et al., 2004), when they considered it to be the cause of swine fever (hog cholera). The importance of the organism as a cause of disease in pigs was neglected when the viral etiology of swine fever was discovered, and a number of years elapsed before *Salmonella choleraesuis* was recognized as a primary pathogen that was capable of causing several different disease syndromes.

2.3.14.2. The Genus *Salmonella*

The genus *Salmonella*, within the family Enterobacteriaceae, is a morphologically and biochemically homogenous group of facultatively anaerobic, non-spore forming, oxidase-negative, catalase-positive Gram-negative rod-shaped bacteria; the rods are typically 0.7-1.5 x 2-5µm in size, although long filaments may be formed. Most strains are motile due to peri-trichous flagella and ferment glucose with production of both acid and gas. Typically, *Salmonella* are non or slow lactose fermenters with some strains fermenting it rapidlythough; adonitol, sucrose, salicin and 2-ketogluconate are not fermented; urea is not hydrolysed; tryptophan and phenylalanine not deaminated; acetoin not produced; hydrogen sulphide (H_2S) is produced from thiosulphate; lysine and ornithin is decarboxylated. Most schemes for the detection of the organism are based on these properties (Grimont et al. 2000). Some strains produce a biofilm, which is a matrix of complex carbohydrates, cellulose and proteins. The ability to produce biofilm can be an indicator of dimorphism, which is the ability of a single genome to produce multiple phenotypes in response to environmental conditions.

Many genetic studies have been done on *Salmonella* species, particularly on serovar typhimurium. Its chromosome is very similar to that of *Escherichia coli* and consists of a single circular DNA molecule consisting of about 4 x 10^6 base pairs with a

molecular weight of 4 x 10^9 and a total length of about 1.4mm. Many of the genes have been mapped. According to the biochemical reactions and growth conditions of *Salmonella* shown above, different selective plating media have been developed. Summarize the appearance of *Salmonella* colonies on a variety of the most frequently used agar nutrient media.

Salmonella can grow within the range 2 to 54°C, although growth below 7°C has largely been observed only in bacteriological media, not in food, while growth above 48°C is confined to mutants or tempered strains. The optimum temperature for growth is 37°C. The natural ecology of most *Salmonella* strains of concern to public health is the gastrointestinal tract of warm-blooded animals (Brenner et al. 2000). The optimum pH for the growth of *Salmonella* is within a range of 6.5-7.5, in liquid nutrient media strains can grow at pH values up to 9.5 and down to 4.05. While growth occurs down to or close to the minimum pH with non-volatile organic acids such as citric acid or mineral acid such as hydrochloric acid, growth stops at higher pH values when volatile fatty acids are used. *Salmonella* grow at aw (water activity) values between 0.999 and 0.945 in laboratory media, down to 0.93 in foods, with an optimum of 0.995.

2.3.14.3. Taxonomy and nomenclature

The classification of the *Salmonella* within this genus has undergone considerable changes in recent decades (Bisping et al., 1988). *Salmonella* taxonomy is complicated (Tindall et al., 2005). Strains of *Salmonella* are classified into serovars on the basis of extensive diversity of lipopolysaccharide (LPS) antigens (O) and flagella protein antigens (H) in accordance with the (Nalbantsoy et al., 2010).

The Kauffman and White classification scheme is a classification system that permits serological varieties of the genus *Salmonella* to be differentiated from each other. This scheme differentiates isolates by determining which surface antigens are produced by the bacterium. First, the "O" antigen type is determined. "O" antigens are the polysaccharides associated with the lipopolysaccharide of the bacterial outer membrane. Having found the "O" antigen group, the "H" antigen is determined. The "H" antigens are proteins associated with the bacterial flagella (singular; flagellum). *Salmonellas* exist in two phases; a motile phase and a non-motile phase. These are

also referred to as the specific and non-specific phases. Non-motile isolates may be "switched" to the motile phase using a Cragie tube - bacteria are inoculated down the center of a hollow tube in a semi-solid nutrient agar. Those bacteria that become motile can then swim out of the bottom of the tube and are recovered from the agar outside of the tube. Pathogenic strains of *Salmonella* typhi carry an additional antigen, "Vi", so-called because of the enhanced **vi**rulence of strains that produce this antigen, which is associated with a bacterial capsule (Le Minor, 1988).

Salmonella nomenclature is not completely standardized. Several synonyms may be used for the same species or subspecies. Under the classification scheme used by the U.S. Centers for Disease Control and Prevention (CDC), World Health Organization (WHO) and some journals, there are now only two species in the genus *Salmonella*: *S. enterica* and *S. bongori* . There are also more than 2500 serovars within both species, which are found in a disparate variety of environments and which are associated with many different diseases.

Salmonella enterica has 6 subspecies: *S.* enterica subsp. enterica, *S.* enterica subsp. salamae, *S.* enterica subsp. arizonae, *S.* enterica subsp. diarizonae, *S.* enterica subsp. houtenae and *S.* enterica subsp. indica. These subspecies are also referred to by a number. *S.* enteric subsp. enterica = subspecies I. *S.*enterica subsp. salamae = subspecies II. *S.*enterica subsp. arizonae = subspecies IIIa. *S.*enterica subsp. diarizonae = subspecies IIIb. *S.* enteric subsp. houtenae = subspecies IV. *S.*enterica subsp. indica = subspecies VI.

However, within this six subspecies are differentiated by genetical, biochemical and, in part, serological methods (Bisping et al. 1988).

The nomenclature used throughout this publication follows that devised by Le Minor which divides the bacterial species *Salmonella enterica* into six subspecies: *enterica*, *salamae, arizonae, diarizonae, houtenae* and *indica*.

The method of naming serovars of subspecies *enterica* differs from that used for the other five subspecies in that the familiar serovar names are assigned to subspecies *enterica* whilst the other subspecies are designated by antigenic structure. Serovars in

S. enterica subsp. *enterica* are referred to by name. The names of these serovars can be shortened from the full name to the genus and serovar. For example, *S. enterica* subsp. *enterica* ser. Enteritidis can be called *Salmonella* ser. *enteritidis* or *Salmonella enteritidis*. Most of the serovars in the other 5 subspecies of *S. enterica*, as well as in *S. bongori*, are referred to by their antigenic formulas. These formulas include:

1. The subspecies/species designation (I, II, IIIa, IIIb, IV or VI for *S. enterica* subtypes; V for

S. bongori)

2. O (somatic) antigens followed by a colon

3. H (flagella) antigens (phase 1) followed by a colon **4**. H antigens (phase 2, if present)

Using this convention, a *S. enterica* subsp. *houtenae* strain with an O antigen designated 45, H antigens designated g and z51, and no phase 2 H antigens would be written as *Salmonella* serotype IV 45:g,z51: (Le Minor, 1988).

2.3.14.4. Salmonella Pathogenicity Island-7 (SPI-7)

Salmonella Pathogenicity Island-7 (SPI-7) is the largest genomic island yet identified in *Salmonella*, comprising up to 134 kb. It was first discovered as a large insertion in the genome of the human restricted pathogen *Salmonella enteric* subsp. *Enteric* serovar Typhi (*S. Typhi*), relative to that of serovar Typhimurium (*S. Typhimurium*) (Liu and Sanderson, 1995). The "major pathogenicity island" was renamed SPI-7 during the annotation of the *S.* Typhi strain CT18 genome, after the discovery of a number of other *S.* Typhi-specific islands. The genome annotation revealed that SPI-7 incorporates approximately 150 predicted genes, systematically numbered STY4521-STY4680. SPI-7 is also carried by *Salmonella enteric* subsp. *Enteric* serovar Paratyphi C (*S.* Paratyphi C) and some strains of serovar Dublin (*S.* Dublin) (Morris et al., 2003).

2.3.14.5. Pathogenesis

Three common conditions caused by Salmonella are gastroenteritis, enteric fever, and bacteremia. *S. typhimurium*, *S. enteritidis*, and *S.* Newport are serotypes associated with human and animal gastroenteritis, S. typhi and the paratyphi species are associated with human enteric fever and *S. choleraesuis* is associated with bacteremia

in pigs. *S. choleraesuis* is found mostly among animals other than humans, yet it is not as deadly in animal hosts as it is in human hosts (Stabel et al., 2002).

2.3.14.6. Antibiotic resistance pattern of *Salmonella sp*

Antibiotics are used in food-producing animals for prophylaxis, to treat diseases or to aid in the growth and development of farm animals. However, animals could be a source of foodborne-resistant bacteria. Several studies carried out have documented the direct transfer of antibiotic-resistant bacteria from animals to humans (Zare et al. 2014). Antimicrobial-resistant Salmonella in food animals could acquire their resistance in animals, which might lead to human infections with foodborne-resistant bacteria through the food chain (Glenn, 2011). Also, they could perpetuate the spread of antibiotic-resistant genes to human by horizontal gene transfer through such mobile genetic elements as plasmids and integrons (Igbinosa et al. 2013). The resistance of *Salmonella* to a single antibiotic was first reported in the early 1960s (Montville and Matthews, 2008). Since then, the isolation frequency of *Salmonella* strains resistant to one or more antibiotics have increased throughout the world. This is due to the increased and uncontrolled use as well as easy accessibility to antibiotics in many countries of the world (Yoke-Kqueen et al., 2007). The resistance towards the traditional first-line antibiotics such as ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole define multidrug resistance (MDR) in *Salmonella enteric* (Crump and Mintz, 2010). This is of great concern because majority of infections with MDR *Salmonella* are acquired through the consumption of contaminated foods of animal origin such as meat of swine, sheep, goat and chicken eggs.

2.3.14.7. Isolation and Identification

Rectal swabs were collected and pre enriched in buffered peptone water, incubated at 37˚C for 16 h. Then one ml of inoculum was transferred into Selenite-cystein broth (Eevuri et al., 2013). A loopful of inoculums plated onto Xylose Lysine Deoxycholate (XLD) medium and incubated at 37˚C for 24 hrs. Black centered colony from XLD was inoculated in Brilliant Green Agar (BGA) and incubated. Red colonies in BGA agar considered as positive for *Salmonella sp.*

2.3.14.8. Morphological characterization by Gram's staining method

A small colony from the representative *Salmonella* colonies was picked up from XLD and BGA plates with a bacteriological loop, smeared on separate glass slide and fixed by gentle heating. Crystal violet was then applied on each smear to stain for two minutes and then washed with running water. Few drops of Gram's iodine was then added to act as mordent for one minute and then again washed with running tap water. Acetone alcohol was then added (acts as decolorizer) for few seconds. After washing with water, safranin was added as counter stain and allowed to stain for 2 minutes. Then the slides were washed with water, blotted and dried in air and then examined under microscope with high power objective (100X) using immersion oil (Nesa et al., 2011).

2.3.14.9. Reaction of the organism in TSI agar slant

The TSI agar slant was used to detect the lactose, sucrose and dextrose fermenters. The medium also helped to determine the ability of the organisms to produce H_2S. A minimum of three black (or black-centered) colonies were inoculated into triple sugar iron slant and incubated for 24 hours at 37°C. Isolates with positive slant reactions were considered as positive as *Salmonella sp* (Pao et al., 2005). The isolates produced Pinkish slant and yellow butt or black slant and yellow butt was recorded as the positive reaction for *Salmonella sp*. Blackening in butt reflected as positive H_2S reaction and air bubble in the butt depicted for gas production in slants.

2.3.14.10. Carbohydrate fermentation test

The carbohydrate fermentation test was performed by inoculating a loopful of NB culture of the organisms into the tubes containing different sugar media (Dextrose, Lactose, Sucrose, Mannitol and Maltose) and incubated for 24 hours at 37°C. The isolates which were unable to ferment lactose and sucrose remain red and gas production was noted by the accumulation of gas bubbles in the inverted Durham's tube were suspected for *Salmonella sp*instructed by (Nesa et al., 2011).

2.3.14.11. Serotyping of *Salmonella sp*

A single species of microorganism could be stratified into different groups of serotypes which share distinctive surface structures. For instance, *Salmonella* bacteria look alike under the microscope but can be separated into many serotypes based on

two structures on their surface. First one is outermost protein or somatic or "O"-antigen and left one is flageller or "H"-antigen. For serotyping, the somatic "O"-antigen of the *Salmonella* isolates were determined with a slide agglutination test as described by Ewing (1986), whereas the flagellar (H) antigens were identified by using a microtechnique that employs microtitre plates (Shipp and Rowe, 1980).

2.3.14.12. Antibiogram of *Salmonella sp*

Susceptibility of the isolated *Salmonella*e to different antibacterial agents was performed through disc diffusion method to determine the drug sensitivity pattern. The antibacterial discs used were erythromycin, amoxicillin, cephalexin, chloramphenicol, co-trimoxazole, kanamycin, ciprofloxacin and nalidixic acid. The disk diffusion test, which is also known as the Kirby-Bauer test, is the most commonly used method for susceptibility testing (Reese et al., 1996). The diameters of the zones of inhibition are measured and compared to interpretive breakpoint values published by CLSI which assign a value of "susceptible", "intermediate" or "resistant" to the isolate (Islam et al., 2013). Antibiotic sensitivity assay of the isolates revealed that many of the *Salmonella* isolates were resistant to colistin (41.66%), nitrofurantoin (70%) and amikacin (51.66%), while all were sensitive to chloramphenicol and imipenem. The sensitivity to other antibiotics varied greatly. Most of the isolates were sensitive to acriflavin (95%), crystal violet (93.33%) and mercuric chloride (96.66%).

2.3.14.13. Epidemiology

Salmonella has been recovered from the intestines of a wide range of animalsincluding fish, reptiles, birds, and mammals (Wray and Sojka 1977). Excretion with faeces can result in contamination of water, soil, other animals and feed. Animals are infected by direct nose-to-nose contact or by contact to the faeces of infected animals or by feedstuff containing contaminated compounds. The main sources of salmonellosis in humans are food animals and their products such as raw eggs, poultry meat and pork (Hald and Wegener 1999).

The reservoir for *Salmonella* is the intestinal tract of warm- and cold-blooded animals. *Salmonella* have mastered virtually all of the attributes necessary to ensure wide distribution including abundant reservoir hosts, efficient fecal shedding from

carrier animals, persistence within the environment, and the effective use of transmission vectors (feed, fomites, vehicles, etc.). In apparent, long-term carriers that can shed *Salmonella* in feces continuously or intermittently, often in high numbers are common in most host species. Shedding of the organism can be exacerbated by a long list of stressors, including commingling of pigs, transportation, concurrent diseases, and food deprivation.

The epidemiology of *Salmonella* infections in swine is two relatively separate problems: *Salmonella* infection of pork carcasses and retail products and infections that cause salmonellosis in swine. Infection of swine by one or more serotypes is common, but primary clinical disease caused by serotypes other than *S.choleraesuis* or *S.typhimurium* is uncommon. It is important to understand that swine can be infected with a variety of serotypes that do not cause disease in swine but do represent a source of infection for pork products.

Extrapolation of epidemiological data from experimental studies where a single serotype with predetermined dose is administered to naive healthy pigs is not likely to represent field situations, where there are multiple serotypes, varying doses, intermittent exposures, variable host resistance, many management variables, and various intercurrent infections and diseases. Similarly, disease prevalence surveys must be carefully scrutinized to be sure that infection is not equated with disease, and that a source of infection is not inaccurately implicated (Schwartz, 1999).

In contrast with poultry, where vertical transmission of some serovars may occur from the layer hen to the egg, horizontal transmission is of major importance in spread of *Salmonella* in pigs. Pigs get infected with *Salmonella* through direct contact with infected pen-mates, via contaminated feed or via the environment. The epidemiology of *Salmonella* is complex, because there is a wide range of hosts and vectors, because *Salmonella* can survive for a long time in the environment and because of the ability to persist in the population due to the carrier state.

The number of sources for *Salmonella* in pig herds is endless (Schwartz, 1999). *Salmonella* infections within pig herds are often clustered at the pen level but outbreaks of salmonellosis can typically spread from pen to pen (Schwartz, 1999).

Nevertheless, because of the presence of different vectors such as water sources, cats, insects, rodents, boots, *Salmonella* may spread to distant pens within the same herd (Barber et al., 2002). The role of cats, birds and rodents in the spread of *Salmonella* between herds needs to be investigated further.

2.3.14.14. Public Health Significance

Typhoid fever is a global problem, with more than 27 million cases worldwide each year resulting in an estimated 217,000 deaths (Crump and Mintz, 2010). *Salmonella enteric* serovar Typhi (S. Typhi) and S. Paratyphi A are the Gram-negative bacteria that cause this debilitating condition. S. Typhi is an exclusively human pathogen causing a bacteremic disease that, unlike many other Gram-negative bacteremias, does not typically manifest with neutrophilia or septic shock (Tsolis et al., 2008).

Poorly nourished animals reared in unhygienic and high humid conditions, reduced immunity, imbalance nutrition, housing, climatic condition, transportation stress etc might have influence in the occurrence of multiple bacterial populations in goats.

Chapter-3: Materials and Methods

Chapter-3: Materials and methods

3.1. Study area

Chittagong has a total area of 168.07 square kilometers (64.89sq mi). The city is known for its vast hilly terrain that stretches throughout the entire district and eventually into India. The city is located at 22°22'0"N 91°48'0" E on the banks of the Karnaphuli River. The samples were collected at S A Quaderi Teaching Veterinary Hospital (SAQTVH) in Chittagong Veterinary and Animal Sciences University (CVASU) located in the heart of the city.

3.2. Study period

The study was conducted during July- December 2015.

3.3. Sample collection

One hundred and fifty goats (n=150) were sampled having pneumonia and/or enteritis during study period. The sample was collected by inserting a sterile swab into RAJ (Recto-Anal Junction) and nostril of the affected animal. The collected swab was placed in a falcon tube (5ml) containing Stuart's transport medium (Oxoid ltd, Basingstoke, Hampshire, UK), and sent to the Poultry Research and Training Centre (PRTC) Laboratory, Chittagong Veterinary and Animal Sciences University for laboratory analysis. Additional demographic and epidemiological information of each sampled animal was collected using a well structured pre-tested questionnaire shown in Annex-I.

3.4. Experimental design

The whole study was separated into three major steps: The first step was collection of samples to the laboratory and inoculation into different culture media. In the second step, isolation and identification of the bacterial isolates was done based on their cultural uniqueness including pigment production, hemolytic activity, Gram's staining character etc. In the third step, characterization of the organism was done using various biochemical tests. Finally the antibiotic sensitivity test of the isolates was performed to observe drug resistance profile of bacterial isolates.

Figure 01: Geographical locations of the study area (SAQTVH, CVASU)

3.5. Bacteriological examination

3.5.1. Isolation and identification of *Staphylococcus spp*

Nasal swab (n=150) from transport media was placed into sterile Buffered Peptone Water (BPW) (Oxoid ltd, Basingstoke, Hampshire, UK) and enriched for 24 hours at 37 °C (Thaker et al., 2013). Both Mannitol salt agar (MSA) medium and Blood agar base were prepared according to the instructions of manufacturer (Oxoid ltd, Basingstoke, Hampshire, UK). Blood agar was prepared by adding 5% citrated-bovine blood in the blood agar base. A loopful of inoculum from enrichment were streaked on Blood Agar (Oxoid ltd, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 hours for detection of hemolysis. Growth of yellow colonies on MSA (Oxoid ltd, Basingstoke, Hampshire, UK) surrounded by yellow zones as a result of fermentation of mannitol after 24 hours of incubation at 37°C indicated a positive result (Kateete et al., 2010). Smear was prepared from the isolated colony on clean grease free microscopic glass slide and stained with Gram's Method of staining. All the positive samples were subjected to coagulase tests for biochemical confirmation of *Staphylococcus sp* as described by (Monica, 1991). After that five such cross-sectional colonies were picked up and transferred to a 10 ml test tube containing 5 ml of brain heart infusion broth (BHIB), were prepared according to the instructions of

manufacturer (Oxoid ltd, Basingstoke, Hampshire, UK), incubated at 37 °C for 6 hours.

3.5.2. Preparation of Horse plasma for Coagulase test

Whole blood from horse was collected into commercially available sterile tubes containing EDTA to perform the test. Then blood was centrifuged at 2600 rpm for 10 minutes using a refrigerated centrifuge device. The resulting supernatant, the plasma, was then immediately transferred to a sterile 1.5 ml eppendorf tube using a sterile tip and stored at -20ºC for future use.

3.5.3. Tube test

From each tube cultivated in BHIB, 50 µl was transferred to sterile tubes containing 50 µl of horse plasma. The incubation was done at a temperature of 37ºC for 6 hours. The presence of coagulates was justified, considering large organized coagulation and coagulation of all the contents of the tube which do not come off when inverted Brasil (2003). A control tube also placed to validate the result.

3.5.4. Slide coagulase test

Staphylococcus spp (which were confirmed by tube coagulase test) were further confirmed by slide coagulase test. One drop of the horse plasma was placed on a clean grease free glass slide. A loopful of suspected culture was mixed with plasma separately and checked for agglutination. The cultures showing agglutination were recorded as positive for coagulase test and thus were confirmed as *Staphylococcus spp.*

3.5.5. Tube catalase test

Nutrient agar slant was prepared according to the instructions of manufacturer (Oxoid ltd, England). Suspected bacterial colonies inoculated into agar slant and incubated at 37°C for 24 hours. After that 1 ml of 3% H_2O_2 was added and rapid ebullition of gas considered as positive reaction of *Staphylococcus spp* (Hogan et al., 1999).

3.5.6. Preservation of the culture

Biochemical test positive isolates were inoculated into BHIB (Oxoid ltd, England), incubated overnight at 37^{o}C and then preserved at -80^{o}C with 50% glycerol in 1.5 ml eppendorf tubes for future investigation.

3.6. Antimicrobial susceptibility profile testing of bacterial isolates (*Staphylococcus spp)*

All biochemical test positive bacterial isolates (*Staphylococcus spp*) were investigated for their diversity in antimicrobial susceptibility profiles by disk diffusion method on Mueller-Hinton agar (Oxoid ltd, Basingstoke, Hampshire, UK) according to the Clinical Laboratory Standards Institute (CLSI). Bauer-Kirby disk-diffusion procedure (Bauer et al., 1966) was used on Mueller-Hinton (MH) agar, prepared according to the manufacturer's instructions (Oxoid ltd, Basingstoke, Hampshire, UK). A bacterial turbidity equivalent of 0.5 McFarland standards was used for each isolate. A 0.5 McFarland standard was prepared by adding 0.5 ml of 1% (11.75g/L) $BaCl2.2H_2O$ to 99.5 ml of 1% (0.36N) H_2SO_4 (Carter and Cole, 1990) (Figure 08). The panel of antibiotics used for three bacterial species along with the sizes of zone of inhibition of them to be considered as resistant (R), intermediately resistant (I) and sensitive (S) against the tested isolates are shown in Table 3.1. These characterizations were based on the recommendations from Clinical and Laboratory Standards Institute (CLSI, 2007).

3.6.1. Disk-diffusion procedure

A sterile swab was dipped into the inoculums, prepared for antimicrobial sensitivity test, and rotated against the side of the tube with firm pressure. Then after removing the excess fluid from the swab the dried surface of MH agar was inoculated by streaking the swab three times over the entire agar surface rotating the plates approximately at 60 degrees for each time to ensure an even distribution of the inoculums. The antimicrobial disks were then placed on the surface of the inoculated agar. A separate forceps was always used to dispense each of the antimicrobial disks.

The disks were placed carefully on the surface of the agar with a gentle pressure to make a complete contact. After dispensing all of the disks the agar plate was incubated at 37^oC for 18 hours. At the end of incubation the size of zone of inhibition around a micro-disk was measured with a digital slide calipers and the result was recorded according to (CLSI, 2007).

Table 3.1: Panel of antibiotics used their concentrations and Zone diameter interpretative standards for *Staphylococcus spp* (CLSI, 2007).

Group of Antimicrobial Agent	Antimicrobial Agent	Disk Contents	Zone Diameter, nearest whole (mm)			Manufacturer
			R	**I**	**S**	
Penicillin	P-G	10 units	≤28	-	≥29	Oxoid Ltd. Basingstoke, Hampshire, England
Penicillin	Ampicillin	10µg	≤28	-	≥29	
β–lactamase inhibitor combination	Amoxicillin–clavulanic acid	20/10µg	≤19	-	≥20	
Cephems	Cefotaxime	30 µg	≤14	15-22	≥23	
Cephems	Ceftriaxone	30 µg	≤13	14-20	≥21	
Cephems	Oxacillin	1 µg	≤10	11-12	≥13	
Cephems	Vancomycin	30 µg	-	-	≥15	
Cephems	Clindamycin	2 µg	≤14	15-20	≥21	
Tetracycline	Tetracycline	30 µg	≤14	15-18	≥19	
Aminoglycosides	Gentamicin	10 µg	≤12	13-14	≥15	
Folate pathway inhibitor	Trimethoprim–sulfamethoxazole	1.25/23.7 µg	≤10	11-15	≥16	

3.7. Detection of resistant genes of *Salmonella spp* by Polymerase chain reaction

3.7.1. Procedure of DNA extraction

At first isolates stocked at - 80°C temp.in 50% glycerol were grown overnight in Blood Agar at 37°C temp. DNA was extracted by boiling method using hot water bath. For each isolate 200 µl deionized water was taken into an autoclaved Eppendorf tube. About 3-4 well isolated colonies of each isolate were picked up by an inoculating loop and mixed with deionized water taken into Eppendorf tube. After proper mixing it was homogenized by using vortex homogenizer. Then the Eppendorf tubes containing homogenized culture were placed in porous cock sheet for boiling. A tiny pore was made over the lid of Eppendorf tubes to facilitate evaporation during boiling. DNA materials were extracted by adjusting the temperature of hot water bath at 97°C for 15 minutes. After boiling Eppendorf tubes were placed in icebox and kept at 4°C in refrigerator for 10 minutes. Ice was prepared by using ice maker. Then Eppendorf tubes were placed in a centrifuge machine adjusted at 12000 rpm for 10 minutes. After centrifugation the extracted materials divided into two phases. The supernatant contained the DNA materials and cellular elements other than DNA formed pellet and deposited at the bottom. About 90 µl of supernatant was transferred to another Eppendorf tube. The extracted DNA containing Eppendorf tubes were then stored at - 20°C until preparation of reaction volume.

3.7.2. Preparation of reaction volume

Reaction volume contains Nuclease free water, Master Mix, Forward Primer, Reverse Primer, and extracted DNA as template. Reaction volume was calculated by following way:

- Total reaction volume was 25 µl for each isolate.
- Master Mix was always half of the reaction volume i.e. 12.5 µl / isolate.
- Forward Primer was 0.5 µl / isolate.
- Reverse Primer was 0.5 µl / isolate.
- Extracted DNA material as template was 1 µl / isolate.
- Rest of the reaction volume was filled by Nuclease free water i.e. 10.5 µl / isolate.

During preparation of reaction volume Master Mix, Extracted DNA material containing Eppendorf tubes, stock solution of Forward & Reverse Primer, all were kept in ice box to maintain 4°C temperature. Forward & Reverse Primer was diluted from stock solution to prepare working solution according to company instructions. At first appropriate amount of Nuclease free water was taken into an Eppendorf tube. Then required amount of Master Mix was mixed with it. The mixer was gently pipetted. Desired amount of Forward & Reverse Primer was mixed with it using Nano tips. Before dispensing into PCR tube the mixture was gently homogenized by vortex homogenizer. For each isolate 24 µl of reaction volume was dispensed to every PCR tube. For each isolate 1 µl of extracted DNA was mixed with 24 µl of reaction volume in a PCR tube. Then the PCR tubes were placed in Thermal Cycler to run PCR.

Table 3.2: Thermal profile of PCR for the detection of *tetA* resistance genes:

Reaction volume	Number of cycle	Stages	Temperature	Time	Reference
25 µl		Initial denaturation	95°C	4 minutes.	(Boerlin *et al.,* 2005)
	35 cycles	Denaturation	95°C	1 minute.	
		Annealing	64°C	1 minute.	
		Elongation	72°C	1 minute.	
		Final elongation	72°C	7 minutes.	
		Holding	4°C	Infinite.	

Table 3.3: Sequences of primers used in the detection of *tetA* resistance genes through PCR:

Genes	Type of primer	Primer sequence	Fragment size (bp)	Reference
tetA	Forward primer 5'-3'	GCT ACA TCC TGC TTG CCT TC	210 bp	Karczmarczyk et al., 2011
	Reverse primer 5'-3'	CAT AGA TCG CCG TGA AGA GG	210 bp	Karczmarczyk et al., 2011

Table 3.4: Thermal profile of PCR for the detection of *bla_{TEM}* ß lactamase gene:

Reaction volume	Number of cycle	Stages	Temperature	Time	Reference
25 µl		Initial denaturation	94°C	3 minutes.	(Chikwendu *et al.*, 2011)
	25 cycles	Denaturation	94°C	1 minute.	
		Annealing	50°C	1 minute.	
		Elongation	72°C	1 minute.	
		Final elongation	72°C	10 minutes.	
		Holding	4°C	Infinite.	

Table 3.5: Sequences of primers used in the detection*bla_{TEM}* ßlactamase gene through PCR:

Gene	Type of primer	Primer sequence	Fragment size (bp)	Reference
bla_{TEM}	Forward primer 5'-3'	TAC GAT ACG GGA GGG CTT AC	716 bp	Belaaouaj et al., 1994
	Reverse primer 5'-3'	TTC CTG TTT TTG CTC ACC CA	716 bp	Belaaouaj et al., 1994

3.7.3. Visualization of PCR Product

Agarose gel (1%) was prepared for electrophoresis of PCR- amplification products. A gel tray was assembled with setting a proper teeth sized comb in the tray. Then 1 % agarose solution (SeakemLEagarose –Lonza) was prepared in 1x TAE buffer by boiling in a microwave oven for 2 minutes. The agarose gel thus prepared was cooled to 40- 50^{o}C in a water bath, having added with 1 drop of ethidium bromide at a concentration of 5µg per ml. Finally, agarose gel was poured into the gel tray and allowed about twenty minutes to stand for solidification of the gel.

The gel was shipped into an electrophoresis tank, already filled in with 50ml of 1x TAE buffer. Then 5µl of each of the PCR products for an isolate was loaded up into gel–holes. One hole was loaded with DNA marker (Thermo Scientific O' Gene Rular 1 kb plus) to compare the amplicon size of the gene product. As a negative control one hole was loaded up with only 5µl of distilled water. As we had no known PCR positive gene isolates from any reference laboratory, so we could not use any positive control during electrophoresis.

Electrophoresis was done at 110 volts and 80 Amp for 20 minutes. After completion of electrophoresis the gel was placed in a water bath for rinsing, just for a while. Finally the gel was examined under an UV transilluminator (BDA digital, biometra GmbH, Germany). Gel electrophoresis was repeated twice with the same PCR products.

3.7.4. Statistical analysis

The data obtained from field and laboratory was entered into spread sheets of the MS Excel-2007 program. Data were checked and sorted in the Excel program before exporting to STATA-11 Statistical software.

Descriptive analysis with univariate analysis, logistic regression and odds ratio calculation was performed for goat related variables and association of risk factors (breed, age, sex, body condition score, transportation history) with prevalence of selective pathogens.

Chapter-4: Results

Chapter-4: Results

4.1. Prevalence of *Staphylococcus spp* in pneumoenteritic goat

Although one hundred and fifty (n=150) samples showed different pattern of bacterial growth on blood agar, but only 42 of them were found to be positive for *Staphylococcus spp* based on their colonial growth characteristics on mannitol salt agar, blood agar plates and also depicted positive in biochemical tests. So the prevalence of *Staphylococcus sp* in the study population was 28.00%.

Bacteria	No. of animal sampled (n)	No of positive (Prevalence %)	95% CI
Staphylococcus sp	150	42 (28)	20.98-35.91

Table 4.1: Overall prevalence of *Staphylococcus sp* in pneumoenteritic goat

CI = Confidence Interval

4.2. Univariate association between factors and prevalence of *Staphylococcus spp* in nasal swab sample

The prevalence of *Staphylococcus spp* was higher in Black Bengal Goat (31.6%) than Crossbred (30.8%) and Jamunapari (14.8%). The differences of the prevalence of *Staphylococcus spp* in different breeds of goat under this study was statistically insignificant (P>0.05) as shown in Table 4.2. In case of sex, female showed (34.3%) more susceptible in *Staphylococcus spp* infection and become infected than male (22.9%) goats. The differences in the prevalence of *Staphylococcus spp* between male and female goats were not significant (P>0.05) as stated in Table 4.2.

Table 4.2: Univariate association between factors and Prevalence of *Staphylococcus* in nasal sample

Factor	Category	N	Staphylococcus		p-value
			Positive (%)	Negative	
Breed	Black Bengal Goat	19	6 (31.6)	13	0.24
	Cross Breed	104	32 (30.8)	72	
	Jamunapari	27	4 (14.8)	23	
Sex	Male	83	19 (22.9)	64	0.14
	Female	67	23 (34.3)	44	
Age	Young	54	17 (31.5)	37	0.57
	Adult	96	25 (26.0)	71	
Body Condition Score	CND	8	5 (62.5)	3	0.02
	CPD	45	7 (15.6)	38	
	GBC	26	10 (38.5)	16	
	RBC	71	20 (28.2)	51	
Vaccination Status	Yes	22	6 (27.3)	16	1.0
	No	128	36 (28.1)	92	
Transportation History	Yes	29	9 (27.3)	20	0.65
	No	121	33 (31.0)	88	

The prevalence of *Staphylococcus spp* was higher (31.5%) in young goats than adult goats (26.0%). Statistically, the relationship of the prevalence of *Staphylococcus spp* in between adult and young goats were insignificant (P>0.05). In case of, Body Condition Scoring (BCS), the prevalence of *Staphylococcus* was more in CND (Cachectic, protruding rib with prominent pin bone, no diarrhea) than from CPD (Cachectic, protruding rib with prominent pin bone, presence diarrhea), GBC (Good Body Condition) and RBC (Rough Body Condition and Ribs are moderately visible). The prevalence of *Staphylococcus spp* in different body condition scored goats was statistically significant (P<0.05) under this study. Goats, those were vaccinated reveal less prevalence (27.3%) in *Staphylococcus spp* than those were not vaccinated (28.1%). The difference of prevalence of *Staphylococcus spp* is not significant in between goats of different vaccination status. Goats that have transportation history

showed less prevalence (27.3%) in *Staphylococcus spp* than that of having no transportation history (31.0%). In Statistical point of view, the differences of the prevalence of *Staphylococcus spp* in history of transportation was not significant (P>0.05).

4.3. Frequency distribution of sensitivity and resistance of different antibiotics along with association of sensitivity and resistance between coagulase positive and coagulase negative *Staphylococcus spp*

Of the tested isolates, coagulase positive Staphylococcus was highly resistant to Penicillin (45%) and less resistant to Cefotaxime (17.6%) among the β-lactams. Others were 39.5%, 39.4%, 32.4% respectively for Ampicillin, Amoxicillin-clavulenic and Oxacillin. Similarly, among the Aminoglycosides, Gentamicin (50%) was mostly resistant for coagulase positive Staphylococcus whereas Clindamicin (29.6%) of fewer resistant. Vencomycin was also 41.4% resistant. Tetracyclin and Potentiated Sulphonamides drug (Trimethoprim & Sulfamethoxazole) as well 37.5% and 12.5% resistant for coaglase positive *Staphylococcus* spp respectively.

On the other hand, amongst B-lactam coagulase positives Staphylococcus was highly sensitive to Oxacillin (87.5%) as well as Ampicillin, Cefotaxime, Amoxicillin-clavulenic was also 75%, 60% and 55.6% respectively and there was no sensitivity to Penicillin. Coagulase positive Staphylococcus was found to be more sensitive to Clindamicin (66.7%) than the other Aminoglycosides. Vancomycin and Gentamicin also 46.2% and 28.6% sensitive respectively. From the tested isolates, 60% of Tetracyclin and 50% of Trimethoprim & Sulfamethoxazole also found to be sensitive. In case of coagulase negative Staphylococcus, Cefotaxime (82.4%) was highly resistant from other B-lactam. Other B-lactam like Oxacillin (67.6%), Amoxicillin-clavulenic (60.6%), Ampicillin (60.5%) and Penicillin (55%) was also found to be resistant.

Similarly, among the Aminoglycosides, Clindamicin (70.4%) was mostly resistant for coagulase negative Staphylococcus whereas Vencomycin and Gentamycin was found to be 58.6% and 50% resistant respectively. Tetracyclin and Potentiated Sulfur drug (Trimethoprim & Sulfamethoxazole) as well 62.5% and 87.5% resistant for coaglase negative Staphylococcus respectively. On the other hand, amongst B-lactam coagulase negative Staphylococcus was highly sensitive to Penicillin (100%) as well

as Ampicillin, Cefotaxime, Amoxicillin-clavulenic, Oxacillin was also 25%, 40%, 44.4% and 125% respectively was also found to be sensitive. In Coagulase negative Staphylococcus there was more sensitivity of Gentamicin (71.4%) than the other Aminoglycosides. Clindamicin and Vancomycin also 33.3% and 53.8% sensitive respectively. Coagulase negative Staphylococcus also sensitive to Tetracyclin (40%) and Trimethoprim & Sulfamethoxazole (50%).

Table 4.3: Frequency distribution of sensitivity and resistance of different antibiotics along with association of sensitivity and resistance between coagulase positive and coagulase negative *Staphylococcus spp*

Major class of antibiotics	Type of Antibiotics	Category	Coagulase Positive	Coagulase Negative	OR (univariate logistic) (Coagulase positive vs coagulase negative)	p-value	95% CI
B-lactam	Penicillin	Resistant	18 (45.0%)	22 (55.0)			
		Sensitive	0	2 (100.0)	Omitted due to presence of 0 cell		
	Ampicillin	Resistant	15 (39.5%)	23 (60.5)			
		Sensitive	3 (75.0%)	1 (25.0)	4.5	0.2	0.4-48.5
	AMOX-CLA	Resistant	13 (39.4)	20 (60.6)			
		Sensitive	5 (55.6)	4 (44.4)	1.9	0.4	0.4-8.5
	Cefotaxime	Resistant	3 (17.6)	14 (82.4)			
		Sensitive	15 (60)	10 (40)	7.0	0.010	1.6-30.8
	Oxacillin	Resistant	11 (32.4)	23 (67.6)			
		Sensitive	7 (87.5)	1 (12.5)	14.6	0.018	1.6-134.1
Aminoglycosides	Gentamicin	Resistant	14 (50)	14 (50)			
		Sensitive	4 (28.6)	10 (71.4)	0.4	0.19	0.1-1.6
	Vancomycin	Resistant	12 (41.4)	17 (58.6)			
		Sensitive	6 (46.2)	7 (53.8)	1.2	0.8	0.3-4.5
	Clindamicin	Resistant	8 (29.6)	19 (70.4)			
		Sensitive	10 (66.7)	5 (33.3)	4.8	0.02	1.2-18.4
Tetracycline	Tetracycline	Resistant	12 (37.5)	20 (62.5)			
		Sensitive	6 (60)	4 (40)	2.5	0.2	0.6-10.7
Potentiated Sulfur drug	Trimethoprim & Sulfamethoxazole	Resistant	1 (12.5)	7 (87.5)			
		Sensitive	17 (50)	17 (50)	7.0	0.08	0.8-63.2

4.4. Univariate logistic regression model

There was no significant difference observed on the prevalence of *Staphylococcus spp* with different variables such as breed and age however the prevalence was significantly ($p<0.05$) higher in CND group than others.

Table 4.4: Output of univariate logistic regression model:

Factor	Category	OR	Confidence Interval	p-value
Body Condition Score	CPD, GBC and RBC	1		
	CND	4.7	1.1 – 20.8	0.04
Breed	Jamunapari	1		
	Pure Black Bengal and Cross	2.6	0.8-7.9	0.10
Sex	Male			
	Female	1.8	0.9-3.6	0.12

4.5. Association between tetA and blaTEM resistant gene of *Salmonella*

Out of twenty five (25) test isolates, eighteen (18) samples was proceeded for detection of resistant gene i.e. tetA and blaTEM of *Salmonella sp*.

50% of the test isolates was found to be tetA positive and blaTEM was positive to 72.2% isolates.

Table 4.5: Association between tetA and blaTEM resistant gene of *Salmonella*

Category	Positive N (%)	Negative N (%)
TetA	9 (50.0)	9 (50.0)
blaTEM	13 (72.2)	5 (27.8)

McNemar significance probability = 0.4531

Chapter-4: Discussion

Chapter-5: Discussion

Pneumoenteritis is one of the most common complications in small ruminants involving both respiratory and digestive systems throughout the world. Respiratory and enteric diseases are concurrently occurred under unhygienic management conditions in goat flocks, affecting groups or individuals and then potentially leading to significant loss (Leite-Browning, 2007). Most of the infectious agents that cause digestive and respiratory disease are pervasive in nature and are normal inhabitants of the naso-pharynx and digestive tracts (Omer et al. 2012).

Staphylococcus spp considered as one of the major cause of respiratory infection and frequently isolated from pneumonic goats. Although a single agent may be the primary determinant of the disease but in most instances the situation is aggravated by secondary invaders (Islam et al., 2006). On the basis of bacteriological culture and biochemical tests 42 (28%) out of 150 samples were found positive for *Staphylococcus spp* in the study population. A study conducted in Nigeria recorded only 0.1% prevalence of pneumonia in goats (Adamu and Ameh, 2007). Emikpe et al. (2009) found 26% prevalence of *Staphylococcus spp* in West African dwarf goats. In another study Tijjani et al. (2012) found 18% prevalence in their study population of goats. This type of variation in isolation of *Staphylococcus spp* might attributable to geographic variation of the region from where the samples were collected, mixed bacterial population in animals, variation of the techniques adopted by different laboratories for conducting the experiments. The finding of present study is close but slightly lower than that of the findings of Adamu et al. (2010) that depicted 30% prevalence in goat population. The prevalence of *Staphylococcus sp* was found to be slightly higher than other breeds, this might be due to breed differences of animals supported by (Sarker and Islam, 2011).

Prevalence was higher in female goats (34.3%) than male (22.9%) which was supported by Momin et al. (2014). This study recorded higher prevalence of pneumonia in young goats (31.5%) as compared to adult goats (26.0%): though the difference was statistically insignificant ($P>0.05$). Kumar et al. (2004) recorded a higher incidence of pneumonia in 3 – 6 month-old goats (25.5%) as compared to >12 months age goats (3.3%) in India. Young animals are more prone to develop pneumonia caused by bacterial agents; however, adult animals are also susceptible, particularly when immuno-compromised (Rimoldi and Moeller, 2013). Body condition score (BCS) was found to be significantly ($p>0.05$) associated with

the occurrence of *Staphylococcus spp* which is in the line with the agreement of Omer et al., (2012) who reported that when the local resistance of respiratory mucus is lowered bacteria from nose and throat extend more down wards leading to multiple bacterial infections.

In addition to *S. aureus*, other (CPS) coagulase-potive staphylococci species can cause severe infections compared with those caused by coagulase-negative staphylococci (CNS) (Sasaki et al., 2007). The pathogenicity of coagulase- positive staphylococci are related to the production of many virulence factors including toxins, and enzymes from which coagulase enzyme was consider as the most important one. Coagulase production was described as one of the most reliable criteria for the identification of pathogenic *Staphylococcus* species. Staphylococci producing coagulase are usually pathogenic (Quinn et. al., 2002).

Forty two *S. aureus* isolates were tested against various antibiotics. The results showed that beta-lactam antibiotic (oxacillin), showed percentage of susceptibility 87.5 % in case of coagulase positive staphylococcus (CPS). This result is agreement to the study of Hanon (2009), who mentioned that the percentage of susceptibility of oxacillin was 52.5%.This differences may be due to the site of sample collection.

In the present study, susceptibility of *Staphylococcus spp* isolates (CPS) to vancomycin was 46.2%. Similar finding were obtained in the study of Hanon (2009) who recorded that 55% of *S. aureus* isolates from bovine were sensitive to vancomycin. On the other hand, all *S. aureus* isolates were 100% sensitive to vancomycin in another study (Panhotra et al., 2005).

The sensitivity of coagulase negative staphylococcus (CNS) to gentamycin was 71.4 %. A similar finding was obtained by the study of AL-Marsomy (2008), who recorded sensitivity of S aureus isolates from mastitis to gentamycin was 76.8%, but the highest sensitivity (100%) was found by the study of (Bendahou et al., 2008).

In the present study, CPS were (29.6%) resistant to clindamycin, These results agree with the study of Bratu et al.(2005), who found the resistant of *S. aureus* isolated from hospital nursery and maternity units to clindamycin during 2003, were 20%.

Isolated *Staphylococcus spp* were showed high a resistance percentage to penicillin which was in agreement with the results of the others (Borges et al., 2011).

Clinically, *Salmonella* infection in goat is typically manifested as watery or bloody diarrhea, and often associated with fever, depression, anorexia, dehydration and endotoxemia. On the other hand *Salmonella* spp. can be localized into the gallbladder of asymptomatic ruminants (Wang Y et al., 2010).

From twenty five isolates, eighteen of the antimicrobial-resistant isolates were investigated for the corresponding resistance genes. High prevalence of the *tet A* (50%), *blaTEM* (72.2%) genes was detected from the antimicrobial-resistant isolates, as shown in previous reports (Miko et al., 2005). This result in agreement to the study of (Truong et al., 2012), who mentioned that the prevalence of resistance genes of *tet* A was 55.2% and *blaTEM* was 90%.This differences may be due to the breed and sample size dissimilarities.

Chapter-6: Conclusion

Chapter – 6: Conclusion

The present study includes the characterization of the selective pathogens of pneumoenteritic goats along with their antibiogram. The prevalence of *Staphylococccus spp* in pneumoenteritic goats was 28%. Among the *Staphylococcus aureus*, the coagulase positive *Staphylococcus spp* was 43% whereas the coagulase negative *Staphylococcus spp* was 57%. Among the farm level risk factors, the body condition score was statistically significant but the others like age, breed, sex, vaccination and transportation were insignificant. The prevalence of *Salmonella sp* was 16.7% in pneumoenteritic goats. The isolated *Salmonella sp* containing TetA gene were 50% of the total isolates whereas the *Salmonella sp* containing blaTEM gene were 72% of the isolates. However, both the Salmonella and Staphylococcus were highly prevalent in pneumoenteritic goats and were resistant to most of the antibiotics used except cefotaxime and potentiated sulphonamides. In conclusion, the study will help selecting proper treatment strategy to control pneumoenteritis in goats and to take necessary steps in preventing the disease as well.

Chapter-7: Recommendation

Chapter – 7: Recommendation

The present study includes the characterization and antibiogram of the selective pathogens of pneumoenteritic goats at Chittagong, Bangladesh suggests the following recommendations –

- Indiscriminate or excessive use of drugs should be restricted through education and motivation of farmers and practicing veterinarians.
- Veterinarians are advised to be more rigorous when prescribing veterinary medicinal products and to become aware of rules for the prudent use of antimicrobials.
- Owners should respect the prescribed withdrawal periods of drugs. It is also necessary to organize seminars on the risk of the excess use of antimicrobial substances.
- This study covered only few pathogens in pneumoenteritic goats at Chittagong district. Therefore, a comprehensive study is required to characterize a series of organisms of pneumoenteritic goats along with antibiogram all over Bangladesh.

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Annex-1

Questionnaire for collecting relevant data from Pneumoenteritic goats

Questionnaire for collecting of clinical samples and relevant data from Pneumoenteritic goats

Date: ☐☐☐ 2. Contact No: ☐☐☐☐☐☐☐☐☐☐

1. Contact address: Village/Ward: .....; PO: .....PS: .....
2. Case ID: ☐☐☐☐☐☐ 3. Sample Code & ID: FS ☐☐☐☐ , NS ☐☐☐☐
4. Study site (SITE): 1. TVH; 2. Govt. Vet Clinics : .....
5. Age (AG): 1. Kid (< 6 month): ☐ 2. Adult (> 6 month): ☐
6. a) Sex (SE): 1. Male ☐ 2. Female ☐ b) Breed (BRE): 1. BBG ☐ 2. JP ☐ 3. CB ☐
7. Weight (WT): ☐ kg
8. Total no of goats of the owner (TGN): ☐
9. Owner's education (EDU) and profession (PRO):
10. Clinical Signs:
    a) Rectal temperature (TEMP) : ☐ °F
    b) Presence of diarrhea (PDI): 1. Yes ☐ 2. No ☐
    c) Duration of diarrhoea (DURD): ☐ day/month
    d) Presence of nasal discharge (PNDIS): 1. Yes ☐ 2. No ☐
    e) Coughing (COU): 1. Yes ☐ 2. No ☐
    f) Difficult breathing (BRA): 1. Yes ☐ 2. No ☐
    g) Any other signs:
11. Feeding Habit:
    a) Only stall feeding (FEE): 1. Yes ☐ 2. No ☐
    b) Any grazing opportunity (GRA): 1. Yes ☐ 2. No ☐
    c) Providing green grass (Daily) (GGR): 1. Yes ☐ 2. No ☐
    d) Jackfruit trees' leave provided (JTR): 1. Yes ☐ 2. No ☐
    e) Source of drinking water (WAT): 1.Pond ☐ 2. Tube-well ☐ 3. WASA-supplied ☐
12. Any other clinical complications / diseases (COD):
13. Duration of current illness (DUI):
14. History of any disease during last 3-4 months (HDI)
15. History of recent transportation (TRA):
16. Use of any antimicrobials in the past 15 days (ANM): 1. Yes ☐ 2. No ☐; if Yes name of antibiotic:
17. History of Vaccination (-----):1. Yes ☐ 2. No ☐
18. Write the name of those vaccines :
19. Deworming of goat (s) (------): 1. Yes ☐ 2. No ☐
20. *Body condition score (BCS): 1. ☐ **2.** ☐ **3.** ☐ **4.** ☐

***Body condition score (BCS):**

(+) = Good body condition (GBC)

(++) = Rough body condition and ribs are moderately visible (RBC)

(+++) = Cachectic, protruding rib with prominent pin bone, no diarrhea (CND)

(++++) = Cachectic, protruding rib with prominent pin bone, presence of diarrhea (CPD)

Annex-2: Picture gallery

Figure 02: Golden yellow colored colony on MSA

Figure 03: β- hemolytic colonies with golden pigmentation due to Staphyloxanthin on BA

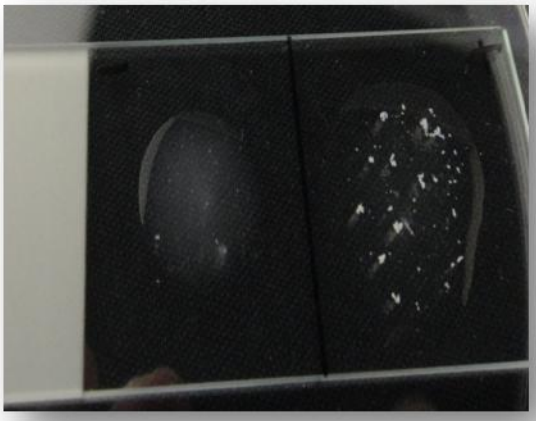

Figure 04: Positive isolates showed agglutination on slide coagulase test.

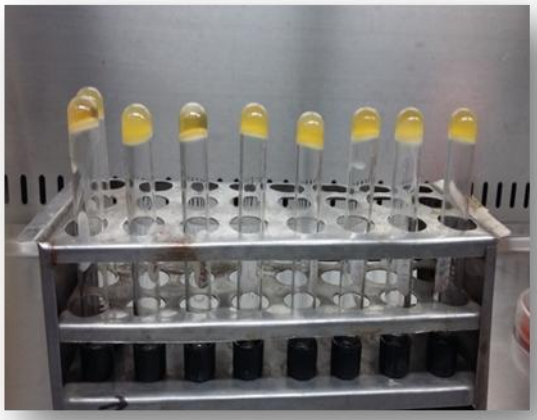

Figure 05: Due to heavy coagulation contents didn't come off even after inverting in tube coagulase test.

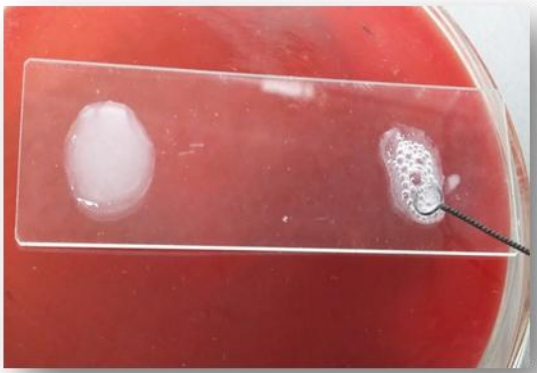

Figure 06: Formation of vigorous bubbles in catalase test

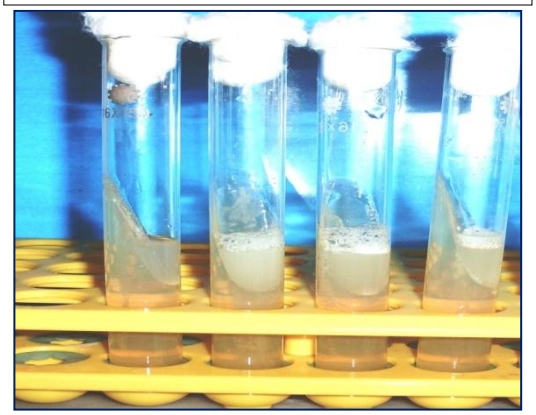

Figure 07: Tube catalase test for confirmation of *Staphylococcus sp*

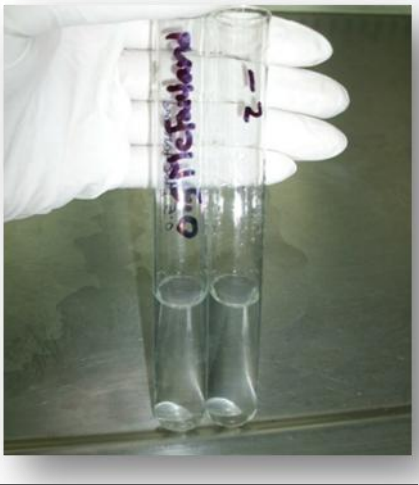

Figure 08: Turbidity checking of inoculums

Figure 09: streaking of bacterial inoculums

Figure 10: Inserting of Antibiotic disk onto the agar plate

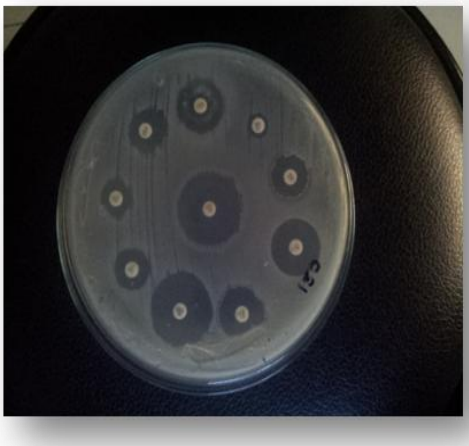

Figure 11: Isolate showed resistance by exhibiting zone of inhibition against using discs.

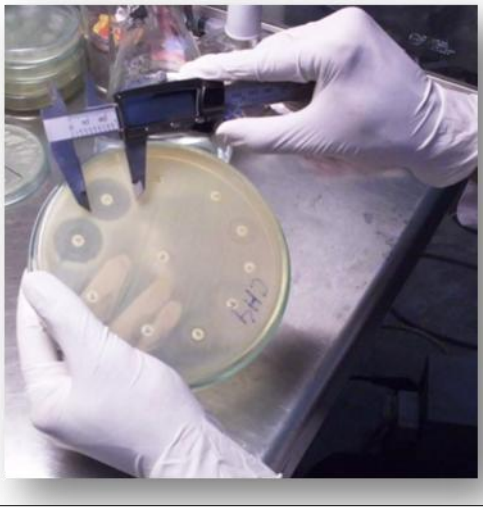

Figure 12: measuring zones of inhibition

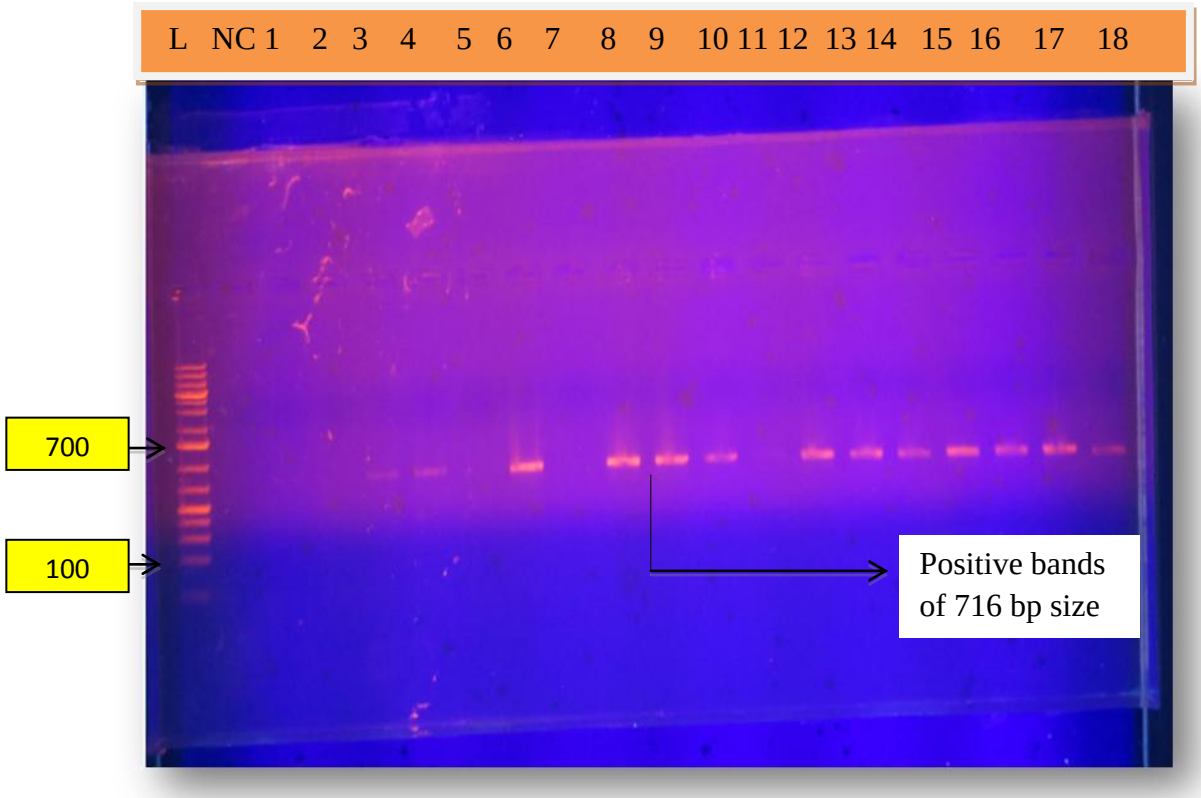

Figure 13: Results of PCR for *blaTEM* gene of *Salmonella sp* Lane L: 1 kb plus ladder; Lane NC: Negative control; Lane 1-18: 716 bp sized amplicons of *blaTEM* gene.

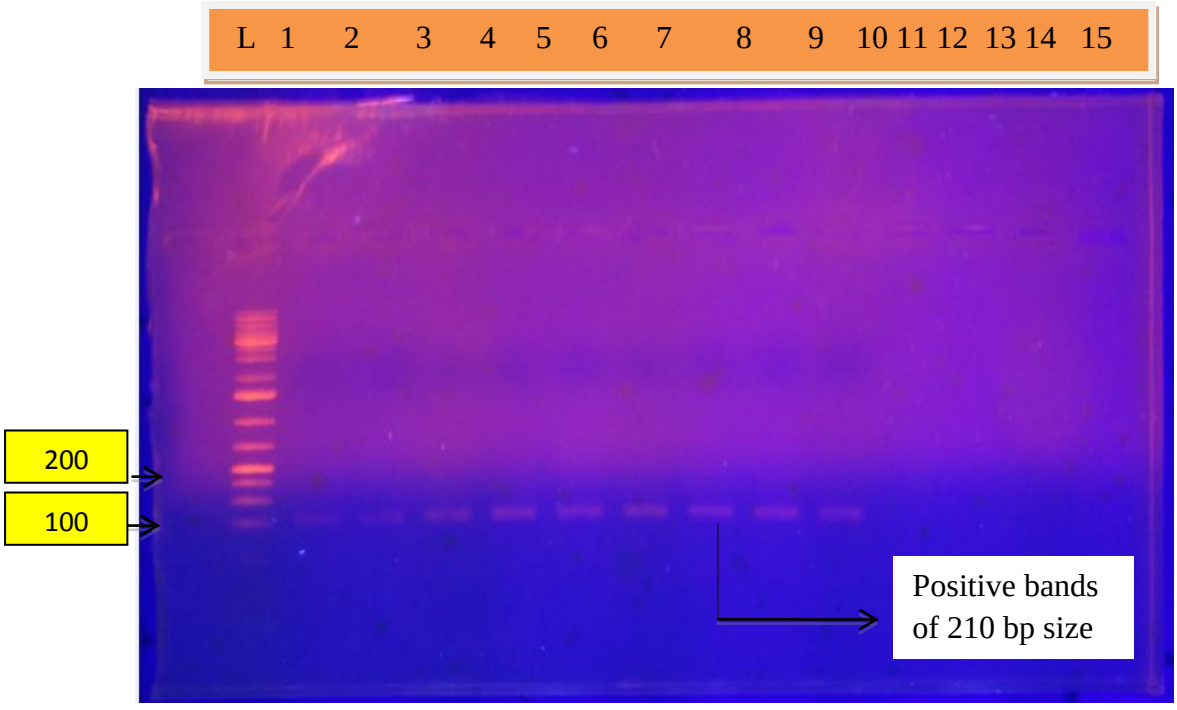

Figure 14: Results of PCR for *tet A* gene of *Salmonella sp* Lane L: 1 kb plus ladder; Lane NC: Negative control; Lane 1-18: 210 bp sized amplicons of *tetA* gene.

Brief Biography

Abu Hena Mostafa Kamal passed the Secondary School Certificate Examination in 2003 followed by Higher Secondary Certificate Examination in 2005. He obtained his Doctor of Veterinary Medicine Degree in 2010 (held in 2012) from Faculty of Veterinary and Animal Science, Sylhet Agricultural University (SAU), Bangladesh. Now, he is a Candidate for the degree of MS in Medicine under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, Chittagong Veterinary and Animal Sciences University (CVASU). He has immense interest to work with characterization of several bacterial pathogens along with antibiogram both in livestock and poultry.