ISOLATION, IDENTIFICATION & ANTIBIOGRAM PROFILING OF STAPHYLOCOCCUS AUREUS FROM FRESH COW MILK SAMPLE

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CERTIFICATE

This is to certify that the thesis entitled, **"ISOLATION, IDENTIFICATION & ANTIBIOGRAM PROFILING OF *STAPHYLOCCUS AUREUS FROM FRESH COW MILK SAMPLE''*** *submitted to the Department of Microbiology & Parasitology, Faculty of Animal Science & Veterinary Medicine, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE (MS) in Microbiology****, embodies the result of a piece of bonafide research work carried out by* ***Tanay Chakrabortty****, Registration No.* ***12-05046,*** *Session:* ***July-December/2018*** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated:
Dhaka, Bangladesh

_____

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DEDICATED TO
ASVM FAMILY

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ABSTRACT

Staphylococcus aureus is an important pathogen causing a wide spectrum of diseases in both humans & animals. *S. aureus* produces a variety of virulence factors that are liable for sub-clinical & persistent intra-mammary infections like mastitis. This research was suspected to isolation, identification & characterization of *S. aureus* causing bovine mastitis in cattle. The bacteria was isolated, identified & characterized by cultural, staining, morphological, & biochemical. The 12 isolates produced β hemolysis on 5% sheep blood agar & fermented on mannitol salt agar & aslo produced yellow colonies. In Gram staining, the organism found as gram positive, grapes like clusters formed by cocci. The isolates showed catalase & coagulase positive that confirmed as *S. aureus.* The antimicrobial susceptibility pattern of *S. aureus* isolates were investigated by disc diffusion method. In this study 42 fresh milk samples were obtained from three different points of Savar, Dhaka, Bangladesh like Savar Hat Area, Shimulia Hat Area & Bagbari Bazar Area & 12 samples yielded positive result for *S. aureus,* given an overall prevalence of 28.57%. The antibiotic susceptibility profile of the 12 *S. aureus* isolates revealed that the majority of the isolates were resistant against penicillin, erythromycin & amoxicillin. The isolates showed 100% resistant to penicillin & amoxicillin, while ciprofloxacin, oxicillin, cloxacillin & neomycin were found the most effective drug for the treatment of *S. aureus*.

Keywords: Isolation, Identification, Antibiogram, Bovine Mastitis, *Staphylococcus aureus*

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LIST OF ABBREVIATIONS

MSA	=	Mannitol Salt Agar
BA	=	Blood Agar
SAU	=	Sher-e-Bangla Agricultural University
µl	=	micro liter
ml	=	Milli liter
NA	=	Nutrient Agar
NB	=	Nutrient Broth
MSA	=	Muller Hinton Agar
Sl. No.	=	Serial No.
Prof.	=	professor
et al.	=	Associates
Vol.	=	volume
Gm	=	Gram
Fig.	=	Figure
S. aureus	=	*Staphylococcus aureus*
Spp.	=	species

CHAPTER 1

INTRODUCTION

Staphylococcus aureus is one of the most important microorganism in the process of fresh milk production & has significance for humans' health as it causes dangerous contamination of dairy production (Berhilevych *et al*., 2017). Food is considered as the most important energy & protein source for all living beings. Most of the foods contain viable microorganisms unless properly treated or it is an important medium for transmission of pathogenic organisms to the consumers. Contamination of food products with infective organisms may influence considerably their harmlessness, endanger to the health of consumers & decrease shelf quality, resulting in food-borne infections, intoxications & economic losses from food spoilage. *S. aureus* is a very important food born pathogen & causes a mild skin infection as well as severe diseases such as pneumonia & septicemia (Lowy *et al.,* 1998). Staphylococcal food poisoning is often associated with the ingestion of manually handled foods that contain one or more highly heat stable staphylococcal enterotoxins. The safety of milk with respect to food borne diseases is of vast concern in the world. This is especially true in developing countries where production of milk often takes place under unhygienic conditions & the consumption of raw milk which is typically produced in small dairy farms under unhealthy conditions is the most common practice (Le *et al.,* 2003). The ability of these microorganisms to survive under adverse conditions & to grow in the presence of low levels of nutrients & at suboptimal temperatures (Knife *et al*., 2007).

S. aureus is that the most prevalent & economically significant pathogen causing mastitis in dairy herds (Akineden *et al.,* 2001; Cabral *et al.,* 2004; Katsuda *et al.,* 2005). The organism is responsible for approximately 30%-40% of all mastitis cases (Asperger and Zangeri, 2003). *S. aureus* can get access to milk either by direct excretion from udders with clinical or subclinical Staphylococcal mastitis or by contamination from the surroundings during handling of raw milk (Scherrer *et al.,* 2004; Jorgensen *et al.,* 2005). When the mammary gland is infected, *S. aureus* may expel into milk in variable numbers starting from 0 to 10^8 CFU/ml (Asperger and Zangeri, 2003). It is the most common reason of community-associated cellulites, endocarditis & bacteremia. *S. aureus* strains were once nearly uniformly susceptible

to semi-synthetic penicillin resistant β-lactams (e.g. methicillin, oxacillin), the most commonly used class of antibiotics for skin infection. The *Staphylococcus aureus* is a Gram positive & produce smooth, convex, circular colonies reaching a size of 0.5-1.5 μm in diameter in Gram's stain. It looks an irregular three dimensional bunch of grapes-like clusters of cells under the microscope. In dependence on growth conditions, the colony pigmentation varies from grey, grey-white with yellowish to orange shades with typical β hemolysis on the blood agar (Deresse *et al.,* 2012; Sushma *et al.*, 2012).

Pathogenic strains are usually coagulase-positive & have been found to cause disease in their hosts throughout the planet. Diseases in cattle caused by *S. aureus* vary from simple abscesses & mastitis to the more severe toxic shock syndrome. Milk is a wonderful growth medium for a large number of micro-organisms, including *S. aureus*. Bacterial contamination of milk sometimes happens throughout the milking process & depends on the sanitary condition of the environment, utensils used for milking & the milking personnel. It could also result from micro-organisms that enter the udder through the teat opening point (Smith *et al.,* 2007). Mastitis caused by *S. aureus* is a serious problem in dairy production & infect animal may contaminate the bulk milk. In addition, human handlers, milking equipment, environment & udder as well as test skin of dairy animals are other likely source of bulk milk contamination. Food handlers carrying enter toxigenic *S. aureus* in theirs or in orifices such as the nose, are regarded as the major source of food contamination. Air, dust, and food contract surfaces can aslo serve as a vehicles for the transmission of *S. aureus* (Argudin *et al*., 2010).

Antibiotic-resistant *S. aureus* isolates cause a severe challenge to both veterinary & human health professions as well as dairy cattle producers because they have a negative impact on medical care. The usage of antibiotics correlates with the emergence & maintenance of antibiotic-resistant traits within pathogenic strains. These traits are coded by particular genes that may be carried on the bacterial chromosome, plasmids, transposons or on gene cassettes that are incorporated into integrons these are simply transferred among isolates. Multiple antibiotic resistant *S. aureus* strains have been isolated from milk obtained from cattle, beef & human samples in many parts of the world. The prevalence of antibiotic resistance usually

varies between isolates from the various source of samples & even between isolates from different herds on the same farm.

Determination of levels of *S. aureus* & an appraisement of the antibiotic-resistant phenotypes of the isolates could serve as a tool for determining the hygiene standards implemented during milking. Data on antibiotic resistance could also be used to characterize these opportunistic pathogens, which may further limit the risks related to the consumption of contaminated milk as well as its products (Wubete, 2004). Studying antimicrobial resistance in humans & animals is important for detecting changing patterns of resistance, implementing control measures on the use of antimicrobial agents & preventing the spread of multidrug resistant strains of bacteria (Ateba *et al*., 2010).

The milk & its products are extremely prone to various type of microorganisms, because of their high nutritive value & complex chemical composition. The biological changes produced by these organisms can be either desirable or undesirable. They may have a useful function in the preparation of soured milk products such as whey & cheese or they may have undesirable effects & produce changes in the odor, color, taste, texture or appearance of the food. In addition, most of these bacteria produce toxins & cause food poisoning frequently (Aycicek *et al.,* 2005). A recent report by International Center for Diarrheal Disease Research, Bangladesh also revealed that 75% of pasteurized milk is unsafe for direct consumption due to heavy bacterial load (Haque *et al*., 2018).

Therefore, the aim of this study was:

- To isolate & identify *S. aureus* from fresh milk samples of cow.
- To evaluate the antibiotic sensitivity pattern of the isolated *S. aureus*.

CHAPTER 2

REVIEW OF LITERATURE

The main purpose of this chapter is to get up-to-date information regarding the research work addressed here.

2.1 Isolation & Identification of *Staphylococcus aureus*

Abdrezzak *et al.* **(**2009) found that eighty-one samples of raw milk, whey & cheese were analyzed for the presence of *staphylococcal* strains. Isolates were identified by Gram stains, tests for coagulase, the API staph system and the Walk Away® 40/96, which also determines the antimicrobial susceptibility profiles.

Ingrid *et al.* **(**2009) studied fifty-four samples of raw milk for cheese making which were tested for the presence of *Staphylococcus aureus*. Eighty strains were identified as *S. aureus*.

Helena *et al.* **(**2010**)** mentioned the occurrence of *Staphylococcus aureus* in milk produced in 37 farms located in the regions of Ribeirao Preto & Sao Carlos, state of Sao Paulo, Brazil. Two-hundred & eight samples of milk from individual cows showing subclinical mastitis, 37 samples of bulk tank milk were analyzed. *S. aureus* strains were detected in 18 (7.3%) milk samples: 14 (6.7%) from samples of individual cows & 4 (10.8%) from bulk tank milk.

Mekonnen *et al.* **(**2011**)** observed the prevalence & distribution as well as characterization of the isolates to determine their ability in synthesizing coagulase from raw bovine milk samples & analyzed about 200 raw bovine milk samples consisting of 100 buckets milk of farms & 100 tanks from milk collection centers & found 33% & 46% prevalence of *Staphylococcus* in buckets milk & tanks milk, respectively with an overall prevalence of 39.5% (79/200). Comparison of the prevalence of Staphylococcus in raw bulk milk samples showed a relatively higher prevalence in tanks milk (46%) than buckets milk (33%).

Deresse *et al.* **(**2012**)** isolated a total of 78 *S. aureus* from milk during this study. The levels of contamination with *S. aureus* were higher in milk obtained from CCP1, CCP2, CCP3, CCP4 and CCP5 at Hawassa area farms 18.0%, 25.6%, 27.0%, 21.8% & 7.7% respectively.

Forough *et al.* **(**2012**)** studied that, a total of 348 raw milk samples from cow, sheep & goat were collected from randomly selected herds in Fars, Chahar Mahal va Bakhtiari & Ghom, provinces, Iran. Overall, 46 raw milk samples (13.2%) were found to be contaminated with *S. aureus*.

Iman *et al.* **(**2012**)** examined total 62 raw milk & human swab samples 18 (29.03%) were positive to *S. aureus*. *Staphylococcus aureus* was isolated from cow & buffalo, throat swabs & skin swabs with the following percentages 30.0 (6/20), 25 (5/20), 27.3 (3/11) and 36.4 (4/11), respectively. The survival of *Staphylococcus aureus* on various surfaces at room temperature suspended in physiological saline & nutrient broth was recorded for at least 35 days. Staphylococcal viability was longest on polyethylene (49 days), stainless steel & glass (42 days).

Matyi *et al.* **(**2013**)** isolated methicillin-resistant *Staphylococcus aureus* strains from milk of dairy cattle in a Paso Del Norte region dairy of the United States. Using physiological based identification schemes, a total of 40 *S. aureus* strains were isolated out of 133 samples analyzed.

Rahimi *et al.* **(**2013**)** studied an experiment on the isolation of *S. aureus* from milk samples from September 2010 to July 2011. A total of 200 cow (n = 50), sheep (n = 40), goat (n = 40), camel (n = 30) & buffalo (n = 40) bulk milk samples were collected from 46 randomly selected herds in Fars provinces, Iran & found that 22 of 200 raw milk samples (11.0%) were contaminated with *S. aureus*. The highest prevalence of *S. aureus* was found in buffalo milk (17.5%), followed by cow (16.0%), sheep (10.0%), goat (7.5%), & camel (3.4%).

Thaker *et al.* **(**2013**)** studied a total of 160 milk & milk product samples collected from different areas around Anand city such as milk collection centre of Co-operative milk dairies, cattle farms, individual household, milk vendors & sweet shops. The

samples were collected under aseptic conditions & were enriched in Peptone Water (PW) followed by direct plating on selective media viz. Baird-Parker Agar. The presumptive *S. aureus* isolates were identified by biochemical tests. Antibiogram pattern of *S. aureus* to antimicrobial agents were evaluated by disk diffusion method. Analysis of result revealed that out of total 160 samples of milk (100) and milk products i.e. curd (30) & pedha (30) resulted in the isolation of 10 isolates (6.25 %) of *S. aureus.*

Baloch *et al.* **(**2018**)** fresh milk samples were collected from cows presenting with clinical mastitis consistent with poor milk yield, color change & udders inflammation. Milk collection process was performed after cleaning the teats, initial streams of milk discarded & teat tips scrubbed with cotton balls moistened with 75% alcohol. Teat cleaning before milking. In total, one milk sample from each cow was collected & 195 cows were obtained from 2 dairy farms during August to December in 2016 in Beijing, China. The samples were isolated by cultural & biochemical test & incubated 37^0C temperature for 24-48 hours. Among them 46.2% showed positive result for *S. aureus.*

Haque *et al*. (2018) an aliquot of each sample inoculated into nutrient broth containing test tube & incubated for 6 hours for enrichment samples were then spread & streaked respectively onto the Staphylococcus medium No. 10 agar (Oxoid, England) & MSA (HiMedia, India), specific media for Staphylococcus spp. Growth & then incubated at 37^0 C for 48 hours. All the isolates were then subjected to subculture on mannitol salt agar for containing pure colony, gram staining & culture on blood agar for morphological confirmation. Catalase & coagulase test were done for biochemical confirmation.

2.2 Cultural, Biochemical & Staining Properties of *Staphylococcus* spp.

Abdrezzak *et al.* **(**2009) showed the predominance of coagulase-negative staphylococci (54 %). Coagulase-positive staphylococci that were identified were divided into 3 groups comprising *S. aureus* (40%), *S. intermedius* (2%) & *S, hyicus* (4%).

Stefano *et al.* **(**2009**)** Mentioned that 122 cultures were positive for the presence of coagulase test.

Helen *et al.* **(**2011**)** performed two-step enrichment for the growth of *S. aureus* on chromogenic agar, A MR-CNS were detected in 48.2% of samples from livestock & chicken carcasses, 46.4% of samples from bulk tank milk & minced meat, 49.3% from human samples. Using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), 414 selected MRCNS strains belonged to seven different species (*S. sciuri*, 32.6%; *S. fleurettii*, 25.1%; *S. haemolyticus*, 17.4%; *S. epidermidis*, 14.5%, *S. lentus*, 9.2%; *S. warneri*, 0.7%; *S. cohnii*, 0.5%). *S. sciuri* & *S. fleurettii* thereby predominated in livestock, BTM & minced meat samples, whereas *S. epidermidis* & *S. haemolyticus* predominated in human samples.

Kumar *et al.* **(**2011**)** reported the coagulase-positive methicillin resistant *Staphylococcus aureus* (MRSA) in healthcare workers & the prevalence of nasal colonization with *Staphylococcus aureus*. Different biochemical tests were done to isolate *S. aureus*. Species confirmation for *S. aureus* was done using the tube coagulase test.

Mekonnen *et al.* **(**2011**)** isolated 8%, *S. aureus* with 6% *S. intermedius*, 6% *S. hycius* & 13% Coagulase Negative Staphylococci (CNS). There was no significant difference ($p>0.05$) among these proportion of isolates in both buckets & tanks milk. All the isolates were tested for the production of coagulase to determine their pathogenicity. Comparison of the prevalence of Coagulase Positive Staphylococci (CPS) showed a relatively higher CPS prevalence in tanks milk (27%) than buckets milk (20%). However, this difference was not statistically significant ($p>0.05$). The high level of Staphylococcus isolate found raw milk samples in the present study represent a poor quality & public health risk to the consumer.

Islam *et al*. (2016) each isolated *S. aureus* samples were performed 10 fold dilution & were streaked onto 5% sheep blood agar (HiMedia®, India), incubated at 37^0 C overnight. Then the presumptive colonies of *S. aureus* were cultured onto mannitol salt agar (MSA) & then sub-cultured to get pure culture. These isolates were preserved for further bacterial identification. The isolates were identified as *S. aureus*

on the basis of Gram staining, colony morphology on mannitol salt agar (MSA) (HiMedia®, India), beta hemolytic patterns on blood agar, biochemical characterization of the isolates on catalase & coagulase tests. The pure colony of *S. aureus* were placed on the clean glass slide using sterile inoculation loop & a drop of respective reagents were added & mixed with the loop, then agglutination tests were performed. For catalase & coagulase tests 3% hydrogen peroxide & fresh rabbit plasma were used respectively. Each positive culture underwent Gram staining.

2.3 Antibiotic Resistance of *Staphylococcus aureus*

Abdrezzak *et al.* **(**2009) described the antibiotic resistance pattern of *S. aureus* from 81 milk samples. The antimicrobial susceptibility-profile of the staphylococcal strains revealed a high incidence of *S. aureus* to penicillin G. In addition, *Staphylococcus lentus* presented considerable resistance to the oxacillin, erythromycin & lincomycin.

Ingrid *et al***. (**2009) identified 80 strains as *Staphylococcus aureus* that were resistance to erythromycin, penicillin & ampicillin. *S. aureus* in raw milk for cheese making may constitute a risk with respect to staphylococcal food poisoning from raw milk products.

Collins *et al***. (**2010**)** established that the levels of contamination with *S. aureus* were higher in milk obtained from the communal farms in Lokaleng & Mogosane (24.6% and 35.4%, respectively) compared to the commercial farms in Rooigrond & Molelwane (17.9% and 22.1%, respectively). A large percentage of the *S. aureus* isolates (39%–100%) from both communal farms was resistant to methicillin (MT), ampicillin (AP), penicillin G (PG), sulphamethoxazole (Smx), oxytetracycline (OT), erythromycin (E), nitrofurantoin (NI) & streptomycin (S), but not vancomycin (V). An even higher percentage (64.2%–100%) of the isolates from both commercial farms was resistant to nitrofurantoin & sulphamethoxazole. A comparably smaller percentage (3.4%–4.7%) of the isolates from both communal farms was resistant to vancomycin, but all isolates from commercial farm milk were susceptible to this drug. When comparing the percentage of antibiotic resistance, a significant positive correlation was observed between the isolates from the commercial farms ($r = 0.966$, $p < 0.01$).

Rhee *et al.* **(**2010**)** isolated and identified 165 *Staphylococcus aureus* strains from different food samples between 2003 and 2006, were tested for antimicrobial susceptibility. Of the 165 *S. aureus* isolates, 150 strains (90.9%) were resistant to at least one antibiotic while no strain was resistant to vancomycin.

Haran *et al.* **(**2012**)** found high prevalence of methicillin-susceptible *S. aureus* (MSSA) was 84%, while MRSA herd prevalence was 4%. A total of 93 MSSA isolates & 2 MRSA isolates were recovered from 150 BTM samples. Antibiotic susceptibility testing of *S. aureus* isolates showed pan-susceptibility in 54 isolates, resistance to a single antibiotic class in 21 isolates, resistance to two antibiotic classes in 13 isolates, & resistance to ≥3 antibiotics classes & thus multi-drug resistance in 5 isolates. The two MRSA isolates displayed resistance to β-lactams, cephalosporins, lincosamides & were multi resistant. Seven isolates, including the two MRSA isolates, produced staphylococcal enterotoxins B, C, D, & E on overnight culture.

Kalmus *et al.* **(**2011**)** identified antimicrobial resistance in *Staphylococcus aureus*, including methicillin resistant *S. aureus (*MRSA), recovered from raw retail meat products purchased in the Washington, D.C., area. From March to August 2008, 694 samples of ground beef (n = 198), ground pork (n = 300), & ground turkey (n = 196) were collected by random sampling from stores of three grocery chains. In total, 200 *S. aureus* isolates (29%) were recovered by direct plating. When tested for susceptibility to 22 antimicrobials, 69% of the *S. aureus* isolates were resistant to tetracycline, 26% to penicillin, 17% to ampicillin, 13% to methicillin, 8% to erythromycin, 4.5% to clindamycin, 1.5% to gentamicin, and 0.5% to chloramphenicol, oxacillin, cefoxitin, or quinupristin dalfopristin. However, 27% of the isolates were susceptible to all tested antimicrobials.

Deresse *et al.* **(**2012**)** examined that all of 78 samples were contaminated with *S. aureus.* All strains were resistant to Penicillin G (PG) (10µg), Ampicillin (AP) (10µg), Amoxicillin-Clavulanic acid (AC) (30µg), Ciprofloxacin (CIP) (5µg), Erythromycen (E) (15µg), Cefriaxone (CRO) (30µg), Trimethoprim-Sulfamethoxa (TMP-SMZ) (25µg) Oxacillin (Ox) (1µg) & Vancomycin (V) (30µg), 67.9%, 70.9%, 30.9%, 0%, 32.1%, 23.1%, 7.7%, 60.3% & 38.5% respectively.

Forough *et al.* **(**2012**)** 46 raw milk samples (13.2%) were found to be contaminated with *S. aureus.* Antibiotic susceptibilities of the isolates were determined against 11 antimicrobial drugs by the disk diffusion assay. Most of the isolates (82.6%) were resistant to one or more antimicrobial agent. Six isolates (13.0%) were resistant to single antibiotic & 16 isolates (34.8%) showed resistance to 2 antimicrobial agents. Multi resistance was found in 34.8% of *S. aureus* isolates. Resistance (resistance and intermediate resistance) to ampicillin was the most common finding (54.3%), followed by resistance to oxacillin (28.3%), tetracycline (26.1%), penicillin G (23.9%), erythromycin (23.9%), trimethoprim-sulfamethoxazole (17.4%) & cephalotin (2.2%). All isolates tested for antibiotic sensitivity were susceptible to methicillin, vancomycin, chloramphenicol & ciprofloxacin.

Geidam *et al.* **(**2012**)** reported the prevalence of multi-drug resistant bacteria in apparently healthy chickens from 3 selected poultry farms in Selangor area of Malaysia. Antimicrobial sensitivity test was monitored with the disc diffusion assay against 12 antimicrobial agents. A total of 96 *S. aureus*, 48 *E. coli*, 7 *Pasteurella sp.* and 6 *Salmonella sp.* were isolated. *E. coli* & *Salmonella* spp. isolates were multi-drug resistant while 77.2% of *S. aureus* & 71.5% of *Pasteurella* spp. isolates were multi-drug resistant.

Mayer *et al.* **(**2012**)** studied the antibiotic resistance pattern of coagulase positive *S. aureus* (CPSA) isolated from nasal swabs of 100 slaughter pigs from one farm in Uruguay. Out of 69 animals, 71 CPSA were collected. No methicillin resistant *S. aureus* were detected. All CPSA were resistant to three or more classes of antimicrobials (i.e. multiresistant), whereby all CPSA were resistant to spectinomycin. Most of the isolates (46%) were resistant to six classes of antimicrobials. Almost all isolates were resistant to penicillin (99%), ampicillin (99%), gentamicin (96%), tetracycline (90%) & tilmicosin (87%).

Thaker *et al.* ***(***2013**)** found the isolated *S. aureus* of which highest sensitivity towards cephalothin (100.00%), co-trimoxazole (100.00%), cephalexin (100.00%) and methicillin (100.00%) followed by gentamicin (90.00%), ciprofloxacin (80.00%), oxacillin (70.00%), streptomycin (60.00%) and ampicillin (60.00%). The pattern clearly indicated that the overall high percent of *S. aureus* isolates were resistant to

Penicillin-G (100.00%) followed by ampicillin (40.00%), oxytetracycline, oxacillin (20.00%), streptomycin & gentamicin (10%).

Berhilevych *et al*. (2017), the of the present study was to establish antibiotics sensitivity profiles of *S. aureus* depending on location of dairy farm. A total 165 samples were collected for investigation during 2014-2014 at Ukraine & Culture in the Baird Parker Agar with Egg Yolk Tellurite Emulsion. The standard disk diffusion method was used to determine sensitivity of *S. aureue* isolates to 10 antibiotics. The result demonasted that that most of *S. aureus* resistant to penicillin, oxaciline & vancomycin.

Baloch *et al*. (2018) in this study, broth dilution method was applied to estimate the antimicrobial susceptibility of all tested isolates using the Biofosum® gram positive panel (Fosum Diagnostics, Shanghai, China) & interpreted by the Clinical and Laboratory Standards Institute (CLSI, 2015). The antimicrobial agents included Ceftior (EFT) (0.25-64 μg/ml), Choloramphenicol (CHL) (0.5-128 μg/ml), Ciprofloxacin (CIP) (0.125-16 μg/ml), Daptomycin (DAP) (0.06-16 μg/ml), Enrofloxacin (ENO) (0.125-32 μg/ml), Erythromycin (ERY) (0.125-16 μg/ml), Gentamycin (GEN) (0.5-64 μg/ml), Penicillin (PEN) (0.06-32 μg/ml) & Tetracycline (TET) (0.25-64 μg/ml) *S. aureus* ATCC™29213 was used as the reference strain for the AST.

Regasa *et al*. (2019) the anti-microbial susceptibility profile of *S. aureus* isolates was performed using disc diffusion method. The diameters of growth inhibition zone were interpreting & recorded as susceptible, intermediate & resistant to the recommended given by CLSI (2017). For the susceptibility testing, the followings antimicrobial drugs (OXOID, England) were used. Amoxicillin (AMX) (25μg), Ampicillin (AM) (10μg), Penicillin (P) (10μg), Tetracycline (TE) (30μg) & Erythromycin (ER) (15μg). Drug selection on the basis of their accessibility & habitual uses.

CHAPTER 3

MATERIALS & METHODS

3.1 Study Period & Working Place

The research work was conducted at the laboratory of the Department of Microbiology & Parasitology, Sher-e-Bangla Agricultural University, Dhaka- 1207, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka during the period of July 2018 to June 2019.

3.2 Materials

3.2.1 Area of the Study & Collection of Samples

A cross-sectional study was designed to investigate the bacteriological analysis of fresh cow milk samples at Savar Hat Area, Shimulia Hat Area & Bagbari Bazar Area from July 2018 to June 2019. A total of 42 fresh cow milk samples were collected. All of these were from hybrid herd, average age of 6-8 years old & the milking technique was manual.

3.2.2 Transportation of the Samples

Approximately 10 ml of fresh milk were collected from three different areas by using sterile test tubes. The sample were transported aseptically wrapped in polythene bags on icebox to the Microbiology Laboratory of the BLRI for analysis. Upon arrival in the Laboratory, Milk samples were kept in an incubator at 37°C until they were cultured in Blood agar (Himedia, India).

Table 1. No. of Milk Samples Collected from Three different Areas of Savar

Sl. No.	Sampling Point	No. Collected
1	Savar Hat Area	16
2	Shimulia Hat Area	14
3	Bagbari Hat Area	12
Total		42

3.3 Brief Description of the Detail Experimental Design Stated in the Flow Diagram

Fresh milk samples were collected from the cows of three different areas of Savar. The samples were first inoculated on to 5% sheep blood agar by spreading method. The isolated organisms found to be β hemolysis were further subculture to get pure culture of *S. aureus*. Later the isolates were characterized by cultural characteristics on Staphylococcal selective media (MSA). Then Gram's staining & biochemical tests. Finally the isolated organisms were subjected to antibiotic sensitivity test to observe the resistant characteristics of organism on some specific antibiotic disk.

Experimental Design: The Following is a Flow Chart of Representing Design of the Experiment

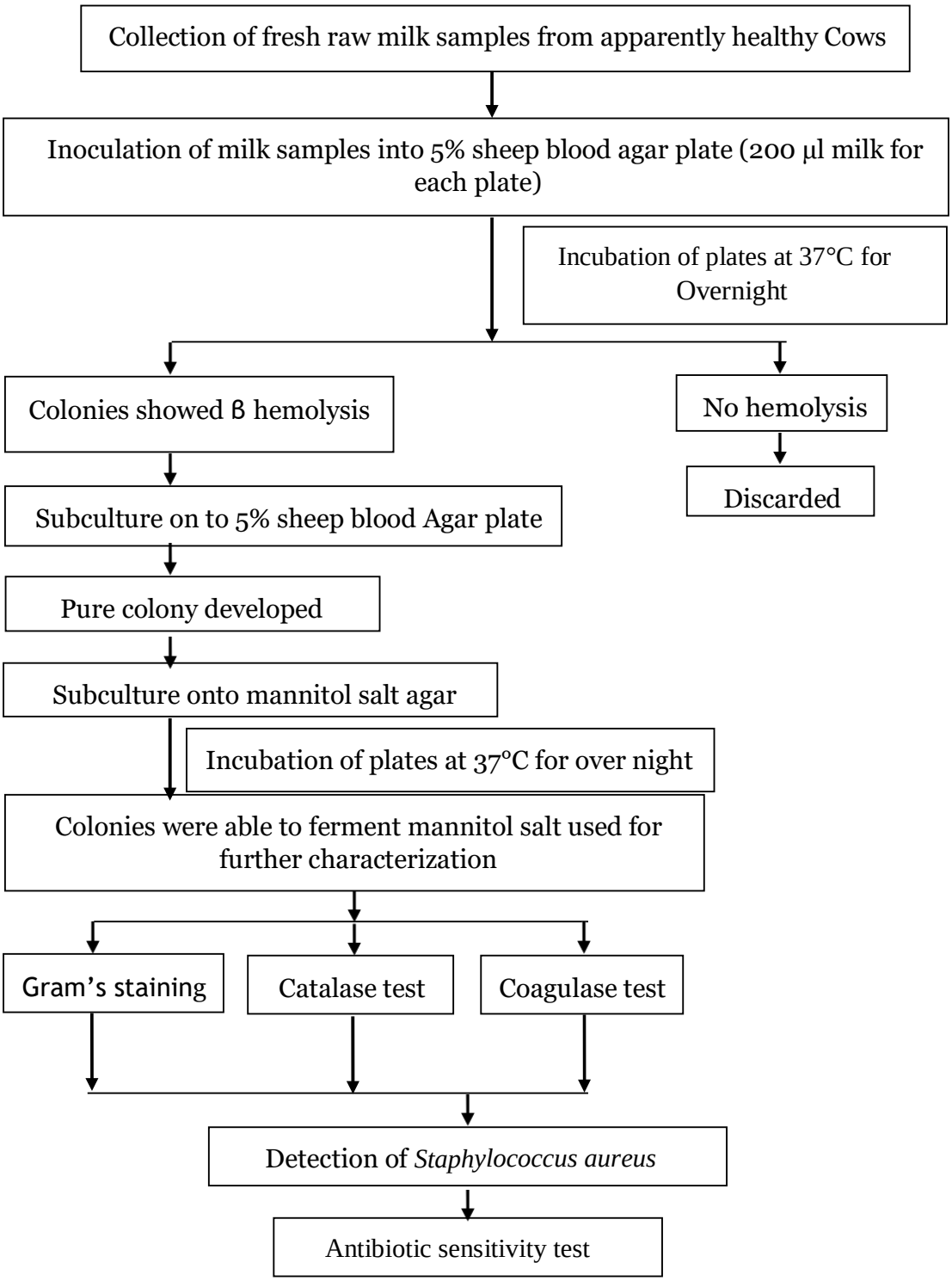

Figure 1. Schematic Illustration of the Experimental Design

3.4 Media for Bacteriological Study

3.4.1 Blood Agar

Blood agar (Himedia, India) enriched with 5% sheep blood was used to observe the hemolytic property of *S. aureus* and other than *S. aureus*. 23 grams of dehydrated blood agar was suspended in 100 ml of distilled water & boiled until dissolved properly. It was then sterilized by autoclave at 121^0 C, 15 minutes less than 15 pounds pressure per square inch (1 kg/cm^2). After autoclaving, the medium was allowed to cool down at 45^0 C in water bath and then 5% defibrinated sheep blood was added. The medium was then poured in the sterile petridishes (75mm diameter) in a volume of 10 ml quantities in each to form thick layer & was kept at room temperature for solidification. After solidification, sterility of the media was checked by incubating at 37^0 C in the incubator for 2 hours & those found sterile were kept at $(4\text{-}8)^0$ C until used.

3.4.2 Mannitol Salt Agar (MSA)

Mannitol salt agar was used as a selective medium for the identification of *S. aureus* organisms. MSA agar media was prepared according to the instruction of (Himedia, India) Manufacturer Company. 11.1grams powder of mannitol salt agar base was suspended in 100 ml of distilled water. The suspension was heated until to boil for few minutes to dissolve the powder completely in water. The medium was autoclaved for 30 minutes, 15 lbs. pressure per square inch (1 kg/cm ^{2} to make it sterile. After autoclaving the medium was put into water bath maintaining 45°C & 10-20 ml of medium was poured into small or medium size sterile petridish to make MSA plates. After solidifying the medium, the plates were kept in the incubator at 37°C for overnight to check its sterility.

3.4.3 Muller Hinton Agar (MHA)

Muller Hinton Agar plates were specially used for the antibiotic sensitivity test which was prepared according to the instruction of Manufacturer Company (Himedia, India). 38 grams of dehydrated Muller Hinton Agar Medium was suspended in 1000 ml cold

distilled water and boiled to dissolve the medium completely. The solution was then sterilized by autoclaving at 121^0 C, 15 lbs. pressure for 15 minutes. The autoclaved materials were allowed to cool to a temperature of 45^0 C in a water bath & distributed to sterile petridishes. After solidification petridishes were placed in an incubator for 24 hours at 37°C to check sterility & then placed in a refrigerator at 4^0 C until use.

3.4.4 Nutrient Broth & Nutrient Agar

Nutrient broth & Nutrient Agar were used for the primary growth of *S. aureus* which were prepared according to the instruction of Manufacturer (Merck Specialties Private Limited) company. For nutrient broth 13 gm of dehydrated nutrient broth (NB) base (Himeda, India) was dissolved in 1000 ml of distilled water, heated gently by an electric heater and then sterilized by autoclaving at 121^0 C , 15 lbs. pressure per square inch ($1kg/cm^2$) for 15 minutes (1 kg/cm^2). 10ml broth was transferred in sterile tubes & then stored at 4°C in the refrigerator until use.

For nutrient agar 2.5gms of Bacto-Nutrient Agar (Difco) was suspended in 100 ml distilled water and boiled to dissolve completely. The solution was sterilized by autoclaving at 121 ^{0} C at 15 lbs. per sq. inch (1 kg/cm^2) for 15 minutes. And autoclaved. The medium was poured in 10 ml quantities in sterile petridishes (75 mm diameter) to form a thick layer and allowed to solidify. The sterility of the medium was checked by incubating overnight at 37^0 C & then the plates were stored at $(4\text{-}8)^0$C for future use.

3.5 Glass Ware & other Necessary Instruments

During the study period following glass ware & other instruments were used:

- ✓ Sterile test tubes
- ✓ Petri-culture dishes
- ✓ Pipette
- ✓ Slides
- ✓ Conical flasks
- ✓ Glass spreader
- ✓ Bacteriological loop
- ✓ Bacteriological incubator
- ✓ Electric balance
- ✓ Spirit lamp
- ✓ Sterilized cotton
- ✓ Light microscope &
- ✓ Immersion oil.

3.6 Chemicals & Reagents

- Phosphate buffered saline (PBS) solution
- Reagents for Gram's staining (Crystal violet, Gram's iodine, Acetone alcohol & Safranin)
- 3% Hydrogen peroxide
- Normal saline solution
- Rabbit plasma
- 50% buffered glycerin
- Alcohol
- Common laboratory chemicals, reagents & distilled water were used for this research.

3.7 Antibiotic Disc

Commercially available antibiotic disc (Oxoid, England) was used for the test to determine the drug sensitivity pattern. The test was performed using disc diffusion method .This method allowed for the rapid determination of the efficacy of the drugs by measuring the diameter of the zone of inhibition that resulted from different diffusion of the agent into the medium surrounding the disc. The following antibiotic discs were used:

Table 2. Lists of Antibiotics & Their Dosage

Name of the antibiotics	**Dose (µg/disc)**
Penicillin (P)	10
Erythromycin (E)	15
Neomycin (N)	30
Amoxicillin (AML)	10
Cloxacillin (OB)	5
Ciprofloxacin (CIP)	5
Oxicillin (OX)	5

µg= micro gram

3.8 Methods

3.8.1 Isolation of *Staphylococcus aureus* from Fresh Cow Milk Sample

Ten-fold serial dilutions were performed using 2% peptone water & aliquots of 200µl from each dilution were spread plated 5% sheep blood agar (Supplied by Hi media company, India). The plates were incubated aerobically at 37°C for overnight. *S. aureus* colonies that produced β hemolysis further purified by sub-culturing onto Blood agar plates & the plates were incubated aerobically at 37°C for overnight. Other than *S. aureus* could not produce β hemolysis were discarded. These isolates were preserved for further bacterial identification & inoculate on to MSA plate for further characterization.

3.8.2 Microscopic Identification by Gram's Staining Method

According to methodology all suspected cultures of *Staphylococcus* spp. were subjected to Gram's staining & observed under a light microscope for Gram's reaction, size, shape & arrangements. The gram-stained smears from typical colonies that showed gram-positive cocci occurring in bunched, grape like irregular clusters & stained as violet color were taken as presumptive *S.* spp. The *Staphylococci* colonies were characterized morphologically using Gram's stain according to the method described (Deresse *et al.*, 2012). Briefly, a small colony was picked up from mannitol salt agar with a bacteriological loop, smeared on separate glass slide with a drop of distilled water & fixed by gentle heating. Crystal violate (Himedia, India) was then applied on each smear to stain for two minutes followed by washing with running water. Few drops of Gram's Iodine (Himedia, India) was then added, which acted as mordant for one minute & then washed with running water. Acetone alcohol (Himedia, India) was then added for few seconds. After washing with water, safranin was added as counter stain & allowed to stain for two minutes. The slides were then washed with water, dried in air & then examined below light microscope with high power objective (100X) using immersion oil.

3.8.3 Biochemical Studies for the Identification of *Staphylococcus aureus* Isolates

Catalase Test:

To perform the test 2-3ml of 3% H_2O_2 was poured into a test tube. Pure culture of the isolates were picked up by a sterile loop from the agar plate & mixed with a drop of 3% H_2O_2 on a clean glass slide. If the organism was positive, bubbles of oxygen were liberated & formed bubble within a few seconds, the negative isolates did not produce any bubbles. The catalase positive cocci were considered as staphylococci.

Coagulase Test:

For the coagulase test, 0.5 ml of rabbit plasma was diluted with sterile physiological saline (1:5) separately in two different test tubes. The tubes contain an equal volume of 24 hours old *Staphylococcal* cultured broth & incubated at 37°C for 4 hours & tubes were examined after 2-4 hours for detecting the presence of clots of plasma. The negative tubes were left at room temperature for overnight & re-examined. A simple slide coagulase test was also performed by mixing an equal volume of freshly cultured broth with rabbit plasma on a glass slide. A positive result was indicated by macroscopically clumping of the bacterial cells within 5 seconds because fibrinolysin enzyme lysis the rabbit plasma. Pathogenic *Staphylococci s*howed coagulase-positive result & formation of clotting occur whereas nonpathogenic *Staphylococci* showed no clotting.

3.9 Antibiotic Sensitivity Test

Antibiotic susceptibility tests were performed against all *S. aureus* isolates to determine their antibiotic-resistance profiles. For susceptibility test, a pure culture of all *S. aureus* were taken & transferred to a tube containing 5ml of sterile normal saline & mixed gently to make homogenous suspension. The bacterial suspension was inoculated onto Mueller Hinton agar by sterile cotton swab. Susceptibilities of the isolates to a panel of seven completely different antibiotic discs (penicillin, erythromycin, cloxacillin, amoxicillin, oxicillin, neomycin, ciprofloxacin) were determined. Antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar to confirm intimate contact with the surface & the plates were incubated

aerobically at 37°C for 24 hours. Inhibition of zone diameters were measured & values obtained from the National Committee on Clinical Laboratory Standards were used to interpret the results obtained. *S. aureus* isolates were then classified as resistant, intermediate resistant or susceptible to a particular antibiotic based on the standard interpretation table (Table 2) updated according to the Clinical & Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013). Multiple drug resistant (MAR) phenotypes were recorded for isolates showing resistance to three & more antibiotics.

Table 3. Zone diameter Interpretive Standards for *Staphylococcus* spp. (According to the CLSI, 2013).

	Resistant	**Intermediate**	**Sensitive**
Amoxicillin	≤ 13	14 - 17	≥ 18
Ciprofloxacin	≤ 15	16 - 20	≥ 21
Erythromycin	≤ 13	14 - 22	≥ 23
Neomycin	≤ 10	11 - 12	≥ 13
Cloxacillin	≤ 12	-	≥ 21
Penicillin	≤ 14	-	≥ 15
Oxacillin	≤ 10	11 - 12	≥ 13

Sl.= Serial, No.=Number, ≤ = Less than or equal, ≥ =Greater than or equal

3.9.1 Inoculation of Test Plates

1. 15 minutes after adjusting the turbidity of the inoculums suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times & pressed firmly on the inside wall of the tube above the fluid level. This removed excess inoculums from the swab.

2. The dried surface of a Muller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, as a final step, the rim of the agar is swabbed.

3.9.2 Application of Discs to Inoculated Agar Plates

1. The predetermined battery of antimicrobial discs was distributed onto the surface of the inoculated agar plate using sterile forceps. Every disc was pressed down to ensure complete contact with the agar surface. Whether the discs ware placed one by one or with a dispensing apparatus, they were distributed equally so that they are no closer than 24 mm from center to center. Ordinarily, no more than twelve discs should be placed on one 150 mm plate or more than five discs on a 100 mm plate. Because some of the drug diffuses almost instantaneously, a disc should not be relocated once it has come into contact with the agar surface. Instead, place a new disc in another location on the agar. During this case seven discs were placed on media containing isolated organisms.

2. The plates were inverted & placed in an incubator set to 35°C within 15 minutes after the discs were applied kept for nightlong incubation.

3.9.3 Reading Plates & Interpretation of Results

1. After overnight incubation, every plate was examined. If the plate was satisfactorily streaked & the inoculums were correct, the resulting zones of inhibition will be uniformly circular & there will be a confluent lawn of growth. If individual colonies are apparent, the inoculums were too lightweight & the test must be repeated. The diameters of the zones of complete inhibition (as judged by the unaided eye) are measured, as well as the diameter of the disc. Zones were measured to the nearest whole millimeter, using a ruler, which is held on the back of the inverted petridish.

2. The zone margin ought to be taken because the area showing no obvious, visible growth which will be detected with the unaided eye. Faint growth of tiny colonies, which can be detected only with a magnifying lens at the edge of the zone of inhibited growth, is ignored. However, discrete colonies growing within a clear zone of inhibition should be sub cultured, re-identified & retested.

3. The sizes of the zones of inhibition were interpreted by referring to Table 4 (Zone Diameter Interpretative Standards) of the CLSI (2013): Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement & the organisms are reported as susceptible, intermediate or resistant to the agents that have been tested.

3.10 Maintenance of Stock Culture

- Stock culture required for the similar analysis. So stock culture was mixed with a medium prepared by 1ml of 50% sterilized glycerol in 1ml of pure culture in nutrient broth & was stored -20^{0}C for further use.
- By this method, bacteria can be preserved with no deviation of its original characters for several years.

CHAPTER 4

RESULTS & DISCUSSION

A total 42 fresh milk samples from three different areas were bacteriologically examined, resulted positive for *Staphylococcus aureus* & other species of *Staphylococcus* bacteria. The results of isolation & identification of *Staphylococcus aureus* are presented under the following steps-

4.1 Results of Cultural Examination

4.1.1 Culture on Blood Agar

Among 42 samples, 12(28.57%) were showed β hemolysis on 5% sheep blood agar with circular, small, smooth raised with yellowish in color colony.

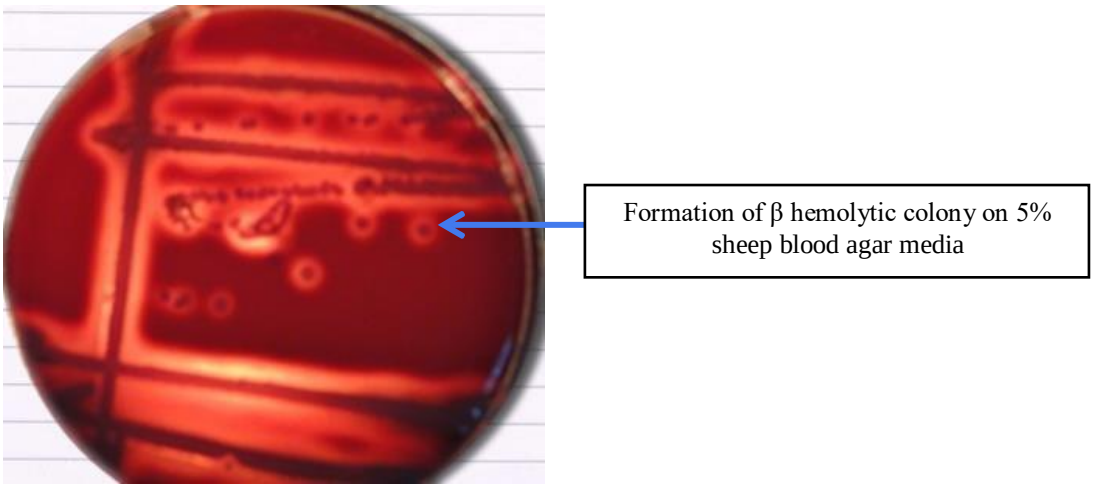

Figure 2: β hemolysis in 5% sheep blood agar by *S. aureus*

4.1.2 Culture on Mannitol Salt Agar Plate

After night long incubation on MSA media, some plates showed yellow colony & some plates showed whitish colony. All the suspected *S. aureus* that produced β hemolysis on 5% blood agar were able to ferment mannitol salt agar characterized by the formation of yellow colony & white colony indicated different *Staphylococcus* spp.

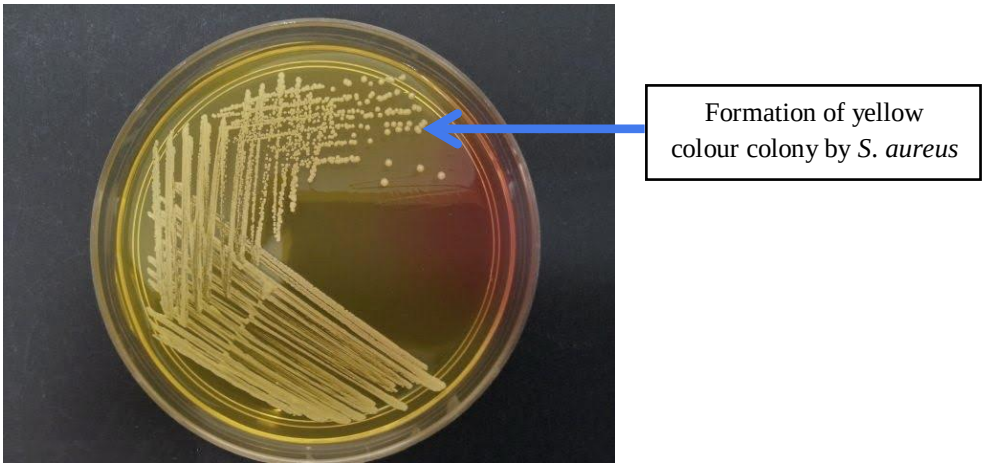

Figure 3: Fermentation of mannitol salt agar by *S. aureus*

4.1.3 Culture in Nutrient Broth

Diffused turbidity in Nutrient broth was characterized by the growth of *Staphylococcus* spp.

4.1.4 Culture on Nutrient Agar

On nutrient agar media small, circular & smooth raised white colonies formed by *Staphylococcus aureus* were observed.

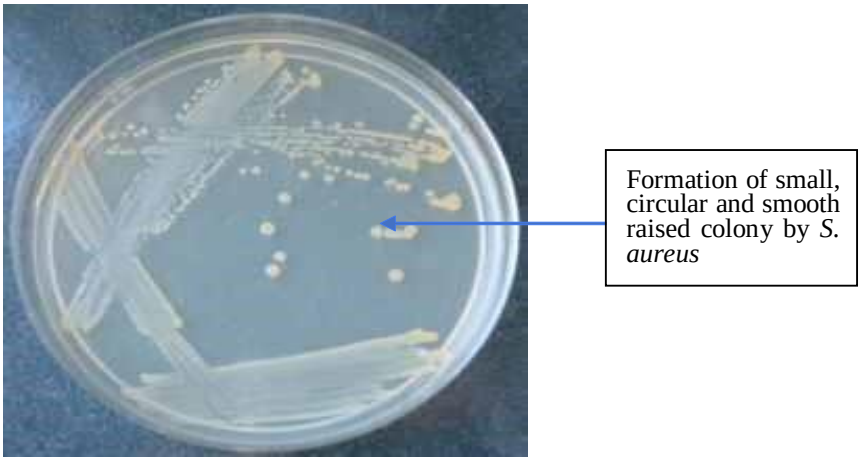

Figure 4: Growth of *S. aureus* on Nutrient agar media

4.2 Results of Microscopic Examination

In Gram's staining, the organism showed Gram positive, violet colored, cocci shaped & arranged in grapes like cluster below the light microscope (100x).

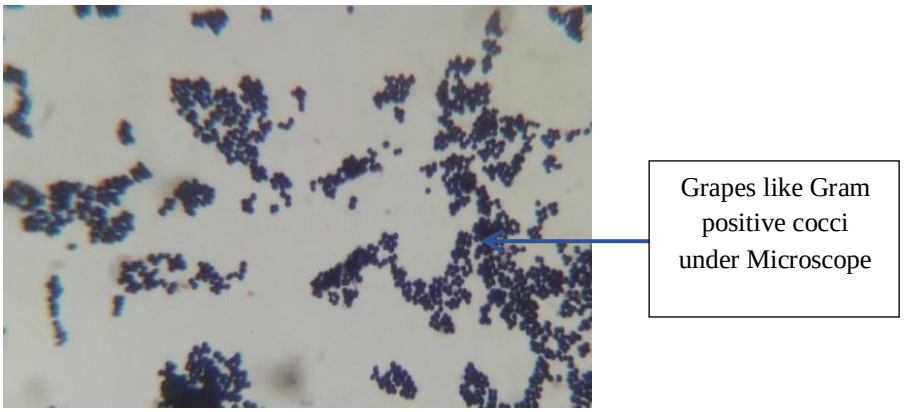

Figure 5: Gram's staining of *S. aureus* (100x)

4.3 Results of Biochemical Tests

4.3.1 Results of Catalase Test

Catalase test was performed to differentiate *Staphylococci* (catalase producer) from *Streptococci* (non-catalase producer). H_2O_2 was breakdown into water & oxygen. Production of oxygen was indicated by bubble formation. All *Staphylococcus aureus* isolates were catalase positive. 12 (28.57%) samples were found catalase positive, whereas the negative control did not turn out any bubble. The catalase test was done by slide & tube method.

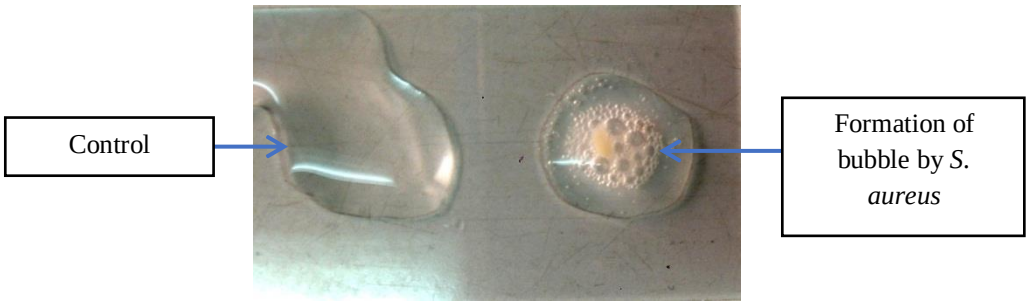

Figure 6: Slide catalase test of *Staphylococcus aureus*

4.3.2 Result of Coagulase Test

A complete of 12(28.57) isolates of *Staphylococci* gave positive reaction in coagulase test that indicated the isolates are *S. aureus.* The positive result was confirmed by the formation of curd like clotting whereas negative control showed no formation of curd like clotting.

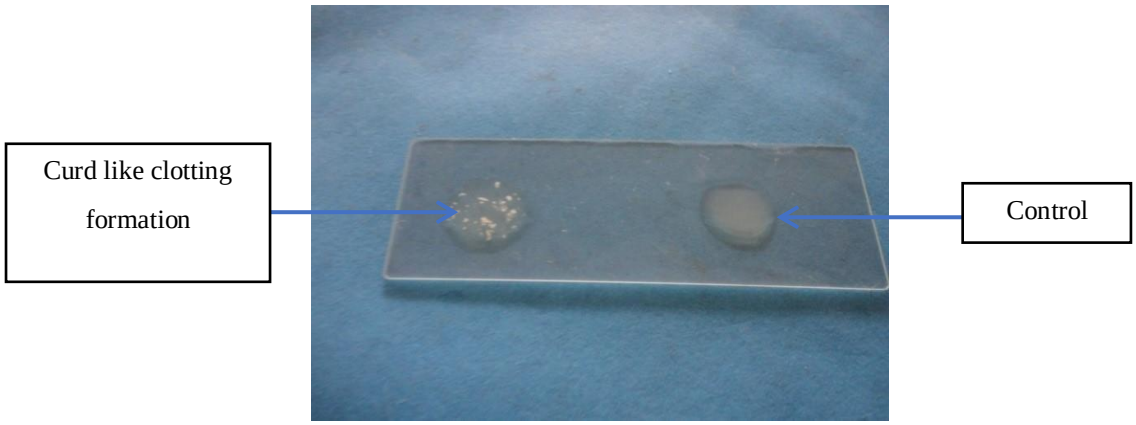

Figure 7: Coagulase test (Slide test).

Left: Coagulation of plasma by *S. aureus*

Right: Coagulase negative

Table 4. The Summary of the results of laboratory examination of *Staphylococcus* spp. in different cultural media

No of samples	Properties of *S. aureus*				
	5% Sheep Blood Agar	**Mannitol Salt Agar**	**Catalase Test**	**Coagulase Test**	**Gram's Staining**
	Small, Circular, smooth raised yellowish colonies with β hemolysis.	**Small, Circular, Yellowish colonies with fermentation**	**Bubble formation**	**Formation of curd like clotting**	**Gram positive, Blue colored, cocci shaped & arranged in grape like cluster under electron microscope**
RM- 1	Do	Do	Do	Do	Do
RM- 2	Do	Do	Do	Do	Do
RM- 3	Do	Do	Do	Do	Do
RM- 6	Do	Do	Do	Do	Do
RM- 9	Do	Do	Do	Do	Do
RM- 10	Do	Do	Do	Do	Do
RM- 20	Do	Do	Do	Do	Do
RM- 21	Do	Do	Do	Do	Do
RM- 26	Do	Do	Do	Do	Do
RM- 33	Do	Do	Do	Do	Do
RM- 36	Do	Do	Do	Do	Do
RM- 41	Do	Do	Do	Do	Do

4.5 Prevalence of *S. aureus*

A total of 42 raw milk samples directly collected from cattle to three different areas of Savar. Out of 42 samples of raw milk were 12 samples (28.57%) were found to be positive for *S. aureus*

Table 5. Prevalence of *S. aureus* in different areas of cattle

Sl. No	Sources & Location	Total no. of Milk Samples Collected	Number of samples positive for *S. aureus*	Prevalence%
1	Savar Hat Area	16	6	37.50%
2	Shimulia Hat Area	14	3	21.43%
3	Bagbari Bazar Area	12	3	25%
Total		42	12	28.57%

The Difference of prevalence in different locations were not statistically significant.

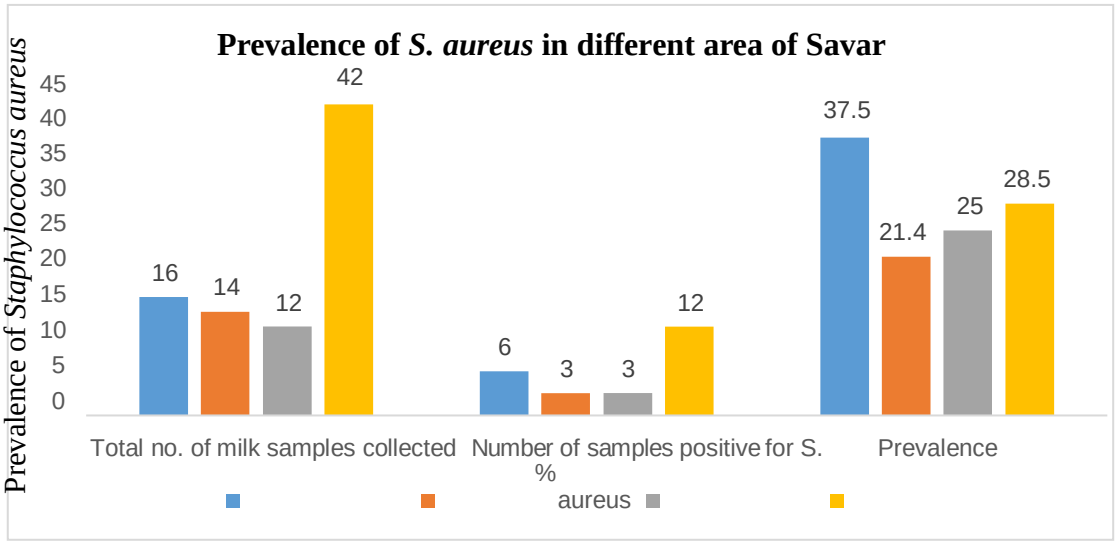

Figure 8: Prevalence of *S. aureus* in different areas cattle of Savar

4.6 Antibiotic Sensitivity Profile

The antibiotic sensitivity profiles are studied for the coagulase positive *S. aureus* that isolates from the raw cow milk samples. According to the CLSI,2013 some *S. aureus* isolates were found resistant against penicillin & amoxicillin (100%), *S. aureus* 75% showed resistant to erythromycin & 25% intermediate sensitive, this pathogen was found to be 33.33% sensitive & 66.67% intermediately sensitive to neomycin respectively. The positive *S. aureus* isolates showed 83.33% sensitivity & 16.67% intermediate sensitivity to cloxacillin antibiotic, 58.33% sensitive, 25% intermediate sensitive & 16.67% resistance to ciprofloxacin antibiotic. Finally, In case of oxacillin antibiotic the isolated *S. aureus* were 100% sensitive.

Table 6. Results of Antimicrobial Susceptibility Test of *S. aureus*

Sample no.	LIST OF ANTIBIOTICS						
	P	**E**	**N**	**AML**	**OB**	**CIP**	**OX**
RM- 1	R	I.S	I.S	R	I.S	S	S
RM- 2	R	R	I.S	R	I.S	S	S
RM- 3	R	R	S	R	S	I.S	S
RM- 6	R	R	I.S	R	S	I.S	S
RM- 9	R	R	I.S	R	S	S	S
RM- 10	R	R	S	R	S	S	S
RM- 20	R	R	S	R	S	S	S
RM- 21	R	I.S	I.S	R	S	R	S
RM- 26	R	I.S	I.S	R	S	S	S
RM- 33	R	R	I.S	R	S	S	S
RM- 36	R	R	I.S	R	S	I.S	S
RM- 41	R	R	S	R	S	R	S

RM= Raw Milk, P= Penicillin, E= Erythromycin, N= Neomycin, AML= Amoxicillin, OB= Cloxacillin, CIP= Ciprofloxacin, OX= Oxicillin and R= Resistant, I.S= Intermediate Sensitive, S= Sensitive.

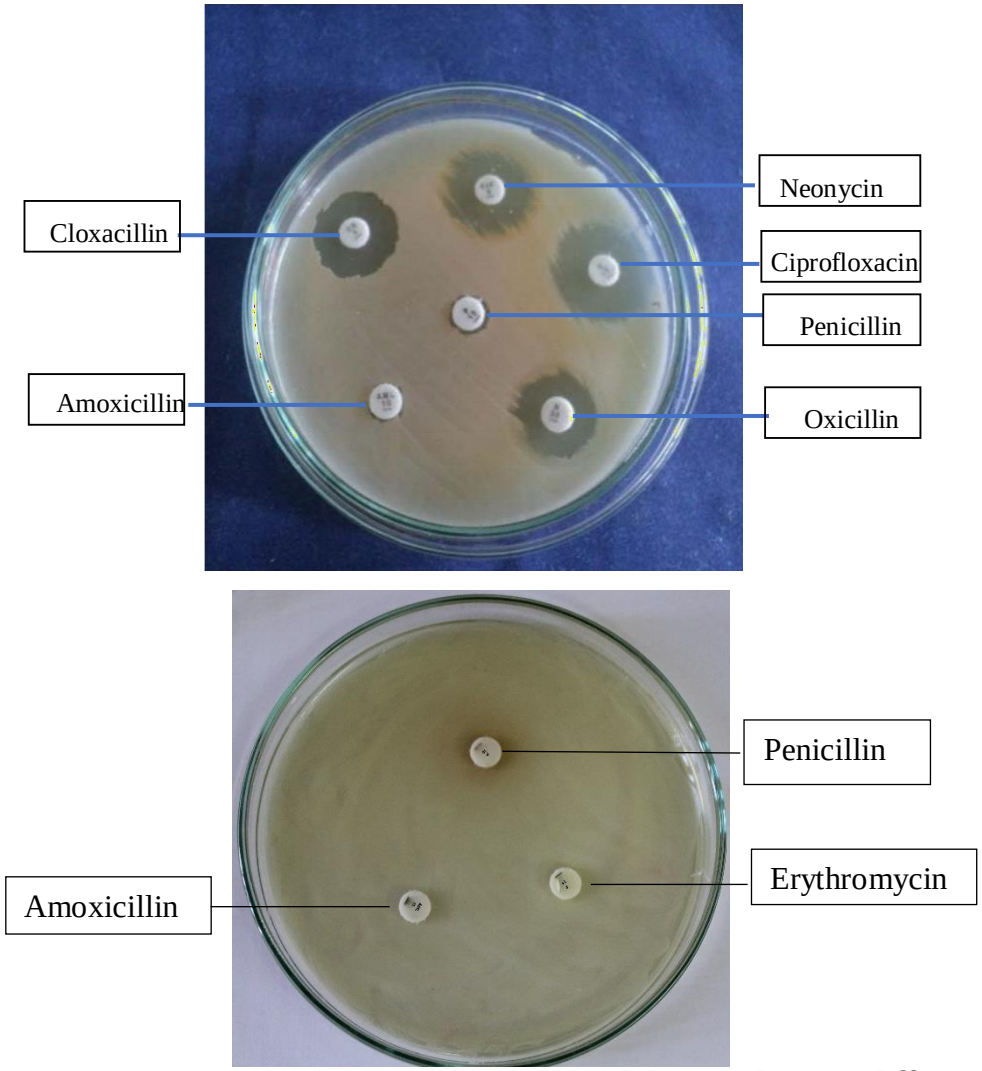

Figure 9: Antimicrobial susceptibility test by agar diffusion method

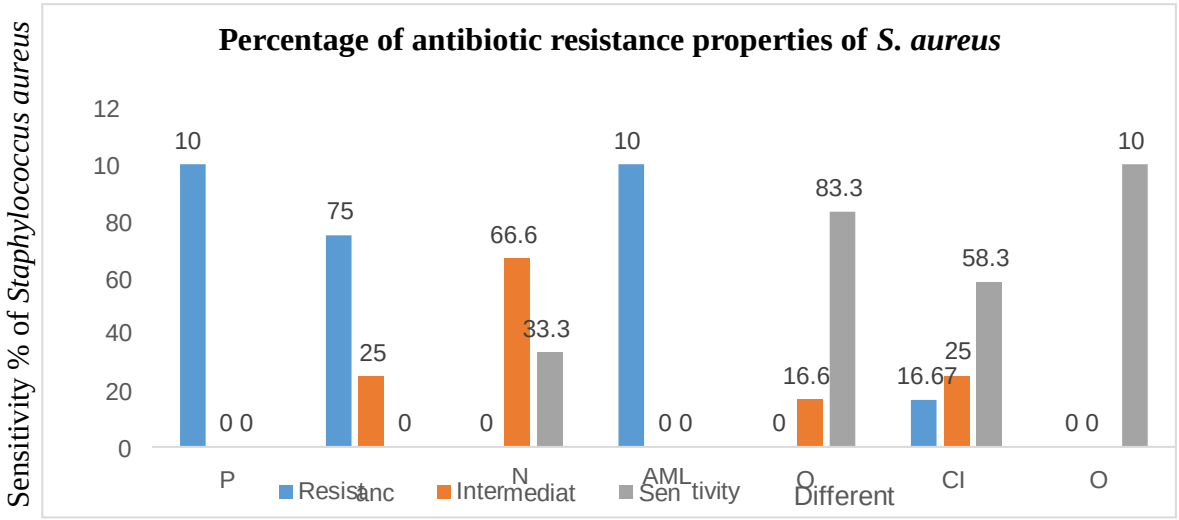

Figure 10: Percentage of antibiotic resistance properties of *S. aureus*

4.7 DISCUSSION

The current findings indicate that, out of 42 samples, 12(28.57%) samples were found to be positive for *S. aureus*. The result shows a high contamination rate, which might be attributed to poor hygienic sanitation & handling improper. A similar report was also made by previous researcher (Abebe *et al*., 2013). That *S. aureus* was 15.5% & 24.2% in fresh milk samples respectively in Ethiopia & in the other parts of the world which is contrary to this; different literatures revealed a very significant isolation rate of *S. aureus* from fresh milk samples (Reta *et al*. 2016). And this result is favorable to the study of (Helena *et al*., 2010), which showed out of 37 cow milk samples, 4 (10.8%) were positive for *S. aureus*, Milk is a favorable media for the growth of many bacteria in which some of them are pathogenic & fresh milk is enriched with pathogenic & nonpathogenic *Staphylococcus* spp. which can be transmitted to human by direct contract & consumption. The purpose of this study is to determine the presence of pathogenic strain of Staphylococcus bacteria in the fresh raw milk. *S. aureus* might be hazardous if proper boiling of milk is not done during consumption. It also cause disease if proper hygienic procedure is not maintained during milking. In Bangladesh (Parveen, 2000) characterize *S. aureus* isolated from human & animal samples (Das, 2012) isolate & identify *Staphylococcus aureus* from laboratory animals, human & also determine antibiogram profile. (Jorgensen *et al*., 2005) stated that the presence of strains assigned to this *S.* spp. in bulk milk or in raw milk products could reflect human contamination. The high incidence of *S. aureus* is indicative of poor hygienic measures during production, handling & distribution, stated in the findings of (Sushma *et al*., 2012). The proper boiling & refrigeration can minimize the chance of infection with *S. aureus*.

In this study, Gram's staining method, created blue colored, smooth, cocci shaped, circular & an irregular colonies as well as formed grapes-like structure. In dependence on growth conditions, the colony pigmentation varies from grey, grey-white with yellowish to orange shades with typical β-haemolysis on the blood agar **(**Deresse *et al.*, 2012; Sushma *et al*., 2012; Alzbeta *et al*., 2013) also recorded similar staining characteristics of *S. aureus.*

The selected organism *S. aureus* gave positive result on catalase test (formation of bubble) & coagulase test (formation of card like clotting) which were closely correlated with (Sasidharan *et al*., 2011)**.**

The isolated *S. aureus* were 100% resistant to penicillin & amoxicillin. Erythromycin showed 75% resistant against this organism. *S. aureus* was sensitive to neomycin, cloxacillin, ciprofloxacillin & oxacillin. These outcomes are more or less correlated to (De Oliveira *et al.*, 2000; Guerin *et al*., 2003) where they analyzed 119 isolates of *S. aureus* collected between 1998 & 2000 in France & the cows with clinical mastitis. In this work, observed that *S. aureus* is constantly resistant against those antibiotic that are commonly applied as a treatment.

CHAPTER 5

SUMMARY & CONCLUSION

The study showed insights into the magnitude & incidence of *S. aureus* from fresh cow milk samples. Food borne pathogens like toxigenic *S. aureus* can easily be transmitted through the food produced from milk under neglected hygienic conditions. The presence of these pathogen in the milk could be attributed to poor hygienic practices during milking. Samples obtained from Savar Hat Area were found to have had the 37.50% of *S. aureus*. It is evident in this study, *S. aureus* isolates exhibit 100% resistant to penicillin & amoxicillin but showed sensitivity to neomycin, cloxacillin, ciprofloxacin & oxacillin. Finding in this study suggest that this organism is alarming for food safety concern. This study also observed that *S. aureus* is becoming increasingly resistant to antimicrobial agents that are commonly employed in human as well as animal medicines & very alarming for human health. Hence, attention should be given to proper handling of the milk during milking & using recent antibiotics in the treatment of this diseases both in humans & animals.

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APPENDICES

APPENDIX I

COMPOSITION OF DIFFERENT MEDIA

1. Nutrient Broth (NB)

Peptic digest of animal tissue	5.00gm
Sodium chloride	5.00gm
Beef extract	1.50gm
Yeast extract	1.50gm
Distilled water	1000 ml
Final pH (at 25°C)	7.4±0.2

2. Mannitol Salt Agar

Lab-Lemco Powder	1.00 gm
Peptone	10.00 gm
Mannitol	10.00 gm
Phenol red	0.025 gm
Agar	15.0 gm
Distilled water	1000 ml

3. Blood agar (BA)

Bactotryptose	10.00gm
Sodium chloride	2.50gm
Beef extract	1.50gm
Glucose	0.15 gm
Dextrin	0.25 gm
Para-aminobenzoic acid	10.00gm
Agar	7.50 gm
distilled water	500 ml
Defibrinated blood	50 ml

1. Muller Hinton Agar

Beef extract	2.00gm
Acid hydrolysate of casein	17.50gm
Starch	1.5gm
Agar	17.00gm
Distilled water	1000ml

2. Nutrient Agar (NA)

Beef extract	3.00 gm
Peptone	5.00 gm
Sodium chloride	5.00 gm
Agar	20.00 gm
Distilled water	1000 ml
Final pH (at 25ºC)	7.1 ± 0.1

APPENDIX II

COMPOSITION OF DIFFERERNT REAGENTS

1. Phosphate Buffered Saline

Sodium chloride	8.00gm
Disodium hydrogen phosphate	2.80gm
Potassium chloride	0.20gm
Potassium hydrogen phosphate	0.20gm
Distilled water	1000ml

2. Gram's stain solution

Stock Crystal violet solution

- Crystal violet 10.00 gm
- Ethyl alcohol (95%) 1000 ml

Stock oxalate solution

- Ammonium oxalate 1.00 gm
- Distilled water 1000 ml

Crystal violet working solution

- Solution no. a 20.00 ml
- Solution no. b 80.00 ml

Gram's iodine solution

- Iodine crystal 1.00 gm
- Potassium iodide 2.00 gm
- Distilled water 300 ml

Acetone alcohol

- Ethyl alcohol 250 ml
- Acetone 250 ml

Safranine working solution

- Safranine dye 2.50 gm
- Ethyl alcohol (95%) 100 ml
- Distilled water 300 ml

3. 3% Hydrogen Peroxide (H_2O_2) for Catalase Test

H_2O_2	3 ml
Distilled water	97 ml