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Isolation, Identification and Characterization of Staphylococcus Aureus from Raw Milk in Different Places of Savar, Bangladesh

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Abstract

Milk and its derivates are considered vehicles of *Staphylococcucs aureus* infection in human. *S.aureus* commonly found on the skin and hair as well as in the noses and throats of people and animals. The bacteria are present in up to 25 percent of healthy people and are even more common among those with skin, eye, nose, or throat infection. *S. aureus* can cause food poisoning when a food handler contaminates food and then the food is not properly stored. Other sources of food contamination include the equipment and surfaces on which food is prepared .These bacteria multiply quickly at room temperature to produce a toxin that causes illness. *S.aureus* is killed by cooking and pasteurization. Present study was carried out from June 2017 to November 2018 .The aim this investigation was to isolate *Staphylococcus aureus* from raw cow milk obtained from different parts of Savar, Ashulia, Dhamrai area distract of Dhaka, Bangladesh. A total of 45 milk samples were collected. Milk samples were subjected to bacteriological and biochemical tests.

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All the characterized isolates were subjected to determine antibiotic susceptibility pattern by disc diffusion method testing such as and using bacteriological and biochemical schemes, 31 out of 45 sample (69%) isolates were identified as *S .aureus* from 45 samples. All the isolates showed growth on MSA and MHA agar. According to antibiogram results of antibiotic sensitivity of *S.aureus*, percentages of sensitivity was observed against different group of antibiotics as follows: chloramphenicol (93%, Gentamycin(93%), Vancomycin (89%), Streptomycin(89%) Ciprofloxacin(64%),Tetracycillin(71%), Oxcillin (57%) Sulfamethoxazole (50%), All of the isolates were found to be resistant against Penicillin (100%) and Azithromycin 100%) . Isolated *S.aureus* showed the resistance pattern to broad spectrum antibiotic. Some people who have tendency to drink without cooking milk and raw milk products, there is high risk of *S. aureus* infection in human health.

Keyword: Raw milk; *Staphylococcus aureus* ; Antibiotic resistance; Human health.

1. Introduction

Milk is a white liquid produced by the mammary glands of mammals. It is the primary source of nutrition for infant mammals (including humans who breastfeed), before they are able to digest other type of food. The milk lactation contains colostrums which carries the mother's antibodies to its young and can reduce the risk of many diseases. It contains many other nutrients including protein and lactose. The quality of milk is determined by aspects of composition and hygiene. Its complex biochemical composition and high water contains milkserves as an excellent culture medium for the growth and multiplication of various microorganisms.The quality of milk may be lowered by a number of factors such as adulteration, contamination during and after milking and the presence of udder infection [1]. Pathogenic organisms in milk can be derived from the cow itself, the human hand or the environment [2]. Diarrhea, Dysentery ,vomiting, nausea, cramping illness, pneumonias, Skin infections or throat wound infection, tuberculosis, brucellosis, typhoid and listeriosis con be transmitted to human through milk.. *Staphylococci* are normal inhabitants of the skin hair and mucous membranes of animals and humans. Pathogenic strains are usually coagulase- positive [3] and have been found to cause diseases in their hosts throughout the world [4, 5]. Diseases in cattle are caused by *Staphylococcus aureus* range from simple abscesses and mastitis to the severe toxic shock syndromes [6, 4, 5]. Mastitis is the persistent, inflammatory reaction of the udder tissue due to physical trauma or microorganism's infection. Pathogenesis mastitis occurs white blood cell are released into the mammary gland, usually in response to bacteria invading on the udder Mastitis causing bacteria including *Staphylococcu aureus Staphylococcu epidermis, Staphylococcu, uberis, Brucella meliensis, Klebsiella pneumonia*. S*taphylococcal* mastitis is the most common and economically the greatest concern wherever dairy farming is practiced. The chief reservoir of this bacterium is an infected udder. A bacterium is shed into milk from infected quarters [7]. The pathogenesis of bacteria causing food- borne poisoning depends on their capacity to produce toxins after ingestion (in the digestive tract) or intoxication (ingestion of preformed toxins in foodstuff). Among the bacteria predominantly involved in these diseases, *Staphylococcus aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food.

Aims and objectives

This kind of research work will help the policy maker to take steps to control the zoonotic diseases related to milk product materials and public health. This research will elucidate the conditions those are highly responsible for intensifying the growth of microbes in foods and milk products with following aims and objectives:

1) To isolate and identify *Staphylococcus aureus* from raw milk sample.
2) To determine the antibiotic resistance pattern of isolated *Staphylococcus aureus.*

2. Methodology

2.1 Sample and Sampling

Raw milk samples were collected during the period from July 2017 to December 2017 at different areas of Savar. Total 45 samples of raw milk were collected. Volumes of each sample were 10 ml. Samples were collected in sterile tubes by maintaning asceptic condition transported in an icebox to laboratory. All the milk samples were immediately transported and tested within three hours of their collection. The microbiological analysis was done in the laboratory of department of microbiology in Gono Bishwabidyalay, Savar, Dhaka.

2.2 Sample collection areas

The sample collection areas from where milk samples were collected include Gono Bishwabidyalay Dairy Farm & Gonoshasthya Kendra, Ashulia, Dhamrai Islampur, Nobinagar, Nayerhat , Nolam. ,

Figure 1: Collected samples in test tube.

2.3 Isolation and characterization

Overnight pre-enrichment was done by peptone water. 1 ml of raw milk and 9 ml of peptone water was mixed together with gentle hand mixing. Overtaxing was not done to keep the milk proteins intact. After incubation a loop full of enriched samples will be streaked on Mannitol salt agar plate for primary isolation of the organism

and incubated at 37°C for 24 hours. Further the isolates were sub cultured onto Mannitol salt agar and Nutrient agar to detect well isolated colony. The *Staphylococci* will ferment lactose producing yellow color colony. The characteristic colony of bacteria was then sub cultured on nutrient agar plate. Finally, biochemical tests, gram staining and antibiotic sensitivity tests were done for characterization of sample.

2.4 Mannitol salt Agar

Mannitol salt agar or MSA is a commonly used selective and differential growth medium in microbiology. It encourages the growth of a group of bacteria while inhibiting the growth of others. This medium is important in laboratories by distinguishing pathogenic organisms in a short period of time .It concentration a high contains of salt (NaCl) about 7.5% - 10%.., making it selective for Gram-positive bacteria eg *Staphylococcoue* and *Micrococcaceae* since this level of salt is inhibitory to most other bacteria. It is also a differential medium for Mannitol-fermenting *Staphylococci,* containing carbohydrate Mannitol and the indicator phenol red, a P^H indicator for detecting acid produced by Mannitol- fermenting *Staphylococci. Staphylococcus aureus* produces yellow colonies with yellow zones, whereas other coagulase negative *Staphylococci* produce small pink or red colonies and no color change to the medium. If an organism can ferment Mannitol, an acidic by product is formed that causes the phenol red in the agar to turn yellow. It is used for selective isolation of presumptive pathogenic (PP) *Staphylococcus* species.Gram positive *Staphylococcus*: fermenting Mannitol: medium turns yellow (*S aureus*) Gram positive : *Staphylococcus*: not fermenting mannitol, medium does not change color (*S. epidermidis),* Gram positive *Staphylococcus:* inhibited growth.

2.5 Nutrient agar

Nutrient agar is a general purpose medium supporting growth of a wide range of non-fastidious organisms.It typically contains (mass / volume): 0.5% peptone this provides organic nitrogen,0 .3% beef extract / yeast extract-the water-soluble content of these contribute vitamins, carbohydrates ,nitrogen, and salts, 1,5 % agar – this gives the mixture solidity, 0.5% Sodium Chloride-this gives the mixture proportions similar to those found in the cytoplasm of most organisms, distilled water serves as transport medium for the agars various substances pH adjusted to neutral 7.0 at 25˚c .These ingredients are combined and boiled for approximately one minute to ensure they are mixed and to sterilize them. Them they are cooled to around 45˚c and poured into Petri dishes which are covered immediately. Once the dishes hold solidified agar, they are stored refrigerated used within 2-3 weeks .Inoculation takes place on warm dishes rather then cool ones: If refrigerated for storage, the dishes must be warmed to room temperature prior to inoculation. Nutrient agar used for subculture .

2.6 Biochemical tests

The following tests were performed for identification and characterization of *Staphylococcus aureus.*

2.7 Coagulase test

Coagulase test is used to differentiate for *Staphylococcus aureus* positive or Negative Coagulase is an enzyme produced by *S. aureus* that converts soluble fibrinogen in plasma to insoluble fibrin. *Staphylococcus aureus*

produces two Slide Coagulase test detects clumping factor (formerly referred as cell-bound coagulase)Clumping factor directly coverts fibrinogen to fibrin causing agglutination. On glass slide organisms is made of suspension and mixed with a drop of plasma. Agglutination a positive test indicates *Staphylococcus aureus* Some species and Its no agglutination Coagulase is negative *Staphylococcus.*Coagulase teses can be positive or Negative results should be confirmed. factor (bound coagulase) differs from free coagulase in that it is cell-bound and requires only fibrinogen.21 The slide agglutination test for clumping factor is very rapid but up to 15% of *S. aureus* strains are negative,11 so isolates negative in slide tests should be confirmed with a tube agglutination test. Some less common species of *Staphylococci*, including *S. schleiferi* and *Staphylococcus* lugdunensis, may give positive results in the slide coagulase test. The test is unsuitable for isolates that are not easily emulsified and clumping factor can be obscured by large amounts of capsule.

2.8 Catalase test

During aerobic respiration microorganisms produce hydrogen peroxide and in some cases an extremely toxic superoxide. Accumulation of these substances will result in death of the organism unless they can be enzymatically degraded. The catalase enzyme serves to neutralize the bactericidal effects of hydrogen peroxide. Catalase expedites the breakdown of hydrogen peroxide (H_2O_2) into water and oxygen. This reaction is evident by the rapid formation of bubbles.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Using sterile technique a wooden stick collect a small amount of organism from a well-isolated 18- to 24-hour colony and place it onto the glass slide to pick up only organism not any agar. Then, place 1 drop of 3% H_2O_2 onto the organism on the glass slide. Do not mix. We observed for immediate bubble formation (O_2 + water = bubbles). Observing for the formation of bubbles against a dark background enhances readability. Positive reactions are evident by immediate effervescence (bubble formation). Place microscope slide over a dark background and use a magnifying glass or microscope to observe weak positive reactions. No bubble formation (no catalase enzyme to hydrolyze the hydrogen peroxide) represents a catalase-negative reaction.

2.9 Indole test

This test was done to determine if bacteria can breakdown the amino acid tryptophan into indole .After incubating the bacteria at least 24 hours then added koves reagent to the culture media, and shacked very well and observation. If red color in the reagent layer indicated indole the red/pink color development of the layer on top of the media is a positive result (indicated the bacteria can breakdown tryptophan to from indole) there is no red layer a Negative result. Or indicated the indole was not formed from tryptophan.

2.10 Methyl red test

In 5 ml of the sterile MR-VP broth and small amount from the pure of the test organism and incubated at 37^0c

24 hours. After incubation 5 drops of methyl red solution was added and mixed then observed for color formation. If development of red color indicated positive result and yellow color negative result respectively (Chees brough, 1985).

2.11 Voges -Proskauer (V-P) test

This test 5 ml of sterile MR-VP broth into the test organisms from pure culture were grown in incubation at 37^{o} c for overnight .After 06 ml of 5% alphanapthol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine was added per ml of broth culture . Shaking well the broth after 3-4 minutes and allowed to stand for 15 minutes to observe the color formation. If development of pin-red color indicated positive result, no color indicated negative results.

2.12 Oxidase test

The oxidase test detects the presence of a cytochrome oxidase system that will catalyse the

transport of electrons between electron donors in the bacteria and a red ox dye- tetra methyl-p-phenylene-diamine. The dye is reduced to deep purple color. This test is used to assist in the identification of Pseudomonas, Neisseria, Alcaligens, Aeromonas, Campylobacter, Vibrio, Brucella and Pasteurella, all of which produce the enzyme cytochrome oxidase. A number of reagents can be used for this test. Kovacs Oxidase Reagent: 1% tetra-methyl-p-phenylenediaminedihydrochloride, in water .Gordon and McLeod's Reagent: 1% dimethyl-p-phenylenediaminedihydrochloride, in water. Gaby and Hadley (indophenol oxidase) Reagent: 1% α-naphthol in 95% ethanol. 1% p-aminodimethylaniline HCL Place 1 or 2 drops of 1% Kovács oxidase reagent or 1% Gordon and McLeod reagent on the organisms. Do not invert or flood plate. 4. When using Kovács reagent, microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds.

2.13 Simmons citrate utilization test

Some organisms are capable of using citrate as the sole source of carbon. The bacteria to convert citrate into oxaloacetate. This test indicates whether or not an organism can utilize citrate (citric acid) as a nutrient. The medium also contains a pH indicator called Bromthymol blue. The indicator Bromthymol blue is blue above pH 7.6 and green at pH values below 7.6. If citrates are utilized, the medium pH will rise and the medium will turn from green to blue.

2.14 Urease test

The samples was inoculated in a tube containing urea broth or slant all these tubes was incubated at 37˚c for 24 hours after incubated ,and one tube without sample as bacteria control. Reddish pink or red colour of test tube was as positive , but absence of red colour is negative result .

2.15 Triple Sugar Iron (TSI) agar test

A loop of bacteria was spread across the surface of the agar, with a needle of bacteria was inserted (stabbed) into the bottom (butt) of the tube. Kept the tubes in incubation for 24 hours at 37˚c for bacterial growth, then examined the culture tubes for the result.

2.16 Microscopic study by Gram's staining method

Gram's staining was performed as per procedures described by Merchant and Packer (1969) to determine the gram positive or gram negative, size, shape and arrangement of bacteria. The gram positive, organisms revealed purple colored round shaped appearance and arranged in cluster suspected as *Staphylococcus aureus.*

2.17 Procedure of Gram Staining

-Take a drop of water and mix one loop full bacteria, by using sterile technique make a smear of isolated bacteria.

-Allowed smears to air-dry and then heat fixed in the usual manner.

-Gently flooded smears with crystal violet and let stood for 1 minute. Then wash the slide properly.

-After that the smears were flooded with Gram's iodine and let stood for 1 minute. Gently washed with tap water.

-The third step is decolorization with 95% ethyl alcohol for 5-7 seconds. Gently washed with tap water.

- Stain the smear with safranin for 45 sec.Gently washed with tap water and blot dry with bibulous paper. Then examine under microscope.

2.18 Antibiotic sensitivity test

2.19 Muller-Hinton agar

Muller-Hinton Media contains Beef Extract, Acid Hydrolysate of Casein, Staarch and Agar. Beef Extract and Acid Hydrolysate of Casein provide nitrogen, vitamins, carbon, amino acid, sulphur and other essential nutrient. Starch is added to absord any toxic metabolites produced.Starch hydrolysis yields dextrose, which serves as a source of energy .Agar is the solidifiying agent. The use of a suitable medium for testing the susceptibility of microorganisms to sulfonamides, and trmethoprim is essential. Antagonism to sulfonamide activity is demonstrated by paraaminobenzoic acid (PABA) and its analogs. Reduced activity of trimethoprim, resulting in smaller inhibition zones and innerzonal colonies, is demonstrated on unsuitable Mueller Hinton medium possessing high levels of thymidine. Both the PABA and thymine / thymidine content in . Mueller hinton Agar are reduced to a minimum, thus markedly reducing the inactivation of sulfonamides and trimethoprim when the media is used for testing the susceptibility of bacterial isolates to these antimicrrobics.

2.20 Procedure of antibiotic sensitivity test

Antibiotic susceptibility and resistance test was done by standard commercial disk of antibiotic on MHA. A fresh *Staphylococcus aureus* culture on Mueller Hinton agar were carried out by disk diffusion method. Antibiotic choices from Clinical and Laboratory Standards Institute CLSI 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA,2014.The plates was incubated at 37^0c for 24 hours inhibition zones in diameter was measured in mm . Results was classified as Resistant(R), Intermediate (I) and susceptible (S) according to the criteria recommended. By the national committee for laboratory standards 2001.

Table 1: The following antibiotic are using in this study.

a) Vancomycin 30 µg	f) Azithromycin 15 µg
b) Penicillin 10 µg .	g) Chorampherical 30 µg
c) Gentamycin 10 µg .	h) Streptomycin 10µg
d Sulfamethoxazole 25µg.	i) Oxcillin µg
e) Tetracycillin30 µg.	j) Ciprofloxacin 5µ

- Mueller Hinton agar plates were place right side up in an incubator for heated to 37˚c for 15 to 20 minutes with the covers adjusted the plates.
- Each of the agar plates were labeled with the name of the test organism and antibiotic.
- Sterile technique, were followed all agar plates with respective test organisms inoculate .
- Using were a sterile cotton swab into the inoculums well mixed test culture saline and removes excess inoculums by processed and rotating the swab against the inner wall of the culture tube.
- The swab, streak were streaking the all over the surface of the medium three times, rotating the plate horizontally ,vertically, through an angle of 60˚ after each application .Finally, pass by a cotton swab round the edge of the agar surface.
- After that all inoculums plates were dried for a few minutes at room temperature with the lid closed. Ensured a heavy growth over the entire surface.
- Then the antibiotic discs were placing the over of the agar surface .Each disc were gently pressed down with sterile forceps to ensure even contact with the medium.
- If dispensers are not available, distribute the individual discs at equal, distances with forceps dipped in alcohol and flamed.
- All cultures plates were Incubated in an inverted position for 24 hours at 37˚c.
- After overnight incubation, the diameter of each zone (including the diameter of the disc)were measured and recorded in mm.
- After incubation, the results were examined the according to the critical diameters of the zones of complete inhibition was measured in mm. showmen in table no 6.
- The zone diameter for individual anti-microbial agents was used to determine susceptible, intermediate, and resistant categories by referring to an interpreting table no 5 (Barry and Thomsberry,1985).

2.21 Measuring of the zone of susceptibility for Staphylococcus aureus resistance profile

Overnight cultures were prepared and used for antibiotic sensitivity tests. An aliquot (100µl) from each isolate suspension was spread plated on Muller Hinton agar (MHA) (Himedia, India). Susceptibilities of the isolates to a panel of 10 different antibiotic discs were determined. Antibiotic discs were gently pressed onto the inoculated Muller Hinton agar ensure intimate contact with the surface and the plates were incubated overnight (Sears, 2003). Inhibition zone diameters were measured and values obtained from the Clinical and Laboratory Standards Institute (CLSI) 950 west Valley Road ,Suite 2500 Wayne ,PA 19087 USA. were used to interpret the results obtained. *S aureus* isolates were classified as resistant, moderately sensitive or susceptible to a particular antibiotic.

3. Results

3.1 Total Number of Samples

Total 45 raw milk samples were collected from seven sampling area.

Table 2: Sample collection site and number

Name of sample collection site	Number of Samples	Number of Positive Samples
Dhamrai, Islampur	15	10
Gono Bishwabidyalay Dairy Farm & Gonoshasthaya Kendra, Ashulia	12	11
Nobinagar, Nayerhat , Nolam.	18	10
Total	45	31

Cultural characteristics

3.2 Mannitol Salt agar

Mannitol salt agar is used as selective media for isolation of *Staphylococcus aureus*. *Staphylococcus aureus* produce yellow colonies with yellow zones, whereas other *Staphylococci* produce small pink or red colonies with no color change to the medium. If an organism can ferment mannitol, an acidic by product is formed that will cause the phenol red in the agar to turn yellow.

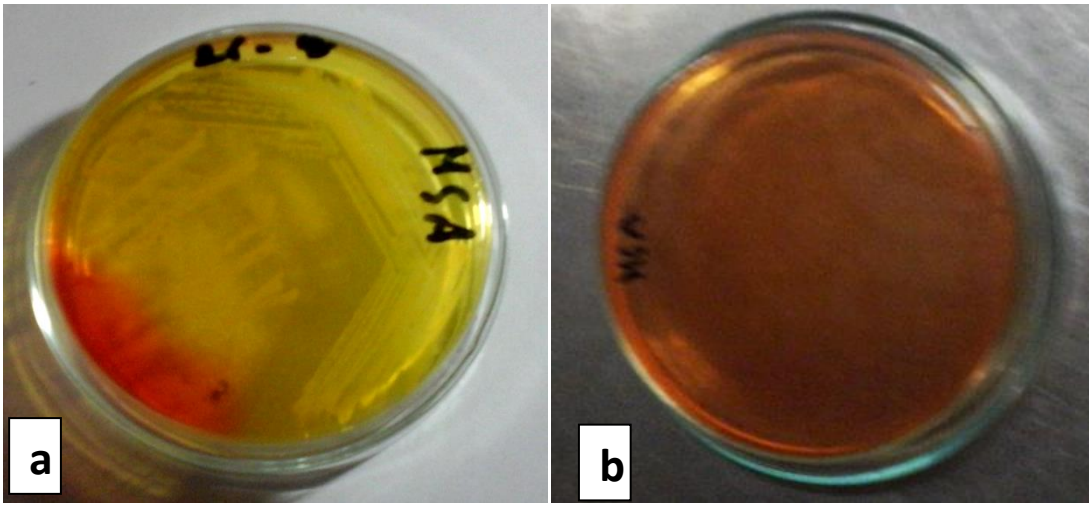

Figure 2: Culture of organisms on MSA Agar producing yellow color (fig a) and fresh MSA agar plate (fig b)

3.3 Nutrient agar

Nutrient agar is used as a general purpose medium for the growth of a wide variety of non-fastidious microorganisms. It consists of peptone, beef extract and agar. This relatively simple formulation provides the nutrients necessary for the replication of a large number of non-fastidious microorganisms. Nutrient agar is used for the cultivation and maintenance of non-fastidious organisms as well as enumeration of organisms in water, sewage, dairy products, feces and other materials. *Staph* produces small whitish colony on nutrient agar.

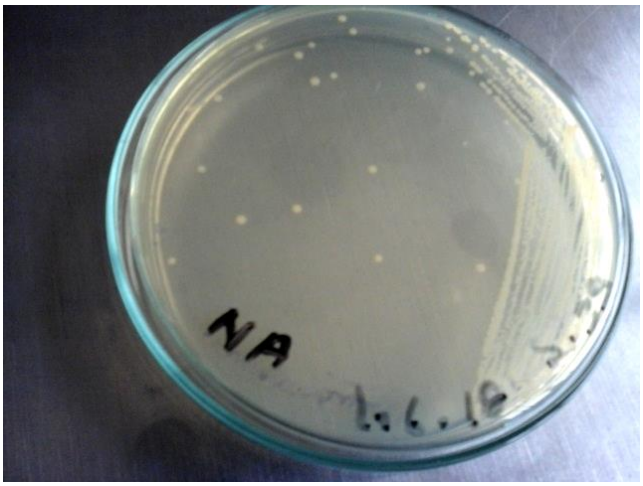

Figure 3: Colony of *Staphylococcus aureus* on Nutrient Agar

3.4 Characterization of the isolates by gram staining

Gram staining was performed for the identification of the organism whether it was Gram positive or negative and shape, arrangement of bacteria .The following as isolated strains were found to be Gram positive and cocci in shape and arrangement was cluster.

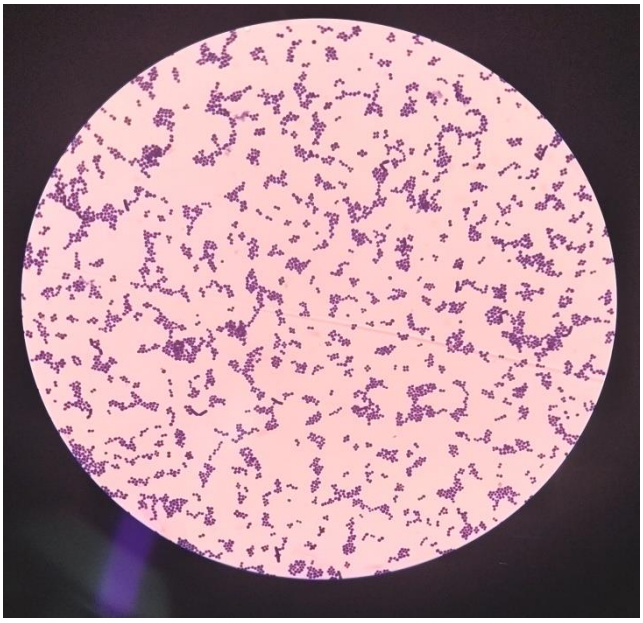

Figure 4: Observation of gram staining result showing gram positive purple color cocci.

Table 3: Result of Gram staining of the isolated bacteria.

Sample no	Gram Staining Reaction	Shape	Arrangement	Comments
1	Purple (+ve)	Cocci	Cluster	Positive
3	Purple(+ve)	Cocci	Cluster	Positive
4	Purple(+ve)	Cocci	Cluster	Positive
5	Purple(+ve)	Cocci	Cluster	Positive
6	Purple(+ve)	Cocci	Cluster	Positive
8	Purple(-ve)	purple	Cluster	Positive
S9	Purple(+ve)	(+ve)	Cluster	Positive
11	purple(+ve)	Cocci	Cluster	Positive
13	purple(+ve)	Cocci	Cluster	Positive
15	purple(+ve)	Cocci	Cluster	Positive
16	purple(+ve)	Cocci	Cluster	Positive
19	purple(+ve)e	Cocci	Cluster	Positive
20	purple(+ve)	Cocci	Cluster	Positive
21	purple(+ve)	Cocci	Cluster	Positive
22	purple(+ve)	Cocci	Cluster	Positive
23	Purple(+ve)	Cocci	Cluster	Positive
S24	purple(+ve)	Cocci	Cluster	Positive
S-27	purple(+ve)	Cocci	Cluster	Positive
S29	purple(+ve)	Cocci	Cluster	Positive
S-31	purple(+ve)	Cocci	Cluster	Positive
S-32	purple(+ve)	Cocci	Cluster	Positive
S-34	purple(+ve)	Cocci	Cluster	Positive
S-36	purple(+ve)	Cocci	Cluster	Positive
S-37	purple(+ve)	Cocci	Cluster	Positive
S-38	purple(+ve)	Cocci	Cluster	Positive
S-39	purple(+ve)	Cocci	Cluster	Positive
S-40	purple(+ve)	Cocci	Cluster	Positive
S-41	purple(+ve)	Cocci	Cluster	Positive
S42	purple(+ve)	Cocci	Cluster	Positive
S-44	purple(+ve)	Cocci	Cluster	Positive
S-45	Purple(+ve)	Cocci	Cluster	Positive

3.5 Biochemical Characterization of organisms

The biochemical identification of the organisms was done by performing the biochemical tests. The Biochemical tests were done as stated on Bergey's Manual of Determinative Bacteriology. Different biochemical test indicates different biochemical properties of the organisms. In this work 11(eleven) biochemical test have been done. Figures of the different biochemical test results shown in table no 4.

Table 4: Result of Biochemical test.

Sl.	Lactose	Sucrose	Indole	MR	VP	Citrate	Catalase	Oxidase	Urease	Coagulase
1	+	+	-	+	-	-	+	-	-	-
3	+	+	-	+	-	-	+	-	-	+
4	+	+	-	+	-	-	+	-	-	+
5	+	+	-	+	-	-	+	-	-	+
6	+	+	-	+	-	-	+	-	-	-
8	+	+	-	+		-	+	-	-	-
9	+	+	-	+	-	-	+	-	-	-
11	+	+	-	+	-	-	+	-	-	+
13	+	+	-	+	-	-	+	-	-	+
15	+	+	-	+	-	-	+	-	-	+
16	+	+	-	+	-	-	+	-	-	+
19	+	+	-	+	-	-	+	-	-	+
20	+	+	-	+	-	-	+	-	-	+
21	+	+	-	+	-	-	+	-	-	+
22	+	+	-	+	-	-	+	-	-	+
23	+	+	-	+	-	-	+	-	-	-
24	+	+	-	+	-	-	+	-	-	+
27	+	+	-	+	-	-	+	-	-	-
29	+	+	-	+	-	-	+	-	-	+
31	+	+	-	+	-	-	+	-	-	-
32	+	+	-	+	-	-	+	-	-	-
34	+	+	-	+	-	-	+	-	-	+
36	+	+	-	+	-	-	+	-	-	+
37	+	+	-	+	-	-	+	-	-	+
38	+	+	-	+	-	-	+	-	-	-
39	+	+	-	+	-	-	+	-	-	-
40	+	+	-	+	-	-	+	-	-	+
41	+	+	-	+	-	-	+	-	-	-
42	+	+	-	+	-	-	+	-	-	-
44	+	+	-	+	-	-	+	-	-	-
45	+	+	-	+	-	-	+	-	-	+

3.6 Methyl Red test

The MR test ''M'' portion of the fore [IMViC] test , is used to identify enteric bacteria based on their pattern of glucose metabolism. All enteric initially produce pyruvic acid from glucose metabolism.Some enteric subsequently use the mixed acid pathway to metabolize pyruvic acid to other acid such as lactic , acetic, and formic acids.These bacteria are called methyl-red positive and include Escherichia coli.Other enteric subsequently use the butylenes glycol pathway to metabolize pyruvic acid to neutral end products.These bacteria are called methyl- red- negative.The isolated bacteria *Staphylococcu aureus* were MR positive.

Figure 5: Methyl Red (MR) test positive result and left showing and a negative

3.7 Voges-Proskauer test

Voges-Proskauer test is a test used to detect aceton in a bacterial broth culture. The test is performed by adding alpha-naphthol and potassium hydroxide to the Voges-Proskauer broth which has been inoculated with bacteria. Acherry red color indicated a positive result, while a yellow-brown color indicated a positive result, while a yellow-brown color indicates a negative result. The isolated bacteria *Staphylococcus aureus* were negative or positive.

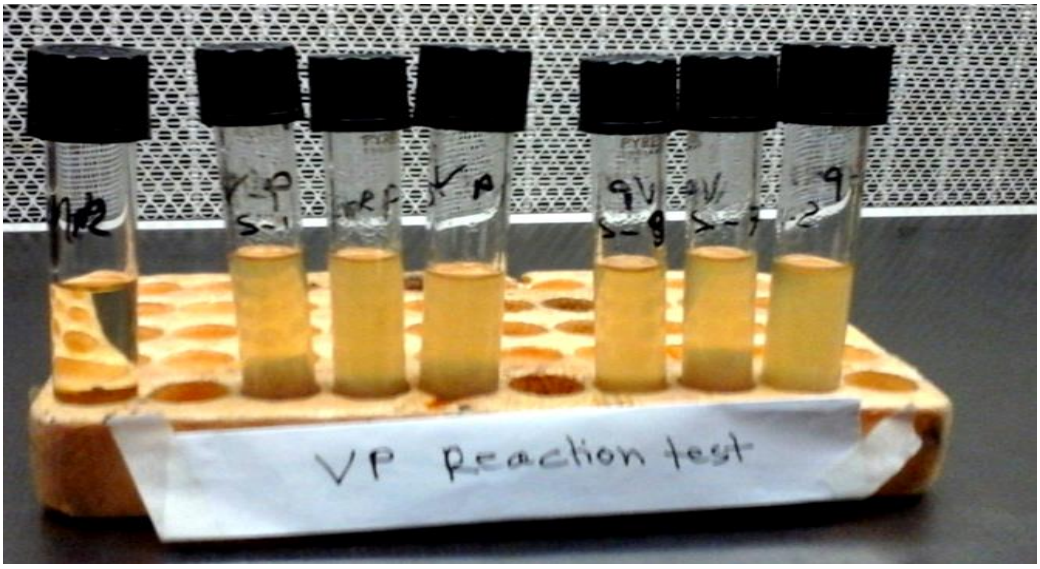

Figure 6: VP reaction test results.

3.8 Coagulase test

Coagulase test is used to differentiate for *Staphylococcus aureus* positive or Negative Coagulase is an enzyme produced by *S. aureus* that converts soluble fibrinogen in plasma to insoluble fibrin. *Staphylococcus aureus* produces two Slide Coagulase test detects clumping factor (formerly referred as cell-bound coagulase)Clumping factor directly coverts fibrinogen to fibrin causing agglutination. On glass slide organisms is made of suspension and mixed with a drop of plasma. Agglutination a positive test indicated *Staphylococcus aureus* Some species and Its agglutination Coagulase is negative *Staphylococcus*.Coagulase teses can be positive or Negative results

Figure 7: Coagulase test showing an agglutination (clumping)

3.9 Catalase test

The catalase test is one of the three main tests used by microbiologists to identify species of bacteria. If the bacteria possess catcalase are catalase-positive when a small amount of bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed .The catalase test is done by placing a drop of hydrogen peroxide on a glass slide. An applicator stick is touched to the colony and the tip is then smeared onto the hydrogen peroxide drop. If the mixture produces bubbles the organism is said to be ‘’catalase-positive’’. If not, the organism is catalase-negative. The isolated bacteria *Staphylococcus aureus* were negative or positive.

Figure 8: Catalase test a positive result

3.10 Citrate test

Bacteria are inoculated on a medium containing sodium citrate and a pH indicator such as bromothymol blue. The medium also contains inorganic ammonium salts, which are utilized as sole source of nitrogen. Use of citrate involves the enzyme citrase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and carbon dioxide (CO_2). Production of sodium bicarbonate ($NaHCO_3$) as well as ammonia (NH_3) from the use of sodium citrate and ammonium salts results in alkaline pH.This results in a change of the medium's color from green to blue. The isolated bacteria *Staphylococcus aureus* were negative.

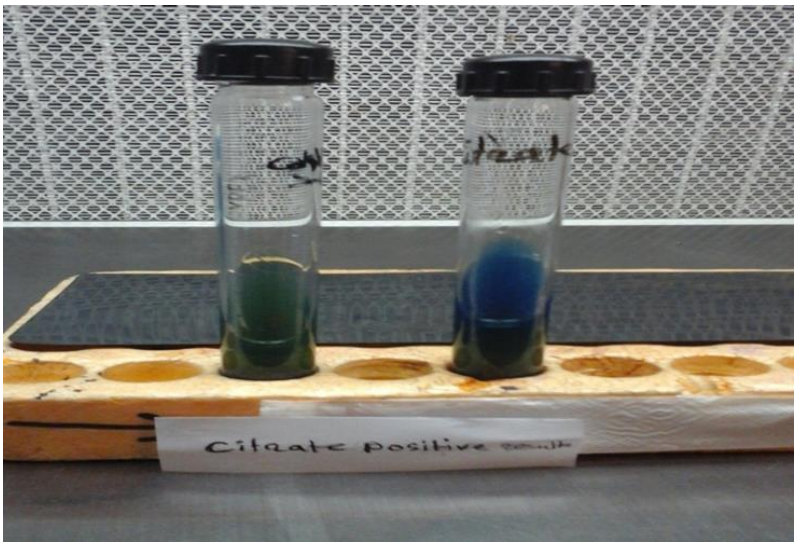

Figure 9: Simmons's citrate test Left showing negative results Right positive control.

3.11 Oxidase test

This test depends on the presence of cytochrome oxidase in bacteria that will catalyze the transport of electrons between electron donors and redox dye. Tetraethyl-p-phenylene diamine dihydrochloride in the reagent is reduced to deep purple color. Oxidase positive organisms give blue color within 5-10 secods, and in oxidase negative organisms, color does not change, the isolated bacteria *Staphylococcus aureus* were oxidase negative.

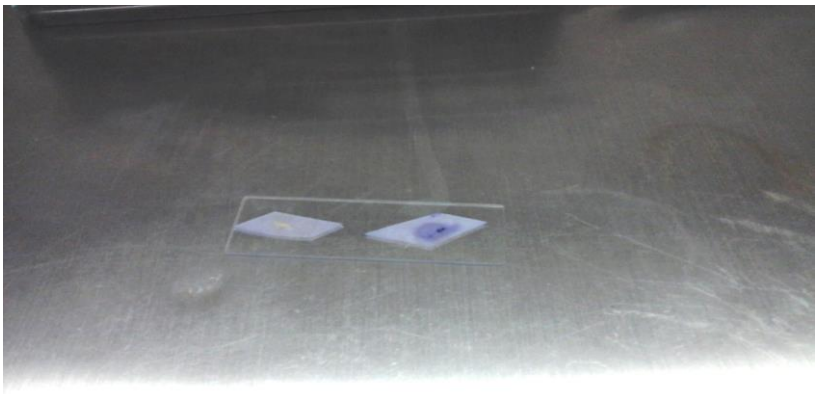

Figure 10: Oxidase Test Right positive control and lift showing a negative result

3.12 Indole test

The indole test is a biochemical test performed on bacterial species to determine the ability of the organism to convert tryptophan into the indole. This division is performed by a chain of a number of different intracellular enzymes, a system generally referred to as ‘’tryptophanase’’. The presence of indole is detectable by adding Kovac;s reagent,which produces a cherry red layer .A positive result is shown by the presence of a cherry red color in the surface layer of the broth. The absence of red coloration demonstrates that the substrate tryptophan was not hydrolyzed and indicates an indole negative reaction. The isolated bacteria *Staphylococcus aureus* were indole negative.

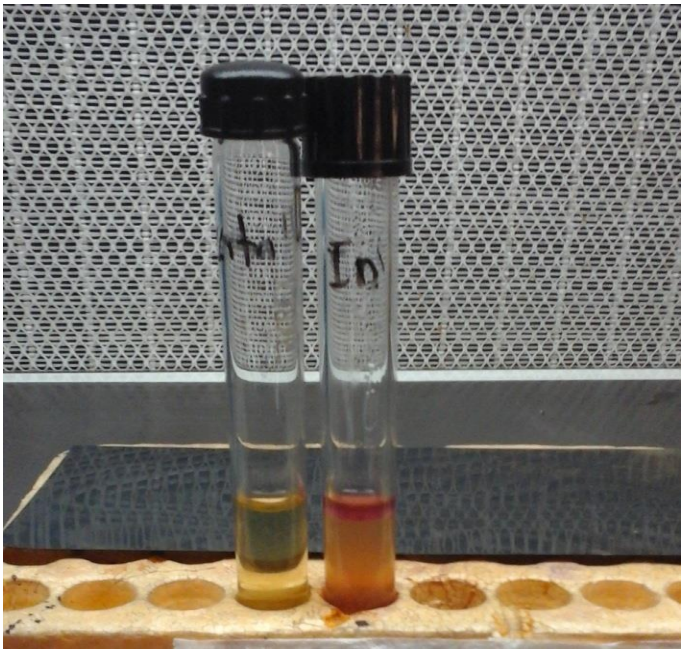

Figure 11: Indole test Right showing a positive control and a negative result

3.13 Urease test

This test is used to determine the ability of an organism to split urea, through the production of an enzyme ,urease. It identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urea-positive bacteria from other. This is a positive reaction a deep pink the presence of urease.It is not a deep pink color a negative result . The isolated bacteria *Staphylococcus aureus* were indole negative.

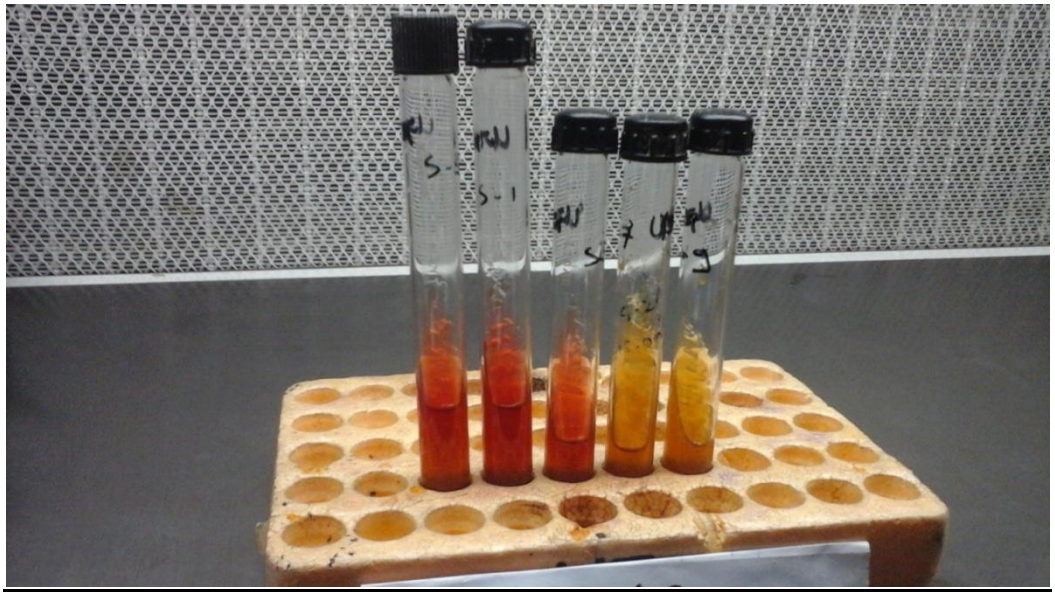

Figure 12: Urease test result.

3.14 Lactose test

The samples was inoculated in a tube containing broth all these tubes was incubated at 37˚c for 24 hours after incubated ,and one tube without sample as bacteria control. The appearance of pinkish or whitish or reddish color was a positive, but absence of colour is negative result. The isolated bacteria *Staphylococcus aureus* were Lactose test positive.

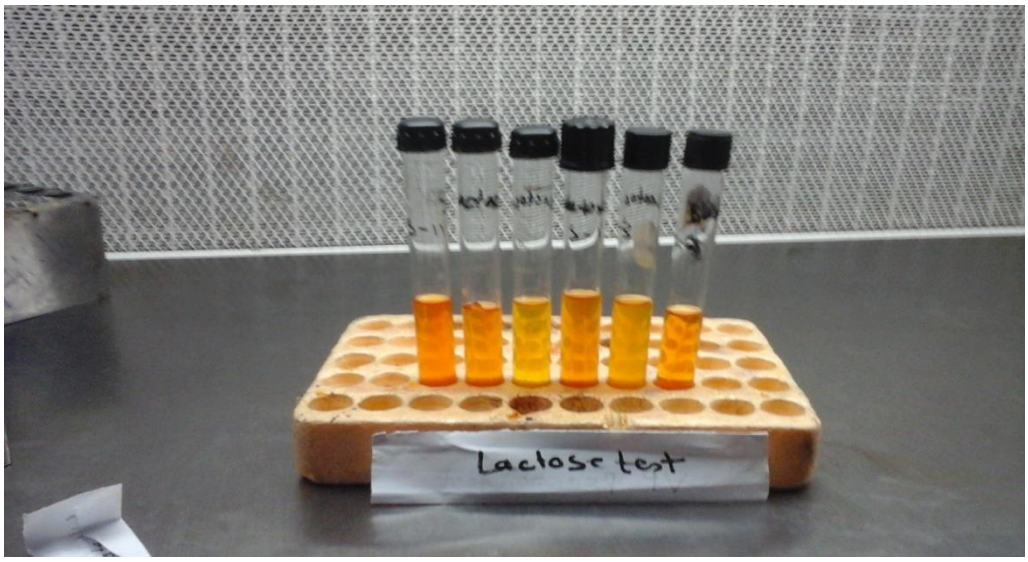

Figure 13: Lactose test showing positive results

3.15 Sucrose test

The samples was inoculated in a tube containing broth all these tubes was incubated at 37˚c for 24 hours after

incubated ,and one tube without sample as bacteria control. A positive result indicates the appearance of yellow color but absence of yellow colour is negative result . The isolated bacteria *Staphylococcus aureus* were Sucrose test positive.

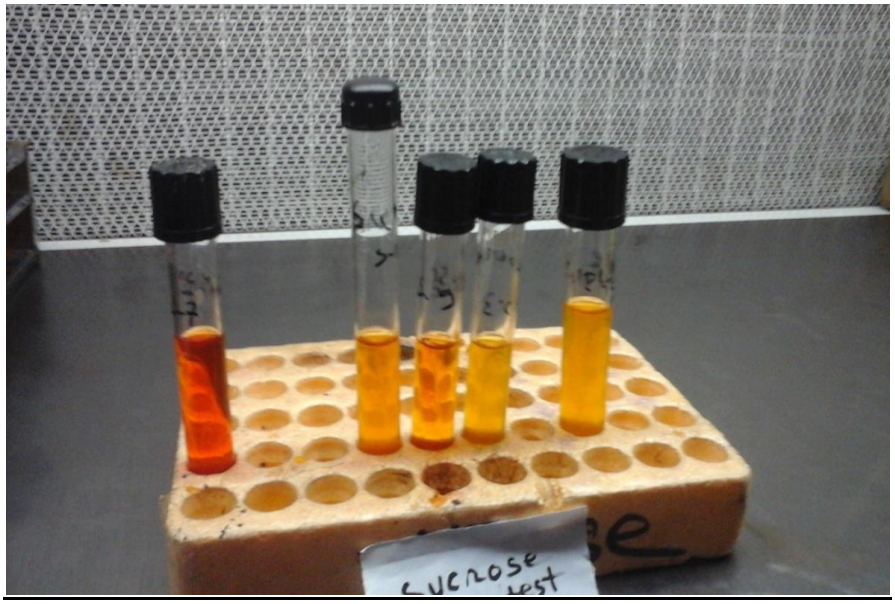

Figure 14: Sucrose test result. Left showing negative control. Right showing positive results

3.16 Antibiotic Sensitivity test

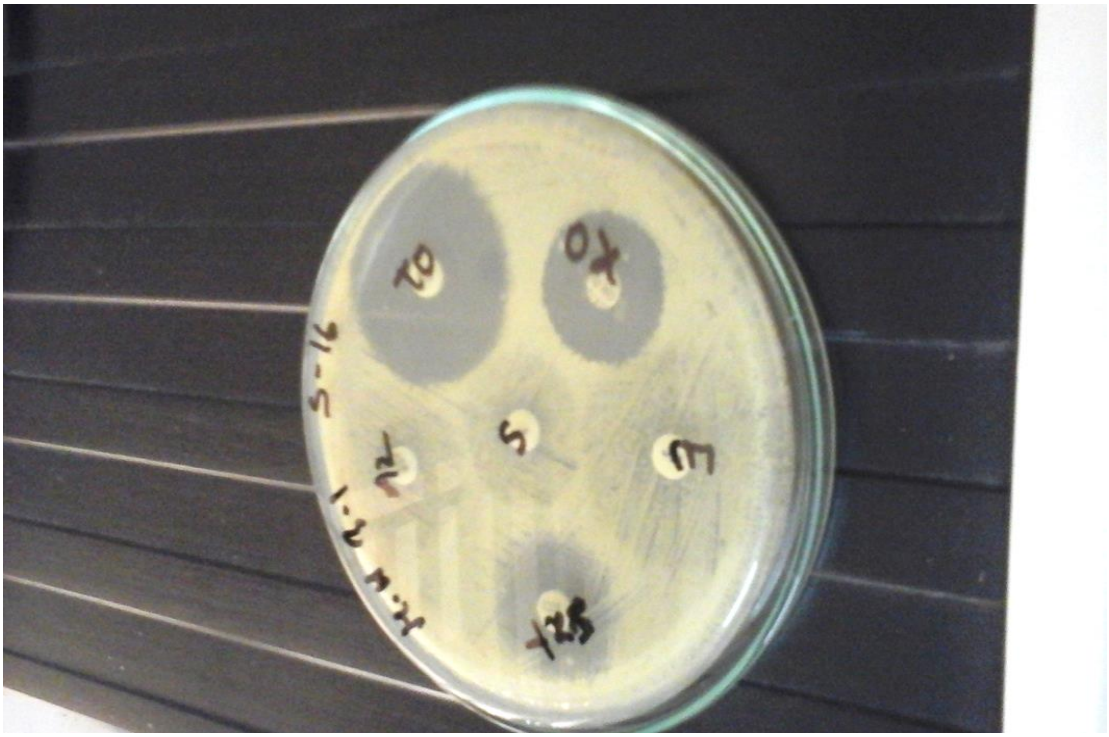

Figure 15: Antibiotic Sensitivity disc test result for *Staphylococcus aureus*

Table 5: Interpretative chart of zone sizes

Name of antibiotic	Disc potency	Susceptible	Intermediate /moderately susceptible	Resistant
Azithromycin	15 µg	≥18mm	14 - 17mm	
Ciprofloxacin	5 µg	≥21mm	16 - 20	≤15
Vancomycin	30 µg	≧15		≤
Chorampherical	30 µg.	≥18	13 - 17	≤12
Gentamycin .	10 µg .	≥ 15	13 – 14	≤ 12
Penicillin.	10 µg	≥ 29	--	≤28
Tetracycillin	30 µg.	≥ 19	15 - 18	≤ 14
Sulfamethoxazole	25µg	≥ 16	11 -- 15	≤10
Streptomycin	10µg	≥ 15	12-	≤11
Oxcillin µg	1 µg	≥22	--	≤ 21mm

CLSI (2007) Performance Standards for Antimicrobial Susceptibility Testing; Seventeenth Informational Supplement, Clinical and Laboratory Standards Institute.

Table 7: Percentages of Sensitivity and Resistances Patterns of *Staphylococcus aureus*

Name of Antibiotics	Percentages of Sensitivity	Percentages of Resistance
Penicillin	-	100 %-
Azithromycin	-	100 %
Sulfamethoxazole	50 %	50 %
Oxacillin	57 %	47 %
Chloramphenicol	93 %	7 %
Gentamycin	93 %	7 %
Vancomycin	89 %	11 %
Streptomycin	89 %	11 %
Tetracycillin	71 %	29 %
Ciprofloxacin	64 %	36 %

Table 6: Antimicrobial Susceptibility test zone diameter interpretive criteria

Isolate number	Azithromycin	Ciprofloxacin	Vancomycin	Chloramphenicol	Streptomycin	Genta-micin	Penicillin	Tetracy-cline	Trimethepr Sulfametho xazole	Oxacillin
0 1	9mm®	28mm(s)	18mm(s)	29 mm(s)	17mm(s)	18mm(s)	24mm®	25mm(s)	14mm®	20mm®
03	11mm®	24mm(s)	16mm(s)	24mm(s)	15mm	12mm®	14mm®	26mm(s)	18mm(s)	16mm®
04	6mm®	15mm®	16mm(s)	24mm(s)	19mm(s)	22mm(s)	14mm®	28mm(s)	12mm®	20mm®
05	10mm®	21mm(s)	19mm(s)	25mm(s)	18mm(s)	21mm(s)	19mm®	27mm(s)	19mm(s)	19mm®
06	10mm®	13mm®	16mm(s)	25mm(s)	15mm	24mm(s)	15mm®	26mm(s)	13mm®	22mm(s)
08	11mm®	24mm(s)	17mm(s)	27mm(s)	18mm(s)	20mm(s)	26mm®	11mm	10mm®)	22mm(s)
09	13mm®	11mm®	22mm(s)	27mm(s)	16mm(s)	20mm(s)	27mm®	19mm(s)	19mm(s)	23mm(s)
11	9mm®	23mm(s)	13mm ®	19mm(s)	19mm(s)	19mm(s)	13mm®	13mm	14mm(I)	19mm ®
13	®	24mm(s)	17mm(s)	25mm(s)	16mm(s)	16mm(s)	19mm®	20mm(s)	20mm(s)	13mm®
15	13mm®	25mm(s)	17mm(s)	22mm(s)	11mm®	20mm(s)	22mm®	28mm(s)	13mm®	20mm®
16	10mm®	20mm(I)	14mm®	16mm(I)	16mm(s)	19mm(s)	21mm®	20mm(s)	17mm(s)	20mm®
19	9mm®	13mm®	17mm(s)	21mm(s)	16mm(s)	13mm(I)	28mm®	29mm(s)	11mm®	23mm(s)
20	6mm®	24mm(s)	15mm(s)	20mm(s)	19mm(s)	19mm(s)	25mm®	28mm(s)	20mm(s)	21mm®
21	15mm®	20mm(I)	17mm(s)	19mm(s)	16mm(s)	14mm(I)	19mm®	18mm(s)	19mm((s)	19mm®
24	12mm®	24mm(s)	18mm(s)	18mm(s)	9mm®	15mm(s)	20mm®	13mm	18mm(s)	20mm®
29	12mm®	21mm(s)	19mm(s)	27mm	18mm(s)	19mm(s)	18mm®	28mm(s)	25mm(s)	21mm®
31	5mm®	19mm(I)	16mm(s)	19mm(s)	18mm(s)	15mm(s)	20mm®	27mm(s)	(®)	22mm(s)
32	8mm®	23mm(s)	18mm(s)	20mm(s)	15mm(s)	16mm(s)	23mm®	25mm(s)	17mm(s)	18mm®
34	11mm®	21mm(s)	13mm®	18mm(s)	21mm(s)	15mm(s)	22mm®	17mm(s)	11mm®	22mm(s)
36	9mm®	21mm(s)	16mm(s)	14mm®	19mm(s)	19mm(s)	24mm®	22mm(s)	9mm ®	21mm®
37	10mm®	20mm(I)	18mm(s)	20mm(s)	18mm(s)	17mm(s)	22mm®	12mm	10mm®	17mm®
38	12mm®	22mm(s)	18mm(s)	21mm(s)	12 (®	17mm(s)	19mm®	19mm(s)	18mm(s)	23mm(s)
39	12mm®	24mm(s)	19mm(s)	22mm(s)	16 mm(s)	24mm(s)	19mm®	22mm(s)	12mm®	23mm(s)
40	12mm®	23mm(s)	17mm(s)	23mm(s)	19mm(s)	20mm(s)	20mm®	17mm	19mm(s)	18mm®
41	9mm®	22mm(s)	11mm(R)	18mm(s)	16mm(s)	15mm(s)	22mm®	25mm(s)	15mm(I)	22mm(s)
42	14mm®	18mm(I)	18mm(s)	19mm(s)	21mm(s)	17mm(s)	19mm®	23mm(s)	17mm (s)	19mm®
44		23mm(s)	16mm(s)	21mm(s)	20mm(s)	20mm(s)	14mm®	17mm	18mm(s)	24mm(s)
45	10mm®	22mm(s)	19mm(s)	22mm(s)	17mm(s)	19mm(s)	20mm®	26mm(s)	20mm(s)	16mm®
Total %	100%(R)	64 % (s)	89 % (s)	93% (s)	89 % (s)	93 % (s)	100 % ®	71 % (s)	50 % (s)	57 % (s0

N.B : S= Sensitivity, I = Intermediated, R = Resistant

4. Discussion

Staphylococcus aureus generally cause illness from raw cow milks with mastitis, and from handlers or from deficient hygiene. High levels of contamination are a favorable condition for *Staphylococcus aureus* growth. The organisms present in food can be risk to human health, and can cause a public health problem because this bacterium produces toxins. Milk can be contaminated by *Staphylococcus aureus* by infection of the mammary gland, by bad hygienic habits or direct contact of human such as handling equipment of milk during or after milking, coughing, sneezing, not washing hands *etc.* The importance of milk is very vital and therefore food value level is important to preserve. Their microbial contamination index can be used to judge the quality, as well as the sanitary conditions of its production and the health of the herd. And hygienic environment also important for milking place. Dung, mud, the utensils used for milk are also the source of various types of bacteria, but the main sources is the contaminated water that is added to milk to increase its quantity. It takes long time to reach the consumer and during that time it becomes highly contaminated because of high temperature which causes the proliferation of bacteria (Hassan *et.al*, 1978). In These studies of 45 samples of raw milk, 31 of them were contaminated by *S.aureus*, corresponding to 69% of samples contaminated. The similar study of *S.aureus* from raw milk in Bahia, Brazil in 50 samples showed 34 contaminations by *S.aureus*, corresponding to 68% of the samples being contaminated (LillanPorto de Cllveira *et.al*, 2001). In another study of *S.aureus* from raw milk in Bangladesh, out of 47 samples 12(25.53%) isolates were confirmed as *S.aureus.* (Mueena *et.al*.2015) .Further study was performed with well characterized isolates and was subjected to antibiotic susceptibility testing. Vancomycin(89%), Chlorampherical(93%). Gentamycin(93%), Streptomycin(89%), Tetracycline(71%), Ciprofloxacin(64%),Sulfamethoxazole (50%),Oxacillin (57%) were antibiotic sensitive and all isolates were found to be antibiotics resistant to Penicillin (100%) and Azithomycin.(100%). This data corresponds to approximately close findings in raw milk isolates in Nagpur India (Preeti G:*et,al*.2011). According to the results of antibiotic sensitivity of *S.aureus* and susceptibility was observed against Chlorampherical (100%). Gentamycin was found to be sensitive against (96%), Streptomycin (91%). A large proportion of isolates were found resistant to be Co-trimoxazole (89%), Penicillin (35%). And other raw milk isolates result in Bangladesh,(Mueena. *et.al.*2015) According to the results the isolates were found as resistant to Penicillin(100%), and Amoxicillin (100%). On the other hand the isolates were sensitive to Ciprofloxacin (83.33%), Oxacillin(100%).

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