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ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA STAPHYLOCOCCUS AUREUS AND SALMONELLA FROM RAW MILK SAMPLES OF DIFFERENT CITIES OF PAKISTAN

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Abstract:

Food-borne diseases are main public health problem throughout the world. Milk is very important component of human diet containing water, fats, proteins, vitamins and minerals. The risk of illnesses is increased when raw milk is consumed on daily basis. Mostly milk is contaminated at the time of its collection, processing, delivery and storage. . Different types of pathogenic bacteria like S. aureus and Salmonella spp. enter in milk and then multiply, after multiplication they become active in causing various diseases. Selective medium xylose lysine deoxycholate agar (XLD) was used for identification of Salmonella spp, while Mannitol salt agar was used for the identification of S. aureus, then further confirmation of these pathogenic bacteria was made through biochemical tests. Salmonella was found in 87% samples of raw milk while 91% samples were contaminated with S. aureus. Isolation of these pathogenic bacteria in milk is an important step towards controlling different diseases in humans.

Keywords: *Staphylococcus aureus; Milk; Pathogenic Bacteria.*

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INTRODUCTION:

Milk is a main component of people's diet. It is complete food containing water, fats, proteins, vitamins and minerals. (Javaid et al., 2009). Milk is consumed by people of all the age groups and quality of milk is very important for human health (Nirwal et al, 2013). It is a major part of human food and play important function in the Pakistani's diet. In Pakistan, approximately total 2 billion litre milk is produced annually and 75% of the milk is collected from cows in rural areas and 25% from other animals. .Milk is consumed in almost 30% in market products such as chocolate, ice cream, butter and cheese etc. In many countries, 5-10% milk provides total calories of the daily human diet. Moreover, milk is a best source of calcium and phosphorus in building the bones and teeth in the body (Pandey., et al, 2011). Milk aids the prevention of different type of disease like hypertension and diabetes. Milk is complex ecosystem for various microorganisms including bacteria. Mostly milk is contaminated at the time of its collection, processing, distribution and storage. At this time microorganism enter in the milk, rapidly grow and then cause different diseases (Pourhassan et al, 2011). Sometimes diseased animals like cows and buffaloes also secrete some pathogenic microorganisms in their milk. S. aureus is mostly present in unpasteurized milk and such contaminated milk product cause food poisoning and gastrointestinal illness. These are responsible for broad variation in texture and taste of milk. It produces many types of toxic enzyme and can facilitate bacterial attack and proliferation in the body of host. Staphylococcal food poisoning occurs when food is consumed that contains toxins produced by S. aureus. Food handlers carrying enterotoxin-producing S. aureus in their noses or on their hands and other main source of food contamination via direct contact or through respiratory secretions (Argudin et al. 2010). Consumption of contaminated milk in different areas of Pakistan results in different diseases such as typhoid fever and several other types of infections. The major causative agents of these diseases are different pathogenic micoorganisms eg. Salmonella and S. aureus etc. A salmonella spp. cause's gastroenteritis infection, therefore the occurrence of Salmonella and other human pathogens in unpasteurized milk cause public Health Hazards. Salmonella food poisoning is most common bacterial and widely distributed disease worldwide, estimated to cause 3 million deaths worldwide (Ohud et al., 2012).

The aim of present study is to analyze raw milk for the presence of Salmonella and S. aureus. These bacteria are involved in causing pathogenicity due to consumption of raw milk, which are responsible for different harmful disease. The aim of study is to isolate pathogenic bacteria (Salmonella and S. aureus) from raw milk. Preventive measures against these pathogenic bacteria can be done on their identification. The aim is also to find out the percentage of occurrence of Salmonella and S. aureus in raw milk samples of different cities of Pakistan. Special measures can be done to eradicate these bacteria from milk. By isolation and identification of S. aureus and Salmonella from samples it would be helpful for policy makers to take safety measures and set some rules and regulations for prevention of food borne diseases. This will help to control outbreak of different harmful diseases due to raw milk consumption and use of raw milk in making various milk products like cheese, yogurt etc.

MATERIAL AND METHODS:

Study Area

This study covers different cities of 3 provinces Punjab, KPK and Sindh as well as Kashmir. Experiments were conducted in research labs of University of Haripur.

Sample Collection

A total of 100 individual raw milk samples were collected from different cities of Pakistan from February 2015 to July 2015. Each sample was collected in a sterile falcon tubes and were directly sent to the laboratory under cold condition then the samples were analyzed within 24hours.

Isolation and identification of S. aureus

Serial dilutions of sample were made up to 10^6 dilutions in peptone water for each sample. About 1 ml of diluted sample was taken from 3rd dilution and it was transferred by pipette into nutrient agar. It was incubated at 37 ºC for 24 hours. The selective medium used of isolation of S. aureus was Mannitol salt agar. A loopful of inoculum from nutrient plate was streak on Mannitol salt agar and incubated for 48 hours at 37ºC. After incubation the colonies were observed and analyzed for morphological and biochemical characteristics of S. aureus.

Morphological Characteristics:-

The smear was prepared from isolated culture on clean glass slide and stained with gram staining. The stained smear was observed under microscope at oil immersion 100X lens.

Slide preparation:

First added one drop of water on glass slide. A loopful of bacterial colony was added from pure culture of S. aureus and mix gently. The smear was

made thin then air dried or fixed with heated flame.

Gram staining:
Added crystal violet on the slide for 1 minute then washed with tap water. After washing, added gram iodine for 1 minute then washed with tap water. Added decolorizing agent on slide waited for 15 second then washed with tap water. At last added, Safranin for 15 seconds then washed with tap water. After gram staining slide was air dried then added oil immersion on slide and observed at 100X lens. S aureus showed blue/purple color in chain or clustered form. For further conformation several biochemical tests were performed.

Biochemical test:-
Biochemical test were performed to confirm S. aureus. These tests were Catalase, Coagulase, Oxidase and Fermentation tests etc. There were three test tubes for each test;
Tube A was not containing bacteria but it contained suitable medium used for test.
Tube B was for the Positive control, while tube C was containing the sample of bacterium.

Catalase test:
Added 1.0 ml of 3% H_2O_2 directly onto an 18- to 24-hour heavily inoculated pure culture grown on a nutrient agar slant. Another recipe was also tried for the Catalse test:
A loopful of colonies of bacteria was added from Mannitol salt agar in 2-3 ml of peptone water in a tests tube and mixed gently. 2-3 drops of H_2O_2 were added in this test tube. Bubbles were produced which showed that S. aureus is present. Catalase test was positive.

Coagulase test: 1-2 ml of normal saline was taken in a test tube. A loopful of colonies of bacteria was added from Mannitol salt agar and then some plasma (5 drops) was added in it. The tube was kept in an incubator at 37° C for 2-4 hours. Agglutination of the sample indicated the presence of S. aureus.

Fermentation test:
10% solution of Glucose was prepared. Added loop full of bacterial colonies in test solution then incubated at 37 ºC for 1-2 hours. After incubation gas bubbles were produced which indicated that test is positive.

DNase test:
Bacterial colonies were taken from culture media then these were streaked on DNase agar plate and incubated at 37 ºC for 24 hours. The appearance of clear zone around colonies indicated that test is positive. (CDC, 2015)

Isolation and identification of Salmonella:
Serial dilutions of sample were made up to 10^6 dilution in peptone water for each sample. About 1 ml of diluted sample was taken from 3rd dilution and it was transferred by pipette into nutrient agar. Plates were inverted in incubator at 37 C^0 for 24 hours. Small part of colonies was taken from nutrient agar with help of sterile wire loop then streaked on selective media incubated at 37 C^0 for 24 hours. After incubation round yellow and black colonies appeared on media.

Morphological characteristics
The smear was prepared from isolated culture on clean glass slide and stained with gram staining. The stained smear was observed under microscope then Salmonella was observed as rod shaped.

Slide preparation:
Slide preparation for Salmonella was done as described in 3.3.1.1.

Gram staining: Added crystal violet on the slide for 1 minute then washed it with tap water. After washing, added gram iodine for 1minute then washed with tap water. Added decolorizing agent on the slide wait for 15 seconds then washed with tap water. Lastly added Safranin for 15 seconds then washed with tap water. After gram staining slide was air dried then added oil immersion on slide and observed at 100X lens. Salmonella was observed as pink color rod shaped form. For further confirmation several biochemical tests were performed.

Catalase test:
Catalase test for Salmonella was performed as described in 3.3.3.

Indole test
About 10-20 ml of peptone water was taken in a test tube containing the bacterial colonies and incubated it at 37 ºC for 24 hours. 0.5 ml of Kovac s reagent was added after incubation. A pink colored ring formation indicates that the test is positive. In the case of Salmonella ring is not produced it indicated that Salmonella showed Indole test negative. (Wikipedia, 2015)

Oxidase test
A filter paper was dipped in solution of tetramethyl p-phenylene-diamine-dihydrochloride. A loop full of bacterial colonies was taken with a clean wire loop then it was rubbed on filter paper piece. Appearance of deep blue or deep purple color within 10 seconds

indicates that the test is positive. But in the case of Salmonella color is not changed it showed that Oxidase test is negative. (Wikipedia, 2015)

Hydrogen Sulfide test

A strip of filter paper was dipped in solution of lead acetate then it was dried. This strip was placed in nutrient broth tube containing bacterial colonies. Incubated it at 37 ºC for 24 hours. Paper color turned black, which indicated that Salmonella shows positive hydrogen sulfide test.

Citrate test

Bacteria have ability to use citrate as its carbon and energy source. Prepared Simmon Citrate agar plates. Pure colony was taken from bacterial culture then streaked it on the surface of slant. Incubated the plate at 37 ºC for 24 hours. The color was changed from green to blue which indicated that the test is positive.

Urease test

First prepared Urease broth and filled in the test tube. Inoculated a bacterial colony in test tubes. Incubated these tubes at 37 ºC for 24 hours. A red color ring is appeared which indicates that test is positive.

Fermentation test

10% Glucose solution was prepared. A loop full of bacterial colony is added in test solution than incubated it at 37 ºC for 1 hour. After incubation gas bubbles were produced which indicated that test is positive.

RESULT AND DISCUSSION:

S. aureus was found in 91% samples of raw milk collected from different cities of Pakistan while 87% samples of raw milk were positive for Salmonella**.** S. aureus showed smooth, round and translucent colonies in nutrient agar plates. The selective medium used for the detection of S. aureus was Mannitol salt agar; it produced round, yellow /pinkish colored colonies. (Fig.1)

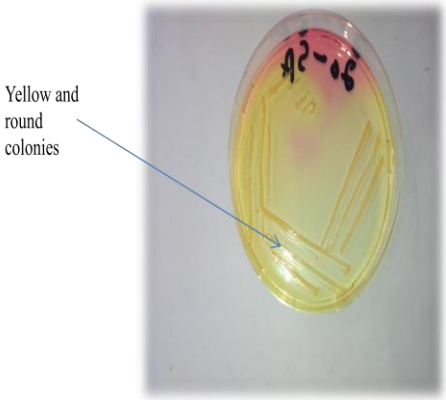

Figure 5: *S .aureus* is present in Mannitol salt agar

S. aureus showed gram positive character when gram staining was performed. It showed purple coloration and clusters of S. aureus appeared under the microscopic examination. For further confirmation biochemical tests were performed for these bacteria. Different bacteria produce different types of enzymes such as S. aureus produces Catalase which breakdown hydrogen peroxide and convert it into water and oxygen , hence Catalase action is very useful in differentiating between groups of bacteria. For example, the morphologically similar Enterococcus (Catalase negative) and Staphylococcus (catalase positive) can be differentiated using the catalase test.

Catalase test was positive for S. aureus in the form of bubbles of oxygen gas when Hydrogen peroxide was added in a test tube containing solution inoculated with S. aureus.

Figure 7: *S. aureus* showed catalase postive test

Some bacteria produce protein enzymes such as S. aureus also produces coagulases that convert Protein into fibrinogen and fibrin (Fig. 8).

Coagulases are enzymes that clot blood plasma by a mechanism that is equal to normal clotting. The coagulase test identifies whether an organism produces this exoenzyme. This enzyme clots the plasma part of blood. The only important disease causing bacteria of humans that produce coagulase enzyme are Staphylococcus aureus. Thus this enzyme is a good indicator of the pathogenic potential of S. aureus.

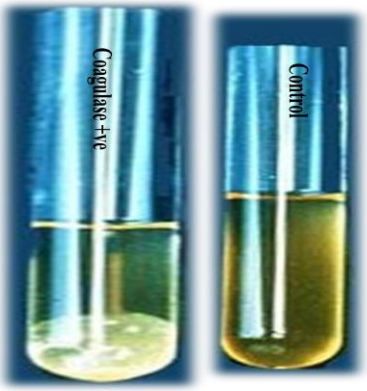

Figure 8: S. *aureus* showed coagulase test postive

S.aureus also has ability to use DNase as carbon energy source for growth. It breaks down DNA into smaller fragments, hence DNase test is also used for the identification of S. aureus.

Salmonella showed circular, smooth and translucent colonies on nutrient agar plates. The Xylose Lysine Deoxycholate agar (XLD) is a selective medium for the detection of Salmonella. It produced round, yellow and black colonies. (Fig.9)

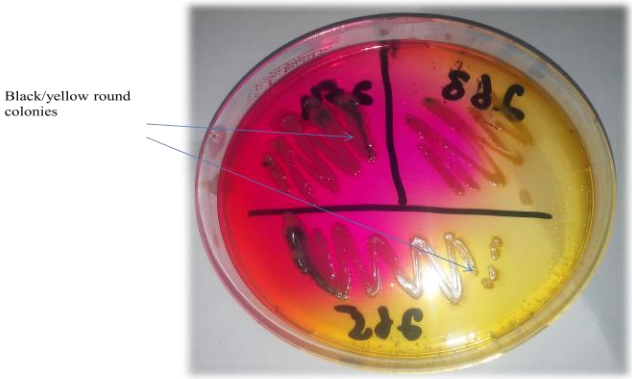

Figure 9*: Slomonella* is present on Xylose Lysine Deoxycholate agar (XLD)

A thin smear was prepared with the colony of Xylose Lysine Deoxycholate agar (XLD) for gram staining. Salmonella showed gram negative character. Pink coloration and rod shaped structure of Salmonella was seen when gram stained samples were observed under the microscope.

For further confirmation of the presence of Salmonella in raw milk samples, biochemical tests were performed. Salmonella also produces catalase like S. aureus; hence it also showed Catalase positive result. Different other biochemical tests are used for the identification of Salmonella like Citritase which breaks down citrate to oxaloacetate and acetate. Salmonella showed positive result for citrate test in the form of blue coloration of the solution.

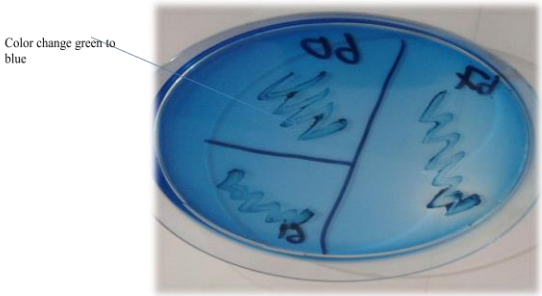

Figure 11: *Salmonella* showed citrate test positive

Indole test is used to determine whether an organism can split indole from tryptophan. Salmonella showed negative Indole test.

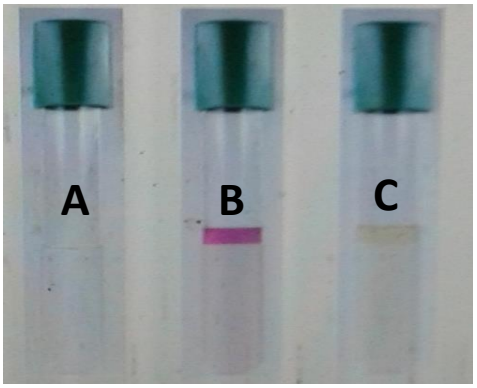

Figure 12: Salmonella showed Indole test negative as depicted in the photograph by sample C. Tube A shows an un-inoculated medium while Tube B shows positive control with E. coli added. Urease (Fig. 13) and Oxidase tests were also negative for Salmonella. Salmonella also has the ability to ferment different Carbohydrates like Maltose, Dextrose etc, hence Fermentation test was also found positive for Salmonella.

Present study showed different percentages of two different types of bacteria S. aureus and Salmonella in raw milk samples collected from different cities of Pakistan. Their presence in raw milk shows that contamination may be added by different sources for example by environment, handling, milk containers, milk handlers, utensils and storage, dirty udders of cows and buffaloes etc. Some diseased animals secrete certain pathogenic bacteria in their milk and when this milk is consumed by humans, it causes toxic effects. Due to the lack of cooling facility, microorganisms multiply in it and then cause different types of diseases. These bacteria cause several different diseases in humans who consume raw milk without heating or boiling. Our policy makers should cause local awareness among people to prohibit the consumption of raw milk without boiling and should use proper hygienic measures in handling of raw milk and its products.

The higher percentage of S. aureus and Salmonella in present research is in accordance with the result of Pourhassan et a., (2010). They explained the spatial distribution of bacterial pathogens in raw milk samples on Malayer city in Iran. Donkor et al., (2007) also conducted a similar study on raw milk samples of Accra and Kumasi cities. They cultured and identified different bacterial strains. They demonstrated that due to poor hygiene condition probable faecal contamination of the milk was mostly caused by different types of bacteria.

The use of unclean milking and transport equipment also contributed to the poor hygienic quality (Bonfoh et al., 2003). Present study showed 91 (91%) samples positive for S. aureus. A similar result was also shown by Oliveira et al., (2011). They showed that 68 % samples of raw milk were positive for S. aureus in Brazil. This difference in results may be due to difference in sampling techniques. Badini et al. (1996) also reported that 50 % samples of raw milk were contaminated with Staphylococcus aureus. Another report was given by Stamford et al., (2006). They found that 77% of milk sold in the state of Pernambuco had entero-toxigenic Staphylococcus.

Routine identification of 2 pathogenic bacteria (S. aureus and Salmonella) was done in the present research by using conventional methods based on the use of Mannitol Salt agar medium, and Xylose Lysine-deoxycholate agar medium followed by biochemical testing of suspected colonies. Gram staining characters were also noticed. However for microbiological safety in food production some others molecular tests are also used for the detection of pathogenic microorganisms in milk and its products. Polymerase chain reaction (PCR method) based on 16SrRNA gene for the detection and identification of pathogenic bacteria is used. In future we plan to do several conventional PCRs (for 16Sr RNA gene, sec for SA and spvC for S) of the suspected raw milk samples for the detection of S. aureus and Salmonella.

Table 1: Biochemical characterization of S. aureus

Biochemical test	Reaction
Catalase	Positive
Coagulase	Positive
Oxidase	Negative
Fermentation	Positive
DNase	Positive

Table 2: Biochemical characterization of Salmonella

Biochemical test	Reaction
Catalase	Positive
Fermentation	Positive
Oxdiase	Negative
Indole	Negative
Urease	Negative
Citrate	Positive
H_2 S	Positive

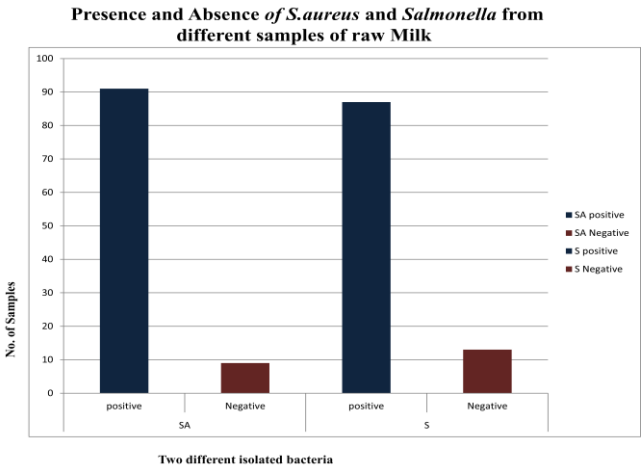

Figure 3: Detection of Salmonella and S. aureus from raw milk samples collected from different cities of Pakistan. SA shows S. aureus while S shows Salmonella.

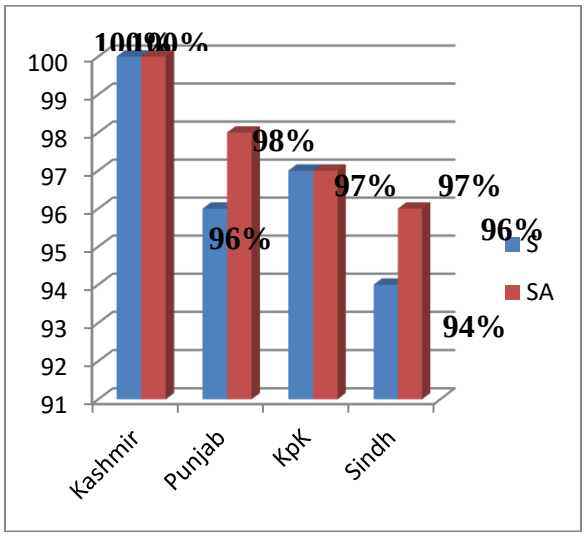

Figure 4: **Distribution on the bases of provinces.**

CONCLUSION:

Majority of the raw milk samples collected from different areas of Pakistan were contaminated with pathogenesis bacteria (S. aureus and Salmonella). The raw milk available to consumers in Pakistan (Feb– June 2015) was highly contaminated with pathogenic bacteria like S. aureus and Salmonella. Different other pathogenic micro-organisms could also be present in raw milk samples. This might be due to unhygienic condition during production,

processing, storage and handling of raw milk. It could also due to the use of raw milk from diseased animals.

Recommendations:

Antibiotic resistance of pathogenic bacteria isolated from milk can be tested further to analyze the severity of these pathogenic microbes. Real time PCR and 16RNA sequencing technique can also be performed to identify and characterize other pathogenic bacteria in milk. Several other strains of Salmonella and Staphylococcus aureus can also be detected by several conventional PCRs. Non culture based identification of bacteria in milk can also done by protein finger printing. The proposed use of protein markers for non-culture-based bacterial identification allows for high-throughput detection of pathogens present in milk samples.

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