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Isolation of *Salmonella* and *Shigella spp* from Spoilt Pasteurized Liquid Milk

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Abstract:- Milk is a major part of human food and plays a prominent role in their nutrition. Spoiled milk is the result of an overgrowth of bacteria that compromises the quality, flavor, and texture of milk. This study was carried out to isolate *S**almonella*** **and *Shigellaspp* from spoilt pasteurized liquid milk. A total of ten (10) tins of pasteurized liquid milk were used for the analysis. The tins were opened using sterilized knives and were not preserved in the refrigerator for 3-4 days. They were later analysed for the presence of spoilage microorganisms using Salmonella Shigella Agar. Out of the 10 samples that were analysed, 7 (70%) samples had *Salmonella spp* while 5 (50%) samples had *Shigella spp*.** The antibiotic susceptibility pattern of the isolates revealed that**five (5) isolates of *Salmonella spp* were sensitive to Ampicillin and Septrin, while only 4 isolates were sensitive to both Augumentin and chloramphenicol. Also, 6 isolates were sensitive to Ofloxacin while all the 7 isolates were sensitive to both Ciprofloxacin and Trimethoprim. However, all the isolates were resistant to Cefoxitin and Perfloxacin. Also, 4 *Shigella*isolates were sensitive to Ampicillin while 5 isolates were sensitive to both Ciprofloxacin and Septrin. All the isolates were resistant to Pefloxacin, Augmentin, Ofloxacin, Chloramphenicol, Trimethoprim and Cefoxitin. This study shows that poor preservation of the milk samples led to the growth of the microorganisms which can be detrimental to human health when consumed. Hence, already opened/exposed pasteurized liquid milk should be refrigerated at a temperature of between 0°C to 4°C to avoid microbial growth. Keeping milk cold is critical to ensure it stays fresh, lasts longer, and keeps its delicious taste.**

Keywords:- *Liquid milk, Pasteurized, spoilage, Salmonella spp., Shigella spp.*

I. INTRODUCTION

Milk is a nutritious white fluid secreted by the mammary gland of mammals [20]. It is known to be a balanced food as well as highly nutritious [15]. It is a major part of human food and plays a prominent role in the diet[9]. Milk has been not only the primary source of nutrition for any newborn in mammalian species, but also an excellent source of nutrient for children's growth and most adults [17]. It is the first food for mammals and provides all the necessary energy and nutrients to ensure proper growth and development, being crucial in respect to bone mass formation[14]. It is a nutrient-dense food consisting of varying amounts of carbohydrate, fat, and protein. The major constituents are water (87.4%) and milk solids (12.6%), which includes vitamins (thiamin, riboflavin, niacin, vitamin B6, B12, folate, vitamin A and vitamin C), minerals (magnesium, potassium, phosphorous and zinc), carbohydrate, fat, and protein which have an important impact on human metabolism. It is also a rich source of iron [2][7][13][19][20]. Cow milk consumption varies around the world with an average of 10 – 212kg per person per year [20].

Milk is very important to human health since calcium is very important for building strong bones, proteins for proper brain function and development of muscles and for normal growth as well. It also helps to prevent cardiovascular diseases, high blood pressure, and Type 2 diabetes [6] [12][16]. Calcium derived from milk intake is beneficial in reducing cholesterol absorption, and in controlling body weight and blood pressure. Lack of milk in the diet can participate in calcium and vitamin D deficiencies and poor health [10]. Studies has shown that milk has a wide range of physiological functionalities including anti-carcinogenic, anti-inflammatory, anti-oxidative, anti-hypertensive, anti-hyperglycemic and anti-osteoporosis properties [20]. Therefore, many dietary guidelines stress milk and dairy foods as an essential component of a healthy diet for all people, regardless of their age [10].

Due to its complex biochemical composition and high water activity, milk serves as an excellent culture medium for the growth and multiplication of many kinds of microorganisms [17]. Spoilage of milk and milk products result from growth of fermentative bacteria when storage temperatures are sufficiently high for psychotrophs. Heat-resistant proteinases of psychotrophic bacteria cause spoilage in processed milk because of enzyme-retaining activity after the heat treatment [3]. Pasteurization destroys disease-causing bacteria and extends the shelf life of milk. However, pasteurized milk can readily spoil and could cause foodborne illness if not properly handled and preserved after use. Over time, these small bacterial communities can multiply and eventually cause the milk to spoil [1]. They are usually spoiled by non-spore forming Gram-negative rods such as *Salmonella* and *Shigella*spp or Gram-positive spore forming bacteria. These organisms when consumed in spoilt milk can be detrimental to human health.

The rate at which milk spoils depends on many factors, including the number of spoilage bacteria present, temperature at which the milk has been stored, and light exposure [11].

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II. MATERIALS AND METHODS

A. Collection of samples

A total of ten (10) tins of pasteurized liquid milk were bought from different shops in Agbani, Enugu state. The samples were taken to the microbiology laboratory of Enugu state university of science and technology for analysis.

B. Sample preparation

The tins were opened using sterilized knives and were not preserved in the refrigerator for 3-4 days. They were later analysed for the presence of spoilage microorganisms.

C. Serial dilution

About 4mls of the spoilt pasteurized liquid milk was added into 3 mls of sterile distilled water in a test tube to form the stock. Five other test tubes containing 9mls of sterile distilled water were arranged in the test tube rack. 1ml of the stock was collected using a sterile pipette and was introduced into the first test tube and from the first test tube 1ml was collected using a sterile pipette into the second test tube up to the fifth test tube respectively i.e. $10^{-}1$, $10^{-}2$, $10^{-}3$, $10^{-}4$ and $10^{-}5$ respectively. The same procedure was carried out on the remaining samples respectively.

D. Isolation of microorganism

Serially diluted samples (from $10^{-}3$, $10^{-}4$ and $10^{-}5$) were inoculated into Salmonella Shigella agar plates and were incubated at 37°C for 24 hours.

E. Identification of bacteria isolates

The isolates were identified based on their gram staining and biochemical tests[6].

F. Gram staining

A smear of each bacteria isolate was made on different clean grease free slides with a sterile wire loop and left to dry and after they were heat fixed and allowed to cool. Then the different smears were stained with crystal violet for 30-60 seconds and rapidly washed off with clean water. Then the smears were stained with lugol's iodine for 30-60 seconds and rapidly washed off with clean water. The smears were decolourized with 75% alcohol for 30 seconds and washed out immediately with clean water. Then the smears were stained with safaranine for 30-60 seconds and washed off immediately with clean water. The stained slides were then allowed to air dry. After drying, a few drops of oil immersion were dropped on the stained smears and viewed under microscope (100 oil objective lens) to check for the microscopic properties of the organism. The gram negative cells appeared red pink in colour while gram positive cells appear purple or blue.

G. Biochemical tests

Several biochemical tests were carried out in order to have a presumptive and further identification of the potential bacteria.

H. Catalase test

Two mls (2mls) of hydrogen peroxide solution was poured into a clean test tube and using a wire loop, a good growth of the test organism was removed and immersed into the hydrogen peroxide solution, active bubbling indicated a positive result while no release of bubble indicated a negative test.

I. Coagulase test

A drop of physiological saline was placed on a clean slide and a loopful of the isolate was emulsified into it then a loopful of plasma was placed on it. It was rocked and clumping indicated a positive result while no clumping indicated negative result.

J. Indole test

The little portion of each of the isolate was inoculated into 5mls of sterile peptone water which was added in different test tubes using wire loop and then the test tubes containing the organisms were left to incubate at 37°C for 48 hours. After incubation, 3-4 drops of indole reagent were added and it was mixed gently. A red surface layer after 10 minutes gave a positive result while no red surface layer after 10 minutes gave a negative result.

K. Citrate utilization test

The test organisms were inoculated into Simmon citrate agar slant and incubated for 24 hours at room temperature. The appearance of growth with blue colour indicated positive result while green colour indicated negative result.

L. Urease test

A 24 hours culture of each of the isolates was streaked into the surface of urea agar slant medium contained in bijou bottle; they were incubated at 27°C for 24 hours. Purple pink colour indicated positive test.

M. Sugar fermentation

The ability of an organism to ferment various sugars or digest carbohydrate is indicated by the production of acid and gas.

- The test organism was incubated in peptone water both containing 1% solution of desired sugar
- Phenol red was added as an indicator
- An inverted durham tube was inserted in the culture tube and was incubated at 37°C for 24 hours.

Acid production was indicated by the change of colour of the medium to yellow. If gas is produced, it collects in durham tubes, which rise up the culture tubes.

N. Antibiotic sensitivity test

Antimicrobial sensitivity test was performed using the standardized diffusion method [6]. The 0.5 Marcfarland standard was used to adjust the turbidity of the inocula for the antimicrobial susceptibility test. The 0.5 Macfarland was prepared by adding 0.5ml of a 1.1775% (wt/vol) barium chloride dehydrate ($BaCl_2$ $2H_2O$) solution into 99.5ml of 1% (vol/vol) sulphuric acid (H_2SO_4). The turbidity standard was then aliquoted into screw capped test tubes identical to those used to prepare the inoculum

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suspensions. The test tubes were then sealed with wax to avoid evaporation.

Inoculating needle was used to pick the isolated colonies and these were transferred into test tubes containing sterile saline. They were vortexed thoroughly. The test tube containing the turbidity standard was also vortexed so that white precipitates of barium could be mixed well. The bacteria suspensions were then compared with 0.5 Macfarland turbidity standard. Those test tubes with inoculum that did not appear to be of the same density as the 0.5 Macfarland turbidity were either added more sterile saline or increased by adding more organisms. Within 15 minutes after adjusting the turbidity of the inoculums suspension, they were inoculated on plates containing Muller Hinton agar and sterile glass spreader was used to streak the inoculum for even distribution of the organisms. Gram negative discs were place on the inoculated plates using sterile forceps and they were incubated at 37°C for 24 hours. Clear zones of inhibition produced by the organisms were observed and measured.

III. RESULTS

In this study, the isolates were identified on the basis of cultural, morphological and biochemical characteristics and their antibiotic sensitivity pattern were also determined. The isolates identified were *Salmonella* and *Shigella spp.* (table 1& 2) Out of the 10 samples, 7 (70%) samples had *salmonella spp* while 5 (50%) samples had *Shigellaspp*(table 3).

The isolated organisms revealed susceptibility to the different antibiotics that were used. Five (5) isolates of *Salmonella spp* were sensitive to Ampicillin and Septrin, while only 4 isolates were sensitive to both Augumentin and chloramphenicol. Also, 6 isolates were sensitive to Ofloxacin. All the 7 isolates were sensitive to both Ciprofloxacin and Trimethoprim. However, all the isolates were resistant to Cefoxitin and Perfloxacin (table 4).

Also, 4 *Shigella*isolates were sensitive to Ampicillin while 5 isolates were sensitive to both Ciprofloxacin and Septrin. All the isolates were resistant to Pefloxacin, Augmentin, Ofloxacin, Chloramphenicol, Trimethoprim and Cefoxitin (table 5).

Sample	Morphology	Microscopy	Suspected Organism
Spoilt pasteurized liquid milk	Colonies are small, about 2 mm in diameter, circular, convex, smooth, opaque or colourless on SSA agar	Small Gram negative rods, 0.3-1µm in diameter and 1-6 µm in length, appearing singly, in pairs and in chains	*Shigella*Spp
Spoilt pasteurized liquid milk	Smooth, colourless colonies with black centre	Gram negative rod and medium sized, 0.7-1.5 µm to 2.2-5.0 µm. They have peritrichous flagella	*Salmonella* Spp

Table 1: Cultural and morphological characteristics of the isolated organisms

Biochemical tests	*Shigella*spp	*Salmonella* spp
Gram reaction	Gram -ve rod	Gram -ve rod
Catalase	+ve	+ve
Citrate	-ve	+ve
Coagulase	-ve	+ve
Oxidase	-ve	-ve
Indole test	-ve	-ve
Urease	-ve	-ve
Sugar fermentation		
Glucose	+ve	+ve
Galactose	+ve	+ve
Sucrose	+ve	-ve
Mannose	+ve	+ve

Table 2: Result of the Biochemical test on the isolates

Organisms identified	Number of Samples	Number of positive samples	Percentage (%)
*Shigella*Spp	10	5	50
Salmonella Spp	10	7	70

Table 3: Prevalence of *Shigella* and *Salmonella spp*isolatedfrom the samples

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Antibiotics	Number of isolates	Number sensitive	Number resistance	Disc potency (µg)	Zone of inhibition (mean)(mm)
Ampicillin	7	5	2	10	18
Augumentin	7	4	3	30	16
Ciprofloxacin	7	7	0	10	25
Perfloxacin	7	0	7	10	0
Ofloxacin	7	6	1	10	25
Septrin	7	5	2	30	23
Trimethoprim	7	7	0	30	20
Chloramphenicol	7	4	3	30	19
Cefoxitin	7	0	7	10	0

Table 4: Antimicrobial susceptibility pattern of *Salmonella*spp

Antibiotics	Number of isolates	Number sensitive	Number resistance	Disc potency (µg)	Zone of inhibition (mean)(mm)
Ampicillin	5	4	1	10	20
Augumentin	5	0	5	30	0
Ciprofloxacin	5	5	0	10	25
Perfloxacin	5	0	5	10	0
Ofloxacin	5	0	5	10	0
Septrin	5	5	0	30	23
Trimethoprim	5	0	5	30	0
Chloramphenicol	5	0	5	30	0
Cefoxitin	5	0	5	10	0

Table 5: Antimicrobial susceptibility pattern of *Shigella*spp

IV. DISCUSSION

Milk is a major part of human food and plays a prominent role in nutrition. When not properly handled, its nutritional quality can always be compromised by spoilage microorganisms which can be detrimental to health. Hence, the need to find better ways of preserving the already used products to avoid human infection.

The results obtained from this study shows that out of ten (10) spoilt pasteurized liquid milk samples that were analysed, 7 (70%) had *Salmonella* spp while 5 (50%) had *Shigella*spp. This could be as a result of poor handling/preservation of the already used product. The rate at which milk spoils depends on many factors including the number of spoilage bacteria present, temperature at which it has been stored and exposure to light. However, these organisms could grow at poor refrigerated storage temperatures. When they are not properly preserved, these organisms create various degradative enzymes that result in spoilage thereby rendering them inedible. Moreover, these harmful organisms can grow at warmer temperatures and the longer the milk is exposed to this temperature the faster the organisms grow and the quality is always compromised. This can cause food poisoning that may result in uncomfortable digestive problems such as stomach pain, diarrhea, vomiting and nausea.

The isolated organisms revealed susceptibility to different antibiotics that were used. Five (5) isolates of *Salmonella spp* were sensitive to Ampicillin and Septrin, while only 4 isolates were sensitive to both Augumentin and chloramphenicol. Also, 6 isolates were sensitive to Ofloxacin. All the 7 isolates were sensitive to both Ciprofloxacin and Trimethoprim. However, all the isolates were resistant to Cefoxitin and Perfloxacin. This is in conformity with the work [8] who reported same sensitivity and resistance to the antibiotics that were used in the study. Also, it agrees with the work of [18] which showed that the *salmonella* isolates were sensitive to both Ciprofloxacin and chloramphenicol.

Also, 4 *Shigella*isolates were sensitive to Ampicillin while 5 isolates were sensitive to both Ciprofloxacin and Septrin. All the isolates were resistant to Pefloxacin, Augmentin, Ofloxacin, Chloramphenicol, Trimethoprim and Cefoxitin. This agrees with the work of [4] who reported sensitivity of *Shigella*isolatesto Ciprofloxacin and Ampicillin.

Spoilage of pasteurized liquid milk results from overgrowth of microorganisms when exposed to poor storage temperatures which encouraged the growth of these organisms. This study shows that poor preservation of the milk samples led to the growth of the microorganisms which can be detrimental to human health. It was observed that *Salmonella* and *Shigella*spp were responsible for the spoilage of the samples. Hence, already opened/exposed pasteurized liquid milk should be properly refrigerated at a temperature of between 0°C to 4°C to avoid microbial growth.

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