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Prevalence of *Staphylococcus aureus* in Bulk Tank Milk Collected from Dairies of District Allahabad, India

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A B S T R A C T

A study was carried out to evaluate the antibiotic sensitivity of *Staphylococcus aureus* isolated from different raw milk samples. Isolation and identification of *S. aureus* were done from 5 types of dairies raw milk samples. A total of 125 milk samples from dairies were cultured for incidence of *S. aureus*. *S. aureus* was isolated from a total of 4 (3.2%) of the 125 milk samples. The antibiotic susceptibility pattern of *S. aureus* isolated from different raw milk samples was performed. *Staphylococcus aureus* strains were found to be susceptible, intermediate and resistant against different antibiotics. Strain1 was found to be susceptible against gentamycin and cotrimoxazole, intermediate against chloramphenicol, tetracycline and cephalothin, and resistant against ampicillin and methicillin. strain2, starin3 and strain4 were found to be susceptible against Gentamycin, Chloramphenicol and tetracycline, and Resistant against Ampicillin, Methicillin, Cotrimoxazole and Cephalothin. The present study provides preliminary information on incidence of antibiotic sensitive *S. aureus* as milk contaminates. The antibiotic residue in raw milk samples were analyzed in terms of Ampicillin by using TLC. According to the total number of 125 samples analysed, the ratio of contamination with antibiotics was detected as 0.8%.

Introduction

Sufficient evidence exists to effect that milk is an excellent source of nutrients for both man (Sharma and Joshi, 1992) and bacteria (Henry and Newlander, 1997). It is therefore required that milk be consumed only after it is pasteurized to make it safe. Unfortunately, recent evidence has revealed post-pasteurization contamination of milk with pathogenic bacteria (Oliver *et al.,* 2005) and antibiotic resistant bacteria. According to Hawkey (2008) antimicrobial resistance is currently the greatest challenge to the effective treatment of infectious globally. Contamination of milk with microbes therefore results in rapid multiplication and this may deteriorate the quality of milk leading to issues of food safety (Frazier and West Hoff, 1986) and public health concern when the microbes are antibiotic resistant (Guerra *et al.,* 2003). Milk is a nutritious food for human beings, acting as good medium for the growth of many microorganisms,

especially bacterial pathogens (Chye *et al.*, 2004). Raw milk is an ideal growth medium for several microorganisms. Milk and its derivates are considered vehicles for *Staphylococcus aureus* for infection in humans (Zecconi and Hahn, 2000). *S. aureus* is an important foodborne pathogen and causes a wide variety of diseases in humans and animals, ranging in severity from a mild skin infection to more severe diseases, such as pneumonia and septicemia (Lowy, 1998). In dairy cattles *S. aureus* is frequently associated with subclinical mastitis (Adesiyun, 1994) and many contaminate milk and other dairy products (Capurro *et al.*, 1999).

Staphylococcus aureus isolates are normal inhabitants of skin and mucus membranes. The coagulase-positive staphylococci constitute the well know pathogenic species of *Staphylococcus aureus* (Mahon and Larsen, 1995). A variety of diseases may be potentially transmitted through milk. The source of pathogenic agents occurring in milk may be either a cow, or a human, and it may be transmitted by both (Seguin *et al.*, 1999).

Staphylococcus aureus has been recognized as a very important virulent and frequently encountered pathogen in clinical practice. It is an endogenous microorganism colonizing in the nasal cavity, skin gastrointestinal, anus and vaginal vulvae of healthy women (Onanuga *et al.*, 2005).

The capacity to produce human diseases had not diminished even with the introduction of the antibiotics. It frequently causes septicaemia, osteomyelitis and bacteraemia (Emmerson, 1994).

Transmission occurs mainly at milking time through contaminated milking machines, clothes, and hands of milkers or machine operators (Radostitis, 2000).

Antibiotics are invariably administered to cattle to control infectious diseases, but their indiscriminate use, without adequate veterinary control, can lead to negative consequences at all levels of dairy productive chain.

Antibiotic susceptibility tests indicated that some isolates were multi-resistant to Ampicillin, Tetracyclline, Chloramphenicol, Gentamycin, Contrimoxazole, Methicillin and cephalothin. Antimicrobial resistant isolates were found in all types of milk sampled. The others were locally produced pasteurized cow milk (20.75%), powered. Most of these organisms are free living, widely distributed in cows, buffaloes, goats, dairy utensils etc.

The source of milk contamination may be due to environment, milking utensils and the personnel. The pathogenic agents, includes staphylococci, occur in milk may be either a cow, or a human and it may be transmitted by both.

Incorrect applying of antibiotics deposits noticeable residue in meat, egg, milk, cheese, butter and other livestock products. Human as a non-target organism of this drugs receives different amounts of them as residue which can cause private changes in his intestine microflora and elimination of some useful bacterial strains. These reasons make it important to effectively control antibiotic residues in milk and therefore, regulatory authorities have enacted maximum residue limits (MRLs) for a number anti-infective agent in milk.

Detectable concentrations of antibiotic residues in milk supplies higher than the MRLs are illegal. Running effective monitoring program requires specific, sensitive and reliable analytical methods that can detect all drug residues below regulated levels. The overall objective is to develop and

validate multi residue methods in order to support the implementation of both existing as well as future regulations in the area of food control (Samanidou *et al.,* 2008).

In this study a simple and fast method was done for detection of antibiotics residue in milk samples. Thin layer chromatography is a sensitive and exact method for monitoring low amounts of different biological and chemicals. Illumination of antibiotics against UV light helps as a simple detector for this mean.

Materials and Methods

Sample collection

A total of 125 milk samples (25 from each dairy) were collected in sterile samples bottle from the following dairies of Allahabad such as Aggies dairy, Raj dairy, Krishna dairy, Paryag dairy private limited, Shyam dairy.

Identification and identification of *S. aureus*

Milk sample was serially diluted in sterile 1% peptone water before plating on to Mannitol Salt Agar to isolate *Staphylococcus aureus*. Samples were then incubated at 37°C for 24 hours and sub culture into Nutrient Agar plates to obtain pure culture. Maximum of 5 colonies were obtained which were examined microscopically for Gram's reaction and for morphology using 24 hours old cultures. Motility and classical biochemical tests were performed. An appropriate positive and negative control was used to make diction between positive and "false-positive" reactions.

The identification was done by cultural, morphological and biochemical analysis as per Bergey's Manual of Systemic Bacteriology (Holt *et al.,* 1984).

Antibiotic susceptibility assay

All the *S. aureus* isolates were subjected to antibiotic sensitivity testing by standard disc diffusion method on Muller-Hinton agar (Merck®, Germany) according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Sensitivity pattern of the isolates to Ampicillin, Tetracycline, Contrimoxazole, Gentamycin, Merthicillin, Chloramphenicol, Cephalothin were determined. Isolates were divided into three groups based on the zone of inhibition produced by the antibiotic disc; susceptible, intermediately susceptible and resistant according to the Clinical and Laboratory Standards Institute (CLSI) guideline; Performance Standards for Antimicrobial Susceptibility Testing (CLSI, 2007).

Assessment of residual antibiotic in milk samples

Thin Layer Chromatography is a technique used to separate the pure components present in a mixture. This separation is possible due to the difference on the adhesion force of the molecules that are present in the mixture to a mobile phase (normally a solvent) and to a stationary phase (called thin layer, silica gel). This difference translates into more or less movement of each individual component, which allows its separation and identification.

As a part of research, 125 milk samples were collected from the same dairies. Attention was paid at the time of milk collection and milk samples were kept in refrigeration (4°C) until analysis was done. Sodium citrate Buffer (pH 3.75), stock solution of standard and TLC solvent were prepared. For extraction of the antibiotic residues of milk samples it was centrifuged at 3000rpm for 10 min and the portion remaining on supernatant after proteins were precipitated was used for analysis (Kaya *et al.,* 2010).

Preparation of silica plates

Slides were washed in acetone bath. For each plate 2 gr of Silica gel mixed in 5 ml distilled water and shaked thoroughly to produce fine paste. Clean slides were coated with silica paste by TLC gel spreader system in 0.25 mm thickness. Silica slides activated in 120°C for two hours. (Boyer, 1993).

Pointing, running and detection

Single drop of prepared antibiotic standard and centrifuged milk samples were pointed on silica plates with the help of capillary tube. Treated plates transferred to TLC solvent system as mobile phase. After receiving of solvent front to end of plates, chromatograms observed on UV light at 256 nm (Thangadu *et al.*, 2002).

Results and Discussion

A total of 125 milk samples from various dairies were Cultured for incidence of *S. aureus*.

From all 125 samples, 4 (3.2%) samples showed growth on Mannitol salt agar. After biochemical characterization which involves, coagulase, and catalase, identified by rapid identification kits (Himedia, India), only 4 samples showed positive result for all the Cultural, Morphological and Biochemical tests.

The Antibiotic susceptibility pattern of *S. aureus* isolated from different raw milk samples were performed in the present investigation. *Staphylococcus aureus* strains were found to be susceptible, intermediate and resistant against antibiotics. Strain1 was found to be susceptible against Gentamycin and Cotrimixozole, Intermediate against Chloramphenicol, tetracycline and Cephalothin, and Resistant against Ampicillin and Methicillin. Strain2, starin3 and strain4 were found to be susceptible against Gentamycin, Chloramphenicol and tetracycline, and Resistant against Ampicillin, Methicillin, Cotrimoxazole and Cephalothin.

Investigation of different raw milk samples showed out 125, 1 milk sample has presence of ampicillin antibiotic in it. Pointing of centrifuge milk samples and ampicillin standards on silica plates and concentrated solvent at different stages of evaporation made detection of antibiotics easy and easier.

Comparison between raw milk samples and ampicillin standards were made it showed that there are no similarities between the peaks of centrifuged milk samples and ampicillin standards except one milk sample. Similarities between Rf of detected peaks from suspected milk samples with antibiotic standards led us to sure there is near link and correlation between them.

The Rf value of milk sample and antibiotic showed correlation obtained is 0.72. Based on the total number of analysis samples the ratio of concentration with antibiotic was detected as 0.8%.

Various studies have been conducted to evaluate the prevalence of *S. aureus* in milk obtained from communal and commercial farms. The results reported in our study are likewise low when compared to those formerly documented (Shitandi and Sternesjo, 2004; Gundogan *et al.*, 2006). Based on observations made throughout the collection of samples, it is therefore reported that improper hygiene and poor dairy management practices contributed to the presence of *S. aureus* in the milk, especially in those from the communal dairy. The *S. aureus* incidence at a considerable high percentage indicates the alarming situation both for dairy farming and for public health as well. The numerous

examples of *S. aureus* causing bacteremia were reported in human with predisposing conditions of dairy (Normanno *et al.,* 2007). *S. aureus* was resistant to multiple classes of antibiotics which can cause serious health problems (Tenover, 2006). In the present study 125 raw milk samples were screened for the incidence of *S. aureus* isolates exhibited multiple drug resistant.

A total of 4 raw milk samples were found positive for the presence of *S. aureus*. Several *S. aureus* isolates from milk samples were found resistant to Nalidixic acid (Kresken and Wiedemann, 1988), Amoxycillin+sulbactam (Liu and Lewis, 1992), Cloxacillin (Akbarzadeh *et al.,* 2010), Erythromycin (Linda *et al.,* 2010), Kanamycin (Virdis *et al.,* 2010) and Vancomycin. On the other hand several isolates were found susceptible to the Ofloxacin, Ampicillin, Tetracycline, Oxacillin, Streptomycin, Sulphafurazole and Ciprofloxacin. In the present study out of 4 strains of *S. aureus* strain all were found to be susceptible against Gentamycin, Chloramphenicol, Tetracyclin and Cotrimixozole and were found resistant to Ampicillin, Methicillin, Cotrimixozole and Cephalothin. On the other hand 1 isolate was found Intermediate against Chloramphenicol, Tetracyclin and Cephalothin.

These susceptible antibiotic drugs will be used as the effective drugs against staphylococcal infections. The present study demonstrated that the resistant strains may be transferred to milk from infected udders, poor farm practices and due to poor handling during milking, it transmitted to the milk utensils, which can be the reason of infection in human beings. The over dose of antibiotics use during farm practices are also responsible for the emergence of antibiotic resistant microorganisms. Regular Health checkups of dairy cattle, sterilization of dairy equipment, washing of utensils, milking workers hands, udders, pasteurization/boiling of milk should be practiced.

Different methods are reported for detection of drugs residue in raw ex-farm products (Gustavson *et al.,* 2002; Kotretsu, 2004; Ramos *et al.,* 2003). Thin layer chromatography is a simple non expensive and exact technique which can execute easily in most laboratories. Among chromatographic techniques HPLC have high accuracy but have some limitations (Choma, 2003). For direct investigation of residues on dairy farms TLC have low costs, is fast and can analyze at least 10 samples at the same time.

In a study carried out by Demet *et al.,* (1992) where they tried to determine the level of penicillin G, ampicilline and penicillin V residues, using HPLC method in 50 milk samples collected from various dairy farms and creameries, 6 of the milk samples demonstrated penicillin G-potassium but none of the samples demonstrated penicillin V and ampicilline. Again in a study carried out by Demet *et al.,* (1992) in 61 milk samples collected from various dairy farms in Konya, in order to detect the level of chloramphenicol using HPLC method, 28 milk samples demonstrated chloramphenicol residues.

Even though the contamination rates discovered in milk samples as a result of residue detection studies carried, are so high, the contamination level detected in this study was only 0.8%. This can be explained by the increased public awareness about food safety and healthy nutrition and efforts of producers to market high quality products after the media started emphasizing the issue.

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