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Prevalence, Identification and Drug Resistance Pattern of *Staphylococcus aureus* and *Escherichia coli* Isolated from Raw Milk Samples of Jaipur City of Rajasthan

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The aim of this study was to isolate *S. aureus* and *E. coli* from raw milk samples supplied in the Jaipur city of Rajasthan along with their antibiogram patterns. Totally 160 samples were collected from different regions of Jaipur city. After aseptic collection and processing of samples, they were screened by direct plating on selective media *viz.* Baird-Parker Agar, EMB agar. The presumptive *S. aureus* and *E. coli* colonies were confirmed by appropriate biochemical tests. Result revealed that from screened 160 milk samples, 65 isolates (40.63 %) were of *S. aureus* and 57 (35.63 %) were confirmed as *E. coli*. Variable antibiotic susceptibility pattern (high to low) were obtained for *S. aureus* and *E. coli* isolates against the used drugs. *E. coli* isolates showed highest resistant towards drug Ampicillin and Penicillin G of 80.76% and 77.19%, respectively. On other side, they were moderately resistance to Nirtofurantoin (42.11%) and Oxacillin (38.60%). In case of obtained *S. aureus* isolates, highest resistant was noticed to Penicillin-G (86.15%), Cefotaxime (84.62%), Nirtofurantoin (81.54), Ampicillin (73.85%) followed by Chloramphenicol (69.23%) and Tetracycline (64.62%). Present findings clearly point out that the individuals consuming such contaminated raw milk and its products from this region are at high risk of getting ill and might to develop antibiotic resistance.

Key words: *S. aureus, E. coli,* Milk, Antibiotic Resistance.

Milk is a complex biological fluid and by its nature, a good growth medium for many microorganisms. Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder. Microbial contamination can generally occur from three main, sources viz. within the udder, exterior of the udder and the surface of milk handling and storage equipment.The prevalent unhygienic conditions, poor commercialization of dairy sector and improper handling practices favours the entrance of these un-desirable and infectious microorganisms, affects the overall nutritional and market value of the product. In this context, bacteria come in priority due to simple physiological and nutritional requirements. As evident from previous investigations, presence of such pathogenic bacteria poses serious health hazards to human and animal's health, as we are highly susceptible and no-doubt provides convenient conditions for their proliferation (Soomro *et al.,* 2003). Raw or processed milk is a well-known good medium for

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the growth of several microbes due to ideal pH, nutrients and water activity. Milk is also considered as a complete food for all age of humans and in Indian sub-continent its products are liked with more preferences over other foods. Consumption of such spoiled product or infectious agent or their toxins caused severe intoxications in consumers (Murinda *et al.*, 2004; Oliver *et al.*, 2005).

The presence of pathogenic bacteria in milk often emerge as a major public healthconcern, especially for those individuals who still drink raw milk.Initial bacterial contamination of milk may occur from milking animal itself, through shedding of microorganisms colonize on its teats canal or an infected udder (clinical and subclinical mastitis) or it gets contaminated later on at various stages from the animal skin, handling persons, equipments used, through extraneous dirt or use of unclean water (Banwart 1989; Hayes *et al.*, 2001),therefore the microbial content of milk is a major feature in determining its quality (Rogelj, 2003).Different microorganisms can make access to milk and milk products, among them *Escherichia coli* and Coliform infections are frequent and they are generally used as marker organisms to check fecal contamination of milk (Diliello, 1982; Soomro et al., 2002; Benkerroum *et al.*, 2004). Although, majority of obtained *E. coli* strains from milk products are non-virulent, but highly pathogenic strains were also reported in past from many milk born outbreaks that have lethal effects on host. As evident, their intoxication may cause severe intestinal and other disorders in humans (Kaper *et al.*, 2004). The ability of bacteria to cause food-borne poisoning depends on their capacity to produce toxins after ingestion (in the digestive tract) or intoxication (ingestion of preformed toxins in foodstuff). However, there are some other bacterial species that are also involved predominantly in many diseases, such as *Staphylococcus aureus*, it is considered as a leading cause of gastroenteritis occurred from ingestion of contaminated food (Loir et al., 2003). As suggested, heating of food products to cooking temperature prior to intake inactivate most of microorganisms but their secondary metabolites (toxins) still remains active for hours and once ingested established the disease (Presscott et al., 2002). The enterotoxins of *Staphylococcus* species are highly heat-stable and as suggested there toxicity increases more in foodstuffs due to shielding effect provided, them in a laboratory medium (Bergdoll 1983).

S. aureus infections range from minor skin defects suchas formation of pimples, boils, cellulites, toxic-shock syndrome, impetigo, and abscesses to life threatening disease such as vomiting, abdominal cramps, pneumonia, meningitis, endocarditis, and septicaemia (Balaban and Rasooly 2000, Soomro et al., 2003). Their infection rate is comparatively higher in India, due to prevalent warm and humid conditions for its favourable growth (Bhatia and Zahoor, 2007).

One of the important and biggest reasons of failure in treating such diseases lies in in-discriminate use of antibiotics without testing there *in vitro* sensitivity of causal organisms. Moreover, this use is higher in milking animals and cattle. On one hand, this non-directive practice increases economic losses, side effects on consumer and on other side it increases new challenges for microbiologist how to tackle this developed resistant in microbes (Owens et al. 1997.) When low doses of antibiotics are used, they inhibit the growth of susceptible bacteria while resistance bacteria thrive and grow. The resistant bacteria present in environments are in contact with human beings and animals. The indiscriminate use of antibiotics has led to the development of multiple antibiotics resistances thereby rendering the antibiotic treatment ineffective. For suitable antibiotic therapy, bacterial isolation and antibiotic sensitivity studies are atmost important. Antimicrobial susceptibility tests help us in selecting the most targeted and appropriate antimicrobial agent for treating infections.

Thus, primary objective of this study was to investigate the occurrence of the pathogen i.e. *E. coli* and *S. aureus* in milk samples, as beside causing various diseases, it also spread antimicrobial resistance in humans and animals due to its high daily intake, than other food products thus in second phase we determined the antibiotic susceptibility pattern of both the screened out isolates.

MATERIALS AND METHODS

Sample collection

In all, 160 raw milk samples, randomly collected from various regions of Jaipur city during the eight months, (March-October) period in year

2013, were employed in this study. The samples were obtained in duplicate from houses, dairy farms, milk collection centres of Co-operative milk dairies and vendors in sterilized screw capped bottles. The samples were transported to laboratory in a refrigerated transportation box and tested for presence of targeted pathogens within 24 h using standard procedures. Upon arrival in the Laboratory, samples were analyzed immediately.

Isolation and Identification of *Staphylococcus aureus* and *Escherichia coli*

Isolation of *S. aureus* was carried out according to standardized method of Singh and Prakash, (2008), with some minor modifications. Prior to isolation, a 10 ml of homogenized milk sample was first enriched with 90 ml (0.1%) of sterile peptone water and incubated for 24 h at 37°C to promote growth. Here, conventional media, Baird Parker Agar (BPA) was used as a selective medium to enumerate *S. aureus* colonies. A loopful of inoculums from enrichment broths were streaked on BPA and incubated for 48 hours at 37°C. The characteristic appearance of jet black colonies (Table-1) with a white halo was considered as presumptive for *S. aureus* in samples. The pure cultures were transferred on Nutrient agar and were further characterized by biochemical tests after incubation for 24 h at 37°C.

Similarly, to isolate *E. coli* strains, the enriched samples were pour plated with MacConkey Agar (MCA), Hi-media, Laboratories, Mumbai-a dual purpose (selective and differential) medium and plates were incubated at 37°C for 24 hours. The appearance of pink coloured colonies on MCA medium was considered as presumptive identification for *E.coli* colonies (Table-1). A single strain was picked and streaked on Eosin Methylene Blue Agar (EMB) medium, Hi-media Laboratories, Mumbai and the plates were allowed to incubate at 37 °C for 24 hours to enumerate growth. The *E. coli* colonies produced characteristic metallic sheen on EMB agar was further taken into nutrient broth for biochemical characterization.

Biochemical examination

The biochemical tests as- Gram staining, catalase test, indole, methyl red, voges- proskauer test, nitrate reduction, citrate utilization and urease productionwereperformed to characterized *E.coli* isolates.For *S. aureus* identification and confirmation, Gram staining, D-mannitolfermentation, catalase, coagulase, DNase, acetoin production,andoxidase tests were performed (Table-2 and 3).

Antibiogram pattern of the isolates to some antimicrobial agents

The susceptibility of obtained isolates to different antimicrobial agents was determined by disk diffusion method of (Bauer et al., 1996) using commercial available disks procured from Hi-media Laboratories, Mumbai. The following antimicrobial agents as -Ampicillin (10µg), Azithromycin (15µg), Cefotaxime (30µg), Cephalexine (30µg), Chloramphenicol (30µg), Co-trimoxazole (25µg), Erythromycin (15µg), Kanamycin (30µg), Nitrofurantoin (300µg), Ofloxacin (30µg), Oxacillin (1µg), Penicillin-G (10 units), Streptomycin (10µg), tetracycline (30µg) were used to check the susceptibility patterns of selected isolates. The diameter of zone of inhibition above and equal to 20 mm were considered as sensitive for a particular antibiotic, while a zone varies from 15 to 19 mm was considered as intermediate or moderately resistance. Completely resistance zones were having a diameter of 14 mm and less. Here, we have chosen these inhibitory zones as a standard criterion given in Clinical laboratory standard institute antimicrobial susceptibility testing manual, USA to test pathogenic microorganisms capable of causing human and animal diseases (CLSI, 2012).

RESULTS

Analysis of our results showed that out of 160 tested milk samples, 65 (40.63%) were found infected with *S. aureus* and 57 (35.63%) for *E. coli*.

Table 1. Morphological and culture characteristics

Isolated bacteria	Gram staining	Specific media	Culture characteristics
E. coli	Gram Negativeshort rods	Mac Conkey Agar, EMB Agar	Pink Coloured colonies Metallic sheen
S. aureus	Gram positive cocci	BP Agar	Jet black colonies surrounded by halo zone

This study has revealed that isolates of *S. aureus* were more resistant towards Penicillin-G, as 56 (86.15%) isolates from 65 showed zones of inhibition equal to or less than 14 mm followed by Cefotaxime55 (84.62%) and Nirtofurantoin53 (81.54%)Table 4.However, some *S. aureus* isolates also showed intermediate sensitivity to Ampicillin 12 (18.46%),Ciphalexin 10 (15.38%) and

Table 2. Biochemical characterization of *E. coli*

Biochemical test	Reaction
Catalase	Positive
Indole	Positive
Methyl red	Positive
Voges-Proskauer	Negative
Citrate utilization	Negative
Nitrate reduction	Positive
Urease	Negative

Table 3. Biochemical characterization of *S. aureus*

Biochemical test	Reaction
Catalase	Positive
Coagulase	Positive
DNase	Positive
Acetoin production	Positive
Oxidase	Negative
D-mannitol Fermentation	Positive

Table 4. Antibiogram pattern of *S. aureus* isolatesfrom milk

Antibiotics	Sensitive	Intermediate	Resistance
Ampicillin	5 (7.69%)	12 (18.46%)	48 (73.85%)
Azithromycin	55 (84.62%)	3 (4.62%)	7 (10.74%)
Cefotaxime	6 (9.23%)	4(6.15%)	55 (84.62%)
Cephalexine	32 (49.23%)	10 (15.38%)	23 (35.38%)
Chloramphenicol	45 (69.23%)	8 (12.30%)	12 (18.46%)
Co-Trimoxazole	52(80.00%)	6 (9.23%)	7 (10.77%)
Erythromycin	46 (70.77%)	5 (7.69%)	14 (21.54%)
Kanamycin	35 (53.85%)	5 (7.69%)	25 (38.46%)
Nitrofurantoin	8 (12.30%)	4 (6.15%)	53 (81.54%)
Ofloxacin	50 (76.92%)	7 (10.77%)	8 (12.30%)
Oxacillin	37(56.92%)	9(13.85%)	19(29.23%)
Penicillin-G	4 (6.15%)	5 (7.69%)	56 (86.15%)
Streptomycin	24 (36.92%)	10 (15.38%)	31 (47.69%)
Tetracycline	15 (23.08%)	8 (12.30%)	42 (64.62%)

Table 5. Antibiogram pattern of *E. coli* isolates from milk

Antibiotics	Sensitive	Intermediate	Resistance
Ampicillin	3 (5.26%)	8 (14.03%)	46 (80.70%)
Azithromycin	34 (59.65%)	11 (19.30%)	12 (21.05%)
Cefotaxime	49 (85.96%)	3 (5.26%)	5 (8.77%)
Cephalexine	37 (64.91%)	6 (10.53%)	14 (24.56%)
Chloramphenicol	49 (85.96%)	4 (7.02%)	4 (7.02%)
Co-Trimoxazole	41 (71.93%)	9 (15.79%)	7 (12.28%)
Erythromycin	29 (50.88%)	11 (19.30%)	17 (29.82%)
Kanamycin	45 (78.95%)	7 (12.28%)	4 (7.02%)
Nitrofurantoin	23 (40.35%)	10 (17.54%)	24 (42.11%)
Ofloxacin	32 (59.65%)	18 (32.58%)	7 (12.28%)
Oxacillin	23 (40.35%)	12 (21.05%)	22 (38.60%)
Penicillin-G	6 (10.53%)	7 (12.28%)	44 (77.19%)
Streptomycin	22 (38.60%)	26 (45.61%)	11 (19.30%)
Tetracycline	25 (43.86%)	13 (22.81%)	19 (33.34%)

Streptomycin 10 (15.38%). Of the 65 isolates maximum sensitivity (zones in mm) was noticed for Azithromycin 55 (84.62%), followed by Co-trimozole 52 (80%), Ofloxacin 50 (76.92%), Erythromycin 46 (70.77%), Chloremphenicol 45 (69.23%), Oxacillin 37 (56.92%).

Likewise to *S.aureus* isolates different antibiotic spectrum was observed for *E. coli*for different drugs Table 5. The highest resistance of *E. coli* isolates was observed toampicillin (80.70%)followed by Penicillin-G (77.19%). Least resistance was noticed for *E.coli* strains to Chloramphenicol 4 (7.02%) and Kanamycin 4 (7.02%). Intermediate resistance pattern was found highest against Streptomycin 26 (45.61%) and least toCefotaxime 3 (5.26%). Maximum isolates were found sensitive to Cefotaxime 49 (85.96%) and Chloramphenicol 49 (85.96%).

DISCUSSION

Various pathogens especially, *E. coli* and *S. aureus* occurred frequently in milk. Such milk samples obtained from organized and unorganized sectors of Jaipur district where people still prefer to buy milk from local vendors (milkman) that may have added bacterial contamination from unhygienic handling during processing and transfer of milk, use of unclean water to do adulteration, poor temperature storage, may be the pooled milk is infected with animal pathogens shed during milking and the use of unclean utensils. So, to check the microbial quality of vendor supplied milk and other collected milk in Jaipur, We tested milk for the presence of *E. coli* and *S. aureus*and their antibiogram pattern. Here, we found contamination of milk samples for both *S. aureus* and *E. coli* microorganisms.

Lingathuri and Vellathuri, (2011), in their investigation found 40% tested milk samples were positive for *S. aureus* infection and strengthens our results. Sharma et al., (2011) tested for different types of raw milk samples (115) from Meerut, India and found 25 samples positive for *S. aureus.*

Similar findings were also reported from other countries, where *S.aureus* was isolated from raw, pasteurized, processed and bulk milk tanks. Recently, de Oliveira et al., (2011) in their investigation analyzed50 raw and 20 pasteurized milk samples from 10 municipalities of Brazilian region and reported the presence of *S. aureus* in 34 (68%) raw and 6 (30%) pasteurized samples. Quintana et al., (2006) in their investigation of Goias states, found that 28.5% of the samples were positive for *S. aureus* agent.

Similar findings were also obtained in a differently placed study of Freitas et al.(2005), where raw milk samples were analyzed from Belem and 71.43% shows the presence of *S. aureus* contamination. Haran et al., (2012) tested nearly 150 pooled bulk milk tank samples collected from 50 farms and found 84% herd prevalence of methicillin-susceptible *S. aureus* while methicillin resistant prevalence was 4%. When compared to *S. aureus, E. coli* contamination was only detected in 57 (35.63%) samples. This high prevalence of *S. aureus*and *E. coli* contamination in milk samples might be due to suffering of animals with sub-clinical mastitis and use of contaminated water for washing of udder and teats. Mammary glands of milking animals are the major site of *S. aureus* infection but some investigators had reported skin as a major reservoir for this organism. Moreover, unhygienic practices may also contribute this microflora.

The appearance of antibiotic resistant among farm animals and its surrounding environment raises many questions to consumer health. As both *E. coli* and *S. aureus* are major causative agents responsible for mastitis and are the primary reasons for use of different antibiotics (Bradley, 2002). Resistance in one microorganism may spread this trait among different infectious genera and species. So, the antibiotic susceptibility of these two microorganisms will be routinely checked to prevent its spread, as well versed from MRSA emergence and is the secondary goal of this study. Here, we have shown the sensitivity of *E. coli* and *S. aureus* against important antibiotics *viz.* Azithromycin, Co-trimoxazole followed by Ofloxacin and Erythromycin. These antibiotics have showneffective results against these bacteria, some of which were also reported in the findings of Thaker et al., (2013). Here highest resistance of *S. aureus* isolates was observed to Penicillin-G, which is in consistence to the findings of Mohanty et al., (2013).

Conversely, *S. aureus* isolates were found sensitive to Azithromycin, Co-trimozole, Erythromycin and Chloremphenicol and the results

were supported by the findings of Moges et al., (2011) and Sarangi et al., (2009b).Very recently, Jeykumatand co-workers(2013), had reported that Penicillin is fully (100%) ineffective against strains of *E. coli, Klebsella, Streptococcus* and *Staphylococcus* species. In a similar, but differently designed study, Harini et al., (2011) and Mobarack et al., (2012) obtained penicillin resistance to most of the employed strains in study. It is suggested that this high resistance among *S. aureus* strains to Penicillin-G might be due to overuse and dosages of this antibiotics that had subsequently led to development of this resistance in bacteria.

Likewise for *S. aureus*, the antibiotic resistance in *E. coli* is of equal concern. As earlier discussed this bacteria gained entry into milk from various routes. Here, varying degree of antibiotic spectrum was noticed for *E. coli* isolates against differently used drugs and were found resistant to Ampicillin and Penicillin G and that is well supported by studies of Arya et al.,(2008). Thaker et al., (2013) reported that, Ampicillin was 100% resistant against *E. coli* isolates obtained from raw milk samples collected from Anand district of Gujrat. Conversely, moderate resistance was observed for Nirtofurantoin (42.11%) and Oxacillin (38.60%) antibiotics. Rehman et al., (2013), reported similar findings, where the isolates showed less resistance towards Chloramphenicol, but the results of Kanamycin sensitivity were in contradiction to present findings. These differences could be due to different biochemical and genotypic properties of bacteria, that lead to drug resistance and it's spread among different genus and species is mediated by horizontal gene transfer (Schwarz et al., 2001).

CONCLUSIONS

Conclusively, the result from this study strongly point out that microbial quality and safety of raw milk produced by local farmers/ dairies and supplied by vendors to socio-economic week to modern households is unsafe for consumption due to prevalence of microorganisms of health concern as mentioned in Indian and American safety standards. They gain entry at different steps from production to distribution at consumer end. Presence of *E. coli* is a clear indicative of faecal contamination of milk that indicates for its reservoir of other serious pathogenic microorganisms that are difficult to isolate in all samples in a very short period. Such milk is unfit for making various products. Overuse of antibiotics for farm practices is a primary reason for resistance development in microorganisms and is arising issue in metro cities. Ironically, there is an immense need for more hygienic practices, proper and regular sterilization of involved equipments, cleaning of utensils and selection and treatment of diseased animals. These strategies cannot be fulfilled without improving the literacy and awareness among the persons involved in dairy business. Moreover, suitable strategies should be adopted at farm level to make proper chilling and pasteurization of milk before its distribution in public. Furthermore, future studies will be required to identify the probable sites where this milk gets contaminated, so that a more directive approach can be prepared to stop its microbial adulterations and disease spread.

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