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Original Article

Antibiogram Profiling and Thermal Inactivation of *Staphylococcus aureus* and *Escherichia coli* Isolated from Milk of Dharan, Nepal

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Abstract

Milk is an excellent medium for the growth of many bacteria. The aim of this study was to determine antibiotic profiling and thermal inactivation of *Staphylococcus aureus* and *Escherichia coli* isolated from raw milk of Dharan. Total viable, total staphylococcal, and total coliform counts were carried out by conventional microbiological methods. Identification was done based on Gram's staining and biochemical tests. The antibiotic susceptibility test was carried out by the modified Kirby-Baur disc diffusion method. Thermal inactivation of the isolates was carried out by subjecting them to thermal treatment in a water bath. Total plate count ranged from 204×10^4 CFU/mL to 332×10^5 CFU/mL. Total staphylococcal and total coliform counts ranged from 14×10^5 CFU/mL to 8×10^6 CFU/mL and 11×10^4 CFU/mL to 3×10^6 CFU/mL respectively. *S. aureus* showed an increasing resistance patterns towards Ampicillin, Cefotixin, Carbenicillin and Cefotaxime. Ciprofloxacin, Erythromycin, Amikacin, Gentamycin, Azithromycin, and Chloramphenicol were found to be effective against *S. aureus*. All the *E. coli* isolates were resistant to Ampicillin and least resistant to Cefotixin. Chloramphenicol, Amikacin, Azithromycin, and Nalidixic acid were found highly effective in *E. coli*. The D-values for *S. aureus* at 56° C, 58° C and 60° C were 1.36 min, 1.19 min, and 1.09 min respectively. The Z-value was 14.92° C. While D-values were obtained as 0.98 min, 0.75 min, and 0.57 min for *E. coli* at 56° C, 58° C and 60° C respectively, and Z-value was 9.75° C. Hence, *S. aureus* was found to be more heat resistant than *E. coli*.

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1. Introduction

Milk is defined to be the lacteal secretion, practically free from colostrums, obtained by the complete milking of one or more healthy cows, five days after and 15 days before parturition, which contains not less than 8.5 percent milk solids-not-fat and not less than 3.5 percent milk fat (USPHS, 1965; Itzerott, 1960).Milk is a complete food, containing proteins, fats, carbohydrates, vitamins, and mineral salts. Milk has been referred to as the "most nearly perfect" food containing proteins, carbohydrates, fats, minerals, vitamins, and water as chief constituents (Park et al., 2007). Milk is also an excellent medium for the growth of many bacteria (Pelczar et al., 2013).

When milk is drawn from the udder of a healthy animal, milk contains organisms from the teat canal. They are mechanically flushed out during milking. Milking under hygienic conditions with strict attention to sanitary practices will result in a product with low bacterial content and good keeping quality (Pelczar et al., 2013). Milk and milk products are highly susceptible to microbial contamination because their composition provides a favourable medium for the growth of a wide variety of microorganisms (De Buyser et al., 2001; Van Kessel et al., 2004). Pathogens like *E. coli*, *Staphylococcus* spp., *Lactobacillus* spp., *Bacillus* spp., *Streptococcus* spp., *Listeria monocytogenes*, etc are commonly found in milk (Maniruzzaman et al., 2010). Among them, *E. coli* and *Staphylococcus aureus* are the most common contaminants. These microorganisms may gain access to milk or the products of the milk through the interior

of the udder, exterior of the cow's body, atmosphere, utensils, milker or handler, and various ingredients added to dairy products (Eckles et al., 2000). *S. aureus* has been known for decades as a bacterium that contaminated milk and causes food poisoning (Farzana et al., 2004). Presence of staphylococcal enterotoxins can cause gastroenteritis. Exotoxins producing *S. aureus* are the most dangerous and harmful for human health (Thaker et al., 2013).

Although, recognized as an opportunistic infective agent in wound, (Halpin-Dohnalek and Martha, 1989; Jablonski and Bohach, 1997), *S. aureus* infection now poses even more challenges associated with the emergence and dissemination of multiple antibiotic resistant *S. aureus* strains. Illness through *S. aureus* range from minor skin infection such as pimples, boils, impetigo to life-threatening disease such as pneumonia, meningitis, endocarditis, and septicaemia (Soomro et al., 2003; Masud et al., 1988).

E. coli is often used as a marker organism. Recovery of *E. coli* is used as a reliable indicator of faecal contamination and indicates the possible presence of enteropathogenic microorganisms which constitute a public health hazard (Bali et al., 2013). Generally, most of the *E. coli* bacteria are harmless, but some are known to be pathogenic bacteria, causing severe intestinal and extra-intestinal diseases in man (Kaper et al., 2004). Clinical infections caused by *E. coli* include urinary tract infection, septic infections of a wound, diarrhoeas, dysentery, septicaemia, pneumonia, neonatal meningitis, and abscesses in a variety of organs (Chakraborty, 2003).

Therefore, this study aimed to determine the sensitivity of *S. aureus* and *E. coli* isolated from raw milk against several antibiotics and to study heat resistance of *S. aureus* and *E. coli* isolated from raw milk samples.

2. Materials and Methods

2.1 Sample collection

Altogether 10 milk samples were collected aseptically in a dry and clean plastic container by random sampling method from households of Dharan, Sunsari, Nepal, and transported to the microbiology laboratory of Central Campus of Technology, Hattisar, Tribhuvan University, Dharan, for the laboratory work from May to August 2016. Samples were processed immediately as soon as possible (within 30 mins) otherwise preserved at 4° C.

2.2 Isolation and identification of S. aureus and E. coli

S. aureus and *E. coli* were isolated on Mannitol salt agar (MSA) and Eosin methylene blue (EMB) agar plates respectively by spread plate techniques followed by serial dilution of the milk samples. Then the inoculated plates were incubated at 37° C for 48 hours. Moreover, total plate count (CFU/mL) was also carried out on plate count agar by pour plate technique (Aneja, 2003).

Golden yellow and orange colonies developed on MSA were selected and streaked on Nutrient agar (NA) where *S. aureus* gave large, round, smooth, raised, shiny, and opaque colonies. For *E. coli*, green metallic sheen colonies developed on EMB were sub-cultured on Nutrient agar and incubated at 37° C for 24 hours.

Identification and confirmation of presumptive *S. aureus* and *E. coli* were done by using standard microbiological techniques as described in Bergey's Manual. After Gram's staining, biochemical tests were carried out for the identification of the organisms. The antibiotic susceptibility test of the isolates was done by the modified Kirby-Baur disc diffusion method as recommended by Clinical and Laboratory Standards Institute by using Muellen-Hinton agar (MHA) (CLSI, 2014). Antibiotics namely, Ampicillin, Azithromycin, Ciprofloxacin, Erythromycin, Gentamycin, Ceftriaxone, Cefotaxime, Amikacin, Carbenicillin, Nalidixic acid, and Chloramphenicol were used for Antibiotic Susceptibility Test.

2.3 Thermal Treatment

Identified bacteria (*S. aureus* and *E. coli*) were sub-cultured into Nutrient agar. The colonies were inoculated in Nutrient broth (NB) and incubated at 37° C for 24 h and finally stored in at refrigerator as a pure culture. For thermal inactivation; Isolated *S. aureus* and *E. coli* were inoculated in NB and incubated at 37° C for 4 h to obtain the turbidity of 0.5 McFarland standards. Then 10 mL of sterile milk was taken in sterile test tubes. 1 mL of each bacterial suspension was poured into the test tubes and subjected to thermal treatment in a water bath. Each heat treatment was conducted triplicate for each of the time/temperature combinations.

To calculate D-value, bacteria containing milk were heated at a certain temperature for five different

times; namely 1 min, 2 min, 3 min, 4 min, and 5 min. Similarly, to calculate Z-value, thermal treatment was conducted at three different temperatures; namely 56° C, 58° C and 60° C.

After heat treatment, test tubes were removed and placed in the ice-water bath. Isolation and enumeration of *S. aureus* and *E. coli* were performed by using spread plate techniques after serial dilution on MSA and EMB agar respectively.

2.4 ***Calculation of D and Z-values***

For the calculation of the D-value, the average of the log number of bacteria was plotted against the treatment time at each temperature. The average slope and standard error (SE) of the resultant curves were obtained using linear regression analysis (Excel), and used to calculate D-values for each temperature treatment using the equation 1.

$$D = -1/slope \quad (1)$$

Z-value was calculated from slope of an individual curve of a plot of D-values against temperature by linear regression of the slopes of the plots. The regression coefficient (R^2) of the slope of the curve was used to check the goodness of fit. *S. aureus* and *E. coli* used in this study were isolated from raw milk.

3. Results and Discussion

Analysis of results showed that out of 10 milk samples, *S. aureus* was found in 2 (20%) samples and *E. coli* was present in 6 (60%) milk samples. Total plate count of raw milk ranged from 204×10^4 CFU/mL to 332×10^5 CFU/mL. The total staphylococcal count ranged from 14×10^5 CFU/mL to 8×10^6 CFU/mL whereas total coliform count ranged from 11×10^4 CFU/mL to 3×10^6 CFU/mL.

Two isolates of *S. aureus* and six isolates of *E. coli* were subjected to antibiotic sensitivity tests. 11 antimicrobial agents belonging to different antibiotic classes were used. The most resistant drug was Ampicillin against all the isolates of *E. coli* and *S. aureus*. Also, all isolates of *E. coli* were found to be more or less susceptible to the rest of the antibiotics. Similarly, all isolates of *S. aureus* were found to be resistant to Cefotixin, Carbenicillin, and Cefotaxime and were more or less susceptible to remaining antibiotics (Table 6).

Table 1: Biochemical characterization of *S. aureus*

Biochemical test	Reaction
Catalase	+
Coagulase	+
Indole	-
Methyl red	+
Voges-proskauer	±
Citrate utilization	-
Nitrate reduction	+
Gelatin liquefaction	+

Table 2: Biochemical characterization of *E. coli*

Biochemical test	Reaction
Catalase	+
Indole	+
Methyl red	+
Voges-proskauer	-
Citrate utilization	-
Nitrate reduction	+
Gelatin liquefaction	-
SIM	+
Urease activity	-
TSI	Acid/acid

Table 3: Total Viable Bacterial Count.

Samples no.	No. of cells/ ml (CFU/ mL)
1	214×10^5
2	184×10^5
3	204×10^4
4	148×10^5
5	220×10^5
6	160×10^5
7	28×10^6
8	248×10^5
9	17×10^6
10	332×10^5

Table 4: Total Staphylococcal Count

Sample No	Number of cells/ml (CFU/mL)
2	14×10^5
10	8×10^6

Table 5: Total Coliform Count

Sample No	Number of cells/ml (CFU/mL)
5	3×10^6
6	5×10^5
7	8×10^5
8	7×10^5
9	11×10^4
10	12×10^5

Table 6: Antibiotic Susceptibilities of *S. aureus*, and *E. coli*

Antibiotics	*S. aureus*(n=2)			*E. coli* (n=6)		
	(S) %	(I) %	(R) %	(S) %	(I) %	(R) %
Ampicillin	-	-	100	-	-	100
Ciprofloxacin	100	-	-	3.3	66.6	-
Erythromycin	100	-	-	6.6	0.5	33.3
Gentamycin	50	50	-	6.6	33.3	-
Azithromycin	-	100	-	3.3	16.6	-
Chloramphenicol	-	100	-	100	-	-
Nalidixic Acid	-	50	50	83.3	16.6	-
Cefotixin	-	-	100	33.3	0.5	16.6
Carbenicillin	-	-	100	-	83.3	16.6
Amikacin	100	-	-	100	-	-
Cefotaxime	-	-	100	66.6	33.3	-

The results obtained for the log D-values at three different temperatures and Z-value are presented in Table 7. The thermal inactivation reactions followed first-order kinetics and goodness of fit (R^2) of 0.92 to 0.99 were obtained.

Table 7: D and Z-value of *S. aureus* and *E. coli*

Organisms	Temp. (°C)	D-value(min)	R^2_D	Z-value (°C)	R^2_Z
S. aureus	56	1.36	0.928	14.92	0.978
	58	1.19	0.967		
	60	1.09	0.945		
E. coli	56	0.98	0.951	9.75	0.995
	58	0.75	0.984		
	60	0.57	0.992		

Note: R^2_D= R^2 for D-value, R^2_Z= R^2 for Z-value.

In this study, the overall isolation rate of *S. aureus* in milk was 20% which seems to be similar to the findings like 17.39% by Tambekar and Bhutda (2010), 18.18% by Ekici (2004), and 18.80% by Santana (2010), and 20% by Singh and Prakash (2008). Besides, the Contamination rate of *E. coli* in milk samples was found to be 60% which is similar to the finding like 57% by Adesiyun (1994) and Soomro (2002).

Antimicrobial resistance has been recognized as an emerging worldwide problem in human medicine (Cohen, 2000) both in developed and developing countries. The susceptibility test results of *S. aureus* in the study showed 100% resistance to Ampicillin. Alian et al., (2012) also reported that resistance to Ampicillin was the most common finding (54.3%). Moreover, this study showed resistance of *S. aureus* to Cefotixin, Carbenicillin, and Cefotaxime (100%), and Nalidixic acid (50%). However, *S. aureus* was found to be intermediately sensitive towards Chloramphenicol (100%) and Gentamycin (50%) which is by the findings of Mekuria et al., (2013) who reported 45.2 % of *S. aureus* isolates were susceptible to Chloramphenicol and 38.1% were susceptible to gentamycin.

Antibiotic resistance in *E. coli* is of particular concern because it is the most common cause of urinary tract infections, bacteraemia (Salvadori et al., 2004) as well as a cause of diarrhea (Kaper et al., 2004). In this study, resistance was observed most often to Ampicillin (100%) which is in agreement with the result of Aly et al., (2012) in which 95% of *E. coli* isolates showed Ampicillin resistance. Similarly, Rasheed (2014) also recorded a high level of Ampicillin resistance among isolates of *E. coli*. About 33.3% of *E. coli* isolates were found resistant to erythromycin followed by Cefotixin and Carbenicillin (both 16.6%).

The D-values found in this study are not in general agreement with the data available in the literature. Furthermore, differences in heat resistance have been described different species of the same genus, and among different strains of the same species. Intraspecies variation in resistance has been studied in several microorganisms, including *Escherichia coli*, *Salmonella enterica*, *Aeromonas hydrophila*, and *Staphylococcus aureus*, among others (Cebrian et al., 2007; Sherry et al., 2004). A representative example is *Salmonella enterica* serovar Senftenberg strain 775W, which shows D_T values 10 times higher than other strains of the same species (Ng et al., 1969; Mañas et al., 2001). Many studies have been published on this topic, and some interesting observations have been reported: wild strains, for instance, are frequently more heat resistant than laboratory strains (Humphrey et al., 1995).The reasons behind this behavior are not always known, and admittedly the final events leading to bacterial cell inactivation by heat are not fully clear. Heat affects diverse cellular structures and functions to a different degree, and those structures are interlinked. This complexity leads to the presence of sub lethally damaged cells with a variety of injuries, which will only be able to recover and resume growth under appropriate environmental conditions. Also, the survival or inactivation of vegetative bacteria exposed to thermal treatments is influenced by many factors, such as growth conditions (temperature, time, composition of the growth medium), previous exposure to stresses, and physicochemical characteristics of the treatment

medium and environmental conditions after the treatment. The influence of these factors may be of great magnitude (Cebrián et al., 2017).

For example, Ugborogho and Ingham (1994) reported D_{56} values of 10.2 min for *S. aureus* ATCC 13565 and 13.2 min for *S. aureus* ATCC 14458. Kennedy (2004) also reported D-value at 56° C in the same range (13-21.7 min). In contrast to it, the present study revealed that the D_{56} value was 1.36 min. In reference data, D_{60} values ranged from 4.8-6.5 min but the D_{60} value obtained was 1.09 min. Similarly, the D_{58} value was also small (1.19 min) in this study than the reference data. In reference data Z-values ranged from 7.7 to 8° C but in this study, Z-value was 14.92°C.

According to Holland and Dahlberg (1940), Pereira (2007), and Read (1961), *E. coli* was found to be considerably more heat resistant than the strain used in this study. Pereira reported D-value was 10.9 min at 55° C while the D_{56} value was 0.98 min in this study. In this study, the D_{58} value for *E. coli* was 0.75 min. However, in the study of Read, Schwartz, and Litsky, the D_{57} value was 1.3 min. In this study, the D_{60} value for *E. coli* from milk sample was 0.57 min while Holland and Dahlberg reported that the D_{60} value for *E. coli* was 0.8 min. Similarly, this study showed 9.75 for the Z-value of *E. coli* whereas that of reported by Holland and Dahlberg was 9.5^{o}C.

The range of heat resistance of vegetative bacterial cells is also wide. It is generally assumed that Gram-positive cells are more resistant than Gram-negative cells (Jay, 1992) and those coccoid cells are more resistant than bacillary cells (Olson and Nottingham, 1980). Some genera are quite heat sensitive, for example, *Aeromonas* and *Campylobacter* (Sagarzazu, et al., 2010), whereas others are thoroughly heat resistant, such as *Enterococcus* (Sörqvist, 2003).

This study provides not only very important information on the antibiotic resistance pattern of *S. aureus* and *E. coli* but also thermal inactivation values of *S. aureus* and *E. coli*. However, the data generated strongly suggest that *S. aureus* is more heat resistant than *E. coli.*

4. Conclusion

In this study, raw milk collected from local areas of Dharan was found to be contaminated with *S. aureus* and *E. coli.* The milk isolates such as *S. aureus* was found to be MDR while *E. coli* showed gaining antibiotic-resistant. This study provides much-needed data on D-value and Z-value regarding both bacteria among which *S. aureus* is more heat resistant than *E. coli*. This study also suggests the need for further study on thermal inactivation of *S. aureus.*

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Conflicts of Interest

The authors declare that there is no conflict of interest with this publication.

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