PREVALENCE AND ANTIBIOTIC RESISTANCE OF BACTERIA IN TWO ETHNIC MILK BASED PRODUCTS

KALSOOM FARZANA¹, SAEED AKHTAR²* AND F. JABEEN³

¹Department of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan
²University College of Agriculture, Bahauddin Zakariya University, Multan, Pakistan
³Quality Control Manager, Jeans Pharmaceuticals, Lahore, Pakistan.

Abstract

Prevalence of food borne pathogens in milk products, khoya (a common ingredient in many traditional Indian sweets made by slowly evaporating milk under heat) and burfi (khoya cooked with sugar until it solidifies) and their sensitivity against different antibiotics was evaluated. Coliform indicated the lowest count (7.5x10³ CFU/g) and the highest (5.3x10⁶ CFU/g) in burfi whereas 6.5x10³ and 5.2x10⁶ CFU/g in khoya for 28 selected samples. Presence of Staphylococcus aureus, Escherichia coli and Klebsiella spp., was also confirmed in a large number in khoya and burfi samples. S. aureus represented the major part of bacterial flora in burfi and khoya. Enterobacter spp., and E. coli spp., constituted ~ 1.2%, in both burfi and khoya. The unidentified microflora comprised 12.56% and 8.41% in burfi and khoya, respectively. E. coli and Enterobacter spp., isolated from both khoya and burfi showed more susceptibility to Septran and Amikin. Ampiclox and Tetracycline exhibited higher degree of sensitivity against these isolates. However, Klebsiella spp., Enterobacter spp., and E. coli were found to be resistant to Urixin. Locally prepared milk products might be a potential source of bacterial contamination which poses a significant clinical threat to consumers through excessive use of various antibiotics against these micro-organisms.

Introduction

The origin of contamination by pathogenic bacteria varies with the type of product and the mode of production and processing. Contamination of milk and dairy products by pathogenic micro-organisms can be of endogenous origin, following excretion from the udder of an infected animal and/or exogenous origin, through direct contact with infected herds or through the environment (e.g., water, personnel). Treatment and processing of milk can inhibit or encourage the multiplication of micro-organisms (Brisabois et al., 1997). Food borne pathogens can survive and thrive in post-pasteurization processing environments, thus leading to recontamination of dairy products. These pathways pose a risk to the consumers from direct exposure to food borne pathogens present in unpasteurized dairy products as well as dairy products that become re-contaminated after pasteurization (Oliver et al., 2005).

Staphylococcus aureus by far is the most frequent pathogen associated with outbreaks (85.5% of the outbreaks), followed by Salmonella (10.1%) (De Buyser et al., 2001). Cooked food products and raw milk were most commonly contaminated with food borne pathogens and many of them were resistant to different antibiotics. Milk products are often contaminated with enterotoxigenic strains of S. aureus (Chao et al., 2007). It is currently not possible to effectively and consistently exclude such multiantibiotic-resistant strains from the human food chain, which means that they continue to pose a significant clinical threat to consumers and concomitant economic threats to the food production and processing industry (Walsh et al., 2005).

*Corresponding author E-mail: saeedbzu@yahoo.com
Presence of enterotoxigenic and antimicrobial resistant strains of *S. aureus* have become remarkably widespread in foods. This requires a better control of food contamination sources and distribution of antimicrobial-resistance organisms (Normanno et al., 2007).

Around 100 to 130 patients suffering from food poisoning and gastroenteritis were daily admitted to emergency wards of all major hospitals in Pakistan in 2007. A large number of children were also hospitalized for eating unhygienic food (Ali, 2007). Contamination of dairy foods with virulent pathogens render them to be a source of public health hazard. The possible contamination sources are either mastitis dairy cow or the milk itself (Carter, 1995). Growing concerns over food safety among the consumers call for the manufacturing and processing of foods under extremely hygienic conditions to avoid possible health challenges. Food safety conditions in Pakistan are not encouraging and milk products, specifically prepared by local manufactures, being unpasteurized, either exposed or improperly packed, are highly contaminated. The objective of the present study was to evaluate the level of prevalence of micoflora viz., *S. aureus*, *Enterobacter* spp., *E. coli* and *Klebsiella* spp., in frequently consumed dairy products and to assess their sensitivity against the most commonly used antibiotics.

**Materials and Methods**

**Collection of samples:** Thirty samples of *burfi* and *khoya* were collected in sterilized glass bottles from retail shops and were brought to the laboratory under low temperature for microbiological assay. The inocula were prepared by homogenizing 10 g of cooled and well-mixed samples in 100 ml chilled sterile normal saline solution containing 0.1 percent peptone.

**Control strains:** *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as control strains in this study.

**Aerobic colony count (ACC):** ACC was carried out by pour plate technique as reported previously (Case & Johnson, 1984). The homogenates were serially diluted in sterilized water, pour-plated in a thin layer of Nutrient Agar (Difco, BD Diagnostic Systems, Sparks, MD, USA) and were incubated at 37°C for 24 h to determine CFU/g. The experiment was repeated twice and reported data represent mean values (CFU/g) of these measurements.

**Coliform count:** *Klebsiella* spp., *E. coli* and *Enterobacter* spp., were enumerated in their selective media as *coliform* count. *Coliform* count was conducted by MPN technique, tubes containing gas in the inverted durham tubes were considered positive for the *coliforms*. To measure number of *coliforms* present in the milk products (*khoya* and *burfi*), dilution was read from MPN table and results were computed by multiplying this number with the dilution factor (Cappuccino and Sherman, 1992).

**Fecal coliform count:** Fecal *coliforms* were obtained by MPN technique. 0.5 ml of *coliform* culture present in the tubes was incubated into 10 ml brilliant green bile broth tubes. The broth tubes were incubated at 44.5°C for 48 hours and the results were recorded from MPN-table. Presences of fecal *coliforms* were confirmed by streaking
from positive brilliant green broth culture on eosin methylene blue agar (EMB) plates. Bacterial colonies developed were considered as fecal *coli forms* and were counted.

**Identification, morphological and biochemical characterization of bacterial strains:** The colonies isolated after purification were initially Gram stained and the isolates were biochemically characterized and identified up to species level by applying Baird parker agar, Manitol salt agar, Dnase test, Coagulase test, Oxidase catalase, Indole, methyl red, Voges-proskauer, simmons citrate, EMB as reported previously (Davidson & Henson, 1995; Holt, 1993; Pelczar *et al*., 1999)

**Antibiotic sensitivity profile**

**Disc Diffusion Susceptibility Test:** *Burfi* and *khoya* isolates; *Enterobacter* spp., *E. coli* and *Klebsiella* spp. were assessed for their sensitivity against different antibiotics viz., Urixin, Chloramphenicol, Ampicillin, Ampiclox, Nitrofurantoin, Tetracycline, Amikin, Amoxil, Augumentin and Septran as reported previously (Bauer *et al*., 1966).

The Disc Diffusion Susceptibility Test was used for each Gram-negative rod on Mueller-Hinton agar (CM337-OXOID) as growth medium. Medium was prepared according to manufacturer’s instructions and sterilized by autoclaving at 121°C for 15 min. These plates were stored at 2-8°C in sealed plastic bags for use within two weeks (Bauer *et al*., 1966). Tryptone soya broth (TSB) (CM129-OXOID) was dispensed in screw-capped test tubes and sterilized by autoclaving at 121°C for 15 min., for inoculum preparation. The test tubes were cooled and kept in an incubator for 24 h at 35°C to confirm sterility. Each isolated clinical strain was inoculated in the sterilized test tubes containing the medium and placed in an incubator overnight at 35°C. The presence of turbidity in broth cultures was adjusted according to 0.5 McFarland standard to obtain standardized suspension by adding sterile saline against a white background according to the methods outlined by National Committee for Clinical Laboratory Standards, NCCLS (Anon., 1993). Inoculum was spread evenly over the entire surface of the Mueller-Hinton agar plates by swabbing back and forth across the agar in three directions to give a uniform inoculum. Then the discs of given potency were applied on the inoculated plates with the help of forceps and incubated at 35°C for 18 h in an inverted position. The results were recorded as zone of inhibition from the standard table.

**Results and Discussion**

The frequency distribution of *burfi* and *khoya* samples in relation to various microbial counts is given in Tables 1 and 2 respectively. The ACC, *coli forms*, fecal *coli forms* and *S. aureus* count examined by Standard Plate Count (SPC), indicated an excessive contamination in both types of dairy products. *Khoya* in general revealed more bacterial contamination as compared to *burfi* samples. The ACC was found to range from $10^6$ to $10^{11}$ CFU/g for both *khoya* and *burfi* and the highest number of samples i.e., 10 of 28, manifested a bacterial count of $10^5$-$10^6$ CFU/g. *Coli forms* were found almost at the similar extent ranging from $10^2$ to $10^5$ CFU/g for both *khoya* and *burfi* samples however, a little variability in the number of samples of *khoya* and *burfi*, exhibiting extent of *coli forms* and fecal *coli forms* was observed (Tables 1 and 2).
Table 1. Microbiological profile (CFU/g) of milk products (khoya).

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of samples</th>
<th>Bacterial range (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPN-C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>MPN-FC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26</td>
<td>4</td>
</tr>
<tr>
<td>S. aureus&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24</td>
<td>-</td>
</tr>
</tbody>
</table>

ACC<sup>a</sup> = Aerobic colony count, C<sup>b</sup> = Coliform, FC<sup>c</sup> = Fecal Coliform.
The values are the mean of two experiments.

Table 2. Microbiological profile (CFU/g) of milk products (burfi).

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of samples</th>
<th>Bacterial range (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
</tr>
<tr>
<td>MPN-C&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>MPN-FC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26</td>
<td>3</td>
</tr>
<tr>
<td>S. aureus&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24</td>
<td>-</td>
</tr>
</tbody>
</table>

ACC<sup>a</sup> = Aerobic colony count, C<sup>b</sup> = Coliform, FC<sup>c</sup> = Fecal Coliform.
The values are the mean of two experiments.

S. aureus count in both khoya and burfi were found to be 10<sup>5</sup> to 10<sup>8</sup> CFU/g (Table 1 and 2) with 50% (12 of 24) samples of khoya indicating 10<sup>5</sup>-10<sup>6</sup> CFU/g as compared to 42% (10 of 24) samples of burfi samples representing the similar extent of bacterial growth. With a little variability, level of contamination detected in both khoya and burfi for this pathogenic microorganism revealed a consistent and identical pattern (Tables 1 and 2). Microbiological assay of khoya and burfi clearly manifested a higher count of ACC (3 to 4 logs) as compared to other bacterial isolates i.e., coliforms and S. aureus (Tables 3 and 4). The degree of prevalence of the micro flora in these dairy products was found to be above acceptable limits and coliforms were found in 93% of the total samples examined. The highest CFU/g were 5.3x10<sup>6</sup>, 5.2x10<sup>6</sup> and the lowest were 7.5x10<sup>3</sup> and 6.5x10<sup>3</sup> in 86% of the total samples tested for coliform and fecal coliform for burfi and khoya respectively (Tables 3 and 4). The average coliform load determined was 4.15x10<sup>4</sup>, 3.51x10<sup>5</sup> in burfi and khoya, respectively (Tables 3 and 4).

Microbiological analysis of khoya and burfi samples revealed that S. aureus was the major part of bacterial flora in burfi (30.5%) and khoya (33.56%) (Fig. 1). The level of contamination with coliforms was found to be 28.49% and 30.95% in burfi and khoya respectively. The overall coliforms were 16.16% and 16.54%; and for Enterobacter spp., E. coli and, Klebsiella spp., the level of contamination with Klebsiella spp., was the highest i.e., ~ 11.17% and 7.6% in burfi and khoya samples respectively (Fig. 1). Prevalence of Enterobacter spp., and E. coli did not exceed 1.2%, in both burfi and khoya. The results of the present study clearly indicated that S. aureus, coliforms and fecal coliforms were the major contaminants of milk products (burfi and khoya).

This study also pointed out major count of coliforms (Enterobacter spp., Klebsiella spp., E. coli), fecal coliforms and S. aureus. Coliforms contamination was shown to be relatively less in both types of tested products as compared to fecal coliforms, Staphylococcus contamination was normally attributed to food handlers, since nasopharyngeal cavity of human beings is the reservoir of microflora from which these bacteria get localized on the skin, especially on hands (Kaplan 2005; Masud et al., 1988; Patel, 1985; Stone et al., 2001).
Table 3. Maximum, minimum and average values of various bacterial count (burfi) (CFU/g).

<table>
<thead>
<tr>
<th>Nature of test</th>
<th>Samples (No.)</th>
<th>Max</th>
<th>Min</th>
<th>Avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC (^a)</td>
<td>28</td>
<td>2.0 x 10(^{11})</td>
<td>1.5 x 10(^{6})</td>
<td>8.50 x 10(^{8})</td>
</tr>
<tr>
<td>MPN-C (^b)</td>
<td>26</td>
<td>5.3 x 10(^{6})</td>
<td>7.5 x 10(^{3})</td>
<td>4.15 x 10(^{4})</td>
</tr>
<tr>
<td>MPN-FC (^c)</td>
<td>26</td>
<td>5.3 x 10(^{6})</td>
<td>7.5 x 10(^{3})</td>
<td>4.15 x 10(^{4})</td>
</tr>
<tr>
<td>S. aureus</td>
<td>24</td>
<td>7.0 x 10(^{7})</td>
<td>2.2 x 10(^{4})</td>
<td>1.45 x 10(^{5})</td>
</tr>
</tbody>
</table>

ACC \(^a\) = Aerobic colony count, C \(^b\) = Coliform, FC \(^c\) = Fecal Coliform.
The values are the mean of two experiments.

Table 4. Maximum, minimum and average values of various bacterial count (khoya) (CFU/g).

<table>
<thead>
<tr>
<th>Nature of test</th>
<th>Samples (No.)</th>
<th>Max</th>
<th>Min</th>
<th>Avg</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC (^a)</td>
<td>28</td>
<td>2.5 x 10(^{11})</td>
<td>1.6 x 10(^{6})</td>
<td>9.25 x 10(^{8})</td>
</tr>
<tr>
<td>MPN-C (^b)</td>
<td>26</td>
<td>5.2 x 10(^{6})</td>
<td>6.5 x 10(^{3})</td>
<td>3.51 x 10(^{5})</td>
</tr>
<tr>
<td>MPN-FC (^c)</td>
<td>26</td>
<td>5.2 x 10(^{6})</td>
<td>6.5 x 10(^{3})</td>
<td>3.51 x 10(^{5})</td>
</tr>
<tr>
<td>S. aureus</td>
<td>24</td>
<td>7.2 x 10(^{7})</td>
<td>2.4 x 10(^{4})</td>
<td>1.56 x 10(^{5})</td>
</tr>
</tbody>
</table>

ACC \(^a\) = Aerobic colony count, C \(^b\) = Coliform, FC \(^c\) = Fecal Coliform.
The values are the mean of two experiments.

The higher Staphylococcal count, as 1.7 x 10\(^6\) for khoya and 1.9 x 10\(^3\) for burfi was observed and the comparable range was present in milk products 10\(^3\) to 10\(^8\) (Gordon & Gibbon, 1999). Our study demonstrated 30.5% and 33.18% S. aureus in khoya and burfi and for Enterobacter spp. isolates, 56.92% and 55.8% were present in burfi and khoya, respectively.

Dust and skin of human beings were also known to contaminate food items with pathogens like S. aureus and Klebsiella spp. Manufacturers contaminate khoya and burfi during the process of sugar mixing and cutting of sweets into small pieces (Hobb's & Gilbert, 1978). S. aureus isolated from the milk products produce enterotoxins strains. Coliforms and fecal coliforms may enter the food through contamination with dust either directly or indirectly through utensils and equipments used in preparation of these milk products.
products. The food handlers and dust, constitute the major sources of microbial contamination of sweets. The food handlers also significantly contribute in contamination of *khoya* than in *burfi* (Masud et al., 1988). However, the presence of *S. aureus* and *Klebsiella* spp., will render these products unfit for human consumption, since sufficient number of these organisms will cause infection and intoxication. Multiplication and production of *S. aureus* would however, depend upon environmental factor like time, temperature, relative humidity and duration of storage and food factors, potential water activity (aw), moisture contents, nutrients present, additives used and associated microflora like *S. aureus* and *coli forms* and fecal *coli forms* (Garg & Mandokhot, 1984).

According to United States Environmental Protection Agency, (Anon., 2003), the presence of *E. coli* in the intestine and feces of warm-blooded animals is an indicator of fecal pollution. The grazing of cattle and land application of animal wastes may lead to the occurrence of enteric pathogens near the surface and ground waters. This potential contamination due to animal husbandry operations can be a serious threat to public health.

The present work reports high count of *S. aureus* in all samples of *khoya* and *burfi* and that can be due to careless handling at various stages of processing. The presence of *coli forms* and fecal *coli forms* like *Enterobacter* spp., *Klebsiella* spp., and *E. coli* shows the unhygienic nature of these sweets prepared from milk (Hobb’s & Gilbert, 1978).

**Antibiotic sensitivity of gram-negative bacteria:** Antibiotics resistance pattern of *E. coli*, *Enterobacter* spp., and *Klebsiella* spp., isolated from *burfi* and *khoya* samples has been shown in Figs. 2 and 3. *E. coli* isolated from *burfi* and *khoya* exhibited 100% resistance against Urixin, Chloramphenicol and Ampicillin. The level of resistance of *E. coli* against Ampiclox, Nitrofurantoin and Tetracycline declined almost with the same magnitude in both types of dairy products. The susceptibility of *E. coli* isolated from *burfi* and *khoya* when tested against Amikin, Amoxil, Septran and Augmentin was 100% (Figs. 2 and 3). *Enterobacter* spp., isolated from *khoya* demonstrated a greater degree of resistance against different antibiotics, particulary Ampicillin and Nitrofurantoin as compared to *Enterobacter* spp., isolated from *burfi* (Figs. 2 and 3). However, Amikin, Septran and Augmentin were still found to be as effective against *Enterobacter* spp., as *E. coli* from both *burfi* and *khoya* samples. One noticeable exception was observed with Amoxil, manifesting the similar efficacy i.e., ~ 29% resistance of *Enterobacter* spp., from both type of dairy products (Figs. 2 and 3).

Urixin, Chloramphenicol, Ampicillin, Ampiclox and Nitrofurantoin, remained ineffective against *Klebsiella* spp. In *khoya* and *burfi* isolates showing a greater variability (100, 64, 61, 36 and 39% resistance level of *Klebsiella* spp., respectively) in their effectiveness. Amikin and Amoxil demonstrated a similar extent of susceptibility i.e., 14% resistance against *burfi* isolates while *khoya* isolates of *Klebsiella* spp., manifested relatively higher resistance (25 and 29% respectively) for these antibiotics. Similarly, *Klebsiella* spp., only showed some low resistance against Septran, among all the tested antibiotics (4%). Tetracycline and Augumentin indicated 100% efficacy against all *burfi* and *khoya* isolates examined in this study (Figs. 2 and 3). The activity of Septran, Tetracycline and Augumentin remained at the maximum for *Klebsiella* spp., *Enterobacter* spp., and *E. coli* isolates and the maximum resistance was observed towards Urixin against the *Klebsiella* spp., *Enterobacter* spp., and *E. coli* isolates of *khoya* and *burfi* (Figs. 2 and 3).
Fig. 2. Resistance of Gram-negative bacteria (%) against different antibiotics. Isolates from burfi were tested against Urixin (white bars), Chloramphenicol (grey bars), Ampicillin (black bars), Ampiclox (vertical lines), Nitrofurantoin (horizontal lines), Tetracycline (downward diagonal), Amikin (dotted lines), Amoxil (upward diagonal). Augumentin and Septran indicated 0% resistance in all isolates and do not appear in the figure.

Fig. 3. Resistance of Gram-negative bacteria (%) against different antibiotics. Isolates from khoya were tested against Urixin (white bars), Chloramphenicol (grey bars), Ampicillin (black bars), Ampiclox (vertical lines), Nitrofurantoin (horizontal lines), Tetracycline (downward diagonal), Septran (large confetti) Amikin (dotted lines), Amoxil (upward diagonal). Augumentin indicated 0% resistance in all isolates and does not appear in the figure.

Dupont et al., (1978) confirmed the efficacy pattern of these antibiotics against Enterobacter spp., E. coli and Klebsiella spp. The researchers investigated different antibiotics for their resistances and found Amikin to be active against Enterobacter spp., E. coli and Klebsiella spp., which is consistent with the present results.
In the present study, *E. coli* was quite resistant to Ampiclox, but *Klebsiella* spp., and *Enterobacter* spp., were sensitive to Ampiclox. Blumberg & Strominger, 1974 investigated the mechanism of antibiotics efficacy substantiating Ampiclox to be effective against *Enterobacter* spp., *E. coli* and *Klebsiella* spp., by inhibiting the synthesis of cell wall mucopptide. The resistance of this antibiotic against Gram-negative bacteria was caused by mutation of acquisition of R-plasmids (Chamberlain, 1976). It was further demonstrated that Amoxil's was highly effective against *Enterobacter* spp., *E. coli* and *Klebsiella* spp. (Stone et al., 2001). Changes in microorganisms lead to the constant evolution of new pathogens, development of antibiotic resistance and changes in virulence of known pathogens. In many countries, as people increasingly consume food prepared outside the home, growing numbers are potentially exposed to the risks of poor hygiene in commercial food service settings (Anon., 2007). Current evidence exists to suggest that not only are such antibiotic resistant strains more difficult to control in terms of human infection, they may also be more resistant to heat processes (Davidson & Henson, 1995). In our study, *Enterobacter* spp., and *Klebsiella* spp., were sensitive but resistance was also reported, when isolated from *burfi* and *khoya*. Roupas & Pitton (1974) studied the resistant strains of *Enterobacter* spp., *E. coli* and *Klebsiella* spp., by forming β-lactamase production. Chamberlain, (1976) suggested that this resistance might be due to the induction, mutation or by acquisition of R-plasmids.

It was noticed that *E. coli* and *Klebsiella* spp., were resistant to Chloramphenicol but *Enterobacter* spp., was less resistant to this antibiotic. Moreover, ribosome located bacterial resistance to Chloramphenicol is uncommon and resistance of Gram-negative bacteria is usually acquired by means of R-plasmids (Chamberlain, 1976). In our study, *E. coli*, *Enterobacter* spp., and *Klebsiella* spp. were found to be resistant to Urixin either isolated from *burfi* or *khoya* samples.

The current results indicated that locally prepared milk products might be a potential hazardous sources of pathogenic *S. aureus*, *Klebsiella* spp., *E. coli* and *Enterobacter* spp., Strict control measures must be applied to minimize and eliminate the contamination possibilities through milk and its products leading to minimized use of various antibiotics which are excessively used and becoming ineffective against such bacterial strains.

References


(Received for publication 3 September 2008)