QUALITATIVE AND QUANTITATIVE ASSESSMENT OF β-LACTAM ANTIBIOTIC RESIDUES IN UNPROCESSED MARKET MILK IN LAHORE, PAKISTAN

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ABSTRACT

Present study was conducted to evaluate the extent of β-lactam (Penicillin G, Ampicillin, and Amoxicillin) antibiotic residues in unprocessed market milk during the year 2013 from four different zones of Lahore city. Two zones (Mall road and Canal road) are located in center of city while other two zones (Multan road and Ferozpur road) exist near to periphery. A total of 120 samples (30 each from 4 zones) were collected from shops selling raw (unprocessed) milk. Qualitative assessment of β-lactam antibiotic residues was done by Bacillus subtilis Qualitative Field Disc Assay. Milk samples producing zone of inhibition were considered having antibiotic residues. Quantification of these drugs residue in positive milk samples was done by High Performance Liquid Chromatography (HPLC). Antibiotic residue was detected in 23% samples on the basis of disc assay. The maximum inhibition on B. subtilis was 14 mm while minimum observed was 3.5 mm. Zone wise distribution of positive samples was 5 (17%), 3 (10%), 9 (30%) and 11 (37%), respectively. All 28 positive samples quantified through HPLC showed that ampicillin, amoxicillin and penicillin were present in 32 % (9/28), 85% (24/28) and 89% (25/28) of positive samples, respectively. About 68% (19/28) of the positive samples, were unfit for human consumption having residues higher than maximum residual limit (MRL’s) standards of EU (4μg/L) and FDA (5 and 10μg/L), respectively. Overall percentage of samples with more than MRL’s was 16 (19/120).

Keywords: antibiotic residue, Bacillus subtilis, β – Lactam, HPLC, milk

INTRODUCTION

Milk is a nourishing healthy food consumed across the globe. Pakistan is fourth largest milk-producing country in the world (ESOP, 2014). Different types of milks are available commercially, out of which bovine milk holds the highest percentage, followed by ovine and caprine milk. In milk residues different type of drugs (antimicrobials, hormones and other chemical contaminants like dioxins

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and pesticides) have been found with their subsequent public concern (Herrera et al., 2009). Dairy animals that have been treated with antibiotics produce milk containing antibiotic residues for a few days after treatment. These residues may cause severe allergic reactions in sensitive consumers and contribute to antibiotic resistant infections in humans. Moreover, drug-resistant bacteria are produced after prolonged exposure to antibiotic residues (Di Corcia and Nazzari, 2002).

β-lactam antibiotics are frequently used in veterinary practice for the treatment of diseases. These products are easily available in market, and their off-label use for prophylactic measures is common. Medicated feeds are also administered orally after mixing with animal feed. Indiscriminate use of antibiotics such as needless use for disease control and avoidance to follow prescribed directions are key reasons for presence of antibiotic residues in milk (Koesukwiwat et al., 2007).

All across the world, monitoring programs have been established by governments to determine the levels of antibiotic residues in food and set a maximum residual level (MRL) for them. Various techniques have been used to determine β-lactam antibiotics including spectrophotometry (Saleh et al., 2003) and liquid chromatography (Samanidou et al., 2003). High performance liquid chromatography (HPLC) has been the most common technique for analyzing antibiotics in different matrices, usually coupled with mass spectrometry (MS) (Blasco et al., 2007).

Keeping in view the harmful effects of antibiotics used in the animals on the human health, the present study was designed to determine the residues of different β-lactam in market (unprocessed) milk in different localities of Lahore.

**MATERIALS AND METHODS**

A total of 120 samples from four zones of Lahore (Mall Road, Canal Bank Road, Ferozpur Road and Multan Road), 30 samples from each zone were collected from raw (unprocessed) milk shops in 50ml sterilized falcon tubes, transported in ice box (4°C) to Lab and stored at -20°C until further processing. Two zones (Mall Road and Canal Bank Road) make central point of city while Ferozpur Road and Multan Road are close to periphery of city. These samples were screened for the presence of β-lactam antibiotic residues by using *Bacillus subtilis* (*B. subtilis*) Qualitative Field Disc Assay (Khaskheli et al., 2008). Culture of *B. subtilis* was taken from Department of Microbiology, UVAS, Lahore. The culture was purified by Four-way/Double streaking method. After purification of the culture, raised, dull, wrinkled colonies of pure *B. subtilis* were obtained which were subsequently inoculated into TSB (Tryptic soy broth) to obtain bulk growth. The optical density value of bacterial suspension was set against that of 0.5 Mac-Forland solutions.

Discs of 6 mm diameter were made from Whatman filter paper with the help of punch machine. The falcon tubes containing milk samples were shaken and the prepared discs were dipped 3 times into the samples and allowed to air dry (one disc/sample). Dried discs were placed on already swabbed petri plates on equal distance (six discs on each plate). The plates were incubated for 24 hours at 37°C. Zone of Inhibition for each disc was examined and measured separately.
Parallel to that, antimicrobial susceptibility standards test discs of Ampicillin, Penicillin G and Amoxicillin (Oxoid™) were processed for comparison.

β-lactam antibiotic residues in market milk were quantified by HPLC (Freitas et al., 2013). The HPLC (Model SP-20A, Shimadzu™) was used with column RP C18 250*4.6 mm with 5μ particle size (Waters™). The column effluent was monitored at a detector wave length of 245nm. Two mobile phases were used: (A) formic acid 0.1% (v/v) in water and (B) formic acid 0.1% (v/v) in acetonitrile. Gradient program was used, at a flow rate of 0.45 mL/ min, 5 min from 97% A to 40% A; 5 min from 40% to 0% A; 10 min from 0% back to 97% A; 12 min 97% A. The column was maintained at 40°C and the injection volume was 20 µL.

Standard antibiotic solution was prepared by dissolving, 100 mg each of ampicillin, amoxicillin and penicillin in 100 ml of methanol in volumetric flask separately for each antibiotic. Subsequently, 1 ml from this solution was dissolved into 100 ml of phase A. Finally, the standard solutions of these three antibiotics were made having concentration of 10 µg/ml. Twenty µl of the standard solutions was then injected into HPLC system.

Samples were prepared for HPLC by taking 2ml homogenized raw milk separately in 20 mL centrifuge tubes. These mixtures were vortexed and kept in the dark for 10 min. Proteins were precipitated and antibiotics were extracted through shaking for at least 20 minutes with 10 ml acetonitrile, followed by centrifugation for 15 minutes at 5000 rpm. The supernatant was collected in a new tube and evaporated until it dried, under a mild stream of nitrogen. The residue was again dissolved with mobile phase A (50 mL), filtered through a 0.45μ polyamide filter paper, transferred to vials and was injected into the HPLC system. Data regarding the residues of β-lactams were analyzed statistically by One-way Analysis of Variance (ANOVA) using SPSS Version 20.0. P-value < 0.05 was considered significant.

RESULTS
Presence of β - lactam antibiotic residues were detected in 23% (28/120) milk samples through Bacillus subtilis Qualitative Field Disc Assay. The distribution of positive samples was 17% (5/30), 10% (3/30), 30% (9/30) and 37% (11/30) in Zone 1, 2, 3 and 4, respectively. The positive samples for antibiotic residues were significantly higher (P<0.05) in Zone 4 than Zone 2, but non-significant (P>0.05) between Zone 1 and Zone 3, while they were slightly higher (P = 0.05) in Zone 3 as compared to Zone 2. In unprocessed market milk samples, the maximum zone of inhibition on B.subtilis growth was 14 mm, while minimum Zone observed was 3.5 mm, however, the mean positive being 9.5 mm.

The HPLC analysis of positive milk samples indicated that 22% (6/28) had one antibiotic residue, 50% (4/28) samples had two antibiotic residues, and 28.5% (8/28) had three antibiotic residues. Zone wise distribution depicted that one type of antibiotic residue was shown by 22% (2/9) and 36% (4/11) samples in zone 3 and 4, respectively; two types of antibiotic residue was shown by 60% (3/5), 67% (2/3), 44% (4/9), and 45% (5/11) samples in Zone 1, 2, 3 and 4, respectively and three types of antibiotic residue was shown by 40% (2/5), 33%, (1/3), 33% (3/9) and 18% (2/11) samples in Zone 1, 2, 3 and 4, respectively. Descriptively the overall percentage of ampicillin, amoxicillin and penicillin was
32% (9/28), 85% (24/28) and 89% (25/28), respectively in positively screened samples.

Positive milk samples having ampicillin residues more than maximum residual limit (MRL) were 20% (1/5), 33% (1/3), 22% (2/9) and 9% (1/11) in Zone 1, 2, 3 and 4, respectively. Positive milk samples having amoxicillin residues more than MRL were 40% (2/5), 67% (2/3), 22% (2/9) and 45% (5/11) in Zone 1, 2, 3 and 4 respectively. Positive milk samples having penicillin residues more than MRL were 80% (4/5), 67% (2/3), 67% (6/9) and 36% (4/11) in Zone 1, 2, 3 and 4, respectively. Table 1 gives mean ± SE of antibiotic residues (µg/L) along with maximum and minimum values.

Rejectable positive samples were 100% (5/5), 100% (3/3), 67% (6/9) and 45% (5/11) in Zone 1, 2, 3 and 4, respectively. Overall, percentage of the rejectable samples out of positive samples was 68% (19/28).

Table 1. Concentration of antibiotic residues above maximum residual limit (MRL) in unprocessed market milk samples collected from different zones of Lahore city

<table>
<thead>
<tr>
<th>Variables</th>
<th>Ampicillin</th>
<th>Amoxicillin</th>
<th>Penicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zone-1</td>
<td>Zone-2</td>
<td>Zone-3</td>
</tr>
<tr>
<td>Samples (n) containing AB residues more than MRL</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mean ±SE values of AB residues (µg/L)</td>
<td>9.9±0.00</td>
<td>4.5±0.00</td>
<td>5.5±0.15</td>
</tr>
<tr>
<td>Minimum values of AB residues (µg/L)</td>
<td>-</td>
<td>-</td>
<td>5.7</td>
</tr>
<tr>
<td>Minimum values of AB residues (µg/L)</td>
<td>-</td>
<td>-</td>
<td>5.4</td>
</tr>
</tbody>
</table>

**DISCUSSION**

This study showed that the antibiotic residues are present in market milk supplied in Lahore. These residues are a major hazard for human health in terms of direct toxicity or antibiotic resistance in humans (Nisha, 2008). In this study 23% milk samples were positive with β-lactam antibiotic residues. A study performed in Hyderabad (Sindh, Pakistan), showed 36% milk samples positive (Khaskheli et al., 2008). In India, Arora and Chhabra (2004) reported 24% milk samples positive with same antibiotic residues. Ardic and Durmaz (2006), found 21.33% of β-lactam antibiotic drug residues in unpacked milk consumed in Turkey (Sanliurfa region). In the northwest Iran, Movassagh and Karami (2010) reported 2.66% positive for β-lactam antibiotic residues out of all collected pasteurized milk samples. Difference in percentages of positive samples for antibiotic residues in different places may be due to indiscriminate use of antibiotics.

The percentage of the positive raw milk samples for antibiotic residues was high in milk shops at periphery of city milk shops Multan road (37%) and Ferozpur road (30%) but this percentage was moderate at mid-city milk shops Mall road (17%) and Canal road (10%). Probably reason is that farmers located in the periphery of the city are not properly facilitated for veterinary services and...
directions regarding use of antibiotics. Mostly milk producers have their own selling point and they don’t bother to discard the milk of diseased or animal under treatment. This higher concentration may also be due to unawareness of farmers regarding antibiotic withdrawal period. In comparison antibiotic residues were less in the mid-city markets where veterinary services are provided by qualified veterinary doctors.

The rejection rate of milk based upon maximum residual limit (MRL) value of antibiotic was 16% of overall raw milk samples. This was highest in samples from Ferozapur road (20%), followed by Multan road and Mall road (16% for both) and least from Canal road (10%). Penicillin and Amoxicillin were more than 20% in raw milk samples but ampicillin was seen in 1% cases. According to Riediker et al. (2001) antibiotic residues, frequently detected were penicillin G and cloxacillin, the later often in combination with ampicillin or amoxicillin.

CONCLUSION
It is concluded from the present study that about a quarter raw milk sold in the Lahore city had antibiotic residues. Penicillin and Amoxicillin residues were found more common among β-lactam group. These residues were more in the samples taken from periphery of Lahore than central zones. The study highlights the need for dairy animals awareness of, owners to use antibiotics in consultation to qualified veterinarian and give consideration to milk withdrawal period after administration of antibiotics to dairy animals.

Conflict of interest statement
The authors declare that they have no conflict of interest.

REFERENCES

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