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Detection of ß - Lactam Antibiotic Residues in Market Milk

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Abstract: The present study was conducted to evaluate the extent of ß - lactam antibiotics residues in unprocessed market milk during the year 2006. Milk samples were randomly collected from Hyderabad city, Latifabad and Qasimabad. Microbial screening test (*Bacillus subtilus* Field Disc Assay) and High Performance Liquid Chromatography (HPLC) methods were used to detect, identify and quantify the ß-lactam residues in milk. A total of 137 milk samples were screened. Among these 63.50% were negative and 36.50% positive for ß - lactam antibiotics residues. The zones size of positive samples appeared between 5.0 and 15.0mm (mean 8.91±0.36mm). Residues level quantified between 0.4 to 400 µg/L for Penicillin G, between 1.0 to 190µg/L for Amoxicillin, between 0.5 to 141µg/L for Ampicillin and between 2.1 to 122µg/L (40.74± 10.59µg/L) for unknown antibiotics. The residues of Penicillin G (mean 59.53µg/L) in unprocessed milk was 14.9 and 11.9 fold, Amoxicillin (mean 36.11µg/L) 9.03 and 3.61 fold, Ampicillin (mean 46.91µg/L) 11.73 and 4.69 fold higher than (MRL's) standards of EU (4µg/L) and FDA (5 and 10µg/L), respectively.

Key words: Milk, Beta lactam, antibiotics, Penicillin residues

Introduction

Antibiotics are vital medicines considered as the ultimate strategy to treat human infections. Their effectiveness is however, threatened by extensive and inappropriate use of these, not only in medicine but also in agriculture. In veterinary practice, antibiotics are utilized at therapeutic levels primarily to treat diseases and to prevent infection. They are also used at sub-therapeutic levels to increase feed efficiency, promote growth and prevent diseases.

The frequent use of antibiotics may result in drug residues that can be found at different concentration levels in products from animal origin, such as milk or meat. Presence of drugs or antibiotics residues in food above the maximum level recognized world wide by various public authorities is illegal (Kempe and Verachtert, 2000). Consumers want to be confident that their food supply is free of contamination by herbicides, pesticides, drugs or antibiotics due to the fact that they may cause potential health hazards, for example allergic reaction, carcinogenicity and promotion of the spread of bacterial resistance to antibiotics used in human medicines. Approximately 5-10 percent of the population is hypersensitive to Penicillin at a concentration as low as 1 ppb or other antibiotics and suffers allergic reactions (skin rashes, hives, asthma, anaphylactic shock). Beside this, antibiotics may interfere with the manufacture of several dairy products. Concentration of 1 ppb delays starter activity during butter and yoghurt making. Antibiotics also decrease the acid and flavor production associated with butter manufacture and they reduce the curdling of milk and cause improper ripening of cheese (Jones, 1999). The USA Food and Drug Administration (FDA) have recently identified approximately 80 drugs which are likely residues in animal-derived human food (Ghidini *et al*., 2002).

ß - lactam is the oldest group of antibiotics which are frequently used for the treatment of sick animals in Pakistan. Milk of such animals is used without any safety measures, causing problems in dairy industries as well as in human health. However, the molecules belonging to the group of ß - lactam have the lowest tolerance in the EU between all the antimicrobials. Consequently the EU regulations 2377/90 set the maximum residues limit (MRL) for some ß - lactam antibiotics in milk, for example, penicillin G 4 µg/L, Ampicillin 4 µg/L, Oxacillin 30 µg/L, Amoxicillin 4 µg/L, Dicloxacillin 30 µg/L, Cephalexin 100 µg/L and Cephairin 60 µg/L (Ghidini *et al*., 2002). Since, no work has been reported on antibiotic residues on market milk in Pakistan. Thus, present study has been planned to evaluate the level of ß - lactam antibiotics in market milk.

Materials and Methods

Screening of milk samples for the presence of ß - lactam antibiotic residues: A total of 137 unprocessed market milk samples from Hyderabad city, Latifabad and Qasimabad were collected and brought to the laboratory of Department of Dairy Technology, Sindh Agriculture University Tandojam for detection, identification and quantification of ß - lactam antibiotic residues. Milk samples were screened for the presence of ß - lactam antibiotic residues using *Bacillus subtilis* Qualitative Field Disc Assay as described by Association of Official Analytical Chemists (AOAC, 2000). The blank disc of filter papers (Whatman 1, 12mm) were completely dipped into milk sample using forcep and placed on the surface of agar medium containing the sensitive test

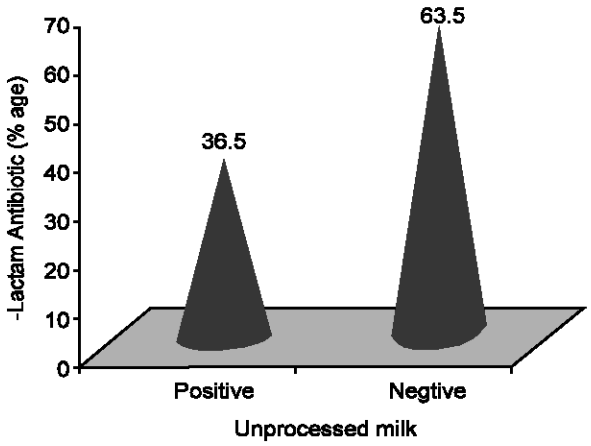


Fig. 1: Screening of unprocessed market milk samples (%age) for ß - lactam antibiotic residues.

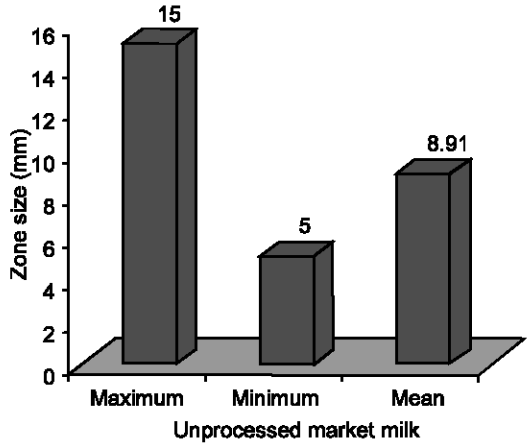


Fig. 2: Diameter of inhibitory zones (mm) determined by microbiological assay of unprocessed market milk.

organism (*Bacillus subtilis*). The plates were incubated at 35°C for 24h. The positive results (the presence of ß - lactam antibiotic residues) were manifested by formation of transparent zones around disc. Parallel to that antimicrobial susceptibility standards test discs of Ampicillin, Penicillin G and Amoxicillin (Oxoid) were processed for comparison purpose.

Quantification of ß - lactam antibiotics residues: ß-lactam antibiotic residues of market milk were quantified by the method as described by Ghidni *et al.* (2002). A High Performance Liquid Chromatography (HPLC) system (Hitachi) was used, which equipped with an autosampler (Model, L-2200), a UV detector (Model, L-2400), a pump (Model, L-2130) isocratic, Column Oven (Model, L-2300) and C18 (ODS-3) column. The column effluent was monitored at a detector wave length of 365nm.

Table 1: HPLC analysis of unprocessed market milk samples (+ve in screened test)

Category of antibiotic residues	Positive over 50 samples (No.)	%age
ß-Lactam Residues		
Penicillin G	32	64
Amoxicillin	28	56
Ampicillin	24	48
Unknown	15	30

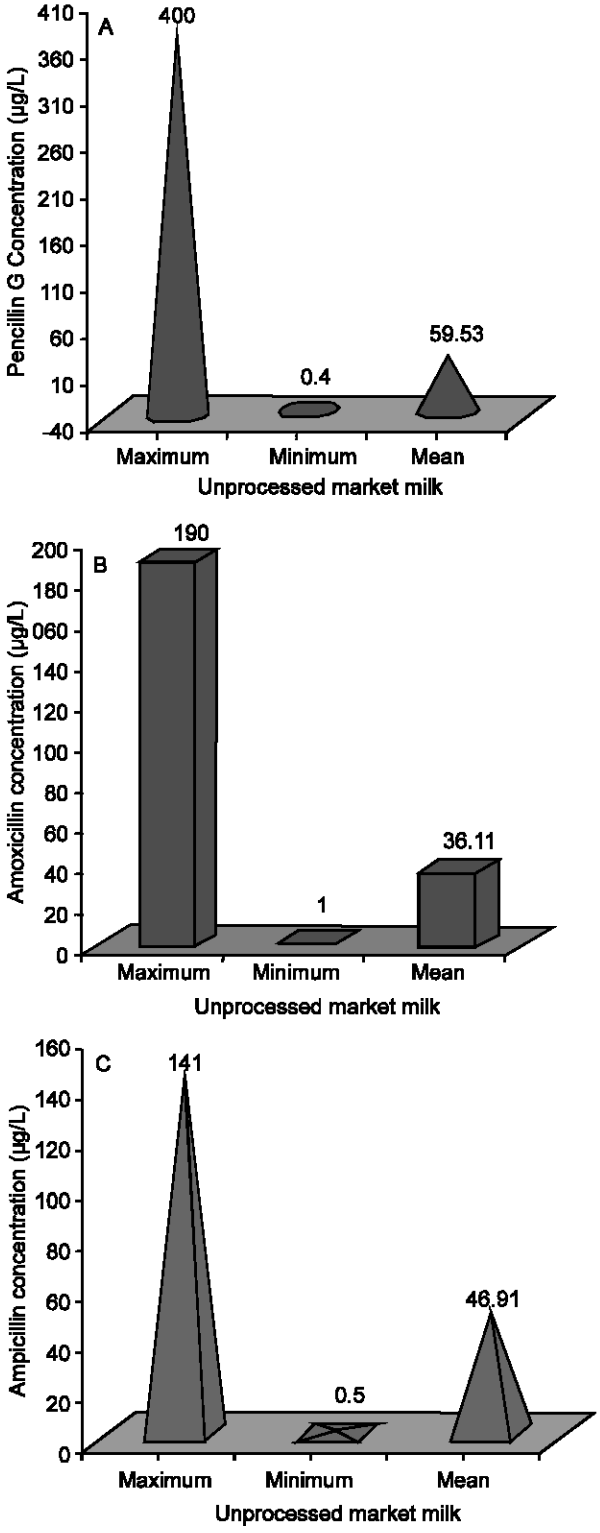
Preparation of sample: Milk sample (5 ml) was taken in 10 ml sterilized pyrex screw cap centrifuge tubes and vortex mixed with 10% aqueous solution of acetic acid (400 µl). The acidified milk was then centrifuged at 3500 rpm for 10 minutes at 4°C in the Backman centrifuge machine. The clear supernatant phase was taken by a disposable syringe, avoiding taking upper fat layer and filtered through a 0.45 µm nylon filter (13 mm diameter) with the back pressure of pump. The filtered extract was put into 2 ml sterilized vials and injected (5µl) into HPLC system. Each sample was prepared in duplicate.

Preparation of standard: Stock standard solutions at a concentration of 100µg/ml of Ampicillin (Sigma), Penicillin G (Sigma) and Amoxicillin (Sigma) were prepared individually in deionized water, previously filtered in 0.45µm filter paper. Mixed standard working solution containing Ampicillin, Amoxicillin and Penicillin G were prepared from the stock solution at concentrations level of 0.2 µg/ml, 0.4 µg/ml, 0.6 µg/ml, 0.8 µg/ml, 1.6 µg/ml, 2.0 µg/ml, 2.4µg/ml, 3.0µg/ml and 4.0µg/ml. The solutions were stored at freezing temperature till use (not more than one month).

Quantification: The quantification of analysis was carried out by injecting standard solution, blank sample and spiked samples. For each ß - lactam analyzed, the factor of response consisting of the ratio between the height of analyte peak and height of internal standard was verified.

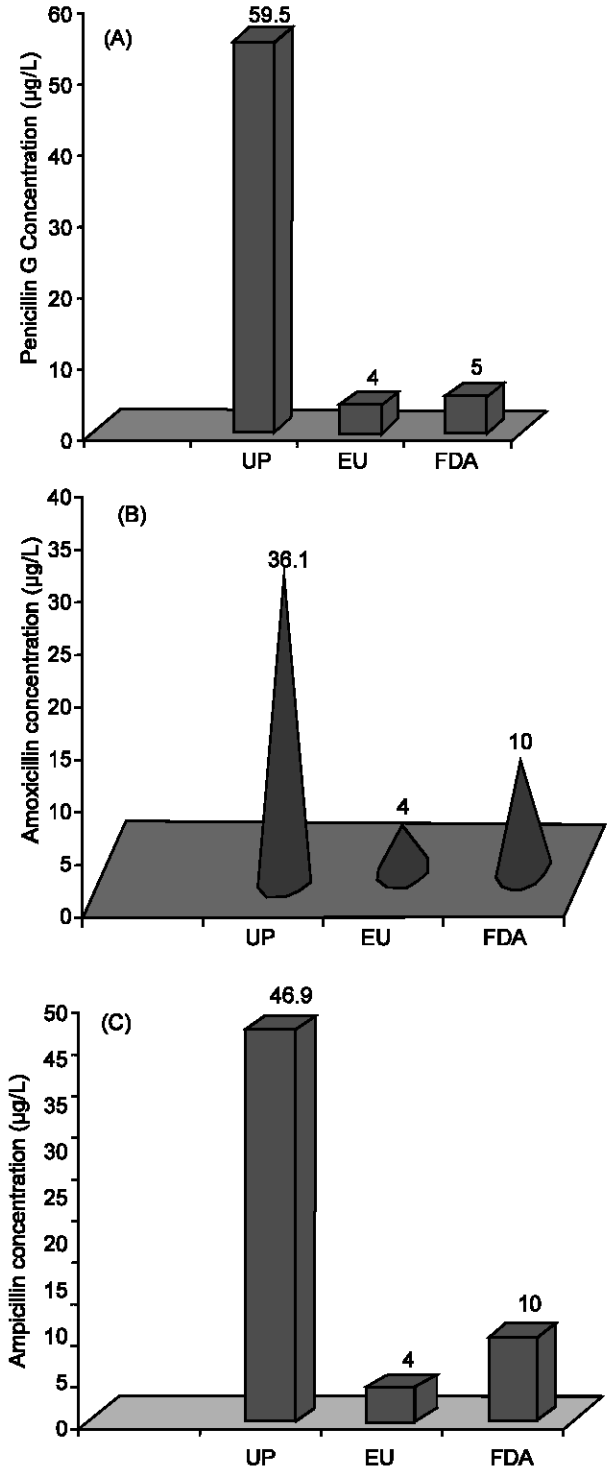
Results and Discussion

Screening of milk samples for ß - lactam antibiotic residues: Raw (unprocessed) milk samples (137) were screened for ß - lactam antibiotic residues through *Bacillus subtilis* Quantitative Field Disc Assay. Majority of samples 87 (63.50%) were negative for ß - lactam antibiotic i.e. no visible transparent zone around disc. While 50 (36.50%) of samples was positive with a zone size ranging from 5.0 to 15.0 mm (mean 8.91±0.37 mm). The present residues limit indicates that milk sold at the vicinity of Hyderabad city, Latifabad and Qasimabad was expected to be at 36.50% risk level for the presence of ß - lactam residues. However, Arora and Chhabra (2004) also reported the prevalence (23.80%) of relatively similar residues in milk samples over the 105 samples analyzed with similar technique at India. While relatively



SE (±) = 17.97, 9.46, 8.39, respectively for A, B and C.

Fig. 3: Penicillin G, Amoxicillin and Ampicillin antibiotics concentration (µg/L) in market milk samples determined by HPLC method.



UP = Unprocessed, EU = European Union, FDA = Food and drug Administration, U.S.

Fig. 4: Penicillin, Amoxicillin and Ampicillin residues in unprocessed market milk v/s Maximum Acceptable Residual Level (MRL standard).

within the similar range to those was reported by Jevinova *et al.* (2003) i.e. 4 to 20mm up to 96 hour after administration of antibiotic to cows suffering from mastitis.

HPLC analysis of market milk samples (+ve in screened test): Milk samples (50 +ve) in screened test were confirmed for ß - lactam antibiotic residues through High Performance Liquid Chromatography (HPLC method) and results are shown in Table 1. The quantification of analyses was limited to Penicillin G, Amoxicillin and Ampicillin due to unavailability of other ß - lactam antibiotic standards at the time of study period. Other peaks appeared were quantified as unknown inhibitors. The result reveals that over the 50 samples analyzed, 32 (64%) were contaminated with Penicillin G residues, 28 (56%) with Amoxicillin, 24 (48%) with Ampicillin and 15 (30%) with unknown inhibitors.

Penicillin G residues in raw market milk ranged between 0.4 to 400µg/L and averaged 59.53±17.91µg/L (Fig. 3A). The results of present study are remarkably lower than maximum residues level of Penicillin G (upto 6240±550µg/L) reported in bovine raw milk (Ghidini *et al.*, 2003). However, the residues are not agreed with the result of maximum residual limits (MRL's) of the European Union regulations (Ghidini *et al.*, 2002) i.e. 4µg/L, Food and Drug Administration (U.S. FDA), (Holstage *et al.*, 2002) i.e. 5µg/L or Junqueira and Brito (2006) i.e. 3.0µg/L. Penicillin residues in market milk were14.9 fold higher than European Union regulation and 11.9 fold than FDA regulations (Fig. 4A).

Amoxicillin residues in market milk varied between 1.0 and 190µg/L and averaged 36.11±9.46µg/L (Fig. 3B). The mean of the Amoxicillin in the present study is within the range of reported values (8.5±1 to 53.7±2.3µg/L) of Cozzani (2005). However, the result of the present study is not in line with the MRL's standards (Ghidini *et al.*, 2002; Holstage *et al.*, 2002) i.e. 4.0 and 10.0µg/L, respectively. Amoxicillin residues level in unprocessed market milk (36.1µg/L) was 9.03 fold higher compared to values recommended by European Union and 3.61 fold to values of FDA regulations (Fig. 4B).

Ampicillin residues level in the market milk samples ranged between 0.5 to 141.0µg/L and averaged 46.91±8.30µg/L (Fig. 3C). The mean value in the present study is 11.72 fold higher than MRL's standards i.e. 4.0µg/L as reported by Ghidini *et al.* (2002) and Holstage *et al.* (2002) i.e. 10µg/L (Fig. 4C).

Conclusion: Among 137 market milk samples (unprocessed) screened, 50 (36.50%) concluded to be contaminated with ß - lactam antibiotic residues. Penicillin residues in market milk samples were dominant followed by Amoxicillin, Ampicillin and unknown antibiotics. Remarkably higher level of Penicillin G, Amoxicillin and Ampicillin residues revealed in raw milk contrast to Maximum Residual Limits (EU and FDA standard). Improper use of antibiotics could be incurred in the vicinity of Hyderabad city, Latifabad and Qasimabad.

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