

Determination of Antibiotic Residues in Milk Samples ^[1]

Seyda ERGIN KAYA * Ayhan FILAZI ** 

[1] This work was summarised from same named PhD thesis

* Vilsan Company, Product Manager, Yenice Koyu Mevkii, TR-06760 Esenboğa, Ankara - TURKEY

** Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Ankara University, TR-06110 Diskapi, Ankara - TURKEY

Makale Kodu (Article Code): KVFD-2009-1174

Summary

In this study, antibiotic residues in raw milk and pasteurized milk products sold in Ankara- Turkey investigated. For this purpose; a total of 240 milk samples, which contains 10 raw and 10 pasteurized milk samples per month collected from various markets in Ankara between April 2003 and March 2004 were analyzed in terms of penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin by using TLC (Thin Layer Chromatography)/Bioautographic method, in which *Bacillus subtilis* ATCC 6633 was used as test microorganism. The minimum detectable concentrations for penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin, as µg/L were 4, 100, 200, 100 and 1000, respectively and recovery rate as percentage were 75.6, 79.7, 80.9, 84.7 and 73.5, respectively. The concentrations found among pasteurized samples were 150.4 µg/L oxytetracycline and 33.5 g/L penicillin G and 7688.4 µg/L of neomycin among raw samples, which are higher than the maximum residue limits in milks accepted in Turkey and European Countries. According to the total number of samples analysed, the ratio of contamination with antibiotics was detected as 1.25%.

Keywords: Milk, Antibiotic, Residue, TLC/Bioautographic method

Sütlerde Antibiyotik Kalıntılarının Belirlenmesi

Özet

Bu çalışmada Ankara'da satılan çiğ süt ve pastörize süt ürünlerinin antibiyotik kalıntılarının araştırılması amaçlandı. Nisan 2003 ile Mart 2004 tarihleri arasında, çeşitli satış yerlerinden her ay 10 çiğ süt ve 10 pastörize süt olmak üzere toplam 240 adet süt örneği toplanarak penisilin G, oksitetrasiklin, gentamisin, streptomisin ve neomisin yönünden analiz edildi. Bunun için test mikroorganizması olarak *Bacillus subtilis* ATCC 6633'ün kullanıldığı İnce Tabaka Kromatografisi /Biyootografi yönteminden yararlanıldı. Buna göre penisilin G, oksitetrasiklin, streptomisin, gentamisin ve neomisin'in en küçük belirlenme miktarları sırasıyla µg/L olarak 4, 100, 200, 100 ve 1000, geri alınma oranları ise yüzde olarak yine sırasıyla 75.6, 79.7, 80.9, 84.7 ve 73.5 olarak belirlendi. Pastörize süt örneklerinin birinde 150.4 µg/L oksitetrasiklin, birinde 33.5 µg/L penisilin G ve bir adet çiğ süt örneğinde 7688.4 µg/L neomisin tespit edilmiş ve bu konsantrasyonların Türkiye ve Avrupa Birliği ülkelerinde sütlerde bulunmasına izin verilen maksimum kalıntı limitlerinin üstünde olduğu belirlendi. Toplam analiz örneği sayısına göre antibiyotikle kirlenme sıklığı %1.25 olarak bulundu.

Anahtar sözcükler: Süt, Antibiyotik, Kalıntı, İnce Tabaka Kromatografisi/Biyootografi

INTRODUCTION

The first use of antimicrobials for treatment of infections in veterinary medicine was in the late 1940s, shortly after their development ¹. In the treatment bovine mastitis, antibiotics are widely used and improper application can lead to the contamination of milk at

farm level. Nowadays, beta lactam (penicillin G etc), aminoglycoside (streptomycin, neomycin, etc) and tetracycline (oxytetracycline, etc) antibiotics are the most frequently used antimicrobials for treatment of mastitis in dairy cows and consequently, the most



İletişim (Correspondence)



+90 312 3170315



filazi@veterinary.ankara.edu.tr

commonly found type residues in milk². Residues are of concern due to their possible adverse effects on people allergic to antibiotics, potential buildup of antibiotic-resistant organism in humans and inhibition of starter cultures used to produce cultured milk products such as yogurt and cheeses^{3,4}. These reasons make it important to effectively control antibiotic residues in milk and therefore, regulatory authorities have enacted maximum residue limits (MRLs) for a number antiinfective agent in milk. National monitoring programs to control the veterinary drug residues in various animal originated foods, including milk, are compulsory in all EC countries and Turkey⁵⁻⁷. Detectable concentrations of antibiotic residues in milk supplies higher than the MRLs are illegal.

Running effective monitoring program requires specific, sensitive and reliable analytical methods that can detect all drug residues below regulated levels. The overall objective is to develop and validate multiresidue methods in order to support the implementation of both existing as well as future regulations in the area of food control⁸.

Various analytical methods have been described to determine antibiotic residues in milk, such as microbiological, chromatographic, immunochemical, receptor and enzyme-based tests. Microbiological tests are commonly applied in dairy and in survey studies^{9,10}. For the detection of antimicrobial residues in milk; microbiological screening tests are used that utilize bacterial test strains such as *Bacillus stearothermophilus* var. *calidolactis*, *Streptococcus thermophilus* and *Bacillus subtilis* ATCC 6633^{11,12}.

The coupling of thin layer chromatography with microbiological detection (TLC/bioautography) has been used for the identification and quantification of several antibiotics. It is considered a simple, cheap and quite sensitive and specific method^{13,14}. The application of bioautography combined with TLC in antibiotic residues detection in milk and animal tissue has been demonstrated previously by Choma et al.¹⁵ and Neidert et al.¹⁶.

The purpose of this study was to analyse residues of streptomycin, penicillin G, oxytetracycline, gentamicin, and neomycin by the using TLC/bioautography; that are frequently seen in raw and pasteurized milk products sold in Ankara-Turkey.

MATERIAL and METHODS

Apparatus and Reagents

Antimicrobial standards penicillin G potassium,

oxytetracycline, streptomycin sulphate, gentamicin and neomycin sulphate were purchased from Sigma-Aldrich Ltd. HPLC grade acetone, glycerine, chloroform, potassium hydrogen phthalate, n-propanol, Whatman cellulose TLC plates (200X200 mm) (Merck), Standard Plate Count Agar and Tryptose Soy Agar (Oxoid) were purchased. Bioplates (sterilized glass plates-245x245 mm) prepared privately.

Impregnation liquid: Mix 0.1 N phthalate buffer pH 3.75 and glycerine (19+1).

Stock solutions of standards were prepared by dissolving each compound in milli-Q water at a concentration of 1 mg/ml and stored in amber vials in a freezer (-20°C). Fresh stock solutions were made every month. Working standard solutions (different concentrations) were made by serially diluting the stock solutions with milli-Q water every time just before use and stored at 4°C in amber glass vials. Method validation samples were prepared at levels of ½, 1 and 2 or 4 times MRL values. All spiked samples (standards and method validation samples) were assayed 6 times in duplicate spread over three working days. The variation coefficient of the within-laboratory reproducibility for method validation samples was calculated.

Sample Collection

As part of the study, between 03.04.2003 and 30.03.2004 (for one year), a total of 240 milk samples, 10 of which were raw milk and 10 were pasteurized milk every month were collected from different points of sales in Ankara-Turkey. Attention was paid to ensure that the milk samples analysed were of different brands and were collected from different locations. Milk samples were kept in the refrigerator (4°C) until analysis and were analysed within two days at most.

Assay organism: *Bacillus subtilis* ATCC 6633 Difco spore solutions were obtained from The Ministry of Agricultural and Rural Affairs, Central Veterinary Control and Research Institute. It cultured on Tryptone Soya Agar (European Pharmacopeia).

TLC solvent system: Acetone-chloroform-n-propanol-impregnation liquid (16 + 20 + 27 + 16)

Preparation of Milk Samples

For extraction of the antibiotic residues of milk samples, methods specific to liquid chromatography developed by Tyczkowska et al.¹⁷ were used. Accordingly, it was extracted 1 ml from each milk sample and placed in centrifuge tubes. In order to precipitate the proteins in milk 1 ml of acetonitrile - methanol, deionized water

(40:20:20) mixture was added on it. After stirring with hand thoroughly, it was centrifuged at 3000 rpm for 10 min and the portion remaining on supernatant after proteins were precipitated was used for analysis.

Thin Layer Chromatography

Cellulose plates were divided into 10 equal channels. And then the 10 µl extracts were applied to the channels. During application, air current was applied on the plate to prevent the sample from decomposing. The plate was left to rest for 5 min in room temperature to dry them. Afterwards, the plates were placed in the development tanks prepared at least one hour in advance, in order to help the ambience reach saturation. Then it is waited for the solution to rise up to 15 cm on the plate. Plate was removed from the tank and dried. The dried plate was then applied to the newly prepared bio-plate.

Bioautography

Seventeen and a half grams of Standard Plate Count Agar was measured and 1 L distilled water was added and was melted in 100°C water for 2 h and was sterilized by keeping for 16 min under 1 atmosphere pressure at 121°C. And by adjusting its pH to 7.2, its temperature was decreased by helping it contact water occasionally up to the temperature (38-40°C) required for *B. subtilis* to reproduce. For every 100 ml agar, 400 µl from *Bacillus subtilis* ATCC 6633 spore solution was added and as a result, 4 ml was added to 1 L agar and it was shaken for 15 sec. to help it diffuse homogenously. 300 ml agar was added to every bio-plate sterilized in advance for one h at 121°C under 1 atmospheric pressure. After waiting for agar to become solid (for around 20 min), the TLC plates that were developed by applying sample extracts or antibiotics standards and dried, were placed on the food-lot surface and was allowed to contact agar for 20 min. At the end of contact period, plates were removed and bio-plates were left for incubation for 16 h at 37°C. The inhibition zones diameters that developed at the end of the period were measured using calliper¹⁶.

Table 1. R_f mean, minimum detectable concentration, recovery and MRL of antibiotics measured by TLC/bioautography

Tablo 1. TLC/Biyootografi ile ölçülen antibiyotiklerin R_f düzeyi, belirlenebilen en düşük konsantrasyonu, geriye kazanç yüzdeleri ve maksimum kalıntı düzeyleri

Antibiotics	Tested Concentrations (µg/L)	Minimum Detectable Concentrations (µg/L)	Recovery Rates (%)	R _f Values	MRLs ^a (µg/L)
Penicillin G	2, 4, 8, 16	4	75.6	0.84	4
Oxytetracycline	50, 100, 200, 400	100	79.7	0.34	100
Streptomycin	100, 200, 400, 800	200	80.9	0.50	200
Gentamicin	50, 100, 200, 400	100	84.7	0.24	100
Neomycin	750, 1500, 3000, 6000	1000	73.5	0.07	1500

a: Maximum Residues Limits (EC directive and Turkish Food Codex)

RESULTS

The R_f means obtained by preparing and using the standards, at certain concentrations, for detecting the streptomycin, penicillin G, oxytetracycline, gentamicin and neomycin residues using the TLC/bioautography method as well as the their low impact densities, their recovery rates and their minimum detectable concentrations are given in the [Table 1](#).

As a result of the analysis in one of the pasteurized milk samples collected in April 2003, 150.4 µg/l oxytetracycline and 33.5 µg/l penicillin in another company's sample was detected, whereas in the raw milk sample obtained from Ankara-Ayaş Gökyayla village, 7688.4 g/l neomycin was detected. Based on the total number of analysis samples, the ratio of contamination with antibiotics was detected as 1.25%.

DISCUSSION

TLC/bioautography method developed by Neidert et al.¹⁶ for detecting antibiotic residues in animal tissue was used. The method developed by Tyckowska et al.¹⁷ for extraction and detection of penicillin G from cow milk by HPLC was used to extract the antibiotic in milk. As a result of the recovery procedure carried out using these methods, 75.6% for penicillin G, 79.7% for oxytetracycline, 80.9% for streptomycin, 84.7% for gentamicin, and 73.5% for neomycin were calculated as recovery rates. In the recovery study in meat conducted by Neidert et al.¹⁶, these values were as follows; 95% for penicillin G, 95-98% for oxytetracycline, 80% for streptomycin, 100% for gentamicin, and neomycin. Accordingly, recovery levels in this study were lower for antibiotics other than streptomycin. The difference is believed to be due to differences between samples. Besides the fact that, TLC/bioautography is not a costly method compared to the other analysis methods, which can be adapted to laboratory conditions and applied easily; it has also certain advantages such as enabling distinction between

antibiotics when appropriate plate and development systems are selected, leading more frequent use.

The degree of contamination of milk and dairy products with anti-bacterial additive residues differs, depending on the level of education, legislations and effectiveness of food inspection in different countries¹⁸. According to the results of the technical report prepared for milk hygiene, by World Health Organization and Joint Expert Committee on Food Additives (JECFA), the rate of contamination of milk and dairy products with anti-bacterial additives in developed countries such as USA, Australia, UK and Scotland was 7-10% until 1969, after that year, the rate of contamination of the same products decreased to 0.5% in USA, 2.1% in Australia, 1.5% in UK and 3.4% in Scotland due to the precautions taken after the given date. The same report indicates that, in underdeveloped and developing countries, which fall behind in terms of increasing the level of awareness of stock breeders, improvement of hygienic conditions and in terms of inspection effectiveness, the rate of contamination for milk and dairy products might be higher¹⁹.

In Turkey, there are only a limited number of anti-bacterial residue studies in milk²⁰⁻²⁷. The most comprehensive is the one related to detection of veterinary medicine residues in foods carried by 5 Veterinary Control and Research Institutes in various cities in Turkey and by the 9 Provincial Control Laboratories, under the leadership of the Ministry of Agriculture and Rural Affairs. In this study, 3084 milk sample were analysed using intertest method for the presence of penicillin, tetracycline and chloramphenicol in which 377 of the samples (12%) were found as positive²¹.

In another study by Ceyhan and Bozkurt²² from a total of 200 milk samples (100 raw milk, 50 pasteurized milk and 50 UHT sterilized milk) collected around Ankara region, 11 of them were reported as penicillin positive (5.5%).

In a study carried out by Aydın et al.²³ in 307 milk samples (204 raw milk and 103 pasteurized milk samples) using intertest and agar diffusion tests with the goal of determining antibiotic and other inhibitor substances, the following results were obtained; with the intertest method, raw milk and pasteurized milk, 44% and 29% positive, 1.5% and 15.5% suspicious and 49.5% and 53% negative respectively; with the agar diffusion method, 15.7% and 14.5% positive, 4.4% and 7.8% suspicious and 80% and 77.7% negative respectively.

In another study carried out by Şanlı et al.¹⁹, in 89

milk samples chloramphenicol contamination was found as 6.9% with the residue level of 1.15 ppm.

In a study carried out by Demet et al.²⁴, where they tried to determine the level of penicillin G, ampicilline and penicillin V residues, using HPLC method in 50 milk samples collected from various dairy farms and creameries, 6 of the milk samples demonstrated penicillin G-potassium but none of the samples demonstrated penicillin V and ampicilline. Again in a study carried out by Demet et al.²⁵ in 61 milk samples collected from various dairy farms in Konya, in order to detect the level of chloramphenicol using HPLC method, 28 milk samples demonstrated chloramphenicol residues.

Dokuzlu and Tayyar²⁶ reported that, using the intertest method, they have detected chloramphenicol residue in 2 of the 150 raw milk samples collected in Bursa Province.

As a result of the study conducted by Önal et al.²⁷, for the detection of chloramphenicol residues on 444 raw and pasteurized milk samples collected from public and private sector establishments in Ankara and the surrounding region, the following rates were obtained; with the Intertest method, 78 positive (17.56%), 65 suspicious (14.63%), 301 negative (67.79%); with the triple plate method, carried out using *B. subtilis*, 24 (5.40%) positive, 1 suspicious (0.22%), 419 (94.36%) negative. It is indicated that this difference is due to the method used, sampling time and the establishments.

Chloramphenicol is an additive that was banned for use in livestock used for milk production and breeding, with the circular dated 19 April 1993 and numbered 419, issued by the Ministry of Agriculture and Rural Affairs due to its certain unwanted affects²⁸. However it is noteworthy and frustrating that it was detected in milk in various residue detection studies conducted after that date.

Even though the contamination rates discovered in milk samples as a result of residue detection studies carried in Turkey in previous years are so high, the contamination level detected in this study was only 1.25%. This can be explained by the increased public awareness about food safety and healthy nutrition and efforts of producers to market high quality products after the media started emphasizing the issue.

As a result the recovery values of the residues of penicillin G, oxytetracycline, gentamicin, streptomycin and neomycin detected using the TLC/Bioautography method in milk were found to be satisfactory. Based on the results of this study and previous studies, it was determined that the probability of detecting antibiotic

in milk during spring and autumn is higher than the probability of detecting it in other months. The values detected are higher than the MRLs accepted in European Countries and Turkey. However, the fact that there is less antibiotic contamination in the milk produced in Turkey, compared with the residue rates obtained in previous studies, is considered to be a positive sign in terms of food safety.

REFERENCES

1. **Mitchell JM, Griffiths MW, McEwen SA, McNab WB, Yee AJ:** Antimicrobial drug residues in milk and meat: Causes, concerns, prevalence, regulations, tests and test performance. *J Food Prot*, 61, 742-756, 1998.
2. **Gustavsson E, Degelaen J, Bjurling P, Sternesjö A:** Determination of beta lactams in milk using a surface plasmon resonance-based biosensor. *J Agric Food Chem*, 52 (10): 2791-2796, 2004.
3. **Jones GM, Seymour EH:** Cowside antibiotic residue testing. *J Dairy Sci*, 71 (6): 1691, 1988.
4. **Seymour EH, Jones GM, McGilliard ML:** Persistence of residues in milk following antibiotic treatment of dairy cattle. *J Dairy Sci*, 71 (8): 2292-2296, 1988.
5. **EC Council Directive:** 96/23/EC of April 1996 on measures to monitor certain substances and residues thereof in live animals and animal product. *Off J Eur Comm*, L125, 10-32, 1996.
6. **EEC Council Regulation:** No. 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *Of J Eur Comm*, L224, 1-8, 1990,
7. **Turkish Food Codex:** Türk Gıda Kodeksi Hayvansal Kökenli Gıdalarda Veteriner İlaçları Maksimum Kalıntı Limitleri Tebliğinde Değişiklik Yapılması Hakkında Tebliğ (No: 2007/17). 9 Mart 2007 tarih ve 26457 sayılı Resmi Gazete.
8. **Samanidou V, Nisyriou S:** Multi-residue methods for confirmatory determination of antibiotics in milk. *J Sep Sci*, 31 (11): 2068-2090, 2008.
9. **Kang JH, Jin JH, Kondo F:** False-positive outcome and drug residue in milk samples over withdrawal times. *J Dairy Sci*, 88 (3): 908-913, 2005.
10. **Knecht BG, Strasser A, Dietrich R, Martlbauer E, Niessner R, Weller MG:** Automated microarray system for the simultaneous detection of antibiotics in milk. *Anal Chem*, 76 (3): 646-654, 2004.
11. **Nouws JFM, Loeffen G, Schouten J, Van Egmond H, Keukens H, Stegeman H:** Testing of raw milk for tetracycline residues. *J Dairy Sci*, 81 (9): 2341-2345, 1998.
12. **Nouws J, Van Egmond H, Smulders I, Loeffen G, Schouten J, Stegeman H:** A microbiological assay system for assessment of raw milk exceeding EU maximum residue levels. *Int Dairy J*, 9 (2): 85-90, 1999.
13. **Petz M, Solly R, Lymburn M, Clear MH:** Thin-layer chromatographic determination of erythromycin and other macrolide antibiotics in livestock products. *J Assoc Off Anal Chem*, 70 (4): 691-697, 1987.
14. **Ramirez A, Gutierrez R, Diaz G, Gonzalez C, Perez N, Vega S, Noa M:** High-performance thin-layer chromatography-bioautography for multiple antibiotic residues in cow's milk. *J Chromatogr B Analyt Technol Biomed Life Sci*, 784 (2): 315-322, 2003.
15. **Chooma IM, Choma A, Staszczuck K:** Determination of flumequine in milk by thin - layer chromatography-bioautography. *J Liq Chrom Rel Technol*, 25 (10): 1579-1587, 2002.
16. **Neidert E, Saschenbrecker PW, Tittiger F:** Thin-layer chromatographic/bioautographic method for identification of antibiotic residues in animal tissues. *J Assoc Off Anal Chem*, 70 (2): 197-200, 1987.
17. **Tyczkowska K, Voyksner RD, Aronson AL:** Development of analytical method for penicillin G in bovine milk by liquid chromatography with ultraviolet-visible detection and confirmation by mass spectrometric detection. *J Chromatogr*, 490 (1): 101-113, 1989.
18. **Allison JRD:** Antibiotic residues in milk. *Br Vet J*, 141 (1): 9-16, 1985.
19. **Şanlı Y, Kaya S, Aydın N, Yavuz H, Akar F, Doğan A:** Süt örneklerinde kloramfenikol kalıntıları. *Ankara Univ Vet Fak Derg*, 38 (3): 402-416, 1991.
20. **Temamogullari F, Kaya S:** Ankara piyasasında satılan sütlerde bazı antibiyotik kalıntılarının ince tabaka kromatografisi ve biyotografik yöntemle saptanması. *Kafkas Univ Vet Fak Derg*, 16 (2): 187-191, 2010.
21. **Gıdalarda Katkı- Kalıntı ve Bulaşanların İzlenmesi:** Gıdalarda veteriner ilaç ve anabolizan maddelerin kalıntı düzeylerinin tespiti. Uludağ Üniversitesi Basımevi, Bursa, ISBN 925-7657-97-2. 1997.
22. **Ceyhan I, Bozkurt M:** Ankara piyasasında satılan sütlerde penisilin araştırması. *Türk Hij Den Biyol Derg*, 44, 1-5, 1987.
23. **Aydın N, Cambazoğlu M, Ayhan H:** İnterest yöntemi ile sütteki antibiyotiklerin ve diğer inhibitör maddelerin saptanması üzerine çalışmalar. *Etlık Vet Mikrobiyol Derg*, 6, 11-22, 1989.
24. **Demet Ö, Acet A, Traş B, Baş L, Eğilmez İ:** Konya'da faaliyet gösteren çeşitli mandıralardan toplanan süt örneklerinde penisilin G, ampisilin ve penisilin V kalıntılarının araştırılması. *Selçuk Univ Vet Fak Derg*, 8, 33-35, 1992.
25. **Demet Ö, Acet A, Traş B:** Konya'da faaliyet gösteren çeşitli mandıralardan toplanan süt örneklerinde kloromfenikol ilaç kalıntılarının araştırılması. *Selçuk Univ Vet Fak Derg*, 8, 35-37, 1992.
26. **Dokuzlu C, Tayar M:** Bursa ve çevresinde çiğ sütlerde antibiyotik varlığının belirlenmesi. *Vet Bil Derg*, 17 (1): 153-157, 2001.
27. **Önal A, Aydın N, Ayaz Y, İşcan D, Savaş N:** Süt ve etlerde bulunan bazı antibiyotiklerin çeşitli yöntemlerle saptanması. *Etlık Vet Mikrobiyol Derg*, 7, 48-51, 1993.
28. **Kaya S:** Antibiyotikler. In, Kaya S, Pirinççi İ, Bilgili A (Eds): Veteriner Uygulamalı Farmakoloji. Cilt 2. Baskı 2, s. 247-408, Medisan Yayınevi. Ankara, 2000.