10.22059/ijvm.2020.284171.1005003

Iranian Journal of Veterinary Medicine
Volume 14- Issue 04

Original Article
Online ISSN: 2252-0554

Comparison of Yogurt Test with Commercial Kit for Detection of Antibiotic Residues in Raw and Pasteurized Milk

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Abstract

BACKGROUND: Antibiotics are widely used for the treatment of livestock. Their inappropriate usage leads to various disorders in humans as a result of consuming animal products. Milk is among the foods that are significantly affected by consuming antibiotics.

OBJECTIVES: The objective of this study was to compare Yoghurt Culture Test (YCT), Four- Plate Test (FPT), and the Copan test for detecting antibiotic residues in raw and pasteurized milk produced in Chaharmahal and Bakhtiari province, Iran.

METHODS: A total of 146 raw milk samples and 54 pasteurized milk samples were selected randomly from dairy farms and dairy products suppliers. The presence of antibiotics was evaluated by YCT, FPT, and Copan test. In addition, the sensitivity of the three tests for tetracycline and penicillin, as the two common antibiotics in the treatment of animals, was compared.

RESULTS: Our findings showed that 8.9% of raw milk and 11% of pasteurized milk samples contained antibiotics. However, the levels of antibiotic residues were higher in 2% of the positive samples than maximum residue levels (MRL). Moreover, significant differences were observed between FPT, YCT, and the Copan test ($P<0.05$). On the other hand, the positive results of YCT and Copan tests were not significantly different ($P>0.05$).

CONCLUSIONS: The results of this study showed that a low percentage of milk samples contained antibiotic residues higher than the permissible limit. Furthermore, YCT could be used as an inexpensive, easy, and sensitive method for identifying the residues of penicillin and tetracycline in milk.

KEYWORDS: Antibiotic residue, Commercial kit, Milk, Yogurt test

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Received: 2020-04-28
Accepted: 2020-08-31

How to Cite This Article

Bonyadian, M., Mahmoodi kordi, F ,A. (2020). Comparison of Yogurt Test with Commercial Kit for Detection of Antibiotic Residues in Raw and Pasteurized Milk. ***Iranian Journal of Veterinary Medicine,*** 14(4), 394-402.

Introduction

Antibiotics are antimicrobial compounds obtained from the secondary metabolism of microorganisms, which could destroy bacteria or inhibit their growth via affecting their structural or metabolic elements needed for survival. In contrast to primary metabolites, secondary metabolites are not essential for growth and reproduction and are produced by microorganisms for various reasons (Welsh *et al.*, 2019).

Long-term consumption of antibiotics at concentrations less than the therapeutic dose (1-10 mg/kg feed) results in an increased growth rate. In such conditions, the yield of feed digestion and weight gain are higher likely due to changes in microbial flora (World Health Organization, 2017). Irregular use of antibiotics for prevention and treatment for an inappropriate time causes the transfer of antibiotics into milk resulting in allergy and pathogenic bacterial resistance which is an important concern (Tumini *et al.*, 2019). Milk is exposed to various chemical contaminations including hormones, antiseptics, nitrites, nitrates, and nitrosamine, insecticides, fungal toxins, toxic metals, dioxins, and antibiotics. These substances enter milk through medicines, rations, and a milking environment (Meyer, 2000).

Antibiotics are used to control systemic and localized infections in dairy herds, among which mastitis is the main reason for consuming antibiotics. In comparison with the water-soluble antibiotics, fat-soluble antibiotics may remain in the udder for a longer time. Scientists found that the addition of antibiotics and anabolic compounds, such as artificial estrogen into feed could elevate the weight gain of livestock. Therefore, antibiotics and estrogens were commercially produced, and the Food and Drug Administration (FDA) permitted the consumption of these compounds (Kang *et al.*, 2005).

Various studies have been conducted to determine the contamination of milk and its products with antibiotics in different countries. Antibiotic residue in milk is not a new concern. Studies conducted before 1960 showed that 6% of milk in the US market contained antibiotics residue. This rate decreased to 3.7% after 1960. However, 7%-15% of milk specimens in 1975 contained detectable levels of antibiotics. A study conducted on 1100 milk samples selected from a milk tank in 1963 in France demonstrated that 3.6% of the specimens had penicillin. In Kenya, studies carried out on pasteurized and non-pasteurized milk collected from local suppliers indicated that 16% of milk samples were contaminated with antibiotics (Kang *et al.*, 2005).

In Iran, many studies have been conducted on antibiotics in milk. An investigation in Qazvin revealed that 43% of the milk samples were contaminated with antibiotic residues. The cause of this high contamination rate was stated to be the irregular use of antibiotics in cattle farms. In addition, another study was conducted in 2014 in different parts of Iran on raw and pasteurized milk samples and it was observed that 30.76% of raw milk and 22.2% of pasteurized milk obtained from milk processing plants and retail shops contained antibiotics, respectively (Razzagh Mahmoudi *et al.*, 2014).

Antibiotics residue in milk is undesirable because it may cause antibiotic resistance by spreading antibiotic-resistant strains. Furthermore, some people, especially infants whose diet is mainly milk might develop an allergic reaction to antibiotics (Priyanka1 *et al.*, 2017). This is supported by the susceptibility of some people to an insignificant amount of penicillin in foods.

In addition, antibiotics in milk destroy the beneficial bacteria used in making cheese yoghurt, and other fermented products. Therefore, they reduce the quality and quantity of products (Priyanka *et al.*, 2017). Milk is an important part of the daily diet and .is easily contaminated with antibiotic residues Given the fact that milk contaminated with antibiotics cannot be used for producing dairy

fermented products, it is mainly utilized for producing pasteurized and sterilized milk resulting in many health problems in the .consumers The aim of this study was to compare YCT, FPT, and Copan test to identify antibiotic residues in raw and pasteurized milk produced in Chaharmahal and Bakhtiari province, Iran.

Materials and Methods

Sampling

A total of 200 milk samples (146 raw milk samples and 54 pasteurized milk samples) were randomly collected from farms and dairy processing plants during Des 2018-June 2019. Samples were transferred to the laboratory on ice.

YCT

First, fresh healthy milk was skimmed by centrifuge, heated, and sterilized at 121°C for 10-15 min. Next, the samples were cooled to 42-44°C and 10-15 gr starter granules containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus* were added to 0.5 L of sterilized milk. The inoculated milk was incubated at 42°C for 3-4 h until acidity reached 100° dormice. Afterwards, it was immediately transferred to the refrigerator. The obtained culture is known as mother culture (the Samples were stored at 5°C).

The samples were homogenized and heated to 85°C for 30 min in a warm water bath (Memmert WNB Co., Germany). The temperature of samples was rapidly reduced to 42-43ºC and 1 mL of fresh well-stirred lactic inoculum (homogenized) was mixed with the milk sample. Next, 0.1 mL of red methyl reagent was added to the samples and were incubated at 42°C for 3 h, and color variations in the samples were checked. An antibiotic-free sample was used as negative control and a sample with antibiotics was applied as a positive control (penicillin and tetracycline, 0.5 ppm) (Khaled, 2004).

Antibiotic-free samples showed pink color after incubation because of the growth of starter bacteria leading to lactic acid production, while positive samples remained orange without acid production. Moreover, the presence or absence of lactic coagulation in samples was obvious from their consistency. The acidity was measured by an electric pH meter (Metrohm, Switzerland). The acidity of suspicious samples was about 10°D less than that of natural milk indicating the presence of antibiotics or other inhibitory compounds. The samples with pH>5.3 were considered positive samples (Tumini *et al.*, 2019).

FPT

The FPT has been recently investigated and its sensitivity as a standard method for identification of antibiotic residue has been approved by the EU. Five classes of antibiotics, including beta-lactam, tetracycline, sulfonamide, aminoglycoside, and a macrolide could be identified by this test (Koenen-Dierick *et al.,* 1995) (Table 1).

Table 1. Identifiable medium and antibiotics by the four-plate test

pH	Bacteria type	Antibiotic type
6	*Bacillus subtilis*	Penicillin class Tetracycline class
7.2	*Bacillus subtilis*	Sulfonamide class
8	*Bacillus subtilis*	Aminoglycoside class Penicillin class
8	*Micrococcus luteus*	Macrolide class

To prepare 100 mL of medium, 0.345 gr of both casein and meat peptone, 0.5 g sodium

chloride, and 1.3 g trimethoprim agar were weighed and dissolved in 100 mL distilled water. Next, 0.24 gr trisodium phosphate was added to the prepared medium. The pH values of the prepared medium were adjusted by a digital pH meter with acetic acid and sodium hydroxide as 6, 7.2, and 8. The media were sterilized at 121°C for 15 min in an autoclave (Zaim Mega F.I.M, China).

The absorbance of the two bacterial cultures *Bacillus subtilis* (Ptcc 1365) and *Micrococcus luteus* (PTCC 1169) was read at 580 nm by a spectrophotometer (Spectronic, Sweden) (Society for General Microbiology, 2006). Afterwards, 0.1 mL of each dilution of *B. subtilis* was added to 100 mL of the medium at pH 7, 7.2, and 8. In addition, 0.1 mL of the dilution of *M. luteus* was added to 100 mL of the medium with pH 8.

Blank disks with a thickness of 2 mm were coated with raw and pasteurized milk samples and were placed on the media along with positive and negative control samples. The plates with four pH values containing *B. subtillis* at 30°C and the plates containing *M. luteus* at 37°C were incubated for 24 h. Next, the inhibition zone was measured by Collis Vernier, and inhibition zones equal and larger than 2 mm in diameter were considered as a positive sample (Gaudin *et al.*, 2004). The inhibition zone in the FPT is only observed when antibiotics residue exceeds the permitted level.

Copan Test

Copan test is used for detecting antibiotics residue in raw, heated, and dry milk samples. Copan kit (Chr. Hansen, Denmark) is used for the qualitative monitoring of antibiotics in milk. It has 96 microplates containing agar with the spores of *B. stearothermophilus* var. *calidolactis,* as well as glucose and Bromocresol purple (Forouzan *et al.*, 2014).

About 100 µl of milk sample was poured on the agar and put in a water bath at 64±1°C for 3 h. In the absence of antibiotics, the spore grows, ferments glucose, and produces acid. Consequently, the color of the medium changes to yellow.

Comparison of Tests for Detecting Tetracycline and Penicillin

First, healthy antibiotic-free milk was obtained from a healthy cow. It was homogenized for 5 min, heated at 85°C for 30 min, and tested for antibiotics by Copan test. Afterwards, dilutions 0.1, 0.5, 1, 2, 5, 10, and 20 mg/L of crystalline penicillin, as well as 0.1, 0.5, 1, 10, 25, 50, and 100 mg/L of tetracycline were added to milk. Negative and positive samples were prepared from antibiotic-free and antibiotic-containing milk, respectively. Finally, the samples were evaluated by YCT, FPT, and Copan test.

Statistical Analysis

Data were analyzed by SigmaStat ٤ software using the Cochran test. P-value<٠,٠٥ was .considered significant

Results

The number of samples containing antibiotics residue is given in Table 1. Eleven (7.5%) and 13 (8.9%) raw milk samples were positive by YCT and Copan test, respectively. Moreover, regarding the samples of pasteurized milk, 5 (9.3%) and 6 (11%) samples were positive with YCT and Copan test, respectively. Three (2%) raw samples and 1 (1.8%) pasteurized milk sample were positive by FPT (Figure 1). In the plate containing *B. subtillis* at pH 6, zones of inhibition >2 mm in diameter were observed indicating the residues of penicillin and tetracycline (Table 2).

The Chi-square test showed no significant difference between YCT and Copan test (P>0.05). However, significant differences were found between FPT and YCT, as well as between FPT and Copan test (P<0.05).

Table 2. Occurrence of antibiotics residue in raw and pasteurized milk producing in chaharmahal and Bakhtiari province-Iran.

Samples	Number	Four plate test	Yogurt test	Copan test
Raw milk	146	3(2%) [a]	11(7.5%) [b]	13(8.9%) [b]
Pasteurized milk	54	1(1.8%) [a]	5(9.3%) [b]	6(11%) [b]

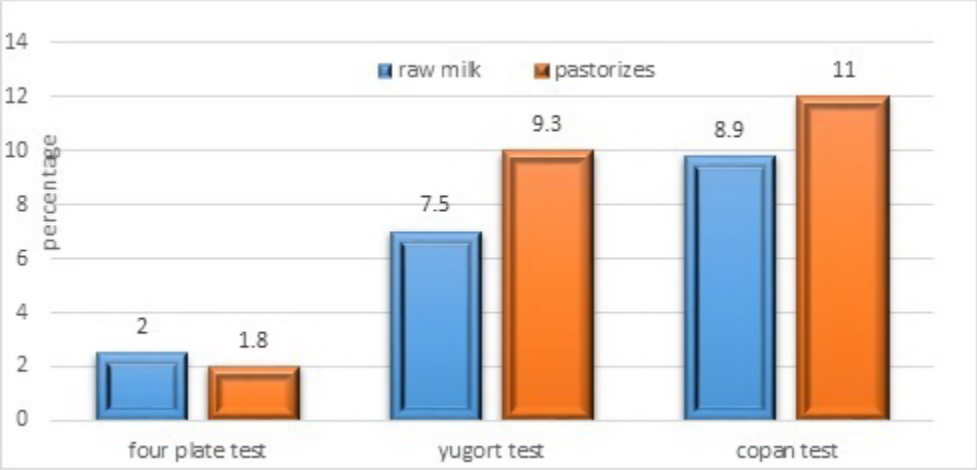

Figure 1. Antibiotic residues in raw and pasteurized milk determined with YCT and Copan tests.

According to our results, the limit of detection of FPT was 313 and 500 ppb for penicillin and tetracycline, respectively. The limit of detection for YCT was 60 ppb for penicillin and 100 ppb for tetracycline. However, for Copan test was 6 and 100 ppb for penicillin and tetracycline, respectively (Table 3).

Table 3. Detection the limit of yogurt, four-plate, and Copan tests forpenicillin and tetracycline in milk.

Antibiotic	Concentration	Four plate test	Yogurt test	Copan test
Penicillin (ppm)	0.006	-	-	+
	0.06	-	+	+
	0.313	+	+	+
	0.63	+	+	+
	1.25	+	+	+
	3.13	+	+	+
	6.25	+	+	+
	12.5	+	+	+
Tetracycline(ppm)	0.1	-	+	+
	0.5	+	+	+
	1	+	+	+
	10	+	+	+
	25	+	+	+
	50	+	+	+
	100	+	+	+

Discussion

According to the findings of the Copan test, 8.9% of raw milk and 11% of pasteurized milk produced in Chaharmahal and Bakhtiari province were contaminated with antibiotic residues. However, 2% of the positive samples were higher than MRL. Other studies have shown that raw milk produced in other parts of Iran contained antibiotic residues. Razzagh *Mahmoudi et al.* (2014) reported that 30.76% of raw milk produced in Ilam province, Iran was contaminated with antibiotic residues. In Pakdasht, Tehran, Iran, Mousavi *et al.* (2011) revealed that 13.3% of raw milk specimens were positive for antibiotics. A similar study in Ilkhchi, Tabriz, Iran using the Copan test showed that 5% of raw milk samples contained antibiotic residues (Movassagh and Karimi, 2010).

We observed that 11% of pasteurized milk was contaminated with antibiotic residues. The presence of antibiotics in pasteurized milk was reported from different provinces of Iran (Moghadam *et al.*, 2016; Aalipour *et al.,* 2013; Razzagh Mahmoudi *et al.,* 2014; Forouzan *et al.*, 2014). Rasouli and Bokaii (2010) found that 8.7% of pasteurized milk samples contained tetracycline and oxytetracycline in Tehran. Furthermore, Ghanavi *et al.* (2013) reported milk contamination with penicillin residues in different parts of Iran by the BetaStar test. Beta-lactam residues were observed in 23.8% of raw and 10.2% of pasteurized samples. The greatest contamination of 50% was related to beta-lactam antibiotics.

Manafi *et al.* (2011) examined raw and pasteurized milk utilizing Delvotest in East Azerbaijan, Iran. Raw milk from 26% of industrial farms, 16% of collection centers, and 30% of pasteurized milk contained antibiotic residues. The high level of antibiotic residues in pasteurized milk could be attributed to the common usage of antibiotic- and preservative-free raw milk for producing fermented dairy products, such as yoghurt, buttermilk, and cheese in dairy processing plants. Moreover, high-quality raw milk is used for preparing ultra-high-temperature milk. Therefore, contaminated and low-quality milk is mainly used for producing pasteurized milk as a basic and widely used dairy product.

Sani *et al.* (2015) studied the residues of tetracycline and sulfonamides by enzyme-linked immunosorbent assay in Mashhad, Iran. The results revealed that 93% of the 42 samples contained sulfonamide. The level of sulfonamides was higher than the Codex Alimentarius of 25 ppm. Furthermore, 93% of the samples were contaminated with tetracycline. Tetracycline residue was lower than the Codex standard. Dabbaghmoghaddam *et al.* (2013) evaluated the residues of tetracycline by high-performance liquid chromatography and Delvo test in Tehran, Iran in 56 specimens of pasteurized milk. Overall, 33.93% of the samples contained tetracycline residues and 8.93% had antibiotic levels exceeding MRL.

Raw and pasteurized milk contamination with antibiotic residues have been reported from other countries. Layada *et al.* (2016) in Algeria found that 65.46% of milk samples contained antibiotic residue. In addition, Khanal *et al.* (2018) in Nepal studied the levels of penicillin and sulfonamide residues in raw milk samples. The results showed that 81% of the samples were positive for amoxicillin (68-802 µg/kg), 41% for sulfamethoxazole (31-69 µg/kg), 27% for penicillin G (13-353 µg/kg), and 12% for ampicillin (0.5-92 µg/kg). In a study conducted by Prado in Brazil, raw milk samples were examined by HPLC indicating that 3% of the samples were contaminated with tetracycline (Prado *et al.*, 2015).

The YCT is based on the fermentation of suspicious samples by lactic thermophile bacteria (starters) and acidity measurement after 3 h incubation. Acidity was compared with the acidity of the control sample. The results of the YCT in the present study indicated that 7.5% of raw and

9.3% of pasteurized milk samples had antibiotic residues.

According to the findings of the present study, the detection limit of YCT for penicillin and tetracycline was 60 and 100 ppb, respectively. The mentioned values are within the MRL of 100 ppb for penicillin and tetracycline (Commission CA, 2015). The sensitivity of FPT for detecting penicillin and tetracycline was 313 and 500 ppb, respectively.

In the previous studies, the lack of clot formation was considered as an indicator of antibiotic level measurement in YCT, whereas in the present study, pH variations were considered as an indicator of the presence of antibiotics. The pH changes in milk begin before clot formation and when pH reaches the isoelectric point of casein protein (4.7) the clot is formed. Therefore, considering pH alterations is a more valid criterion than clot formation in YCT.

Conclusion

It could be concluded that antibiotic residues in milk specimens produced in Chaharmahal and Bakhtiari province are not high, compared to other studies. Furthermore, YCT based on pH changes as the main criterion could be recommended as a cost-efficient, rapid, and .reliable test for antibiotics detection in milk

Acknowledgments

The authors thank the deputy of research of Shahrekord University for supporting this research.

Conflict of Interest

The author declared no conflict of interest.

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10.22059/ijvm.2020.284171.1005003

Iranian Journal of Veterinary Medicine
Abstracts in Persian Language

Online ISSN 2252-0554

مجله طب دامی ایران، ۱۳۹۹، دوره ۱۴، شماره ۴، ۳۹۴-۴۰۲

مقایسه آزمون ماست با کیت تجاری برای تشخیص باقیمانده آنتی‌بیوتیک در شیر خام و پاستوریزه

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(دریافت مقاله: ۰۹ اردیبهشت ماه ۱۳۹۹، پذیرش نهایی: ۱۰ شهریور ماه ۱۳۹۹)

زمینه مطالعه: از آنتی‌بیوتیک‌ها به طور گسترده برای درمان حیوانات مزرعه‌ای استفاده می‌شود. از آنجا که انسان‌ها از محصولات تولیدشده از حیوانات استفاده می‌کنند، استفادهٔ نامناسب از آنتی‌بیوتیک‌ها در حیوانات باعث اختلالات متعدد در انسان می‌شود. شیر از جمله این مواد غذایی است که در صورت استفاده از آنتی‌بیوتیک تحت تاثیر قرار گرفته و آنتی‌بیوتیک در آن باقی خواهد ماند.

هدف: این مطالعه برای ارزیابی باقیمانده آنتی‌بیوتیک در شیر خام و پاستوریزهٔ تولیدشده در استان چهارمحال و بختیاری به‌وسیلهٔ آزمون‌های چهار بشقابی (Four plate Test)، آزمون ماست (Yogurt Test) و آزمون کوپن (Copan Test) به اجرا در آمد.

روش کار: به این منظور ۱۴۶ نمونه شیر خام و ۵۶ نمونه شیر پاستوریزه به‌طور تصادفی از گاوداری‌ها و کارخانه‌های تولید فراورده‌های لبنی اخذ گردید. حضور باقیمانده آنتی‌بیوتیک در نمونه‌ها به‌وسیلهٔ آزمون‌های چهار بشقابی، آزمون ماست و آزمون کوپن ارزیابی شد. همچنین حساسیت این آزمون‌ها برای آنتی‌بیوتیک‌های پنی‌سیلین و تتراسایکلین مقایسه شد.

نتایج: در مجموع ۸/۹ درصد از شیرهای خام و ۱۲ درصد از شیرهای پاستوریزه حاوی باقیمانده آنتی‌بیوتیک بودند که از این بین در ۲ درصد از آنها میزان باقیماندهٔ آنتی‌بیوتیک از میزان مجاز بیشتر بود. با توجه به نتایج بین آزمون چهار بشقابی و آزمون‌های ماست و کوپن اختلاف آماری معنی‌داری برای تشخیص پنی‌سیلین و تتراسایکلین در شیر وجود داشت ($P<۰/۰۵$) ولی آزمون آماری نشان داد که بین آزمون ماست و آزمون کوپن اختلاف آماری معنی‌داری برای تشخیص این آنتی‌بیوتیک‌ها در شیر وجود ندارد ($P>۰/۰۵$).

نتیجه‌گیری نهایی: بر این اساس می‌توان از آزمون ماست به‌عنوان یک آزمون با حساسیت مناسب، ارزان و آسان برای تشخیص باقی‌ماندهٔ آنتی‌بیوتیک‌های پنی‌سیلین و تتراسایکلین در شیر استفاده کرد.

واژه‌های کلیدی: آزمون ماست، کیت تجاری، باقی‌ماندهٔ آنتی بیوتیک، شیر

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