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Efficacy of Newly Developed Screening Test (D-Saft1) For Detection of Antibiotic Residues in Raw Milk Samples Collected from Khartoum State, Sudan

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Abstract

Aim: In this study a newly antibiotic screening test (D-SAFT1) was developed, it is based on activation of Lactobacillus casi (DSM 38124) embedded in dried milk particles.

Methods: For the preparation of the test mixture: powdered milk, lactose, standard bromocerol green indicator solution and 0.1 ml of 1.5 × 10^7 Lactobacillus casei MRS culture were added to each Universal bottles containing specific amount of antibiotic standard. The mixtures were frozen in a deep freezer at -20^{0}C for 24 hours. Then the bottles were lyophilized (–60^{0}C) and kept at 4-5^{0}C until used. Field samples of raw milk (200 cows, 50 camels and 50 goats) were collected from Khartoum State and examined for antibiotic residues using D-SAFT1 against two other approved methods that include Trisensor antibiotic test and the Modified One Plate Test.

Results: Tri sensor, Modified one plate test and the new detection method (D-SAFT1) revealed the same results that 80 (40%) of cow milk samples were positive to antibiotics residues, while all camel and goat milk samples were negative.

Conclusion: The antibiotic new detection method should be looked upon as an alternative screening method by encouraging its improvement, use and application as a field test, this especially because it can be prepared locally in addition to its low cost.

Keywords: Antibiotic residues; Detection; D-Saft1; Efficiency; Field application; New developed method; Screening tests

Introduction

The presence of antibiotic residues in milk can be attributable to a number of different causes such as the misuse of antibiotics during treatment of lactating cows, disease prevention, failure to observe the withdrawal period and the illegal use of antibiotics as growth promoters [1,2]. Milk and milk products may be contaminated with antibiotics residues such as sulphonamids, beta-lactam, nitrofurans, which are widely used at high dosage for the treatment of diseases in many cattle [3].

Consumers demand for residue free and in other aspects safe animal-derived food products are high, and to guarantee that food of animal origin is toxicologically safe, withdrawal times and maximum residue limits have been established is increasing [4,5]. It is necessary to monitor the presence of antimicrobial drug residues from not entering the human food supply [1,6-10]. The best way to monitor commingled milk is through the use of residue screening tests [2,7,9,10] using rapid and reliable microbiological [2,10,11], immunological [12,13] or physico-chemical screening methods [14,15]. These tests are rapid, qualitative, and can detect a broad range of antibiotic residues [9]. Screening tests are used to prevent the introduction of the contaminated milk into food chain and, therefore they are frequently used by regulators and food producers [16]. The most commonly used screening tests include microbial growth inhibition assays, microbial receptor assays, receptor binding assays, immunologic assays, and enzymatic assays [17,18]. Biosensors have revolutionized diagnostics for use in point-of-care testing [19]. Moreover screening tests can decrease the danger of residue contamination at violative levels if they are reliable to detect them at the concentrations found in bulk and tanker truck milk [20].

Microbiological and immunoassay methods used for determination of these antibiotics were validated according to the guidelines laid down by European Commission Decision 2002/657/EC [21]. Effective monitoring program requires specific, sensitive and reliable analytical methods that can detect all drug residues below regulated levels [5,8]. The overall objective is to develop and validate multi-residue methods in order to support the implementation of both existing as well as future regulations in the area of food control [5].

A new detection method for antibiotics (D-SAFT1) that was developed initially by researchers from the Department of Food Hygiene and Safety, University of Khartoum, which was a rapid microbiology screening test, was evaluated during this study. Its validation and efficiency against other two approved methods: Trisensor antibiotic test and Modified One Plate test were assessed.

Materials and Methods

Antibiotic residues testing

Disk assay method: Modified one plates test utilizing Bacillus subtilis was performed as described by Koenen-Dierick et al. [22] to detect the residues of antibiotic in the milk samples obtained from three species of animals (cow, goat and camel). After impregnated sterile paper discs (using sterile forceps) into the milk sample to be tested, the paper disc was put into the central of a Petri dish of nutrient agar that containing an overnight broth culture of Bacillus subtilis. The plates were incubated at 370C for 24 hours and then examined. If inhibition zone was found around the paper disc, the result was considered positive.

Tri sensor antibiotic test: This multiplex dipstick test is a lateral flow assay using specific receptors and generic monoclonal antibodies. The procedure was perfumed as described in the company

technical manual. About 100 µl of milk to be tested were added into reagent micro well and incubated for 3 minutes at 400C. One dipstick was dipped into the reagent micro well and then incubated further for 3 minutes. Finally, the test lines color intensities were compared with the control line for the interpretation of the result (Plate 1). The results are visualized at the 3 specific capture lines by the use of colloidal gold-conjugates. The control line serves also to establish a threshold value limit for each test line (Plate 1).

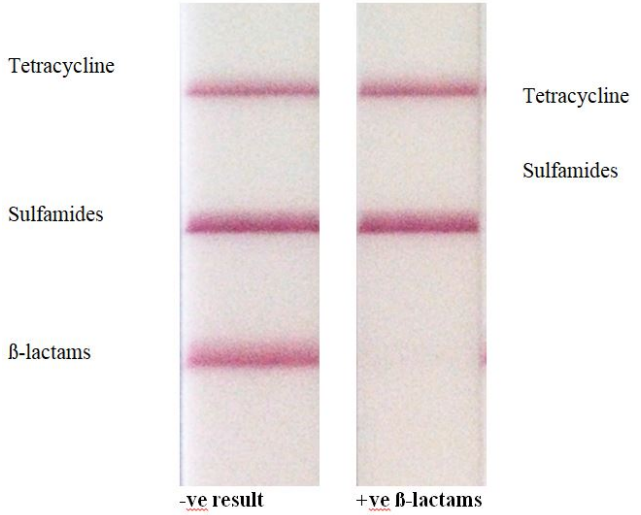

Plate 1: Trisensor method for antibiotics detection showing β-lactams positive result.

The D-SAFT1 test

Preparation of the test organism: Lactobacillus casi sub spp. Lactobacillus casi DSM 38124 was selected as a test organism and obtained from German type culture collection (DSMZA) due to its high survival rate in dry matrices [23] and its fast production of lactic acid.

Lactobacillus casei dried culture was activated in MRS broth and incubated for 24-28 then sub cultured in MRS agar to obtain visible colonies. the colonies were dispersed aseptically in tubes containing sterile distilled water and serial dilutions in distilled water was carried to reach specific concentration using the McFarland turbidity standard; Mc 500 million colony/ml [24].

Preparation of the test mixture: For each test tube; powder milk (0.5 gram) lactose (0.02 gram), bromocerol green indicator solution (0.5 ml) and 0.1 ml of 1.5 × 107 Lactobacillus casei MRS culture were added to each test tube containing specific amount of antibiotic standard. The color changes from white to blue. The pH measurements were recorded for each concentration at one hour intervals during three hours. If there was no change in the milk color this indicates negative result (Plate 2 and 3).

Plate 2: The dry test tube showing the new developed antibiotic residue detection method (D-SAFT1).

Plate 3: Positive and negative results for D-SAFT1 antibiotic residue detection method.

Designing of the final form of the test: Universal bottles (n=300) containing powder milk (0.5 gram) and 0.02 gram of lactose, 2 drops of bromocerol green indicator and 0.1 ml of 1.5×10^7 Lactobacillus casei MRS culture were frozen in a deep freezer at -200C for 24 hours. Then the mixture in the bottles was lyophilized using cold drying apparatus at –600C. After that the test was ready for use and can be kept at room temperature however, cold ad dry place is preferred.

Results and Discussion

Efficiency of the new antibiotic residue detection test (D-SAFT1)

The D-SAFT1 method utilized Lactobacillus casei as a microbiological screening test (Plate 2 and 3). Previously other bacterial test strains such as Bacillus stearothermophilus var. calidolactis, Streptococcus thermophilus and Bacillus subtilis ATCC 6633 were used [25,26]. The assays with Bac. stearothermophilus as the test microorganism are routinely used in milk industry worldwide based on inhibition zone [27]. Moreover in the microbial growth inhibition methods test, local isolated culture of Bacillus subtilis was

used as a test microorganism due to its high sensitivity to detect a wide range of antibiotics commonly used in animal disorders [28].

The incubation period of D-SAFT1 detection method is short compared to the modified one plate test, which is also nonspecific microbiological detection method [Table 1]. It takes about 2 to 3 hours, which is relatively longer time in comparison with Trisensor test, which is easy to perform, samples are applied directly and the incubation period is very short (6 minutes) and the results can be read visually or instrumentally. Trisensor helps producers and processors to save time and to prevent expenses by combining three single tests in one for the screening of residues of three different families of anti-infectious agents simultaneously in milk such as β-lactam, tetracyclines and sulfanomides drugs [29]. The results obtained by the three methods (Tri sensor, modified one plate test and the new detection method) revealed the same results as shown in Table 2, which indicate the validity and efficiency of D-SAFT1. The same result for the milk samples screened using Tri sensor method and the modified one plate test [6]. The most important advantage of the Trisensor test (Plate 1) is that the test can distinguish between tetracycline, Sulfamides and B-lactam antibiotics. However the D-SAFT1 detection method is not specific towards differentiation of different types of antibiotics residues as it gives only a positive or negative result [Table 1], (Plate 2 and 3) and comparatively, it needs more sample preparation and cleaning. Never the less it could be used at the industrial or small scale production levels where the alternative tests could not be found or expensive [Table 1] especially in Sudan where performing of one Trisensor test cost approximately about 50 Sudanese pound and the most important advantage of D-SAFT1 detection method is its low cost; as one test cost approximately about 1.5 Sudanese pound when conducting the experiment. It is important to improve this new developed detection method for more specificity.

Test	Time	Cost	Advantage	Disadvantage
Trisenor	6 minutes	1 kit=50 USD	Distinguish between tetracycline, sulfamides and beta-lactam antibiotics rapid test	High cost and unavailable
				It might missed some positive results if antibiotic present is not belong to the stated families
Modified one plate	48 hours	1 kit=5 USD	Low cost	Not rapid and needs more preparations
				Not specific for different type of antibiotic
New developed method (D-SAFT1)	2-3 hours	1 kit=2 USD	Low cost and relatively rapid	Not specific for different type of antibiotic
			Can be produced locally	

Table 1: Comparison of efficiency of D-SAFT1 detection method against two different types of antibiotic screening tests.

Sources of milk	Numbers of examined samples	Numbers of positive samples		
		Modified one plate method	Trisenor method	D-SAFT1 method
Cows	200	80 (40%)	80 (40%)	80 (40%)
Goats	50	0	0	0
Camels	50	0	0	0
Total	300	80 (26.67%)	80 (26.67%)	80 (26.67%)

Table 2: Detection of antibiotics residues in milk samples collected from Khartoum State suing the three different methods of antibiotic screening.

Prevalence of antibiotics residues in milk samples suing the three different methods of antibiotic screening

About 80 (26.67%) milk samples of the collected milk showed positive results and all were found in the samples collected from cows (40%), whereas all the milk samples collected from goats and camels showed negative results for the antibiotics residues [Table 2]. Safe milk should not contain residues of antibiotic [30]. This because the presence of antibiotics residues in milk promoting the spread of resistance to antibiotics [31-33]. Further the antibiotic resistant may spread to other microbial populations with threat hazards to human and animal health [34].

The variations of milk samples collected from different animals that contaminated with antibiotics residues [Table 2] might be due to the different location of sale points and collection points [6]. In addition to other different factors including animal breed, diseases in the area and the way of treatment, different management practice and the level of workers awareness on how to deal with antibiotics treated animals [7,35]. The use of antibiotics and other antimicrobial agents at sub-therapeutic levels in dairy animals have always been considered as one of important reasons for the presence of residues [36]. Also the lack of pasteurization and cooling facilities might encourage producers to adulterate their milk by using antibiotics and other chemical preservatives in order to increase the shelf lives of the milk [6,30,32,37]. The residues of antibiotics are harmful when transfer to human through milk resulting in therapy failure and development of antibiotics resistant organisms [34,38,39]. However the present result [Table 1], showed higher values compared to those which found that 16 (6.66%) and 30 (12.25%) of the milk samples were contaminated with antibiotic and sulphanomide [32]. The samples contaminated by tetracyclines were found as 34% and 31% and sulfonamides were 31% and 29% in raw and heated milk respectively, in Nyala city at South

Darfur State [7]. Moreover the values were also higher than that reported by Movassagh and Karami who found 5% of raw milk samples were positive for antibiotic residues [40]. They indicated that the variations might be due to the differences of drug that used in the study areas and also variation in the drug withdrawal period of the antibiotics used.

All the milk samples with antibiotics residues [Table 2] were found to be beta-lactam [Figure 1]. The beta-lactams, sulfamides and teteracyclines are widely used in veterinary medication due to their broad spectrum activity and low cost [41]. The beta-lactam (penicillin G etc.) and tetracycline (oxytetracycline, etc.) antibiotics are the most frequently used antimicrobials for treatment of mastitis in dairy cows and consequently, the most commonly residues found in milk [42]. Moreover the antibiotics given mostly to cows commonly are penicillin, oxytetracycline, sulfadiadine, metronidazole, chloramphanicol, cephalosporin, streptomycin and rifampicin; among them the antibiotics which are commonly excreted through milk are oxytetracycline, chloramphenicol and streptomycin [43].

Consumption of animal products contaminated with antibiotic residues can cause allergic reactions in humans and reduce the efficacy of antibiotics for treatment human infections. The resulting increase in antibiotic-resistant bacteria is a public health concern [44]. In a previous study, the resistant of some beta-lactam antibiotics in mastitic milk in Khartoum State, Sudan [34,38].

The obtained prevalence [Table 2] was in line with those that found out of 127 samples of milk, 64 were contaminated with beta-lactam residues, and 24 with sulphonamide residues [45]. However in the Netherlands and Germany, only 0.81% and 1.6% positive samples, respectively were reported for the presence of sulphonamide residues [10,46]. Also alkane analyzed 46 samples using HPLC confirmatory method, and confirmed the presence of sulphonamide residues only in a single sample being above the maximum limit [47]. However 51.3% of sulphonamide contaminated samples were found in Mexico [48].

The present research found that all camels and goats were free from antibiotics residues whereas some of cow milk samples revealed positive results. So vaccination programs for epidemic diseases in dairy cows should be applied in order to minimize the need for antibiotics treatment; however, education programs in the uses of antibiotics and their withdrawal period should be implemented for farms owners and labors.

Conclusion

The present study concluded that the D-SAFT1 as a new developed detection method for antibiotics residue has low cost and its incubation period has reasonable time (2-3 hours). Research should be focus on the new detection method so as to shorten its incubation period and to improve its specificity. It is also important to encourage the farmers, milkmen and factories to utilize this local and low cost product, whereas the other screening tests were too expensive and unavailable.

Ethical Approval and Informed Consent

No ethical approval was required, however all the farmers from whom the milk samples were collected agreed to participate in this study. Moreover a property right was issued locally in Sudan.

Authors' Contributions

Suzan M. Ibrahim conducted the laboratory work for the designed and validation of the developed test, Limya Warsma carried out the experimental field and laboratory efficiency of the test and conducted the statistical analysis of the data and drafting the manuscript. Nazik Mustafa developed the test idea, co-supervised the laboratory work and revised the manuscript. Ibtisam El Zubeir supervised the field and laboratory work and designed the experimental field work and manuscript preparation and writing.

Competing Interests

All authors declare that they have no competing interests.

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