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Detection of antibiotic residues in milk and milk products of cattle in dairy Farms in Baghdad region

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Abstract
The aim of this study was to evaluate the general hygienic status of the dairy farms, meat and milk products through detecting the antimicrobial residues by using local bacterial isolate *Bacillus stearothermophilus* which was isolated and diagnosed previously from different soil regions in Baghdad, Iraq.
The local bacterial isolate *B stearothermophilus* was selected based on its high sensitivity to antibiotics and rapid growth within 2hrs at high temperatures to detect the antibiotic residues in milk and milk products.
Results revealed that *B stearothermophilus* has abroad spectrum to verify the presence of a multitude antimicrobial substance in milk. However, apart from the specified sensitivities for penicillin G and Sulfadiazine, a substantial number of other antibiotics, sulfonamides and inhibitory substances can be detected at or close to the levels that are defined by the Maximum Residue Level.

Keywords: Antimicrobial residue, milk, maximum residue level, *B. stearothermophilus*

1. Introduction
Common management practice for dairy animals worldwide includes antibiotic therapy. Residues of these antibiotics—whether infused, injected, or added to the diet—may enter the milk supply from the treated animals. Regulations for use of antibiotics require that milk from treated animals withdrawn from sale for a prescribed time. When proper procedures for use of a drug and withdrawal of the milk are not followed, milk containing drug residues may be sent to the marketplace.
Antibiotics have been used in the dairy industry for more than five decades in dairy cattle production to treat or prevent disease and to increase milk production or improve feed efficiency [1]. Residual antibiotics in milk can seriously affect consumers' health causing allergic reactions and developing resistant strains. Antibiotic contamination in milk can also cause significant economic losses for producers and manufacturers of milk and milk products. Although antimicrobial drugs are useful for treatment of human infections, their occurrence in milk causes adverse public health effects such as drug resistance and hypersensitivity that could be life threatening [2, 3]. The use of antibiotics therapy to treat and prevent udder infections in cows is a key component of mastitis control in many countries. Due to the widespread use of antibiotic for treatment of mastitis in dairy cows, much effort and concerns have been directed towards the proper management and monitoring of antibiotics usage in treatments in order to prevent contamination of raw milk. As widespread use of antibiotics has created potential residue problems in milk and milk products that are consumed by the general public. Because of the public health significance, milk and milk products contaminated with antibiotics beyond a given residue levels, are considered unfit for human consumption [4]. The good quality of milk must contain no harmful or toxic residues, such as antimicrobial drugs. The extra-label use of these antimicrobial treatments, insufficient withdrawal period and lack of records are the most common causes of theses residue in milk, which lead to the increase of these residues in milk above the acceptable maximum residue limits (MRLs). The (MRL) is defined as the maximum concentration of a residue, resulting from the registered use of an agricultural or veterinary chemical that is recommended to be legally permitted or recognized as acceptable in or on a food, agricultural commodity, or animal feed. The concentration is expressed in mg/kg of the commodity or mg/L in the case of a liquid commodity or ppm/ppb [5]. The MRL is based on the Acceptable Daily Intake (ADI) for a given compound, which is

the amount of a substance that can be ingested daily over a life time without appreciable health risk. MRLs are fixed on the basis of relevant toxicological data including information on absorption, distribution, metabolism and excretion [6]. In addition the lack of good veterinary practice and illegal use of veterinary drugs by farmers will increase this problem [7-9].

To detect antibiotic residues in milk different methods were developed and are applied in laboratory analysis. These consist of screening and chromatographic techniques to detect as many antibiotics as possible. The screening method is generally performed by microbiological, enzymatic and immunological methods. The screening methods are based on various susceptibilities of bacteria to different antibiotics. The antibiotic residue detection assays that are currently available use different methods and test microorganisms [10]. Microbiological assays for the detection of antibiotic residues utilize bacteria such as *Bacillus stearothermophilus* because of its high sensitivity to the majority of antibiotics. Both microbiological and chromatographic methods have been described for monitoring antibiotics in milk and animal tissues. Although the microbiological assay techniques have been recommended as official and conventional methods because of their simplicity, the bioassay methods lack specificity and provide only semi-quantitative measurements of residues detected and sometimes produce false positives [11, 12]. Regulatory limits for antibiotic residues have been imposed on the dairy industry in many countries [13, 14]. Accordingly, the aim of the study was to evaluate the general hygienic status of the dairy farms in Baghdad State, to detect any contiminants or residues in milk and milk product with antibiotics by using local strain of *B. stearothermophilus*

2. Materials and Methods

Period of study

Period of study during May/ 2016-March/ 2017

2.1 Test Organism

Bacillus stearothermophilus var. calidolactis isolated from soil in Baghdad, Iraq. The isolates were cultured on brain heart infusion agar. All plates were incubated for 24h at 75°C. The isolates were identified in laboratories of central health public laboratory.Isolates maintained on brain heart infusion agar and stored at 4 ºC, and were sub cultured once very two-week [15].

2.2 Sensitivity test

2.2.1 Minimum inhibitory concentration: MIC was determined by using broth dilution assay method. In the tube dilution assay, standard bacterial suspension (1.5×10^8 CFU/ml) was added to tubes containing 10 ml Nutrient broth with different concentration (0.1, 0.2, 0.4, 0.6, 0.8, 10.0 and 200 ng/ml). One tube contains nutrient broth served as positive control. After 24 h incubation at 37 ºC, the tubes were examined for growth [16, 17].

2.3 Rapid growth bacterial isolation: To test the activity and the rapid growth of bacterial isolation, spore suspension was prepared, according to the method described by [18] and estimating the number of spore in 1 ml of suspension by using (Specto-20). Then 0.1 ml of spore suspension was transfered to each of the 42 tube containing 9 mL of nutrient broth then the pH and transmittancy was examined before incubation as considered zero time, then all tubes incubated at 60 ºC. Re-examination was done every half hour and up to 6 hours and left three tubes to be tested after 24 hours, as well as to noting the proportion of spore formed [17].

2.4 Prepare test analysis: The test devices were consisted of tubes contains a solid and buffered agar medium including all the required nutrients trypton, glucose, a standardised number of spores (5×10^8 spore/ml) for the test organism *Bacillus stearothermophilus var. calidolactis*. An antifolate trimethoprim and a purple coloured pH indicator bromocresol purple were used. The tests storage upright, in the dark at a constant temperature below 8°C prevented from freezing. The principle of the test was based on diffusion of possible inhibitory substances through the agar. This reduced the growth of the test organism and delayed or prevented the agar from changing colour from purple to yellow when incubated in the test device containing the milk sample at a temperature of 65 °C.

2.5 Sample collection

2.5.1 Milk samples: 180 fresh milk (raw) samples were collected from cows, buffaloes, sheep and goats received from the teaching hospital for the College of Veterinary Medicine and the College of Agriculture / University of Baghdad and supermarket. Twenty pasteurized milk samples, twenty five milk powder for children and fifteen samples for adults.

2.5.2 Dairy products samples: twenty five samples were collected from the local cheese from different markets in Baghdad and ten samples of local cream.

2.5.3 Meat samples: One hundred meat samples were collected from sheep, cows and goats from local markets in Baghdad.

2.6 Sample analysis: Milk samples were mixed well and the formation of air bubbles or foam was avoided. 100 µl of milk sample was transferred to the tube containing nutrient agar embedded with *Bacillus stearothermophilus var. calidolactis* spores and Bromocresol purple indicator and incubated in water bath for 2-3 hours at 65 ºC. A clear color change from purple to yellow indicated that the antimicrobial compounds were below the detection limits. A purple color indicated the presence of antibiotics at or above the detection limits of the test.

2.7 Stability of antibiotics in milk samples: Raw, inhibitor free milk samples were spiked with the selected antibiotics: penicillin G, ampicillin, cloxacillin, and ceftiofur at the levels of 1 × MRL, 1.5 × MRL and 2 × MRL, and oxytetracycline at the levels of 1 × MRL (100 ppb), 500 and 700 ppb. The samples were stored at 4 ± 2 ºC and –18 ± 2 ºC, and were tested every day or every week, respectively. All tests were performed in duplicates. The results were evaluated visually, by comparison with colour scale manual. The MRL values for the tested substances and detection levels of the used method was presented in (Table 3).

2.8 Experimental cows and treatment: The study was conducted in a dairy farm of college of veterinary medicine. Animals that needed to be treated for mastitis were placed in a separate pasture lot and manually milked twice a day. Treatment of each animal consisted of daily intramuscular injections with a commercial suspension containing 8.000.000 UI of penicilin G (75% penicilin G-procaine, 25% potasium penicillin), for a total of 4 days. For a 500 kg cow, this was approximately equivalent to daily dosages of 12.000 UI/kg

PPG, 4000 UI/kg potasium penicillin. The product was reconstituted immediately before use in 20 mL sterile saline, and injected deep intramuscularly in two equal volumes of 10 mL at each buttock.

2.8.1 Experimental design: The experimental design administrations were started on day 1 for each cow and continued until day 4. On day 7, the withdrawal time for milk ended according to the product labeled instructions of 3 days following the last administration. On day 10, and for the purpose of the study, cows that were manually milked for an additional 3 days past the usual time of withdrawal returned to their original destination lot. Milk samples were collected every 12 hours between 60 and 144 hours after the last administration and submitted for residue analysis of pre- and post-heating treatment.

2.8.2 Milk collection and residue analysis: All four quarters were manually milked by farm operators in stainless steel buckets from which a composite sample was directly collected into 10 mL sterile plastic vials. To avoid potential contamination between samples, buckets and hands were washed with an iodine solution and thoroughly rinsed between cows. The samples were kept at 4°C for a maximum of 2 days prior to analysis. Sample was retested after milk was subjected to heat treatment of 82°C for 5 minutes. This treatment has been shown to be a fast, simple, and inexpensive way to remove false-positive results due to natural inhibitors and has no effect on positive samples containing most antimicrobials [19].

3. Results and Discussion

3.1 Identification of *Bacillus stearothermophilus:* Isolate of *B. stearothermophilus* were isolated from soil samples in Iraqi- Baghdad using nutrient agar plates at 55°C, isolate show heavy growth after one day incubation period that was recognized as thermophilic *Bacillus* spp. when they were cultured at 75°c for 24hrs using the same medium among other isolates (Fig.1).

Fig 1: Growth of Thermophilic *Bacillus* spp. on NA plate at 75°C/24hrs.

The morphological features of the colonies were flat, opaque, rough surface and they had circular irregular edge. The isolate was gram positive, rod shaped; spore forming bacteria after microscopical examination. The results of biochemical tests shown in (Table 1) they cannot produce indole, can grow very well at 75 °C and in the presence of 3% NaCl, they can hydrolyze starch and gelatin, can produce acid and they cannot produce gas from glucose fermentation [20, 21].

Table 1: Cultural and Biochemical tests of Local Isolate of *B. stearothermophilus*

Test	Local Isolate	Standard
Growth at 55°c	+	+
Growth at 75°c	+	+
Growth at 3%Nacl	+	+
Indole Production	-	-
Starch hydrolysis	+	+
Gelatin lique.	+	+
Acid production	+	+
Gas production	-	-
Motility	+	+
Gram staining	+	+

3.2 Determination of MIC: The results of MIC estimated by tube dilution method showed that β-lactam and cephalosporins antibiotics relatively had low MIC (0.004 ug/ml) and (0.003 ug/ml) respectively, while for Chlorotetracycline, Minocycline and Gentamycin is (200 ng/ml). The results of MIC estimated by tube dilution method showed that β-lactam relatively had low MIC (0.004 ug/mL) while for Chlorotetracycline, Minocycline and Gentamycin is (200 ng/mL). This result is agreement with [22], which found *B. stearothermophilus* disc assay was the most sensitive to penicillins [Minimum Inhibiting Concentration in mcg/ml, MIC, between 0.001 and 0.004), cephalosporins (MIC between 0.003 and 0.09, apart from Ceftazidime, 0.3) and aminoglycosides (MIC between 0.03 and 0.6). Since β lactam antibiotics, dominantly penicillin, are most widely used in treatment of the bacterial diseases of cattle, therefore the tests for the detection of beta lactam residues are most widely used in control of milk for antibiotics residues. Even though they are useful tool for the prevention of use of residue contaminated milk, simultaneously they carry numerous disadvantages, on top of them is their ability to detect residues bellow the maximum tolerated concentration [23].

3.3 Rapid growth bacterial isolation: The result of activity and the rapid growth of bacterial isolation, (Fig. 2).Three readings rate of the value of pH and the percentage of the transmittances of light, at zero time passage of light (85%) with pH of (6.82). Over time periods a gradual decrease in the pH value and transmittances of light to after (2) hours of incubation (5.1) and (32%) respectively. Observed changes in pH are typical of *Bacillus* Species cultured in aerated media. All the specifications that are available in the bacterial isolation under study of the speed of growth and high sensitivity to antibiotics at high temperatures (65-70 C°) as well as being unsatisfactory can be considered features or encouraging factors to be used in tests detecting residues of antimicrobial and any other inhibitors. The result of activity and the rapid growth of bacterial isolation agreed with [24] who found growth and sporulation data for the five strains of *B. stearothermophilus* a burst of growth followed an extended stationary phase. Sporulation paralleled growth so that the majority of spores were produced within a 2-hr interval. Observed changes in pH are typical of *Bacillus* Species cultured in aerated media. All the specifications that are available in the bacterial isolation under study of the speed of growth and high sensitivity to antibiotics at high temperatures (65-70 ºC) as well as being unsatisfactory can be considered features or encouraging factors to be used in tests detecting residues of antimicrobial and any other inhibitors.

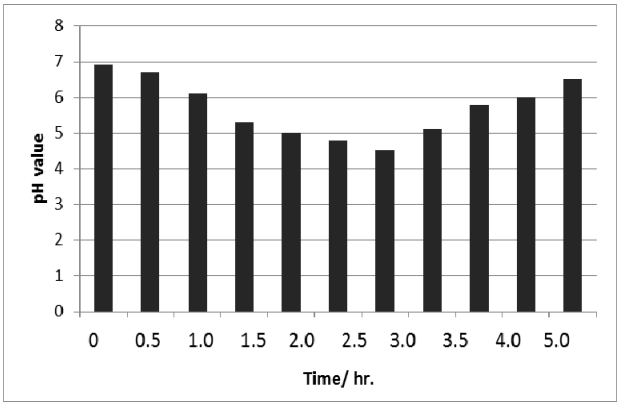

Fig 2: Rapid growth of bacteria and change the pH value with time

3.4 Sample analysis: The samples of milk and different food product obtained from different areas of Baghdad were examined for the contamination of antimicrobial drugs (Table2).

Table 2: Number and percentages of positive and negative samples for the presence of antimicrobial residues in milk and different food product.

Samples	No.	Positive	Dubitable	Negative
Raw milk Cow	90	48 53.3^{b}	2 ^{b}2.2	40 ^{b}44.4
Raw milk Buffalo	40	15 37.5^{c}	3 ^{a}7.5	22 55^{b}
Raw milk sheep	30	17 56.6^{b}	- 0.0	13 43.4^{b}
Raw milk goat	20	8 40.0^{c}	1 ^{a}5.0	11 55^{b}
pasteurized milk	20	4 20.0^{d}	- 0.0	16 ^{a}80.0
Milk powder for children	25	4 15.0^{c}**d**	2 ^{b}8.0	19 ^{a}76.0
Milk powder for adult	15	6 ^{c}40.0	2 ^{a}13.3	7 ^{b}46.6
Local Cheese	25	5 ^{d}20.0	2 ^{a}8.0	18 ^{a}72.0
Local cream	10	2 ^{d}20.0	1 ^{a}10.0	7 ^{a}70.0
Cow meat	40	25 62.5^{c}	- 0.0	15 37.5^{b}
Sheep meat	40	32 ^{a}80.0	1 ^{a}2.5	7 ^{c}17.5
Goat meat	20	6 ^{d}30.0	3 ^{a}15.0	11 ^{b}55.0

- Positive: yellow color, Negative: purple color
- Significant difference between different letters [vertically comparing].

3.5 Stability of antibiotics in milk samples: The results of the analyses of the frozen samples were summarized in (Table 4). The lowest durability was observed for penicillin G and oxytetracycline. The first antibiotic was detected up to 1–10 weeks, and the second one for 10–16 weeks, depending on the method of analysis and its initial concentration. In the samples containing cloxacillin at the initial level of 30 ppb, the antibiotic was detected only for 3 weeks using receptor test and up to 20 weeks with the microbiological method. The samples containing higher initial concentrations were positive for 23–34 weeks. The highest stability was identified for ampicillin and ceftiofur. These substances were detected for 24–35 weeks.

Table 3: MRLs for the analysed antibiotics and detection levels of the used tests

Substances	MRL values [ppb]	Tests sensitivity [ppb]
Penicillin G	4	2 - 3
Ampicillin	4	3-4
Cloxacillin	30	28-30
Ceftiofur	100	24
Oxytetracycline	100	400 - 800

Table 4: The results of the detection of antibiotics in milk samples stored at –18 ºC

Antibiotics	Initial concentrations [ppb]	Last positive results [week of experiment]
Penicillin G	4	10
	6	10
	8	10
Ampicillin	4	23
	6	35
	8	35
Cloxacillin	30	22
	45	35
	60	34
Ceftiofur	100	24
	150	35
	200	34
Oxytetracycline	100	10
	500	10
	700	10

The data obtained in the study indicate, that stability of antibiotics in milk samples depends on the conditions of storage, type and initial concentrations of these substances, as well as on the method used for analysis. The acidification of milk stored at 4 C was possibly not crucial for chromatographic methods; however, it has become an obstacle when the receptor tests and the microbiological method were used. There is no available data to which the results obtained can be referred. Using the HPLC method with fluorescence, [25] did not observe changes of ampicillin concentration in milk samples with initial level of 20 ppb stored at 4 ºC for 6 days. According to [26], after 72 h of storage at 4 ºC, the concentration of tetracyclines in milk samples decreased about 18%. [27] (Riediker *et al.,* 2004) using the LC-ESIMS/MS method observed over a 50% decrease of five β-lactam antibiotic concentrations in milk samples stored under the same conditions for 6 day. Other data has been reported for tissue samples [28]. O'Brien *et al.,* (1981) used the inhibition zone diameters to establish antibiotic concentrations, and observed a 76.05%-100% decrease in ampicillin level in meat samples stored at 4 ºC for 6 weeks. The concentration of oxytetracycline decreased in 7.4% and sulphadimidine in 20.1% under the same conditions.

Generally, the stability of antibiotics is much higher during storage at –20 ºC in comparison with 4 C [28-31]. The decrease in the quinolones activity in frozen stock solutions stored at –20 ºC did not exceed 10%, whereas the levels of β-lactams did not change during 3 months of storage [32]. Freitas *et al.* (2012) [33] noted that amoxicillin may persist in chicken meat stored at –20 ºC almost for one year. The best stability of antibiotics in different samples was observed during storage at –75 ºC [34, 35]. Although antibiotics are relatively stable in frozen food samples, the necessary analyses of their residues should be always performed as soon as possible.

3.6 Experimental cows: Table 5 shows the numbers of positive and negative milk samples for each one of the screen assays in all 212 milk samples that were taken throughout the study. The test detected 60 (28.30%) positive samples, 20 of which become negative when retested following heat treatment of the 40 (18.86%) remaining positive samples.

Table 5: Number of milk samples that were positive and negative for residues by assay throughout the entire study [n=212].

Detection method	Milk sample			
	Positive	**%**	**Negative**	**%**
Test	60	28.30	152	71.70
Test –post heating	40	18.86	172	81.14

The percentage of cows (n=37 animals) that were positive at each time when the test was not preceded by heat treatment the percentage of cows still yielding positive results at 12, 24, 36 48 and 60 hours past the recommended withdrawal time (at 72 hr post-administration] was 21% (8/37), 19% (7/37), 16% (6/37), 14% (5/37) and 8% (3/37) respectively. However, when the test was preceded by heat treatment only 10% (4/37) were positive for one more milking (at 84 hr post-administration).

The objective of this study was to assess the performance of common screening tests used for the detection of antimicrobial drug residues in individual milk from cows treated for subclinical mastitis with one of several dozen commercially available procaine penicillin-G products (PPG) in the Iraqi market. The test specific for beta-lactams were selected based on being the two most routinely employed assays by the Iraqi dairy industry. The results showed that the labeled withdrawal time of 3 days after the last administration was adequate in 35 of 37 cows, and only 2 animals yielded milk positive for residues for an additional day. However, the test when used as instructed, that is, without heat treatment, resulted in a high number of false positive results. Of a total of 60 positive samples to the test, only 40 remained positive when retested after heating at 82 ºC for 5 minutes.. It has long been known that cows with mastitis yield milk with natural microbial growth inhibitors, such as lysozyme and lactoferrin [36, 37]. In particular, tests such this test, which is a detection assay based on microbial growth, have been shown to yield false positive results due to the presence of such natural inhibitors [38]. In general, drugs with milk withdrawal periods of more than 4 days are not approved for dairy cows in lactation. In Iraq In, there are numerous procaine penicillin-G products that are registered for use in lactating cows, and the times of milk withdrawal for the majority are 2-3 days, and exceptionally 4 days. For the dosage and duration of treatment used in this study, the withdrawal time was not expected to exceed 3 days. In fact, the FDA (Food and Drug Administration) recommends withdrawal times for IM PPG products [not exceeding 10 mL per injection site) of 2, 3, 4 and 5 days for dosages of 6,600, 14000, 21.000 and 28,000 UI/kg respectively [39].

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