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A Study on the Use of the Yogurt Starter Culture Bacteria for the Detection of Antibiotic Residues in Milk

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Submitted in partial fulfillment of the requirements for the degree of Master of Science in Food Science
Faculty of Graduate Studies

University of Jordan

May, 1994

This thesis was successfuly defended and accepted on May 28th, 1994.

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TABLE OF CONTENTS

DEDICATION

To My Dear Late Father
Who I Love deeply
Who I Miss Sadly
Who Waited My Happy Moments Longly
& Faithfully

&

To My Mother Who Made It Possible
With Respect

ACKNOWLEDGMENTS

I would like to express my sincere appreciation to my advisor Dr. Mohammed I. Yamani for his great efforts in supervising the entire work and for his useful remarks.

Thanks are also due to the examination committee members Dr. Mohammed A. Humeid, Dr. Salma Tukan, Dr. khalil Shawabkeh and Dr. Malik Haddadine.

Appreciation is extended to all staff members, collegues, friends and employees at the Nutrition and Food Technology Department for their encourgement.

Thanks are also due to all the employees of the Jordan Dairy Plant, the Danish Dairy Plant and Al Raa'i Dairy Plant for help.

Finally my deepest thanks to my sister, brothers, and husband for their patience, support and continous encouragement.

Abstract

A Study on the Use of the Yogurt Starter Culture Bacteria for the Detection of Antibiotic Residues in Milk

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The sensitivity of yogurt culture bacteria (*Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus*) to antibiotics was used to develop a test for the detection of antibiotic residues in milk. In order to standardize milk fermentation by yogurt culture and to fix the time needed for curdling of the milk, different combinations of culture concentration (2,3,4, and 5%) and milk pH values (6,6.2, and 6.4) were tried to find out the shortest possible time for curd formation. The best combination was the culture concentration 4% and milk pH 6. The developed test, Yogurt Culture Test (YCT), could be done by lowering the pH of 96 ml of the tested milk to 6 using 1 N HCI, adding 4 g of yogurt culture and incubation at 42°C for 2.5 h. Negative result (absence of inhibitory substances in the milk) is indicated by curd formation and change in the color of the pH indicator chlorophenol red (0.2% in 50% ethanol), of which 1 ml is added before or after the incubation.

The lowest concentration of penicillin enough to inhibit curd formation by yogurt culture bacteria in YCT was 0.03

IU/ml, whereas for chloramphenicol, oxytetracycline, tetracycline, ampicillin, erythromycin and cloxacillin were: 2.0; 0.1; 0.2; 0.1; 0.3 and 0.3 µg/g, respectively. YCT was more sensitive to chloramphenicol and oxytetracycline than Delvotest-P® and was of comparable sensitivity to tetracycline and erythromycin, but of less sensitivity to penicillin and ampicillin.

YCT was used to examine 618 samples of milk received by the largest three dairy plants in Jordan for antibiotic residues. 15% of the samples gave positive results after 2.5 h of incubation. When extending the incubation time to 4 h only 2.1% of the samples were positive, indicating the presence of other inhibitory substances in about 13% of the samples in marginal concentrations. When testing the positive samples to YCT after 2.5 and 4 h by Delvotest-P®, only 12.3% were positive after 2.5 h and the results were similar to YCT after 4 h.

YCT is a simple and of low cost test. It has comparable reliability to Delvotest-P®. Testing milk samples for antibiotic residues by the same culture used in the processing is an advantage, because it reflects the actual suitability of the tested milk for processing as yogurt. Thus YCT could be recommended as an alternative to the commercial testing methods.

I. INTRODUCTION

Antibiotics are widely used in the therapy and control of bacterial diseases in dairy cattles . Whatever their route of administration, antibiotics may be secreted in varying concentrations in milk (Jurdi and Asmar, 1981). The problem of antibiotic in milk arises chiefly from the failure of some dairy farmers to withhold the milk of treated animals from marketing until such drugs have been completely secreted. There is also the suspicion that antibiotics are sometimes added directly to the milk to retard spoilage. The presence of antibiotics in milk is objectionable both for public health reasons and for their deleterious effects on the manufacture of cultured dairy products(Jurdi and Asmar, 1981).

Lactic acid bacteria which are used in the processing of many cultured dairy products are very sensitive to different antibiotics. The effect varies from stopping to retarding the growth and the activity (Balbi and Hartman, 1985). This could be noticed in the processing of yogurt and other cultured dairy products. In addition, contaminated milk with antibiotics may have some health concerns especially to those allergic to antibiotics (Bishop and White, 1984).

Because antibiotic residues in milk is a major problem for the dairy industry in the whole world, including Jordan, several commercially available tests have been developed for the detection of antibiotic residues in milk. These tests vary with respect to the range of antibiotics detected, their sensitivity, mode of action, the detection time and cost (Senyk *et al.*, 1990). The application of testing methods routinely by regulatory and dairy

personnel have resulted in a significant reduction in antibiotic adulterated milk (Albright *et al.*, 1961).

However, some of the available commercial tests have limited sensitivity to antibiotics while others have a long detection time or lack simplicity. Furthermore, the available tests are expensive and could make the routine examination of milk for antibiotic residues a financial burden for most of the producers in the developing countries, especially when the number of milk suppliers to the dairy plants is high as in Jordan.

The conventional curdling of milk by yogurt starter culture bacteria could be used as a test for the detection of antibiotic residues. Its use would be most appropriate in Jordan where yogurt and the products based on it such as labaneh and jameed are largely produced . This research was conducted to:

1- Develop a simple and a rapid test, based on the modification of milk curdling by yogurt starter culture bacteria, which is of low cost .

2- Measure the sensitivity of the developed test to the most available and used antibiotics in dairy cattle management in Jordan.

3- To survey cattle milk recieved by the three largest dairy plants in Jordan for antibiotic residues using the developed test.

II. LITERATURE REVIEW

A- Inhibitory substances in milk

A wide range of inhibitory substances are present in milk, these potential inhibitors can be classified into the following :

1- Natural inhibitors

There are various natural antimicrobial systems present in milk, their major role is the protection of the suckling animals against infection and disease. The inhibitory compounds, known as lactenins, are heat sensitive and are destroyed by heating the milk to 68-74 $^\circ$C. Another bactericidal component found naturally in milk is the peroxidase system, which consists of lactoperoxidase/ thiocyanate / hydrogen peroxide [LP / SCN$^-$ / H$_2$O$_2$ abbreviated as LP system]. LP is synthesized in mammary gland, SCN ions are derived from the rhodanese catalized reaction with thiosulfate in the liver and kidney, H$_2$O$_2$ results from the metabolic activity of certain streptococci . In this system the inhibitory compound is the result of an oxidation reaction where the LP enzyme combines with H$_2$O$_2$ to oxidize SCN$^-$, however, the inhibition is reversible in the presence of some reducing compounds, e.g. cysteine. LP system is inactivated by heating milk at 85 $^\circ$C for 16 seconds.

Other inhibitory systems that may warrant some consideration are the bacterial agglutinin which can cause agglutination of the starter organisms, thus affecting their metabolic activity and growth; Leucocytes in mastitic milk may cause inhibition of lactic starters through phagocytosis. Heat

treatment of such milk brings no significant improvement (Tamime and Robinson, 1985).

Lysozyme and Lactoferrin, which are natural antibacterial substances, are present at higher concentrations in mastitic milk and in colostrum than in normal milk. They can cause false positive results in antibiotic assays (Carlsson and Bjorck, 1989a; Geleta *et al.*, 1984; Schiffmann *et al.*, 1992).

2- *Bacteriophages*

Bacteriophages are viruses which can attack and destroy bacteria. They are species and/or strain specific. Certain bacteriophages attack and destroy yogurt culture bacteria and the resultant failure of lactic acid production leads to poor coagulation of the processed milk (Tamime and Robinson, 1985). The public health aspect of the presence of phage is not critical as it is when antibiotics exist in milk, but when any thing slows the lactic acid fermentation of milk, growth of undesirable becteria may be encouraged (Kosikowski, 1982). If milk is the origin of phage contamination, then heat treatment at 85 °C for 20 minutes assure their destruction (Tamime and Robinson, 1985).

3- *Chemical inhibitors*

a- Detergent and disinfectant residues

Detergent and disinfectants are widely used in the dairy industry for the cleaning and sanitizing of dairy equipment in the farm and the dairy plant. The detergents formulations contain alkali compounds such as sodium hydroxide, whereas the sanitizing agents are quaternary ammonium compounds (QAC)

or iodine or chlorine-based compounds. Inorganic compounds are also used for cleaning and disinfecting purposes (Kosikowski, 1982; Tamime and Robinson, 1985). Residues of these compounds in milk can be attributed to the negligance, bad management or a faulty cleaning-in- place (CIP) system. The presence of such compounds in milk is not recommended for public health reasons, and also because they can adversely affect or totally inhibit the growth of starter cultures (Tamime and Robinson, 1985).

b- Antibiotic residues

   1- Sources of antibiotic residues in milk

     Antibiotics have been used in dairy cattle management for more than three decades. They are an important component of herd health programs for the prevention and control of diseases affecting dairy cattles, especially mastitis and other bacterial diseases (Oliver *et al.*, 1990). They are administered to cattle by any of the following four general routes (Bishop *et al.*, 1985; Lampert, 1975; Livingston, 1985):

a. Infusion into the udder for treatment of mastitis.

b. Injection: Intramuscular, intravenous, or subcutaneous for treatment of numerous diseases.

c. Orally: for treatment of diseases or as feed additives, to increase the efficiency of feed utilization and promote growth to shorten the time needed to bring an animal to market weight.

d. Reproductive "flush" for uterine, cervical or vaginal infections. Such uses could lead to the contamination of milk and milk products with antibiotics. Food andDrug Administration (FDA) surveys indicated that improper use of antibiotics in the

control of mastitis is the major source of antibiotic contamination of the milk supply (Jones and Seymour, 1988). When the mammary glands of dairy cattles are treated with antibiotic preparation in an effort to control or eliminate infections, the milk from such udders will contain objectionable quantities of these drugs for several milkings (Doan, 1956) .

The presence of antibiotic residues in milk could be due to the following reasons (Chagonda and Ndikuwera, 1989; Mcewen *et al.*, 1991; Semour *et al.*, 1988a) : 1. Not observing the full recommended withholding time of milk which could be attributed to the lack of knowledge of the importance of withholding time or absence of records for cows on treatment. 2. Contaminated milking equipments. 3. Prolnged drug clearance due to excessive or multiple dosing. 4. Drugs not used according to label directions. 5.Ppurchase of treated cows.

The use of antibiotics as an animal feed additive accounts for about 40% of the antibiotics manufactured in North America, which is considered as a source of antibiotic residues in milk (Collins- Thompson *et al.*,1988). There is a considerable conflict in the research data available as to the influence of antibiotics-supplemented feeds on the milk production of dairy cows. Some of the results of these researchs indicates that introducing antibiotics to dairy cattles have a positive reaction on milk production, while others are not. So there is no clear conclusion (Lassiter, 1963). There is a possibility that antibiotics may be directly added to milk in an effort to reduce the number of viable bacteria (Doan, 1956).

2- Persistence and duration of antibiotic residues in milk

To treat dairy cattle animals for any bacterial disease, the quantity of antibiotic administered to a dairy cattle varies according to (Albright *et al.*, 1961):

a. The type of antibiotic used.

b. The severity of the infection.

c. The frequency with which the antibiotic is to be administered during the course of treatment.

d. The judgement of the person administering the therapeutic dosage .

Once the antibiotic is administered to the dairy cow, much of the antibiotic (about 20%) is excreted in the first milking after therapeutic use. Diminishing quantities are excreted in subsequent milkings (Booth and Harding, 1986; Foster *et al.*, 1957), therefore milk must be withhold according to the drug manufacturer's instructions. The withholding time is defined as the time from the last injection or infusion to the last recorded milk sample taken from any quarter of the treated animal in which penicillin and/ or other antibiotics was detected (Johnson *et al.*, 1977a). That part of the antibiotic that is not absorbed by the tissues of the udder when antibiotics are infused is generally eliminated in the milk over a period of about three days (Lampert, 1975). Albright *et al.*, 1961, reported that studies conducted by FDA in 1953 showed that after intramammary infusion of one udder quarter with 100,000 international units (I U) of penicillin, it's level for the first and second milkings was 8.15 IU/ ml; for the fifth and sixth milkings was 0.042 IU / ml; and no detectable amount on subsequent milkings. The recommendation that milk must be discarded for three days following treatment came from

this study. The concentration of antibiotics and the duration of time they persist in milk following treatment is dependent upon the following factors (Oliver *et al.*, 1991; Olson and Krawczyk, 1963; Ormiston *et al.*, 1960):

a. Type and concentration of antibiotic used.

b. Stage of lactation and milk production level of the animal.

c. Type of suspension vehicle (whether it is present in the form of aqueous or oil suspension) .

d. Physical condition of the udder and health state of the animal.

Elimination is slower in animals suffering from mastitis than in healthy cows and also slower in low than from high producers (Jones and Seymour, 1988). Because of these variables, exact statements as to how soon after the antibiotic has been injected the milk will be suitable for manufacturing use are not possible. The common recommendation however, is to withhold the milk for the first 72 h after the last injection (Foster *et al.*, 1957).

A study was conducted to determine the persistence of antibiotic residues in milk beyond the recommended withdrawal period. Analysis indicated that 21% of milk samples were positive for residues beyond the recommended withholding period (Seymour *et al.*, 1988b). Persistence of some drugs (antibiotics) beyond their recommended withholding time could be attributed to that most of the currently available antibiotic intramammary products were developed and introduced to the market many years ago, before comprehensive and specific testing methods were developed, also the currently applied analytical methods is more sensitive than methods used in the past, which can detect the traces of antibiotics persisting in milk

from the treated quarter for long periods (Kosikowski, 1961; Seymour *et al.*, 1988b).

Antibiotics used for the treatment of dairy cattle by infusion in the udder can be transferred from the treated quarter to the untreated one, whether this transfer is significant or not, there are many studies regarding this case, and its difficult to assess the relative importance and length of time to discard milk from untreated quarters (Albright *et al.*, 1961; Cosgrove and Etgen, 1960). The pattern of distribution in untreated quarters indicates that some mechanism other than direct secretion from the blood stream could be involved, but no enough data are available to accurately determine the exact pathways (Rollins *et al.*, 1970). A study was conducted to determine the possible incidence of penicillin transfer from treated to untreated quarters of cows' udders after direct udder infusion of penicillin for treatment of mastitis. Penicillin concentration in untreated quarter or quarters was greatest in low-producing animals and least in higher-producing animals. Therefore the relative rates of excretion of penicillin from the untreated quarter was greatest in high-producing animals and least in low producing animals (Evans and Stern, 1960). **442392**

Some dairy cows are being treated during their dry period with intramammary infusion of antibiotic preparations to aid mastitis prevention. To ensure complete removal of antibiotics from the milk, it shoud be highly recommended to dump the milk from the treated cows for the reommended number of milkings during and after the treatment (Johnson *et al.*, 1977b; Oliver *et al.*, 1984). Generally, the incidence of inhibitory substances varied among

manufacturing problems result from the presence of antibiotics in the above mentioned products, health problems are apparent (Albright *et al.*, 1961). As little as 0.0017 IU penicillin / ml can cause partial inhibition of dairy starter activity, 0.005 IU / ml can cause significant inhibition whereas 0.007 IU / ml can cause 50% or greater inhibition of starter cultures (Balbi and Hartman, 1985), and 0.1 IU /ml is sufficient to inhibit completely the sensitive strains of the bacteria used in starter cultures (Lampert, 1975). One international unit or 1USP unit, is defined as the equivalent of 0.6 μg of benzyl penicillin sodium, one milligram has the potency of 1667 units (Lampert, 1975).

Antibiotic residues in milk affects the validity of certain quality control tests in milk (Bishop and White, 1984). One of the problems arising from antibiotics in milk is their interference with the enzyme phosphatase activity in it and therefore, they affect the validity of the phosphatase test that is used to monitor pasteurization efficiency and/or adequacy. The reasons why antibiotics inhibit phosphatase activity are not clear (Manolkidis and Alichanidis, 1971).

Residues in milk should be avoided because of the following three distinct concerns (Bishop and White, 1984; Bishop *et al.*, 1984; Bishop *et al.*, 1985; Brady and Katz, 1988):

a. They are illegal.

b. The milk from treated cows may still contain large numbers of potential pathogens.

c. They may have biologically active metabolites in milk.

Milk which contains antibiotics is considered by public health officials to be adulterated and a potential health hazard (Albright *et al.*, 1961). This is because certain individuals are allergic to

antibiotics, especially penicillin (Albright *et al.*, 1961). Between 5-10% of all American adults are hypersensitive to antibiotics. As little as 0.003 units of penicillin / ml of milk may cause allergic responses after ingestion, including skin rashes, asthma, hives, anaphylactic shock, idiosyncratic reactions or even death (Brady and Katz, 1988; Jones and Seymour, 1988; Seymour *et al.*, 1988a). Minute amounts of drug residue can be carcinogenic, teratogenic, mutogenic, cause enzyme induction and inhibition and interact with other environmental chemicals (Seymour *et al.*, 1988a).

It is now well recognized that the residual effects of continued consumption of antibiotics present in the animal products, upsets the normal bacterial flora relationships of the human body. It permits antibiotic resistant organisms, previously harmless, to multiply, flourish, acquire virulence, and to establish themselves as invaders. Also antibiotic residues in milk may contain unusual antibiotic- resistant strains of infectious bacteria capable of causing illness in humans (Albright *et al.*, 1961; Doan, 1956), and so, treatment with the antibiotic concerned would be ineffective and it would be difficult to be treated (Doan, 1956; Lampert, 1975).

The current regulation of the FDA specify a zero- tolerance for antibiotics in milk and milk products. From a practical point of view, however, it is the lowest concentration of the antibiotic which is detectable by the prevailing technique which determines the adulteration status (Kornfeld, 1977). The WHO/FDA guidelines for antibiotic residues in milk for human consumption limit most residues to < 0.2 ppm (i.e. 0.2×10^{-3} mg/ml) (Collins-Thompson *et al.*, 1988). Table 1 presents the maximum

acceptable concentrations of antibiotic residues in milk by the World Health Organization, 1969. As reported by Booth and Harding, 1986 the major concerns must be considered in evaluating the safety of antibiotic residues in milk are (Livingstone, 1985):

1. The level, nature and safety of drug -related residues in milk.
2. The development of adequate procedures for setting withdrawal periods and assuring that residues do not exceed the safe level in actual use .

4- Control of antibiotic residues in milk

The best practical way to institute residue control for antibiotics is to institute a solid test program (Kosikowski, 1961). Continuous laboratory testing and regulatory actions torwards antibiotic adulterated milk reduced the incidence of inhibitors in milk from 5.2% during 1950-1959 period to 0.5% during the period of 1971-1972, a ten fold decrease (Messer, 1973). However results of the tests available for antibiotics detection are obtained too late to be of much use in deciding to accept or reject a particular lot of milk. One of the limitations of testing is that a supply which was satisfactory one day, may contain antibiotic the next day, as a result of antibiotic treatment. Likewise, milk unsatisfactory today may be satisfactory tomorrow because the antibiotic has been eliminated to a sufficient degree (Foster *et al.*, 1957). The use of on-farm test kits for residues will reduce antibiotic residue risk (Mcewen *et al.*, 1991), and consequently prevent financial penalties that the dairy man in some countries will be subjected to when milk

Table1. Acceptable maximum antibiotic residue concentraions in milk according to World Health Organization.

Antibiotic	Concentration (ppm)
Ampicillin*	0.010
Bacitracin	1.200
Cephalosporins*	0.010
Chloramphenicol	0.000
Chlorotetracycline	0.020
Cloxacillin**	0.020
Dihydrostreptomycin	0.200
Erythromycin	0.040
Framycetin**	0.150
Nafcillin*	0.020
Neomycin	0.150
Novobiocin	0.150
Nystatin	1.100
Oleandomycin	0.150
Oxytetracycline	0.100
Penicillins	0.006
Polymixin B	5.000
Streptomycin	0.200
Sulphonamides+	0.100
Tetracycline	0.100
Tylosin	0.000

Added by veterinary Products Comittee in:
**1982   *1983   +1984
(Booth and Harding, 1986).

fails to pass the prescribed test for antibiotic residues (Larocque and Neville, 1985). The FDA has attempted to reduce adulteration by limiting the quantity of antibiotic in each preparation to be used for mastitis therapy and by a requirement that a warning against the use of milk from recently treated animals be placed on the preparation (Bishop and White, 1984; Lampert, 1975).

It was claimed that heat treatment can be used for inactivating antibiotic residues in milk. Residues in milk would not be inactivated by normal pasteurization procedures (71°C for 15 s) and so it can not be relied on to inactivate residues of even the more heat sensitive antibiotics such as penicillin and the tetracyclines. More severe heating as in processing for canning will inactivate some of the more heat sensitive compounds, but others such as neomycin and possibly streptomycin, will survive this temperature. Such temperatures are not recommended to use because they tend to caramelize the milk, alter its curd qualities, and render it unfit for the products in question (Doan, 1956; Moats, 1988). The breakdown products formed from antibiotics during the heating procedures have not been described. Even if antimicrobial activity is destroyed, the compounds formed might still produce an allergic response in sensitive persons (Moats, 1988).

Many chemicals can destroy antibiotic activity but their use in milk would constitute adulteration, or add a toxic substance. Some farmers in the USA are suspected of deliberately adding commercial β-lactamases to milk in order to make the penicillin control impossible by inactivating β-lactam drugs. Penicillinase

addition is an adulteration and contrary to law (Guay *et al.*, 1987; Kosikowski, 1961; Lampert, 1975).

The following are recommendations collected from literature for preventing antibiotic residues in milk (Schoech, 1977):

1. Know the kind of drug being used and what its effects are expected to be. Do not use questionable preparations.

2. Read the lable for dosage requirements. Do not exceed the prescribed dosage.

3. Read the label to determine the length of time milk must be withheld from sale. Do not cut this time depending on dilution effect.

4. Identify all cows treated either by herdman or veterinarian.

5. Treated cows should be milked last.

6. Even if only one quarter has been treated, discard all milk from that treated cow.

7.One person should be responsible for administering all drugs and identifying all treated animals.

8. Milk from lactating cows just purchased or under treatment should be checked before adding the milk to the supply.

9. Treated cows should be milked completely. The more completely a cow is milked, the faster any drug will clear out.

10. Intramuscular injections of antibiotics are absorbed into the blood stream and eventually contaminate the milk. They generally require a longer withholding period than udder infusion. withhold this milk from market for the prescribed period .

time, temperature, cost , and sensitivity, some of them developed and are now considered as standard methods.

1- Cylinder plate method

It is a microbiological based on the following principle(Bishop and white, 1984; Merck, 1987): The culture medium is inoculated with the relevant test strain and poured into plates. Defined quantities of milk sample under examination and an antibiotic standard are applied into cylinders (sterile glass cups cut from tubing, warmed and set lightly on the agar). Upon incubation (time and temperature are dependent on the test microorganism), inhibition zones develop around the site of application, there is no microbial growth within these zones and their diameter is a measure of the concentration of the antibiotic in the milk sample. The concentration of the antibiotic in the milk sample can be determined by comparing the diameter of its inhibition zone with that of the antibiotic standard.

several microorganisms have been suggested for use in the cylinder plate procedure such as: *Bacillus subtilis; Staphylococcus aureas* and *Sarcina lutea*.

Larocque and Neville, 1985, used the cylinder plate method to assay quantitatively the presence of penicillin, streptomycin, neomycin, and polymyxin B-sulfate in milk. They used *B. stearothermophilus* var. *calidolactis* (ATCC 12980) to assay penicillin with incubation at 64 °C for 4.5 h. *Microccoccus lutea* (formerly named *Sarcina lutea)* (ATCC 9341) was also used to detect penicillin with incubation at 30 °C for 18 h. On the other hand streptomycin was assayed by this method using *B. subtilis* (ATCC 6633) with incubation at 37°C for 18 h. whereas

neomycin was assayed using *Staphylococcus epidermis* (ATCC 12228) with incubation at 34 °C for 16-18 h. Polymyxin assayed using *Bordetella bronchiseptica* (ATCC 4617) with incubation at 37 °C for 16-18h. In all cases penicillin interference was checked by the use of penicillinase which inactivate penicilin, and consequently detects all the other non-penicillins(Larocque and Neville, 1985).

The *Sarcina lutea* cylinder plate method was modified and standardized for the detection of penicillin in non-fat dry milk by adding a 10 ml of medium no. 1 to each sterile petri-dish (base layer); after hardening the appropriate amount of culture (*S. lutea* ATCC 9341) is added to medium no. 4. The appropriate amount of culture is determined by trial plates to seed agar to obtain the best zones of inhibition. 4-ml of the inoculated agar is added to each plate receiving 10 ml of the base layer and left for hardening. For the examination of non-fat milk powder,1 gm milk powder is reconstituted with 9 ml , phosphate buffer. Place six stainless steel cylinders outside diameter of 8 mm, inside diameter 6 mm and 10 mm long on each assay plate; two samples can be tested per plate. Two cylinders are filled with 0.05 IU penicillin / ml as a reference standard, and two cylinders are filled with the test sample. The plates are incubated for 16-18 hr at 30 ± 1°C. After incubation, the plates are inverted to remove the cylinders and to measure the clear inhibition zones produced. Any test sample producing an average zone of inhibition of 9.1 mm or greater is a presumptive positive test and must be confirmed by using penicillinase, to emphasize the presence of penicillin or other inhibitory substances. The sensitivity of this

assay was normally 0.01 IU / ml (Bruhn *et al.*, 1985; Messer, 1984).

A survey of commercial milk samples obtained from New York State during the year of the 1976 were analyzed for antibiotic residues, which detected by the *Sarcina lutea* cylinder plate method, revealed a high incidence (72%) of non-specific inhibition. Penicillin was detected by penicillinase assay in 3.1% of the samples. However, substances inhibitory to *S. lutea* could develop normally in some milks during marketing (Ledford and Brown, 1977). The addition of egg-white lysozyme to the *S. lutea* seeded agar caused the production of larger zones of inhibition than the use of *S. lutea* seeded agar without the addition of egg-white lysozyme. The increased zone size enables detection of lesser quantities of the antibiotic. The addition of lysozyme to the agar enabled the detection of 0.0075 unit penicillin per ml. It was suggested that this modification enables the test to detect low levels of the antibiotic (Kornfeld, 1977). Although *S. lutea* cylinder plate procedure combines both reliability and fair sensitivity (Katz and Fassbender, 1978), It is considered slow and requires considerable skills to carry it out, for this reasons it has not met with general acceptance (Ginn *et al.*, 1982 a).

2- Disc assays

Some methods of microbiological assays used for the detection of antibiotic residues in milk have utilized the Bacillus genus of microorganisms because of their high sensitivity to the majority of antibiotics (Larocque and Neville, 1986). Disc assays

utilizing Bacillus genus can be divided according to the species used into:

a- *Bacillus subtilis* disc assay.

b- *Bacillus stearothermophilus* disc assay.

c- *Bacillus megaterium* disc assay.

a- Bacillus subtilis disc assay

This method is used for the detection of antibiotic residues in milk using *Bacillus subtilis* (ATCC 6633), in the form of spore suspension. The agar medium and the spore suspension are mixed at a specific ratio in order to get the required spore numbers per milliliter. The medium used is the antibiotic medium no. 1 according to Bruhn *et al.*, 1985. Other researchers used whey agar medium and Penassay agar medium (Bishop and White, 1984). The amount of the seeded agar used must be specified according to the petri-dish size to get a uniform agar thickness. Filter paper discs (diameter: 12.7 mm) are touched to the milk sample to absorb by capillary action a specific amount of the sample, and are immediately transferred to the petri-dish and incubated invertedly for a time-temperature combination according to the spore concentration. When the spores concentration in the petri-dish is 10^5 spores /ml, incubation conditions will be at 34 °C for14-24 hours, while for spores concetration 10^6 spores /ml, incubation conditions will be at 35° C for 5-7 hours, whereas for spores concentration 5×10^6 spores/ml, incubation conditions will be at 37°C for 3-4 hours (Bruhn *et al.*, 1985).

The important thing is to make a control disc (containing 0.05 IU penicillin /ml) is required along with the samples discs. If

the control yielded an inhibition zone, the incubation is arrested, otherwise the incubation must continue. Inhibition zones can be measured after incubation using a caliper to the nearest 0.1 mm (Bruhn *et al.*, 1985). According to the Association of Official Analytical Chemists (AOAC) official methods of analysis, *B. subtilis* disc assay method can detect penicillin qualitatively at a concentration of 0.05 IU /ml or higher (AOAC, 1980; AOAC, 1990).

The sensitivity of the *B. subtilis* disc assay method could be increased by preloading the disc papers with critical concentrations of penicillin, so that minute quantities of antibiotic residues in milk above the critical concentrations would produce zones of inhibition. Preloading disc papers with 0.01 IU penicillin /ml can increase the sensitivity of the test from 0.05 to 0.015 IU/ml (Balbi and Hartman, 1985).

b- *Bacillus stearothermoplilus* disc assay

In this method *Bacillus stearothermophilus* var. *calidolactis* (ATCC 10149) is used employing antibiotic medium no. 4. A specific amount of seeded agar is put in each petri-dish, so as to have a uniform agar thickness. Filter paper discs (diameter 12.7 mm) are used to absorb milk samples by capillary action, and then placed on the surface of the seeded agar. Plates are incubated for 2.5 h at 64 °C or for 4 h at 55 °C, until a well defined zone of inhibition (17-20 mm) around the control disc is obtained (Bishop and White, 1984; Bruhn *et al.*, 1985; Kaufmann, 1977; Messer ,1984). A zone of 15 mm is considered by others as a positive result. This assay could detect as little as 0.005 IU /ml of milk (Bishop *et al.*, 1985). According to Bruhn

et al., 1985, this method could be used for qualitative and quantitative detection of antibiotic residues in milk. When the *B. stearothermophilus* spores concentration is 10^6 spores /ml, the test can detect penicillin in concentrations as low as 0.008 IU/ml milk (Bruhn *et al.*, 1985).

The Difco disc assay method using *B. stearothermophilus* var. *calidolactis* was evaluated and compared to *B. subtilis* disc assay method and the cylinder plate method (Ginn *et al.*, 1978). In the Difco method a color indicator was incorporated to facilitate reading the results in addition to the zone of inhibition. It was found that the Difco disc assay method was more sensitive than the *B. subtilis* disc assay method (Ginn *et al.*, 1978). A collaborative study was performed on *B. stearothermophilus* paper disc method designed to detect residual levels of four antibiotics in marketed whole milk. Whole milk samples spiked at low levels with ampicillin, cephapirin, cloxacillin, and penicillin G were sent frozen to 11 collaborating laboratories with instructions to assay them promptly according to the method provided. Five of the laboratories reported inconclusive results due to technical difficulties encountered with the method. The six remaining laboratories all detected levels of 0.005-0.008 IU /ml for penicillin G, ampicillin, and cephapirin and 0.05-0.08 IU/ml for cloxacillin (Ouderkirk, 1979).

The inhibition zone can be measured using Vernier caliper, or by using an overhead projector which magnifies the diameter of the inhibition zone by five fold. This procedure considerably reduces the time required for reading the zone diameters as compared with the direct measurement of zones using calipers and with similar accuracy (Jarvis, 1966).

B. stearothermophilus qualitative disc method is considered one of the AOAC official methods of analysis, it is applicable to a level $\geq$ 0.008 IU penicillin G/ ml (Anonymous, 1982; AOAC, 1980; AOAC, 1990). It was found that the sensitivity of the test could be enhanced by preloading the disc papers with penicillin. Preloading the discs at a concentration of 0.002 IU penicillin/ml increased the sensitivity of *B. stearothermophilus* disc assay, from 0.005 to 0.003 IU /ml (Balbi and Hartman, 1985).

β-lactam residues in raw milk can be assayed quantitivily in addition to the qualitative assay using *B. stearothermophilus* disc assay method. Quantitative estimates above or below the reference level of antibiotic are computed through a paired-t statistical test. The AOAC standardized the quantitative *B. stearothermophilus* disc method . They used 90 µL milk samples or a reference standard (0.016 IU/ml) (Anonymous , 1982 ; AOAC , 1980 ; AOAC , 1990 ; Ginn *et al* ., 1982 b).

Absorption of the milk sample by capillary action of the disc paper (moistening method) is a potential source of variation , because the absorbed amount is related to the disc mass . To reduce the variability , 90µl of the milk sample is transferred into each paper disc using a micropipetor . It was found that the moistening method and the 90 µl method yielded the same zone diameter sizes and have similar degree of variability , but still the 90 µl method is less subjective , less tedious , and requires approximately half the time required by the moistening method (Ryan *et al* ., 1988) .

Oliver *et al* .(1990) used the *B . stearothermophilus* disc assay using penicillin-in-milk indicator (PMI or PM) agar , they considered an inhibition zone of more than 16 mm as a positive

result, and they used disc papers of 14 mm. They found that the sensitivity of the *B. stearothermophilus* disc assay for detection of the antibiotics Novobiocin, Cephapirin benzathine, Amoxicillin, Oxytetracycline were: <0.62; 0.01; 0.006; >0.2 μ g/ml, respectively, and for Penicillin G it was 0.005-0.008 IU/ml. Several studies were conducted to evaluate and compare two different agar media that were used in *B. stearothermophilus* disc assay, these were penicillin-in-milk (PM) agar and antibiotic medium number 4(A4). For this purpose a collaborative study was conducted on *B. stearothermophilus* paper disc (12.7 mm) method to detect residual inhibitors in milk (β-lactams). 18 participating collaborators assayed raw milk samples spiked with a β-lactam (Penicillin G). They demonstrated that either antibiotic medium no. 4 or PMI agar was suitable for use in the assay (Messer *et al* ., 1982). Others compared the two media at incubation temperatures of 55°C , and 64°C regarding their sensitivity to β-lactam residues . They found that A4 at 64°C shows the best detection (i.e. higher percent of positive values) than the other combinations (Peeler *et al* ., 1983) . Another study was conducted to find out the sensitivity for the detection of four β-lactam antibiotics in several milk products using the two media . The sensitivity was highest using PM agar (Rajkowski *et al* ., 1986 ; Rajkowski *et al* ., 1988) . Another study compared the two media at 64°C incubation temperature for five antibiotics , these were : ampicillin , cephapirin , erthromycin , neomycin and penicillin G , A4 was better for cephapirin , while ampicillin showed no differences regarding the two media (peeler *et al* ., 1989) . The AOAC considers the PM indicator agar satisfactory and adopted it (AOAC , 1990).

C - *Bacillus megaterium* disc assay

It is a disc assay procedure using the spore-forming *Bacillus megaterium* , which is sensitive to eight sulfa drugs , bacitracin and eight other antibiotics . It was used for the detection of sulfa drugs and other antibiotics in milk . The count of *B. megaterium* (ATCC 9855) is calculated to give a final concentration of about 5×10^4 spores / ml of agar , was mixed with the Mueller-Hinton agar , 4-ml of the inoculated culture are placed in a petri-dish . The test is performed by dipping 12.7 mm filter paper discs in the milk sample and placing them on the agar surface followed by incubation at 37 °C for 4 - 5 h . This incubation temperature affords maximum sensitivity to sulfa drugs . Clear zones around the discs are considered positive results , provided appropriate controls are used . A 12.7 mm disc impregnated with 50 µg of para - aminobenzoic acid (PABA) may be used to identify sulfa drug inhibitors , this overcomes the inhibitory properties of sulfa drugs up to concentrations of 5 mg per disc (Bruhn *et al* ., 1985).

3- *Delvotest-P*®

It is an agar diffusion test which was introduced in 1974. It consists of two parts:

a- The agar medium containing the spores of *Bacillus stearothermophilus* var. *calidolactis* (ATCC 10149) in a stabilized sporulated form, which is available either in an ampoule or in a plate. This microorganism has been chosen

Penicillinase is used to confirm the presence of β-lactam antibiotics. Positive results that remains after treatment with penicillinase is an indication for the presence of antibiotics other than penicillin such as tetracyclines (AOAC, 1990).

Delvotest-P® kits are available in two forms:

1- An ampoule test, for one or few samples.

2- A plate test, for a large number of samples, each plate contains 96 cups and can be divided into 6 blocks, 16 cups each . It is called Delvotest-P® Multi.

The ampoule test is suitable for testing a limited number of samples. Whereas Delvotest- P® Multi can be used for lagre numbers of samples. By using a special delivery pipette, one person can handle 400 samples per hour. The sensitivity and test duration of both the ampoule and multi test are similar (Van Os and Beukers, 1980).

A concentration of 0.005 IU penicillin /ml milk can be detected, while that of of 0.002 IU/ml gives negative result. Concentrations between 0.002-0.005 IU/ ml give doubtful results (Anonymous, 1982; AOAC, 1990). The Delvotest-P® has been used to screen both individual cow milk samples and farm bulk tank samples for possible antibiotic residues, so it could be considered as an on-farm antibiotic screening test (Jones and Seymour, 1988). Delvotest-P® can also detect a broad spectrum of antibiotics as shown in Table 2.

Raw milk samples from 73 individual milk producers were examined for the presence of antibiotics using the Delvotest-P®. It was found that 5.5% of the samples contained more than 0.005 IU penicillin/ml milk . 2.8% samples contained antibiotics other than penicillin as these samples remained positive after treatment

with penicillinase. Also in the same study it was found that a withholding period of 7-8 days was necessary to obtain milk free of detectable residues (Chagonda and Ndikuwera, 1989). During a collaborative study at a large number of milk-receiving stations in the Netherlands, in which penicillin was added in concentrations of 0, 4, 5, 6, 7, 8, and 10 IU/L to milk samples, the Delvotest-P® method gave reliable results in almost all instances with no difficulties encountered. (Pater, 1977).

Delvotest-P® method was evaluated for its reproducibility and sensitivity, and it was concluded that this test is particularly simple, rapid and sensitive but it has a problem in interpretation of the doubtful (+/-) result. Some laboratories experienced uncertainty over the interpretation of this result (Huhtanen et al., 1977). A collaborative study was performed on a rapid B.stearothermophilus agar diffusion ampoule method to detect low levels of penicillin G in seven types of fluid milk products. A multitest technique for testing a large number of samples simultaneously was also studied. The level of detection for the different fluid milk products ranged from 0.005-0.007 IU/ml for the ampoule test, and from 0.004-0.007 IU/ml in the multitest. Both techniques were adopted as an official method by the AOAC (Kelley, 1982).

It was postulated, however, that changes in the pH of milk samples due to transport and storage may influence Delvotest-P® results (Seymour et al., 1988a). Also it was claimed that natural inhibitors in milk and colostrum may produce false positive Delvotest-P® reactions (Seymour et al.,1988a). The Delvotest-P® Multi plate test was evaluated by screening 100 milk samples for total antibiotic residues (penicillin G, streptomycin, and

Table 2. Sensitivity of Delvotest-P® to different antibiotics

Antibiotic	Sensitivity Range		Lowest Detection Concentration[c]
	1[a]	2[b]	
Penicillin (IU/ml)	0.002-0.004	0.002-0.004	0.004
Cloxacillin (μg/ml)	0.015-0.025	0.020-0.025	0.025
Ampicillin (μg/ml)	0.003-0.005	0.004-0.005	0.003
Streptomycin (μg/ml)		4.000-6.000	8.000
Neomycin (μg/ml)	0.500-1.000	1.000-2.000	6.000
Tetracycline (μg/ml)	0.150-0.250	0.200-0.400	0.200
Oxytetracycline (μg/ml)	0.150-0.250	0.200-0.400	0.300
Erthromycin (μg/ml)	0.300-0.400		1.750
Cephapirin (μg/ml)			0.008
Norobiocin (μg/ml)			0.800
Gentamycin (μg/ml)	0.100-0.500		0.500
Spectinomycin(μg/ml)			12.500
Oleandomycin (μg/ml)	2.000-5.000		
Nafcillin (μg/ml)	0.005-0.010	0.008-0.010	
Chlorotetracycline (μg/ml)	0.400-0.700	0.5-1.0	
Chloramphenicol (μg/ml)	5.000-8.000	5.0-8.0	
Kanamycin (μg/ml)	5.000-15.000	10.0-15.0	
Sulpha compounds (μg/ml)		50.0-100.0	
Dihydrostreptomycin (μg/ml)	2.000-3.000		
Rifamycin (μg/ml)	0.050-0.200		

(a) Van Os and Beukers, 1980.

(b) Gist-Brocades-Holland, 1993.

(c) Jones and Seymour, 1988.

neomycin). The samples were taken in conjunction with an antibiotic depletion study in milk derived from six cows treated with a multiple antibiotic intramammary infusion product. Within the limits of sensitivity of the Delvotest-P®, only penicillin G persisted in milk samples taken beyond 60h, whereas in some samples, the other antibiotics appeared to be depleted as early as 48h. More sensitive tests, however, could detect neomycin in 50% of samples taken at 60h and streptomycin at 14.5 days after discontinuation of infusion. Therefore it was concluded that although the Delvotest-P® is rapid, convenient and apparently a reliable screening method for detecting residues of penicillin G in milk, it can not be relied upon to give a true qualitative result for total antibiotic residues in milk because it gives misleading results for antibiotics other than penicillin (Larocque and Neville, 1986).

4- Charm Test

a- Charm test I- This assay is based on the specific, irreversable affinity of beta- lactam antibiotics for certain enzyme sites on the cell walls of microorganisms. ^{14}C- labled penicillin and *Bacillus stearothermophilus* are added to milk samples. Antibiotics in the sample compete with ^{14}C penicillin for binding sites. The amount of bound ^{14}C is counted and compared with the control to determine the presence or absence of β-lactam antibiotics (Anonymous, 1982; Bruhn *et al.*, 1985). The assay is conducted by taking a 5-ml milk sample in a test tube. Specific amounts of ^{14}C reagent and *Bacillus stearothermophilus* suspension are mixed well with the sample and incubated at 90°C for 3 min.

After centrifugation at 1200 xg., milk is decanted and the fat ring is carefully swabed out using cotton swabs, then the tube is carefully rinsed twice with distilled water so as not to disturb the preciptate at the bottom. The precipitate is resuspended with distilled water and placed on an aluminum planchet on the surface of a hot plate maintaned at 400 °C to let the material in the planchet dry. Finally the result is read-out by placing the dry planchet into the penicillin analyser and radiation is measured from the ^{14}C chanel for 8 min. The count is compared with a predetermined control point to findout if the sample is positive or negative (Anonymous, 1982; Bruhn et al., 1985). The average of 10-zero standards is taken to determine the control point . Control point = 0.80 x av. count. Test samples fall below control point, contain β-lactam antibiotic(s).

In a collaborative study, 11 laboratories correctly distinguished 10-coded zero penicillin G samples and 10-coded 0.01 IU/ ml samples. The proposed test is qualitative (i.e. positive or negative) and can detect the presence of β-lactam antibiotics at 0.01 IU/ml milk (Charm and Chi, 1982).

b- Charm test II : is a later version of the microbial receptor assay (Charm test I), which makes detection and identification of 7 different antibiotics possible including tetracycline, streptomycin, erythromycin, sulfa drugs, and chloramphenicol using ^{3}H-labeled reagents binding to specific receptor sites on added microbial cells (Bruhn et al., 1985; Carlsson and Bjorck, 1989b). Screening the antibiotic family requires 10-12 min, while a quantitative assay requires 15 min or more (Charm et al., 1985; Suhren and Heeschen, 1987).

Modification of charm test I is applied to the sample preperation regarding its incubation conditions, because the counts of some antibiotic determinations were sensitively influenced by the incubation conditions. The following incubation conditions proved to be suitable: 9 min incubation period for all antibiotics tested and incubation temperatures of 50 °C for penicillin, tetracycline and erythromycin and 30 °C for streptomycin, novobiocin, chloramphenicol and sulfonamides. Also after centrifugation, scintillation fluid is added, mixed, and measured in a scintillation counter on ^3H channel for tetracycline, streptomycin, sulfonamide; ^{14}C channel for β-lactams, erythromycin and chloramphenicol. The control point for the modified method is calculated either as a 3 standard deviations from the average of 6-zero samples or for convenience, substract a percentage specified for each antibiotic from the average counts of 6 zero samples (Anonymous, 1988; AOAC, 1990; Brady and Katz, 1988; Bruhn et al., 1985; charm and chi, 1988). The sensitivity of the method corresponds, in the case of β-lactam antibiotics,with those of microbiological test systems with Bacillus stearothermophilus, while in the case of other antibiotics (tetracycline, erythromycin, streptomycin, novobiocin, chloramphenicol and sulfonamides) higher sensitivities are obtained. The sensitivity of charm test II to different antibiotics is tabulated in Table 3.

Sixty four milk samples were examined in a survey in the USA over a 3-month period representing different milk brands and bottling plants using charm test II. Sixty three percent of milk samples contained one or more residues. Tetracycline and sulfonamides were the most predominant ones detected. A subsample of milk was used

to confirm the qualitative presence of residues using microbial assays. All 9 presumptive tetracycline positive samples were confirmed, whereas 3 out of 4 presumptive streptomycin - positive were confirmed (Brady and Katz, 1988). In another study, 214 consumer milk samples from across North America were examined for antibiotic residues by means of the *B. stearothermophilus* disc assay and the charm test II procedure. Sulphamethazine and tetracycline detected by charm test were found the most predominant residues. The *B.stearothermophilus* disc assay procedure was unable, in most cases, to detect these residues possibly due to the lower sensitivity of this test. Further comparative tests between charm test II and other methods of similar sensitivity

Table 3. Detection limits of Charm test II

Antibiotic	Detection limits from different studies			
	a	b	c	d&e
Penicillin (μg/ml)	0.003-0.007	0.002	0.008	0.003
Tetracycline (μg/ml)	0.600	0.400	0.5	0.600
Erythromycin(μg/ml)	0.02-0.04	0.02	0.065	0.02
Streptomycin(μg/ml)	0.02-0.05	0.01	0.16	0.010
Novobiocin(μg/ml)	0.005-0.010	0.01	-----	0.010
Sulfonamide (μg/ml)	0.05	-----	0.0125	-----
Sulfamethazin(μg/ml)	-----	0.005	-----	0.005
Chloramphenicol(μg/ml	0.2-0.32	0.08	0.05	0.08
Gentamycin(μg/ml)	-----	0.02	-----	-----

a- Bruhn *et al.*, 1985; *b*- Collins-Thompson *et al.*, 1988; *c*- Brady and Katz, 1988; *d*- Anonymous, 1988; *e*- AOAC, 1990.

are recomended to confirm these findings (Collins-Thompson *et al.*, 1988).

The sensitivity and effeciency of charm test II were tested both under laboratory conditions on milk from 5 different dairy farms and on tanker milk. The results showed low detection limits for the antibiotics tested. It was also indicated that the charm test II gives a high precision and sensitivity in the detection of the major types of antibiotics used in mastitis therapy. The charm test II should preferably be used as a confirmatory test and as a complement to microbial screening methods (Carlsson and Bjorck, 1989b; Carlsson and Bjorck, 1991). The charm test is adopted by the AOAC as a method for the detection of antibiotics and / or antimicrobials (AOAC, 1990; Charm *et al.*, 1988).

5-*Other methods*
a- Dye Detection method

A simple procedure for screening large numbers of milks to detect traces of penicillin is outlined . Detection of 0.002 IU pencillin/ ml within 4-6 hours was demonstrated . Peptone-sugar nutrients , spores of *Bacillus subtilis* and triphenyl tetrazolium chloride dye are freeze-dried on paper discs . The discs are placed individually in small cups of a disposable plastic tray resting in warm water at 40 °C . No agar is used . A small volume of test milk added to each cup containing a disc remains openly exposed until almost total evaporation of milk occurs , concentrating any penicillin present . Color changes in the disc , depending upon spore growth , indicate the presence or absence of inhibitory substances (Palmer and Kosikowski, 1967).

b- Dye Marker method

An indirect control of antibiotic residues in milk can be acheived by incorporation of a marker dye with the antibiotic preperation such that the excretion rate of the dye correlates , as far as possible , with that of the preperation administered (Van Os, 1978) . The infusion of dye marked mastitis preperations result in production of colored milk , which in turn acts as a strong deterrent to the producer to include contaminated milk in his shipment (Macintosh and Vilim, 1977). Added dyes , usually were Brilliant Blue F.C.F (" Food Blue 3 ") and Green S (" Food Green 4"). The most suitable was Brilliant Blue F.C.F . It showed a reasonably parallel rate of excreation for the dye and penicillin . Dye detection methods were all based either on visual techniques or ion exchange resin method at a low dye concentration (Macintosh and Vilim, 1977 ; Vilim *et al.*, 1979). The potential advantage of dye marking is that it immediately alerts the dairy farmer to antibiotic contamination by visually discoloring milk . Dye marking technique has been introduced for mandatory use in several countries , such as in the States of Victoria (Australia), South Africa , and Japan (Novak *et al.*, 1984). Dye marking technique on the other hand was considered unnecessary and undesirable because it showed many disadvantages such as (Van Os, 1978):

1. Marked deviation of some dyes between the rate of excretion of the dye and the antibiotic .

2. other routes of antibiotic administration such as subcutaneous and intramuscular can not be detected by dye-marker technique

3. Addition of the dye itself leads to a residue problem .

4. The use of the dye marker increases the cost of the antibiotic preperations .

5. Dye-markers contaminate the clothes and hands of the farmers, who are unpleased to work with them.

c- The Spot test

Angenics , Incorporation has developed a 6-min test for antibiotic residues in milk (the spot test) . The test is immunologically - based using current monoclonal antibody technology (Bishop and White, 1984) . The spot test is a receiving station screening test for detecting 0.01-0.02 IU/ml penicillin , cephapirin , and cloxacillin residues in raw milk . However , it is not recommended as an on-farm screening test because the procedure is complex. Furthermore the interpretation of the results may be difficult for the untrained or for those who use the procedure infrequently (Jones and Seymour , 1988) . For samples with 15.6-16.8 mm inhibition zone diameters on disc assay , the agreement with comingled samples was 77- 87 % . For test results greater than 17.0 mm , the agreement was 100 % . The spot test could be considered as a rapid and reliable method for detecting penicillin , cephapirin , and cloxacillin residues in raw milk at cocentrations that will produce a 16 mm zones (Ryan et al. 1986).

d- The Penzyme test

It is an enzymatic colorimetric screening method for the rapid detection of β-lactam antibiotics in milk (Bishop and White, 1984) . It involves the enzyme DD carboxy -peptidase, which is inactivated by β-lactam antibiotics and produces a yellow color .

An orange (pink) color is produced when β-lactams are not present . The test requires 20 mins to be completed but it was not recognized by the AOAC as an official test . The manufacturers suggested that the test would detect residues of 0.004-0.012 IU/ml (Jones and Seymour, 1988). The enzyme carboxy-peptidase , rapidly forms a stable , inactive complex with these antibiotics . Five - minutes incubation of a specific amount of enzyme with a milk sample (0.2 ml) containing sufficient antibiotic resulted in the inactivation of the enzyme . If inactivation occured , no further reaction took place when the substrate was added and the vial incubated for a further 15 min, the contents of the vial remained yellow , indicating the presence of β-lactam antibiotics. If no antibiotics were present , the enzyme remained active and reacted with the substrate , resulting in a pink or orange color (Seymour *et al.*, 1988 a). The major disadvantages of this test were the difficulty in color detection, interpretation the reuslts and the narrow spectrum of antibiotics to which it is sensitive , the β- lactams . The primary advantage was its 20-min speed (Jones and Seymour, 1988).

e-The Triphenyltetrazolium method

Used for the detection of inhibitory substances in milk which is based on the conversion of 2,3,5-tripheyltetrazolium chloride (TTC) to formazan by the actively growing becterial cells. This conversion is accompanied by a color change from the yelowish leucoform to red. *A Streptococcus thermophilus* culutre was used and incubated at 37 °C for 2.5h. The test detected the presence of 0.04 IU penicillin /ml milk, but sanitizing agents were found to

interfere with the test (Bishop and White, 1984; Tamime and Deeth, 1980).

f-Valio T101 test

It is a new microbiological assay test for detection of antibiotic residues in milk . The T101 test is based on the use of *Streptococcus thermophilus* T101-strain, which is used as a starter in Swiss cheese and yogurt . Ten small vials containing the T101 bacteria strain and a color indicator in a freeze dried form are presented along with 10 special disposable pipettes and a small incubator in each kit set. 2 ml of the milk sample is pipetted, turned upside down and set into a water bath for heating at 90-100 °C for 5 min. The sample is then transfered into the vial after cooling to room temperature and shaken . The sample is incubated for 4.5 h at 42 °C and the color is compared after shaking the vial to a color chart. The yellow color indicates the absence of antibiotics, whereas the blue color indicates the presence of anitiotic residues. A green / yellow-green color is considered a doubtful result. The sensitivities of the test to Tetracyclin, Streptomycin, Erythromycin, Neomycin, Spiramycin, Sulfadimidine were 0.2-0.3; 1.0-1.5; 0.05-0.1; 0.3-0.5; 0.15-0.30 and 0.5-1.5 µg/ml respectively, and for Penicillin it was 0.004-0.006 IU/ml. (Valio Ltd-Finland, 1994).

C. Yogurt starter culture

1- Bacteriology of yogurt starter culture

The yogurt starter organisms are thermophilic lactic acid bacteria capable of growing at 40-45°C, they include *Lactobacillus delbruekii* subsp. *bulgaricus* (formerly

Lactobacillus bulgaricus) and *Streptococcus thermophilus* (Balows *et al.*, 1992; Sneath *et al.*, 1986). There is an association between the growth of the two microorganisms (Tamime and Robinson, 1985).

The growth of the starter bacteria depends upon adequate supplies of suitable sources of nitrogen and carbon. If the starter organisms posses a lactose-hydrolyzing enzyme, the carbon source is not limiting. However, this is not so with respect to the nitrogen source because free amino acids and peptides are present only to a limited degree in milk. Starter bacteria have limited biosynthetic capabilities and hence they require most of the amino acids for growth. Lactic acid bacteria used as starter must have two essential features. First: possession an efficient proteolytic system. Second: having the ability to ferment lactose rapidly to lactic acid. Since the quantities of free amino acids and peptides present in milk are below the minimum level needed for rapid growth of starter bacteria, a complement of proteinases and peptidases is essential for the degradation of protein. The proteolytic ability of lactic acid bacteria is dependent upon species and strains. The proteolytic enzymes of *L. bulgaricus* degrade casein with the liberation of low molecular weight peptides and amino acids. The amino acids arising from this proteolytic activity have been identified as specific growth stimulants for *S. thermophilus* (Rajagopal and Sandine, 1990). A study was conducted to assess the proteolytic activity of different strains of *L. bulgaricus* and *S. thermophilus* and combinations of them. It was found that *lactobacilli* were proteolytic and *S.thermophilus* were less proteolytic. Mixed cultures, with the exception of one combination, liberated more

acid and tyrosine than the sum of the individual cultures (tyrosine was a measure of proteolytic activity) (Rajagopal and Sandine, 1990). Because of the associative growth of *S.thermophilus* and *L. bulgaricus* , the rate of lactic acid production, growth and number of both microorganisms is greater than that which occurs in single strain cultures (Mitchell and Sandine,1984;Pette and Lolkema,1950a;Tamime and Robinson,1985). Also an increase in the production of acetaldehyde ,the chief volatile flavor component of yogurt produced by *L. bulgaricus* was observed when this bacterium is grown in association with *S. thermophilus* (Mitchel and Sandine,1984;Pette and Lolkema,1950d). It is also noticeable that the rate of acid development of *S. thermophilus* and *L. bulgaricus* increases with increase in incubation temperature up to the maxima of 40 °C and 45 °C respectively. *S.thermophilus* is initially more active than *L. bulgaricus* in relation to acid production . Although the activity of mixed strains is optimum at 45 °C , it is recommended that in order to maintain and/or achieve a ratio of 1:1 between *S. thermophilus* and *L. bulgaricus* cells or chains, organisms should be propagated at 42 °C (Hamann and Marth ,1984; Pette and Lolkema,1950 c;Radke-Mitchell *and Sandine,* and Robinson,1985). A proper balance between *S. thermophilus* and *L.bulgaricus* must usually maintained for proper fermentation , especially in the manufacture of high quality yogurt (Kulshrestha and Marth, 1974; Mitchell and Sandine, 1984).

This symbiosis is supported by the production of stimulatory factors from both microorganisms which take place during the incubation period. Many researchers postulated several theories

about the nature and types of stimulatory factors produced by both microorganisms. It can be concluded from these theories that *L.bulgaricus* provide the essential nutrients , i.e amino acids which are insufficiently present in milk (Valine being the most stimulatory) for *S. thermophilus* (Pette and Lolkema, 1950b),while *S. thermophilus* produce formic acid - like compounds which promote the growth of *L. bulgaricus* (Tamime and Robinson, 1985). However, *S. thermophilus* strains were found to produce carbon dioxide during incubation that stimulate the acid production by *L.bulgaricus* (Mitchell and Sandine,1984).

Although a symbiotic relationship between *S. thermophilus* and *L. bulgaricus* is assumed, not all strains are compatible and an imbalance in growth may occur. Some rod-coccus culture combinations were found to be inhibitory, stimulatory or neutral regarding the rate of lactic acid production as compared to single strain cultures. This may result in a stale, empty (no aroma) yogurt in the case of predominance of *S. thermophilus* or an over acidification in the case of predominance of *L. bulgaricus* (Mitchell and Sandine, 1984).

S. thermophilus grows quickly at first, making use of the essential amino acids produced by *L. bulgaricus*, it produces lactic acid which lowers the pH to a more optimal level for growth of *L.bulgaricus* along with lesser amounts of formic acid which stimulate the growth of *L. bulgaricus*. The growth of *S. thermophilus* then slows down while *L.bulgaricus* lowers the pH even further by producing lactic acid (Hamann and Marth, 1984).

2- Sensitivity to antibiotics

Antibiotics in milk can either inhibit the growth (microbiocidal) or reduce the activity (microbiostatic) of yogurt starter cultures. The minimum antibiotic concentration which is required to prevent total growth, is refered to as "the bacteriostatic antibiotic concentration" while antibiotic concentrations that are lower than the bacteriostatic concentration are refered to as "the subbacteriostatic concentrations". Microbial growth may be initiated at subbacteriostatic antibiotic concentrations, however, under such a condition, the growth rate might be lowered (Yondem et al., 1989). Fourty two different strains of lactic streptococci, lactobacilli, leuconostoc, staphylococci, and other dairy bacteria were tested for their sensitivity to 30 antibiotics and antimicrobial agents. Almost all the microorganisms investigated, except for S. thermophilus, exhibited definite resistance to eight different sulfonamides. Strains of S. thermophilus, on the other hand, were inhibited by several sulfa drugs. Most of the strains of starter streptococci and L. bulgaricus were sensitive to all 16 antibiotics and antimicrobial agents used for mastitis control (Reinbold and Reddy, 1974).

Antibiotics inhibit microorganisms by interference with the cell membrane structure and permeability,as well as with the cellular metabolism of proteins, carbohydrates and lipids, the energy yielding transformations in the cell, the inhibition of various enzymes and phosphorylation systems, and by blocking the synthesis of DNA and RNA during cell division (Tamime and Robinson, 1985). Under balanced growth conditions, cell walls are synthesized at the same rate as microbial growth rate. When

penicillin G is introduced into a culture, its effect will depend on the growth phase and growth rate of the bacteria. The number of cell wall-synthesizing microorganisms is higher in an actively growing culture, and hence they are expected to be more susceptible to the pencillin G effect than those growing at a lower rate (Yondem *et al.*, 1989).

Antibiotics appear to affect seriously the fermentation rate of mastitic milk of post antibiotic treatment milkings.(Keys *et al.*, 1976). The sensitivity of *S. thermophilus* and *L. bulgaricus* to different antibiotics are presented in Table 4.

Table 4. Sensitivity of yogurt starter culture to different antibiotics[a]

Antibiotic (per ml)	S.thermophilus	L.bulgaricus
Penicillin	0.004-0.010 IU	0.02-0.100 IU
Streptomycin	1.00-21.00µg	0.10->0.80µg
Tetracycline	0.130-0.500 µg	0.34-2.000 µg
Chlorotetracycline	0.060-1.00 µg	0.060-1.000 µg
Oxytetracycline	0.310-1.000 µg	0.310-1.000 µg
Cloxacillin	0.340-0.700µg	0.260- > 1.000µ
Bacitracin	0.040-0.120 IU	0.040-0.100 IU
Erythromycin	0.300-1.300 mg	0.070-1.300 mg
Chloramphenicol	0.800-13.000mg	0.800-13.00 mg

[a] (Tamime and Deeth, 1985)

3- Use of yogurt starter culture as a tool for detection of antibiotic residues in milk

The suitability of Laban (yogurt) fermentation (Jurdi and Asmar, 1981) as a test system for the detection of antibiotics in milk was assessed. Milk was heated to 93 °C for 3 min, cooled to 40 °C and inoculated with 2-2.5 % fresh starter or its equivalent amount of lyophilized starter; it was then incubated at 40 °C for 3 h. Yogurt fermentation was used to test the sensitivity of the bacterial system to different antibiotics. Fermentation inhibition was assessed by monitoring coagulum firmness and by measuring pH values.

Laban fermentation was significantly affected by levels of 0.005 IU penicillin/ml, 1µg streptomycin/ml, 1µg dihydrostreptomycin/ml, 10µg chloramphenicol/ml and between 0.05 and 0.5 µg oxytetracycline/ml. Although the method was not standardized, it was used to check 858 milk samples in Lebanon,11.20% of which were found to contain lactic acid bacterial inhibitory substances (Jurdi and Asmar, 1981).

III - MATERIALS AND METHODS

A- Establishment of the optimum starter culture concentration and pH for the Yogurt Culture Test (YCT)

In order to shorten the time of curd formation by yogurt starter culture, different combinations of milk pH and yogurt starter culture concentrations were tried. Furthermore, different pH-indicators were used to make the test results easier to read.

Sampling

Fresh milk was procured from a single cow from a private farm, which was transported within one hour to the lab using a private car. The cow was not treated with any medicaments including antibiotics nor the durg was added to the feed during the time of the experiment.

Starter culture

Each starter culture of three dairy plants were prepared by transfering 1g from a well mixed freshly prepared yogurt to 99 g of reconstituted nonfat dry milk (10% solids w/v) that was previously pasteurized at 95°C for 5min, then it was mixed well and incubated at 42°C for 4h. The culture was kept at 5°C until used on the following day.

Procedure

Appropriate amounts of milk were dispensed into four well cleaned glass jars. The pH of the milk in three jars was lowered to 6.4, 6.2 or 6.0 using 1N HCl, while the pH of the fourth jar was not adjusted to serve as a control. The milk in each jar was

divided into four equal portions. Each portion was warmed to 45 °C in a microwave (Toshiba ER 692). Yogurt starter culture was added to each pH treatment to give 2%, 3%, 4% and 5% (w/w) inoculum rate and mixed well. Ten ml aliquots were transferred to 8 test tubes (10 x 1.5 cm) and were warmed in a water bath at 42°C.

The pH and acidity % were immediately determined after mixing the starter culture with the milk and after 1.5, 2.0 and 2.5h of incubation in the water bath. Curd formation was also observed.

pH

pH- measurment was done using a Hana instruments pH meter (Model HI 8416) to determine the pH value by immersing the electrode in the sample.

Titratable acidity (T.A%)

Titratable acidity was determined by titration against 0.1N NaOH according to the Standard Methods for the Examination of Dairy Products (Case *et al.*, 1985). Five grams of the sample were transferred into 100 ml conical flask and diluted with 10 ml CO_2-free distilled water and mixed well. 0.5-ml of phenolphthalein indicator (1%) was added and the mixture was titrated with 0.1 N NaOH to the first permanent (30 sec) color change to pink. Titratable acidity expressed as % lactic acid (LA) was calculated according to the following equation:

$$\%acidity = \frac{ml\ of\ NaOH\ X\ N\ NaOH}{weight\ of\ sample} \times LA\ eq.\ wt \times 100$$
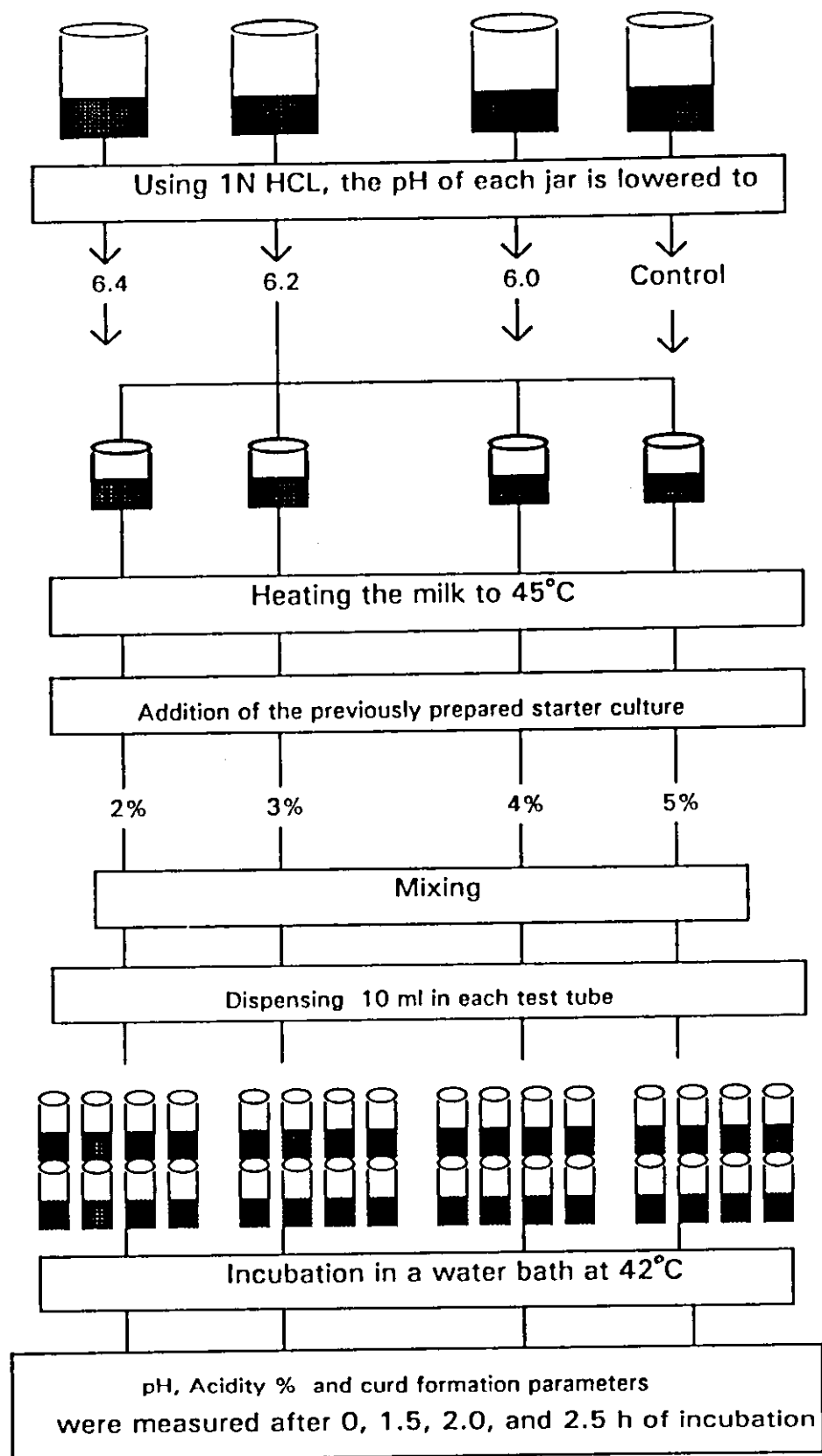
Figure 1. Flow chart showing the procedure of the YCT

Curd formation

Curd formation was assessed by tilting the test tube at about 45°and observing curdling of the milk in its first stages.

pH - indicator

After establishing the optimum pH value and starter culture concentration which give the shortest time for curd formation, 0.1 ml of Bromocresol Green (0.2% in 50% ethanol), Methyl Red (0.2% in 50% ethanol) , Chlorophenol Red (0.2% in 50% ethanol) or Bromocresol Purple (0.2% in 50% ethanol) was added to the milk directly at the end of the incubation. The shifts in the color of each indicator and the degree of distinction of the color were observed. The incorporation of the pH indicator were tried again and added before incubation. The difference between the addition of the pH-indicator before or after incubation was monitored.

B-Sensitivity of the developed test to different antibiotics used in dairy cattle managment

The sensitivity of Yogurt Culture Test (YCT) to different antibiotics was assessed. Eight different antibiotics were tested, these were Penicillin (Biochemiegmbh, Austria), Chloramphenicol (Sigma-chemical company LTD, England), Oxytetracycline (Biochemie, Austria) , Tetracycline (Helmaagg, Germany), Ampicillin (Biochemiegmbh, Austria), Erythromycin (Sanofi, France), and Cloxacillin (Medilife, Italy) Each antibiotic was diluted with milk to give the desired antibiotic concentration as shown in Table 5.

Table5. Antibiotic concentrations in testing the sensitivity of yogurt starter culture

		Antibiotics						
		Penicillin	Chloramph-inicol	Oxytetracy-line	Tetracycline	Ampicillin	Erythrom-ycine	Cloxacillin
First Dilution	Antibiotic (g)	0.06	0.01	0.05	0.10	0.02	0.1	0.04
	Milk (g)	100	1000	1000	1000	1000	1000	1000
	Antibiotic concentration (µg/g)	60	10	50	100	20	100	40
Stock Solution B	A(g)	1	1000	10	8	10	5	10
	Milk(g)	999	0	990	992	990	495	990
	Antibiotic concentration (µg/g)	0.06	10	0.5	0.8	0.2	1	0.4
Dilutions used for testing 1	B(g)	100	100	100	100	100	60	100
	Milk (g)	0	0	0	0	0	40	0
	Antibiotic concentration (µg/g)	0.06 (0.1 IU/g)	10	0.5	0.8	0.2	0.6	0.4
2	B(g)	50	50	50	75	75	50	87.5
	Milk (g)	50	50	50	25	25	50	12.5
	Antibiotic concentration (µg/g)	0.03 (0.05 IU/g)	5	0.25	0.6	0.15	0.5	0.35
3	B(g)	10	40	40	50	50	40	75
	Milk (g)	90	60	60	50	50	60	25
	Antibiotic concentration (µg/g)	0.006 (0.01 IU/g)	4	0.20	0.4	0.1	0.4	0.30
4	B(g)	5	30	30	37.5	37.5	30	62.5
	Milk (g)	95	70	70	62.5	62.5	70	37.5
	Antibiotic concentration (µg/g)	0.003 (0.005 IU/g)	3	0.15	0.30	0.075	0.3	0.25
5	B(g)	1	20	20	25	25	20	50
	Milk (g)	99	80	80	75	75	80	50
	Antibiotic concentration (µg/g)	0.0006 (0.001 IU/g)	2	0.10	0.2	0.05	0.2	0.2
6	B(g)	0	10	10	12.5	12.5	0	0
	Milk (g)	100	90	90	87.5	87.5	100	100
	Antibiotic concentration (µg/g)	0	1	0.05	0.1	0	0	0
7	B(g)	—	0	0	0	—	—	—
	Milk (g)	—	100	100	100	—	—	—
	Antibiotic concentration (µg/g)	—	0	0	0	—	—	—

1N HCl was added to the different antibiotic-spiked milk dilutions to lower the pH to 6, and the milk was then warmed to 45°C in a microwave oven. 4% of the previously prepared starter culture and 1% Chlorophenol Red indicator were added. After mixing well, 10-ml amounts of milk were dispensed into test tubes, and incubated in a water bath at 42°C. pH and acidity % were determined at 0, 1.5, 2.0, 2.5 and 4h of incubation. color change and curd formation were also noticed.

C- Survey of fresh milk for antibiotic residues

Six hundred and eighteen milk samples from the three largest dairy plants in Jordan were tested over a period of three months using the developed test (YCT).

Sampling

100-ml milk were taken in a screw capped glass bottle from a composite sample taken by the plant for the routine examination of the milk of each supplier and transferred to the laboratory within 1h. The temperature of each sample was kept between 2-7 °C. pH of each sample was measured and recorded.

Test procedure

Two hundred grams plastic cups like those used for marketing yogurt in Jordan were marked from the outside to about the volume of 96g milk. One milk sample was taken into one plastic container to the mark. HCl (1N) was added to each milk sample to lower the pH to 6, and then warmed in a microwave oven to 45°C. Four grams of the starter culture and 1.0 ml of chlorophenol Red (0.2% in 50% ethanol) were added to the milk.

A stainless steel spoon, which when filled gives 4g of the culture, was used for transfer. After mixing, the containers were placed in an incubator at 42°C. Starter cultures were that routinely used in the dairy plant from which the milk samples were taken.

To check the suitability of the starter cultures used, a control was included in each run in the form of antibiotic free skim milk solution (10%) that was treated in the same way as the milk samples.

Color change and curd formation were observed after 2.5 and 4h. Samples without curd formation or with any change in color after 2.5 and 4h were suspected to be contaminated with antibiotics (a positive test). These samples were tested by Delvotest-P® for comparison and/or confirmation.

Delvotest - P®

The Delvotest-P® kit was obtained from Gist-Brocades nv Delft, Holland. The procedure recommended by the manufacturer was followed. The ampoules were opened and 0.1 ml of the milk sample suspected to contain antibiotics, was transfered after the addition of the nutrient tablet present in the kit. Ampoules were incubated at 64°C in a water bath for 2 .5 h and examined for color change. A yellow color over the entire solid medium indicated a sample free of detectable residues. A purple color over the entire solid medium indicated a penicillin concentration of at least 0.005 IU/ ml of milk; and a partly purple color of the solid medium indicated a penicillin concentration of between 0.002-0.005 IU/ ml of milk.

IV - RESULTS

A-Establishment of the optimum inoculum level of starter Culture and pII for the YCT

The combinations of the starter culture concentration and the pH which resulted in curd formation after 2.5h of incubation with the starter cultures of the three dairy plants were 4% and pH 6.0, 5% and pH 6.0 and 5% and pH 6.2. Table 6. Curd formation began at pH 4.8 and at acidity of 0.5% (lactic acid). The combination 4% and pH 6.0 was used in the developed test(YCT).

pH - Indicator

Table 7 shows the results of incorporation of the four pH indicators in the modified curd system. Chlorophenol red was the best for distinguishing positive results indicating curd formation because the shift in its color from light violet to biege yellow was easier to note than the color shifts of the other indicators. Thus it was used in the developed test.

Addition of chlorophenol red (0.2%) before or after incubation did not result in any differences in the colors and had no effect on curd formation.

B - Sensitivity of the YCT to Antibiotics

Table 8 shows the minimum concentrations of the different antibiotics tested which gave positive results in the developed test, i.e- no curd formation and no shift in the color of chlorophenol red after 2.5h and 4h. YCT was shown to be most

Table 6. pH and acidity of milk inoculated with different inoculum levels of yogurt starter cultures from three dairy plants at different milk pH values

Culture conc.	Dairy plant		pH 6			pH 6.2			pH 6.4			Control (pH 6.6-6.7)		
			time (h)			time (h)			time (h)			time (h)		
			1.5	2.0	2.5	1.5	2.0	2.5	1.5	2.0	2.5	1.5	2.0	2.5
2%	A	a	5.64	5.30	4.97	5.70	5.28	4.87	5.84	5.42	4.98	5.96	5.59	5.22
		b	0.34	0.43	0.55	0.33	0.44	0.56	0.30	0.41	0.58	0.27	0.36	0.50
	B	a	5.84	5.49	5.22	5.94	5.58	5.30	6.03	5.69	5.39	6.17	5.83	5.43
		b	0.26	0.32	0.40	0.26	0.32	0.39	0.23	0.29	0.38	0.21	0.27	0.35
	C	a	5.78	5.41	4.87	5.93	5.72	5.07	5.75	5.69	5.14	6.10	5.83	5.27
		b	0.26	0.31	0.44	0.24	0.29	0.44	0.27	0.29	0.42	0.20	0.26	0.39
3%	A	a	6.02	6.13	6.09	5.58	5.52	4.94	5.61	5.37	4.87	5.67	5.41	4.98
		b	0.28	0.26	0.25	0.36	0.48	0.61	0.34	0.40	0.65	0.33	0.47	0.60
	B	a	5.48	5.39	5.07	5.72	5.40	5.11	5.79	5.47	5.12	5.94	5.63	5.25
		b	0.36	0.38	0.48	0.31	0.38	0.47	0.29	0.37	0.47	0.27	0.35	0.42
	C	a	5.49	5.02	4.65*	5.60	5.03	4.66*	5.80	5.16	4.81*	5.90	5.44	4.91
		b	0.53	0.45	0.56	0.30	0.46	0.51	0.27	0.40	0.54	0.24	0.35	0.46
4%	A	a	5.32	5.08	4.70*	5.04	4.86	4.58*	5.26	5.04	4.77*	5.59	5.34	4.96
		b	0.54	0.62	0.59	0.63	0.66	0.68	0.57	0.61	0.66	0.47	0.55	0.61
	B	a	5.46	5.15	4.70*	5.55	5.21	4.80	5.66	5.28	4.88	5.76	5.35	4.91
		b	0.36	0.43	0.50	0.34	0.41	0.48	0.32	0.40	0.49	0.32	0.38	0.46
	C	a	5.13	4.57	4.34*	5.30	4.78	4.43*	5.42	4.85	4.54*	5.43	4.84	4.48*
		b	0.39	0.50	0.55	0.36	0.47	0.54	0.32	0.48	0.58	0.34	0.49	0.56
5%	A	a	5.26	4.82	4.45*	5.34	4.89	4.47*	5.28	4.80	4.45*	5.24	4.85*	4.43*
		b	0.54	0.55	0.63	0.55	0.60	0.65	0.58	0.71	0.67	0.57	0.68	0.66
	B	a	5.40	5.01	4.67*	5.50	5.14	4.70*	5.60	5.21	4.79*	5.71	5.27	4.87
		b	0.37	0.45	0.52	0.34	0.43	0.51	0.34	0.42	0.51	0.34	0.41	0.49
	C	a	5.09	4.74	4.39*	5.68	5.31	4.79*	5.80	5.32	4.79	5.57	5.00	4.56*
		b	0.39	0.38	0.57	0.28	0.35	0.51	0.27	0.37	0.35	0.30	0.46	0.54

a- pH   b- % Acidity   * The begining of curd formation.

Table 7. The four pH indicators used in the study and their characteristics[a]

pH-Indicator and Concentration	pH-Range	Color before incubation	Color after incubation	Comments
Bromocresol Green (0.2%)	5.4-3.8 blue-yellow	Bright blue	Light green	Difficult to distinguish +ve from -ve results
Methyl Red (0.2%)	6.3-4.2 yellow-red	Very light peach	Creamy	Very difficult to distinguish +ve from -ve results
Chlorophenol Red (0.2%)	6.4-4.8 purple-yellow	Light violet	Beige yellow	Easy to distinguish +ve and -ve results
Bromocresol Purple (0.2%)	6.8-5.2 purple-yellow	Bright green	Bright yellow	Can be distinguished

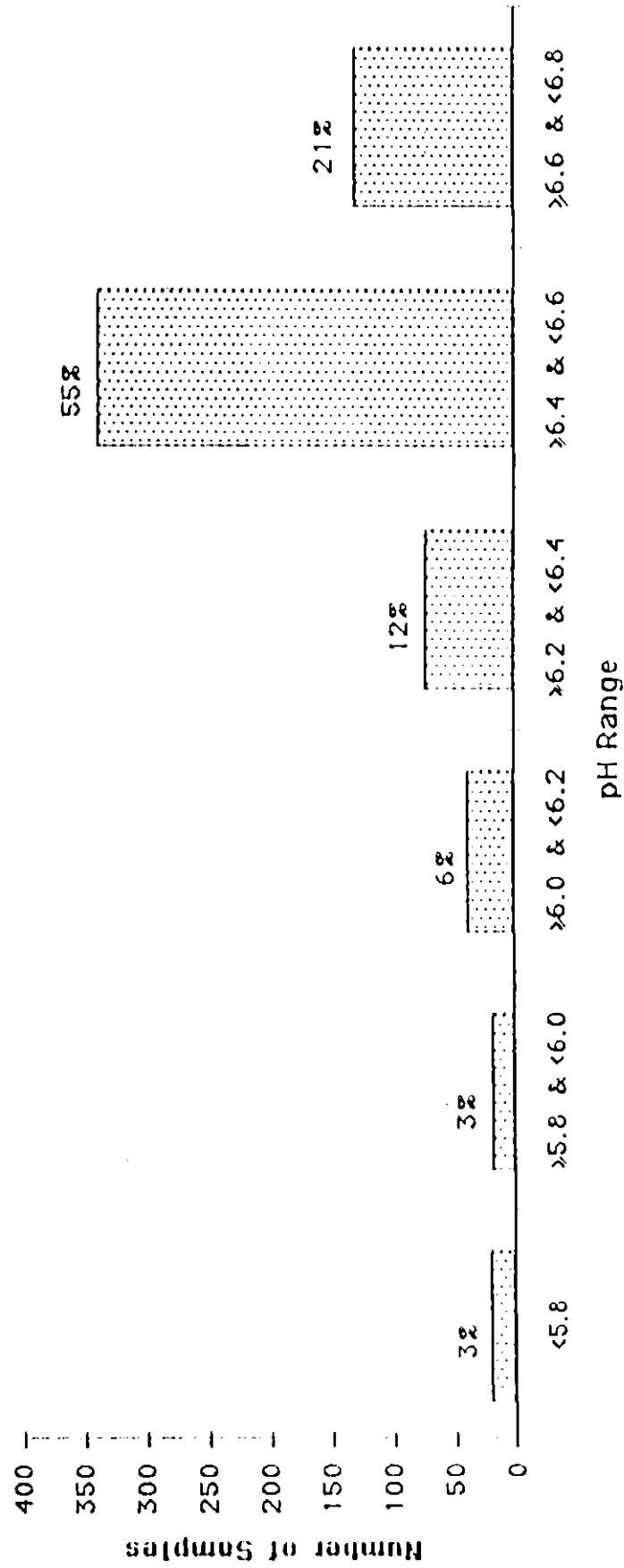(a) Each pH indicator is added to the milk at a concentrations of 1%.

Figure 2. Frequency distribution of milk samples tested for antibiotic residues by YCT according to their pH values.

Table 9. Numbers and (%) of milk samples from different dairy plants which gave positive results to YCT and Delvotest -P® when tested for residues:

Sources of samples	Numbers of samples	No. and (%) of positives by YCT after 2.5h	* No. and(%) of ± by Delvo - test-P®	No. and (%) of positives by YCT after 4h	No. and (%) of positives by Delvo- test-P®
A	184	34 (18.5)	27 (14.7)	6 (3.3)	6 (3.3)
D	311	29(9.3)	21(7.7)	7(2.3)	7(2.3)
E	123	30(2.4)	28(2.3)	0(0)	0(0)
Total %	618 100	93 (15.05)	76 (12.3)	13 (2.1)	13 (2.1)

*Only samples which gave positive results to YCT after 2.5 and 4h were subjected to Delvotest-P®.

V-DISCUSSION

A- Sensitivity of yogurt bacteria to antibiotics when used in YCT

The sensitivity of yogurt bacteria to the antibiotics mostly used in treatment of mastitis and other cattle diseases, as compared to other detection methods and to the results of related studies are shown in Table 10. YCT was more sensitive than Delvotest-P®, to chloramphenicol and oxytetracycline but of comparable sensitivity to tetracycline and erythromycin, and of lower sensitivity to penicilin and ampicillin. YCT was in general less sensitive than charm test, which is probably the most sensitive test for antibiotic residues (Collins-Thompson *et al.*, 1988). With some antibiotics Charm test has sensitivity to concentrations much lower than needed to inhibit the growth of some lactic acid bacteria starter cultures, Table 10. As could be seen in Table 10, no single test for detecting antibiotic residues is superior in sensitivity to all antibiotics generally used in veterinary medicine and animal production.

The mixed culture of *Lactobacillus delbruekii* subsp. *bulgaricus* and *Streptococcus thermophilus* in YCT showed less sensitivity to antibiotics than when each bacterium is singly present, Table10. The symbiosis of these bacteria, which is utilized in yogurt production, is well known (Mitchell and Sandine, 1984; Pette and lolkema, 1950a; Tamime and Deeth, 1980). This symbiosis seems to make the bacteria more tolerant to antibiotics than when present singly.

Table 10. Comparison of the sensitivity of yogurt bacteria and other detection methods to different antibiotics.

Antibiotic	YCT 2.5 h (per g)	YCT 4 h (per g)	Fermentation test (Yogurt Culture) (per ml)	Valio test[m] S. therm. (per ml)	S.therm[l]. (per ml)	L.bulg[l]. (per ml)	Delvotest-p[b,c,g,h,n] (per ml)	Charm test[a,b,c,e,f,d] (per ml)	Cylinder Plate method[b,c] (per ml)	B.subtilis disc assay[b,c] (per ml)	B.stearother mophilus disc assay[b,c,j,k] (per ml)
Penicillin, IU	0.05	0.1	0.005	0.004-0.006	0.004-0.01	0.02-0.10	0.002-0.005	0.002-0.008	0.01	0.05	0.008
Chloramphenicol, µg	2	4	10	NA	0.80-13mg	0.80-13mg	5-8	0.05-0.3	NA	NA	NA
Oxytetracycline, µg	0.1	0.25	0.05-0.5	NA	0.310-1.000	0.310-1.000	0.15-0.40	NA	NA	NA	0.2
Tetracycline, µg	0.2	0.4	NA	0.02-0.3	0.130-0.50	0.34-2.00IU	0.15-0.40	0.5-0.6	NA	NA	NA
Ampicillin, µg	0.1	0.15	NA	NA	NA	NA	0.003-0.005	NA	NA	NA	0.005-0.008
Erythromycin, µg	0.3	0.4	NA	0.05-0.1	0.3-1.3mg	0.07-1.3mg	0.03-1.75	0.02-0.065	NA	NA	NA
Cloxacillin, µg	0.3	0.35	NA	NA	0.340-0.70	0.260->1.00	0.015-0.025	NA	NA	NA	0.05-0.08

a- Anonymous, 1988

b- AOAC, 1980

c-AOAC, 1990.

d-Brady and Katz, 1988.

e-Bruhn et al., 1985.

f-Collins-Thompson et al., 1988.

g-Gist Brocades - Holland, 1993.

h- Jones and Seymour, 1988.

i-Jurdi and Asmar, 1981

j-Oliver et al., 1990.

k-Ouderkirk, 1979.

l- Tamime and Deeth, 1980.

m-Valio, 1994.

n-Van Os and Beukers, 1980.

The YCT was developed as a means for the detection of the presence of antibiotics in amounts high enough to cause inhibition of yogurt culture bacteria. In this way, it is a method for testing the suitability of milk for processing as yogurt, the most important fermented dairy product in Jordan and in many other Arab countries. If the need arises to test milk for antibiotic residues at very low concentrations, which is not enough to cause faulure of milk fermentation, other available tests of higher sensitivity could be used. This type of testing is not usually done in dairy plants, where the main concern of the processors is to check the suitability of milk for production of fermented dairy products using lactic acid bacteria cultures.

B-YCT for testing antibiotic residues in milk

Curdling of milk by starter culture bacteria was described for detection of antibiotic residues (Bishop and White, 1984; Jurdi and Asmar, 1981; Valio, LTD-Finland, 1994). In this study optimization and standarization of the fermentation of milk curdling by yogurt lactic acid bacteria (LAB) was put forward in order to make the test more reliable. Adjusting the pH of milk before the addition of cultures eleminates the effect of pH of fresh milk on curd formation. As shown in Figure 2, it is worthnoting that there are considerable differences in milk pH of different supplies in Jordan. Furthermore, lowering the pH of milk to 6.0 makes the milk more favorable for the growth of yogurt culture bacteria which, as other lactic acid bacteria, prefer low pH for growth. The increase of the yogurt culture concentration leads generally to decrease in the time needed for curd formation (Pette and Lolkema, 1950c). This objective was acheived in all yogurt

cultures tested, (Table 6). Lowering the pH and increasing the culture concentration resulted in better control of the time of testing and made it possible to fix the test reading time to 2.5h. All these modifications could make the test results reproducible between different laboratiories. The use of the pH indicator chlorophenol red is useful in confirming the results of the test. This is a clear advantage when testing large number of samples.

A positive test within 2.5h means for sure the presence of inhibitory substances in milk. Extending the incubation time to 4h helps in giving the examiner general information about the inhibitory effect of the residues. Failure of curd formation after 4h indicates the presence of residues of strong inhibitory effect, while negative curd formation after 2.5 h and positive curd formation after 4h indicates the presence of the inhibitory substances in marginal concentrations. This situation is similar to reading the results of Delvotest-P®, in which color change is examined after 2.5 h of incubation. A yellow color over the entire solid medium indicates a sample free of detectable residues (less than 0.002 IU/ml). A purple color over the entire solid medium indicates a penicillin concentration at least 0.005 IU/ml of milk, and partly purple color of the solid medium indicates a penicillin concentration of between 0.002-0.005 IU pencillin/ ml of milk (Gist Brocades, 1993).

YCT could be used as an alternative to the commercial tests for the dairy plants to test the incoming milk for antibiotic residues. This test is simple reliable and needs no skill to perform. Most important, that it is not expensive. It does not need any special equipments. All of its reagents and glassware are available at any lab, and it doesen't add any expenses to the

processing budjet. The sensitivity showes that it could be as reliable as Delvotest-P®. The use of yogurt culture from the plant in which milk is to be processed in testing milk samples, is an obvious advantage, as it reflects the suitability of the tested milk for processing such as yogurt; the most important cultured dairy product in Jordan and in many other Middle Eastern countries.

C- Antibiotic residues in Jordanian milk as tested by YCT

The results of surveying 618 fresh milk samples for antibiotic residues using YCT and as confirmed by Delvotest-P® Table 9, showed that 2.1% of the samples gave positive result to antibiotic residues test. This indicates that antibiotic residues could be a problem for the milk producers and dairy plants in Jordan; this calls for more attention and care from both sides (Milk producers and processors). Milk producers should read the label for dosage requirments and not exceed it. They must also withhold the milk from sale for the specified length of time written on the label, and identify all cows treated. Even if only one quarter has been treated they must discard all milk from that treated cow. Furthermore only one person should be responsible for administering drugs and identifying all treated animals. Treated cows should be milked completely. Milk from lactating cows just purchased or under treatment should be checked before adding the milk to the bulk (Schoech, 1977).

Many dairy plants, as a result of experience, are aware that fresh milk could contain inhibitory substances as absence of residues in milk of suppliers can not be guaranteed all the time. Accordingly it is generally recognised that testing of milk for antibiotic residues is a necessity if milk is to be processed using

cultures of lactic acid bacteria. But none of the plants do the testing routinely for all milk samples they receive due to many reasons. Choosing the right testing method from the many commercially available ones is not easy, because each method has its advantages and limitations, which could not be known without at least trying the methods. Among the most prominant limitations of the available testing methods is that most of them have lengthy procedures, and that they have variable sensitivities, some are complicated and require qualified personnel to operate the tests procedures. Table 11 shows the commercially available tests with their advantages and limitations. But the most important reason for not making the antibiotic testing on regular basis by the dairy plants in Jordan and many other developing countries is the high running cost which is aggravated by the high number of milk suppliers to each dairy plant, who deliver their milk directly to the plant and not via milk collecting centers. Reducing the number of the suppliers by the introduction of milk collecting centers in which milk of a number of farmers is sampled, tested (by YCT) and then mixed may help in this aspect.

Having a program for controlling antibiotic residues in milk, which includes the routine examination for antibiotic residues, led to lowering the positive cases of antibiotic contaminated milk in many countries (Albright et al., 1961). Such a program is urgently needed in Jordan. Because of its obvious advantages, YCT could be the method of choice to be used in this program for detecting antibiotic residues.

Table 11. Advantages and limitations of the commercially available tests for antibiotic residues in milk.

Test	Advantages	Limitations
I. Cylider Plate assay	has relatively low cost	- requires long time of incubation (12-16h) - requires different preperations from the day before. - requires considerable skills.
II. Disc assays: a] *Bacillus stearothermophilus* disc assay	- easy to operate and does not require qualified personnel. - has relatively low cost	- requires different preperations from the day before.
b] *Bacillus subtilis* disc assay	- does not require qualified personnel. - used as qualitative and quantitative test. - has relatively low cost	- requires different preperations from the day before.
III- Delvotest-P®	- results can be very easily read. - could be easily operated without the need of qualified personnel. - needs relatively short time (2.5h) to read results.	- high cost especially when used routinely. - uncertainty could arise in interprestation of +/- result. -less sensitive towards antibiotics other than penicilin.
IV- Charm test	- can detect and identify different antibiotics. - requires a very short time (15 min). - has relatively higher sensitivity to antibiotics other than β-lactams.	- requires highly trained and qualified presonnel. - has a very complicated procedure. - very expensive and requires costy equipments.

VI . CONCLUSIONS AND REMARKS

1. The high cost of milk testing for antibiotic residues and the large number of milk suppliers to each dairy plant, are the most important reasons for not testing the milk routinely for antibiotic residues in Jordan.

2. Antibiotic residues in milk is a problem especially for the dairy plants in Jordan. Therefore, testing milk for antibiotic residues is a must if milk is to be used for cultured products.

3. It is advisable to lower the number of the suppliers to each dairy plant through the introduction of milk collecting centers.

4. YCT is simple, of low cost and comparable to Delvotest-P® in sensitivity and reliability. It could be used reliably to test the suitability of milk for the processing into yogurt or other fermented dairy products.

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الملخص

دراسة استعمال بكتيريا باديء اللبن الرائب للكشف عن بقايا

المضادات الحيوية في الحليب

إعداد

لينة "محمد عدنان" درويش الكردي

إشراف

الدكتور محمد عصام اليماني

تم في هذه الدراسة تطوير اختبار يعتمد على حساسية بكتيريا باديء اللبن الرائب (Lactobacillus delbruekii subsp. Bulgaricus and Streptococcus thermophilus) للمضادات الحيوية وفشل تخثر الحليب في حالة وجود مضادات بتراكيز كافية تقتل أو تمنع نمو هذه البكتيريا. لجعل اختبار الترويب أكثر قياسية ولتثبيت عامل الزمن، تم تجريب توافيق تراكيز مختلفة لمزرعة البادي (٢،٣،٤،٥%) والرقم الهيدروجيني للحليب (٦، ٦٫٢، ٦٫٤) التي تعطي تخثراً للحليب في أقصر زمن ممكن. تبين أن أفضل هذه التوافيق هو تخفيض الرقم الهيدروجيني الى ٦ واضافة مزرعة البادي بنسبة ٤% (و/و)، وبذا فان طريقة الفحص المطوره تكون بخفض الرقم الهيدروجيني للحليب باستعمال حمض الهيدروكلوريك احادي العيارية الى ٦ واضافة مزرعة البادي بتركيز ٤% (و/و) والحضن على درجة ٤٢ °م لمدة ساعتين ونصف،ويتم بعدها قراءة النتيجة عن طريق ملاحظة تكون الخثرة وتغير لون كاشف الرقم الهدروجيني كلوروفينول رد (٠٫٢% في ايثانول ٥٠%) الذي يضاف بمقدار ١% عند بداية الاختبار او عند نهاية الحضن.

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وبينت دراسة حساسية بكتيريا باديء اللبن الرائب لأهم انواع المضادات الحيوية المستعملة في الطب البيطري والانتاج الحيواني عند استعمالها في فحص الترويب المعدل، أن اقل تراكيز للبنسلين،والكلورامفنيكول،والاوكسيتتراسيكلين، والتتراسيكلين،والامبسلين،والايريثرومايسين، والكلوكساسيلين كافية لمنع تخثر

الحليـب هـي ٣ .،. ، ٢،. ، ١ ،.. ، ٢ ،.. ، ١ ،.. ، ٣ ،.. ، ٣ ،.. ، مـايكروغرام/ غـرام على التوالي. كان هذا الاختبار اكثر حساسية مـن اختبار الدلفو (®Delvotest-P) المستعمل تجاريـاً فـي حالـة الكلورامفينيكـول والاوكسيتتراسـيكلين ومسـاو فـي الحساسـية للتتراسـيكلين والايريثرومايسـين واقـل حساسـية فـي حالـة البنسـلين والامبسلين.

عند استخدام اختبار الترويب المصنع للكشف عن بقايا المضـادات الحيويـة في الحليب المورد لاكبر ثلاثة مصانع ألبان في الأردن وذلك بفحص ٦١٨ عينـة حليب خلال فترة ثلاثة شهور، وُجـد أن ١٥٪ مـن العينـات اعطت نتيجـة ايجابيـة للاختبـار، مقابل ١٢,٣٪ عند استخدام فحص الدلفو، أمـا فـي حالـة اطالـة مـدة الحضن باستعمال الاختبار المطور الى اربـع ساعات انخفضت هـذه النسبة الـى ١,٢٪، وكانت نتيجـة تـأكيد هـذه النسبة باختبـار الدلفو مطابقـة تمامـاً للاختبـار المطـور، وبـذا يسـتنتج ان ١٣٪ مـن العينـات كـانت تحتـوي علـى مثبطـات للنمـو بتراكيز حدية غير كافية لتثبيط نمو بكتيريا اللبن الرائب تماماً.

ويتضح من مقارنة الاختبار المطور مع اختبار الدلفو وهو مـن الاختبـارات المستعملة على نطاق واسع لفحص بقايا المضادات الحيوية، ان الاختبار المطـور ذو حساسية مشابهة لاختبار الدلفو بل ويمتاز عنه بأنه قليـل التكلفـه وأكثـر سـهوله ولا يحتـاج اجراؤه كفـاءة علميـة عاليـة. كمـا وان استعمـال بكتيريـا بـاديء اللبـن الرائب المستخدمة لإجراء الاختبار المطور يُفيد المنتج في التعـرف إلـى صلاحيـة الحليب المستلم لعمل لبن رائب، وهذه ميزة غير متوافرة في كثير مـن الاختبـارات التي تعتمد على حساسية أحياء دقيقة أخرى غير بكتيريا باديء اللبن الرائب.