Detection of microbial load in Imported UHT milk in Baghdad Markets

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Abstract

The aim of this study was concerned on the evaluation of microbial load of imported UHT milk sold in different markets and stores in Baghdad. A total of 50 milk samples were collected, analyzed and processed during February till March 2011. A methylene blue reduction time, resazurin reduction time and 2,3,5-triphenyltetrazolium chloride (TTC) reduction test were used for detection and enumeration of microbial loadaccording to the standard protocols of Food and Drug Administration (FDA) and International Organization for Standardization (ISO) with some modifications.In this study, TTC was added to Plate Count Agar (PCA) for enumeration of microorganisms in UHT milk samples, with the aim to verify the frequency of microorganisms that are unable to reduce TTC.Milk samples were decimally diluted in phosphate buffered saline and pour-plated in PCA plus 0.015% TTC. Colonies were counted after 24h and 48h of incubation at 35^{o}C. From a total of 50 samples, a 7 (14%) were contaminated with many types of bacteria according to results of tests used in this study especially gram negative rods such as *Escherichiacoli* as well as high percentage of microorganisms unable to reduce TTC in UHT milk, which cannot be detected by laboratory procedures based on the formation of red colonies. These findings suggest presence of high microbial load that contaminates some UHT or sterilized milk samples in Baghdad city, so it is strongly recommended that these imported products are monitored carefully.

التحري عن الحمولة الميكروبية للحليب المستورد والمعالج بالحرارة الفائقة في أسواق بغداد

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الخلاصة

استهدفت الدراسة التحري عن الحمولة الجرثومية للحليب المستورد والمعالج بالحرارة الفائقة المباع في الأسواق المحلية في مدينة بغداد للمدة من شباط لغاية أذار 2011. جمع 50 أنموذجا من مناطق مختلفة من مدينة بغداد، وعوملت النماذج حسب الطرائق القياسية لهيئة الأغذية والأدوية والمنظمة العلمية للتقييس مع بعض التحويرات بأستعمال أختبارات الأختزال اللونية غير المباشرة. أظهرت النتائج تلوث بعض النماذج بحمولة ميكروبية مرتفعة بواقع 7 نماذج 14% لاسيما بجراثيم الأشيريكيا القولونية ذات مستعمرات الفورمزان الحمراء ونسبة مرتفعة من المستعمرات عديمة اللون والموجبة لصبغة كرام. نستنتج من هذه الدراسة تلوث بعض نماذج الحليب المعقم بالحرارة الفائقة بجراثيم مختلفة وينصح بمراقبة هذه المنتجات لضمان الصحة العامة.

Introduction

Commercial sterility is defined as a condition in which equipment and packages do not contain viable microorganisms of public health significance or microorganisms of non-health significance, which could reproduce under normal storage and distribution conditions (1). In the canned food industry, commercial sterility is achieved by heat treatment of a food product inside a sealed container. Aseptic processing uses separate systems to sterilize the product and package. The sterile product is filled into sterile

packages within the sterile zone of an aseptic packaging system (1). The UHT treatment and aseptic package protects dairy foods from bacteria and external contamination. The shelf life of milk is extended from 21 days in traditional pasteurization to over four months with UHT and aseptic technology (2). Challenges are present in all aspects of food processing and UHT is not an exception. According to (3), major problems associated with UHT-processed milks include age gelation, component separation, flavor degradation, post-process contamination, slow packaging speeds, and limited shelf life knowledge. Currently, effective UHT processing for products containing particulates has not been achieved due to solids settling and over processing risks (4). UHT processing requires substantial management knowledge and operator skill. Two dyes are commonly employed in this procedure to estimate the number of viable organisms in suitable products: methylene blue and resazurin. To conduct a dye-reduction test, properly prepared supernatants of foods are added to standard solutions of either dye for reduction from blue to white for methylene blue; and from slate blue to pink or white for resazurin. The time for dye reduction to occur is inversely proportional to the number of organisms in the sample (5). Dye-reduction tests have a long history of use in the dairy industry for assessing the overall microbial quality of raw milk. Among their advantages are that they are simple, rapid, and inexpensive; and only viable cells actively reduce the dyes (5). Disadvantages are that not all organisms reduce the dyes equally, and they are not applicable to food specimens that contain reductive enzymes and the existenceof inherent reductive substances unless special steps are employed (5).2,3,5-triphenyltetrazolium chloride (TTC) is a dye largely used for enumeration of microbial colonies in solid culture media (6,8). This dye is a key component of the dry rehydratable film system used for microbiological analysis of food (7,9). TTC is colorless in the oxidized form and red when reduced. Living microorganisms reduce TTC by enzymatic action, originating formazan which is kept inside granules in the cells, which become red (8). Some factors, like pH, temperature, light and concentration of the dye, also interfere in TTC reduction. The reduction of TTC is more intense at high pH (9).The concentration of TTC added to the culture medium is very important because high levels may have a deleterious effect (3). Consequently, the concentration of TTC used in culture media should be low enough to avoid inhibition of growth, but high enough to allow color development (10). The objective of this study was to survey the frequency of microbial load in our imported UHT milk samples in Baghdad markets in order to determine the efficiency and hygienic measurements of these products to ensure healthy aspects impact and processing environment.

Materials and Methods

- **Collection of UHT milk Samples:** This study was conducted on UHT milk sold in different locally markets in Baghdad markets.A 50 UHT milk tetra packs or packages were collected into sterile plastic bags and transported aseptically to the laboratory of food hygiene in Baghdad Veterinary College during February till March 2011. Each sample was divided into three parts: two parts for methylene blue and resazurin reduction time and third part for 2,3,5-triphenyltetrazolium chloride (TTC) reduction test , were processed according to standard protocols of Food and Drug Administration (FDA) and International Organization for Standardization (ISO) with some modifications.
- **Methylene blue and Resazurin reduction time:** The methylene blue reduction test is based on the fact that the color imparted to milk by the addition of a dye such as methylene blue will disappear more or less quickly. The removal of the oxygen from milk and the formation of reducing substances during bacterial metabolism cause the color to disappear. The agencies responsible for the oxygen consumption are the bacteria. Though certain species of bacteria have considerably more influence than

others, it is generally assumed that the greater the number of bacteria in milk, the quicker will the oxygen be consumed, and in turn the sooner will the color disappear. Thus, the time of reduction is taken as a measure of the number of organisms in milk although actually it is likely that it is more truly a measure of the total metabolic reactions proceeding at the cell surface of the bacteria.The necessary equipment consists of test tubes with rubber stoppers, a pipette or dipper graduated to deliver 10 ml of milk and a water bath for maintaining the samples at 35 to 37^{o}C. The bath should contain a volume of water sufficient to heat the samples to 35^{o}C within 10 minutes after the tubes enter the water and should have some means of protecting the samples from light during the incubation period. If a hot-air chamber is used, the samples should be heated to 35^{o}C in a water bath since warm air would heat the milk too slowly. The dry tablets contain methylene blue thiocyanate and may be obtained from any of the usual laboratory supply houses. They should be certified by the Commission on Standardization of Biological Stains. The solution is prepared by autoclaving or momentarily boiling 200 ml of distilled water in a light resistant (amber) stoppered flask and then adding one methylene blue tablet to the flask of hot water. The tablet should be completely dissolved before the solution is cooled. The solution may be stored in the stoppered, amber flask or an amber bottle in the dark. Fresh solution should be prepared weekly (11).

- **Procedure in Testing:** The following procedures are recommended.

1. Sterilize all glassware and rubber stoppers either in an autoclave or in boiling water. Be sure all glassware is chemically clean.
2. Measure 1 ml of the methylene blue thiocyanate solution into a test tube.
3. Add 10 ml of milk and stopper.
4. Tubes may be placed in the water bath immediately or may be stored in the refrigerator at 0 to 4 ^{o}C for a more convenient time of incubation. When ready to perform the test, the temperature of the samples should be brought to 35 ^{o}C within 10 minutes.
5. When temperature reaches 36 ^{o}C, slowly invert tubes a few times to assure uniform creaming. Do not shake tubes. Record this time as the beginning of the incubation period. Cover to keep out light.
6. Check samples for decolorization after 30 minutes of incubation. Make subsequent readings at hourly intervals thereafter.
7. After each reading, remove decolorized tubes and then slowly make one complete inversion of remaining tubes.
8. Record reduction time in whole hours between last inversion and decolorization. For example, if the sample were still blue after 5 hours but was decolorized (white) at the 2.5 hour reading, the methylene blue reduction time would be recorded as 2 hours. Decolorization is considered complete when four-fifths of the color has disappeared.

- Classification: The suggested classification is listed (11).

Class 1. Excellent, not decolorized in 8 hours.
Class 2. Good, decolorized in less than 8 hours but not less than 6 hours.
Class 3. Fair, decolorized in less than 6 hours but not less than 2 hours.
Class 4. Poor, decolorized in less than 2 hours.

The resazurin test is conducted similar to the methylene blue reduction test with the judgment of quality based either on the color produced after a stated period of incubation or on the time required to reduce the dye to a given end-point. Numerous modifications have been proposed. The two most commonly used are the "one-hour test" and the "triple-reading test" taken after one, two, and three hours of incubation. Other modifications have value in specific applications.The procedure for making the resazurin test is as follows: Prepare resazurin solution by dissolving one resazurin tablet (dye content/ tablet, approximately 11 mg, certified by Biological Stain Commission) in 200 ml of hot distilled water as was done in the methylene blue test. Place one ml of dye

solution in a sterile test tube, and then add 10 ml of sample. Stopper the tube, place in the incubator and, when the temperature reaches 36^{o}C, invert to mix the milk and dye. Incubate at 36^{o}C. Tubes are examined and classified at the end of an hour in the"one-hour test" or at the end of three successive hourly intervals in the "triple reading test."The resazurin test may be a valuable time saving tool if properly conducted and intelligently interpreted, but should be supplemented by microscopic examination (11).

The following relationships of color and quality are generally accepted:

Color of Sample: Quality of Milk (11).

1. Blue (no color change): Excellent
2. Blue to deep mauve: Good.
3. Deep mauve to deep pink: Fair.
4. Deep pink to whitish pink: Poor.
5. White: Bad.

- **2,3,5-triphenyltetrazolium chloride (TTC) reduction test:** TTC was freshly prepared with Plate Count Agar (PCA) in final concentration 0.015% and stored in sterile dark bottles in which each UHT milk sample was submitted to decimal dilutions in sterilePhosphate Buffered Saline (PBS) by micropipettes, and 1 ml of each dilution was pour-plated, in duplicates, in PCA containing 0.015% TTC . PCA plates were incubated at (35-37)^{o}C. Colony Forming Units (CFUs) were counted after 24 hours and 48 hours of incubation, the number of CFU/ml in each sample was determined according to Swanson et al. (12). Colonies were classified according to color: colorless, white, cream or yellow were considered TTC non-reducers and pink or red were considered TTC reducers. Colonies that changed color between the two counting were also recorded.After 48 hours of incubation, hundreds of colonies considered as non-reducers were streaked on PCA with 0.015% TTC and on Blood Agar. Plating was repeated until pure cultures were obtained. From these, 50 were selected for Gram staining (9).

Results and Discussion

Dye-reduction tests have a long history of use in the dairy industry for assessing the overall microbial quality of raw milk. Among their advantages are that they are simple, rapid, and inexpensive; and only viable cells actively reduce the dyes. Disadvantages are that not all organisms reduce the dyes equally, and they are not applicable to food specimens that contain reductive enzymes unless special steps are employed (5).Results revealed that out of the 50 samples, 7 samples (14%) were contaminated with different types and species of microbes with some problems associated with UHT processed milk samples include component separation and acid elevation below pH 5 as showed in this study. In methylene blue reduction test, samples of UHT milk were reduced and decolorized in less than 6 hours and in reassuring test within 3 hours and that revealed high microbial load of these sterilized long shelf life samples which indicate either insufficient heat treatment or mostly post pasteurization contamination. Besides facilitating the counting of colonies, TTC is also a powerful tool to distinguish colonies from food particles, which don´t react with the dye. Use of TTC is highly recommended for milk testing, because the opacity of the plates, especially those containing the less diluted samples, makes the counting inaccurate (9).Colonies in PCA plus 0.015% TTC were different in their ability to reduce the dye between 24 and 48 hours as well as in size, shape and texture with converting ability from no reduced to reduced colonies within 48 hours and in Gram stain and morphology under the microscope especially *Escherichia coli* that encountered in this study on McConkey agar. Gram positive microorganisms, like *Micrococcus*, *Coryneforms* and some heat resistant *Bacilli* are often present in raw milk and are part of remaining micro flora in pasteurized milk. In counterpart, Gram negative microorganisms are much less common in raw milk. This suggests that the TTC non-reducing Gram positive bacteria detected in the pasteurized milk samples are those that survived to the heat treatment. Their incapacity to reduce TTC after the pasteurization suggests that a possible heat-injury that affected the

capacity of microorganisms to reduce TTC was irreversible (9).Table (1) revealed these study facts.

Table (1). Reduction time and tests in UHT milk samples

UHT milk samples	Methylene blue reduction time		Resazurin reduction time		TTC reduction test Within (24-48) hours	
	Before 6 hours	After 24 hours	Within 3 hours	After 24 hours	Reduced red formazan colonies	Non reduced colonies
50	7 (14%)	43 (86%)	7 (14%)	43 (86%)	10 (20%)	30 (60%)

In conclusion, since microbes were found to a large extent in our environment with post heat treatment contamination during processing, packaging, transport, distribution and storing, it should be mandatory to perform routine controls to screen imported milk for detection of microbial load and types. Our findings suggest that microbes contaminate some UHT milk samples in Baghdad markets; so that it is strongly recommended however that these products are monitored carefully. The implementation of Hazard Analysis and Critical Control Points (HACCP) Programs in milk industries is essential for addressing food safety for population of Baghdad, as well as intervention and education of people involved in the production, processing and monitoring of these products.

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