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DETECTION OF *Escherichia coli* IN DAIRY AND MILK-RELATED PRODUCTS USING DIRECT PLATING AND THE 3-STAGE METHOD

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ABSTRACT

Escherichia coli is a frequent foodborne contaminant that negatively affects the quality and safety of dairy products. The present study aimed to examine the presence of *E. coli* in dairy and milk-related products using two detection methods namely, "direct plating" and "3-stage method". Sixty-nine samples of yoghurt, Domiati cheese, Kariesh cheese, UHT milk, dried skim milk, dried ice-cream, dried whey proteins, creamchantee, infant milk formulas, and "sahlab" were collected from 3 Egyptian governorates and examined for the presence of *E. coli*. A total of 250 suspected *E. coli* isolates could be cultured from these samples and subjected to biochemical identification that characterized 35 of these isolates as potential *E. coli* cultures. Further biochemical testing of these 35 cultures using the Microbact MGNB-12A miniaturized identification system confirmed their belonging to the *E. coli* species. These isolates were variably recovered from yoghurt (8 isolates), Kariesh cheese (12 isolates), UHT milk (1 isolate), dried ice-cream (2 isolates), creamchantee (1 isolate), infant milk formulas (5 isolates), and sahlab (6 isolates). The 3-stage method showed higher efficiency in detecting *E. coli* in these products than direct plating. This was attributed to the ability of the 3-stage method to detect injured cells generated on exposure to sub-lethal stress factors including those applied during food manufacture and preservation. The 3-stage method outlined in this study is, therefore, recommended for reliable detection of *E. coli* in dairy and milk-related products.

Keywords: *Escherichia coli*, detection, injured cells, dairy products.

INTRODUCTION

E. coli is a microorganism that widely exists in nature. It is also a frequent contaminant of foodstuffs including dairy products (Bell & Kyriakides 1998, Batt 2000, Fernandes 2009). *E. coli* can be involved in undesirable fermentations leading to quality defects in dairy products (Fernandes 2009). Some strains of *E. coli* are also pathogenic particularly the *E. coli* O157:H7 strain that has been implicated in several foodborne outbreaks of gastrointestinal diseases worldwide (Bell & Kyriakides 1998, Chart *et al.* 2000). Developing effective detection methods for this micro-organism is therefore necessary to ensure the quality and safety of dairy products.

The detection of *E. coli* in foods is challenged by the emergence of injured cells of this bacterium under sub-lethal stress conditions including those adopted during the preparation and preservation of foodstuffs, e.g. heating, fermentation, salt addition, etc.. (Mackey 2000, Wu 2008). Injured cells can be defined as cells that survive a stress, but lose some of their distinctive qualities, including their resistance to selective agents in

differential media used for their detection in foods (Fricker 1987, Mackey 2000, Wu 2008). However, injured cells have the ability to repair their cellular damage (resuscitation) and regain its ability to grow and proliferate when surrounding conditions are improved. Therefore, determining the presence of injured microorganisms is critical to the quality and safety of final food products The present study aimed to examine the presence of *E. coli* in dairy products and milk-related products using two detection methods. Within this context, the efficacies of these methods for detecting the organism were evaluated as to devise a reliable detection strategy.

MATERIALS AND METHODS

Isolation of *Escherichia coli* from Dairy and Milk-Related Products
Collection of Samples

Sixty-nine samples of dairy and milk-related products were collected from 3 Egyptian governorates: El-Dakahlia, Alexandria and El-Behara. These samples included 10 samples of yoghurt, 4 samples of pickled Domiati cheese, 6 samples of fresh Domiati cheese, 9 samples of Kariesh cheese, 5 samples of UHT milk, 10 samples of dried skim milk, 1 sample of dried whey proteins, 15 samples of dried infant milk formulas, 3 samples of each of dried ice-cream, "creamchantee", and "sahlab".

Detection Methods

Two detection methods namely "direct plating" and "3-stage method" were applied for the detection of *E. coli* in collected samples.

Direct Plating Method

Twenty-five grams of each sample were mixed with 225 ml saline solution (0.85% NaCl), from which loopfuls (approximately 100 µl) were streaked onto plates of MacConkey agar (Oxoid, Basingstoke, UK), followed by incubation at 37°C for 18-24h.

3-Stage Method

This method involved 3 consecutive steps: pre-enrichment, enrichment and plating as follows:

Pre-enrichment

All samples except those of dried skim milk were pre-enriched in tryptone soy broth (TSB) by mixing 25 g as a representative sample with 225ml tryptone soy broth (TSB) (Oxoid, Basingstoke, UK), followed by incubation at 37°C for 24 h. Dried milk samples were pre-enriched by suspending 25 g of dried milk sample into 225 ml of sterile distilled water followed by incubation at 37°C for 24 h.

Enrichment

Ten ml of the pre-enriched sample were transferred into 90 ml of the *Enterobacteriaceae* Enrichment (EE) broth (Oxoid, Basingstoke, UK) followed by incubation at 37°C for 24 h.

Plating

Two to three Loopfuls (approximately 100 µl) from each enriched sample were streaked onto plates of MacConkey agar medium. The inoculated plates were incubated at 37°C for 24h.

Morphological and Biochemical Identification

Suspected colonies of *E. coli* developing red color with bile precipitation on MacConkey agar were picked up and examined by Gram-staining. Suspected cultures showing short rod shape and Gram-negative reaction were maintained for further biochemical examinations including the catalase test, oxidase test, Kliger iron agar (KIA) test, lysine iron agar (LIA) test, and sulfate, indole and motility (SIM) test. These testes were conducted as described by the FDA's bacteriological analytical manual (FDA/BAM 2009).

Biochemical Identification by the Microbact Gram-Negative Bacteria (12A) Miniaturized System

Isolates producing typical *E. coli* reactions in the above tests were further identified using the Microbact Gram-Negative Bacteria (MGNB-12A) identification system (Oxoid, Basingstoke, UK). This system includes strips, each of which consists of 12 wells containing culture media for conducting 12 biochemical identification tests of lysine decarboxylase (LYS), ornithine decarboxylase (ORN), hydrogen sulfide production (H2S), glucose fermentation (GLU), mannitol fermentation (MAN), xylose fermentation (XYL), orthonitrophenyl galactosidase (ONPG), indole production (IND), urea hydrolysis (UR), Voges-proskauer reaction (V.P), citrate utilization (CIT), and tryptophan deaminase (TDA). The system was used according to the manufacturer's instructions (Oxoid, Basingstoke, UK).

RESULTS AND DISCUSSION

Isolation of *Escherichia coli* from Milk and Dairy products

Sixty-nine samples of milk and dairy products were collected from three Egyptian governorates and examined for the incidence of *Escherichia coli*. Table 1 shows the number of collected samples and the locations, from which they were gathered. Two cultural methods were used for isolating *Escherichia coli* from the collected samples. The first isolation method, termed "direct plating", involved mixing 25 g of each sample, with 225 ml saline solution (0.85% NaCl), from which loopfuls (approximately 100 µl) were streaked onto plates of MacConkey agar. Whereas, the second isolation method, termed "3-stage method", consisted of 3 successive steps of pre-enrichment, enrichment and plating. The pre-enrichment stage was performed by mixing 25g of each sample, with 225 ml of sterile tryptone soya broth (TSB), or 225 ml sterile distilled water in the case of dried milk samples. Samples were pre-enriched for 24 h followed by selective enrichment in *Enterobacteriaceae* Enrichment (EE) broth for another 24 h. Loopfuls (approximately 100 µl) from each EE broth were finally streaked onto plates of MacConkey agar. Suspected colonies of *E.coli* that developed red color with bile precipitation were picked up, purified, and subjected to morphological and biochemical identification.

Table 1: Locations and Numbers of Examined Dairy and Milk-Related Products Samples.

Samples	Governorate			Total Number of Samples	Numbers of Suspected *E. coli* Isolates*
	El-Dakahlia	Alexandria	El-Behara		
Yoghurt	10	0	0	10	38
Fresh Domiati Cheese	6	0	0	6	21
Pickled Domiati Cheese	3	1	0	4	6
Kariesh Cheese	4	2	3	9	53
UHT Milk	5	0	0	5	18
Dried Skim Milk	10	0	0	10	12
Dried Whey Proteins	1	0	0	1	3
Dried Ice-cream	3	0	0	3	12
Creamchantee	3	0	0	3	11
Infant Milk Formula	14	1	0	15	59
Sahlab	1	1	1	3	17
Total	60	5	4	69	250

*Suspected *E.coli* isolates are those developing red colonies with bile precipitation on the MacConkey agar.

Morphological and Biochemical Identification of Suspected *E. coli* Isolates.

As shown above in table 1, a total of 250 suspected *E. coli* isolates could be recovered from dairy products samples examined in this study. These isolates were morphologically screened using Gram staining and were found to be Gram-negative, short rods. They were then examined for their biochemical activities employing a series of examinations including the catalase test, oxidase test, KIA test, LIA test and SIM test (Table 2). Isolates were successively subjected to these tests that only isolates producing a typical reaction of *E. coli* in one test were further examined in the next one. For instance, all of the 250 suspected isolates were catalase-positive, which is a typical reaction of *E. coli*. Therefore, these isolates were all examined within the next oxidase test. The latter test showed that 237 out of 250 isolates were oxidase negative. Given that *E. coli* does not typically produce oxidase, these 237 isolates were further subjected to the KIA test. By completing the rest of examinations and comparing their results with the typical reactions of *E. coli*, 35 isolates were finally identified as potential *E. coli* cultures. These isolates were catalase positive, oxidase negative, could ferment glucose and lactose, produced lysine decarboxylase and indole, showed motility, but could not produce H_2S (Table 2). These isolates were further identified using the Microbact Gram-Negative Bacteria (MGNB-12A) miniaturized identification system.

Table 2: Biochemical Identification of Suspected *E. coli* Isolates Cultured from Dairy and Milk-Related Products Samples.

Test	Reaction	Number of Examined Isolates	Positive	Negative	Typical Reaction of *E.coli*
Catalase	Catalase Production	250	250	0	+
Oxidase	Oxidase Production	250	13	237	-
KIA	Glucose Fermentation	237	80	157	+
	Lactose Fermentation	237	80	157	+
	H_2S production	237	157	80	-
LIA	Lysine Decarboxylation	80	66	14	+
	H_2S production	80	14	66	-
SIM	H_2S production	66	31	35	-
	Indole Production	66	35	31	+
	Motility	66	35	31	+

Table 3 shows the results of 12 biochemical identification tests carried out on the 35 potential *E. coli* isolates using the Microbact MGNB-12A miniaturized identification system. It could be seen that all of the examined isolates could produce lysine decarboxylase, ferment glucose and manitol, express □-galactosidase as indicated by the ONPG test, produce indole. The isolates could not produce hydrogen sulfide (H_2S) or urease and were unable to utilize citrate. All isolates gave negative reactions in the Voges-Proskauer test. However, the isolates showed variable results in the ornithine decarboxylase, xylose fermentation, and tryptophane deaminase (TDA) tests. Out of the 35 *E. coli* isolates, 22 isolates produced ornithine decarboxylase, 31 isolates fermented xylose, and only 2 isolates were able to produce tryptophan deaminase. The results of all these reactions were used to calculate the "profile code" of each isolate which indicated that all the 35 isolates could be identified as *E. coli* with probabilities of approximately 68%- 96%.

Evaluating the Efficacies of Direct Plating and the 3 Stage Method in Detecting *E. coli* in Dairy and Milk-Related Products.

Table 4 shows the occurrence of confirmed *E. coli* isolates in the examined dairy and milk-related products. These isolates could be variably recovered from yoghurt (8 isolates), Kariesh cheese (12 isolates), UHT milk (1 isolate), dried ice-cream (2 isolates), creamchantee (1 isolate), infant milk formulas (5 isolates), and sahlab (6 isolates). No confirmed *E. coli* isolates could be detected in other samples including those of fresh Domiati cheese, pickled Domiati cheese, dried skim milk and dried whey proteins. However, the outcome of detecting *E. coli* in positive samples depended on the detection method. The 3-stage method showed higher efficiency in detecting the organism than direct plating (table 4). While no *E. coli* isolates could be detected in yoghurt samples using the direct plating method, 5 of these samples were found to be contaminated with the organism using the 3-stage method. This method could also detect *E. coli* in 4 Kariesh samples, whereas direct plating detected the organism in 3 of these samples. The 3-stage method was also more efficient than direct plating in isolating *E. coli* from other dried milk-related products including dried ice-cream, creamchantee, infant milk formulas and sahlab.

Table 3: Identification of Potential *E. coli* Isolates Using the Microbact Gram-Negative Bacteria (12A) Identification System.

Isolates	Microbact ™ GNB 12A Identification Tests													
	Lysine	Ornithine	H_2S	Glucose	Manitol	Xylose	ONPG	Indole	Urease	V-P	Citrate	TDA	Profile Code	%Probability of *E. coli*
1	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
2	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
3	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
4	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
5	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
6	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
7	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
8	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
9	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
10	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
11	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
12	+	+	-	+	+	-	+	+	-	-	-	-	6660	76.77%
13	+	+	-	+	+	-	+	+	-	-	-	-	6660	76.77%
14	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
15	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
16	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
17	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
18	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
19	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
20	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
21	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
22	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
23	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
24	+	-	-	+	+	-	+	+	-	-	-	+	4661	67.95%
25	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
26	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
27	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
28	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
29	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
30	+	-	-	+	+	-	+	+	-	-	-	+	4661	67.95%
31	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
32	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%
33	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
34	+	+	-	+	+	+	+	+	-	-	-	-	6760	96.39%
35	+	-	-	+	+	+	+	+	-	-	-	-	4760	78.16%

The higher efficiency of the 3-stage method in detecting *E. coli* in the examined products compared to direct plating could be attributed to its ability to recover injured cells. As mentioned above, these cells are generated on exposure to sub-lethal stress factors including those applied during food manufacture and preservation. Due to their being injured, these cells can not resist inhibitory agents in selective culture media such as MacConkey agar (Fricker 1987, Mackey 2000, Wu 2008). Therefore, the inclusion of a pre-enrichment step, in which samples are inoculated into a nutritious, non-selective medium could aid the resuscitation of injured cells into intact, healthy cells that could resist selective agents. Since pre-enrichment also promotes the growth of other microorganisms competing with *E. coli*, a second step of selective enrichment has been recommended before plating on selective, differential agar media. This represents the rationale behind the 3-stage method that was found to improve the detection of *E. coli* in the present study. Previous studies conducted in our laboratory have also shown the higher effectiveness of the 3 stage method in detecting *Salmonella* serovar Typhimurium and *Enterobacter sakazakii* in dairy products compared to direct plating (Nassib *et al.* 2003, El-Sharoud *et al.* 2008). The present results, therefore, confirm the inadequacy of relying on direct plating on selective differential media for examining the presence of *E. coli* in dairy and milk-related products. A 3 stage method like that presented in this study could be recommended for reliable detection of this bacterium in these products.

Table 4: Detection of *E. coli* in Dairy and Milk-Related Products Using Direct Plating and the 3-Stage Method

Samples	Samples Numbers	Confirmed *E. coli* Isolates	Number of Positive Samples	
			Direct Plating	3-Stage Method
Yoghurt	10	8	0	5
Fresh Domiati Cheese	6	0	0	0
Pickled Domiati Cheese	4	0	0	0
Kariesh Cheese	9	12	3	4
UHT milk	5	1	0	1
Dried Skim Milk	10	0	0	0
Dried Whey Proteins	1	0	0	0
Dried Ice-cream	3	2	0	1
Creamchantee	3	1	0	1
Infant Milk Formulas	15	5	0	4
sahlab	3	6	0	1
Total	69	35	3	17

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الكشف عن وجود ميكروب الإيشريشيا كولاي في منتجات الألبان والمنتجات المرتبطة باللبن باستخدام التلقيح المباشر علي الأطباق وطريقة المراحل الثلاثة

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ميكروب الإيشريشيا كولاي هو ملوث ميكروبي متكرر الظهور في منتجات الأغذية ولـه تـأثيرات سلبية علي جودة وسلامة المنتجات اللبنية. ولقد أستهدفت هذه الدراسة الكشف عن وجود الإيشريشيا كـولاي في منتجات الألبان والمنتجات المرتبطة باللبن وذلك بإستخدام طريقة "التلقيح المباشر علي الأطباق" و طريقة "المراحل الثلاثة". وللقيام بذلك تم تجميع ٦٩ عينة من اليوجورت والجبن الدمياطي والجبن القريش واللـبن المعامل علي حرارة عالية (UHT) واللبن الفرز المجفف ومسحوق الآيس كريم وبروتينات الشرش المجففة والكريم شانتيه وألبان الأطفال والسحلب وذلك من ٣ محافظات مصرية. ولقد أمكـن عـزل ٢٥٠ مزرعـة مشكوك في أن تكون إيشريشيا كولاي من هذه العينات، وبفحص هذه المزارع بواسطة اختبارات التعريـف الكيموحيوية تبين أن ٣٥ منها يحتمل أن تكون مزارع إيشريشيا كولاي. وبـإجراء إختبـارات كيموحيويـة إضافية لهذه المزارع المحتملة بواسطة نظام التعريف المصغر "ميكروباكت" تم التأكد من أن هذه المـزارع وعددها ٣٥ تنتمي جميعا إلي نوع الإيشريشيا كولاي. ووجد أن مصادر هذه المزارع هـي اليوجـورت (٨ مزرعة)، الجبن القريش (١٢ مزرعة)، واللبن UHT (مزرعة واحدة)، ومسحوق الآيس كريم (٢ مزرعة)، والكريم شانتيه (مزرعة واحدة)، وألبان الأطفال (٥ مزارع) والسحلب (٦ مزارع). ولقد أظهـرت طريقـة "المراحل الثلاثة" كفاءة أكبر في عزل الإيشريشيا كولاي من هذه المنتجات مقارنة بطريقة "التلقيح المباشـر علي الأطباق". وتم تفسير ذلك بقدرة طريقة "المراحل الثلاثة" علي الكشف عن الخلايا المجروحة التي تنـتج عند التعرض لعوامل إجهاد غير مميتة مثل تلك المستخدمة عند تصنيع وحفظ الأغذيـة. ولـذلك فتوصـي بإستخدام طريقة "المراحل الثلاثة" الموضحة في هذه الدراسة عند القيام بعملية يمكن الوثوق فيها للكشف عن الميكروب في المنتجات اللبنية والمنتجات المرتبطة باللبن.

قام بتحكيم البحث

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