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GROWTH AND SURVIVAL OF *ESCHERICHIA COLI* CARRYING MULTIDRUG RESISTANCE PLASMIDS DURING PREPARATION AND STORAGE OF YOGHURT WITH REFERENCE TO THEIR PUBLIC HEALTH HAZARD

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ABSTRACT :

Through an investigation of bovine subclinical mastitic milk samples, *E.coli* strains were isolated and biochemically identified, they were untypable after testing against available nine serotypes. *E. coli* isolates were subjected to antimicrobial susceptibility testing against 13 antimicrobial agents (cefobid, ofluxacillin, claforan, streptomycin, spiramycin, cephradin, cloxacillin, ampicillin, unasyn, amoxycillin, penicillin G, ciprocin and polymexin B.sulphate). Plasmid profile analysis was carried out to 10 multidrug resistance *E.coli* strains, of which, only four isolates were carrying different plasmids with molecular sizes (5.3-7.5 kb). An *E.coli* strain carrying four plasmids was selected to be inoculated into sterile milk (pH 6.6) with initial dose of 1.3×10^7 cells/ml after adding the yoghurt starter cultures and incubation at 37±1° C. In the finish product, *E.coli* count was 3.2×10^6 and the pH was 5.24. The yoghurt with its control were stored at 4±1° C for 7 days. Slow decline in *E.coli* count was observed, where the *E.coli* strains survived till the end of the week and persisted in large numbers (3.7×10^5 cells/ml) and resisted the acidity of pH 4.03. Public health hazard of yoghurt contamination with such *E.coli* strains was discussed. An experiment was carried out to study the probability of plasmid transmission from *E.coli* strains to starter organisms. Yoghurt was prepared from two types of starters. One of them lived and stored with the tested *E.coli* strain and the other was clean starter. Minimum inhibitory concentrations of unasyn, cephradin, claforan and cloxacillin- which showed resistance against the tested *E.coli* strain- were added to sterile milk after inoculation of the two types of starters separately. The obtained data declared that *E.coli* plasmids could not be transmitted to the starter organisms, also, the results were discussed.

INTRODUCTION

The main object in terms of food hygiene is to avoid risks resulting from the presence of either pathogenic, potentially pathogenic or toxogenic organisms in food (Pazakova *et al.*, 1997) rather than its devoiding of any microorganisms carry antibiotic resistance genes (plasmids) as these plasmids may be transmitted to other human intestinal commensal flora (Wagner and Hahn, 1999).

The multidrug resistance organisms even those which are non pathogenic may constitute reservoirs for disseminating antibiotic resistance plasmids to other pathogens in the community (Kessie *et al.,* 1998). Subsequently, Stephan and Schumacher (2001) recommended periodic surveillance of antibiotic resistance testing for both pathogenic and non pathogenic bacteria in humans, livestocks, foodstuffs and environments to detect the emergence of these resistant genes among different bacterial species. They added that simillar plasmids which carry the same antibiotic resistance patterns establish the genetic exchange between strains living in a close vicinity.

Furthermore, non pathogenic microorganisms may pass from animals to humans via food products and when they are carrying transconjugative plasmids, the latter may be transferred to pathogenic or indigenous flora of human body (Moro *et al.,* 1998). It was found that the coagulase negative staph.spp. of human which are known to be non pathogenic but opportunistic organisms may become a serious problem when they express gene encoded antibiotic resistance (Bagado *et al.,* 2001).

Contamination of dairy products by *Escherichia coli* has been used for a long time as an index of fecal pollution and unsanitary manufacturing or handling practices and their growth in such products may be responsible for defects in texture and flavour (Ernstorm, 1954). However, since the implication of certain strains of *E.coli* in several cases of foodborne illness outbreaks (Marier *et al.,* 1973), the presence of *E.coli* in the dairy products has become of a public health hazard. Several studies have been conducted to characterize Enteropathogenic *E.coli* (EEC) and *E.coli* O157:H7 in fermented skim milk and yoghurt (Goel *et al.,* 1971; Frank and Marth, 1977; Prasad *et al.,*1980; Mohanan *et al.,* 1985; Ahmed, 1990 and El-Hawary and Aman, 1998).

This study was planned to evaluate the growth and fate of non pathogenic *E. coli* strain isolated from subclinical mastitic milk samples) which was carrying plasmids encoding multidrug resistance during the manufacture and storage of yoghurt and its public health hazard. Also to study the probability of transferring of plasmids encoding multidrug resistance from the inoculated *E.coli* strain to the yoghurt starter cultures.

MATERIAL AND METHODS :

1- E.coli isolates:

***E.coli* strains were isolated from milk samples of subclinical mastisis affecting Friesian Holstein cows. Isolation and identification of *E.coli* was carried out according to the method recommended by Quinn *et al.* (1994). The *E.coli* isolates were subjected to serotype identification using nine available antisera (O:26ab, O:55, O:86a, O:111, O:119, O: 124, O: 125 ac, O:126 and O:128) produced by (Difico) and following the manufacturer instructions.**

2-Antimicrobial susceptibility testing:

It was carried out using disc diffusion method on nutrient agar plates against 13 antimicrobial agents (cefobid, 75 μg, ofloxacillin 100 μg, caloforan 30 μg, streptomycin 10 μg, spiramycin 100 μg, cephardine 30 μg, cloxacillin 5 μg, ampicillin 10 μg, unasyn 30 μg, amoxicillin 10 μg, penicillin 10 μg, ciprocin 5 μg and polymexin B-sulphate 300 IU - Oxoid limited- England & UCCMA-Egypt.).

Judgement and categorizing for susceptibility were based on diameter of

inhibition zone measurements and according to Beuer-Kirby scale (Atlas, 1995).

3- Plasmid profile analysis:

Screening the existance of plasmid DNA in *E.coli* strains was done by the alkaline lysis technique. The extracted DNA for each strain was subjected to electrophoresis running through 0.7% agarose gel stained by ethiolium 0.5 μg/ml gel and running 100 A- 120 V. with marker of *E.coli* V. 517 according to David *et al.* (1991).

4- Survival of *E.coli* in yoghurt:

a-Culture:

An untypable *E.coli* strain, recovered from a subclinical mastitis milk sample which was characterized by multidrug resistant pattern against eight antimicrobial agents (ampicillin, unasyn, penicillin G, calforan, spiramycin, cephradin and cloxacillin) and also was carrying four plasmids with molecular sizes 5.3, 5.5, 7 and 7.5 Kb- (Fig. 1) was selected to be inoculated into sterile milk used for yoghurt manufacture. The test organism was grown in brain heart infusion broth (Oxoid), and incubated at 37°C for 24 h. Starter cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) grown in sterile skim milk were obtained from the department of Food Science, Faculty of Agriculture, Assiut University.

b-Preparation and sampling of youghurt:

Two lots of yoghurt were prepared from sterile milk. The milk was inoculated with starter cultures according to Lampert (1975), and divided into two portions. The first portion was inoculated with the test organism (*E.coli)* to provide 1.3×10^7 cells/ml. The other portion was taken as a control (free from *E.coli)*. The two lots of milk were incubated in an adujustable water bath at 37°C ± 1°C to allow a slow fermentation and production of yoghurt. The infected yoghurt and its control were kept in a refrigerator at 4°C±1°C. To determine *E.coli* count and pH value, Samples were taken from milk after inoculation, from prepared yoghurt and daily thereafter up to 7 days. The samples were prepared for examination according to standard methods (A.P.H.A, 1978).

c- Enumeration of *E.coli*:

The method suggested by Speck *et al.* (1976) was employed. Samples were surface plated onto trypticase soy agar (Oxoid).The plates (duplicate plates for each dilution) were held for one hour at room temperature followed by adding a layer of violet red bile agar (Oxoid), then were incubated at 37°C for 24 hours.

d- pH detemination:

The pH value of milk and yoghurt was determined by using pH meter Jenway model 350 supplied with standard combination electrod.

5-Assessment of plasmids (encoding multidrug resistance) transference from *E.coli* into starter cultures of yoghurt:

Two lots of sterile milk were prepared. The first lot was inoculated with a culture from yoghurt previously infected with *E.coli* carrying plasmids encoded multidrug resistance and stored for 7 days. Then the milk was divided into four portions; each of them was inoculated with the minimum inhibitory concentration (MIC) of specific antibiotics; unasyn (16 μg), claforan (6.25 μg), cloaxacillin (12.5 μg) and cephradine (6.25 μg) according to National Committee of Clinical Laboratory Standards (Nccls, 1993). The second lot of the sterile milk

was inoculated with a starter culture of yoghurt free from the test organism (*E.coli*), and then the milk was divided into four portions, each was prepared to contain the same concentrations of the specified antibiotics as mentioned above. The eight portions of milk were made yoghurt according to Lampert (1975). A control yoghurt was prepared from a sterile milk free from added antibiotics for each type of starter culture used.

RESULTS :

The results were recorded in Tables (1&2) and Fig. (1).

Table (1): Survival of untypable *E.coli* carrying multidrug resistance plasmids in yoghurt during its preparation at 37°C and storage at 4±1°C.

Days of storage	Count of *E.coli*/gm	pH value
inoculum of milk	1.3×10^7	6.6
0 time (Finished product)	3.2×10^6	5.24
1	3×10^6	4.40
2	2.7×10^6	4.20
3	2×10^6	4.15
4	1×10^6	4.15
5	6×10^5	4.10
6	6×10^5	4.03
7	3.7×10^5	4.03

Table (2): Production of yoghurt from milk containing minimum inhibitory concentration (MIC) of the selected antibiotics.

Milk used	Yogurt production	
	Starter A*	Starter B**
I- Added antibiotics:		
Cloxacillin 16 µg/ml	failed	failed
Cifradine 6.25µg/ml	failed	failed
Claforane 6.25 µg/ml	failed	failed
Unasyn 16 µg/ml	failed	failed
II- Antibiotic free sterile milk (Control)	+ Ve	+ Ve

*Starter A: a starter culture previously lived and stored with the tested E.coli strains carrying multidrug resistance plasmids.

**Starter B: Clean starter culture.

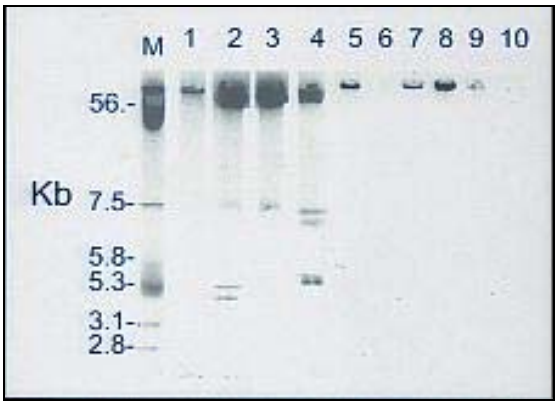

Fig. (1): Plasmid profile analysis of multidrug resistance *E.coli* strains recovered from bovine subclinical mastitis.

DISCUSSION :

In recent years, there is an alarming increase in the rate of human infections with antibiotic resistant microorganisms (Salvat *et al.* 2001). Two main categories of bacteria carrying genes encoding for antibiotic resistance may be transmitted from animals to humans via food products. The first category is the obligate infectious pathogens as Salmonella enterica, while the second one is the facultative pathogenic species as *E.coli* (Wagner and Hahn, 1999), the matter we concerned in the present work. The public health hazard of *E.coli* strains when they are present in human gut is manifested not only in being faculatative pathogens, but also in disseminating their multidrug resistance plasmids and infecting other microorganisms since the transference of these plasmids can take place and the exchange may occur even between gram +ve and gram -ve bacteria through conjugational genetic transfer (Mazodier and Davis, 1991 and Kessie *et al.*, 1998).

1-Antimicrobial susceptibility and plasmid profile analysis:

The obtained results proved that *E.coli* strains recovered from bovine subclinical mastitis were untypable as they are negative for serotype reactions against the nine available antisera. Through screening of plasmid existance, only four strains out of ten multidrug resistant *E.coli* were carrying plasmids with molecular sizes ranged from 5.3-7.5 kilo base pairs (kb)- (Fig.1). The highly antibiotic resistant strains carrying no plasmids indicated that the encoding genes were located chromosomally giving rise to perminant non transferable high level of resistance (Thomas *et al.,* 1999). *E.coli* strain No. 4 was selected for yoghurt inoculation process which was multidrug resistant carrying four plasmids (Fig.1).

2- Survival of *E.coli* in yoghurt :

As recorded in Table (1), *E. coli* decreased in numbers from 1.3×10^7 to 3.2×10^6 cells/ ml during preparation of yoghurt (at 37±1°C). The organism began to lose its viability very slowly during refrigerated storage at 4±1°C and reached a minimum of 3.7×10^5 cells/ml. The data in Table (1) indicated that there was no significant change in the number of *E.coli* during the 96 hours following yoghurt preparation. The numbers began to decrease slowly during the storage, and the organism survived until the end of the week at a population of 3.7×10^5 cells/ml.

There was a slow drop in the pH value of yoghurt during its preparation and storage to reach its minimum value (4.03) by the end of the week. No significant changes in the pH value of yoghurt during the 3rd, 4th and 5th day were observed

It is obvious from the obtained results (Table 1) that *E.coli* could survive until the end of 7 days and existed in large numbers (3.7×10^5 cells/ml) however, the pH value was unfavourable. This could be explained on the fact that the low fermentation temperature (37°C) used for preparation of yoghurt allows slow production of lactic acid which may creat the opportunity for the organism to adapt such acid medium. Also, the survival of such organism could be attributed to the nature of the strain used which may had the ability to resist acid medium. Such phenomenon was observed by Gibson *et al.* (2002) who proved that *E.coli* O157 could survive and increased in numbers in yoghurt prepared at temperature below 40°C (25-37°C) despite the continued decline in acidity of milk. Also, they found that when yoghurt prepared at temperatures ranged from 40-43°C, *E.coli* O157 died quickly and could not be detected by the end of storage (4 days) at 4°C. Ahmed (1990) obtained different results and proved that Enteropathogenic *E.coli* O125/B15 survived in yoghurt kept at 5 ±1°C for ten days and could not be detected by the end of storage (<10/ ml). Furthermore, El-Hawary and Aman (1998) stated that *E.coli* O157:H7 could survive in yoghurt for 9 days reaching to a minimum count of 40 cells/ml and the organism failed to recover by the end of the 11th day. The variation in such results could be attributed to many factors including nature of the strain used, temperature of initial incubation (during preparation), storage temperature which may control multiplication of the organism but the inoculation dose at storage is quite important.

The obtained results indicated that the fermentation acids were not effective against such *E.coli* carrying multidrug resistance plasmids and could posses a potential health hazard. It has been stated that antibiotic resistant non pathogenic organisms in an animal may be passed to, and colonize humans carrying R-plasmids (transmissible) into

human environment. These R-plasmids may subsequently be transferred to human pathogens or flora (Levey 1992). Their transamission via food products and their zoonotic importance were established and widely discussed (Singh *et al.*,1992 & Wagner and Hahn 1999).

3-Assessment of plasmids (encoding multidrug resistance) transference from *E.coli* into starter culture of yoghurt:

As shown in Table (2), yoghurt failed to be produced from sterile milk containing the added MIC of the tested antibiotics either by using starter cultures lived with *E.coli* carrying multidrug resistance plasmids or by using clean yoghurt starter culture. Fortunately, these results indicated that *E.coli* strains failed to infect starter cultures of yoghurt with such plasmids. The failure of plasmid transference may be attributed to the very small molecular sizes of plasmids carried by the tested *E.coli* strain (5.3-7.5kb) as the smallest conjugative plasmids in *Enterobacteriaceae* detected to be transferred are about 30 kb (Scott, 1984; Thomas and Smith, 1987 and Bennett & Howe, 1998). Also, it has been stated that the plasmids can be transferred between strains of the same species and between strains of different species, whereas the frequency of transfer is reduced in fusion between different species somewhat from that of intraspecific fusion (Bennett & Howe, 1998).

In conclusion, contamination of yoghurt or other dairy products by *E.coli* from the view point of public health should not be ignored. The development of populations of *E.coli* with transmissible drug resistant plasmids in food posses a potential threat to public health. Also, it is concluded that fermentation acids are not effective against the drug-resistant *E.coli*, therefore if pasteurized milk is contaminated with such organism, the fermented products posses a potential health risk.

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نمو وبقاء ميكروب الايشيريشيا كولاى الحامل للبلازميدات الخاصة بمقاومة العديد من المضادات الحيوية أثناء إعداد وتخزين الزبادى مع الإشارة إلى خطورته على الصحة العامة

محمد محمد عبد الحفيظ ، آمال على عبد الحليم

بفحص عينات من لبن أبقار مصابة بالتهاب الضرع الخفى تم عزل عترات من ميكروب الايشيريشيا كولاى وتم تصنيفها بيوكيميائيا وبفحصها سيرولوجيا باستخدام ٩ من مضاد الأمصال المتاحة تبين أنها غير مصنفة سيرولوجيا. وكذلك تم إجراء اختبارات الحساسية لعدد ١٣ مضاداً حيوياً (سيفوبيد، اوفلوكساسيللين، كلافوران، ستربتومايسن، سبيراميسين، سيفرادين، كلوكساسيللين، أمبيسلين، يوناسين، أموكسيسيلين، بنسلين ج، سيبروسين، بوليمكسين ب سلفات). وتم اختبار عشرة عترات من هذه الميكروبات لها المقاومة المتعددة لهذه المضادات الحيوية وذلك لاستقصاء وجود البلازميدات الخاصة بها وباستخدام التحليل الالكتروفوريسى تبين أن أربعة منها كانت تحمل بلازميدات متعددة. وقد اختلفت أحجامها الجزئية وتراوحت من ٥،٣ –٧،٥ كيلوبيز بير (Kb). تم اختيار إحدى هذه العترات وكانت تحمل عدد ٤ بلازميدات للحقن فى لبن معقم بجرعة ابتدائية ١،٣× ١٠^{٧} ميكروب/مل بعد إضافة بادئ الزبادى وتم التحضين عند درجة ٣٧±١° م. وعند إتمام التخمر كان العدد البكتيرى ٣،٢ × ١٠^{٦}ميكروب/مل ودرجة الأس الهيدروجينى ٥،٢٤. وقد تم التخزين عند درجة ٤±١°م ولمدة ٧ أيام. وقد أسفرت النتائج عن تواجد الميكروب فى نهاية الأسبوع بجرعة كبيرة ٣،٧× ١٠^{٥} مقاوما للحموضة حيث بلغ الأس الهيدروجينى حينئذ ٤،٠٣ ، ولقد تمت مناقشة مدى خطورة تواجد هذه الميكروبات الحاملة للبلازميدات فى الزبادى ومنتجات الألبان على الصحة العامة للإنسان. ولدراسة مدى احتمال انتقال بلازميدات المقاومة البكتيرية للمضادات الحيوية إلى ميكروب البادئ تم إجراء تجربة إعداد زبادى من بادئات جديدة ، وكذلك من بادئات الزبادى المصابة بميكروب العترة المختبرة فى ألبان معقمة مضافا إليها جرعات من بعض المضادات الحيوية

المقاوم لها العترة المختبرة (يوناسين، سيفرادين، كلافوران، كلوكساسيللن) بأقل جرعة مثبطة كل على حدة . وأسفرت النتائج عن عدم إمكانية انتقال هذه البلازميدات إلى ميكروبات بادئ الزبادى وتمت مناقشة النتائج.