Annals of Tropical Medicine & Parasitology, Vol. 103, No. 1, 39–52 (2009)

Antimicrobial resistance and pathogenicity of *Escherichia coli* isolated from common dairy products in the Lebanon

I. SALEH[*], O. ZOUHAIRI[†], N. ALWAN[‡], A. HAWI[§], E. BARBOUR[¶] and S. HARAKEH[†]

[*]*Dubai International School, P.O. Box 15495, Dubai, United Arab Emirates*
[†]*Biology Department, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon*
[‡]*Forschungsinstitut Senckenberg, Sektion Ichthyologie, Senckenberganlage 25, D-60325 Frankfurt am Main, Germany*
[§]*Dubai Chamber of Commerce and Industry, P.O. Box 1457, Dubai, United Arab Emirates*
[¶]*Animal and Veterinary Sciences Department, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon*

Received 28 May 2008, Revised 25 September 2008, Accepted 29 September 2008

In a recent study, bacteria have been isolated from popular Lebanese dairy products, which had been collected in the Beqaa Valley, in north–eastern Lebanon. The foods investigated were two cheeses (*shankleesh* and *baladi*) and a dried fermented mixture of yogurt and wheat grains (*kishk*). Bacterial colonies on McConkey and sorbitol–McConkey agar that showed the morphology of *Escherichia coli* were biochemically tested and then classified, using PCR-based assays, into the various strains of pathogenic and non-pathogenic *E. coli*. Some of the confirmed *E. coli* isolates were proven to be pathogenic, including two identified as *E. coli* O157:H7. When the pathogenic isolates were tested for their susceptibility to 10 different antibiotics (all commonly used, by clinicians and veterinarians, for the treatment of infections with Gram-negative bacteria), each tested isolate was found to be highly resistant to at least one antibiotic. It therefore appears that, in Lebanon, some popular dairy products pose a public-health hazard, acting as vehicles for the transmission of drug-resistant pathogens.

Each year, bacterial contamination causes millions of cases of food-borne illness worldwide (Prier and Solnick, 2000; Lamps, 2003). Milk and dairy products are highly susceptible to microbial contamination because their composition provides a favorable medium for the growth of a wide variety of micro-organisms (De Buyser *et al.*, 2001; Van Kessel *et al.*, 2004).

Virulent strains of *Escherichia coli*, which are usually classified into five main groups according to their molecular characteristics, are the most common cause of diarrhoeal infections and outbreaks (O'Ryan *et al.*, 2005). Enterotoxigenic *E. coli* (ETEC) are characterised by the expression of genes coding for heat-stable and heat-labile enterotoxins (Steinsland *et al.*, 2003). The bacteria that carry the *E. coli* attachment and effacement gene *eaeA* are known as enteropathogenic *E. coli* or EPEC (Evans and Evans, 1996). Carriage of the gene (*ipa*H) coding for the invasion plasmid antigen, which enables the pathogens to invade epithelial cells, distinguishes entero-invasive *E. coli* (EIEC) from the other groups of *E. coli* (Matar *et al.*, 2002). Among the pathogenic varieties of *E. coli*, only entero-aggregative *E. coli* (EAEC) carry a pAA plasmid. This plasmid codes for the aggregative adherence fimbrae that facilitate the pathogens' adherence to intestinal mucosa (Wanke, 1995; Henderson *et al.*,

Reprint requests to: S. Harakeh.
E-mail: sharakeh@gmail.com; fax: +961 5468476.

DOI: 10.1179/136485909X384965

1999; Sheikh *et al.*, 2002). Enterohaemorrhagic *E. coli* (EHEC), which are a very common cause of diarrhoea and food-borne outbreaks world-wide, are genetically characterised by the *eaeA* gene, which codes for the protein intimin.

The O157:H7 serogroup is the most broadly studied of the EHEC because of its direct involvement in many serious outbreaks of human illness, including some associated with the ingestion of milk or dairy products (Heuvelink *et al.*, 1999). Each strain of *E. coli* O157:H7 produces 'shiga-like' toxins 1 and/or 2 (Griffin and Tanke, 1991; Li and Drake, 2001; Fraser *et al.*, 2004; Tarr *et al.*, 2005).

Since milk is rich with all kinds of nutrients, it is a convenient medium for bacterial growth. Foods made from unpasteurized milk should therefore be considered as possible vehicles for the transmission of various types of pathogenic bacteria (Gardner, 1997). Of the 183 food-borne outbreaks of human illness caused by *E. coli* O157:H7 that were reported by the United States Centers for Disease Control (CDC) between 1982 and 2002, 4% were associated with the consumption of dairy products (Rangel *et al.*, 2005). More recent outbreaks caused by *Escherichia coli* O157:H7 in the U.S.A. have been linked to the consumption of raw milk (Anon., 2008). Cheese has been implicated in many food-borne outbreaks world-wide, especially in areas where cheese is frequently made with unpasteurized milk. In France, for example, where 20% of the cheese produced is made from raw milk, eight of the 10 outbreaks of food-borne illness reported between 1983 and 1994 were found to be caused by cheese made from raw milk, with two of the eight caused by EHEC (De Buyser *et al.*, 2001). In Italy, a recent outbreak of gastro–enteritis was blamed on the consumption of pecorino cheese made from unpasteurized sheep's milk, with EAEC in the cheese the possible cause (Scavia *et al.*, 2008).

The contamination of food with human pathogens becomes an even greater (potential) problem when the pathogens involved are drug-resistant. The emergence of such pathogens is mainly the result of the indiscriminate and excessive use of antimicrobial drugs in human and veterinary medicine, especially in countries, such as Lebanon, where the distribution of such drugs is uncontrolled (Cizman, 2003). If drug resistance is acquired by both pathogenic and non-pathogenic bacteria, the clearance of the pathogens (e.g. by the host's immune system) may not prevent future infective pathogens from acquiring resistance from the non-pathogenic forms. Resistant bacteria may colonize soil, sewage, surface water and food, as well as the intestinal tracts of humans and other animals (Reinthaler *et al.*, 2003). *Escherichia coli* is known to acquire resistance in many ways, including the production of β-lactamase and the exchanging of mobile elements such as plasmids (Kallman *et al.*, 2003; Soto *et al.*, 2003). The study of antimicrobial resistance in clinical or environmental isolates is crucial to preventing the spread of multi-drug-resistant strains of bacteria (Van Duijkeren *et al.*, 2003).

There appear to have been no previous studies on the microbiology of Lebanese dairy products made from unpasteurized milk. The main aim of the present study was to help fill this gap in knowledge, by investigating some of the bacteria in the three most common dairy products in the Beqaa Valley: *baladi*, *kishk* and *shankleesh*. *Baladi* is a white, semi-soft cheese, usually made at home. *Shankleesh* is a mould-ripened cheese prepared from sheep's yogurt that is heated in a steam-jacketed kettle and defatted. The defatted yogurt is left to drain for 48 h, salted, rolled into balls, left to dry in the open air for 3 days, and then stored in jars for 20–30 days until completely covered with wild mould. After the surface mould is scraped off, each ball of cheese is coated with melted butter and rolled in powdered thyme (Toufeili *et al.*,

1995). The finished *shankleesh* is then eaten immediately or after storage in olive oil. *Kishk* is a dried mixture of fermented yogurt and wheat grains. The wheat is simmered in water until soft, then washed, dried in the open air, ground, mixed with yogurt, strained yogurt (*labneh*) and salt, and left to ferment. Every second day, more yogurt, strained yogurt and salt are added. Fifteen days later, when the fermentation is complete, the product is cut into pieces, partially dried in the sun, milled, sieved, dried further, and finally stored in glass jars.

In the present study, the prevalence and antimicrobial resistance of pathogenic and non-pathogenic *E. coli* in samples of *baladi*, *kishk* and *shankleesh* were assessed, as a first step in the control of milk-borne disease in Lebanon.

MATERIALS AND METHODS

Sample Collection

Samples of *baladi*, *kishk* and *shankleesh* were bought, between the August and December of 2004, from supermarkets, small markets and homes in communes in the north–eastern part of the Beqaa Valley, in Lebanon. Only samples offered for sale to consumers were purchased. The collector wore a new pair of sterile gloves for collecting each sample, and the person selling each sample was asked to place it directly inside a sterile plastic bag (which was held open by the collector). Each bag holding a sample was closed carefully and kept, in a small refrigerator, at 4°C until it could be transferred to a larger refrigerator in the research laboratory. Each collection trip lasted 6–8 h, and most samples were analysed within 24 h of their arrival in the laboratory.

The communes in the study area were divided into four size categories according to number of inhabitants, with samples of dairy food collected from 20%–100% of the communes in each category (Table 1). The majority of samples from the smallest communes, which had no supermarkets or markets, were homemade. The initial plan was to collect approximately 220 samples, with the number of samples collected in each study commune roughly proportional to the size of the commune (Table 2). Some of the small communes selected for study could not be visited, however, because bad weather made the roads to them impassable. In addition, homemade dairy products were available only intermittently, and sometimes not during a collection trip. The total number of samples collected (164 — 83 of *kishk*, 45 of *baladi* and 36 of *shankleesh*) therefore fell below the target value.

Bacterial Isolation and Bacteriological Analysis

Aerobic plate counts (APC) and total coliform counts were used as indicators of the microbiological quality of each sample. Any *E. coli* present were isolated and studied. For the microbiology, a 25-g portion of each food sample was placed aseptically in a sterile stomacher bag (Seward, Worthing, U.K.) with 225 ml sterile 1% peptone water

TABLE 1. *The sizes and numbers of communes in which dairy products were collected*

Size of commune (no. of inhabitants)	No. of communes in study area	Combined population	% of population in study area	No. of communes and (% of the communes in size-class) selected for study
<1000	181	67,730	19.6	36 (20)
1000–5000	75	16,3300	47.2	34 (45)
5000–10,000	5	31,000	9.0	5 (100)
>10,000	4	84,000	24.3	4 (100)
Any	265	346,030	100	79

(Hi-Media, Mumbai, India) and macerated in a stomacher for 3 min. Ten-fold serial dilutions of the resultant homogenate (10^{-1}–10^{-6}) were then prepared, using more sterile peptone water (Harakeh *et al.*, 2005). For the APC, each of the six dilutions was inoculated onto plate-count agar (Oxoid, Basingstoke, U.K.) and incubated at 37°C for 24 h (Hayes *et al.*, 2001). Only the three lowest dilutions were inoculated onto plates of McConkey (MC) agar (Oxoid), with incubation at 37°C for 24 h, and onto plates of sorbitol–McConkey (SMC) agar (Oxoid), with incubation at 42°C for 18–24 h, for total coliform counts and the selection of pathogenic *E. coli*, respectively (Mead and Griffin, 1998; Galland *et al.*, 2001; Hayes *et al.*, 2001; Ramesh *et al.*, 2002; Velázquez Ldel *et al.*, 2005).

Further bacteriological analyses were performed according to the *Compendium of Methods for the Microbiological Examination of Foods* (Downes and Ito, 2001) and *Official Methods of Analysis of AOAC International* (Horwitz, 2000).

Colonies developing on the plate-count agar were counted so that the number of colony-forming units (cfu)/g of each sample could be determined. Samples that showed no bacterial growth or growth of fewer than 30 colonies were grown in enrichment medium. Each colony showing a colour and morphology indicative of *E. coli* was simultaneously patched onto clean plates and cultured in 5 ml brain–heart infusion (BHI) broth (Oxoid). The number of suspected *E. coli* colonies investigated/sample depended on the number of *E. coli* cfu/g. In general, only two colonies were selected from each sample showing <100 cfu/g but four were selected from each sample showing 100–10,000 cfu/g, and eight from each sample showing >10,000 cfu/g. When colonies on a plate showed different colours and/or morphologies, at least two colonies of each variant were selected. Plates and tubes were incubated at 37°C for 24 h. The plates were then stored at 4°C, while a 0.5-ml subsample of each broth culture was mixed with 0.5 ml of sterile 5.43 M glycerol, in a cryotube, and then stored at −70°C (Sprong *et al.*, 2001) until further investigation.

Recovery and Reproducibility of Bacterial Counts

Reference cultures of *E. coli* obtained from the American Type Culture Collection (via the American University Hospital, Beirut) were grown in BHI broth overnight. The bacterial cells were harvested by centrifugation and then resuspended in sterile 0.1 M phosphate-buffered saline at pH 7.2 (PBS; Lonza, Verviers, Belgium) to give a suspension ('solution A') to be used for seeding. Subsamples (1 ml) of solution A were added to tubes that each contained 9 ml BHI broth and then the bacteria in each of these suspensions were counted, using an appropriate selective medium, to determine the number of bacteria in solution A. Simultaneously, 10 tubes, each containing 9 ml sterile milk, were each inoculated with 1 ml of solution A. A 0.1-ml sample from each of these 10 tubes was then plated on selective medium. All plates were incubated for 24 h at 37°C before the counts were recorded (Bottero *et al.*, 2004). In order to

TABLE 2. *Numbers of samples collected on each sampling trip*

Size of commune (no. of inhabitants)	No. of communes visited	No. of markets visited/commune	No. of samples collected/commune
<1000	3 or 4	1	3 or 4
1000–5000	3 or 4	3	9–12
>5000–10,000	3	1	3
>10,000	3	2	6

test the sensitivity of the PCR-based assay used to type the *E. coli* isolates from the dairy products (see below), the DNA of the bacterial colonies recovered from the milk cultures was extracted and tested in the assay (Bottero *et al.*, 2004).

Biochemical Analysis

Colonies with a pink to violet colour on MC agar and those that were colourless or grey on SMC agar were selected as suspected *E. coli* and tested by Gram staining (Velázquez Ldel *et al.*, 2005). All the Gram-negative bacilli were checked for their ability to use citrate and ferment lactose; these two tests help to characterise *E. coli* since this species is the only known species within the Enterobacteriaceae that is citrate-negative and lactose-positive (Mead and Griffin, 1998; Saida *et al.*, 1998; Fujisawa *et al.*, 2000; Niemela *et al.*, 2003). Colonies suspected to be *E. coli* were finally confirmed using the API 20E biochemical system (bioMérieux, Marcy l'Étoile, France) and then molecularly characterised in a PCR-based assay (Neubauer *et al.*, 1998; McLellan *et al.*, 2001; see below).

DNA Extraction

DNA was extracted from *E. coli* colonies using a commercial kit (GFX genomic blood DNA purification kit; Amersham Biosciences, Little Chalfont, U.K.). Bacterial cultures grown overnight were centrifuged, lysed with proteinase K buffer [12 mM Tris-HCl (pH 8.0), 6 mM EDTA, 3.46 mM sodium dodecyl sulphate], and resuspended in proteinase K solution [20 mg/ml, in 10 mM Tris-HCl (pH 8.0)]. RNase A (20 mg/ml) was then added, followed by the addition of the extraction solution. The mixture was filtered through a GFX column before the DNA in the column was washed using wash solution (Tris–EDTA buffer and absolute ethanol). The extracted DNA was eluted using 70 µl pre-heated, sterile 1× TE (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and stored at −20°C prior to its use in the PCR-based assay (Matar *et al.*, 2002).

PCR-based Assays

For the adequate identification of bacterial species, techniques based on molecular biology, such as PCR, are now often the preferred choice (Lukásová *et al.*, 2004; Lukinmaa *et al.*, 2004). In the present study, PCR-based assays were used to characterise the *E. coli* isolates. The first assay, using primers (Eco 223 and Eco455) based on a highly conserved and species-specific region of the DNA coding for the 23S ribosomal RNA of *E. coli* (Riffon *et al.*, 2001), was used to distinguish the *E. coli* colonies from those of other species. The second set of assays was based on primers (Amresco, Solon, OH) that allow *E. coli* to be identified as EIEC, ETEC, EAEC, EPEC or EHEC (Table 3). The final assay was based on primers that amplify the *flic* gene that characterises *E. coli* O157:H7 (Table 3). The sequences of all the primers used and the sizes of the corresponding amplicons are shown in Table 4.

Each PCR was performed using a 25-µl reaction mixture containing 1.25 µl of each primer (10 pm/µl), 3 µl purified DNA solution (50 ng/µl), 2.5 µl 10× PCR reaction buffer, 1.875 µl $MgCl_2$ (25 mM), 0.5 µl of each deoxynucleoside triphosphate (100 mM), 0.2 µl Taq DNA polymerase (5 U/µl; ABgene, Epsom, U.K.) and sterile double-distilled water. Negative controls (no DNA template) and positive controls (DNA from appropriate reference samples of *E. coli*) were included in each set of assays (Harakeh *et al.*, 2005).

For the sizing of the amplicons, 10 µl of the PCR products were mixed with 2 µl loading dye (6×; Bio-Rad) and run on a 1.5%-agarose gel containing 0.25 µg ethidium bromide/ml. The amplicons were visualized by ultra-violet trans-illumination and photographed (Harakeh *et al.*, 2005).

TABLE 3. *The primers used in the PCR-based assays for the identification and categorization of* Escherichia coli, *and their targets*

Identified bacteria	Primers	Amplified target	Reference
Escherichia coli	Eco 223 and Eco 455	16S and 23S rRNA	Riffon *et al.* (2001)
Entero-invasive *E. coli* (EIEC)	IPAH15B and IPAH8B	Invasion plasmid antigen (*ipaH*)	Matar *et al.* (2002)
Enterotoxigenic *E. coli* (ETEC)	LTIb-1 and LTI-2	Heat-labile toxin (LT)	Matar *et al.* (2002)
	STI-1 and STI-2	Heat-stable toxin (ST)	Matar *et al.* (2002)
Entero-aggregative *E. coli* (EAEC)	PCVD432/START and PCVD432/STOP	Plasmid that mediates the entero-aggregative adherence to HEP-2 cells (pCVD432S)	Matar *et al.* (2002)
Enteropathogenic *E. coli* (EPEC)	eaeA-F and eaeA-R	Intimin protein for bacterial attachment.	Reischl *et al.* (2002)
Enterohaemorrhagic *E. coli* (EHEC)	eaeA-F and eaeA-R	Intimin protein for bacterial attachment.	Reischl *et al.* (2002)
	LP30 and LP31	Shiga toxin 1 (*stx*1)	Osek and Gallien (2002)
	LP43 and LP44	Shiga toxin 2 (*stx*2)	Osek and Gallien (2002)
	MFS1F and MFS1R	Plasmid enterohaemolysin gene (E*hly*)	Matar *et al.* (2002)
E. coli O157:H7	Flic-F and Flic-R	Gene coding for H7 flagella (*flic*)	Osek and Gallien (2002)

Antimicrobial Resistance

Each isolate identified as *E. coli* was tested for its susceptibility to antimicrobial drugs commonly used to treat infections with Gram-negative bacteria (Webber and Piddock, 2001; Cizman, 2003; Cole *et al.*, 2003; Murakami and Yamaguchi, 2003), using the disk-diffusion method (Anon., 2004). Each isolate was cultured overnight in 5 ml BHI broth, in a shaking waterbath at

TABLE 4. *The sequences of the primers used in the PCR and the sizes of the expected amplicons*

Primer	Sequence (5′–3′)	Size of amplicon (bp)
Eco 223	ATCAACCGAGATTCCCCCAGT	232
Eco 455	TCACTATCGGTCAGTCAGGAG	
IPAH15B	GCCGGTCAGCCACCCTCTGAG	620
IPAH8B	GTTCCTTGACCGCCTTTCCGA	
LTIb-1	TCTCTATGTGCATACGGAGC	320
LTI-2	CCATACTGATTGCCGCAAT	
STI-1	TTAATAGCACCCGGTACAAGCAGG	147
STI-2	CTTGACTCTTGAAAAGAGAAAATTAC	
PCVD432/START	CTGGCGAAAGACCGTATCAT	630
PCVD432/STOP	CAATGTATATAAATCCGCTGTT	
eaeA-F	GACCCGGCACAAGCATAAGC	364
eaeA-R	CCACCTGCAGCAACAAGAGG	
Stx1-F	CGATTAATGTCGTGGCGAAGG	348
Stx1-R	CACCAGACAATGTAACCGCTG	
Stx2-F	ATCCTATTCCCGGGAGTTTACG	584
Stx2-R	GCGTCATCGTATACACAGGAGC	
MFS1F	ACGATGTGGTTTSTTCTGGA	166
MFS1R	CTTCACGTCACCATACATAT	
Flic-F	GCTGCAACGGTAAGTGAT	984
Flic-R	GGCAGCAAGCGGGTTGGT	

37 °C. A 0.1-ml subsample of each culture was then inoculated onto a plate of Mueller–Hinton agar (Bio-Rad) before disks impregnated with the drugs (bioMérieux) were placed on the plate. The drugs tested were chloramphenicol (30 μg), tetracycline (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), gentamicin (10 μg), ciprofloxacin (5 μg), cefotaxime (30 μg), cefuroxime (30 μg), ofloxacin (5 μg), nalidixic acid (30 μg) and ampicillin (10 μg). Zones of inhibition around each disk were measured after incubation at 37°C for 18–24 h. For each drug, each isolate was classified as highly resistant (not inhibited), moderately resistant (incompletely inhibited) or susceptible (totally inhibited), according to the guidelines of the National Committee for Clinical Laboratory Standards in the U.S.A. (Harakeh *et al*., 2005).

Statistical Analysis

The data were analysed using version 11.0 of the SPSS software package (SPSS Inc., Chicago, IL). Kurtosis showed non-normality within the distribution of the counts of colony-forming units, and Bartlett's test (for comparison of variances) demonstrated that there were statistically significant differences between the standard deviations recorded. The latter result not only violates one of the assumptions underlying analysis of variance (ANOVA) but also invalidates most standard statistical tests. Kruskal–Wallis tests were used to compare median counts for the different foods, communes, and collection dates. To check if any of the three types of dairy product was significantly more likely to harbour pathogenic *E. coli* than either or both other types, χ^2 tests were used to compare the proportions. Finally, Friedman and Nemenyi tests were performed to see if resistance to any one of the 10 antimicrobial drugs investigated was significantly more common than resistance to any of the other nine drugs.

RESULTS

Bacteriological Counts and Microbiological Quality of the Tested Dairy Products

On plate-count agar, every food sample tested gave a count of at least 1000 cfu/g but the *baladi* had significantly greater densities of coliform bacteria ($P<0.01$) and *E. coli* ($P<0.01$) than the *kishk* or *shankleesh* (Table 5). In terms of microbiological quality, the *kishk* appeared to be the best of the dairy products.

In terms of bacterial counts, there were no statistically significant differences between

TABLE 5. *The counts made of aerobic bacteria, coliforms and* Escherichia coli *in samples of the three dairy products*

	No. of colony-forming units/g								
	0	$1–10^3$	$>10^3–10^4$	$>10^4–10^5$	$>10^5–10^6$	$>10^6–10^7$	$>10^7–10^8$	$>10^8–10^9$	$>10^9$
AEROBIC BACTERIA IN:									
Kishk (% of samples)	0	0	2.4	12	22.8	32.5	20.4	8.4	1.2
Shankleesh (% of samples)	0	0	0	44.4	11.1	16.6	11.1	11.1	5.5
Baladi (% of samples)	0	0	0	17.7	28.8	17.7	17.7	8.8	8.8
TOTAL COLIFORMS IN:									
Kishk (% of samples)	85.5	3.6	7.2	3.6	0	0	0	0	0
Shankleesh (% of samples)	61.2	0	11.1	27.7	0	0	0	0	0
Baladi (% of samples)	17.7	2.2	20	31.1	17.7	6.6	4.4	0	0
Escherichia coli IN:									
Kishk (% of samples)	92.7	2.4	2.4	2.4	0	0	0	0	0
Shankleesh (% of samples)	72.2	0	16.6	11.1	0	0	0	0	0
Baladi (% of samples)	33.3	4.4	17.7	22.2	13.3	6.6	2.22	0	0

the (mostly homemade) products collected in the smallest communes and those sold (mostly in supermarkets and large markets) in the larger communes investigated. The season in which the sample was collected also appeared to have no significant effect on the bacterial counts (data not shown).

According to the guidelines of the Lebanese Standards Institution (Libnor) — which state that *kishk* must be completely free of coliforms, including *E. coli*, and that Lebanese cheese must meet the microbiological standards set internationally (Gilbert *et al.*, 2000) — 66.4% of the *baladi* samples tested in the present study but only 14.4% of the *kishk* samples were unfit for human consumption (Table 6).

Recovery Test Results

In the recovery test, the number of *E. coli* colony-forming units appeared almost identical whether the bacteria were suspended in culture medium (BHI broth) or sterile milk, indicating that milk had no inhibitory effects on bacterial recovery. The colonies of the reference isolates of *E. coli* used showed the expected band, of 232 bp, when tested in PCR with the Eco 223 and Eco 455 primers.

Biochemical Identification of *E. coli*

Colonies of all morphologies were selected from the MC and the SMC plates and patched. Out of the 340 patches produced, only 173 showed the morphologies expected of *E. coli*. Although all 173 '*E. coli*-like' isolates were found Gram-negative, just 102 were not only found citrate-negative and lactose-positive but also gave results indicating that they were *E. coli* when tested in the API20E biochemical system. DNA from each of the 102 isolates identified biochemically as *E. coli* was then investigated in the various PCR-based assays.

TABLE 6. *The percentages of the samples of dairy products that did not meet Lebanese microbiological standards because they contained too many coliform bacteria (of any type) and/or* Escherichia coli

	% of samples failing to meet standard		
Standard	*Baladi*	*Shankleesh*	*Kishk*
Total coliforms	60.0	27.7	14.4
Escherichia coli	66.4	28.5	7.2

Identification and Classification of *E. coli* by PCR

In the initial assays, which were based on the species-specific set of primers (Eco 223 and Eco 455), 94 of the 102 isolates checked each gave a clear band of 232 bp, indicating that they truly were *E. coli* (Fig. 1). When these 94 confirmed isolates of *E. coli* were tested using other PCR-based assays, 36 (38.3%) were found to be pathogenic. With the PCVD432/START and PCVD432/STOP primers, three isolates each gave the 630-bp amplicon indicating that they were EAEC. Another 23 isolates were identified as ETEC, with 21 each giving a 320-bp amplicon with the LT set of primers, indicating the presence of the gene coding for the heat-labile toxin (Fig. 2), and three isolates (including one positive for the heat-labile toxin) each giving a 147-bp with the ST set of primers, indicating the presence of the gene coding for the heat-stable toxin. None of the isolates was classified as EIEC but 11 each gave a 364-bp amplicon in assays with the eaeA-F and eaeA-R primers, indicating that they were EPEC or EHEC. As only five of these 11 isolates each gave a 166-bp amplicon when the primers used were MFS1F and MFS1R, the other six isolates were classified as EPEC. When, simultaneously, the same 11 isolates were tested for the presence of the shiga-toxin-1 gene (*stx*1), the shiga-toxin-2 gene (*stx*2) and the *flic* gene that codes for the H7 flagella, four of the isolates gave positive results with the *stx*2 set of primers (each giving a 584-bp) amplicon and were therefore identified as EHEC (none of the isolates tested carried the *stx*1 gene). Finally, two of the four EHEC were found

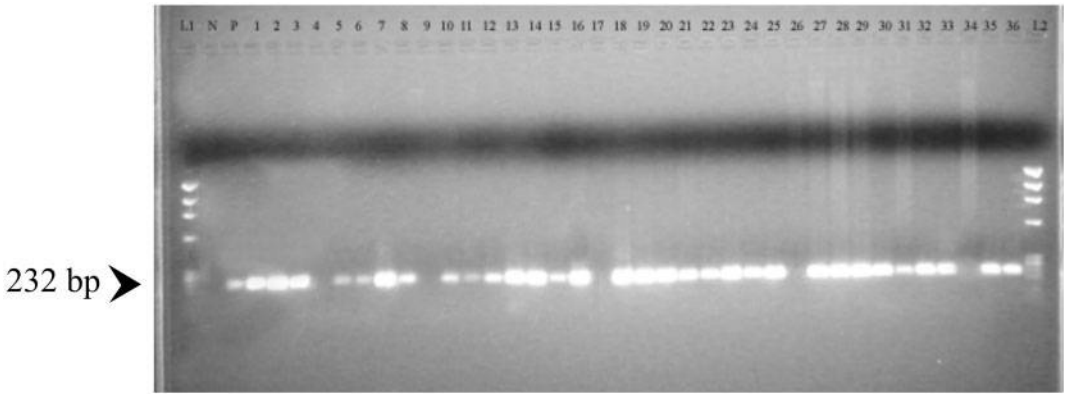

FIG. 1. The results of a PCR-based amplification using a set of primers (Eco 223 and Eco 455) that are species-specific for *Escherichia coli*. The samples run, in 1.5% agarose, were a DNA ladder (L1 and L2), a negative control (N), a positive control of DNA from a reference strain of *E. coli* (P), and DNA extracted from *E. coli* isolated from samples of Lebanese *baladi* (lanes 1–5, 12–20, 34 and 36), *kishk* (lanes 6–10, 22–29, 33 and 35) and *shankleesh* (lanes 11, 21 and 30–32). Thirty-one of the 36 isolates from dairy products gave a positive result, each giving an amplicon of 232 bp.

positive for the *flic* gene, indicating that they were *E. coli* O157:H7.

In summary, of the 94 confirmed *E. coli* isolates, four (4.3%) were identified as EHEC [including two (2.1%) as *E. coli* O157:H7], six (6.4%) as EPEC, 23 (24.4%) as ETEC, and three (3.2%) as EAEC.

The highest frequency of contamination with pathogenic *E. coli* (46.6%) was found in the *baladi* samples ($P<0.05$). Just 15.6% of the *kishk* samples tested positive for pathogenic *E. coli* (and then usually only after being grown on enrichment medium) and only 8.3% of the samples of *shankleesh* were found to harbour pathogenic *E. coli*. Most of the ETEC detected came from the *baladi* whereas most of the EAEC, EPEC and EHEC came from the *kishk* (Table 7).

Antimicrobial Susceptibility of the Confirmed *E. coli* Isolates

Both the pathogenic and non-pathogenic isolates of *E. coli* from the dairy products were tested for their susceptibility to 10 commonly used antimicrobial agents (Fig. 3). Every tested isolate was found resistant to at least one of the antibiotics, with resistance to some of the drugs significantly more common than resistance

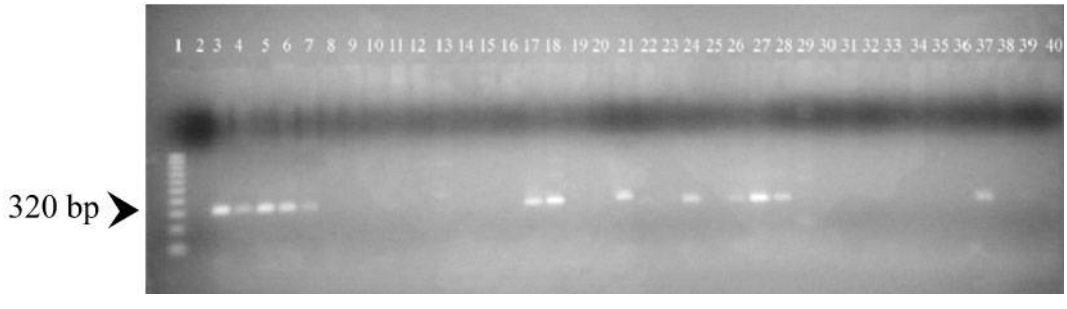

FIG. 2. The results of a PCR-based amplification using a set of primers (LTIb-1 and LTI-2) that are specific for the gene coding the heat-labile toxin of enterotoxigenic *Escherichia coli*. The samples run, in 1.5% agarose, were a DNA ladder (lane 1), a negative control (lane 2), a positive control of DNA from a reference strain of enterotoxigenic *E. coli* (lane 3) and DNA extracted from *E. coli* isolated from samples of Lebanese *baladi* (lanes 4–27), *shankleesh* (lanes 28–33) and *kishk* (lanes 34–40). Thirteen of the 31 isolates from dairy products gave a positive result, each giving an amplicon of 320 bp.

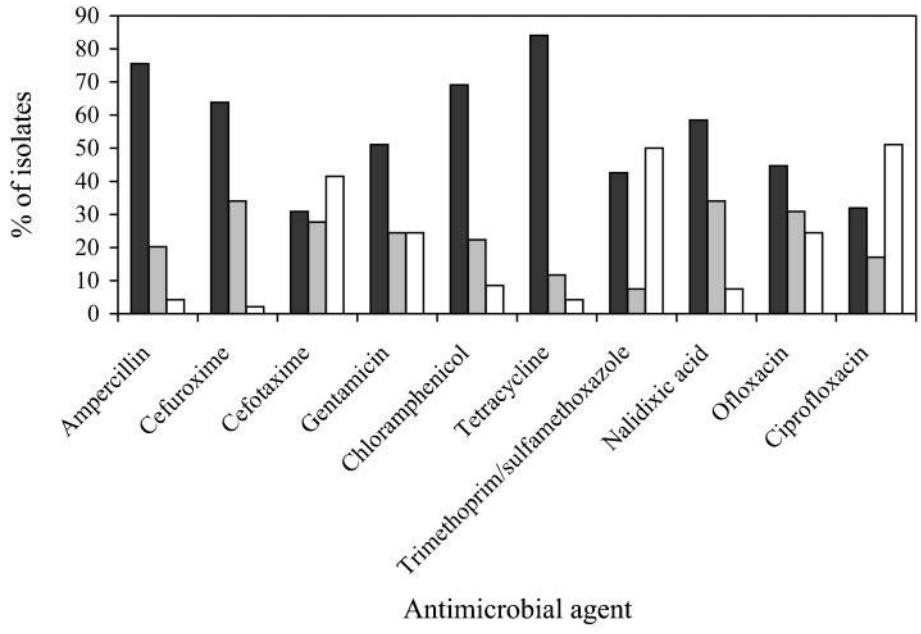

FIG. 3. Percentages of the *Escherichia coli* isolates found highly resistant (■), moderately resistant (■) or susceptible (□) to each of the 10 antimicrobial drugs investigated.

to the others ($P<0.05$). Significantly more (84%) of the isolates were resistant to tetracycline, for example, than were resistant to cefotaxime, gentamicin, trimethoprim/sulfamethoxazole, ofloxacin or ciprofloxacin. The rarest forms of resistance — those to cefotaxime (seen in 31% of isolates) and ciprofloxacin (32%) — were significantly rarer than all the other forms of resistance investigated except resistance to trimethoprim/sulfamethoxazole. Among the β-lactames, resistance to ampicillin (seen in 72% of isolates) and cefuroxime (64%) was significantly more common than resistance to cefotaxime (31%). In the case of the quinolones, resistance to ciprofloxacin (32%) was significantly rarer than resistance to nalidixic acid (59%) or ofloxacin (45%).

TABLE 7. *The percentages of the samples of dairy products found contaminated with entero-aggregative* Escherichia coli *(EAEC), enterotoxigenic* E. coli *(ETEC), enteropathogenic* E. coli *(EPEC), enterohaemorrhagic* E. coli *(EHEC) and/or* E. coli *O157:H7*

	% of samples harbouring:				
Product	EAEC	ETEC	EPEC	EHEC	O157:H7
Kishk	5.4	13.5	13.5	8.1	2.7
Baladi	0	37.8	2.7	5.4	2.7
Shankleesh	2.7	10.8	0	0	0

DISCUSSION

In the present study, the overall microbiological quality of the dairy products investigated was evaluated using two indicators — aerobic plate counts and counts of total coliforms. Aerobic plate counts were used to estimate the total number of viable aerobic bacteria present in each sample (Maturin and Peeler, 1998). As the presence of coliform bacteria in food indicates contamination and the possibility of the presence of harmful organisms, total coliform counts were used as an indicator of the overall hygienic status of the food products (Madigan *et al.*, 2000). The results indicated alarming levels of contamination.

The majority of samples collected for this study were home-made or produced in

farms by traditional techniques, and often made using raw milk that has been heated, for a few minutes, to a temperature that is not enough to kill all types of pathogenic bacteria. The possible sources of contamination with EHEC and ETEC include, first, the cow providing the milk. Milk from infected animals can be easily tainted with pathogens (Vautor *et al.*, 2003). The next possible cause is the milking procedure. The exterior of the cow's udder is usually colonized by faecal coliforms and other pathogens and hence serves as a source of contamination (Vautor *et al.*, 2003). Moreover, some of the samples collected were made in factories where cows are milked mechanically. The continuous use of the same milking machine for several cows, without little if any cleaning between one cow and the next, may easily allow the spread of pathogens among cows and into the raw milk that is collected. Other important sources include the farm workers who handle the milk and produce the milk-based foods, and contamination by organisms from soil and water (Kyozaire *et al.*, 2005). Contamination can occur at any step of the production of *baladi*, *kishk* and *shankleesh*. One of the crucial steps in the production of *kishk* and *shankleesh* is the drying of the foods in the open air. At this point, microbial contamination is likely to occur. Production of *kishk* also involves grinding of the mix by hand, which provides further ample opportunity for bacterial contamination. *Baladi* is also handled at several stages of its production.

Differences in the way *baladi*, *kishk* and *shankleesh* are made and processed are probably largely responsible for the observed differences in the microbiological quality of the three products. For example, after *shankleesh* is prepared, it is covered with powdered thyme and sometimes stored in olive oil. As thyme has been shown to have inhibitory effects against bacteria (Duke, 1987), use of the herb may protect *shankleesh* and improve its microbiological quality. The storage of *shankleesh* in oil provides anaerobic conditions that favor only the growth of certain anaerobic bacteria, although some of these may be involved in food-borne disease (Toufeili *et al.*, 1995). Furthermore, the acidic nature of *kishk* (with a pH of about 3.8), its low moisture content (<10%), and its high salt level (about 2.8 g NaCl/100 g of the final, dried product) may all generally suppress bacterial growth (Tamime and McNulty, 1999).

Escherichia coli is a faecal coliform that may often be present in raw milk and, therefore, in dairy products produced from unpasteurized milk (Jayarao *et al.*, 2006). In some dairy products, however, *E. coli* may be so rare as to go undetected unless cultured on enriched media. In the present study, for example, most of the *kishk* samples found positive for pathogenic *E. coli* (15.6%) were only found positive after enrichment. EAEC, EPEC and EHEC were all more common in the *kishk* samples than in the two other dairy products investigated. The presence of EHEC in the *kishk* is probably a reflection of the ability of this group of *E. coli* to grow in an environment of low water activity (A_w), a feature that most non-pathogenic *E. coli* do not share. The isolation of *E. coli* O157:H7 from 2.1% of the samples is a particular cause for concern, since this serogroup is involved in many outbreaks of human illness, and human infection with it may to lead to severe complications such as haemolytic–uraemic syndrome (Gilbert *et al.*, 2000).

The emergence of drug-resistant bacteria is common in areas, such as Lebanon, where the use of antimicrobial drugs is not carefully controlled, especially in animal husbandry. It may become so common and severe that previously useful antibiotics are no longer effective. Although *E. coli* are known to acquire resistance via many mechanisms, just one mechanism, the production of β-lactamase, may give resistance to a wide variety of commonly used antimicrobial drugs (Sturenburg and Mack, 2003).

In Lebanon, attempts to determine the antimicrobial susceptibility of Gram-negative bacteria have been largely confined to clinical isolates (Araj, 2000). Very little is known about the antimicrobial-resistance patterns of environmental isolates from the country. In the present study, it was striking that every one of the tested *E. coli* isolates, from Lebanese dairy products, was found resistant to at least one antimicrobial drug. This high prevalence of resistance is probably largely the result of the frequent (and often indiscriminate) antibiotic treatment of livestock, by local farmers, as a preventive measure against Gram-negative bacteria.

Many of the pathogenic *E. coli* isolates investigated in the present study showed multi-drug resistance. The exact mechanisms involved in such resistance remain to be elucidated. The results of recent studies on bacterial genomic DNA indicate that Gram-negative bacteria, such as *E. coli*, have a large number of drug exporter genes. The resistance-nodulation-cell division-type drug exporters, including the AcrAB-Tolc complex, are the most common drug exporters in *E. coli*, and have been demonstrated to be the reason for antimicrobial resistance in many *E. coli* isolates (Kallman *et al.*, 2003; Murakami and Yamaguchi, 2003). The AcrAB-Tolc system has a wide substrate range, including quinolones, tetracyclines, chloramphenicol, ampicilin and rifampicin. Bacteria may also acquire resistance through mobile elements, such as transposons, gene cassettes and integrons, or through mutations that lead to amino-acid substitutions within the active sites for some antimicrobials (Webber and Piddock, 2001; Soto *et al.*, 2003).

The common presence of multidrug-resistant strains of pathogenic *E. coli* in dairy products from the Beqaa Valley is alarming, as such strains are serious dangers to public health. There is clearly a need to reduce the contamination of such products, by improving the levels of hygiene in their manufacture. There is also a need to restrict the use of antibiotics by local farmers and so extend the useful life of the drugs that are currently effective. Additional research is, however, required to define better the ecology and evolution of bacterial resistance to antimicrobial agents, in the environment as a whole.

REFERENCES

Anon. (2004). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*. Approved Standards M7-A4. Wayne, PA: National Committee for Clinical Laboratory Standards.

Anon. (2008). *Escherichia coli* O157:H7 infections in children associated with raw milk and raw colostrum from cows — California, 2006. *Morbidity and Mortality Weekly Report*, **57**, 625–628.

Araj, G. F. (2000). Available laboratory tests to guide antimicrobial therapy. *Journal Medical Libanais*, **48**, 199–202.

Bottero, M. T., Dalmasso, A., Soglia, D., Rosati, S., Decastelli, L. & Civera, T. (2004). Development of a multiplex PCR for the detection of pathogenic genes of *Escherichia coli* in milk and milk products. *Molecular and Cellular Probes*, **18**, 283–288.

Cizman, M. (2003). The use and resistance to antibiotics in the community. *International Journal of Antimicrobial Agents*, **21**, 297–307.

Cole, E. C., Addison, R. M., Rubino, J. R., Leese, K. E., Dulaney, P. D., Newell, M. S., Wilkins, J., Gaber, D. J., Wineinger, T. & Criger, D. A. (2003). Investigation of antibiotic and antibacterial agent cross-resistance in target bacteria from homes of antibacterial product users and nonusers. *Journal of Applied Microbiology*, **95**, 664–676.

De Buyser, M., Dufour, B., Maire, M. & Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology*, **67**, 1–17.

Downes, F. P. & Ito, K. (Eds) (2001). *Compendium of Methods for the Microbiological Examination of Foods*, 4th Edn. Washington, DC: American Public Health Association.

Duke, J. A. (1987). *Handbook of Medical Herbs*. Boca Raton, FL: CRC Press.

Evans, D. J. & Evans, D. G. (1996). *Medical Microbiology*. Galveston, TX: University of Texas Medical Branch at Galveston.

Fraser, M. E., Fujinaga, M., Cherney, M. M., Melton-Celsa, A. R., Twiddy, E. M., O'Brien, A. D. & James, M. N. (2004). Structure of shiga toxin type 2 (stx2) from *Escherichia coli* O157:H7. *Journal of Biological Chemistry*, **279**, 27,511–27,517.

Fujisawa, T., Sata, S., Aikawa, K., Takahashi, T., Yamai, S. & Shimada, T. (2000). Modification of Sorbitol MacConkey medium containing cefixime and tellurite for isolation of *Escherichia coli* O157:H7 from radish sprouts. *Applied and Environmental Microbiology*, **66**, 3117–3118.

Galland, J. C., Hyatt, D. R., Crupper, S. S. & Acheson, D. W. (2001). Prevalence, antibiotic susceptibility, and diversity of *Escherichia coli* O157:H7 isolates from a longitudinal study of beef cattle feedlots. *Applied and Environmental Microbiology*, **67**, 1619–1627.

Gardner, I. A. (1997). Testing to fulfill HACCP (Hazard Analysis Critical Control Points) requirements: principles and examples. *Journal of Dairy Science*, **80**, 3453–3457.

Gilbert, R. J., de Louvois, J., Donovan, T., Little, C., Nye, K., Ribeiro, C. D., Richards, J., Roberts, D. & Bolton, F. (2000). Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. *Communicable Disease and Public Health*, **3**, 163–167.

Griffin, P. M. & Tanke, R. V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *Escherichia coli*, and the associated hemolytic uremic syndrome. *Epidemiological Reviews*, **13**, 60–98.

Harakeh, S., Yassine, H., Gharios, M., Barbour, E., Hajjar, S., El-Fadel, M., Toufeili, I. & Tannous, R. (2005). Isolation, molecular characterization and antimicrobial resistance patterns of *Salmonella* and *Escherichia coli* isolates from meat-based fast food in Lebanon. *The Science of the Total Environment*, **341**, 33–44.

Hayes, M. C., Ralyea, R. D., Murphy, S. C., Carey, N. R., Scarlett, J. M. & Boor, K. J. (2001). Identification and characterization of elevated microbial counts in bulk tank milk. *Journal of Dairy Science*, **84**, 292–298.

Henderson, I. R., Kicks, S., Navarro-Garcia, F., Elias. W. P., Philips, A. D. & Nataro, J. P. (1999). Involvement of the enteroaggragative *Escherichia coli* plasmid-encoded toxin in causing human intestinal damage. *Infection and Immunity*, **67**, 5338–5344.

Heuvelink, A. E., Zwartkruis-Nahuis, J. T., Beumer, R. R. & De Boer, E. (1999). Occurrence and survival of verocytotoxin-producing *Escherichia coli* O157 in meats obtained from retail outlets in The Netherlands. *Journal of Food Protection*, **62**, 1115–1122.

Horwitz, W. (Ed.) (2000). *Official Methods of Analysis of AOAC International*, 17th Edn. Gaithersburg, MD: AOAC International.

Jayarao, B. M., Donaldson, S. C., Straley, B. A., Sawant, A. A., Hegde, N. V. & Brown, J. L. (2006). A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in Pennsylvania. *Journal of Dairy Science*, **89**, 2451–2458.

Kallman, O., Fendukly, F., Karlsson, I. & Kronvall, G. (2003). Contribution of efflux to cefuroxime resistance in clinical isolates of *Escherichia coli*. *Scandinavian Journal of Infectious Diseases*, **35**, 464–470.

Kyozaire, J. K., Veary, C. M., Petzer, I. M.& Donkin, E. F. (2005). Microbiological quality of goat's milk obtained under different production systems. *Journal of the South African Veterinary Association*, **76**, 69–73.

Lamps, L. W. (2003). Pathology of food-borne infectious diseases of the gastrointestinal tract: an update. *Advances in Anatomic Pathology*, **10**, 319–327.

Li, W. & Drake, M. A. (2001). Development of a quantitative competitive PCR assay for detection and quantification of *Escherichia coli* O157:H7 cells. *Applied and Environmental Microbiology*, **67**, 3291–3294.

Lukásová, J., Abraham, B. & Cupáková, S. (2004). Occurrence of *Escherichia coli* O157 in raw material and food in Czech Republic. *Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health*, **51**, 77–81.

Lukinmaa, S., Nakari, U., Eklund, M. & Siitonen, A. (2004). Application of molecular genetic methods in diagnostics and epidemiology of food-borne bacterial pathogens. *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, **112**, 908–929.

Madigan, M. T., Martinko, J. M. & Parker, J. (2000). *Brock Biology of Microorganisms*, 9th Edn. Englewood Cliffs, NJ: Prentice Hall.

Matar, G. M., Abdo, D., Khneisser, I., Youssef, M., Zouheiry, H., Abdelnour, G. & Harakeh, H. S. (2002). The multiplex-PCR-based detection and genotyping of diarrhoeagenic *Escherichia coli* in diarrhoeal stools. *Annals of Tropical Medicine and Parasitology*, **96**, 317–324.

Maturin, L. J. & Peeler, J. T. (1998). Aerobic plate count. In *Food and Drug Administration Bacteriological Analytical Manual*, 8th Edn (version A), ed. Merker, R. L. pp. unnumbered. Gaithersburg, MD: AOAC International.

McLellan, S. L., Daniels, A. D. & Salmore, A. K. (2001). Clonal populations of thermotolerant Enterobacteriaceae in recreational water and their potential interference with fecal *Escherichia coli* counts. *Applied and Environmental Microbiology*, **67**, 4934–4938.

Mead, P. S. & Griffin, P. M. (1998). *Escherichia coli* O157:H7. *Lancet*, **352**, 1207–1212.

Murakami, S. & Yamaguchi, A. (2003). Multidrug-exporting secondary transporters. *Current Opinion in Structural Biology*, **13**, 443–452.

Neubauer, H., Sauer, T., Becker, H., Aleksic, S. & Meyer, H. (1998). Comparison of systems for identification and differentiation of species within the genus *Yersinia*. *Journal of Clinical Microbiology*, **36**, 3366–3368.

Niemela, S. I., Lee, J. V. & Fricker, C. R. (2003). A comparison of the International Standards Organisation reference method for the detection of coliforms and *Escherichia coli* in water with a defined substrate procedure. *Journal of Applied Microbiology*, **95**, 1285–1292.

O'Ryan, M., Prado, V. & Pickering, L. K. (2005). A millennium update on pediatric diarrheal illness in the developing world. *Seminars in Pediatric Infectious Diseases*, **16**, 125–136.

Osek, J. & Gallien, P. (2002). Molecular analysis of *Escherichia coli* O157 strains isolated from cattle and pigs by the use of PCR and pulsed-field gel electrophoresis methods. *Veterinary Medicine*, **47**, 149–158.

Prier, R. & Solnick, J. V. (2000). Foodborne and waterborne infectious diseases. *Postgraduate Medicine*, **107**, 245–255.

Ramesh, A., Padmapriya, B. P., Chrashekar, A. & Varadaraj, M. C. (2002). Application of a convenient DNA extraction method and multiplex PCR for the direct detection of *Staphylococcus aureus* and *Yersinia enterocolitica* in milk samples. *Molecular and Cellular Probes*, **16**, 307–314.

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M. & Swerdlow, D. L. (2005). Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982–2002. *Emerging Infectious Diseases*, **11**, 603–609.

Reinthaler, F. F., Posch, J., Feierl, G., Wust, G., Haas, D., Ruckenbauer, G., Mascher, F. & Marth, E. (2003). Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research*, **37**, 1685–1690.

Reischl, U., Youssef, M. T., Kilwinski, J., Lehn, N., Zhang, W. L., Karch, H. & Strockbine, N. A. (2002). Real-time fluorescence PCR assays for detection and characterization of Shiga toxin, intimin, and enterohemolysin genes from Shiga toxin-producing *Escherichia coli*. *Journal of Clinical Microbiology*, **40**, 2555–2565.

Riffon, R., Sayasith, K., Khalil, H., Dubreuil, P., Drolet, M. & Lagace, J. (2001). Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *Journal of Clinical Microbiology*, **39**, 2584–2589.

Saida, H., Ytow, N. & Seki, H. (1998). Photometric application of the Gram stain method to characterize natural bacterial populations in aquatic environments. *Applied and Environmental Microbiology*, **64**, 742–747.

Scavia, G., Staffolani, M., Fisichella, S., Striano, G., Colletta, S., Ferri, G., Escher, M., Minelli, F. & Caprioli, A. (2008). Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. *Journal of Medical Microbiology*, **57**, 1141–1146.

Sheikh, J., Czeczulin, J. R., Harrington, S., Hicks, S., Henderson, I. R., Le Bouguenec, C., Gounon, P., Phillips, A. & Nataro, J. P. (2002). A novel dispersin protein in enteroaggregative *Escherichia coli*. *Journal of Clinical Investigation*, **110**, 1329–1337.

Soto, S. M., Lobato, M. J. & Mendoza, M. C. (2003). Class 1 integron-borne gene cassettes in multidrug–resistant *Yersinia enterocolitica* strains of different phenotypic and genetic Types. *Antimicrobial Agents and Chemotherapy*, **47**, 421–425.

Sprong, R., Hulstein, F. & van der Meer, R. (2001). Bactericidal activities of milk lipids. *Antimicrobial Agents and Chemotherapy*, **45**, 1298–1301.

Steinsland, H., Valentiner-Branth, P., Gjessing, H. K., Aaby, P., Molbak, K. & Sommerfelt, H. (2003). Protection from natural infections with enterotoxigenic *Escherichia coli*: longitudinal study. *Lancet*, **362**, 286–291.

Sturenburg, E. & Mack, D. (2003). Extended-spectrum β-lactamases: implications for the clinical microbiology laboratory, therapy, and infection control. *Journal of Infection*, **47**, 273–295.

Tamime, A. Y. & McNulty D. (1999). Kishk — a dried fermented milk/cereal mixture. 4. Microbiological quality. *Lait*, **79**, 449–456.

Tarr, P. I., Gordon, C. A. & Chandler, W. L. (2005). Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet*, **365**, 1073–1086.

Toufeili, I., Shadarevian, S., Artinian, T. & Tannous, R. (1995). Ripening changes and sensory properties of bovine, caprine and ovine shankleesh. *International Dairy Journal*, **5**, 179–189.

Van Duijkeren, E. V., Wannet, W. J. B., Houwers, D. J. & van Pelt, W. (2003). Antimicrobial susceptibility of *Salmonella* strains isolated from humans, cattle, pigs, and chickens in the Netherlands from 1984 to 2001. *Journal of Clinical Microbiology*, **41**, 3574–3578.

Van Kessel, J. S., Karns, J. S., Gorski, L., McCluskey, B. J. & Perdue, M. L. (2004). Prevalence of *Salmonella*, *Listeria monocytogenes*, and fecal coliforms in bulk tank milk on US dairies. *Journal of Dairy Science*, **87**, 2822–2830.

Vautor E., Abadie G., Guibert, J. M., Huard, C. & Pépin, M. (2003). Genotyping of *Staphylococcus aureus* isolated from various sites on farms with dairy sheep using pulsed-field gel electrophoresis. *Veterinary Microbiology*, **96**, 69–79.

Velázquez Ldel, C., Barbini, N. B., Escudero, M. E. & de Guzmán, A. M. (2005). Resistance of *Yersinia enterocolitica*, *Escherichia coli* O157:H7 and natural microflora against acidic conditions and freezing–thawing in fresh sausages. *Central European Journal of Public Health*, **13**, 89–95.

Wanke, C. A. (1995). Enteropathogenic and enteroaggregative strains of *Escherichia coli*: clinical features of infection, epidemiology, and pathogenesis. *Current Clinical Topics in Infectious Diseases*, **15**, 230–252.

Webber, M. & Piddock, L. J. V. (2001). Quinolone resistance in *Escherichia coli*. *Veterinary Research*, **32**, 275–284.