SURVEY ON THE PRESENCE OF ENTEROVIRULENT *ESCHERICHIA COLI*, INCLUDING *E. COLI* O157, IN CHEESE MANUFACTURED IN THE ITALIAN ALPINE REGION

INDAGINE SULLA PRESENZA DI *ESCHERICHIA COLI* ENTEROVIRULENTI, INCLUSO *E. COLI* O157, IN FORMAGGI PRODOTTI NEL COMPRENSORIO ALPINO ITALIANO

T. CIVERA*, L. DECASTELLI [1], M. MARTORANA [1], A. DALMASSO, D. NUCERA and M.T. BOTTERO

Dipartimento di Patologia animale, Università degli Studi di Torino, 10095 Grugliasco, Italy

[1] Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, 10154 Torino, Italy

*Corresponding author. Tel. +39 0116709214, Fax +39 0116709212, e-mail: tiziana.civera@unito.it

ABSTRACT

The aim of the present study was to investigate the prevalence of enterovirulent *Escherichia coli* strains in cheese manufactured in the Italian Alpine Region. Cheese samples obtained from raw and pasteurised milk were collected from cheese-making plants. Enumeration of *E. coli* and the presence of verocytotoxigenic (VTEC), enteropatho-

RIASSUNTO

La presente ricerca è stata eseguita con lo scopo di osservare la prevalenza di ceppi enterovirulenti di *Escherichia coli* in formaggi prodotti sul territorio alpino italiano. Dai caseifici alpini sono stati raccolti campioni di formaggi prodotti sia da latte crudo che pastorizzato. Per ogni campione si sono determinate le cariche di *E. coli* totali e la presenza di

- Key words: cheese, pathogenic, *E. coli*, PCR -

genic (EPEC) and enterotoxic (ETEC) *E. coli* were carried out. A multiplex-PCR for the amplification of the sequences of VT1, VT2, *eae*, *ehxA*, ST, LT genes was used to identify pathogenic *E. coli* strains. The presence of *E. coli* O157 was investigated using an immunomagnetic separation technique. *E. coli* O157 was not recovered in cheese made from raw or pasteurised milk. The multiplex PCR approach detected at least one virulence determinant in only ten cheese samples, in four of them, a verotoxin-producing gene (VT2 and/or VT1) was detected and in the remaining six only the attaching and effacing lesion genes (*eae*) were found. No ETEC strains were detected. The statistical analyses of the results demonstrated significant associations between the use of raw milk in cheese production and the presence of detectable virulence genes in cheese. Moreover, high *E. coli* counts were also associated with the use of raw milk in fresh and ripened cheese production. The results revealed the survival and recovery of pathogenic strains even after a 60-day ripening period, highlighting the importance of the hygienic and sanitary requirements for milk used in the production of both fresh and ripened cheese.

ceppi verocitotossici (VTEC), enteropatogeni (EPEC) ed enterotossici (ETEC). I ceppi patogeni sono stati identificati mediante l'utilizzo di una PCR multipla per l'amplificazione di frammenti dei geni VT1, VT2, *eae*, *ehxA*, ST ed LT. La ricerca di *E. coli* O157 è stata eseguita utilizzando la tecnica della separazione immunomagnetica. I risultati hanno permesso di evidenziare l'assenza di *E. coli* O157, mentre in 10 formaggi la PCR ha messo in evidenza la presenza di geni di patogenicità: in 4 campioni sono stati evidenziati i geni codificanti per la produzione di verotossina (VT1 e/o VT2), nei rimanenti si è evidenziato solo il gene responsabile delle attaching and effacing lesions (*eae*), mentre non si sono evidenziati ceppi ETEC. L'analisi statistica dei risultati ha permesso di evidenziare che la presenza di geni di virulenza è associata a formaggi prodotti da latte crudo. Inoltre è stata osservata un'associazione tra alte cariche di *E. coli* e prodotti a latte crudo (freschi e stagionati). I risultati della presente ricerca hanno evidenziato la sopravvivenza di ceppi patogeni anche in formaggi stagionati per periodi superiori ai 60 giorni, sottolineando l'importanza delle condizioni igienico-sanitarie del latte utilizzato per la produzione sia di formaggi freschi che stagionati.

INTRODUCTION

Escherichia coli, is physiologically present in the intestines of humans and warm-blooded animals. Although usually harmless, it was defined as a foodborne pathogen in 1971, when nearly 400 people became ill after eating contaminated cheese (MARIER *et al.*, 1973). At present, at least five virulence groups of *E. coli* are recognized with respect to disease syndromes, characteristics, serological grouping and effects on certain cell cultures (JAY *et al.*, 2005). Verotoxin-producing *E. coli* (VTEC) are often associated with the onset of foodborne diseases. These strains may produce two kinds of verotoxins (VT1 and VT2). More than 200 VT-producing *E. coli* serotypes have been isolated, but only a few have caused severe human diseases; the O157 serotype has been responsible for most of these infections (CAP-

)tossici (VTEC), enteropa-
ed enterotossici (ETEC).
eni sono stati identificati
lizzo di una PCR multipla
azione di frammenti dei
., *eae*, *ehxA*, ST ed LT. La
oli O157 è stata eseguita
tecnica della separazio-
agnetica. I risultati han-
i evidenziare l'assenza di
nentre in 10 formaggi la
in evidenza la presenza
)genicità: in 4 campioni
enziati i geni codificanti
one di verotossina (VT1
manenti si è evidenziato
)onsabile delle attaching
sions (*eae*), mentre non
:iati ceppi ETEC. L'ana-
ei risultati ha permesso
he la presenza di geni di
)ciata a formaggi prodot-
o. Inoltre è stata osser-
zione tra alte cariche di
i a latte crudo (freschi e
ultati della presente ri-
denziato la sopravviven-
)geni anche in formag-
periodi superiori ai 60
ando l'importanza del-
:nico-sanitarie del latte
produzione sia di for-
ıe stagionati.

:teristics, serological
ffects on certain cell
al., 2005). Verotoxin-
li (VTEC) are often as-
ıe onset of foodborne
strains may produce
)toxins (VT1 and VT2).
T-producing *E. coli* se-
:n isolated, but only a
severe human diseas-
type has been respon-
these infections (CAP-

RIOLI *et al.*, 2005; MALDONADO *et al.*, 2005). Two virulence factors may affect the pathogenicity of VTEC: the *eae* and *ehxA* genes. The former is responsible for the production of attaching and effacing lesions (AE lesions) in the intestinal mucosa; the latter encodes for the haemolysin called EHEC enterohaemolysin (*ehxA*) (BEUTIN *et al.*, 2004).

While consumption of undercooked beef meat has long been considered the main cause of this infection, *E. coli* O157 and VTEC strains have often been detected in the environment (MALDONADO *et al.*, 2005). In addition, raw milk, as well as cheese and yogurt, have also been associated with outbreaks and sporadic cases of illnesses due to VTEC contamination (GILLEPSIE *et al.*, 2003; LIPTAKOVA *et al.*, 2004; HUSSEIN and SAKUMA, 2005).

E. coli O157 are currently the most common VTEC in many regions of the world (ARMSTRONG *et al.*, 1996). However, other serotypes, such as O26, O103, O111, O118 and O145 have also been recovered from infected patients and are considered a serious threat to public health (CAPRIOLI *et al.*, 1997; ALLERBERGER *et al.*, 2003).

Some non-VTEC strains, named enterotopathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC), can induce sporadic diarrhoea. The former group causes AE lesions in the human small intestine causing diarrhoea, but does not generally produce enterotoxins (CORTÉS *et al.*, 2005). The latter group is a major cause of sporadic diarrhoeic diseases in humans, affecting mainly children in developing countries (NATARO and KAPER, 1998). ETEC strains have also been detected in Europe, in particular Switzerland, in 20% of the raw milk and dairy products (ALLMANN *et al.*, 1995).

At present, conventional microbiological diagnostics in food only include the determination of the *E. coli* count per gram without requiring any further characterization of the isolated strains; the analyses on serogroups and detection of pathogenic determinants are only carried out when foodborne outbreaks occur. Therefore, information about the presence of potentially pathogenic *E. coli* in dairy products is lacking, particularly regarding traditional cheeses produced in specific geographic areas.

The aim of this study was to investigate the occurrence of all potentially enterovirulent *E. coli* (VTEC including *E. coli* O157, EPEC, ETEC strains) in raw and pasteurised milk cheeses manufactured in the Italian Alpine Region, which is known for its high standards in dairy production. The investigation was carried out by coupling the traditional immunoseparation technique used to detect O157 with the newer multiplex PCR technique used to detect other pathogenic *E. coli* strains.

MATERIALS AND METHODS

Sampling

Two-hundred and three soft and ripened cheeses were collected over a 17-month period from 50 cheese-making plants in the Italian Alpine Region. The samples included 143 (70.4%) raw milk cheeses and 60 (29.6%) pasteurised milk cheeses. Soft cheeses (less than 10-days maturation) and ripened cheeses (more than 10-days maturation) were the most frequent cheese types. All of the ripened and most of the soft cheeses analysed were obtained by enzymatic coagulation. Table 1 reports the sample distribution according to ripening time (days), cheese texture, heat treatment and dairy species of origin of the milk used in cheese production. After collection, samples were refrigerated and transported to the laboratory where the samples were stored at 4°C and then processed the following day.

Enumeration of *E. coli*

The total *E. coli* count was done according to the UNI method (2002). Serial dilutions of peptone water were prepared and 1 mL of each dilution was poured onto Tryptone Bile Glucuronide Medium (TBX, Oxoid Italiana, Garbagnate Milanese, Milan, Italy). Plates were incubated at 44°C for 18-24 h and *E. coli* were counted on plates with typical blue/green colonies.

Detection of pathogenic *E. coli* strains

For each sample, 25 g of product were added to 225 mL of modified Tryptic Soy Broth (Oxoid Italiana, Garbagnate Milanese, Milan, Italy) supplemented with novobiocin (20 μg/mL, Oxoid Italiana, Garbagnate Milanese, Italy) and incubated at 41.5°C for 6 h. In compliance with the ISO (2001) procedure, two 50-μL aliquots of broth were streaked onto one plate of Sorbitol MacConkey agar (SMAC, Oxoid Italiana, Garbagnate Milanese, Milan, Italy), and one plate of Sorbitol MacConkey agar supplemented with Cefixime tellurite (CT-SMAC, Oxoid Italiana, Garbagnate Milanese, Italy), in order to increase the selectivity of the medium for *E. coli* detection. Both media were incubated at 37°C for 18-24 h.

If present, five sorbitol-negative and five sorbitol-positive colonies from each plate were inoculated into tryptone water for 24 h at 37°C. These suspensions were sub-cultured onto Nutrient agar (Oxoid Italiana, Garbagnate Milanese, Milan, Italy), emulsified in 5 mL of sterile saline solution, inoculated onto API 20E strips (BioMérieux, Rome, Italy), and incubated at 37°C for 24 h. The same suspensions were also processed to prepare DNA templates for multiplex PCR targeting *E. coli* virulence factors, following the published protocol (BOTTERO *et al.*, 2004) for DNA extraction and amplification. However, in order to effectively identify pathogenic *E. coli* strains, further investigations were performed when multiplex-PCR detected at least one virulence factor when tryptone water-suspended colonies were tested. These were sub-cultured on TBX and at least five isolates per plate were analysed singularly with the same multiplex-PCR method and serotyping.

Isolation of *E. coli* O157

All samples were examined for the presence of *E. coli* O157 following the ISO (2001) procedure and using an immunomagnetic separation (IMS) technique. Each sample underwent pre-enrichment in modified Tryptic Soy Broth (Oxoid Italiana, Garbagnate Milanese, Milan, It-

Table 1 - Cheeses sampled according to days of ripening (A); number of raw and pasteurized milk cheeses according to cheese texture and species of milk origin (B).

Ripening period (days)	Sampled cheeses
<10	74
10-30	45
31-60	47
61-90	28
>90	9

A

	Texture		Species of origin of milk				
Cheeses	Soft	Ripened	Cow	Goat	Cow/Goat	Cow/Sheep	Cow/Goat/Sheep
Raw milk	50	93	115	11	11	4	2
Pasteurised milk	29	31	56	1	2	0	1

B

pplemented with Cefix-
'-SMAC, Oxoid Italiana,
inese, Italy), in order to
ctivity of the medium for
Both media were incu-
r 18-24 h.
e sorbitol-negative and
itive colonies from each
lated into tryptone water
These suspensions were
ito Nutrient agar (Oxoid
nate Milanese, Milan, It-
in 5 mL of sterile saline
ated onto API 20E strips
ne, Italy), and incubated
. The same suspensions
sed to prepare DNA tem-
plex PCR targeting *E. coli*
, following the published
RO *et al.*, 2004) for DNA
amplification. However,
tively identify pathogen-
s, further investigations
when multiplex-PCR de-
ne virulence factor when
suspended colonies were
ere sub-cultured on TBX
isolates per plate were
arly with the same mul-
iod and serotyping.

. *coli* O157

were examined for the
li O157 following the ISO
re and using an immu-
aration (IMS) technique.
derwent pre-enrichment
otic Soy Broth (Oxoid Ita-
ate Milanese, Milan, It-

rigin of milk	
Cow/Sheep	Cow/Goat/Sheep
4	2
0	1

aly) supplemented with novobiocin (20 µg/mL, Oxoid Italiana, Garbagnate Milanese, Milan, Italy) as described above. Immunomagetic separation was performed according to the manufacturer's instructions (Dynal, Oslo, Norway); however, the IMS was repeated after an additional 24 h of incubation.

In compliance with the ISO (2001) procedure, two 50-µL aliquots of the IMS-enriched broth were analyzed as described previously. Suspected *E. coli* O157 colonies were tested for indole production using Kovacs reagent (Merck & Co, Milan, Italy).

Serotyping

Serotyping was performed by means of slide agglutination with an *E. coli* O157 Latex test kit (Oxoid Italiana, Garbagnate Milanese, Milan, Italy) on API 20 E identified *E. coli* isolates, which revealed at least one virulence gene when tested with multiplex PCR. The results were confirmed by tube agglutination of heat-treated cultures (Denka Seiken Co., Ltd., Tokyo, Japan).

Data analysis

In the present study three associations were evaluated with the heat treatment of milk used in the cheese-making process: the first was the total *E. coli* count, the second the presence of virulence genes in colonies isolated from cheese samples and the third the presence of the VT gene in isolated colonies. In order to analyse the data, microbiological and PCR results were coded as binary variables; for *E. coli* count, a value of 1 was attributed to samples where the *E. coli* count was greater than 10^3 cfu/g, and 0 was assigned if the count was less. To evaluate the presence of a virulence gene, a value of 1 was given when at least one isolated colony from the cheese samples showed the presence of at least one virulence factor amplification product with multiplex PCR. Similarly, when the association between milk heat treatment and the presence of the VT gene was evaluated a value of 1 was given to all the samples that had at least one isolated colony with VT1 and/or VT2. The heat treatment of milk used in the cheese-making process was also coded as 1 and 0 for raw and pasteurised milk, respectively. For the evaluation of the association between heat treatment of milk and *E. coli* count, a total of 200 cheese samples were analysed. Since cheese type is associated with both *E. coli* count in cheese and the heat treatment of the milk used, samples were divided into fresh (n=83) and ripened (n=117) cheeses and a stratified analysis was performed in order to check for the confounding effect of cheese type. On investigating the other two associations, 195 cheese samples were analysed, dividing them into fresh (n=78) and ripened (n=117) cheeses. In all the contingency tables the Fisher Exact Probability test (F.E.P.) and the Mantel-Haenszel χ^2 were used to assess the significance of the results. Mantel-Haenszel Odds Ratio (O.R.) values and 95% Confidence intervals (95% C.I.) were evaluated as measures of association.

RESULTS

Enumeration of *E. coli* and pathogenic *E. coli* identification

The *E. coli* counts are reported in Table 2. In the raw milk cheese samples less than 10 cfu/g of *E. coli* were present in 70 cheeses (49% of the samples). Among these samples, 21 (30%) and 49 (70%) were soft and ripened cheeses, respectively. In contrast, 55 (92%) pasteurised milk cheeses had *E. coli* counts less than 10 cfu/g.

Presumptive *E. coli* colonies on SMAC and CT-SMAC were recovered in 123 (61%) and 93 (46%) cheese samples, respectively. When subsequently tested

Table 2 - *E. coli* distribution in cheeses related to heat treatment of the milk used for cheese production.

	E. coli counts (cfu/g)					
Cheeses	< 10	10-10^2	10^2-10^3	10^3-10^4	10^4-10^5	>10^5
Raw milk	70	14	19	15	16	9
Pasteurised milk	55	2	2	1	0	0

with API 20 E, these colonies were identified as *E. coli*. When multiplex PCR was performed on tryptone water from isolated SMAC/CT-SMAC colonies, the presence of at least one virulence gene was detected in 16 (8%) cheeses. In particular, when PCR was performed on SMAC colonies, 6 soft (3%) and 8 ripened (4%) cheeses showed amplification products of at least one virulence factor. Pathogenicity determinants were detected when PCR was performed on tryptone water from isolated CT-SMAC colonies, in 5 (2.5%) soft and 4 (2%) ripened cheeses. Frequencies of virulence determinants in the different kinds of cheeses are reported in Table 3. In particular, the VT2 gene was detected in 3 (2%) of the positive SMAC samples, whereas the VT1 and VT2 genes, respectively, were detected in 2 (2%) and 3 (3%) of the positive CT-SMAC samples. Both genes were detected in only one positive CT-SMAC sample. The *eae* was the most commonly amplified gene, whereas the *ehxA* gene was only detected in 3 isolates in association with VT2 or VT1 genes, detected in three and two isolates, respectively. Tryptone water inoculated with PCR positive samples were sub-cultured on TBX, and both multiplex PCR and serotyping were performed on 5 isolated colonies. Virulence determinants were only detected in 10 samples. Table 4 shows the presence of virulence determinants in these 10 cheese samples: 9 raw milk, ripened cheeses, and one pas-

Table 3 - Number of cheeses with isolated *E. coli* colonies on SMAC (A) or CT-SMAC (B) and with multiplex PCR positive results, according to cheese texture and heat treatment of the milk. The percentage of positive results calculated over the total sample size for each type is reported in parentheses.

	Cheeses	n	SMAC (%)	*ehxA* (%)	*eae* (%)	ST (%)	LT (%)	VT1 (%)	VT2 (%)
Soft	Raw milk	52	28 (53)	3 (6)	3 (6)	------	------	------	------
	Pasteurised milk	31	12 (39)	------	1 (3)	------	------	------	------
Ripened	Raw milk	91	63 (69)	3 (4)	4 (6)	------	------	------	3 (4)
	Pasteurised milk	29	12 (41)	------	------	------	------	------	------
	Total	203	123 (61)	6 (3)	8 (4)	------	------	------	3 (1.5)

A

	Cheeses	n	CT-SMAC (%)	*ehxA* (%)	*eae* (%)	ST (%)	LT (%)	VT1 (%)	VT2 (%)
Soft	Raw milk	52	27 (52)	5 (10)	2 (4)	—	—	1 (2)	1 (2)
	Pasteurised milk	31	7 (23)	—	—	—	—	—	—
Ripened	Raw milk	91	47 (52)	2 (2)	2 (2)	—	—	1 (1)	2 (2)
	Pasteurised milk	29	12 (41)	—	—	—	—	—	—
	Total	203	93 (46)	7 (3.5)	4 (2)	—	—	2 (1)	3 (1.5)

B

ıe milk used for cheese produc-

fu/g)		
^{3}-10^{4}	10^{4}-10^{5}	>10^{5}
15	16	9
1	0	0

:as the VT1 and VT2 genes, ere detected in 2 (2%) and positive CT-SMAC samıes were detected in only Γ-SMAC sample. The *eae* commonly amplified gene, ıxA gene was only detects in association with VT2 detected in three and two ctively. Tryptone water in-CR positive samples were n TBX, and both multiplex rping were performed on 5 s. Virulence determinants cted in 10 samples. Table esence of virulence deter-se 10 cheese samples: 9 ıed cheeses, and one pas-

or CT-SMAC (B) and with mulıent of the milk. The percentage reported in parentheses.

(%)	LT (%)	VT1 (%)	VT2 (%)
-----	------	------	------
-----	------	------	------
-----	------	------	3 (4)
-----	------	------	------
-----	------	------	3 (1.5)

T(%)	LT(%)	VT1(%)	VT2(%)
—	—	1(2)	1(2)
—	—	—	—
—	—	1(1)	2(2)
—	—	—	—
—	—	2(1)	3(1.5)

Table 4 - Cheeses with at least one *E. coli* colony isolated from SMAC or CT-SMAC that showed positive reactions to one or more virulence gene targeted in the Multiplex PCR. The total *E. coli* count is also reported for each sample.

	E. coli count	Multiplex PCR				
Cheese	cfu/g	*ehxA*	*eae*	VT1	VT2	*uidA*
1	<10	+	-	-	+	+
2	<10	-	-	-	+	+
3	<10	+	+	+	+	+
4	230	+	+	+	-	+
5	1,100	-	+	-	-	+
6	<10	-	+	-	-	+
7	<10	-	+	-	-	+
8	160	-	+	-	-	+
9	43,000	-	+	-	-	+
10	<10	-	+	-	-	+

teurised, fresh milk cheese. Four of these isolates were characterized as VTEC, and none as ETEC, since no LT or ST genes were amplified.

Isolation of *E. coli* O157

When samples were tested with the ISO immunoconcentration technique (2001) the presence of *E. coli* O157 was not detected.

Serotyping

When serotyping was performed on ten cheese samples from which isolates with pathogenic determinants were recovered, three isolates, O26, O113, and O146, were identified, the rest were not able to be typed. Interestingly, only the attaching and effacing lesion encoding gene was detected in the O26 isolate, whereas in the O113 strain, isolated from a 70-day ripened cheese, both VT2 and *eae* genes were detected. In the O146 strain, isolated from a mixed raw cow-sheep milk cheese, all four virulence genes (*eae*, *ehxA*, VT1, VT2) were detected.

Measure of association

The use of raw milk for cheese production was significantly associated with *E. coli* counts in cheeses when the stratified analysis was performed (Mantel-Haenszel $\chi^2 = 17.79$; $p<0.001$). In particular, cheeses produced from raw milk were more associated with an *E. coli* count greater than 10^3 cfu/g than cheeses made from pasteurised milk (Mantel-Haenszel O.R.= 29.74; 95% C.I.: 3.63-531.53).

The association between raw milk cheeses and the presence of at least one virulence gene in isolated *E. coli* colonies was also statistically significant (Mantel-Haenszel $\chi^2 = 3.95$ $p<0.05$), indicating that cheeses made from raw milk are more associated with positive PCR results than the ones produced with pasteurised milk (Mantel-Haenszel O.R.=2.31 95% C.I.: 1.11-5.05). In contrast, the presence of the VT gene was not associated with the use of raw milk for cheese production (F.E.P. >0.05).

DISCUSSION

The aim of the present study was to monitor the presence of pathogenic *E. coli* strains and related virulence determinants (*eae*, *ehxA*, VT1, VT2, ST, LT) in raw- and pasteurised-milk cheese produced in the Italian Alpine Region, an area that has high dairy production standards. Conventional techniques for the differential detection of *E. coli* virulent strains in dairy products are not applicable due to the absence of known phenotypic traits related to strain discrimination. Therefore, in the last few years, biomolecular approaches have been extensively explored as strain differentiation tools as an alternative to conventional phenotypic methods.

Genes encoding for virulence determinants, such as ST, LT (ALLMANN *et al.*, 1995), VT1, VT2, *ehxA*, and *eae* (PIERARD *et al.*, 1997; BOUVET *et al.*, 2001;

CHAPMAN *et al.*, 2001) have been used for PCR detection of VTEC, including *E. coli* O157, EPEC, and ETEC. The usefulness of these PCR assays has long been debated. In fact, even if PCR assays can be applied for *E. coli* pathogenic strain detection in human faecal samples, the applicability of these techniques on environmental samples is severely limited by the widespread presence of virulence genes in *E. coli* strains that are only rarely associated with human diseases.

However, despite these limitations, PCR might allow strains to be identified that are not usually detected by conventional techniques. Some VTEC strains belonging to serogroups O26, O103, O111, O145 and O157 isolated from human diseases such as HC and HUS (CAPRIOLI *et al.*, 1997; BEUTIN *et al.*, 2004), were not detected by traditional techniques and were investigated only after the occurrence of food poisoning outbreaks (BEUTIN *et al.*, 2004). In this context, the use of PCR for monitoring *E. coli* virulence factors is important for identifying potentially dangerous foods as part of the HACCP plans, in order to detect and avoid the spread of pathogenic *E. coli*, before food poisoning outbreaks occur.

The results of this research revealed that in 98.3% of the pasteurised-milk cheeses and in 72% of the raw-milk cheeses the *E. coli* counts were less than 10^3 cfu/g, the maximum level allowed (Commission Regulation No 2073/2005) in cheeses made from heat-treated milk. The significantly low *E. coli* count in the Italian Alpine cheese sampled in this investigation, is undoubtedly related to the strict hygiene procedures used in milk and cheese production. However, the analysis of the association between raw milk cheeses with *E. coli* counts greater than 10^3 cfu, and positive multiplex PCR isolated colonies showed a statistically significant association. These results indicate the importance of milk quality and hygiene prior to processing, for both fresh and ripened cheese production.

In the present investigation, the presence of pathogenic *E. coli* in cheeses was evaluated by combining the traditional ISO (2001) procedure for *E. coli* O157 detection, with the detection of *E. coli* virulence determinants through multiplex PCR after pre-enrichment of cheese samples. This approach could detect EPEC, ETEC and VTEC by PCR amplification of virulence determinants, revealing the presence of potentially pathogenic *E. coli* strains (besides O157) in cheeses which might not be detected by standard identification procedures. However, to effectively identify pathogenic *E. coli* strains, other investigations (e.g. serotyping) were performed when multiplex-PCR detected at least one virulence factor when tryptone water-suspended colonies were tested. Using this approach pathogenic *E. coli* were isolated in only 10 cheese samples out of 16 which presented multiplex PCR amplification products. None were serotyped as O157, three were identified as O26, O113, and O146. Four of the isolates were VTEC, but none were ETEC. These findings suggest that by combining the two methods pathogenic *E. coli* isolates, which are likely to pass undetected when only traditional methods are used, can be detected. In particular, the pathogenic *E. coli* levels are usually lower than those of non pathogenic *E. coli* and, when plated, the colonies share the same morphology, thus making it difficult to isolate the pathogenic strains. Hence, from a practical point of view, the presence of such small concentrations of pathogens might be important for the monitoring process needed in HACCP plans in order to control the spread of these strains. In fact, low levels of pathogenic *E. coli* can also be a serious hazard for consumers' health. The infectious dose of strains containing VT2, that is well associated with human disease (HUSSEIN and SAKUMA, 2005), is quite low. TILDEN *et al.* (1996) suggested that the infectious dose evaluated in an *E. coli* O157:H7 outbreak from dry fermented salami consumption

sent investigation, the presogenic *E. coli* in cheeses was y combining the traditional rocedure for *E. coli* O157 de- the detection of *E. coli* virminants through multiplex e-enrichment of cheese samproach could detect EPEC, TEC by PCR amplification determinants, revealing the otentially pathogenic *E. coli* les O157) in cheeses which detected by standard idencedures. However, to effec- pathogenic *E. coli* strains, gations (e.g. serotyping) were nen multiplex-PCR detected virulence factor when trypspended colonies were test- approach pathogenic *E. coli* in only 10 cheese samples h presented multiplex PCR products. None were sero-7, three were identified as nd O146. Four of the iso-EC, but none were ETEC. s suggest that by combining ods pathogenic *E. coli* isore likely to pass undetected ditional methods are used, d. In particular, the pathovels are usually lower than athogenic *E. coli* and, when onies share the same mormaking it difficult to isogenic strains. Hence, from nt of view, the presence of ncentrations of pathogens ortant for the monitoring d in HACCP plans in order spread of these strains. In s of pathogenic *E. coli* can us hazard for consumers' ctious dose of strains conat is well associated with e (HUSSEIN and SAKUMA, low. TILDEN *et al.* (1996) the infectious dose eval-. *coli* O157:H7 outbreak nted salami consumption was less than 50 bacteria, and STRACHAN *et al.* (2001) estimated that an infectious dose could be as low as 4-24 ingested organisms.

Enterovirulent *E. coli* contamination of milk and dairy products (HUSSEIN and SAKUMA, 2005) has been investigated in a limited number of studies. Detection of VTEC strains in raw milk, milk filters and cheeses was reported in Belgium, Canada, Germany, the UK and the U.S.A., but only a few isolates were identified as belonging to serogroups known to cause human illness. CONEDERA *et al.* (2004) analysed 2948 Italian dairy products and *E. coli* O157:H7 was detected in only one sample. Similarly, no O157 VTEC were isolated when 1011 raw cow milk samples were examined in the Netherlands (HEUVELINK *et al.*, 1998).

Likewise, these research results showed a very low prevalence of pathogenic *E. coli* strains in cheeses: no O157 strains and no ETEC were detected. The virulence genes (VT1, VT2, *eae*, and *ehxA*) were detected in only 1 of the 10 isolates presenting virulence determinants. The source of this isolate was a 60-day-old raw-milk cheese, that had an *E. coli* count of less than 10 cfu/g. Similarly, considering multiplex PCR results, most of the genes coding for toxin production were detected in isolates from raw-milk ripened cheeses, which were also characterized by low levels of total *E. coli* contamination. The absence of any correlation between the *E. coli* count and the presence of *E. coli* O157, as well as other VTEC and ETEC strains, has already been observed (CHAPMAN *et al.*, 2001; CONEDERA *et al.*, 2004). These findings suggest that VTEC and ETEC strains could be more resistant than other *E. coli* strains to stress conditions (fermentation and maturation of cheeses) as already observed for *E. coli* O157 (KASRAZADEH and GENIGEORGIS, 1995; REITSMA and HENNING, 1996).

The association between the presence of virulence genes and the heat treatment of milk used in the cheese-making process was also evaluated, in particular the use of raw milk was associated with the presence of virulence genes for both fresh and ripened cheese, indicating that the ripening process alone (even if 60 days) does not guarantee the absence of pathogenic *E. coli* strains. Hence, contrary to what was claimed by QUINTO and CEPEDA (1997), both soft and ripened cheeses should be considered as possible vehicles of infection.

The results of this investigation demonstrate the presence of *E. coli* virulence determinants in raw milk cheese regardless of the ripening process. *E. coli* virulence testing is therefore needed particularly in all raw milk products or other dairy products processed at temperatures below 45°-48°C. This testing is currently not included in routine diagnostic activities. Therefore, to address consumer protection issues there must be a careful control of all the cheese production phases, starting from milking procedures, as well as more detailed information on product labels that indicates the heat treatment of the milk used in production.

ACKNOWLEDGEMENTS

This study was supported by the grant from "Ministro della Salute" as Ricerca Corrente Anno 2000 (IZS PLV 008/00). We also thank Dr. Stefano Morabito and Dr. Alfredo Caprioli (Istituto Superiore di Sanità, Roma) for serotyping the isolates.

REFERENCES

Allerberger F., Friedrich A.W., Grif K., Dierich M.P., Dornbusch H.J., Mache C.J., Nachbaur E., Freilinger M., Rieck P., Wagner M., Caprioli A., Karch H. and Zimmerhackl L.B. 2003. Hemolytic-uremic syndrome associated with enterohemorrhagic *Escherichia coli* O26: H infection and consumption of unpasteurised cow's milk. Int. J. Infect. Dis. 7: 42.

Allmann M., Hofelein C., Koppel E., Luthy J., Meyer R., Niederhauser C., Wegmuller B. and Candrian U. 1995. Polymerase chain reaction (PCR) for detection of pathogenic microrganisms in bacteriological monitoring of dairy products. Res. Microbiol. 146: 88.

Armstrong G.L., Hollingsworth J. and Morris Jr.J.G. 1996. Emerging foodborne pathogens *Escherichia coli* O157:H7 as a model of entry of a new pathogen into the food supply of the developed country. Epidemiol. Rev. 18: 29.

Beutin L., Krause G., Zimmermann S., Kaulfuss S. and Gleier K. 2004. Characterization of Shiga Toxin-Producing *Escherichia coli* strains isolated from human patients in Germany over a 3-year period. J. Clin. Microbiol. 42: 1099.

Bottero M.T., Dalmasso A., Soglia D., Rosati S., Decastelli L. and Civera T. 2004. Development of a multiplex PCR assay for the identification of pathogenic genes of *Escherichia coli* in milk and milk products. Mol. Cell. Probes. 18: 283.

Bouvet J., Bavai C., Rossel R., Le Roux A., Montet M.P., Ray-Gueniot S., Mazuy C., Arquilière C. and Vernozy-Rozand C. 2001. Prevalence of verotoxin-producing *Escherichia coli* and *E. coli* O157:H7 in pig carcasses from three French slaughterhouses. Int. J. Food Microbiol. 71: 249.

Caprioli A., Tozzi A.E., Rizzoni G. and Karch H. 1997. Non-O157 Shiga-Toxin-Producing *Escherichia coli* infections in Europe. Em. Infect. Dis. 4: 578.

Caprioli A., Morabito S., Brugère H. and Oswald E. 2005. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet. Res. 26: 289.

Chapman P.A., Cerdan Malo A.T., Ellin M., Ashton R. and Harkin M.A. 2001. *Escherichia coli* O157 in cattle and sheep at slaughter, on beef and lamb carcasses and raw beef and lamb products in South Yorkshire, UK. Int. J. Food Microbiol. 64: 139.

Commission Regulation No 2073/2005 of 15 November 2005. Microbiological criteria for foodstuffs. Official Journal of the European Union L338: 1.

Conedera G., Dalvit P., Martini M., Galiero G., Gramaglia M., Goffredo E., Loffredo G., Morabito S., Ottavini D., Peterlini F., Pezzotti G., Pisanu M., Semprini P. and Caprioli A. 2004. Verocytotoxin-producing *Escherichia coli* O157 in minced beef and dairy products in Italy. Int. J. Food Microbiol. 69: 67.

Cortés C., De la Fuente R., Blanco J., Blanco M., Blanco J.E., Dhabi G., Mora A., Justel P., Contreras A., Sànchez A., Corrales J.C. and Orden J.A. 2005. Serotypes, virulence genes and intim types of verotoxin-producing *Escherichia coli* and enteropathogenic *E. coli* isolated from healthy dairy goats in Spain. Vet. Microbiol. 110: 67.

Gillespie I.A., Adak G.K., O'Brien S.J. and Bolton F.J. 2003. Milkborne general outbreaks of infectious intestinal disease, England and Wales, 1992-2000. Epidemiol. Infect. 130: 461.

Heuvelink A.E., van den Biggerlaar F.L.A.M., Zwartkruis-Nahuis J.T.M., Herbes R.G., Huyben R., Nagelkerke N., Meichers W.J.G., Monnens L.A.H. and de Boer E. 1998. Occurrence of verocytotoxin-producing *Escherichia coli* O157 on Dutch dairy farms. J. Clin. Microbiol. 36: 3480.

Hussein H.S. and Sakuma T. 2005. Prevalence of shiga toxin-producing *Escherichia coli* in dairy cattle and their products. J. Dairy Sci. 88: 450.

ISO. 2001. Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Escherichia coli* O157. ISO 16654. The International Organization for Standardization, Geneva, Switzerland.

Jay J.M., Loessner M.J. and Golden D.A. 2005. Foodborne gastroenteritis caused by *Escherichia coli*. Ch. 27. In "Modern Food Microbiology" 7th ed. Jay J. M., Loessner M.J. and Golden D.A. (Ed.). p.637. Springer. New York, NY.

Kasrazadeh M. and Genigeorgis C. 1995. Potential growth and control of *Escherichia coli* O157:H7 in soft Hispanic type cheese. Int. J. Food Microbiol. 25: 289.

Liptakova A., Siegfried L., Rosocha J., Podracka L., Bogyiova E. and Kotulova D. 2004. A family outbreak of haemolytic uraemic syndrome and haemorrhagic colitis by verocytotoxigenic *Escherichia coli* O157 from unpasteurised cow's milk in Slovakia. Clin. Microbiol. Infect. 16: 576.

Maldonado Y., Fiser J.C., Nakatsu C.H. and Bhunia A.K. 2005. Cytotoxicity potential and genotypic characterization of *Escherichia coli* isolates from environmental and food sources. Appl. Environ. Microbiol. 71: 1890.

Marier R., Wells J.G., Swanson R.C., Callahan W. and Mehlman I.J. 1973. An outbreak of enteropathogenic *Escherichia coli* foodborne disease traced to imported French cheese. Lancet. 2: 1376.

Nataro J.P. and Kaper J.B. 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11: 142.

Pierard D., Van Damme L., Moriau L., Stevens D. and Lauwers S. 1997. Virulence factors of verocytotoxin-producing *Escherichia coli* isolated from raw meats. Appl. Environ. Microbiol. 63: 4585.

Quinto E.J. and Cepeda A. 1997. Incidence of toxigenic *Escherichia coli* in soft cheese made with raw or pasteurised milk. Lett. Appl. Microbiol. 24: 291.

Reitsma C.J. and Henning D.R. 1996. Survival of enterohemorrhagic *Escherichia coli* O157:H7 during the manufacture and curing of Cheddar cheese. J. Food. Protect. 59: 460.

Strachan N.J., Fenlon D.R. and Ogden I.D. 2001. Modelling the vector pathway and infection of humans in an environmental outbreak of *Escherichia coli* O157. FEMS Microbiol. Lett. 203: 69.

Tilden J.Jr, Young W., McNamara A.M., Custer C., Boesel B., Lambert-Fair M.A., Majkowski J., Vugia D., Werner S.B., Hollingsworth J. and Morris J.G. Jr. 1996. A new route of transmission for *Escherichia coli*: infection from dry fermented salami. Am. J. Public Health. 86: 1142.

UNI. 2002. Microbiology of food and animal feeding stuffs – Routine horizontal method for the enumeration of *Escherichia coli* β-glucuronidase-positive – Colony-count tecnique at 44°C. UNI 10980. Ente Nazionale Italiano di Unificazione, Rome, Italy.

Revised paper received October 23, 2006 Accepted February 22, 2007