

# The Significance of *Escherichia coli*-induced Mastitis in Cows Associated with the Presence of Virulence Genes and Wide Range-resistance to Twenty Antimicrobials

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F5/rfbE genes, *E. coli*, Mastitis.

## ABSTRACT

The significance of *Escherichia coli*-induced mastitis in cows, associated with the presence of virulence genes and wide range resistance to 20 antimicrobials, is concluded. The targeted surveillance included 67 randomly chosen cows on 10 rural farms, distributed among four out of the five provinces of Lebanon. There was a high frequency of California Mastitis Test (CMT)-positive milk samples in the targeted cows, equivalent to 77.6%, with recovered *E. coli* from 31% of the CMT- positive samples. A significant correlation existed between the *E. coli* count (Y) and the CMT score (X) in a regression equation of  $Y = 0.6691X - 0.2454$  ( $P < 0.05$ ). The respective frequencies of three genes, in randomly chosen 75 biochemically confirmed *E. coli* isolates, recovered from CMT- positive milk samples, were: *eae* gene (8.0%), F5 gene of K99-*E. coli* (1.3%), and rfbE of O157 serotype of *E. coli* (0%). The nucleotide sequence of the F5 gene of K99-*E. coli* recovered from one cow revealed a 4% difference from that of the *E. coli* vaccine strain used on that herd. The range of percent resistance of the 75 *E. coli* isolates to 20 antimicrobials was 1-100%. The presence of an average resistance to 20 drugs of 37.9% in Intimin (author, please check spelling) (*eae*) positive-*E. coli* is of high significance in chemotherapeutic programs applied on *E. coli*-induced mastitis in cows.

## INTRODUCTION

Bovine Mastitis is the most unmanageable disease in dairy herds (Bramely and Dodd 1984). The California Mastitis Test (CMT) provided a useful tool for farmers and veterinarians for measuring the level of inflammation in the udder (Barnum and Newbould 1960). There is a meager literature related to research on mastitis in dairy cows present in tropical and sub-tropical countries of the Middle East. The determination of clinical, bacteriological, and epidemiological aspects of clinical mastitis is searched in most

countries of the world, including a 4 year study in the Middle East, that was concluded in Israel (Shpigel et al 1998). This study showed that out of 1,089 cows, 1,190 udder quarters were affected with clinical mastitis. Bacterial isolation from the infected quarters resulted in an association of the clinical mastitis with coliform bacteria (60.2%), environmental streptococci (18.6%) and coagulase-negative staphylococci (8.7%). It is worth mentioning that 90% of the isolated fecal coliforms in this study were *E. coli*.

Another study related to environmental bovine mastitis pathogens was performed in west central Iran. This study showed that out of 620 milk samples, collected from four districts in Iran, 180 were CMT positive. The percentage of samples with recovery of different bacterial population were: 3.88% coagulase-negative staphylococci, 8.33% streptococci, and 9.44% *E. coli* (Ebrahimi et al 2007).

A documented study, performed in the Kingdom of Saudi Arabia, correlated the CMT scores to bacterial counts in milk of the *Camelus dromedarius* (Barbour et al 1985). Virulence genes, present in *E. coli*-induced mastitis, include the *eae* gene. Enteropathogenic *E. coli* strains (EPEC) can colonize the intestine and cause attaching and effacing (A/E) lesions.

Intimin is a protein encoded by *eae* gene (Ghanbarpour and Oswalt 2010). It facilitates the adherence of attaching and effacing *E. coli* to the intestinal epithelial cells. It is proven that the *eae* gene in *E. coli* plays a definite role in induction of cattle mastitis (Correa and Marin 2002). Another virulence factor in K99-*E. coli* is coded by the F5 gene. A study conducted by Ok et al (2007) showed a high rate of detection of the F5 gene in diarrheic calves (13.41%) compared to a lower frequency in healthy calves (5.55%), incriminating the k99-*E. coli* as an etiologic agent of calf diarrhea. The calf diarrhea causes huge economic losses in the veal industry around the world (Saeed et al 1993). In addition, ruminants are important reservoir of *E. coli* O157:H7. This sero-

type has a zoonoses nature, associated with hemorrhagic colitis, bloody diarrhea, and Hemolytic Uremic Syndrome in humans (Tarr et al 2005). The rfbE specific gene is used for identification of *E. coli* O157:H7 serotype (Bilge et al 1996).

Many drug resistant bacteria, involved in animal diseases, are becoming of paramount importance due to their significant negative impact on animal and human health. The multi-antimicrobial-resistant (MAR) bacterial strains are continually identified, carrying more than one antimicrobial resistant determinant on plasmids, transposons, and gene cassettes (Carattoli 2001; Hall and Collis 1995). In Serbia a high percentage of MAR- *E. coli* isolates were recovered from cattle, broiler hens, and swine (Knezevic and Petrovic 2008).

A study performed in Korea by Unno et al (2010) found that the antimicrobial resistant-*E. coli* strains could transfer their resistance to other microorganisms, including zoonotic pathogens. This study emphasized that drug resistant bacteria emerge due to the use of subtherapeutic levels of antimicrobials in disease prophylaxis and growth promotion protocols.

To our knowledge, this is the first targeted surveillance that attempted to correlate the *E. coli* count to CMT scores in cow's milk, while pointing at the significance of detected virulence-associated genes of eae, F5, and rfbE in the *E. coli* etiologies of mastitic milk, and the uncovering of high resistance in the eae positive-*E. coli* etiologies to 20 most commonly used antimicrobials in veterinary and/or human medicine.

## **MATERIAL AND METHODS**

### **Experimental Design of the Targeted Surveillance**

A total of 67 cows, randomly chosen from 10 rural farms located in four out of five provinces of Lebanon, were included in this targeted surveillance. The inclusion number of cows in the design was based on about 50% of the herd size. An average of 1.4 teats /cow were sampled, resulting in a

total of 96 milk samples. The frequency of this sampling was based on the presence of signs of inflammation in the chosen teats. The following parameters were assessed in each individual milk sample, namely, the score of the California Mastitis Test (CMT), *E. coli* count, isolation, and biochemical characterization of *E. coli* recovered from milk samples, presence of virulence genes in *E. coli*-isolates and their susceptibility to 20 antimicrobials. Details of the procedures, related to sampling and assessment of the different parameters, are shown below.

### **Milk Sampling and CMT Scoring**

Milk sample collection was performed by choosing randomly 5-10 lactating cows on each farm. The udder was washed thoroughly with water, dried with a paper towel, disinfected with iodine solution, and the foremilk was discarded. This was followed by collection of around 4 mL of milk from the sampled teat, and its placement in one compartment of the CMT paddle. A volume of 2 mL of CMT reagent is added over the 4 mL of milk, and mixed by swirling of the CMT paddle for 30 seconds, followed by tilting the paddle to a 45° angle, observing the CMT product, and scoring it in a range of 0 to 3, according to a previous description by Mellenberger (2001).

A volume of 10 mL of milk is collected aseptically from the CMT- tested teat, put in a sterile cup, and transported in an ice chest to the Animal and Veterinary Sciences Department Laboratory at the American University of Beirut.

### ***E. coli* Count, Isolation, and Biochemical Characterization**

An amount of 1 mL of each collected individual milk sample was serially diluted, with a dilution factor of 1/10, using sterile saline diluent. An amount of 0.1 mL of each dilution was spread over a McConkey agar plate. The plates were incubated between 18 to 24 h at 37°C. The bacterial colonies were counted and five lactose fermenting colonies were randomly selected, and each colony was subcultured at 37°C for 18 to 24 h, on Triple Sugar Iron (TSI) Agar slant.

The obtained TSI cultures were covered by sterile Tryptose Phosphate broth, and stored at -80°C.

All stored lactose fermenting cultures were later subjected to:

- biochemical tests, on Triple Sugar Iron agar (sugar fermentation and H<sub>2</sub>S production)
- Sorbitol McConkey agar (for sorbitol fermentation)
- Peptone water (for indol production)
- Voges-Proskauer broth (for acetoin production), and

- Simmons Citrate agar (for utilization of citrate as the only source of carbon).

The confirmation of the *E. coli* identity was according to matching biochemical results documented by Genta and Heluane (2001).

### Presence of Virulence Genes in *E. coli* Isolates

The presence of three virulence genes in *E. coli*, namely *rfbE*, *eae*, and F5, was examined on the extracted genome of the isolated *E. coli* strains from milk samples, and the application of optimized PCR protocol for each gene.

**Table 1.** PCR protocols for *rfbE*, *eae*, and F5 genes

PCR steps	PCR protocols for three genes		
	<i>rfbE</i>	<i>eae</i>	F5
DNA concentration	46.2 pmol/μL	46.2 pmol/μL	46.2 pmol/μL
Volume of DNA	2.0 μL	2.0 μL	16.0 μL
REDTaq®Ready Mix <sup>1</sup>	25.0 μL	25.0 μL	25.0 μL
PCR mixture volume	50.0 μL	50.0 μL	50.0 μL
Reaction Mixture Buffer	20.0 mM Tris HCl	20.0 mM Tris HCl	20.0 mM Tris HCl
Thermal cycler <sup>2</sup>	iCycler	iCycler	iCycler
Electrophoresis of amplicon	2% agarose gel, 100 V, 500 mA, 60 min.	2% agarose gel, 100 V, 500 mA, 60 min.	2% agarose gel, 100 V, 500 mA, 60 min.
Amplicon stain	Ethidium Bromide, 0.5 μg/ml in Tris-EDTA buffer	Ethidium Bromide, 0.5 μg/ml in Tris-EDTA buffer	Ethidium Bromide, 0.5 μg/ml in Tris-EDTA buffer
Band visualization	GelDoc system <sup>3</sup>	GelDoc system	GelDoc system
Forward primer (volume)	O157AF of 5 pmol/ μL (2 μL)	IntAF of 5pmol/ μL (4 μL)	K99F of 5pmol/ μL (2 μL)
Reverse primer (volume)	O157AR of 5 pmol/ μL (2 μL)	IntAR of 5pmol/ μL (4 μL)	K99R of 5pmol/ μL (2 μL)
Amplicon size	497 bp <sup>4</sup>	570 bp <sup>5</sup>	314 bp <sup>6</sup>
Cycling conditions	1 cycle (95° C for 5 min), followed by 35 cycles (each set at 94° C for 30s, 66° C for 30s, and 72°C for 30 s)	25 cycles (each set at 94° C for 30s, 50° C for 30s, and 70°C for 90 s)	25 cycles (each set at 94° C for 30s, 50° C for 30s, and 70°C for 90 s)

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<sup>4</sup>Desmarchelier et al. 1998

<sup>5</sup>China et al. 1996

<sup>6</sup>Ok et al. 2007

## DNA Extraction

DNA extraction from the biochemically confirmed-*E. coli* culture was performed using the protocol of the Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare UK Limited, Buckinghamshire, HP7 9NA, UK). The extracted DNA concentration is quantified by spectrophotometry at 260 nm (Barnstead International 2,555, Kerper Boulevard, Dubuque, Iowa 52001, USA) (Heaton 1999). The purified genomic DNA of each *E. coli* colony is stored at -40°C for application of optimized PCR protocols related to the respective three genes.

## Optimized PCR protocols for three genes

The PCR protocols for *rfbE*, *eae*, and F5 genes are presented in Table 1. The PCR optimization of *rfbE* gene was performed on O157:H7 *E. coli* reference strain provided by the American Type Culture Collection (ATCC) (10801 University Boulevard Manassas, VA 20110 USA). The sequence of the forward primer O157AF was 5' AAG ATT GCG CTG AAG CCT TTG 3' and for the reverse primer O157AR was 5' CAT TGG CAT CGT GTG GAC AG 3' (Desmarchelier et al 1998). Other specifics of this optimized PCR protocol are presented in Table 1.

The optimized protocol of PCR for *eae* gene, in reference *E. coli* O157:H7 strain, is described in Table 1. The sequence of forward primer IntAF was 5' ATATCCGTTT-TAATGGCTATCT 3' and that of the reverse primer IntAR was 5' AATCTTCTGCG-TACTGTGTTCA 3' (China et al 1996). The rest of the optimized PCR protocol specifications are presented in Table 1.

The Scourguard 4KC vaccine (Pfizer Inc, New York, 10017, USA) was used for amplification of the F5 fimbriae gene present in the inactivated K99-*E. coli*. Separation of the bacterial-aqueous portion from the oil emulsion of the bacterin, containing *Clostridium perfringens* Type C, and K99-*Escherichia coli*, was performed according to the following steps: A volume of 2 mL of the oil emulsion vaccine was added over 2 mL of acetone, followed by vortexing

for 1 minute (Vortex Mixer, Industries Inc. Bohem, NY, USA). Centrifugation is applied on the vortexed material at a speed of 15,557 x g for 15 minutes. The upper liquid layer is discarded, and the pellet was kept in the centrifuge tube. A volume of 1 mL sterile saline is added over the pellet for reconstitution, followed by vortexing for a period of 1 minute, and centrifugation at a speed of 15,557 x g for 15 minutes. The washing by saline is repeated for three times, ending with a suspended pellet in 1 mL of sterile saline. The F5 fimbriae gene was amplified according to the optimized protocol described in Table 1, relying on previous work reported by Bertin et al (1996). The sequence of the forward primer K99F was 5' -TATTATCTTAGGTG-GTATGG- 3' and that of the reverse primer K99R was 5' -GGTATCCTTTAGCAG-CAGTATTC- 3' (Bertin et al. 1996).

The optimized PCR protocols for *rfbE* and *eae* genes amplification, using reference ATCC isolate of O157: H7, and for the F5 gene amplification using the Scourguard 4KC bacterin, were adopted for application on *E. coli* isolates recovered from milk samples .

## Sequencing of Amplified F5 Gene of K99-*E. coli*

Sequencing of the amplified nucleotides of F5 gene present in K99- *E. coli* of the Scourguard 4KC bacterin, and in the field *E. coli* was performed according to the following protocol: Four µL of Dynamic ET (Amersham, Uppsala, Sweden) was added in a 200 µL capacity PCR tube. A volume of 4 µL of 5 pmol of reverse primer (K99R) was added. Four µL of the DNA template with a concentration of 10 ng/ µL were added to the mixture. Nuclease free water was added to complete the volume to 20 µL. The F5 gene amplicon was sequenced using 3,100 Avant Genetic Analyzer-ABI PRISM (Applied Biosystems, Hitachi). The percentage of difference in the nucleotide sequences of F5 gene between the K99 *E. coli* in the vaccine versus the escape mutant *E. coli* is calculated by dividing the number of mutated nucleotides by the total number of the

sequenced amplicon nucleotides, multiplied by 100.

### **Susceptibility Testing of *E. coli* to Twenty Different Antimicrobials**

The single disc diffusion assay (Bauer et al 1966) was used to assess the susceptibility of *E. coli* isolates to 20 antimicrobials. Briefly, a loopful amount of each isolate was sub-cultured into 0.5 mL Tryptose Phosphate Broth, and incubated for 2-3 h until a log phase was reached. An amount of 0.1 mL of this bacterial suspension, diluted to match the turbidity of McFarland tube number 2 was spread on Mueller Hinton Agar (MHA) (HiMedia Laboratories Pvt. Ltd. Mumbai – 400086, India). Four MHA plates were used for each *E. coli* isolate, with five different antimicrobial discs applied on each. The 20 antimicrobial discs used for susceptibility testing and their potency were as follows:

- Amikacine (30µg)
- Amoxicillin Clavulanic Acid (30µg)
- Ampicillin (10µg)
- Aztreonam (30µg)
- Cefamandole (30µg)
- Cefixime (5µg)
- Cefotaxime (30µg)
- Cefoxitin (30µg)
- Cefepime (30µg)
- Ceftazidime (30µg)
- Cefuroxime (30µg)
- Cephalothin (30µg)
- Ciprofloxacin (5µg)
- Gentamicin (10µg)
- Imipenem (10µg)
- Nitrofurantoin (300µg)
- Norfloxacin (10µg)
- Piperacillin/Tazobactam (85µg)
- Sulphamethoxazole Trimethoprim (25µg), and
- Tobramycin (10µg).

The MHA plates were incubated at 37 °C for 24 h, and the diameter of the inhibition zone around each disc is measured in millimeter. The interpretation of susceptibilities was based on a chart provided by the anti-

microbial disc-manufacturer (Oxoid Ltd., Wade Road, Basingstoke, Hampshire, RG24 8PW, England).

### **Statistical Analysis**

Correlation between the CMT score and the log *E. coli* count was performed by multiple regression at a significance level of  $P \leq 0.05$ . Susceptibilities of the 75 *E. coli* isolates recovered from milk of dairy cows to 20 antimicrobial agents were compared by Chi-square test.

## **RESULTS AND DISCUSSION**

The following are the results of and discussion of *E. coli*-induced mastitis in targeted surveillance of rural dairy cows:

### ***E. coli*-Induced Mastitis**

An average of 1.4 teats/cow was sampled from 67 cows resulting in a total of 96 milk samples. The respective percentages of *E. coli*-positive cows and *E. coli* positive milk samples were 24% and 17.7%, respectively. The high percentage of CMT- positive milk samples (77.6%), associated with *E. coli* recovery of 31%, is indicative of the significance of the *E. coli* etiology in mastitis. This is in agreement with documented literature showing that *E. coli* is among the most common infectious agents isolated from severe mastitis cases in modern dairy farms (Bradley et al 2007; Hogan et al 1989). *E. coli* has been reported to be the most common cause of clinical mastitis in well-managed dairy herds with low milk somatic cell counts (SCC) in the United Kingdom (Bradley 2002). According to Bradley et al (2007), *E. coli* was isolated from 19.8 % of the clinical mastitic milk samples collected from 97 farms that were located in England and Wales. Moreover, *E. coli* has been isolated from 6% of quarter milk samples taken from clinical mastitis cases in Finland, with higher frequency of isolations in Spring and Summer seasons (Koivula et al 2007).

The correlation between the log<sub>10</sub> *E. coli*- counts (Y) and the CMT scores (X) resulted in a significant regression equation of  $Y=0.669X-0.245$ , ( $p<0.05$ ); the CMT positive milk samples out of the 96

tested samples was 77.6%, with 31% of the CMT- positive samples resulting in *E. coli*- recovery. The positive significant correlation between the Log<sub>10</sub> *E. coli* count and the CMT score obtained in this study is in agreement with the results obtained by Dingwell et al (2003), reporting a positive correlation between CMT score and *E. coli* count recovered from cow milk samples. A study performed by Devriese and Keyser (1980), investigating the involvement of different bacterial species in mastitis of dairy cows, revealed a percentage of 7% staphylococcus infection in the CMT negative quarters compared to 21 % in the CMT positive quarters.

Another study performed in dairy herds in California, investigating the occurrence of clinical mastitis over 3-years period, revealed the detection of coliforms and environmental streptococci in 60% of the total clinical mastitis cases, compared to lower frequencies in mastitis-negative samples (Gonzalez et al 1989). The significant correlation proven in this study confirms the significance of the *E. coli* etiology in causing mastitis in a high percentage of the rural

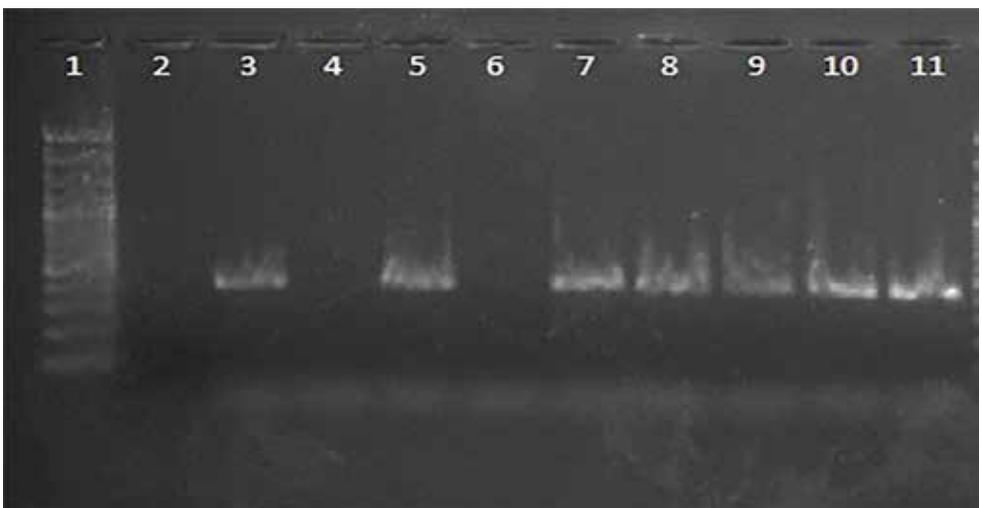
cows subjected to this targeted surveillance.

### Frequency of Virulence Related Genes

The respective frequencies of presence of three virulence related-genes in randomly chosen 75 biochemically confirmed-*E. coli* isolates, recovered from CMT- positive milk samples, were: *eae* gene (8.0%), F5 gene of K99-*E. coli* (1.3%), and *rfbE* gene of O175 serotype of *E. coli* (0.0%). The banding of the *eae* gene amplicons, in comparison to positive control of the reference ATCC O157 *E. coli* and negative controls deprived of *E. coli* DNA, is shown in Fig. 1. The banded amplicons of *eae* gene were consistently positioned at 570 bp, while this band was absent in negative controls.

The presence of *eae* Intimin gene in *E. coli* involved in mastitis of dairy cows is of paramount importance (Fig.1). The length of the amplified part of the Intimin gene is located at 570 bp, which is in agreement with a previously reported data (China et al 1996). *E. coli* with Intimin gene are able to form small microcolonies on the surface of infected epithelial cells, followed by localized degeneration of the microvilli cumulating in an attaching and effacing (A/E)

**Figure 1.** Electrophoretic pattern of PCR amplification of the *eae* (Intimin) gene recovered from six out of 75 *E. coli* isolates present in mastitic milk samples. Lane 1= 100 base pair ladder, lane 2 = negative control, lane 3= positive control of ATCC reference isolate of O157 *E. coli*, lanes 5,7,8,9,10, and 11= positive samples for *eae* gene, lanes 4 and 6= *eae* negative samples, with PCR mixes deprived of DNA substrate.



**Figure 2.** Electrophoretic pattern of PCR amplification of the F5 gene of K99-*E. coli* recovered from one out of 75 *E. coli* isolates. Lane 1= 100 base pair ladder, lane 2= negative control, lane 3= positive control of Scourguard 4KC vaccine isolate of K99 *E. coli*, lane 4 = positive sample for F5 gene.



lesions. In the developing nations, these lesions result in high morbidity and mortality in children, as a result of *E. coli* induction of violent diarrhea (Kenny et al 1997).

Intimin expresses an outer membrane protein needed for intimate adherence (Jerse et al 1990). This gene participates in reorganization of the underlying host cytoskeleton, at the site of accumulation of actin, directly below the attached infected cell. This adherence stimulates a second wave of signal transduction inside the mammalian

cell, including tyrosine phosphorylation of phospholipase C $\gamma$  (Kenny and Finlay 1997). Consequently, the inositol triphosphate and Ca<sup>++</sup> fluxes will be increased as a signaling event leading to destruction of the brushborder of the infected intestinal epithelial cells. The exact mechanism of destruction is not well understood (Truper and Shleifer 2006).

The banding of the F5 gene amplicon of K99-*E. coli*, detected in one out of the 75 *E. coli* isolates, is shown in Fig. 2. The amplicon is positioned at 314 bp, a band that was absent in the negative controls deprived of *E. coli* DNA, and present in the positive control of K99 *E. coli* present in the Scourguard 4KC bacterin. The length of the amplified part of the F5 gene in the *E. coli* recovered from one of the cows (Fig. 2, lane 4), at the 314 bp position, is in agreement with that reported by Bertin et al (1996). The presence of F5 gene in K99-*E. coli* causes a fatal syndrome in the veal calf and it is characterized by anorexia, fever, bloody or non-bloody diarrhea, hemorrhagic diathesis on the conjunctival surface of the eyes, and the mucus surfaces of the mouth and nose (Harrison 1978; Espinasse et al 1973). This disease causes huge economic losses to the veal calf producers, and it has become of major concern to the veal calf industry in the mid Western United States (Saeed et al 1993).

The attempts in amplification of the rfbE gene in 9 *E. coli* isolates, which were non-fermenting of the sorbitol sugar, are shown in Fig. 3. The rfbE gene of the positive control of ATCC reference O157 *E. coli* was successfully amplified, resulting in a band situated at 479 bp, while the amplicon of the negative control, deprived of the reference strain DNA, was absent. The attempt to amplify the rfbE gene in sorbitol-non-fermenting colonies of *E. coli* failed in this work (Fig. 3). The search for the rfbE gene in *E. coli* involved in mastitis should be included in routine continuous and not exclusively in targeted surveillances, due to the fact that *E. coli* O157 strain is found in the normal flora of the cows, and it has a zoonotic potential

**Figure 3.** Electrophoretic pattern of PCR amplification of the reference *rfbE* gene of O157-*E. coli* on agarose gel. Lane 1 = 100 base pair ladder, lane 2 = negative control for *rfbE* gene, lane 3 = positive control for *rfbE* gene amplicon, lanes 4-12 = negative results for the *rfbE* gene in sorbitol-non fermenting isolates.



in humans, causing hemorrhagic colitis, bloody and non-bloody diarrhea and hemolytic uremic syndrome (Wong et al 2000; Dean-Nystrom et al 1997). *E. coli* O157 strains are also associated with pathogenesis in neonatal pigs and calves (Francis et al 1989; Tzipori et al 1988).

#### **Comparison of F5 Gene Sequence in Vaccine Isolate vs Field Escape Mutant**

The comparison of the nucleotide sequence of the F5 gene of the K99 isolate of *E. coli* present in the Scourguard 4K vaccine (Pfizer Animal Health, NY, USA) and that of the escape mutant of *E. coli*, recovered from one mastitic milk, sampled on the farm that uses the Scourguard 4K vaccine, are shown in Table 2. The percentage of difference in the nucleotide sequences of F5 gene between the K99 *E. coli* in the vaccine versus the escape mutant of *E. coli* is 4%. The presence of the F5 gene in field *E. coli* strain recovered from the same farm that has been using the vaccine for almost 4 years against K99-*E. coli*, adds to the proof that vaccination eliminates the disease, but does not eliminate its etiologic agents. This is mainly because of the immune pressure created by the vaccine that help in neutralizing field

strains that are homologous to the vaccine strain, while selecting for replacement in the field by escape mutants of *E. coli*-K99, that cannot be neutralized (Mizoguchi et al 2003). This 4 % mutation in the escape mutant that is recovered in this study will be the subject of future study for its inclusion in modified vaccine against this ailment in dairy herds.

#### **Susceptibility of the 75 *E. coli* Isolates Recovered from Mastitic Milk to Twenty Antimicrobials**

The susceptibility of the 75 *E. coli* isolates recovered from mastitic milk to 20 antimicrobials is shown in Table 3. The percent resistance of

the 75 isolates to the tested antimicrobials ranged from 1- 100%. It is worth noting that 9 out of the 20 antimicrobials were ineffective in vitro against more than 50% of the *E. coli* isolates. The percent resistance of the *E. coli* isolates to each of the 9 antimicrobials, in decreasing order, is as follows:

- Amoxicillin clavulanic Acid (100%)
- Cephalothin (100%)
- Nitrofurantoin (100%)
- Cefotaxime (97%)
- Tobramycin (88%)
- Gentamycin (77%) Cefuroxime (65%)
- Amikacine (65%), and
- Cefepime (53%).

The high resistance in the 75 *E. coli* isolates to the 20 antimicrobials is alarming. This situation could have emerged due to the non-controllable use of drugs against mastitis. Actually, the maintenance of antimicrobial drug resistance is governed by continued exposure to antimicrobial drugs (Jorgensen and Halling-Sorensen 2000), plasmid addition mechanisms (Engelberg-Kulka and Glacer 1999), or close genetic linkage to other

**Table 2.** Comparison of nucleotide sequences\* of F5 gene of Scourguard 4KC vaccine K99 isolate versus the escape mutant of *E. coli* recovered from mastitis milk

Escape mutant of <i>E. coli</i>	K99 isolate of Scourguard 4K vaccine
CCTTCTCGCTGTCNTCTTAAAACCNT NAACTGTGCATCGAATCTCGTACCAGTC AATACGAGCATTGTTTTTCGCTAGGCAG TCATTACTGCCGGGCGCTGGTTTTAGTTT AAAATCCACTACAGTGCCATGACCACTA ATAGCAGCCTGCCAAGATCTATAGTTGA TGTACGATTACCATTGACCTCAGGCTCAA TTGTACAAGTAGCACTCGTTATTTTTGC- CATTGAAGTTAATAGTACCTGTATTC- GCAGAAGCATGGTAGTCGAAAAGC- CATACCACC	CCNTGCCGCTGTTNTCTTA- CAACCTCAACTGTTTCATAGAAT- CAGACCAGTCAATACGAGCATT- GTTTTTCGCTAGGCAGTCATTACT- GCCGGGCGCTGGTTTTAGTTTA- AAATCCACTACAGTGCCATGAC- CACTAATAGCAGCTGCCCAA- GATCTATAGTTGATGTACGATTAC- CATTGACCTCAGGCTCAATTGTA- CAAGTAGCACTCGTTATTTTTGC- CATTGAAGTTAATAGTACCTG- TATTCGCAGAAGCATTGGTAGTCG- CAAAGCCATACCACC

\*N=Non-identified nucleotide

selectively advantageous genes (Kehrenberg and Schwarz 2001).

A study to determine the prevalence of antimicrobial resistance in *E. coli*, performed in South Korea, showed a large percentage of drug resistance in *E. coli* isolates (72,3%). This resistance was to more than one antimicrobial agent (Unno et al 2010). The blame in this global increase in resistance to antimicrobial agents was related to excessive use of drugs (Harakeh et al 2005, von Baum and Marre 2005, Aerestrup and Wegener 1999). It is worth noting that the respective average percent resistance to the 20 antimicrobials in Intimin-positive *E. coli* versus Intimin-negative *E. coli* were 37.9% and 62.8% (P<0.05). The presence of an average resistance of 37.9% in Intimin (eae) positive-*E. coli* to the 20 antimicrobials is of high significance in the therapeutic approaches to control the mammary gland of cows from these highly pathogenic infecting bacteria. The Intimin gene (eae), found in the *E. coli* bacteria, is chromosomally encoded (Schmidt et al 1999, Birch et al 1996). A study conducted in India for the identification of virulence genes in antimicrobial resistant *E. coli* isolates revealed a percentage of 6.4% isolates possessing the Intimin gene (Khan et al 2002). A study focusing on determination of antimicrobial resistance in

relation to the bacterial phage, serotype and virulence genes was performed in Spain.

The study revealed an association between a higher level of multiple drug resistance in Shiga toxin-producing *E. coli* and the presence of the virulence genes, namely eae and stx1 (Mora et al 2005). Another study performed in Greece, studying the occurrence of virulence genes and antibiotic resistance of *E. coli* O157 isolated from bovine, caprine, and ovine raw milk, revealed a percentage of 41.4% of the *E. coli* O157 strains that were positive for the virulence genes stx and eae. Moreover, all the *E. coli* isolates with the virulence genes stx and eae showed resistance to a higher number of antimicrobials than those which were stx-negative (Solomakos et al 2009). A study performed in the US, aimed at characterization of the eae gene from *E. coli* isolates recovered from healthy and diarrheic calves, revealed a high presence of antimicrobial resistant isolates containing eae gene in diarrheic calves (Holland et al 1999).

## CONCLUSION

In conclusion, this targeted surveillance of rural dairy farms confirmed the significance of *E. coli* infection in mastitis of cows. There was a clear significant correlation between the CMT scores and the *E. coli*

**Table 3.** Susceptibility of 75 *E. coli* isolates recovered from mastitic milk of dairy cows to 20 antimicrobial agents

Antimicrobial agents (potency)	Susceptibility of 75 <i>E. coli</i> isolates to drugs (%)		
	Resistant	Intermediate	Susceptible
Amoxycillin Clavulanic Acid (30µg)	75 (100%) <sup>a</sup>	0 (0%) <sup>g</sup>	0 (0%) <sup>g</sup>
Cephalothin (30µg)	75 (100%) <sup>a</sup>	0 (0%) <sup>g</sup>	0 (0%) <sup>g</sup>
Nitrofurantoin (300µg)	75 (100%) <sup>a</sup>	0 (0%) <sup>g</sup>	0 (0%) <sup>g</sup>
Cefotaxime (30µg)	73 (97%) <sup>a</sup>	0 (0%) <sup>g</sup>	2 (3%) <sup>g</sup>
Tobramycin (10µg)	66 (88%) <sup>b</sup>	9 (12%) <sup>e,f</sup>	0 (0%) <sup>g</sup>
Gentamicin (10µg)	58 (77%) <sup>b,c</sup>	14 (19%) <sup>d,e</sup>	3 (4%) <sup>f,g</sup>
Cefuroxime (30µg)	49 (65%) <sup>c,d</sup>	0 (0%) <sup>g</sup>	26 (35%) <sup>e</sup>
Amikacine (30µg)	49 (65%) <sup>c,d</sup>	20 (27%) <sup>d</sup>	6 (8%) <sup>f</sup>
Cefepime (30µg)	40 (53%) <sup>d,e</sup>	33 (44%) <sup>c</sup>	2 (3%) <sup>g</sup>
Piperacillin/Tazobactam (85µg)	35 (47%) <sup>e,f</sup>	0 (0%) <sup>g</sup>	40 (53%) <sup>d</sup>
Cefixime (5µg)	32 (43%) <sup>e,f</sup>	0 (0%) <sup>g</sup>	43 (57%) <sup>d</sup>
Sulphamethoxazole Trimethoprim (25µg)	24 (32%) <sup>f,g</sup>	0 (0%) <sup>g</sup>	51 (68%) <sup>d</sup>
Ampicillin (10µg)	22 (29%) <sup>g,h</sup>	5 (7%) <sup>f</sup>	48 (64%) <sup>d</sup>
Cefoxitin (30µg)	21 (28%) <sup>g,h</sup>	0 (0%) <sup>g</sup>	54 (72%) <sup>d</sup>
Cefamandole (30µg)	20 (27%) <sup>g,h,i</sup>	0 (0%) <sup>g</sup>	55 (73%) <sup>c</sup>
Aztreonam (30µg)	12 (16%) <sup>h,i</sup>	46 (61%) <sup>b</sup>	17 (23%) <sup>e</sup>
Norfloxacin (10µg)	12 (16%) <sup>h,i</sup>	0 (0%) <sup>g</sup>	63 (84%) <sup>a,b</sup>
Ciprofloxacin (5µg)	11 (15%) <sup>i</sup>	5 (6%) <sup>f</sup>	59 (79%) <sup>b</sup>
Ceftazidime (30µg)	2 (3%) <sup>j</sup>	73 (97%) <sup>a</sup>	0 (0%) <sup>g</sup>
Imipenem (10µg)	1 (1%) <sup>j</sup>	6 (8%) <sup>e,f</sup>	68 (91%) <sup>a</sup>

<sup>a-j</sup>Frequencies in a column followed by different alphabet superscripts are significantly different ( $P < 0.05$ )

count, which strengthen the conclusion of *E. coli* involvement in mastitis of cows. The Intimin (eae) and F5 virulence genes were present in 8.0% and 1.3% of the recovered *E. coli* isolates respectively, while the O157 (rfbE) gene was absent in the recovered *E. coli* isolates. The sequencing of the vaccine vs field strain of K99 revealed the presence of a field escape mutant with differences in nucleotide sequence of 4% compared to the used vaccine strain used on the same farm. There was a high antimicrobial resistance to nine drugs in more than 50% of the 75 *E.*

*coli* isolates, including an average resistance of 37.9% to the 20 antimicrobials in the Intimin-positive *E. coli*.

It is recommended in disease-control programs of dairy to study the *E. coli* involvement in mastitis, and to include in the surveillance the detection of virulence genes that are decisive in economic losses. In addition, the *E. coli* susceptibility testing to antimicrobials that are used in veterinary and/or human medicine, will help in future evaluations of drug-resistance control programs.

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