Applicability of RIDA®QUICK Verotoxin/O157 Combi kit for detection of Shiga toxin producing *Escherichia coli* O157:H7 in raw milk

[1]Kamal, R. M., [2]Merwad, A. M., [3]Ali, S. A., [4]Saber, T. M. and [1*]Bayoumi, M. A.

[1]Food Control Department, Faculty of Veterinary Medicine, Zagazig University, Egypt
[2]Department of Zoonoses, Faculty of Veterinary Medicine, Zagazig University, Egypt
[3]Department of Food Science and Nutrition, College for Women, Kuwait University, Kuwait
[4]Department of Medical Microbiology and Immunology, Faculty of Medicine, Zagazig University, Egypt

Abstract

Many methods have been developed for the rapid and sensitive detection of *Escherichia coli* O157:H7 in milk and other foodstuffs. This study was designed to evaluate the application of RIDA®QUICK Verotoxin/O157 Combi kit, an immuno-chromatographic detection method, for the detection of this pathogen in raw milk. RIDA®QUICK kit showed appreciable results in detecting *Escherichia coli* O157:H7 in raw milk, as it was able to detect as low as 100 CFU/ml of raw milk, and as low as 10 CFU/ml of sterilized milk. Raw milk's microflora negatively affected the detection sensitivity, which could be overcome through a prior antibiotics' enrichment step. Comparable results between this new method and a reference detection method were also obtained. Obtained data suggests the applicability of RIDA®QUICK kit in detection of *Escherichia coli* O157:H7 in raw milk.

Introduction

Haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) are two serious human life-threatening diseases caused by the release of potent shiga toxins (ST1 and ST2). These toxins are produced from certain *Escherichia coli* (*E. coli*) strains that are named accordingly as shiga toxin producing *E. coli* (STEC), or according to the disorders, which they cause as enterohemoarrahgic *E. coli* (EHEC) (Nataro and Kaper 1998). Recent classification of STEC has led to more than 500 serotypes (Johnson *et al*., 2006; Mathusa *et al.*, 2010), although few serotypes were associated with HC and HUS (Jayarao *et al*., 2006).

STEC usually find their way into foods of animal origin, especially raw milk (Erickson and Doyle 2007; Martin and Beutin, 2011) or other foods which may come into contact with sewage and manure (Caprioli *et al*., 2005; Fremaux *et al.*, 2008). Many foods have been associated with many STEC outbreaks, as raw milk (Paton and Paton, 1998; Denny *et al*., 2008), soft cheeses (Espie *et al*., 2006), dairy products (Rey *et al*., 2006) and meat and meat products (Cieslak *et al*., 1997; Vogt and Dippold, 2005).

Over the last several years, detection methods of STEC in foods have been significantly developed from culture-based methods into DNA-based and immune assays (Derzelle *et al.*, 2011), with each method having its strengths and weaknesses. While culture-based methods were considered as the standard of detecting STEC, they are laborious and time consuming. A major drawback of the culture-based methods is their inability to detect injured or viable but non-culturable cells (VBNC) (Liu *et al*., 2008). Contrarily, DNA-based methods offer rapid and accurate alternatives (Nielsen and Andersen, 2003; Oses *et al*., 2010). However, many shortcomings have been associated with these methods as they require initial DNA extraction from food matrices which may have inhibitory effects on PCR (Demeke and Jenkins, 2010). In addition, DNA based methods demand for specific laboratory equipment, may constitute a financial burden especially in developing countries.

Very recently, immuno-chromatographical method, RIDA®QUICK Verotoxin/O157 Combi (RQV) kit was developed for the detection of potentially zoonotic STEC organisms in feces. This method has many advantages as the simultaneous detection of the O157 antigen and shiga toxins, which may offer the instant differentiation between the most frequent *E. coli* O157:H7 and other non O157 STEC (Burgos and Beutin, 2012). This study was carried out to explore the suitability (specificity and sensitivity) of RQV kit in detecting *E. coli* O157:H7 in naturally and artificially contaminated milk samples.

Materials and Methods

Samples

For the artificial inoculation study, two types of cow's milk were used; raw and pre-sterilized

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*Corresponding author.
Email: *mohammad.bayoumi@gmail.com*
Tel.: +201159293625

(Ultra High temperature sterilized milk). An initial microbiological testing was performed on raw milk to determine total mesophilic aerobic count (Freitas *et al.*, 2009). Raw milk was tested to ensure its freedom from contaminating *E. coli* O157:H7 using previously described method (Solomakos *et al.*, 2009). Briefly, each sample was initially enriched for 4 h with trypticase soy broth (TSB, Becton Dickinson BD, Sparks, MD) supplemented with VCC supplement (cefixime, 0.05 mg/L; cefsulodin, 10 mg/L and vancomycin, 8 mg/L) (Sigma Aldrich, Germany) at 37°C followed by another enrichment with modified trypticase soy broth, and then plating onto sorbitol MacConkey agar (SMAC, BD) supplemented with 0.05 mg/L cefixime (Sigma Aldrich) and 2.5 mg/L potassium tellurite (Sigma Aldrich). Any raw milk sample was found positive for the presence of *E. coli* O157:H7 (indol positive colorless colony and serologically confirmed using *E. coli* O157:H7 latex test, Oxoid, Hampshire, UK) was excluded from the study. The pre-sterilized milk samples were collected and microbiologically checked for sterility. A total of 60 naturally contaminated raw milk samples (10 mL, each) were aseptically collected from collection centers and villagers' homes (all samples were freshly hand milked and stored in small sized tanks or cans at refrigeration temperature) and transferred into sterile sampling tubes. All samples were kept at 4°C until further analysis.

Sample spiking and preparation

As reference strains, *E. coli* O157:H7 ATCC 43894 (positive for *Stx1* and *Stx2*) and a local isolate of *E. coli* O157:H7 (isolated from raw milk and previously molecularly confirmed for the presence of *eaeA* and *Stx1* genes) were used for the artificial inoculation study. In order to determine the inoculated count, *E. coli* O157:H7 was initially cultivated on Luria Bertani Broth (LB, BD) at 37°C for 18 h. Decimal dilutions were then prepared from the overnight culture and optical densities (OD600) and counting of *E. coli* O157:H7 were determined for each dilution to obtain standard growth curve. Four inoculation ratios were used in this study; ≤10, 10- ≤ 10^2, 10^2 - ≤10^3 and 10^3 - ≤10^4 CFU/mL. Following spiking, each sample of raw and pre-sterilized milk was divided into two parts; one to be enriched using TSB supplemented with cefixime (0.05 mg/L), cefsulodin (10 mg/L) and vancomycin (8 mg/L) for 4 h at 37°C (selective antibiotics' enrichment), and the other one to be tested directly without this enrichment step.

One hundred µL of each sample of inoculated milk (for the artificial inoculation study) and raw milk

Table 1. Amplified genes and amplification conditions

Gene	Amplicon size	Amplification conditions
Eae A	384	- Initial denaturation at 94°C for 10min,
Stx 1	180	Followed by a 35-cycle protocol as follows: first 10 cycles - 1 min denaturation at 94°C - 2 min annealing at 65°C
Stx 2	255	- 1.5 min elongation at 72°C; followed by a touchdown protocol: reduction of annealing temperature by 1°C for each cycle till reach 60°C. - Final 10 min elongation cycle.

(of the naturally contaminated samples' study) were inoculated into tubes containing 4 ml of modified Tryptic Soya broth (mTSB, BD) supplemented with 0.5 µg/mL mitomycin C (Sigma Aldrich, Germany). Tubes were incubated aerobically at 37 °C for 18 h with shaking at 160 rpm. The incubated broth was centrifuged for 12 min at ~1200 ×*g* to obtain clear supernatants, which were then mixed with the supplied extraction buffer (1:2 ratio) in clean test tubes. Similar protocol was applied to raw milk samples for the natural contamination experiment with the selective antibiotics' enrichment step.

RQV testing procedure

For each sample, a test strip of RQV (R-Biopharm AG, Darmstadt, Germany) was aseptically removed from the original package and immersed into tubes containing mixed supernatant with the extraction buffer following the manufacturer's protocol. Immersed strips were kept at room temperature for 15 min.

Cultural detection of E. coli O157:H7

This was carried out following detection protocol of Solomakos *et al.* (2009), using SMAC supplemented with 0.05 mg/L cefixime and 2.5 mg/L potassium tellurite).

Molecular characterization of E. coli O157:H7 isolates

Colonies of *E. coli* O157:H7 isolates from naturally contaminated raw milk samples were selected for molecular detection of *eaeA*, *Stx1* and *Stx2* genes. Two colonies from each positive sample were serologically, biochemically identified and mixed. These selected colonies were grown overnight in Brain Heart Infusion Broth at 37°C. Bacterial DNA was then extracted according to DNA Purification Kit (Qiagen) procedure. Multiplex PCR was employed to detect previously mentioned genes following the method (amplification profile was supplied a, table 1) previously described by Normanno *et al.* (2004). Six primers were used in this study (*eaeA* F; GACCCGGCACAAGCATAAGC, *eaeA* R; CCACCTGCAGCAACAAGAGG, *stx1* F; ATAAATCGCCATTCGTTGACTAC, *stx1*

R; AGAACGCCCACTGAGATCATC, *stx2* F; GGCACTGTCTGAAACTGCTCC and *stx2* R; TCGCCAGTTATCTGACATTCTG). Following amplification, PCR products (180 bp for *Stx1*; 255 for *Stx2* and 384 for *eaeA* genes) were electrophoreted on 1% agarose gel, stained with 0.5 mg/mL ethidium bromide and UV visualized and imaged.

Results and Discussion

Three replicates were carried out in the spiking experiment. In order to validate the results, negative control strains (*E. coli* nissle, Mutaflor®; *E. coli* DH5α (Invitrogen) and *Salmonella enterica* serovar Typhimurium SA941256) were used. According to the manufacturer's interpretation guide, three bands should appear on each strip. A Blue band serves as a control and its absence invalidates the test, while a green band indicates the presence of *E. coli* O157 antigen, and a red band indicates the presence of shiga toxin(s). Bands were considered positive in case of developing of the respective color with any intensity and a band color score of 1 (faint) to 4 (very obvious) was recorded.

Whereas many dairy producers still depend on raw milk in manufacturing of many dairy products, especially cheese, to impart rich flavor and aroma intensities to meet consumer demands, developing of a quick, sensitive and reliable method for detection of food borne pathogens is considered an urgent demand. Among these pathogens, STEC represent a major prominent zoonotic hazard, especially since dairy cattle are considered the primary reservoir of *E. coli* O157:H7 and thus the main route of its infections in human via consumption of contaminated milk, meat and their products (Hussein and Sakuma, 2005). Although detection methods of food-borne pathogens in food have been recently improved, particularly in aspects of accuracy and results' achievement promptness, STEC detection in food is still constituting a problem (Burgos and Beutin, 2012), especially in case of foods harboring high numbers of competing microflora as raw milk.

Artificial spiking study

In this study, RQV was proven very effective in the detection of low numbers of *E. coli* O157:H7 contaminating milk, especially in case of raw milk with low number of microflora or sterilized milk, taking into consideration that the selective antibiotic enrichment step has been applied. All inoculated levels of *E. coli* O157:H7 (of both reference strains) into sterilized milk were successfully detected with RQV (Table 2), with bands' intensities of score 4

Table 2. Artificially spiked sterilized milk samples' detection profile using RQV

Inoculation level of *E. coli* O157:H7§ CFU/mL	Detection	
	Sterilized milk	
	With selective enrichment step	Without selective enrichment step
≤10	+	+
10 - ≤10²	+	+
10² - ≤10³	+	+
10³ - ≤10⁴	+	+

+ Positive results for both O157 antigen and Stx.
§ Similar results were achieved for both reference strains.

Table 3. Artificially spiked raw milk samples' detection profile using RQV

Mean aerobic plate count CFU/mL	Inoculation level of *E. coli* O157:H7§ CFU/mL	Detection	
		Raw milk	
		With selective enrichment step	Without selective enrichment step
5x10⁴	≤10	+	-
	10 - ≤10²	+	+
	10² - ≤10³	+	+
	10³ - ≤10⁴	+	+

+ Positive results for both O157 antigen and Stx.
§ Similar results were achieved for both reference strains.

in all inoculation levels. However, when raw milk (with aerobic count of 10^4 CFU/mL) was used, RQV could not directly detect low numbers of inoculated *E. coli* O157:H7 (Table 3). Furthermore, the band color score was 2 in case of non-enriched spiked raw milk with 10 - ≤10² CFU/mL. Although, after the selective enrichment step, RQV retained its detection's sensitivity and was able to detect as low as 10 CFU/mL with band color intensities of scores 3 and 4 at inoculation levels of 10 CFU/mL and other levels, respectively. From this figure of detection, we could infer that milk microflora were found to have a negative impact on test's sensitivity. Although we have observed that higher numbers of *E. coli* O157:H7 could be detected in samples even without enrichment, the antibiotics selective enrichment step (selective enrichment) was found to be a prerequisite for sensitive detection of *E. coli* O157:H7 in raw milk.

While regarding specificity of the RQV kit, none of the tested negative control strains (*E. coli* nissle, Mutaflor®; *E. coli* DH5α (Invitrogen) and *Salmonella enterica* serovar Typhimurium SA941256) was detected using RQV. In this regard, a previous study has employed RQV in testing of 134 different strains including *E. coli* O157:H7; non-O157 and other non *E. coli* shiga toxin producing strains, and RQV demonstrated a great specificity in identifying O157 antigen without any false-positive or -negative result (Burgos and Beutin, 2012). Depending on those results, we could clearly conclude the highest comparable sensitivity and specificity of the RQV kit in detection of STEC O157 strains.

Burgos and Beutin (2012) tested different inoculated counts of STEC O157 strains harboring
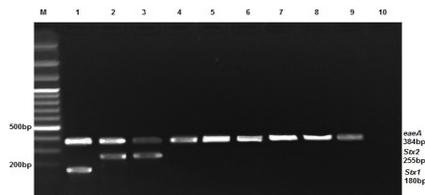
Figure 1. Molecular characterization of *Escherichia coli* O157:H7 isolates of naturally contaminated raw milk samples for the presence of *eaeA*, *Stx1* and *Stx2*

M, 100 bp DNA ladder; lanes 1-10, *E. coli* O157:H7 isolates.

different *stx* genes, and they found that RQV kit was able to detect as minimum as 10 CFU/25 g of inoculated food samples (vegetables), without any false positive results, which further affirms the sensitivity and the specificity of this kit. However, Burgos and Beutin (2012) recommended more tests before validation of this kit in detection of STEC in different foods.

Naturally contaminated raw milk survey

In our study, exploiting all associated benefits, RQV was used to screen the presence of STEC organisms in raw milk samples in order to evaluate the ability of this kit in detecting these organisms among other competitive microflora. In addition to testing with RQV kit, all samples were subjected to the reference detection protocol using selective SMAC method for comparing the results accordingly.

Using cultural SMAC method, ten samples yielded corresponding colonies of *E. coli* O157:H7 (smooth colorless colonies, which were then serologically confirmed to react positively to anti O157 serum). Same culturally positive samples were also found to be contaminated with *E. coli* O157:H7 using RQV. Among them, seven were found to be positive for O157 antigen and three were positive for both O157 antigen and shiga toxin(s). While regarding band color intensities, it was noticed that the two samples which were positive for both O157 antigen and shiga toxin(s) (green and red bands), have color intensities of both bands of scores 3 and 4, respectively. Other positive samples varied in their band color intensities' scores from 2 to 4.

Interestingly, this matching of results between the two methods (cultural and RQV) certainly revealed the adequacy of using the new method as an equivalent protocol. Additionally, a major benefit of RQV is the simultaneous differentiation between STEC O157 and other STEC strains. Moreover, molecular characterization of *E. coli* O157:H7 isolates has yielded interesting results, whereas *eaeA*, *stx1* and *stx2* genes were detected in strains isolated from the same raw milk samples, which were confirmed with RQV to have O157 antigen and

produce shiga toxin (two samples contained *stx2*, one contained *stx1* and all the three samples contained *eaeA* genes). Furthermore, other samples that did not found to produce shiga toxin (using RQV) were also molecularly found to carry neither *stx1* nor *stx2* genes (Figure 1). However, *eaeA* gene could be detected in six isolates among the rest seven samples. Only one sample was found to not harboring any of these virulent genes.

Epidemiologically, reports regarding STEC prevalence in Egypt are, unfortunately, scarce, so we were unable to have a reasonable comparison. However, in a previous survey concerning the prevalence of *E. coli* O157:H7 using cultural method, it was found that 3 out of 50 examined Egyptian raw milk samples were contaminated with *E. coli* O157:H7 (Abdul-Raouf *et al.*, 1996), which nearly corresponded with our detection incidence percentage. In another study (Arimi *et al.*, 2005), 264 samples of raw milk sold in Kenya (a developing country with environment similar to Egypt) were surveyed for the presence of STEC and only 2 isolates of *E. coli* O157:H7 could be detected using cultural and serological identification method and only one isolate was confirmed by PCR to harbor *stx* gene. Worldwide reports regarding *E. coli* O157:H7 in raw milk are numerous. Lately, New Zealand raw milk samples were analyzed for the presence of food-borne pathogens including STEC O157:H7 using cultural methods and *E. coli* O157:H7 was found in 1% of samples, although all of the isolates were missing toxin genes; *stx1* and *stx2* (Hill *et al.*, 2012). In another survey, *E. coli* O157:H7 was isolated from 21 out of 950 examined raw cow's milk samples, and 7 among the isolated *E. coli* were shiga toxin positive (Solomakos *et al.*, 2009).

Succinctly, taking into consideration the promising results achieved in this study and in Burgos and Beutin (2012) findings, RQV seems to offer an adequate alternative to the cultural reference method for detection of STEC in raw milk. Practically, especially in developing countries, application of this method may contribute to the improvement of product safety via detection of contaminated raw milk and thus preventing its use, as this method provides quick, cost effective and reliable approach for detecting Shiga toxin producing *Escherichia coli* in raw milk especially whereas well-equipped laboratories are considered unavailable. However, in order to formally validate this method in microbial food analysis, more comprehensive tests on different STEC strains (as non O157 STEC), cells' conditions (as VBNC) and food systems are required.

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