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COMPARISON BETWEEN TRADITIONAL METHODS AND REAL TIME PCR FOR DETECTION OF *E .COLI* IN BOVINE MEAT PRODUCTS

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ABSTRACT

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This study was performed to compare the prevalence of *E .coli* in minced meat and sausage using traditional methods and real time PCR. A total of 100 samples of minced meat and sausage (50 of each) were collected from different supermarket in Giza and Assiute governorates. The results using traditional methods recorded as 25% and 15% as positive *E .coli* from minced meat and sausage respectively, while the incidence of *E. coli* O157 recorded as 4% and 2% from minced meat and sausage respectively. The incidence of *E. coli*O 157 by using real time PCR recorded as 8% and 6% from minced meat and sausage respectively. The melting curve appeared at (82 ° C) for *E. coli* O 157 by using SYBR green Real time PCR.

***Key words**: Real time PCR sybrgreen, Ecoli O157*

INTRODUCTION

Meat and meat products are considered as an excellent source of high quality animal protein, vitamins especially B complex and certain minerals, especially iron (Mohammed, 2011). They are considered as an ideal culture medium for growth of many organisms because of high moisture content, high percentages of nitrogenous compounds. Meat products may be contaminated with microorganisms from meat handlers, which carry of pathogenic microorganism during the processes of manufacturing, packing and marketing. (Fratmico *et al.,* 2005). Contamination of raw meat is one of the main sources of food borne illnesses (Bhandare *et al.,* 2007; Podpecan *et al.,* 2007). *E. coli* are considered the most commensally living microorganism in the elementary tract of nearly all animal and wild animals as well as human. *Escherichia coli* is a predominant facultative anaerobe of the human colonic flora. The organism is typically colonises the infant gastrointestinal tract within hours of life, and thereafter *E. coli* and the host derive mutual benefit (Nataro and Kaper, 1998).

Most *E. coli* live commensally in the gastrointestinal tract of most mammals (Rahimi *et al.,* 2012) also do not cause disease in human, but certain types may cause diarrhea or more serious forms of illness. *E. coli* is one of the most frequent causes of some of the many common bacterial infections of man such as urinary tract infection, neonatal meningitis, cholecstitis, bacteremia, cholangitis, traveler's diarrhea and pneumonia (Ochoa and Cleary, 2003; Ammar, 2005), *E.coli*O157: H7 is the most common member of a group of pathogenic *E .coli* strains (Rahimi *et al.,* 2012) and other serotypes of Shiga toxin producing *E. coli*, are food borne pathogens of primary concern (Mor-Mur and Yuste, 2010). *Escherichia coli* O157:H7 has emerged as an important foodborne pathogen of considerable public health concern (Kansouzidou and levidiotou, 2003), the severity of infection which it causes and an infectious dose which may be as low as 10 organisms. In an outbreak study reported by (Willshaw *et al.,* 1994) contamination levels in an implicated product were reportedly as low as 2 cells per 25 g. This pathogen has been implicated in a number of high-profile outbreaks in the USA (Bell *et al.,* 1994), Scotland (Michino *et al.,* 1998) as well as in many sporadic cases of infection. Cattle are still regarded as one of main reservoirs of *E. coli O157:H7,* with the pathogen occurring in the faeces (Chapman *et al.,* 1997), rumen (Van Donkersgoed *et al.,* 1999), hide (Elder *et al.,* 2000). *E. coli O 157:H7* also act as etiological agents of haemorrhagic colitis. In some cases, complications may occur, such as haemolytic uremic syndrome and thrombotic thrombocytopenic purpura. EHEC other than *E. coli O157* have been increasingly associated with such complications. The severity of the illness and the low infective dose (<100 organisms) make *E. coli O157*:H7 among the most serious food borne pathogens (Acheson, 2003; Meng *et al*., 2007) meat can be contaminated during the slaughter operation and processing (Juneja and Marmer, 1999; El-Gohary, 1993).

At the end of the human food chain, numerous studies have reported the presence of *E. coli O157: H7*usually at low prevalences, on retail meats. A recent study in France (Vernozy-Rozand *et al.,* 2002) reported 0.12% (4/3450) samples positive for *E. coli O157:H7* in large-scale processed minced beef, while studies of butcher's shops in a range of countries have reported higher values i.e. 3.8% (6/160) in Argentina (Chinen *et al.,* 2001); 2.3% (5/211) in Switzerland (Fantelli and Stephan, 2001); and 1.1% (36/3216) in the UK (Chapman *et al.,* 2000). In the Netherlands, (Heuvelink *et al.*, 1999) reported an overall prevalence of 1.1% (6/571) *E. coli O157:H7* in minced beef products. Other studies found very different results, ranging from 16.8% (50/296) *E. coli O157:H7* samples in Washington State, USA (Samadpour *et al.,* 2002) to a study by (Tarr *et al.,* 1999) which did not recover the pathogen from 1400 retail ground minced beef samples from six stores in Seattle, USA. With the exception of the study of Vernozy-Rozand *et al.* (2002) the above studies merely determined presence or absence of *E. coli O157:H7*, usually after enrichment, and in general, little data are available on the numbers of this pathogen in retail meat products.

Traditional culture methods for detecting microorganisms in food are based on the incorporation of the food sample into a nutrient medium in which the microorganisms can multiply. These conventional test methods easily adaptable, very practical, and generally inexpensive (Chart, 1998).

Although not lacking in sensitivity, they can be laborious and depend on the growth of the microorganisms in different culture media (pre-enrichment, selective enrichment, selective plating, identification), which may require several days before results are known. Products that are minimally processed have an inherently short shelf life, which prevents the use of many of these conventional methods. Therefore, extensive research has been carried out over the years to reduce assay time through the use of alternative methods for detecting foodborne microorganisms and reduce the amount of manual labor by automating methods whenever possible (Feng, 2007; Betts and Blackburn, 2009; Jasson *et al.,* 2010).

Long duration enrichments are often used due to the low number of pathogenic microorganisms that tend to be present in food samples. Although previous enrichment is a limitation in terms of assay speed and precludes quantification of the original contaminant, it provides essential benefits, such as diluting the effects of inhibitors, allowing the differentiation of viable from nonviable cells, and allowing for the repair of cell stress or injury that may have resulted during food processing (Jantzen *et al.,* 2006; Wu 2008). Hence, it would be difficult to completely eliminate enrichment culture from the process of pathogen detection in foods (Feng, 2007).

SYBR Green l is a DNA-intercalating dye that is widely used for real-time PCR applications (Monis PT *et al*, 2004; Stampi *et al.,* 2004). There are advantages and disadvantages to using SYBR Green I for real-time PCR detection. The advantages include high detection sensitivity and speed (30 -40 min per 40 cycles). The rapid real time PCR based method was performed very well compared to the conventional method. It is fast reproduciblethe results can be obtained quickly (in about 3 h) (Bottari *et al.,* 2006), simple, specific and sensitive way to detect nucleic acids, which could be used in clinical diagnostic tests in the future (Pochop *et al.,* 2012). The disadvantage is that it binds to any double-stranded DNA (Epsy *et al.,* 2006).

Compared with the classical urine culture methods, real time PCR is more rapid and can detect smaller number of bacteria; which would otherwise undetectable (Yoshimasa, 2002). Real-time PCR has been used for the rapid and reliable detection of *E. coli* O157 in retail red meats (Suo *et al.,* 2010; Perelle *et al.*, 2007). The culture in these methods is time consuming (up to 3-5 days) and not suitable for routine screening of large samples (Savoye *et al.,* 2012). More recently, real time PCR is being increasingly used as a rapid sensitive and specific molecular diagnostic technique for the testing and identification of food pathogens from biological and environmental samples (Bellin *et al.,* 2001).

MATERIALS and METHODS

Materials and methods

Samples Collection: One hundreds of minced meat and sausage samples (50 of each) were collected random from different supermarket in Assuite and Giza governorates. They were placed in containers and put in refrigerated vehicles during transportation. Samples are taken randomly from boxes outside of our region and conveyed respecting the freezing temperature of -18 °C to the Regional Veterinary Laboratory (Gershwin, 1990). Ten g of samples + 90 ml MacConkey broth after incubation at 37° C for 24 hours were inoculated onto macConkey agar and incubated at 37°C for 24 hours, Pink colonies of a lactose fermenting organism appeared. Single typical well isolated lactose fermenting colonies were tested by culturing on Eosin Methylene blue (EMB), and incubated at 37°C and 44°C for 24 hours. The greenish metallic sheen by reflected light and dark purple centers by transmitted light. Similarity isolated lactose fermenting colonies were tested for sorbitol

fermentation by culturing on sorbitol MacConkey agar and incubated at 37° C over night. On sorbitol MacConkey ,colourless or pale colonies were considered as non fermenters of sorbitol and pinkcolonies as sorbitol fermenters. Morphological examination were done according to (Finegold and Martin, 1982).

Culture and Isolation of *Escherichia Coli* O157:
Enrichment cultures for each sample were carried out by combining 25 g of each sample with 225 ml of buffered peptone water supplemented into a stomacher bag, homogenized for two min and incubated at 37°C for 16–18 h. After incubation, the isolates belonging to *E. coli* were cultured on Sorbitol Mac Conkey medium for detecting non sorbitol variants. This test required the identification guides to the enterohemorrhagic *E .coli* (EHEC) strains. The strains metabolizing sorbitol are excluded, however the rest of the strains are tested for their immunological confirmation by the method of (Koneman, 1992).

Serological Identification: All biochemically identified non sorbitol fermenting colonies from the sorbitol-Mac Conkey Agar (SMAC) were subjected to slide agglutination with the *E. coli* O157 antisera (Difco) and the agglutinating colonies were further processed for definitive confirmation (Quinn, 1994; Edwards, 1972; Jasson *et al.,* 2010).

E. coli O157: H7 antigen determination all sorbitol non-fermenting, indole positive, 4-Methylumbeliferyl-B-D glucuronide (MUG) negative colonies were examined by latex agglutination (Wellcolex, Merseyside, UK). These beads are coated with antibodies which bind to any O157: H7 antigens on the test organisms, forming a visible antigenantibody precipitate (De Boer and Heuvelink, 2000). Colonies giving a precipitation reaction were confirmed as *E. coli O157* positive.

DNA extraction. DNA was extracted from the meat samples use extraction DNAeasy Tissue Kit (JenaBiosience cat # pp-206s) Kit protocol for the extraction of DNA from tissuse. The extracted DNA stored at -20°c until use in real time PCR assays.

Real-time PCR
Methods are the simplest and least expensive method and enables products to be identified by their different Tm, by analysing the melting curve of the amplicon post-PCR besides detection of target DNA during amplification. (Washington *et al.,* 2004; Malorny *et al.*, 2004).

Amplfication and detection were carried out in Stratagene the maxima SYBR Green qPCR master mix (2x) Rox solution provided (#K 0251) was used in all. Amplifications were performed using a Stratagene MX 3000 P with software version 4. For SYBR-Green amplifications, a dissociation step was added to improve amplification specificity. TaqMan and SYBR-Green reactions were carried out in a final volume of 25 µl with appropriate final concentrations of primers, primer use in *E .coli* O 157 in real time PCR as in (Table 1) the primer concentrations were as follows: 10 pmol/ul. (Paton and Paton, 1998). The reaction was run and melting of the amplification products curve analysis. To evaluate the efficiency of the amplification, a standard curve was constructed using the threshold cycle (*CT*) Each reaction was run with the following cycle conditions (Table 2). The specificity of the reaction is given by the detection immediately after the last reaction cycle. The melting curve was visualized with the software.

Table 1: Primer used in detection of (*E .coli* O157)

Primer	organism	Tm C	Sequence 5 _____ 3	Position from start of *E .coli* gene
O157F	*E. coli O157*	56.9	TCTGCGCTGCTATAGGATTAGC	701-722
O157A		56.0	CTTGTTTCGATGAGTTTATCTGCA	926-903

Table 2: Real time PCR condition for amplification of *E.coli O157.*

Program PCR sybr green1 protocol	Target temperature °C	Hold time (s)	No. of cycle
Denaturation	95	600	One cycle
Amplification	95	15	35 cycle
	54	5	
	72	12	
Melting curve analysis	95	1	One cycle
	65	15	
	95	1	
	40	30	

RESULTS

From the analysed meat samples, 40 out of 100 recorded as sorbital positive *Escherichia coli* were characterized by classical phenotypic tests in all samples. Whereas, only 3 % sorbitol negative *Escherichia coli* was identified and isolated, rod, gram negative nonsorbitol fermenting and produce indole. The agglutinating test showed that this strain is belonging to the serotype of *Escherichia coli O157.*

Table 3: The Incidence of *E .coli O157* in meat products samples

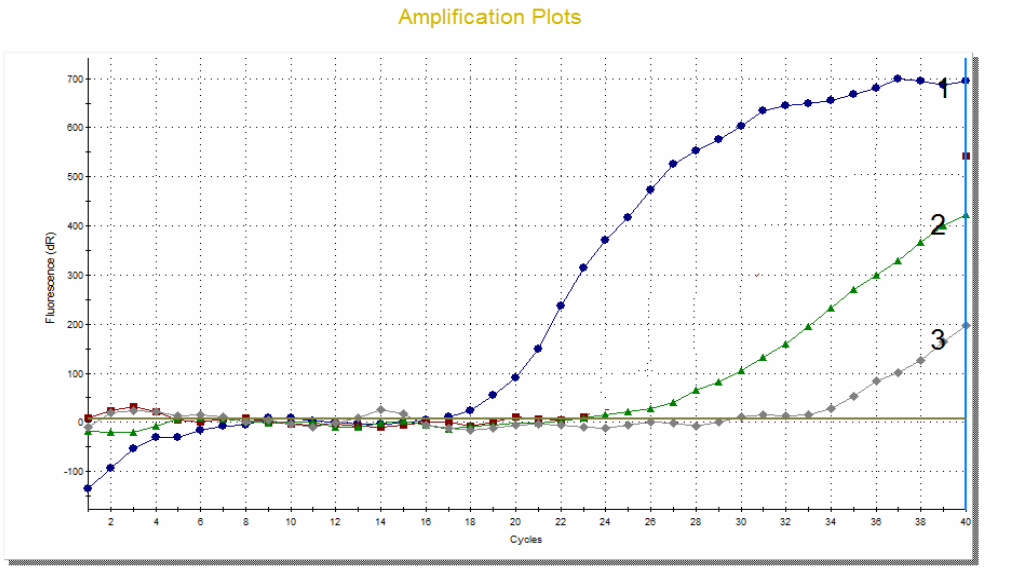
Type of samples	Number of ex samples	Positive results using traditional methods		Positive results of *E .coli O 157* using Real time PCR	
		No of			
		E .coli	*E .coli* O 157	No	%
Minced meat	50	25	2	4	8
Sausag	50	15	1	3	6
Total	100	40	3	7	7

From data in (Table 3) recorded that 40 out of 100 samples (minced meat and sausage 50 of each) recorded as *E. coli* but 3 only out of 40 recorded as *E.coli O157.*

By using the real time PCR from minced meat samples recorded that 4 out of 50 (8%) as *E. coli O 157* while 3 out of 50 (6%) of sausage samples recorded as *E .coli O 157.*

From Fig (1) Using sybrgreen appeared the amplification curve appeared from the 22 cycles as in minced meat and sausage samples because the amount of DNA is sufficient enough to appeared at 22 cycles comparison with control positive native organ (serology unit in AHRI Dokki, Giza), while the melting curve appeared at 82° C as in Fig. (2)

Fig. (1) Amplification of samples comparison with positive control (native organ from AHRI serology unit)



- **1-Control +ve*E coli*O157 native strain from serological unit in AHRI 2-Minced meat samples 3-sausage samples**

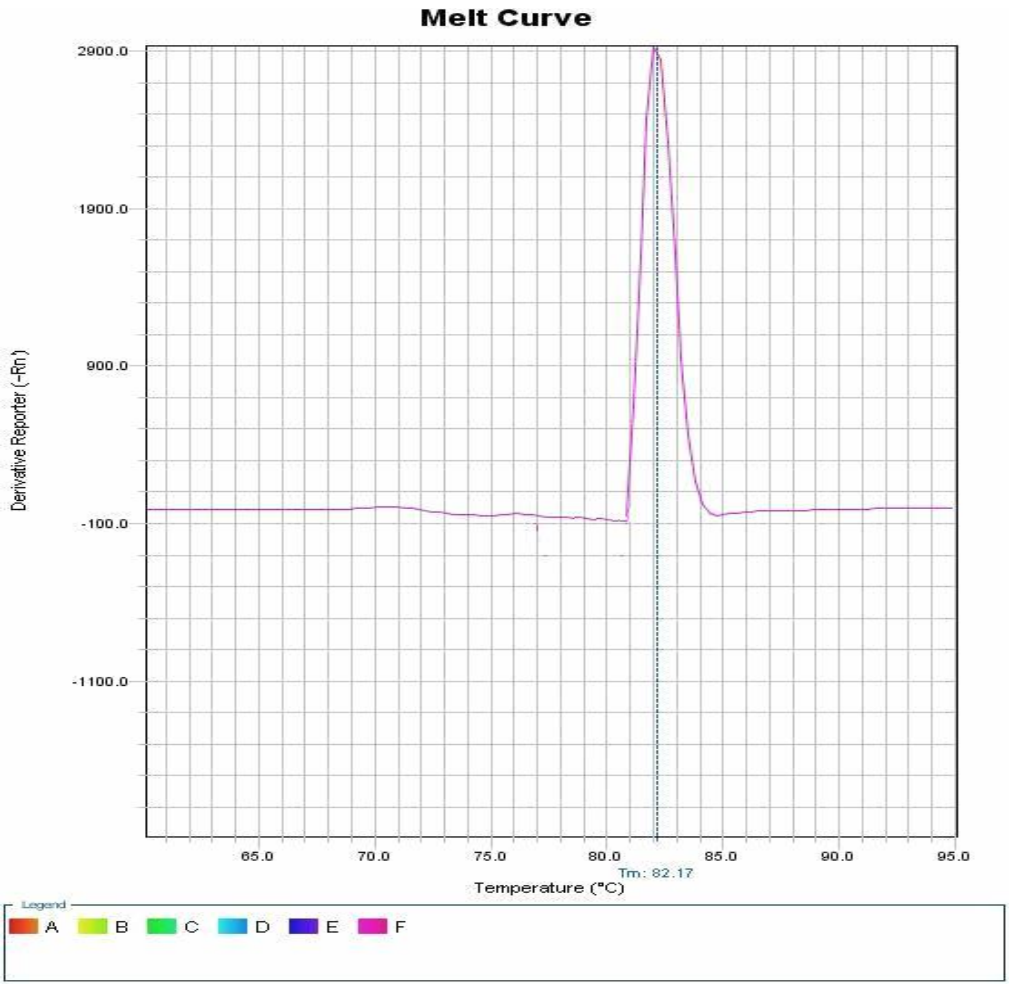


Fig. (2) Melting curve analysis of all positive reactions revealed that variation in melting temperatures were low for *E .coli* O157 specific PCR fragments (82° C)

DISCUSSION

Meat has been linked to consumer health problems, Meat products are recognized as a major source of food born pathogens that cause food poisoning in humans. The most important pathogens associated with meat products are *E .coli* (Borch *et al.,* 1996).

Currently the most important pathogens associated with meat products are *Escherichia coli* (Darwish, 1991; Borch *et al.,* 1996). Meanwhile, all examined meat products samples showed high contamination level of *E .coli* in examined meat products may indicates unsanitary conditions of raw meat production, preparation and handling. Undercooked meat products have caused many food poisoning incidents associated with *Escherichia coli* which is present in the faeces, intestines and hide of healthy cattle from where it can potentially contaminate carcass meat during the slaughtering processes (Duffy *et al*., 2003).

Because of the low dose of *E. coli* O157 needed to cause infection, sensitive and rapid detection methods for *E. coli* O157 in food samples are necessary in food industry to ensure a safe supply of foods. Several studies have reported the prevalence of *E.coli* O 157 in meat product as (Dontorou *et al.,* 2003) who recorded *E. coli* O 157 from 0.5% foods including minced meat and sausages.

The sensitive detection of *E. coli* O157 has been developed over recent years with the use real time PCR. There are different reference methods for detecting *E. coli* O157 in foods (United states Department of Agriculture Food Safety and Inspection Service 2010 a, b; Feng and Weagant 2011; AOAC International 2011).

By using traditional methods, out of 100 samples of meat products, 40 samples (40%) were positive for Escherichia coli. The obtained results was higher than values reported in studies conducted in France (0.12%) (Vernozy-Rozand *et al.,* 2002) and the UK (1.1%) (Chapman *et al.,* 2000; Fantelli and Stephan, 2001) (2.3%) and higher than reported in a study in Argentina (3.8%) (Chinen *et al.,* 2001); (Seran *et al.,* 2012) who recorded the percentage as 8.82%. And higher than El-Taher-Omyma (1998) (25%); Saleh (2001) (16%).

On the otherhand, the data of the present study is nearly similar to the data reported by El-Mossalami (2003) (40%). The prevalence in minced meat indicated that the presence of this pathogen in red meats indicates improper or insufficient hygiene management both at the farm and during the slaughtering and meat handling process Seran *et al.* (2012).

Moreover the incidence of *Escherichia coli* in the examined sausage samples (30%) was lower than the findings recorded by Zaki-Eman (1990) and El-Khateib *et al*. (1994) they recorded *E. coli* in sausage with incidence 44% and 48% respectively, but higher than the incidence which obtained by (Mohamed, 2011) who recorded 12% *E .coli* from sausage. The variation in the results obtained by different investigators may be due to difference in manufacturing practices, handling and difference in time of exposure.

In our study *E .coli* O 157 was detected as 8% from minced meat and 6 % from sausages. In comparison with other countries, the prevalence reported in this study is slightly higher than reported from Greece 0.5% (Dontorou *et al.,* 2003), Morocco 2.8 % (Badri *et al.,* 2009; Abd El–Atty and Meshref, 2007).

In our study the interest was based on the search of *E. coli* O157 in meat considering that consumption of red meat and especially beef' has risen steadily and the stock continues decreasing in Algeria, in order to know if the cold chain was observed during the transport of foods. Apart from the study by Chahed *et al.* (2006) on the contamination assessment levels in bovine carcasses with this bacteria in Algeria, where the author has detected two strains of serotype O157: H7, Allerberger *et al.* (2001).

By using real time PCR, the incidence of *E.coli* O 157 in minced meat is reached to 8 %. The obtained result is higher than the results (0.17% and 1.4 %) that obtained by Walsh *et al.* (1997) and Duffy *et al.* (1998), respectively on the otherhand our result is consistent with finding recorded by Lindqvist *et al.* (1998), Little and de Louvois (1998) and Tarr *et al.* (1999).

Table (3) showing that the Percentage of *E. coli* in examined samples using real time is higher than that recorded using traditional methods, this difference may be attributed to the higher sensitivity of the real time PCR and propability of presence of dead organism in the samples. Using real-time PCR, proved that it was able to detect very small per reaction of *E. coli O157* (Elizaquível and Aznar, 2008 and Narang *et al.,* 2009).

SYBR Green Real Time PCR was determined by the definition of the melting temperature of the PCR product obtained (De Medici *et al*., 2003). The limit of quantification of real-time PCR with food samples is around 10^{3} –10^{4} CFU/g (Rodriguez-Lazaro and Hernandez, 2006; Navas *et al.,* 2006; Jasson *et al.,* 2010). Most samples taken from throughout the food supply chain are usually contaminated with fewer pathogen cells (normally less than 100 CFU/g). These new detection methods are the necessary technologies

that will substantially improve our food safety once integrated in the HACCP (Bhunia, 2008). Microbiological analysis of foods is based on the detection of microorganisms by visual, biochemical, immunological, or genetic means, either before enrichment (quantitative or enumerative methods) or after enrichment (qualitative methods, also known as presence/absence tests).

The complexity of food materiles remains the major obstacle to the development of effective sampling and rapid testing methods (Feng, 2007). Long duration enrichments are often used due to the low number of pathogenic microorganisms that tend to be present in food samples. The presence of a single peak indicates the specificity of the reaction and when more than one amplicon is obtained, they can be distinguished by differences of at least 1°C in Tm Lipsky *et al.* (2001); Ririe *et al.* (1997). Identical amplicons were detected by real time method are similar to findings by (Catarame *et al.,* 2003). The melting curve of *E. coli* is 82° C nearly to (Hanlon *et al.,* 2004) who recorded it at 80.2 °C. Meat products should be properly handled, and packed in sterile polyvinyl wrappers (Abong'o BO and Momba MN 2009).

Future possibilities for this work is to successfully Not only does this technique provide tools for highly sensitive and specific detection of the organism in clinical specimens, but certain characteristics including virulence, toxins and antimicrobial resistant genes may also be determined (Ram *et al*., 2008).

CONCLUSION

It is very important detection method for *E. coli* is necessary for the health of the consumers. Hygienic awareness should be applied for personnel whom involved in handling and preparing of food at factories, home or restaurants. Careful handling and through cooking of raw and frozen meat, regardless of market source by the consumers is required to prevent food born illness. Finally, Hazard Analysis Critical Control Point (HACCP) procedures should be adopted during all steps of manufacture, handling as well as storage of meat products to produce safe and high quality products as well as ensuring compliance with legislation.

The real-time PCR reaction can be easily used in the qualitative and quantitative detection of these bacteria after an anterior enrichment or even directly from the alimentary matrix, as long as the bacterial quantity is detectable by the used instrument, however the costs of the reagents and of the necessary tools are pretty high. The molecular methods are specific, sensitive and reproducible, which makes it easier for the specialists who work in laboratories.

The sensitivity of detection with SYBR-Green may therefore be compromised by the formation of primer-dimers, lack of specificity of the primers, primer concentration and the formation of secondary structures in the PCR product. All of these factors could lead to the creation of unexpected double-stranded DNA products, which would incorporate SYBR-Green and register a fluorescent signal.

The dissociation curves showed that the amplification product was very specific. The result indicated that real-time PCR was 1000 times more sensitive than traditional methods.

The process of selecting an appropriate method must consider the main criteria of the sensitivity of analysis, the time of detection, and the specificity of the test (Table3). The cornerstone of any method is its accuracy. This consists of the sensitivity and the specificity. The intent in developing a rapid assay is to reduce the time required to obtain an accurate result. Qualitative detection (presence/absence) tests are used if information concerning the presence of an organism in a specified quantity of food is required. The sensitivity of these tests is then defined by the quantity of food examined (Stannard, 1997; Jasson *et al.,* 2010). In many cases, the requirement of detection is less than one cell per 25 g of food, as small numbers of some pathogens may be sufficient to cause disease. Sensitive quantitative detection is usually achieved by traditional culture methods (Table 3). Thus, rapid method still lack sufficient sensitivity for direct testing (Feng, 2007). Traditional culture methods may require many days, resulting in very long assay durations. Most rapid methods for the detection of pathogens or toxins can be done in a few minutes to a few hours or at the utmost 1 day (Table 3).

However, many detection systems need an enrichment, and positive results must be confirmed by the appropriate official method, which involve culturing, in many instances (Feng, 2007). In spite of this, commercially available rapid detection methods, such as ELISA, LFD, and PCR, have substantially shortened the total time of the detection assay when compared to conventional methods (Table 3) (Leon-Velarde *et al.,* 2009). They are, therefore, of great use in the rapid analysis of food with the goal of ensuring that only negative samples or lots are sent to market (Bohaychuk *et al.,* 2005). A major disadvantage of alternative methods over culture methods is that most rapid methods involve damaging the cells. Therefore, viable cells for confirmation and further characterization can only be obtained by carrying out repeat analyses using standard culture procedures (Feng, 2007).

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مقارنة بين الطرق التقليدية وتفاعل انزيم البلمرة المتسلسل ذو الوقت الحقيقى فى كشف الاشريشيا كولاى فى منتجات اللحوم البقرية

إيمان مجدى زخارى ، مريم فؤاد منسى

اجريت هذة الدراسة لمقارنة مدى انتشار الاشيرشيا كولاى في اللحم المفروم والسجق باستخدام الأساليب التقليدية وتفاعل انزيم البلمرة المتسلسل ذو الوقت الحقيقى. وتم جمع ١٠٠ عينة من اللحم المفروم والسجق (٥٠ لكل منهما) من سوبر ماركت مختلفة في محافظات الجيزة وأسيوط. وكانت النتائج *E.coli O157* باستخدام الطرق التقليدية قد سجلت ٢٥% و ١٥% إيجابية من اللحم المفروم والسجق على التوالي. بينما سجلت النتائج PCR كنسبة ٨ % و٦% من اللحم المفروم والسجق على التوالي باستخدام تفاعل انزيم البلمرة المتسلسل ذو الوقت الحقيقى. حين بدأ منحنى الذوبان في (٨٢ درجة مئوية) كولاي ١٥٧ باستخدام سيبر الخضراء