Detection of virulence genes of enterohaemorrhagic \( E. \) \( \text{coli} \) isolated from some meat products by polymerase chain reaction.

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\textbf{A B S T R A C T}

A grand total of 105 meat product samples of minced meat, sausage and luncheon (35 of each) were duplicated bacteriologically examined to detect Enterohaemorrhagic \( E. \) \( \text{coli} \) prevalence and some virulence genes. One replicate was processed for \( \text{EHEC} \) non \( O_{157} \) by using conventional method for isolation and identification of \( E. \) \( \text{coli} \) and the other for \( E. \) \( \text{coli} \) \( O_{157}:H_7 \), then serological typing and PCR technique for specific \( stx_1, stx_2, cvcC \) and \( hlyA \) genes from 6 random samples were applied. \( E. \) \( \text{coli} \) was isolated from 12 samples (34%), 9 samples (25.7%) and 11 samples (31%) of the examined minced meat, sausage and luncheon, respectively. The isolated serotypes of \( \text{EHEC} \) were \( O_{26} \) (5 strains) 15.6\%, \( O_{111} \) (3 strains) 9.4\% and \( O_{157} \) (3 strains) 17.6\%. The incidence of \( \text{EHEC} \) \( O_{26} \) were (2 strains) 5.7\%, (2 strains) 5.7\%, (1 strain) 2.85\%, incidence of \( O_{157}:H_7 \) were (2 strain) 5.7\%, (1 strain) 2.85\%, 0\% in minced meat, sausage and luncheon, respectively. The incidence of \( O_{111} \) was 2.85\% from each the type meat products. PCR results indicated that \( stx_2 \) and \( cvaC \) virulence genes were detected in the same studied strain (\( O_{157}:H_7 \) from minced meat sample), while \( stx_1 \) and \( hlyA \) genes were not detected. Accordingly, meat products may constitute an important reservoir for \( \text{EHEC} \) and PCR technique is the most sensitive and efficient approach for detection of \( \text{EHEC} \) genes.

\textbf{Keywords:} Enterohaemorrhagic \( E. \) \( \text{coli} \), Shigatoxins, PCR.

1. \textbf{INTRODUCTION}

\( E. \) \( \text{coli} \) is commonly non virulent but some strains have adapted pathogenic or toxigenic virulence factors that make them virulent for man and animals (Malik and Memona, 2010). Pathogenic \( E. \) \( \text{coli} \) strains are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigen profiles into six categories: Enteroaggregative (\( EAEC \)), (\( EHEC \))/Shiga toxin-producing \( E. \) \( \text{coli} \) (\( STEC \)), Enteroinvasive (\( EIHEC \)), Enteropathogenic (\( EPEC \)), Enterotoxigenic (\( ETEC \)), and diffuse adherent (\( DAEC \)) (Nataro and Kaper, 1998 and Parry and Sharon, 2002). \( \text{EHEC} \) is defined as a subgroup of \( VTEC/STEC \) associated with human diseases which in addition to the verocytotoxin/shigatoxin producing capacity harbors additional genes that are important in virulence. Verocytotoxin producing \( E. \) \( \text{coli} \) (\( VTEC \)) is a term used to describe strains of \( E. \) \( \text{coli} \) characterized by the ability to produce verocytotoxin(s) (VT), or just verotoxins that are capable of killing Vero cells, a tissue culture line of monkey kidney cells. In addition to \( E. \) \( \text{coli} \) \( O_{157} \), \( \text{EHEC} \) includes over 100 serotypes causing food borne illness, such as \( O_{26}, O_{111}, O_{113} \) and \( O_{121} \) (FAO and WHO, 2011). Detection of \( E. \) \( \text{coli} \) \( O_{157}:H_7 \) is based on phenotypic differences from most other serotypes: its inability to ferment sorbitol on MacConkey sorbitol agar and
absence of β-glucuronidase activity in most strains (Adams and Moss, 2008). Shiga toxins (stxs) are considered to be the major virulence factor of VTEC and comprise a family of structurally related cytotoxins with similar biological activity. The two main groups consist of stx1, which is nearly identical to the toxin of S. dysenteriae type 1, and stx2, which shares less than 60 % amino acid sequence with stx1 (Chelsa and O’Brien, 1998). Colicins are antimicrobial proteins produced by one strains of E.coli to suppress the growth of other relative strains of E.coli (Diez-Gonzalez, 2007). PCR is a powerful molecular biology technique that was introduced to facilitate the detection of the E.coli virulence factors by using DNA probes that detect specific virulence factors (Nataro and Kaper, 1998).

2. MATERIAL AND METHODS

2.1. Samples collection

A grand total of 105 samples (35 each of minced meat, sausage and luncheon) were collected from small scale shops with different sanitation levels at El-Menofiya governorate and transferred in an ice box directly to laboratory with a minimum delay to be bacteriologically examined.

2.2. Samples collection

Samples were analyzed by duplicate. One replicate was processed for EHEC nonO157 isolation and the other for E. coli O157:H7 screening.

2.2.1. Isolation and identification of E. coli

The technique recommended by APHA (1992) by using MacConkey broth for enrichment then subculture on MacConkey agar and Eosin Methylene Blue (EMB) agar media. Suspected colonies (dark colonies with metallic sheen) for E. coli were picked up and sub cultured for purification.

2.2.2. Isolation and Identification of Enterohaemorrhagic E.coli O157: H7

A 25 g of each meat product were blended with 225 ml of (mTSB) modified tryptic soya broth supplemented by Novobiocin (20 mg/l). Subculture was done on Sorbitol MacConkey Agar (SMAC) with Cefixime and Tellurite. All plates were incubated for 24-48 hours at 37ºC. Non sorbitol fermenting colonies (N.S.F), transparent colonies were picked up and sub cultured for purification.

2.2.3. Identification of suspected E.coli isolates

The purified colonies were morphologically identified by Gram stain and biochemical tests (Quinn et al., 2002).

2.3. Antibacterial sensitivity test

All the suspected isolates were serologically identified by slide agglutination according to Kok et al., (1996) by using rapid diagnostic E.coli antisera sets (DENKA SEIKEN Co., Japan). while Non-sorbitol fermenting (NSF) isolates used monovalent O157 and H7 antisera.

2.4. Antibacterial sensitivity test

Primers used for detection of four virulence genes that may play a role in virulence of EHEC (Table 1).

Table (1): Primer sequences for virulence genes amplification of EHEC

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers sequences (5’-3’)</th>
<th>product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1</td>
<td>ACACTGGATGATCCTGTTCTG</td>
<td>614</td>
<td>Dipineto et al., 2006</td>
</tr>
<tr>
<td></td>
<td>CTAATCCCCCTTCATTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>CATGAAACGGGAGCACTGTT</td>
<td>779</td>
<td>Dipineto et al., 2006</td>
</tr>
<tr>
<td></td>
<td>CCGTCAACTGAGCACTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hly A</td>
<td>ACGATGTTGTGTTATTCTGGA</td>
<td>165</td>
<td>Dipineto et al., 2006</td>
</tr>
<tr>
<td></td>
<td>CTCACGTGACCCATACAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cva C</td>
<td>CACACACACAGGGAGCTGTT</td>
<td>760</td>
<td>Yaguchi et al., 2007</td>
</tr>
<tr>
<td></td>
<td>TTCCCGGGACATAGTCCCAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Detection of virulence genes of enterohaemorrhagic E. Coli isolated from some meat products

These genes were shiga toxins (stx1, stx2), haemolysin (hlyA) and colicin V production col V gene (cva C). PCR technique was applied on six random isolates (O26 from minced meat sample and luncheon sample; O111 from luncheon and sausage sample; O157 from minced meat and sausage, two isolates for each) following QIAamp® DNA Mini Kit instructions (Catalogue no.51304): Emerald Amp GT PCR Master mix (Takara) Code No. RR310A and agarose gel electrophoresis (Sambrook et al., 1989).

3. RESULTS

Table (2): Prevalence of E.coli and N.S.F E.coli isolated from the examined meat product samples (n=35)

<table>
<thead>
<tr>
<th>Type of examined meat products</th>
<th>Positive samples of E.coli No. %</th>
<th>Positive samples of N.S.F. E.coli No. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minced Meat</td>
<td>12 34</td>
<td>7 20</td>
</tr>
<tr>
<td>Sausage</td>
<td>9 25.7</td>
<td>6 17</td>
</tr>
<tr>
<td>Luncheon</td>
<td>11 31</td>
<td>4 11.4</td>
</tr>
<tr>
<td>Total</td>
<td>32 30.5</td>
<td>17 16</td>
</tr>
</tbody>
</table>

% were calculated according to the type of examined meat product sample

Table (3): Serotypes of EHEC non O157 and N.S.F. E.coli isolates

<table>
<thead>
<tr>
<th>Serotypes of EHEC non O157</th>
<th>Isolates</th>
<th>O26</th>
<th>O111:H4 negative</th>
<th>O157 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>isolates</td>
<td>5</td>
<td>3</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>%</td>
<td>15.6</td>
<td>9.4</td>
<td>75</td>
<td>17.6</td>
</tr>
<tr>
<td>E.coli for N.S.F. isolates</td>
<td>14</td>
<td>17.6</td>
<td>82.4</td>
<td>3</td>
</tr>
</tbody>
</table>

Table (5): Prevalence of EHEC among the examined meat products samples (n=35)

<table>
<thead>
<tr>
<th>Type of products</th>
<th>No. of positive samples</th>
<th>% of total EHEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minced Meat</td>
<td>5</td>
<td>14.28</td>
</tr>
<tr>
<td>Sausage</td>
<td>4</td>
<td>11.4</td>
</tr>
<tr>
<td>Luncheon</td>
<td>2</td>
<td>5.7</td>
</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>10.5</td>
</tr>
</tbody>
</table>

% were calculated according to the type of examined meat product sample

Concerning the conventional methods for identification and isolation of E.coli isolates from meat samples, E.coli appeared as pink colonies on MacConkey agar, gave characteristic green sheen colonies on EMB. While N.S.F. E.coli were transparent on SMAC. E.coli strains were seen as Gram-negative, rods, arranged singly, pairs and groups, non-spore forming. Different biochemical reactions were done for confirmation of all suspected colonies: positive methyl red reaction and produced indole. They did not cause break down of urea and didn't grow in citrate medium. Reactions in TSI agar slant revealed yellow slant and butt with gas but no production of hydrogen sulphide gas was observed. Meanwhile The results showed that E.coli was recovered in 32 samples with an incidence rate 30.5% represented; 34%, 25.7%, 31%, while N.S.F.E.coli was isolated with percent 16%represented ; 20%,17%,11.4% from minced meat, sausage and luncheon, respectively, table (2). Data in table (3) revealed that the serologically identified 32 E. coli isolates for EHEC nonO157 were 8 (25%) isolates gave positive results with polyvalent antisera (2) more over 24 (75%) isolates were negative by using the monovalent antisera The most commonly detected serogroups (O26 and O111) represented as 5 strains were serotyping O26 (15.6%); 3 strains O111 (9.4%), while typing of 17 N.S.F E. coli isolates were 3 (17.6%)isolates can be identified serologically as O157:H7. while 14(82.4%) were negative.
After phenotyping and genotyping of isolates the prevalence of EHEC serogroups was as following:

Fig. (1) PCR detection for virulence genes stx1 and stx2 of EHEC, the stx2 (779bp) gene. stx2: shiga toxin 2 gene. Lan L: 100-1500bp DNA Ladder Neg: Negative control. Pos: Positive control (at 779bp) Lane 1, 2, 3, 4, 5, 6: Enterohaemorrhagic E.coli (Negative). Lane 5: Enterohaemorrhagic E.coli O157 (Positive). The stx1 (614 bp). Stx1: shiga toxin 1 gene. Lane L: 100-1500bp DNA ladder. Neg.: Negative control. Pos.: positive control (at 614bp), Lane 1; 2; 3; 4; 5 & 6: EHEC. (Negative).

Fig. (2) PCR detection for virulence genes hlyA and cvaC genes of EHEC, hlyA (165 bp) gene. hlyA: Haemolysin gene. Lane L: 100-1500bp DNA Ladder. Neg.: Negative control. Pos.: positive control (at 165bp). Lane 1; 2; 3; 4; 5&6: EHEC (Negative). The cvaC (760 bp) gene cvaC: colicine V production colV gene. Lan L:100-1500bp DNA Ladder Neg: Negative control. Pos: Positive control (at 760bp) Lane 1, 2, 3, 4, 5: Enterohaemorrhagic E.coli (Negative). Lane 5: Enterohaemorrhagic E.coli O157 (Positive).

The prevalence of E.coli O26 was 2/35 (5.7%), 2/35(5.7%), 1/35(2.85%) from minced meat, sausage and luncheon respectively with overall 5 samples (4.7%) (Table 4). The prevalence of E.coli O111 was 1/35 (2.85%) from each type of meat products; with overall 3/105 (2.85%) from all samples. The prevalence of EHEC O157:H7 was 3/105 (2.85%); represented as 2/35 (5.7%), 1/35(2.85%) from minced meat and sausage, respectively, but in luncheon failed to recovered (Table 4). Total EHEC were isolated from 11 samples with an incidence rate (10.5%); represented as 5/35 (14.28%); 4/35(11.4%); 2/35(5.7%) from minced meat; sausage; Luncheon, respectively (Table 5).

Table (6): The results of PCR amplification of different used genes of EHEC

<table>
<thead>
<tr>
<th>Sample</th>
<th>I.D of EHEC strains</th>
<th>stx1</th>
<th>stx2</th>
<th>hlyA</th>
<th>CvaC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luncheon</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Minced meat</td>
<td>O26</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sausage</td>
<td>O111</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Luncheon</td>
<td>O111</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Minced meat</td>
<td>O157</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Sausage</td>
<td>O157</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PCR results, table (6) showed that (stx2) and (cvaC) was detected in 1serogroup (O157) isolated from minced meat sample. The stx2 gene was giving product of (779 bp) and (cvaC) was giving product of (760bp). Moreover, the stx1 and hlyA genes were not detected in all studied strain. Fig. (1and 2).

4. DISCUSSION

EHEC was a subset of pathogenic E. coli causing diarrhea or hemorrhagic colitis in humans. Hemorrhagic colitis occasionally progresses to (HUS), an important cause of acute renal failure in children and morbidity and mortality in adults. In the elderly, the case fatality rate for hemolytic uremic syndrome (HUS) can be as high as 50%. The infectious
dose was very low, which increased the risk of disease (CFSPH, 2009).

There is no single technique that can be used to isolate all EHEC serogroups. So the samples were analyzed by duplicate. In the present study, (table 2) revealed that the incidence of E.coli (form minced meat, sausage and luncheon samples) were nearly agreed with Mousa et al., (1993) , Fathi et al., (1994) and Sayed et al., (2001). Higher incidence was reported by Abou-Hussein (2004) and Reda et al., (2015). However, lower incidence rate was documented by Rabie (2014) with rates of 28%, 16% and 4% from minced meat, sausage and luncheon. The variation of the results between different authors may be due to the differences in manufacture practices, handling from producers to consumers, storage and the effectiveness of hygienic measures applied during production.

The species of E.coli are serologically divided into serogroups and serotypes on basis of their antigenic composition (somatic or O antigens for serogroups and flagella or H antigens for serotypes) (Griffin and Tauxe, 1991).

Therefore, the prevalence of EHEC O26 (table 4) were nearly similar to Ghoniem (1992) and O’Hanlon et al.,(2005) .Meanwhile, other results were different to us reported by Hazarika et al., (2004) and Stefen et al., (2007) , Regarding, serogroup O111 (table 4) the obtained results nearly agreed with Ghoniem (1992) who detected E.coli O111 from 2% luncheon but disagree with Ramadan (2015) who isolated E.coli O111 from examined sausage and luncheon samples in higher prevalence rate 8% and 12%, respectively.

The prevalence of O157:H7 in minced meat was nearly similar to Abdul-Raouf et al. (1996), Abd El-Aziz (2004) and Mewafy (2012). Moreover, the obtained result was higher than Fantelli and Stephan (2001) in Switzerland and lower than Mora et al. (2007) and Hejazi (2013). Regarding sausage, the prevalence of EHEC O157:H7 in sausage (table 4) came parallel with Magwira (2005) and Hussein (2007). On the other hand, higher isolation rate of reported by AbuKhadra (2010) and Hejazi (2013).In some studies sausage have found to be free from EHEC O157 as Fayed (2006) and Mewafy (2012). Regarding luncheon E.coli O157:H7 failed to be detected in the all samples. These results go parallel with Sayed et al. (2001), Elsabagh (2010) and Mewafy (2012). This may be attributed to the competency of the organisms with other microorganisms in the food or heat treatment and preservation.

This percentage of isolation of EHEC indicated the role of this group of E.coli as potentially important food borne pathogen in Egypt. These findings were in line with Abdul-Rouf et al., (1993) who indicated that the food of animal origin have been described as primary sources of EHEC infections.

On the other side, Saleh (2001) isolated EHEC in 16% of the examined meat product samples. There are many factors may affecting the differences in prevalence rates among studies such as type, source, initial bacterial load and the methodology used.

The PCR results showed that stx2 was detected in one serogroup O157 recovered from minced meat sample, while stx1 was not detected in all samples .It has been reported that O157:H7 strains that express stx2 alone are more likely to be associated with progression to HUS than are strains producing stx1 alone or, curiously, both stx1 and stx2 (Pickering et al., 1994). These results go parallel with Blanco and Blanco (1996) who detected one EHEC O157:H7 strain produced only VT2. Murphy et al., (2005) mentioned that non O26 isolates harbor stxs. Dambrosio et al., (2007) stated that none of all E.coli O26 isolates harbor stx1 or stx2 genes while Elsabagh
(2010) found that E. coli O₁₁₁ is positive for VT₁ and VT₂, but O₂₆ is only positive for VT₁. Gomez-Aldapa et al. (2013) reported that none of the O₁₅₇:H₇ strains had stx₁ or stx₂. The PCR results (fig. 2) showed that cvaC virulence gene was detected in the same O₁₅₇:H₇ serogroup, whoever hly A genes were not detected in all studied samples. Moreover, These result disagree with Chinen (2001) recorded that all E.coli O₁₅₇ isolates harbored EHEC-hlyA gene, Oteiza et al., (2006) stated that O₂₆ strains harbored EHEC hly A gene and Dambrosio et al. (2007) who recorded that one EHEC O₂₆ isolate harbor hly A gene.

**Conclusion:** From the above mentioned results, this study recorded the high prevalence rate of EHEC especially non O₁₅₇:H₇. This indicated the role of this group of E.coli as potentially important food borne pathogen in Egypt. Moreover, the results cleared that not all EHEC harbored shiga toxins or other virulent genes.

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