Determination of Virulence Gene Markers and Antimicrobial Resistance in *Escherichia coli* Isolated from Rabbit in Egypt

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Abstract: Fecal samples from 48 diarrheic rabbits, acquired from commercial vendors and households at Qena governorate, Egypt, were cultured for *Escherichia coli*. A total of forty *E. coli* strains were recovered and twenty-nine of these, were serotyped, tested with polymerase chain reaction (PCR) for the presence of virulence gene markers of enteropathogenic and toxigenic *E. coli* (*eae*, *bfp*, *stx1*, *stx2*, *LT*, *ST*, *cnf1*, *cnf2*) and investigated for antimicrobial resistance. Detection of drug susceptibility was determined using disc diffusion method to ampicillin (AMP), amoxycillin (AMX), chloramphenicol (CHL), enrofloxacin (ENR), fluomycin (FLU), streptomycin (STR), sulphamethoxazole (SMX) and tetracycline (TET). Serological identification led to recognition of O26, O55, O103, O128 and O145 serogroups. Amplification of various genes analyzed showed that, 31% (9 of 29) of the tested *E. coli* strains, possess *eae* gene and three displayed toxin genes (one each of *stx1*, *cnf1*, *cnf2*). Gene markers of BFP, LT, ST and Stx2 could not detected. Result of antibiotics have shown that all the isolates were sensitive to different antimicrobials tested. Resistance to one or more antimicrobials was exhibited by 8 strains of the 29 strains examined. Single resistance was found against SMX (three strain), AMP (two strains) and STR (one strain). Multiple resistance was observed to SMX, TET, AMP, CHL, STR (one strain) and SMX, TET, CHL, STR (one strain). Generally, the study demonstrated that rabbit *E. coli* isolates express *eae* gene encoding for intimin among and rare toxin displayed. Rabbit *E. coli* showed general sensitivity to antimicrobials, however some exhibited antimicrobial resistance. Presence of pathotype marker genes, serotypes and resistance in rabbit *E. coli*, could constitute potential source of transmission to human.

Key words: Rabbit diarrhea · Virulence markers · Enteropathogenic *E. coli* · Toxigenic *E. coli* · Serotypes · Antimicrobials PCR

INTRODUCTION

In recent years, there has been increasing commercial production of rabbits as a source of protein. In Egypt, increasing demand for rabbits meat due to its palatable test and popularity. In addition to this commercial value, these animals are used as very important models for medical research and as pets [1]. Rabbits are highly susceptible to diseases and intestinal infections are among the most serious health problems of industrial rabbit production. From an etiological aspect, bacteria, viruses and protozoa are involved. According to Peleters (1984) enteropathogenic *E. coli* bacteria together with clostridia and cimeria, are most commonly implicated in the etiology of diarrhea in rabbits [2]. The enteric diseases that associated with *E. coli* pathogenic strains, are often attributable to intestinal colonization by *E. coli* [3].

*Escherichia coli* is the most predominant facultative anaerobe of the colonic flora. Diarrheagenic *E. coli* strains include five major groups: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterocoeaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC) and enteroendesive *E. coli* (EIEC) [4,5].

The enteric diseases due to EPEC strains are considered a major cause of weight loss, watery diarrhea and high mortality rates in rabbits. The hallmark of the infection due to EPEC is the attaching and effacing (A/E) histopathologic condition, which is characterized by effacement of microvilli and intimate adherence between the bacterium and the epithelial cell membrane. Intimin, an outer membrane protein, is an adhesin encoded by the *eae* gene (for *E. coli* A/E).

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EPEC is currently subdivided into typical and a typical subgroups. While typical EPEC carry the EPEC adherence plasmid (pEAE) that encodes the bundle-forming pilus (BFP) and a complex regulatory of various virulence genes (Per) [6], atypical EPEC is devoid of pEAF (or does not express a functional BFP) [6,7]. Enteropathogenic E. coli (EPEC) and the majority of clinical isolates of Shiga toxin (Stx)-producing E. coli (STEC) harbor the “locus of enterocyte effacement” (LEE), a pathogenicity island that is responsible for the phenotype of attaching-and-effacing (A/E) lesions [6,8].

Rabbit EPEC (REPEC) bacteria constitute a subset of the EPEC pathotype. The virulence properties of rabbit EPEC (REPEC) strains seem to be related to the presence of the eae gene, encoding for the protein intimin that is required for the development of the Attaching-Effacing lesions and to the presence of the AF/R1 and AF/R2 fimbriae [9,10].

In rabbits, most food transformation processes occur in the caecum, which is densely populated with bacteria and constitute a favorable culture system. Digestive disorders are the predominant cause of mortality in commercial rabbits [11]. These diseases have been usually treated by administration of antimicrobial substances. However, such management while decreasing the mortality rates, may allow for selection and spread of plasmid-born resistances among the strains [12]. Of high concern, transmission of drug-resistant strains carrying transmissible genetic elements from animals to humans and stable introduction of novel drug-resistance genes in human pathogens represents a constant, major threat to humans.

E. coli is a frequent component of the gut flora in healthy rabbits, although usually at low rates [13]. Thus, isolation and serogrouping alone are not a sufficient way to the diagnosis of strains pathogenic for rabbits. Furthermore, the experimental reproduction of the disease in healthy rabbits with each field isolate is time-consuming and labor work. Furthermore, in Egypt, there is lack in the information about the virulence characteristics of E. coli associated with diarrhea in rabbits.

The present study aim to determine the virulence characteristics of rabbit E. coli strains using PCR technique for the presence of virulence gene markers of enteropathogenic E. coli (EPEC) and toxigenic E. coli pathotypes and to investigate the rabbit E. coli for their antimicrobial susceptibility.

**MATERIALS AND METHODS**

**Samples and Bacteriology:** Fecal samples from 48 diarrheic rabbits belong to one of commercial vendors and households at Qena governonate in Egypt were incubated overnight at 37°C in buffered peptone water.

Microbiological isolation of E. coli was obtained with selective agar medium including MacConkey agar and eosin methylene blue agar (EMB). The E. coli suspected colonies were identified by standard biochemical methods [14] and kept at 4±1°C in trypticase soy agar (TSA). Culture media were obtained from BiloLab, Hungary. The twenty nine E. coli strains a representative of forty strains were used in further analysis.

**Serological Identification:** A selected E. coli strains were subjected to slide agglutination with polyvalent antiserum (SIFIN, Berlin, Germany) against somatic antigens of EPEC and toxigenic E. coli (O26, O55, O78, O103, O114, O119, O126, O128, O145, O153, O157).

**Antimicrobial Susceptibility Test:** The selected isolates were tested by disk diffusion method [15]. The bacterial suspension were adjusted in sterile 0.9% saline to 0.5 McFarland standard (10⁶ cfu/ml) and spread on Mueller-Hinton agar. The antibiotics tested were chosen based on their use as growth promoters and in human and animal therapeutics, including: ampicillin (AMP-25 μg); amoxicillin (AMX-25 μg); chloramphenicol (CHL-25 μg); enrofloxacin (ENR-5 μg); Flumequine (FLU-30); sulphanethoxazole (SMX-25 μg), streptomycin (STR-10); and tetracycline (TET-30) (MASTDISCS–Mast Diagnostics Ltd., UK). Isolates were evaluated as sensitive, intermediate or resistant according to the inhibition zone diameter [16].

**DNA Isolation and Detection of Virulence Genes:** Bacterial DNA was isolated using boiling method as previously described [17]. Gene regions coding for the following pathogenic properties were amplified for each bacterial isolate: Shiga-like Toxin 1 and 2 (str1, str2), enteropathogenic attachment and effacement (eaeA), bundle-forming pilus (bfp), heat-labile toxin (LT), heat-stable toxin (ST), cytotoxic necrotizing factors 1 and 2 (cnp1, cnp2) gene, using specific primers. The amplification was done in a 25 μl containing template DNA, 1.5 mM MgCl2, 10 mM Tris HCL, 50 mM KCL, 0.2 mM of dNTPs, 1 U Taq polymerase. The specific primer sequences and the predicted size of the amplified products for the
Table 1: Primer pairs used for detection of the pathotype marker genes used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Target gene</th>
<th>Primer sequence (5’ to 3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>eae-1</td>
<td>Intimin (eae)</td>
<td>AGGTTCAGCATGCGTAACCT</td>
<td>815</td>
<td>[22]</td>
</tr>
<tr>
<td>eae-2</td>
<td>Intimin (eae)</td>
<td>GATGGCAGCAGAGTTCCATCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFP-a</td>
<td>bundle-forming pilus (bfp)</td>
<td>ATGGGATCCGCTGGGCTGCTGC</td>
<td>326</td>
<td>[23]</td>
</tr>
<tr>
<td>BFP-b</td>
<td>bundle-forming pilus (bfp)</td>
<td>GGCGCCTTTATCCCAACTGCTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STX1-F</td>
<td>Shiga-toxin 1 (stx1)</td>
<td>AAATCGACCATCGTGAACACTCTCTC</td>
<td>366</td>
<td>[18]</td>
</tr>
<tr>
<td>STX1-R</td>
<td>Shiga-toxin 1 (stx1)</td>
<td>TGCCATTTGCAACTCGDAATGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STX2-F</td>
<td>Shiga-toxin 2 (stx2)</td>
<td>CGATCGTCATTCTCACTGTTTCACTCA</td>
<td>282</td>
<td>[18]</td>
</tr>
<tr>
<td>STX2-R</td>
<td>Shiga-toxin 2 (stx2)</td>
<td>GGATATCTTCGCCGACTGTGACACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LT-A</td>
<td>Heat-labile toxin (LT)</td>
<td>TGTTCGACCTTTCTTCTTAG</td>
<td>258</td>
<td>[19]</td>
</tr>
<tr>
<td>LT-B</td>
<td>Heat-labile toxin (LT)</td>
<td>TATCCCTGTTAAGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ST-a</td>
<td>Heat-stable toxin (ST)</td>
<td>TCTGATATTATCTGGTCCTTCCTC</td>
<td>186</td>
<td>[20]</td>
</tr>
<tr>
<td>ST-b</td>
<td>Heat-stable toxin (ST)</td>
<td>ATACATGCAAGCAACAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNF-1-A</td>
<td>Cytotoxic necrotizing factor 1 (cnf1)</td>
<td>GAACTTTAAGGATAGT</td>
<td>543</td>
<td>[21]</td>
</tr>
<tr>
<td>CNF-1-B</td>
<td>Cytotoxic necrotizing factor 1 (cnf1)</td>
<td>CATTTATGAAAGCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNF-2-A</td>
<td>Cytotoxic necrotizing factor 2 (cnf2)</td>
<td>AATCTAATTAGAAAGAAAC</td>
<td>543</td>
<td>[21]</td>
</tr>
<tr>
<td>CNF-2-B</td>
<td>Cytotoxic necrotizing factor 2 (cnf2)</td>
<td>CATCCTTTTATATCTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plasmid Extraction: Plasmid DNA was extracted by alkaline lysis method as previously described [24] and electrophoretic migration was carried out in 0.7% agarose gels and the plasmids were detected by staining with ethidium bromide.

RESULTS AND DISCUSSION

Bacteriology: A worldwide non-coccidial enteric infection of commercially fattened rabbits was increasingly developed [25]. These enteric diseases are often attributable to intestinal colonization by _E. coli_ [3]. In this study, all isolated and identified bacteria possessed the morphological and biochemical characteristics of _E. coli_. A total of forty strains were recovered from diarrheic rabbits and twenty-nine of these strains were further characterized. In 1977, Cantey and Blake [26] were the first to isolate from a rabbit a noninvasive strain of _E. coli_ (RDEC-1) capable of causing severe diarrhea in this animal species. Previous study in Spain reported that 231 _E. coli_ recovered from diarrheic rabbits from different area in Spain and were serotyped, tested for the presence of the _eae_ gene and toxin production [3], meanwhile other [27] isolated 105 _E. coli_ strains from small intestine, caecum or liver of rabbits coming from a rabbitry located in the province of Teramo (Abruzzo, central Italy) and twenty of these strains were further characterized. In Egypt, bacterial examination resulted in isolation and identification of _E. coli_ in 27.64% of diarrheic rabbits [28].

O-Serogroups: Serogroups found in the 29 _E. coli_ strains investigated are shown in Table 2. Using sero-identification, five different serogroups could be demonstrated among the isolates including O26 (one strains), O55 (two strains), O103 (six strains), O128 (three strains) and O145 identified in one isolates, meanwhile the rest of the isolates could not be typable. The O103 and O128 serogroups are included among the

Table 2: Serogroups and virulence marker genes of _E. coli_ isolates from rabbit in Egypt (n=29)

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>No. of isolates (%)</th>
<th>Gene (n° of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O26</td>
<td>1 (3.4)</td>
<td>eae</td>
</tr>
<tr>
<td>O55</td>
<td>2 (6.9)</td>
<td>eae (1)</td>
</tr>
<tr>
<td>O103</td>
<td>6 (20.7)</td>
<td>eae (3)</td>
</tr>
<tr>
<td>O128</td>
<td>3 (10.3)</td>
<td>eae (1)</td>
</tr>
<tr>
<td>O145</td>
<td>1 (3.4)</td>
<td>cnf1</td>
</tr>
<tr>
<td>NT</td>
<td>16 (55.2)</td>
<td>stx1 (1), cnf1 (1), eae (3)</td>
</tr>
</tbody>
</table>

NT: not typable
Table 3: Antibiotic resistance patterns showed by E. coli strains isolated from rabbits in Egypt (n = 29)

<table>
<thead>
<tr>
<th>Antimicrobials</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMX</td>
<td>3 (10.3)</td>
</tr>
<tr>
<td>AMP</td>
<td>2 (6.9)</td>
</tr>
<tr>
<td>STR</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>SMX, TET, CHL, STR</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>SMX, TET, AMP, CHL, STR</td>
<td>1 (3.4)</td>
</tr>
<tr>
<td>Single type resistance</td>
<td>6 (20.7)</td>
</tr>
<tr>
<td>Multiple type resistance</td>
<td>2 (6.9)</td>
</tr>
</tbody>
</table>

AMP (ampicillin), CHL (chloramphenicol), TET (tetracycline), SMX (sulfamethoxazole)

Of serogroups which were more frequently detected in E. coli strains isolated from diseased rabbits. Similarly, previous investigation reported that the majority of the E. coli strains obtained from diarrheic rabbits belong to only four serotypes, whereas the 231 E. coli strains isolated from diarrheic rabbits that were investigated, nearly half were of the O103 serogroup [3]. Furthermore, other study in France, showed that More than half of the 275 E. coli strains isolated from the fecal contents of weaned rabbits in French commercial farms belonged to serogroup O103 and 70% were of one of five serogroups (O2, O26, O103, O128 and O132) [25]. One E. coli strain in this study belongs to EPEC O145 serogroup. Cattle and other ruminants are the main reservoirs of E. coli O145 suggested that rabbits may be infected from cattle via contaminated green fodder. In recent report, E. coli strain belonged to O145 serogroup were isolated from rabbits, suggested that rabbits may encountered and become infected with EHEC and EPEC strains from rabbit colony contaminated with cattle feces [29].

Experimental infections have shown that E. coli strains involved in rabbit enteritis belong to different serotypes [11, 30, 31]. Thus, serotype O109: K2:H2 is mainly associated with yellow diarrhea in suckling rabbits, whereas other serotypes (O2:K1:H6, O15:K2:H2, O20:K2:H7, O26:K2:H11, O103:K2:H2, O109:K2:H7, O128:K2:H2, O132:K2:H2 and O153:K2:H7) are associated with diarrhea in weaned rabbits [32]. However, the epidemiology of EPEC from rabbits remains incomplete, because of the limited number of surveys performed. EPEC strains of serotype O15:K2:H2 are frequently isolated in Belgium, The Netherlands and the United States [11, 30, 33], whereas rhamnose-negative O103:K2:H2 strains are predominant in France and Spain [25, 34, 35]. In Egypt, serological identification of E. coli isolates from rabbits led to recognition of O26:K60, O55:K59, O125:K70, O126:K71, O128:K67 [28].

Detection of Drug Resistance: By evaluation of the antimicrobial susceptibility, All isolates were susceptible to FLU, AMX and ENR. Resistance to one or more antimicrobials was exhibited by 8 strains of the 29 strains analyzed. Single resistance was found against SMX (three strain), AMP (two strains) and STR (one strain). Multiple resistance was observed to SMX, TET, AMP, CHL, STR (one strain) and SMX, TET, CHL, STR (one strain) (Table 3).

Rabbit E. coli strains had a good sensitivity against quinolones (FLU, ENR) [36]. In our investigation, all isolates exhibited 100% sensitivity to flumequine (FLU), enrofloxacin (ENR) and amoxicillin (AMX). Similarly, others determined a high sensitivity to quinolones (enrofloxacin and norfloxacin) [37, 38]. However, in the same study, determined the highest resistance percentage against amoxicillin [38]. The data evidenced resistance of rabbit E. coli strains against sulphamethoxazole, ampicillin, streptomycin, chloramphenicol and tetracycline. Our data similar with regard to these antibiotics to the previously reported [37]. In contrary, [38], reported a preserved sensitivity against chloramphenicol.

Virulence Genes: E. coli is a normal component of rabbit digestive flora and it does not always exert direct pathogenic activity in rabbits. Stress or other pathogens may trigger its overgrowth in the gut environment, which can result in diarrhea or death [39]. In addition, pathogenicity of some strains may be enhanced by the presence of virulence genes. In this study, we have used the PCR technique as an approach to detect genes encoding intimin, bundle-forming pilus, enterotoxins, Shiga-toxins and cytotoxic necrotizing factors.

Not all E. coli strains that cause diarrhea in farm animals synthesize LT or STa enterotoxins. These animal enteropathogenic E. coli strains attach to and efface the microvilli of gut epithelium [3]. In rabbits, only this type of E. coli enteritis is known to be important and it is caused by EPEC strains, also called AEEC [40]. Our results revealed negative detection of genes encoding LT and ST enterotoxins. Similarly, past investigation showed that non of the E. coli strains produced thermostable or thermolabile enterotoxins [34]. For instance, the eae gene, that encodes for intimin, is required to allow the development of Attaching-Effacing lesions (A/E) [41, 42].

The eae gene responsible for the A/E lesions are located on an 35-kb pathogenicity island, known as the locus of enterocyte effacement (LEE). In the present study, amplification of the eae gene showed that 31% (9 of 29) of the E. coli strains isolated from diarrheic
rabbits, possess this virulence determinant (Table 2), (Fig 1). Our results are in agreement with the previous studies on rabbits [3, 37, 41, 43] and showed that eae gene, specific for EPEC, was frequently detected among rabbit E. coli strains. Sequences homologous to the eae gene have been detected in atypical enteropathogenic E. coli (AEEC) strains isolated from diarrheic rabbits [41, 43].

Enteropathogenic E. coli (EPEC) is currently subdivided into typical and atypical subgroups. Typical EPEC harbor an additional 60 MDa plasmid, the EPEC adherence factor (EAF) plasmid [44], that is not present in atypical EPEC (ATEC) strains [7, 45]. The EAF plasmid harbors the bundle-forming pilus (bfp) operon, encoding the type IV pili responsible for localized pattern of adherence (LA). ATEC strains harbor homologues of the LEE pathogenicity island but, due to the lack of the EAF plasmid [45, 46], they most often expresses the LA-like pattern (adhere in a diffuse pattern). Our results confirm those previously reported [47] and show that, to date, no rabbit EPEC (REPEC) strains harboring the bfp genes responsible for the localized adherence of human EPEC has been identified. However, one study reported one rabbit E. coli strain, belonging to serogroup O103, harbored a self-transferable 117-kb plasmid encoding both antibiotic resistance and virulence determinants [48].

Atypical E. coli (AEEC) strains that cause diarrhea in rabbits apparently display a pathogenic mechanism similar to that displayed by human EPEC and enterohemorrhagic E. coli strains, some authors have suggested that AEEC strains can synthesize verotoxins (also called Shiga-like toxins) or other types of related cytotoxins [49].

From all of the E. coli strains screened, only three were toxigenic: one Shiga-toxin-producing E. coli strain (STX1) and two enterotoxigenic E. coli strains (CNF1 and CNF2) (Table 2) (Fig 2, 3).

Our results indicate that E. coli strains from rabbits rarely produce Shiga-tosins (STX1 and STX2) nor the recently discovered cytotoxic necrotizing factors (CNF1 and CNF2) elaborated by clinical E. coli isolates of human and bovine origin [21, 50, 51, 52]. In previous studies, toxigenic E. coli strains were rarely identified among rabbit E. coli strains. Of a collection of 40 rabbit E. coli isolates examined, one O26:B13 strain which reacted
with a VT1 probe [43]. Also, O128:B30 strain has been identified as a producer of cytotoxins distending toxin [32].

Of relevance to the *E. coli* strains of serotypes O26 and O55 isolated from rabbits with diarrhea. These two serotypes are considered enterohemorrhagic *E. coli* (EHEC) rather than EPEC serotypes, because many *E. coli* O26 and O55 strains that cause diarrhea in humans and animals produce verotoxins [4, 53]. These two *E. coli* serogroups isolated from rabbits in this study, were negative for VT1 and VT2 by PCR (Table 2), however, displayed *eae* gene similar to human EPEC strains. Our results are in accordance with the previously reported [3] and showed that rabbit O26 strains presented the *eae* gene but not any of verotoxin encoding genes. In Egypt, one study on rabbits diarrhea, detected O26:K60, O55:K59 and O126:K71 serogroups [28]. On the other hand, previous report identified verotoxin and intimin positive *E. coli* in rabbits and indicated that rabbits are a new reservoir host of EHEC that may pose a zoonotic risk for humans [29].

In summary, of the virulence gene markers screened, the *eae* gene was most commonly detected and rarely the toxins encoding genes. Also, when evaluating antimicrobial susceptibility, quinolons drug were shown to be efficient against rabbit *E. coli*. In addition, the demonstration of some *E. coli* serotypes, pathotypes and resistant strains among rabbit *E. coli*, may represent a potential source of human infection.

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### REFERENCES


42. Agin, T.S., J.R. Cantey, E.C. Boedecker and M.K. Wolf, 1996. Characterization of the *eae* gene from rabbit enteropathogenic *Escherichia coli* RDEC-1 and comparison to other *eae* genes from bacteria that cause attaching effacing lesions. FEMS Microbiology Letters, 144: 249-258.


