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Genetic Typing and Antigenic Characterization of Egyptian Field *Shiga*-Toxigenic *Escherichia coli* Isolates with Regard to Profile of Virulence Proteins

[1]Nesreen A.T. Allam, [2]Sahar G. Abd Ellatif and [3]R.H. Hamouda

[1]Department of Parasitology and Animal Diseases,
Veterinary Research Division, National Research Center, Dokki, Giza, Egypt
[2]Department of Immunology, Animal Health Research Institute,
Agriculture Research Center, Dokki, Giza, Egypt
[3]Mastitis and Neonatal Diseases Research Department,
Animal Reproduction Research Institute, Agriculture Research Center, El-Haram, Giza, Egypt

Abestract: *Shiga* toxin-producing *Eschericia coli* are diverse organisms inducing sever gastrointestinal and systematic diseases recognized worldwide. Ten serogroups were isolated, morphologically identified and biochemically characterized from 281 samples (fecal swabs and internal organs) of diarrheic field cases collected from cattle and ostrich private farms. Haemolysis, enterocytotoxicity, pathogenicity and antibiogram patterns were determined. All isolates were *Stxs* producers according to the cytopathic effects noticed in inoculated Vero cells monolayers with CD_{50} activity titers calculated 32 (O1 and bovine O157), 8 (O8 and O25), 64 (O20), 4 (O78) and 128 (O127, O146, O153, Ostrich O157) CD_{50} /100 µl. Dominant protein bands with apparent molecular masses of 106 to109 kDa, 80-83 kDa, 54-56 kDa, 41 kDa, 37 kDa, 32 kDa, 26-25 kDa, 22 kDa and 7.8 kDa were separated by SDS-PAGE of *E coli* cultures supernatants. These bands were not uniformly expressed nor equally presented by all serotypes under study. In addition, 346 bp *stx*2 specified sequence were amplified by PCR in five serotypes only. None of the isolates were positive for *stx*1 coding sequences. It is clearly noticed that some serogroups are more virulent than others. Virulence properties depend on less characterized factors than *Stx*1 and *Stx*2 which has been proved from profiles of secretory proteins. It was concluded that STEC virulence is multifactorial process. However, *Shiga* toxins production, in particular *Stx*2, is a minimum requirement. Other secreted virulence proteins such as EspA (26-25 kDa), EspB (37 kDa), EspD (41 kDa), EspE (80-83 kDa), EspP (106-109 kDa) and *Clostridium difficile*-like toxins (32 kDa) are putative candidates involved in disease production their contribution in pathogenicity of isolated strains should be identified on both structural and functional levels.

Key words: PCR · Virulence Proteins · Somatic Antigen · *Shegilla*-Toxins Producing *E. coli*.

INTRODUCTION

Multiple O-antigen groups of *Escherichia coli* occur commensally in the colons of humans and animals [1] and contaminated food products [2, 3]. However, many of these serotypes are associated with a number of disease syndromes in humans [4, 5] and farm animals as cattle, sheep, pigs, poultry and rabbits [3-8] Such a subset of serotypes often referred to as pathotypes, causing painful bloody diarrhea, urinary tract infections, meningitis, or systemic disease [4, 9]. O serotyping thus has been useful in understanding the epidemiology of infections and allows differentiation between pathotypes [10, 11]. Recent advancements of molecular techniques have led to adaptation of bacterial typing to various formats, including multiplex assay formats, which are generally more rapid and simpler than conventional methods [12].

The Shiga toxins family which are structurally and biologically very similar, produced by *Shigella dysenteriae* type 1 and certain strains of *E. coli* (STEC), are all AB5 subunit protein toxins [13], exhibit several

Corresponding Author: Nesreen A.T. Allam, Department of Parasitology and Animal Diseases,
Veterinary Research Division, National Research Center, Dokki, Giza, Egypt

dramatic biological effects [11]. Variation in receptor specificities among *Stxs* may be the reason for different disease syndromes in different host species [14, 15]. The A subunits of all three toxins (*Shiga* toxin, *Stx*1 and *Stx*2) are enzymatically active as N-glycosidases, cleaving a specific adenine residue in the 28S rRNA of the 60S ribosomal subunit and cause inhibition of protein synthesis in eukaryotic cells [11, 16]. The B subunits of each toxin mediate binding of the toxin to cellular receptor (globotriaosylceramide), which is a surface glycolipid containing a terminal galactose al-galactose disaccharide [9, 17]. *Stx*1 and Shiga toxin differ in only one amino acid in the A subunit and possess identical B subunits [18, 19]. *Stx2*, although immunologically distinct from *Stx1* and Shiga toxin, shares the same binding specificity and biological activity despite its variants (*Stx2*, *Stx2v*, *Stx2vha*, *Sxt2vhb* and *Stx2va*) [20]. Secretion of other virulence factors is characteristic feature of STEC, like enteropathogenic *E.coli*, which enable this group to adhere tightly to the gut epithelium, efface the surrounding microvilli and induce actin-based pedestal formation underneath the eukaryotic membrane at the site of attachment (attaching and effacing lesion) [20].

The current work characterized field verotoxin producing *E. coli* (VTEC) of variant serotypes which were, collected from diseased species. Also, typing of STEC virulence properties at both expressed virulence proteins profile and genetically by targeting *stx*1 and *stx*2 *coding sequences* in isolated colonies was another target.

MATERIALS AND METHODS

Bacterial Strains and Media: *E.coli* field isolates were recorded on streaked MacConkey agar (Oxoid) and Eosin Methylene blue agar (Oxoid) after 24 hour aerobic incubation at 37°C from 281 samples (fecal swabs and internal organs) of diarrheic field cases collected from cattle and ostrich private farms. Symptoms were noticed on individuals less than three years age and for both sex. Intestinal samples were tied off from both ends and sent to laboratory as soon as possible.

Morphological Identification and Biochemical Confirmation: Suspected colonies were picked up and subjected for morphological and biochemical identification according to the standard procedures [21, 22].

Serological Typing: *E.coli* isolates were serotyped by slide agglutination test (somatic antigen "O") (Denka Seiken Co. Ltd., Tokyo, Japan). Test was done as outlined by Edwards and Ewing [23] using standard polyvalent and monovalent *E. coli* antisera.

Haemolysin Production: All *E. coli* isolates were streaked on sheep blood agar plates, incubated at 37°C for 24hours then examined for the developed haemolysis [24].

Heat Stable Enterotoxin Production and Infant Mice Assay: each 25 ml of toxins production media were inoculated with sole typed bacterial isolate then shaking incubated (Shellab) at room temperature at 200 rpm for 48 hours. 0.1 ml of each bacterial filtrate was injected intra abdominal into milk filled stomach of 3 mice/isolate which are 2-4 days old. After 4 hours all mice were killed and the entire intestines were removed and weighted. The assay was considered positive for enterotoxin if the ratio of combined weight of the intestines of the three inoculated mice/isolate to combined weight of the remaining body weight was >0.083 [25, 26].

Mice Pathogenicity Test: Fifty five Albino white mice aged 30-37 days weighted 18-20 grams were used and 100 µl of each *E.coli* isolate/mouse equals 9×10^8 CFU/ml were injected [27]. The internal organs, heart, liver, spleen, intestine and kidneys, were divided into two parts, one was sent for histopathological examination while the other part was used for re-isolation of *E.coli* strains followed by serotyping with standard antisera [28].

***Shigella*-Like Toxins Production and Vero Cell Assay:** *E. coli* supernatants were prepared according to the method described by Gentry and Dalrymple [30]. 100 µl aliquot of each two fold dilution/E.coli solate were transferred in to 96-wells tissue culture microtiter plates (Coaster) containing Vero cell (Green Monkey Kidney Cells) monolayers (4×10^4 cells/well [29]. Cytotoxic effects were determined and cytotoxic dose titer (CD_{50} unit) was defined [30].

Antibiotic Susceptibility Patterns: Antibiotic susceptibilities of isolates were determined by disk diffusion method on Muller Hinton agar plate (Oxoid) [31]. The following commercial antibiotic disks (Oxoid) were used in illustrated concentration: Amikacin (30 µg), Ampicillin (10 µg), Cefadroxil (30 µg), Chloramphenicol (30 µg), Colistin Sulphate (50 µg), Erythromycin (15 µg), Gentamicin (10 µg), Oxytetracycline (30 µg), Nalidixic

acid (30 µg), Novofloxacin (10 µg), Trimethoprim and Sulphamethoxazol (1.25 µg and 23.75 µg). Inhibition zone diameter of each antibiotic disk was measured [22] and compared with standard zone chart according to manual of the supplier [32].

Oligonucleotide Primers Design: PCR primers pairs were designed with reference to annotated sequence for both *E.coli* O157:H7 accession numbers AE005174v2-1.gbk and AE005174v2-2.gbk [33] and *stx1* and *stx2* [34, 35] they were synthesized by Metabion International AG (Martinsried/Deutschland). These primers had been previously utilized in PCR for *stx* producing *E.coli* detection [36, 37].

DNA Isolation: Templates DNA were extracted from serotypes broth grown over night in Luria broth (Oxiod) at 37°C with agitation (Shellab) at 200 rpm according to Gilmour *et al.* [12].

PCR Protocol: Each PCR mix was prepared in 50 µl with 2 µl of template (100 ng), 50 pmoles of each primer, 45 µl of Ready TaqMix Complete (Mater Mix, AllianceBio, USA) and nuclease free water (Qiagen, Germany) to complete the total volume of the reactions. PCRs were performed in a PTC-100™ Thermal Cycler (MJ Research Inc., USA) using the following cycling protocol: initial denaturation at 95°C for 5 min and then 40 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. Final extension was carried out at 72°C for 7 min. A reagent blank (containing all the components of the reaction mixture with water instead of template DNA) and a VT-negative *E. coli* were run as controls in every PCR procedure. Cultures showing positive results by PCR were retested on two further occasions several days later to examine the reproducibility of PCR testing. Amplified products from the PCRs were electrophorised on 2% agarose gels and stained with ethidium bromide. A 100 bp ladder (Jena Bioscince, GmbH, Germany) was used with each gel.

SDS-PAGE: Proteins where Precipitated and concentrated from cultures [38]. Total proteins contents of each preperation was determined [39], compared to standard protein curve where concentration of Bovine serum albumin Fraction V (BSA, Sigma-Aldrich) ranged from 0.5 to 1000 µg/100 µl. *E.coli* secreted proteins were separated by 12% SDS-PAGE gel run in Mini-PROTEAN II Dual Slab Cell (Bio-Rad) [40]. Then, gel was stained with 0.5% Coomassie brilliant blue in 40% methanol (Sigma-Aldrich).

RESULTS

***Escherechia coli* Isolation:** Out of 281 clinical laboratory samples of Cattle and Ostrich 32.38% specimens were positive for *E.coli*, precisely. The incidence of pathogenic *E. coli* was the highest in fecal samples, followed by large intestine, small intestine, liver, gizzard and crop, recording 39.6, 36.8, 34.2, 28.9, 23.7 and 21.1% prevalence rate, respectively.

Serotypes Prevalence: Serotypes of the 91 isolates of *E.coli* obtained were grouped under 9 serovars designed: O1, O8, O20, O25, O78, O127, O146, O153, O157 with prevalence rates of 9.9, 12.1, 11, 13.2, 12.1, 9.9, 16.5, 8.8 and 6.5%, respectively.

Haemolysis, Enterocytotoxicity and Pathogenicity Patterns: Results of are presented in Table 2.

***Shigella*-Like Toxins Production:** All isolates were *Stx* producers according to the recoded cytopathic effects (CPE) in inoculated monolayers. Cytotoxicity of culture supernatants with CD_{50} titers calculated 32 (O1 and bovine O157), 8 (O8 and O25), 64 (O20), 4 (O78) and 128 (O127, O146, O153, Ostrich O157) CD_{50} /100 µl.

Antibiogram Pattern: It was observed that variant *E.coli* serotypes are resistant to Ampicillin and Trimethoprim and Sulphamethoxazol (100%). In contrast, they were highly sensitive to Gentamicin (100%), Chloramphenicol (100%), Amikacin (90%), Cefadroxil (90%) and Norfloxacin (90%) which were the drugs of choice on recommendations for treatments.

Table 1: PCR primers

Primers	Sequence (5`-3`)	Target Sequence	Amplicon size (bp)
stx-1F	5`-GAAGAGTCCGTGGGATTACG-3`		
stx-1R	5`-AGCGATGCAGCTATTAATAA-3`	nt 1119-1320	130 bp
stx-2F	5`-TTAACCACACCCACGGCAGT-3`		
stx-2R	5`-GCTCTGGATGCATCTCTGGT-3	nt 426-771	346 bp

nt= nucleotide bp= base pair F= forward R= reverse

Table 2: Pathogenicity, Enterocytotoxigenicity and Haemolysis Results

		Pathogenicity		Enterocytotoxigenicity		Haemolysis		
E.coli Serotypes	Number of Isolates	+Ve*	%	+Ve*	%	+Ve*	Type	%
O1	5	4	80	5	100	5	α	100
O8	5	5	100	5	100	5	α	100
O20	5	2	40	1	20	5	γ	100
O25	5	3	60	4	80	5	γ	100
O78	5	5	100	5	100	5	γ	100
O127	5	3	60	4	80	5	α	100
O146	5	3	60	3	60	5	α	100
O153	5	4	80	3	60	5	γ	100
O157	6	6	100	6	100	3" 3 ?	α β	5050

*: The percent was calculated according to number of mice in each group

": Bovine O157 serotype of *E.coli* isolates

?: Ostrich O157 serotype of *E.coli* isolates

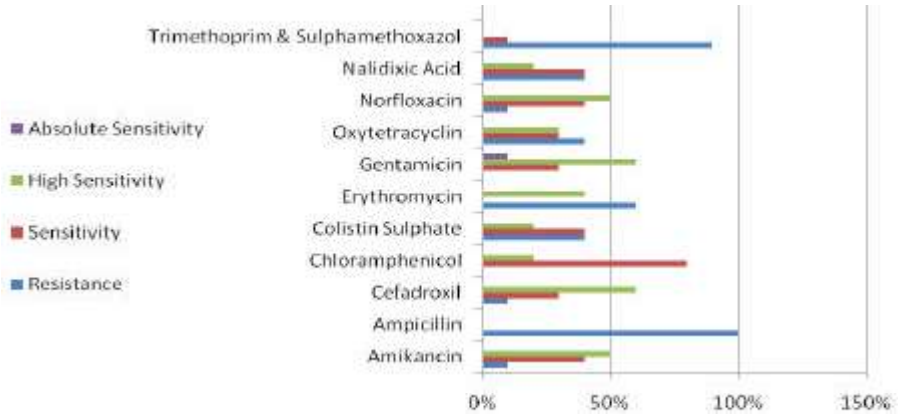

Chart 1: Overall Antibiotic Susceptibility Pattern in Different E.coli isolates

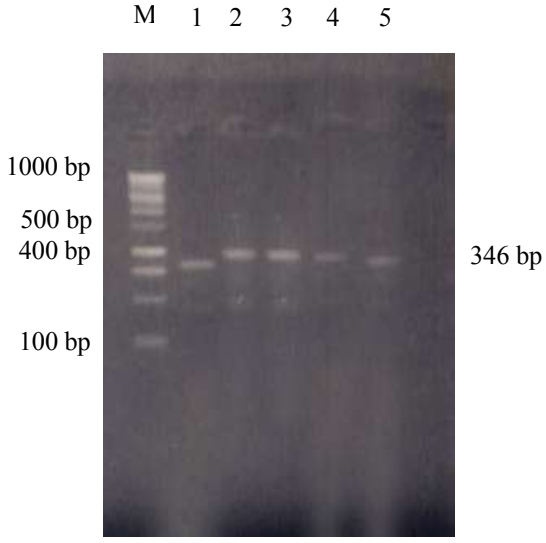

Fig. 1: PCR product of E.coli Cultures representing stx2 specified amplicon of 346 bp molecular size noticed in lanes 1, 2, 3, 4, and 5 representing O20, O127, O146, Ostrich O157 serotype and Bovine O157 serotype. Lane M. Molecular weight marker.

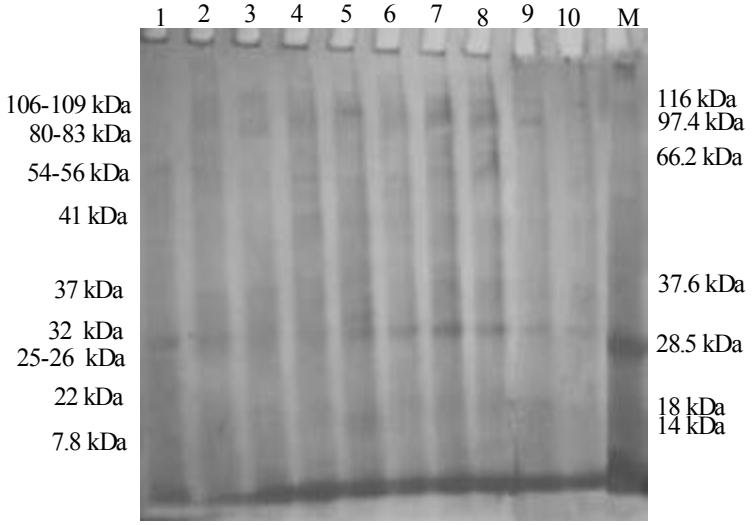

Fig. 2: SDS-PAGE Results of E.coli Cultures Supernatants representing the Secretory Virulence Proteins Profiles. Lane 1. O1, Lane 2. O8, Lane 3. O20, Lane 4. O25, Lane 5. O78, Lane 6. O127, Lane 7. O146, Lane 8. O153, Lane 9. Ostrich O157, Lane 10. Bovine O157, and Lane M. Molecular weight marker.

While different susciptibiliy were recorded with Colistin Sulphate (60%), Oxytetracyclin (60%), Nalidixic acid (60%) and Erythromycin (40%), Chart 1.

PCR Typing of *Stx* Producing *E.coli*: Discrete bands corresponding to the expected *stx*2 amplicons were seen in agarose gel representing the PCR assays products. Controls as expected were negative for *stx*1 and *stx*2 PCR amplification. DNA fragments of 130 bp molecular size could not be recorded in lanes representing any of the strains indicating that coding sequence for *stx*1 gene is missed. *stx*2 specified PCR products of 346 bp molecular size were noticed in lanes representing O20, O127, O146 and all O157 serotypes, subsequently, they own the corresponding sequences of *Stx*2, Figure 1.

SDS-PAGE: Nine dominant protein bands with apparent molecular masses of 106 to109 kDa (O8, O25, O78, O127, O146 and O153), 80 to 83 kDa (O8, O25, O146, O153 and Ostrich O157), 54 to 56 kDa (O25, O78, O127, O146 and O153), 41 kDa (O147 and O153), 37 kDa (O78, O146 and O153), 32 kDa (O1, O8, O20, O78, O127, O146 and all O157), 26-25 kDa (O8 and O78, O146, O153 and all O157), 22 kDa (O20, O25, O78 and O127) and 7.8 kDa (O20, O127 and all O157). As well as a variety of minor bands were detected in the supernatants of the bacterial cultures. These bands were not uniformly expressed nor equally presented by all serotypes used in this study (Figure 2).

DISCUSION

Serological O-antigen typing is an established method used in routine laboratories to identify bacteria on isolation from specimens. It is based on the highly immunogenic variable lipopolysaccharides (LPS) presented on the bacterial cell surface of which about 200 forms are found in *E. coli* [20] Epidemiological studies had shown that livestock, particularly dairy and beef cattle are natural reservoirs of these organisms [41, 42]. In Egypt, previous publications had illustrated the prevalence's of variant *E.coli* serotypes isolated from different specimens of human and veterinary origin. Studies were carried out on cattle byproducts, poultry including Ostrich and diarrheic individuals [42-46]. Previously recorded serotypes; O1, O8, O25, O78, O146 and O157 incidence rates were not different from the calculated percentages in the present study reflecting stability in infectivity, pathogenicity and antibiogram pattern of these serovars [42-46]. On the other hand, serotypes O20, O127 and O153 with prevalence rates 11, 9.9 and 8.8%, respectively, are new records in *E.coli* epidemiology in Egypt. Which is indicating changes in the dynamics of *E.coli* population and higher risk factors for disease susceptibility in national livestock that should be taken in consideration during diagnosis and treatment of similar diseases conditions and vaccination programs [3]. However, their pattern in hemolysis, enerotoxicity and pathogenicity were less than the records of already

established isolates [42-46]. In contrast, *Stxs* activity titers were higher in these new serovars than characterized serotypes, indicating more virulence properties and higher mortality and morbidity rates [20]. Unfortunately, on molecular level *stxs* were not presented by all examined isolates, only serotypes O20, O127, O146, Ostrich O157 serotype and Bovine O157 serotype hold the specified coding sequence for *stx*2 but not *stx*1. The remaining O1, O8, O25, O78 and O153 were negative for both types of *stxs* coding sequences. It is not the first time to record *Stx* activity by isolates not processing the corresponding genes and/or coding locus. Explanations depending on mutations in coding sequences, less availability of target fragment due to less copy number of plasmids carrying coding sequences and other excretory virulence proteins that enhance the cytotoxic effect on Vero cell are all proposed [7, 11]. All could be correct, thus, it was decided to look for other virulence secretory proteins expressed by the serotypes under study [38].

When further characterization was done to illustrate the variant components of the extracellular proteins secreted by STEC in to supernatant media; as trial to mimic *in vivo* conditions, the results indicated nine dominant protein bands with apparent molecular masses of 106 to109 kDa, 80 to 83 kDa, 54 to 56 kDa, 41 kDa, 37 kDa, 32 kDa, 26-25 kDa, 22 kDa and 7.8 kDa. As well as a variety of minor bands were detected in the supernatants of the bacterial cultures. These bands were not uniformly expressed nor equally presented by all serotypes used in this study. However, similar molecular weight bands were previously recorded and functionally characterized [38]. P106-109, P80-83, P41, P37, P26-25 were previously designed as being similar to EspP, EspE, EspD, EspB and EspA, respectively, of EPEC, STEC O157:H7 and other attaching and effacing *E.coli* [38]. EspP (P106-109) belong to serine protease family that contributes to mucosal hemorrhage [47]. EspE (P80-83) acts as receptor to an outer protein which is responsible for attaching/effacing phenomena (Catalase/Peroxidase activity) [47]. EspD, EspB and EspA referring to P41, P37 and P26-25, respectively, are important for signal transduction events and early bacterial attachment to epithelial cells [38]. While P32 and P7.8 are believed to be the A and B subunits of *Stx*2 that is expressed by O20, O127, O146, Ostrich O157 serotype and Bovine O157 serotype holding the specified coding sequence for *stx*2 [38]. But in case of O78, the 32 kDa proteins band tends to be *Clostridium difficile*-like toxin in function [47]. P22 and P54-56 are component of media used for toxins production since have not been documented before. In continence, all the previously mentioned proteins bands need to be sequenced and aligned with homologous protein domain family confirming the previous functional conclusions. The current data reinforce the theory of Whittam [47] that the pathogenic *E.coli* strains have a clonal population structure with board host ranges and wide geographic distribution.

CONCLUSION

STEC Virulence is multifactorial process rather than being dependent on a single gene or gene products. However, Shiga toxins production *Stx*1 and *Stx*2, in particular *Stx*2, is a minimum requirement. Other secreted virulence proteins such as EspA, EspB, EspD, EspE, EspP and *Clostridium difficile*-like toxins are putative candidates involved in disease production their contribution in pathogenicity and epidemiology of the recorded new isolates should be identified on both structural and functional levels.

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