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ORIGINAL ARTICLE

Phenotypic and genotypic analysis of pathogenic *Escherichia coli* virulence genes recovered from Riyadh, Saudi Arabia

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Abstract The current study was carried out to evaluate the phenotypic and genotypic characterization of avian pathogenic *Escherichia coli* recovered from Riyadh, Saudi Arabia. During the period of 10th February–30th May 2015, 70 *E. coli* strains were isolated from chicken farms located in Riyadh, Saudi Arabia. All strains were tested phenotypically by standard microbiological techniques, serotyped and the virulence genes of such strains were detected by polymerase chain reaction (PCR). Most of the recovered strains from chickens belonged to serotype O111:K58 25 strains (35.7%), followed by serotype O157:H7 13 strains (18.57%), followed by serotype O114:K90 10 strains (14.29%), then serotype O126:K71 9 strains (12.9%), serotype O78:K80 8 strains (11.43%) and in lower percentage serotype O114:K90 and O119:K69 5 strains (7.14%). The virulence genotyping of *E. coli* isolates recovered from broilers revealed the presence of the *uidA* gene in all the field isolates (6 serovars) examined in an incidence of 100%, as well as the *cvaC* gene was also present in all field isolates (6 serovars), while the *iutA* gene and the *iss* gene were detected in 5

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out of 6 field serovars in an incidence of 81.43% and 64.29%, respectively. Phenotypical examination of the other virulence factors revealed that 65 isolates were hemolytic (92.9%), as well as 15 isolates (21.42%) were positive for enterotoxin production. Meanwhile, 21 isolates (30%) were positive for verotoxin production, 58 isolates (82.86%) for the invasiveness and 31 isolates (44.29%) for Congo red binding activities of the examined serotypes.

1. Introduction

Strains isolated from localized and systemic disease processes in poultry are designed as avian pathogenic *Escherichia coli* (APEC), causing a number of diseases in domestic poultry, ultimately leading to disease and death, or a decrease in egg production or condemnation of carcasses. Among these diseases is a severe systemic form termed coliseptecemia, which is characterized by the presence of *E. coli* in the blood and colonization of the organism in organs including the heart, liver and spleen (Barnes et al., 2003).

The disease inducing potential of these isolates has been explained by the occurrence of specific virulence factors. Many virulence factors have been associated with avian pathogenic *E. coli* (APEC) strains, although their role in the pathogenesis is not well known (Mellata et al., 2003). Despite extensive literature on virulence factor *E. coli* bacteria, unambiguous markers of virulence have not been identified yet. The relationship between serotyping and virulence is not straightforward either and raises the question whether *E. coli* infections of poultry should mainly be considered as opportunistic (Landman and Cornelissen, 2006). The main goal of this study was to investigate the characteristics of APEC in the boiler chickens collected from different farms located in Riyadh, Saudi Arabia.

2. Materials and methods

2.1. Samples

During the period of 10th February–30th May 2015, samples from the blood and different organs (liver, yolk sac, spleen, ovary and joint) were aseptically collected from 110 broiler chickens and showed signs of colisepticemia in poultry farms, located in Riyadh, Saudi Arabia. Samples were transferred to the laboratory in an ice box to be cultured immediately.

2.2. Isolation and identification of E. coli

Samples were primarily inoculated in pre-enrichment media then streaked on MacConkey agar medium and incubated aerobically at 37 °C. After an overnight incubation, a part of single typical well isolated lactose fermenting colony was tested for sorbitol fermentation by culturing on sorbitol MacConkey agar and sorbitol phenol red agar media, then incubated at 37 °C overnight. Morphological, cultural and biochemical examinations were carried out according to Murray et al. (2003). Isolates that were primarily identified by biochemical tests were then subjected to serological identification using diagnostic polyvalent and monovalent *E. coli* antisera (Welcome diagnostic antisera). Diagnostic *E. coli* O157 antisera (Difco) and H7 anti-sera (Difco) were used for serological identification of *E. coli* O157:H7.

2.3. Detection of virulence factors by polymerase chain reaction (PCR)

Extraction of DNA of each culture by the boiling method was performed according to Croci et al. (2004) PCR design and amplification conditions were performed using PCR primer pairs with reference to published sequence data for the *uidA* gene of *E. coli* (Croci et al., 2004) encoding b-glucuronidase specific for *E. coli* (Heininger et al., 1999), and increase in serum survival of iss gene of *E. coli* (Yaguchi et al., 2007), aerobactin *iutA* of *E. coli* (Delicato et al., 2003), and the *cvaC* gene (Rocha et al., 2008). Details of the nucleotide sequence, the specific gene region amplified, and the size of the PCR product for each primer pair are listed in Table 1. PCR products were visualized by agarose gel electrophoresis according to Sambrook et al. (1989).

2.4. Phenotypic detection of virulence factors of E. coli isolates

Hemolytic activity (hemolysin) was tested using 5% defibrinated sheep blood agar. The ability to produce heat stable

Table 1 Primer sequences used for PCR amplification.

Target gene	Sequence (5–3)	Amplicon size bp
uidA gene of *E. coli*	F(ATC ACC GTG GTGACG CATGTCGC) R(CAC CAC GAT GCC ATG TTC ATC TGC)	468
Iron uptake transport gene *iutA*	F(GGC TGG ACA TGG GAA CTG G) R(CGT CGG GAA CGG GTA GAA TCG)	300
Colicin v *cvaC*	F(CAC ACA CAA ACG GGA GCT GTT) R(CTT CCC GCA GCA TAG TTC CAT)	680
Increased serum survival gene *ISS*	F(ATG TTA TTT TCT GCC GCT CTG) R(CTA TTG TGA GCA ATA TAC CC)	266

enterotoxin was assayed by the infant mouse test as described by Rohins-Brown et al. (1993). The ability of *E. coli* isolates to invade epithelial cells were tested in rabbit eye model "Sereny test". Detection of cytotoxin activity of *E. coli* strains using Vero cells was investigated according to Giugliano et al. (1982). Congo red binding was adopted as a virulence test among *E. coli* isolates according to Berkhoff and Vinal (1986).

3. Results

Seventy *E. coli* strains (63.6%) were isolated from 110 collected samples. Results of serotyping in the present study of the *E. coli* isolates showed that, 25 strains (35.7%) belonged to O111:K58, 13 strains (18.57%) O157:H7, 10 strains (14.29%) O114:K90, 9 strains (12.9%) O126:K71, 8 strains (11.43%) O78:K80 and 5 strains (7.14%) O119:K69.

The virulence genotyping of *E. coli* isolates recovered from broilers chickens revealed the presence of *uidA* gene (468 bp fragment) in all the field isolates (6 serovars) examined in an incidence of 100%, as well as the *cvaC* gene was also present in all the field isolates (6 serovars) in an incidence of 97.14% as shown in Table 2. The *iutA* gene was detected in 5 serovars out of 6 field serovars (81.43%)and the absence of O126:K71, while *iss* gene was detected in 5 serovars out of 6 field serovars (64.29%) and the absence of O111:K58 is shown in Figs. 1 and 2.

In addition to virulence genotyping, phenotypical examination for other virulence factors revealed that 65 isolates were hemolytic with a percentage of 92.9% using 5% defibrinated sheep blood agar and belonging to serotypes O111:K58 (25 strains), O157:H7 (13 strains), O126:K71 (9 strains), O78:K80 (8 strains), 5 strains of O114:K90, and 5 strains of O119:K69 serotypes. Also 15 isolates (21.42%) belonging to serotypes O126:K71 (9), O114:K90 (4) and O119:K90 (2) were positive for enterotoxin production, while 21 isolates (30%) were positive for verotoxin production belonging to 0111:K58 (10 strains), O157:H7 (5 strains), O126:K71 (5 strains) and O78:K80 (1 strains). Whereas, Table 2 and Figs. 1 and 2 represent the invasiveness of 58 isolates (82.86%) belonging to serotypes belonging to O111:K58 (15 strains), O157:H7 (13 strains), O114:K90 (8 strains), O126:K71 (9 strains), O78:K80 (8 strains) and O119:K69 (5 strains). The same table represents 31 isolates (44.29%) positive for the Congo red binding activities of the examined serotypes.

4. Discussion

Avian pathogenic *E. coli* (APEC) strains cause a great diversity of diseases in birds and are responsible for great economic losses in the avian industry van der Westhuizen and Bragg, 2012). To date, several studies have been carried out to better understand the APEC pathogenesis for the possible development of tools which could prevent the economics losses caused by these strains (Barnes et al., 2008; Nakazato et al., 2009). Results of the present study showed that 63.6% *E. coli* isolates were recovered from 110 collected broiler samples belonging to O111:K58 (25 strains), 13 strains of O157:H7, 10 strains of O114:K90, 9 strains of O126:K71, 8 strains of O78:K80 and

Table 2 The relation between the virulence genes detected by PCR and other virulence factors detected phenotypically.

Serovars	No. of serovars	Virulence genes detected by PCR			Virulence factors detected phenotypically				
		Colicin v *cvaC*	*iss*	*iutA*	Hemolytic activity	Enterotoxin production	Verotoxin Production	Invasiveness	Congo red binding
O111:K58	25	24	25	0	25	0	10	15	9
O157:H7	13	13	13	13	13	0	5	13	8
O114:K90	10	9	10	10	5	4	0	8	6
O126:K71	9	9	0	9	9	9	5	9	6
O78:K80	8	8	4	8	8	0	1	8	0
O119:K69	5	5	5	5	5	2	0	5	2
Total positive No.	70	68 (97.14%)	57 (81.43%)	45 (64.29%)	65 (92.9%)	15 (21.42%)	21 (30%)	58 (82.86%)	31 (44.29%)

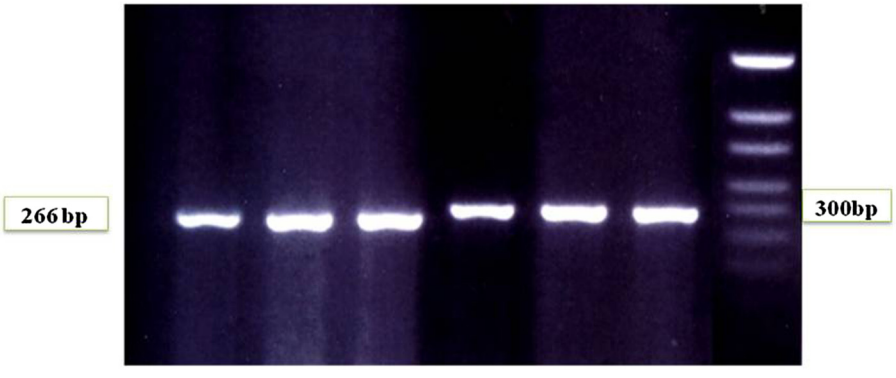

Figure 1 Agarose gel electrophoresis showing amplification of 300 base pair fragments of Iron uptake transport *iutA* gene and amplification of 266 base pair fragments of increased serum survival gene *ISS*.

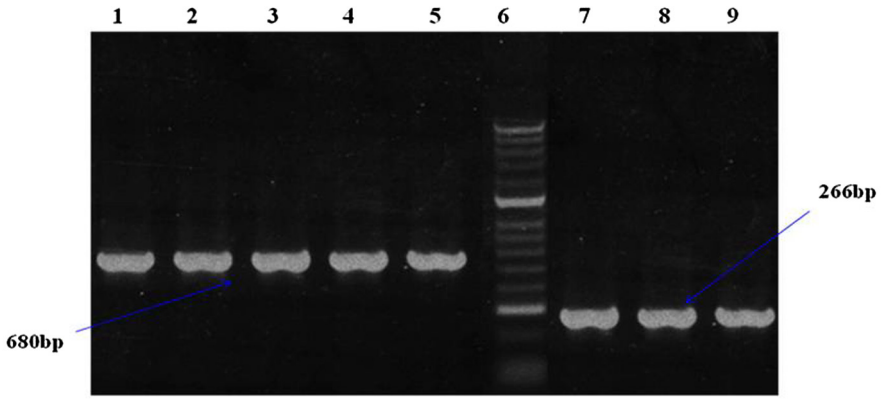

Figure 2 Agarose gel electrophoresis showing amplification of 680 base pair fragments of Colicin v *cvaC* gene and amplification of 266 base pair fragments of increased serum survival gene *ISS*.

5 strains of O119:K69 were recorded in a percentage of 35.7%, 18.57%, 14.29%, 12.9%, 11.43% and 7.14%, respectively. From the obtained results, it is recognized that O111:K58, O157:H7 and O114:K90 were the most prevalent.

In a study of Knöbl et al. (2004), 11 serogroups were identified: O2, O6, O8, O21, O25, and 46, O78, O88, O106, O111, and O143. Serogroup O6 was the most frequent, representing 62% of the total number of strains. Serogroups O2, O21, and O78, commonly found in poultry affected by colibacillosis, as well as Da Silveira et al. (2002) identified 2.3% of APEC serogroup O6 among isolates from chicks with omphalitis.

In the present study, virulence genotyping of *E. coli* isolates recovered from broiler chickens revealed the presence of *uidA* gene (468 bp fragment) in all field isolates (6 serovars) examined in an incidence of 100%.

The result concerning the *cvaC* gene indicated its presence in all field isolates (6 serovars) in an incidence of 97.14% as shown in Table 2. Only Mcpeake et al. (2005) found similar results (99.1%), while other authors obtained a lower percentage. Blanco et al. (1997) reported 22% and Rodriguez-Siek et al. (2005) reported 66.8%.

It is clear from the obtained result that *cvaC* gene showed the highest percentage (97.14%), followed by the *iutA* gene and the *iss* gene (81.43% and 64.29%, respectively). In a study of Rocha et al. (2008) for the presence of some virulence genes such as the *iss* gene showed the highest percentage (73.8%), followed by the *iutA* gene and the *cvaC* gene (45.9% and 23%, respectively).

Phenotypical examination for the other virulence factors revealed that 65 isolates were hemolytic with a percentage of 92.9%. Enterotoxigenic *E. coli* (ETEC) were detected in 15 (21.42%) *E. coli* were found positive for their enterotoxigenicity. It can be speculated that the toxic effects could be due to the heat-labile (LT) toxin (Yamamoto and Yokota, 1983).

The obtained result showed that 21 isolates (30%) were positive for verotoxin production. Tracing literature in this concern Blanco et al. (1997) reported that 20 strains produced a cytotoxic response in HeLa but not in Vero cells. However, cytotoxic activities on Vero cells were observed by Emery et al. (1992) and Fantanatti et al. (1994),

while 58 isolates (82.86%) were positive for the invasiveness and 31 isolates (44.29%) for Congo red binding activities of the examined serotypes. The Congo red linkage capacity in agar medium has been observed among APEC strains. Some authors have purposed the utilization of this characteristic as a virulence marker to APEC strains (Berkhoff and Vinal, 1986).

5. Conclusion

Very little information about the production of virulence factors by avian *E. coli* strains exists in Saudi Arabia, so the existence of APEC strains in KSA poultry farms must be investigated by epidemiological surveys, with particular attention to virulence factors of serotype isolates.

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References

Barnes, J.H., Vaillancourt, J.P., Gross, W.B., 2003. Colibacillosis: Diseases of Poultry, 11th ed. Iowa State University Press, Ames, pp. 631–652.

Barnes, J.H., Nolan, L.K., Vaillancourt, J.P., Saif, Y.M., Saif, A.M., Fadly, J.R., Glisson, L.R., McDougald, L.K., Nolan, Swayne, D. E., 2008. Colibacillosis. Diseases of Poultry, 12th ed. Blackwell Publishing, Ames, pp. 691–737.

Berkhoff, H.A., Vinal, A.C., 1986. Congo red medium to distinguish between invasive and non invasive *Escherichia coli* pathogenic for Poultry. Avian Dis. 30 (1), 117–121.

Blanco, J.E., Blanco, M., Mora, A., lanco, J., 1997. Production of toxins (Enterotoxins, verotoxins, and necrotoxins) and colicins by *Escherichia coli* strains isolated from septicemic and healthy chickens: relationship with in vivo pathogenicity. J. Clin. Microbiol. 35 (11), 2953–2957.

Croci, L., Delibato, E., Volpe, G., Medici, D.D., Palleschi, G., 2004. Comparison of PCR, electrochemical enzyme-linked immunosorbent assays, and the standard culture method for detecting *Salmonella* in meat products. Appl. Environ. Microbiol. 70, 1393–1396.

Da Silveira, W.D., Ferreira, A., Brocchi, M., et al, 2002. Biological characteristics and pathogenicity of avian *Escherichia coli* strains. Vet. Microbiol. 85 (1), 47–53.

Delicato, E.R., de Brito, B.G., Gaziri, L.C., Vidotto, M.C., 2003. Virulence-associated genes in *Escherichia coli* isolates from poultry with colibacillosis. Vet. Microbiol. 94, 97–103.

Emery, D.A., Nagaraja, K.V., Shaw, D.P., Newman, J.A., White, D. G., 1992. Virulence factors of *Escherichia coli* associated with colisepticemia in chickens and turkeys. Avian Dis. 36, 504–511.

Fantanatti, F., Silveira, W.D., Castro, A.F.P., 1994. Characteristics associated with pathogenicity of avian septicaemic *Escherichia coli* strains. Vet. Microbiol. 41, 75–86.

Giugliano, L.G., Mann, G.F., Drasar, B.S., 1982. Response of mammalian cell lines to the toxins of *Escherichia coli*. J. Med. Microbiol. 15, 531–539.

Heininger, A., Binder, M., Schmidt, S., Unertl, K., Botzenhart, K., Döring, G., 1999. PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. J. Clin. Microbiol. 37, 2479–2482.

Knöbl, T., Gomes, T.A.T., Vieira, M.A.M., Bottino, J.A., Ferreira, A. J.P., 2004. Detection of pap, sfa and fim adhesin-encoding operons in avian pathogenic *Escherichia coli*. Inter. J. Appl. Res. Vet. Med. 2, 135–141.

Landman, W.J., Cornelissen, R.A., 2006. *Escherichia coli* salpingitis and peritonitis in layer chickens. Tijdschr. Diergeneesked. 15, 814–822.

Mcpeake, S.L., Smyth, J.A., Ball, H.J., 2005. Characterization of avian pathogenic *Escherichia coli* (APEC) associated with colisepticaemia compared to fecal isolates from healthy birds. Vet. Microbiol. 110, 245–253.

Mellata, M., Dho-Moulin, M., Dozois, C.M., Curtiss III, R., Brown, P.K., Arné, P., Breé, A., Desautels, C., Fairbrother, J.M., 2003. Role of virulence factor in resistance of avian pathogenic *Escherichia coli* to serum and in pathogenicity. Infect. Immun. 71, 536–540.

Murray, P.R., Baron, E.J., Jorgensen, J.H., Pfaller, M.A., Yolken, R. H., 2003. Manual of Clinical Microbiology, 8th ed. American Society for Microbiology, Washington, DC, pp. 110–122.

Nakazato, G., De Campos, T.A., Stehling, E.G., Brocchi, M., 2009. Virulence factors of avian pathogenic *Escherichia coli* (APEC). Pesqui. Vet. Bras. 29 (7), 479–486.

Rocha, A.C.G.P., Rocha, S.L.S., Lima-Rosa, C.A.V., Souza, G.F., Moraes, H.L.S., Moraes, L.B., Salle, C.T.P., 2008. Genes associated with pathogenicity of avian *Escherichia coli* (APEC) isolated from respiratory cases of poultry. Pesqui. Vet. Bras. 28, 183–186.

Rodriguez-Siek, K.E., Giddings, C.W., Doetkott, C., Johnson, T.J., Nolan, L.K., 2005. Characterizing the APEC pathotype. Vet. Res. 36, 241–256.

Rohins-Brown, R.M., Takeda, R.M., Fasano, T., 1993. Assessment of enterotoxin production by *Yersinia enterocolitica* and identification of a novel heat stable enterotoxin produced by a non invasive *Yersinia enterocolitica* strain isolated from clinical material. Infect. Immun. 16 (2), 764–767.

Sambrook, J., Fritscgh, E.F., Meniates, T., 1989. In: Molecular Cloning. A Laboratory Manual, Vol. 1. Cold Spring Harbor Laboratory Press, New York, pp. 54–60.

Van der Westhuizen, W.A., Bragg, R.R., 2012. Multiplex polymerase chain reaction for screening avian pathogenic *Escherichia coli* for virulence genes. Avian Pathol. 41, 33–40. http://dx.doi.org/10.1080/03079457.2011.631982.

Yaguchi, K., Ogitani, T., Osawa, R., Kawano, M., Kokumai, N., Kaneshige, T., Noro, T., Masubuchi, K., Shimizu, Y., 2007. Virulence factors of avian pathogenic *Escherichia coli* strains isolated from chickens with colisepticemia in Japan. Avian Dis. 51, 656–662.

Yamamoto, T., Yokota, T., 1983. Plastids enterotoxigenic *Escherichia coli* HI0407: evidence of two heat stable enterotoxin genes and a conjugal transfer system. J. Bacteriol. 153, 1352–1360.