Archives of Razi Institute, Vol. 72, No. 1 (2017) 1-8

Original Article

Detection of three virulence genes and antibiotic resistance profiles in *Escherichia coli* isolates from commercial broilers with colibacillosis in Tabriz, Iran

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Received 01 October 2016; accepted 31 October 2016
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ABSTRACT

Colibacillosis caused by *Escherichia coli* is one of the most common diseases in commercial broiler chickens, which is associated with a significant financial loss in the poultry industry due to morbidity, mortality and high costs of antibiotic overuse. Diseases caused by *E. coli* mostly present as secondary or opportunistic pathogens, while certain strains of these bacteria with specific virulence factors could act as primary pathogens. This study aimed to detect three virulence genes and determine antibiotic resistance profiles in the *E. coli* isolates of commercial broilers with colibacillosis in Tabriz, Iran using multiplex polymerase chain reaction (PCR) and disc-diffusion method, respectively. According to the results, out of 71 bacterial isolates, 38 (53.5%), 25 (35.2%) and 35 cases (49.3%) carried the iron-repressible protein (*irp*2), pyelonephritis-associated pili (*pap*C) and temperature-sensitive hemagglutinin (*tsh*) genes, respectively. Moreover, 16 isolates contained the *irp*2 gene only, eight isolates had the *pap*C gene only, 13 isolates carried the *tsh* gene only, and 12 isolates lacked all the three genes. In 39 isolates, 2-3 genes were identified simultaneously. In this study, most of the isolates were resistant to enrofloxacin, doxycycline, sultrim, erythromycin and danofloxacin, while they were sensitive to colistin only. Frequency of the isolates susceptible to fosfomycin, neomycin and florfenicol was 45.9%, 45.2% and 48.3%, respectively. Therefore, it could be concluded that high rate of antibiotic resistance and prevalence of some important virulence genes in avian pathogenic *E. coli* isolates might be a serious hazard for the poultry industry and public health.

Keywords: *Escherichia coli* virulence genes, Broiler chickens, Colibacillosis, Antibiotic resistance, Iran

Détection de 3 gènes virulents et des profils de résistances aux antibiotiques chez les isolats d'*Escherichia coli* issus de poulets de chair affectés par la colibacillose à Tabriz, Iran

Résumé: La colibacillose causée par l'*Escherichia coli* est l'une des maladies les plus répandues chez les poulets de chair commerciaux et engendre des pertes financières considérables dans l'industrie avicole. Ces pertes sont principalement générées par les taux élevés de morbidité, de mortalité et de consommation d'antibiotiques liés à cette infection. Les maladies causées par l'*E. coli*sont souvent induites par desagents pathogènes secondaires ou opportunistes, alors que certaines souches virulentes de cette bactérie peuvent agir comme pathogènes primaires. L'objectif de cette étude était de déterminer la présence des trois gènes de virulence et les profils de résistance aux antibiotiques des isolats d'*E. coli*prélevés à partir de poulets de chair affectés par la colibacillose à Tabriz (Iran). A cet effet, une méthode de réaction en chaîne de la polymérase (PCR) multiplex et des antibiogrammes standards par diffusion ont été respectivement utilisés. Selon nos résultats, parmi les 71 isolats bactériens analysés, 38 (53,5%), 25 (35,2%) et 35 (49,3%) cas comprenaient

respectivement les gènes irp2 (iron-repressibleprotein), papC (pyelonephritis-associatedpili) et tsh (temperature-sensitive hemagglutinin). De plus, 16 isolats contenaient uniquement le gène irp2, 8 isolats présentaient seulement le gène papC, 13 isolats incluaient exclusivement le gène tsh, alors que 12 isolats ne portaient aucun des trois gènes. Enfin, dans 39 isolats, 2 à 3 gènes étaient simultanément présents. La plupart des isolats étaient résistants aux antibiotiques enrofloxacine, doxycycline, sultrim, erythromycine et danofloxacine et montraient une sensibilité à la colistine. Les fréquences respectives de susceptibilité des isolats à la fosfomycine, la néomycine et au florfénicol étaient de 45,9%, 45,2% et 48,3%. Par conséquent, le taux important de résistance aux antibiotiques observés dans cette étude et la prévalence de certains gènes de virulence chez les isolats de l'*E. coli*pathogénique aviaire, peuvent constituer un risque sérieux pour l'industrie avicole et la santé publique.

Mots clés: *Escherichia coli*, Gènes de virulence, Poulet de chair, Colibacillose, Résistance antibiotique, Iran

INTRODUCTION

Colibacillosis in poultry refers to the localized or systemic infections entirely or partly caused by avian pathogenic Escherichia coli (APEC). Some of these infections include air sacculitis, pericarditis, peritonitis, salpingitis, synovitis, osteomyelitis, swollen-head syndrome, and yolk sac infection (Barnes, 2003; Nolan, 2013). Mortality rate of colibacillosis ranges between 5-50% in chickens, turkeys and ducks. A common route of colibacillosis transmission is the inhalation of the dust contaminated with feces, which spreads to other target organs through blood circulation after respiratory infection (Barnes, 2003). Host tissue damages are associated with various disorders, such as acute coli septicemia with high mortality, fibrinous, purulent serositis and coligranuloma. Furthermore, colibacillosis might be exacerbated by primary factors (e.g., viral and mycoplasma infections), cold weather or dust. Some studies have denoted the ability of APEC to act as primary pathogens (Nolan, 2013). Colibacillosis is a common disease in commercial chickens, which is associated with a significant financial loss in the poultry industry due to its adverse effects on the performance of birds, increased mortality, slaughter condemnation and high costs of antibiotic overuse (Barnes, 2003; Nolan, 2013). Virulence of invasive bacterial strains depends on their ability in mucosal adherence, toxin production, iron acquisition and other virulence factors. E. coli bacteria are among the normal microflora of the intestinal tract and environment of commercial chickens. E. coli mostly induce diseases as secondary or opportunistic pathogens. Meanwhile, certain pathogenic strains with specific virulence factors have been shown to act as primary agents to cause colibacillosis in poultry and other diseases in human (Janben, 2001; Nolan, 2013). Ferric yersiniabactin uptake and iron-repressible protein (irp2) genes are responsible for coding the proteins involved in iron acquisition, which were first identified in Yersinia. These genes were described in human septicemic and enteroaggregative *E. coli* isolates (Karch, 1999) and subsequently found in avian *E. coli* isolates by (Gophna, 2001) and (Ewers, 2005), who demonstrated that pathogenic APEC is correlated with the aerobactin iron uptake system. Adherence of bacteria to tissue surfaces is an important initial event in bacterial infections. In *E. coli*, P-fimbriae, which mediates bacterial colonization in the respiratory epithelium, is coded by the pyelonephritis-associated pili (pap) (papC) gene. In addition to tissue adhesion, P-fimbriae protects *E. coli* from the antibacterial activity of neutrophils (Pourbakhsh, 1997). In bacteria, a periplasmic serine protease is encoded by the temperature-sensitive hemagglutinin (tsh) gene, which consists of two subunits (Nakazato, 2009). The large subunit of this protein is essential to the attachment of bacteria to host cells in the membrane binding process. This subunit can adhere to fibronectin, hemoglobin, red blood cells, extracellular matrix proteins and collagen IV (Arabi, 2013; Kwon, 2008). Furthermore, this molecule plays a key role in the formation of lesions and fibrin precipitation in air sacs, which increases

colonization at this site, thereby leading to lesions and ulcers (Arabi, 2013; Dozois, 2000). Since the mid 1950s, antimicrobial agents have been routinely used to reduce the losses associated with colibacillosis. However, parallel with the extensive use of antibiotics, antibiotic resistance has developed in APEC isolates in different regions of the world (Barnes, 2003; Nolan, 2013). Transmissible drug-resistance factors could be transmitted to human pathogens from the bacteria in animal foods. On the other hand, effectiveness of new antibiotics used in the poultry industry against poultry diseases has diminished due to the acquired drug resistance. This study aimed to detect three virulence genes (papC, irp2 and tsh) in the E. coli isolates from commercial broilers with colibacillosis in Tabriz, Iran using multiplex polymerase chain reaction (PCR). In addition, in-vitro drug sensitivity of the isolates was determined by the disk-diffusion method.

MATERIALS AND METHODS

Samples. During 2012-2013, live and dead birds of commercial broiler chicken flocks with respiratory diseases and increased mortality were submitted to the veterinary laboratories of Tabriz (Iran) and examined by routine diagnostic procedures. *E. coli* isolation was specifically targeted in these specimens. To do so, chickens' heart and liver that were diagnosed with pericarditis and perihepatitis, respectively were subjected to swabbing.

Bacteriology. Bacterial isolation was carried out in accordance with standard bacteriological methods (Swayne, 1998). Initially, swab samples were cultured on MacConkey agar. Biochemical properties, such as IMViC tests, were used to identify the bacterial isolates.

DNA extraction. For the molecular detection of three virulence genes, DNA of E. coli isolates was extracted using the phenol-chloroform extraction method. Then, 100 µl of the harvested bacterial suspension was added in equal volumes to the lysis buffer and incubated at the temperature of 56 °C for 4 h. Afterwards, 200 µl of saturated phenol was added and centrifuged at 13,000 rpm (15,700 g) for 20 min. Upper phase was transferred to the next tube, and the mixed phenol-chloroform was added at an equal volume (1:1). After centrifugation at 13,000 rpm for 20 min, the aqueous phase was transferred and added to an equal volume of pure chloroform and centrifuged at 13,000 rpm for 5 min. Upper phase was mixed with 1/10 volume of acetate sodium and precipitated with a two-fold volume of cool, absolute ethanol. After final precipitation by 70% ethanol, DNA was dried and resuspended in 50 µl of TE buffer at the temperature of 4 °C and used for PCR.

Primers. Virulence genes (tsh, papC and irp2) were simultaneously detected by the specific primers published for each gene using multiplex PCR (Ewers et al., 2005) (Table 1).

Table 1. Primer pairs used in multiplex PCR to co-amplify three APEC virulence genes and their respective product sizes (Ewers et al., 2005)

Virulence gene	Primer Sequences (5'-3')	Size (bp)
Irp2	**AAGGATTCGCTGTTACCGAC** **AACTCCTGATACAGGTGGC**	413
***Pap*C**	**TGATATCACGCAGTCAGTAGC** **CCGGCCATATTCACTAA**	501
Tsh	**ACTATTCTCTGCAGGAAGTC** **CTTCCGATGTTCTGAACGT**	824

Multiplex PCR parameters and optimization. DNA amplification was performed in a total volume of 25 µl, containing 2 µl of DNA, 1 µl of each primer (100 picomol), 1 µl of dNTP mix (10 mM) (CinnaGen Inc.), 2 µl of MgCl2 (50 mM) (CinnaGen Inc.), 2.5 µl of PCR buffer (10X) (CinnaGen Inc.), 0.8 µl of Taq DNA polymerase (2 units) (CinnaGen Inc.), and 14.7 µl of sterile distilled water. Thermocycling of the reaction mixtures was performed 25 times, which started with an initial denaturation at the temperature of 94 °C for 3 min. Temperature and time profile of each cycle was as follows: 94 °C for 30 sec (denaturation), 58 °C for 30

sec (annealing), and 68 °C for 3 min. Moreover, PCRs were finished with a final extension step at the temperature of 72 °C for 10 min.

Amplicon electrophoresis. At this stage, 10 µl of the aliquot of each PCR product was mixed with 2 µl of loading buffer (6X) and separated via electrophoresis (90 volts for 1.5 h) in 1% agarose gel stained with SYBR Safe (Invitrogen). Afterwards, PCR products were visualized following UV transillumination.

Antimicrobial drug sensitivity test. In total, 60 *E. coli* isolates were examined in terms of drug sensitivity, as described by (Bauer, 1966) and guidelines of the Clinical and Laboratory Standards Institute (2014). Turbidity of the samples was adjusted to a 0.5 McFarland standard by dilution. Immediately after dilution, a sterile swab was dipped into the inoculum and streaked over the entire surface of the Mueller-Hinton agar three times. In this process, we applied the following antibiotic discs (n=9) (Padtan Teb, Tehran, Iran): florfenicol (30 µg), enrofloxacin (5 µg), doxycycline (30 µg), colistin (10 U), sulfamethoxazole/trimethoprim (1.25/23.75 µg), erythromycin E15 (15 µg), fosfomycin (200 µg), danofloxacin (5 µg), and neomycin (30 µg).

RESULTS

In total, 71 *E. coli* bacteria were isolated from the broiler chickens suspected of colibacillosis. Among the samples, 38 (53.5%), 25 (35.2%) and 35 isolates (49.3%) contained the *irp*2, *pap*C and *tsh* genes, respectively. In addition, 16 isolates had the *irp*2 gene only, eight isolates carried the *pap*C gene only, and 13 isolates contained the *tsh* gene only. In 39 bacterial isolates, 2-3 genes were identified simultaneously (Figure 1) (Table 2). According to our findings, the majority of *E. coli* isolates were resistant to enrofloxacin, doxycycline, sultrim, erythromycin, and danofloxacin, while they were only sensitive to colistin. Susceptibility of the bacterial isolates against fosfomycin, neomycin and florfenicol was estimated at 45.9%, 45.2% and 48.3%, respectively (Table 3).

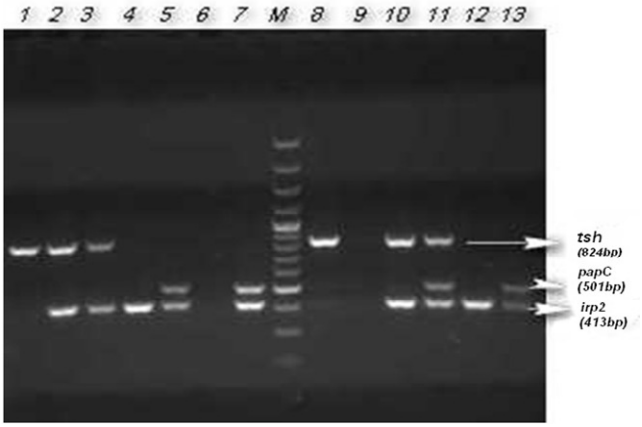

Figure 1. Agarose gel electrophoresis of multiplex PCR products (lanes M: 100 bp DNA ladder, lanes 6: negative control, other lanes: APEC isolates; *tsh* gene with 824 bp size is observed in lanes 1, 2, 3, 8, 10 and 11; *pap*C gene with 501 bp size is observed in lanes 5, 7, 11 and 13; *irp*2 gene with 413 bp size is observed in lanes 2, 3, 4, 5, 7, 10, 11, 12 and 13.).

Table 2. Number and percentage of virulence genes in *E. coli* isolates (n=71)

Virulence genes	N (%)
*Irp*2 (only)	16 (22.5)
*Pap*C (only)	8 (11.3)
Tsh (only)	13 (18.3)
*Irp*2 and *tsh*	22 (31)
*Irp*2 and *pap*C	5 (7)
*Pap*C and *tsh*	3 (4.2)
*Irp*2, *pap*C and *tsh*	9 (12.7)
Total *irp*2	38 (53.5)
Total *pap*C	25 (35.2)
Total *tsh*	35 (49.3)
Without genes	12 (16.9)

Table 3. Results of antibiotic resistance test in *E. coli* isolates

Antibiotics	Sensitivity (%)	Intermediate (%)	Resistance (%)
Florfenicol	48.3	10	41.7
Erythromycin	1.7	20.3	78
Fosfomycin	45.9	4.9	49.2
Enrofloxacin	8.3	36.7	55
Doxycycline	24.2	9.7	66.1
Sultrim	44.3	4.9	50.8
Colistin	75.4	6.6	18
Danofloxacin	24.6	14.7	60.7
Neomycin	45.2	38.7	16.1

DISCUSSION

Until recently, APEC isolates were believed to infect poultry only (e.g., chickens, turkeys and ducks). However, current findings are suggestive of the possibility of APEC implications in extraintestinal human infections as well. It seems that *E. coli* isolates from birds with more virulence-associated genes have higher pathogenicity for both poultry and human. Further investigation is required in order to assess the relationship between virulence factors and pathogenicity of avian isolates (Nolan, 2013). Previous studies have evaluated the frequency of different virulence genes in APEC strains, the results of which are in line with the current research. In our study, out of 71 APEC strains, 49.3% contained the *tsh* gene, while 53.5% and 35.2% carried the *rip*2 and *pap*C genes, respectively. In a study in this regard, (Moon, 2006) reported that out of 118 APEC strains, 55% had the *tsh* gene. Moreover, in the research conducted by (Kwon, 2008) in Korea, it was proposed that out of 120 APEC strains, 94% carried the *tsh* gene, 67% contained the *irp*2 gene, and 11% had the *pap*C gene. In the third study performed in Korea, out of 118 APEC strains, 55% were reported to carry the *tsh* gene, and 38% contained the *irp*2 gene (Won, 2009). Furthermore, results of a study in Brazil indicated that out of 61 APEC strains, 55% and 24% carried the *tsh* and *pap*C genes, respectively. In another research performed in Iran, (Arabi, 2013) reported that among 28 APEC strains obtained from broiler chickens, 96.4% contained the *tsh* and increased serum survival (*iss*) genes, 82.1% had the *pap*C gene, and 53.5% carried the *irp*2 gene. Moreover, out of 77 non-APEC strains, 64.9% carried the *tsh* and *iss* genes, 66.2% contained the *pap*C gene, and 80.5% had the *irp*2 gene. Therefore, it was concluded that the presence of *tsh* and *iss* genes could be a key component to distinguish between APEC and non-APEC strains. In this regard, (Nateghi, 2010) evaluated the virulence genes of APEC and uropathogenic *E. coli* (UPEC) strains in human extraintestinal *E. coli* strains, aiming to support the hypothesis that these genes originate from APEC strains in human UPEC. Findings of the mentioned study suggested that the *irp*2 gene with the frequency of 33% in UPEC and 54% in APEC strains is possibly the most important pathogenic factor in both *E. coli* strains. Since an autotransporter hemagglutinin is encoded by the *tsh* gene, it seems to be involved in the mechanisms of adhesion to the respiratory tract of birds (Dozois, 2000; Kwon, 2008)}. Findings of the study conducted by (Janben, 2001) in Germany indicated that 85.3% of the APEC strains carried the *tsh* gene, which is consistent with similar studies regarding the extensive distribution of this gene in APEC strains (Dozois, 2000). In addition, results of the mentioned research reflected the in-vivo distribution of the *tsh* gene since the bacterial strains were only isolated from the birds that had died due to colibacillosis. As such, it was concluded that the *tsh* gene is a specific genetic marker for APEC strains (Janben, 2001). Moreover, the researchers claimed that 68.0% and 30.0% of the isolates were positive for the *irp*2 and *pap*C genes, respectively (Janben, 2001). In general, septicemic *E. coli* isolates of human and animal origin appear to belong to only a few clonal lineages (Cherifi, 1994). In addition to virulence factors, high resistance rates of APEC strains against antibiotics is an important issue for the poultry industry and public health. According to the results obtained by (Madadi, 2014), most of the *E. coli* isolates obtained in Urmia city (Iran) were resistant to tetracycline, oxytetracycline, sultrim, flumequine and erythromycin, while susceptible to enrofloxacin, florfenicol and lincospectin. Our findings regarding the level of enrofloxacin resistance are inconsistent with the study by (Madadi, 2014). In the mentioned research, most of the bacterial isolates were sensitive to enrofloxacin, while the resistance of these isolates to this antibiotic was higher in the present study. Findings of (Zahraei Salehi, 2006) are partly in congruence with the current research. In the mentioned study, the highest level of antibiotic resistance was observed against erythromycin (97%), oxytetracycline (95%), chlortetracycline (95%), tetracycline (94%), doxycycline (88%), difloxacin (83%), neomycin (81%),

sulfamethoxazole/trimethoprim (80%), enrofloxacin (76%), norfloxacin (68%), ciprofloxacin (67%), and chloramphenicol (67%). On the other hand, low levels of antibiotic resistance were observed against florfenicol (27%), lincospectin (15%), cefixime (14%), ceftizoxime (7%) colistin (6%), and gentamicin (0%). Among the antibiotics used in the present study, florfenicol and fosfomycin are relatively new antimicrobial agents, which have been used extensively in the prophylactic treatment of colibacillosis due to early effectiveness against *E. coli* infections. However, resistance levels of bacterial strains against florfenicol and fosfomycin were relatively high in the current study (41.7% and 49.2%, respectively). In this regard, findings of (Rahimi, 2013) were also indicative of the high frequency of antibiotic resistance against enrofloxacin (79.2%), ciprofloxacin (67.5%) and norfloxacin (77.9%). According to the results of the present study, resistance rates of enrofloxacin and danofloxacin were 55% and 60.7%, respectively. These rates are comparable to those previously reported for the APEC strains obtained from commercial chickens in Tabriz (Iran), as well as other regions in Iran and other countries (Khoshkhoo, 2005 ; White, 2000; Yang, 2004); Zahraei Salehi, 2006). However, these findings are not in line with the studies conducted in Urmia city (Iran) {Madadi, 2014). In the study by (Madadi, 2014) total enrofloxacin resistance was reported to be 9-28% during 2006-2011. In addition to the compatible results of the aforementioned studies, our findings regarding high-level fluoroquinolone resistance (e.g., enrofloxacin) in APEC isolates could be confirmed for several reasons. First, this class of antibiotics has been overused in the Iranian poultry industry for decades. Second, as far as the clinical aspects are concerned, fluoroquinolones are currently less effective against the majority of colibacillosis cases in the Iranian poultry industry compared to the past. In the study performed in Tabriz (Iran), level of resistance against florfenicol was reported to be 27%, while it was 41.7% in the present study (Zahraei Salehi, 2006). This discrepancy could be because florfenicol has been widely used in the poultry industries of Tabriz since 2004 (Zahraei Salehi, 2006), and long-term consumption of this antibiotic has led to a higher resistance rate. In another research performed in five different provinces of Iran, 34.5% of the *E. coli* isolates, which were mostly obtained from the poultry industry, were reported to be resistant to florfenicol (Ghaniei, 2011). Findings of the present study regarding florfenicol resistance are in congruence with the report by (Rahimi, 2013). Correspondingly, florfenicol resistance was reported to be relatively high (62.3%), while the resistance rate of APEC isolates against fosfomycin was relatively low (30.5%) in Kermanshah province. To the best of our knowledge, no further data have been published on the resistance rate of fosfomycin in broiler chickens in Iran. Similar to the previous studies in this regard, a high percentage of the *E. coli* isolates in the present research were resistant to common and even new antibiotics used in the poultry industry of Iran. Increasing antibiotic resistance of *E. coli* and other pathogenic bacteria due to overuse or inappropriate consumption of antibiotics in animals and human may transmit inside and between various species directly or indirectly. These events are associated with significant economic and public health concerns, and adoption of proper strategies for antibiotic use is of paramount importance. Therefore, discreet changes in the methods of antimicrobial use are inevitable in the poultry industry (Nolan, 2013; Smith, 2007). To raise the efficacy of the strategies against colibacillosis in poultry, further information is required on the antibiotic resistance of APEC isolates from the Iranian poultry industry. Moreover, emphasis on the application of other effective methods in this regard is recommended; such examples are vaccination, herbal medication, use of probiotics and prebiotics, observance of proper hygiene, biosecurity measures for farms, and lowering the slaughter age of broilers.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

This Study was financially supported by Razi Vaccine and Serum Research Institute, under grant no. 2-18- 18-92110.

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