Frequency of selected virulence-associated genes in intestinal and extra-intestinal *Escherichia coli* isolates from chicken

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Abstract:

BACKGROUND: Although *Escherichia coli* (*E. coli*) is a part of intestinal normal microflora of warm-blooded animals, including poultry, outbreaks occur in poultry raised below standard sanitation and during the course of respiratory or immunosuppressive diseases. Avian pathogenic *E. coli* (APEC) harbors several genes associated with virulence and pathogenicity. APEC strains are responsible for some diseases in poultry including colibacillosis, swollen head syndrome, yolk sac infection, omphalitis and coli granuloma. **OBJECTIVES:** The aim of this study was examination of the presence and frequency of three important virulence genes in intestinal and extra-intestinal (liver) *E. coli* isolates from chicken of Khuzestan province in the southwest of Iran. **METHODS:** Totally 120 (60 intestinal and 60 liver) *E. coli* isolates were examined by polymerase chain reaction (PCR) for the presence of aerobactin (iutA), temperature sensitive hemagglutinin (tsh) and fimbriae type 1 (fimH) genes. **RESULTS:** The results showed that tsh, iutA and fimH are respectively present in 78.3%, 70% and 61.7% of liver isolates while in intestinal ones the frequency of these genes was 21.7%, 41.7% and 41.7% respectively. The most prevalent genotypes in extra intestinal and intestinal isolates were tsh+fimH+iutA+ and tsh-fimH-iutA-respectively. **CONCLUSIONS:** It seems that these sets of virulence genes are significantly more prevalent ($P<0.05$) in extra intestinal isolates and probably these genes play an important role in the pathogenesis of APEC isolates in the southwest of Iran. Although these virulence genes were not present in all APEC isolates their frequencies were high and using the products of these genes in vaccines may be effective in protecting against infections caused by this bacterium.

Introduction

Escherichia coli (*E. coli*) strains are a part of intestinal normal microflora of warm-blooded animals, including humans and poultry (Brzuszkiewicz et al., 2011; Salehi 2014). Although *E. coli* is a normal inhabitant of the intestine of poultry, outbreaks occur in poultry raised below standard sanitation and during the course of respiratory or immunosuppressive diseases (Kheirandish et al., 2012). The pathogenic *E. coli* strains, known as avian pathogenic *E. coli* (APEC), can cause localized or systemic infections in poultry, such as acute fatal septicemia or subacute pericarditis and airsacculitis (Cavicchio et al., 2015). APEC has a broad range of virulence factors similar to other extra-intestinal pathogenic *E. coli* (ExPEC) strains include adhesins (F1, P, stg fimbriae, curli and EA/I), iron acquisition system (aerobactin, iroprotein, yersinibactin), autotransporters (tsh, vat, AatA), the phosphate transport system, sugar metabolism and the Ibex protein (Wang et al., 2015; Schouler et al. 2007).

APEC strains infect poultry by initial respiratory tract colonization followed by systemic spread (Wang et al., 2014). An important aspect of pathogenesis in several diseases starts with bacterial adhesion to host cells that can result in internalization by bacterial-induced endocytosis (Ramirez et al., 2009). *E. coli* colonization in host tissue is mediated by fimbrial adhesions. Type 1 fimbriae, expressed by APEC, have the ability to bind to D-mannose and thus to many kinds of eukaryotic cells such as lung, intestinal, bladder and kidney epithelial tissues (La Ragion et al. 2002), and so is associated with *E. coli* colonization in extra intestinal tissues (Mcpeak et al. 2005). APEC strains can survive in environments with low iron availability such as inside the host by expression of iron acquisition system. This system includes production of sidrophores such as aerobactin which acts as iron chelates in the host (Nakazato et al. 2009). Temperature sensitive hemagglutinin (tsh) has a hemagglutinin activity in APEC at 26-30ºC and is repressed at 42ºC (La Ragion et al. 2002). This protein is encoded by the tsh gene that is located in high molecular weight plasmids (Dizois et al. 2003). The tsh is a serine protease auto transporter protein and to date, its role in avian coli septicemia is still to be elucidated (Nakazato et al. 2009).

The purposes of the study were to determine the presence and frequency of three important virulence genes (tsh, iutA and fimH) in fecal and extra-intestinal infection *E. coli* isolates from chicken of Khuzestan province in the southwest of Iran.

Materials and Methods

Bacterial isolates: Totally 120 *E. coli* were isolated from either the liver of chickens with clinical signs of colibacillosis (60 isolates), or from feces of apparently healthy chickens (60 isolates) from different poultry farms of Khuzestan province in the southwest of Iran. The isolates were cultured on sheep blood agar and their pure cultures were identified morphologically and biochemically (Markey et al, 2013). All isolates were stored at −60ºC in skimmed milk broth to which 15% glycerol was added after growth.

The reference *E. coli* strains J96 and Mg1655 were used as positive and negative controls for all probes, respectively.

Polymerase chain reaction (PCR): *E. coli* isolates were examined by PCR for the presence of aerobactin (iutA), temperature sensitive hemagglutinin (tsh) and fimbriae type 1 (fimH) genes. The specific sequence of these genes was downloaded from GenBank and analyzed for specific primers of the genes using primer 3 software. Description, primer sequences and sizes of amplified fragments for the characteristics studied have been summarized in Table 1.

Template DNA of all isolates was prepared

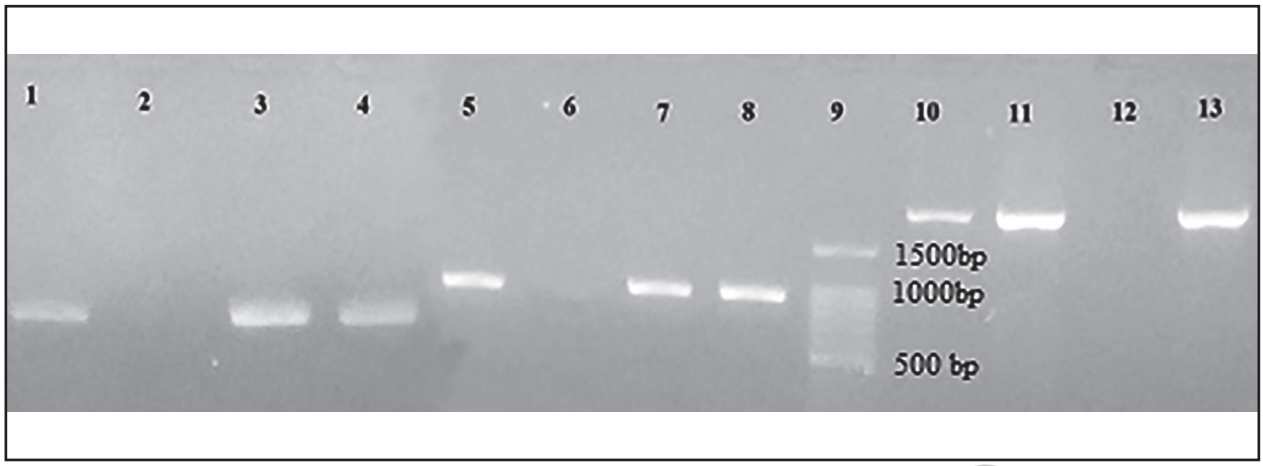

Figure 1. Agarose gel electrophoresis of the PCR products obtained from tsh, fimH and iutA genes of *E. coli* (Lane 1-4: 750bp PCR product of tsh gene; Lane 5-8: 902bp PCR product of fimh gene; Lane9: 100 bp ladder and Lane 10-13: 2272bp PCR product of iutA gene. Lane 2, 6 and 12 are negative controls).

Table.1. Sequence of oligonucleotide primers for amplification of three virulence genes of avian isolates of *E. coli*.

Primer	Target gene	Sequence	Product length (bp)
FimHf & FimHr	Fimbriae type 1 (fimH)	Forward: 5′-ATGAAACGAGTTATTACCCTGT-3′ Reverse: 5′-TTATTGATAAACAAAAGTCACGCCA-3′	902
IutAf & IutAr	Aerobactin (iutA)	Forward: 5′-CAGAGTTTTGTTTCTGACGGTCC -3′ Reverse: 5′-ACGTGCAACCTGGTAACCA-3′	2272
Tshf & Tshr	Temperature Sensitive Hemagglutinin (tsh)	Forward: 5′-ATGATGATAAGCAAAAAGTATACGC -3′ Reverse: 5′-TCAGAACAGCACAGAGTAGTTCAG -3′	750

Table 2. The frequency of different genotypes of three virulence genes in 60 intestinal and 60 extra intestinal *E. coli* isolates from chicken.

Genotype	No of positive fecal isolates	No of positive liver isolates	Total
tsh+iutA+fimH+	5/60	21/60	26/120
tsh+iutA+fimH-	4/60	12/60	16/120
tsh+iutA-fimH+	1/60	8/60	9/120
tsh-iutA+fimH+	9/60	7/60	16/120
tsh+iutA-fimH-	3/60	6/60	9/120
tsh-iutA+fimH-	7/60	2/60	9/120
tsh-iutA-fimH+	10/60	1/60	11/120
tsh-iutA-fimH-	21/60	3/60	24/120

by boiling (Delicato et al. 2003) and the DNA was stored at −60ºC until used. Presence of the iutA, fimH and tsh genes was verified by simplex PCR analysis. PCR was performed with a thermal cycler (Mastecycler Gradient, Eppendorf, Germany) in a 25 µl reaction containing 12.5 µl master mix (Ampelicon, Denmark), 5.5 µl PCR water, 1 µl (10 Pico mole) of each primer (Bioneer, South Korea) and 5 µl of template DNA. DNA polymerization was performed using thermal cycler and J96 strain of *E. coli* as positive and Mg 1655 strain as negative controls. Amplification of fimH was obtained with an initial denaturation step at 95ºC for 5 minutes followed by 35 cycles involving denaturation at 95 ºC for 30 seconds, annealing at 55ºC for 30 seconds, and synthesis at 72 ºC for 1 min. The final extension was down for 5 min at 72ºC. For amplification of iutA gene the conditions were the same as fimH

with the exception that annealing step was at 60ºC for 1 min and extension at 72 for 2.5 min. Amplification of tsh gene was done as fimH but annealing was at 60ºC for 1 min. PCR products were electrophoresed on 1.5% (w/v) agarose gels. Each 60 ml gel contained 1.7 µl of safe stain (Cinnagene, Iran). Gels were run for approximately 60 min at 100 v. Products were visualized using a UV trans-illuminator (UVtech, England) and size determination was achieved using a 100 base pair (bp) ladder (Cinnagene, Iran).

Statistical analysis: Statistical analysis was performed using SPSS software (version 22) and Chi square test. Significance was accepted when the p-value was < 0.05.

Results

The monoplex PCR results obtained for some APEC and non-APEC strains are shown in Figure 1. Frequency, patterns and combinations of three virulence-associated genes for all 120 isolates of *E. coli* are summarized in Table 2. The iutA gene was present in 42 (70%) liver and 25 (41.7%) fecal isolates and this difference was statistically significant (p=0.002). Frequency of fimH gene in liver isolates was 61.7% (37 isolates out of 60) while this gene was present in 41.7% (25 isolates out of 60) of fecal isolates and this difference was also significant (p=0.022). Our data also indicated that tsh was present in 47 (78.3%) liver and in 13 (21.7%) intestinal isolates. Statistical analysis revealed that tsh is also more frequent in liver isolates ($p<0.001$). The fimH+iutA+tsh+ genotype was most prevalent (35%) among all liver isolates, while the fimH-iutA-tsh-genotype was the greatest (35%) genotype among fecal isolates.

Discussion

In this study the 120 including 60 fecal and 60 liver *E. coli* isolates from chicken were investigated for the presence of three virulence-associated genes (iutA, tsh and fimH) described for APEC. According to the results, at least 95% of liver isolates possess one of the examined virulence genes, whereas 65% of fecal *E. coli* isolates were considered positive for at least one of the examined genes. In agreement with our results Kafshdouzan et al. (2013) by examination of avian pathogenic and fecal *E. coli* isolates for 6 virulence associated genes also reported that 85% of APEC and 66% of isolates from apparently healthy birds possess at least one of the examined genes. McPeake et al. (2005) showed that APEC virulence associated genes may be present in *E. coli* isolates from apparently healthy birds.

In this study 70% of APEC isolates were identified positive for iutA while 41.7% of fecal isolates were positive for this gene. Similar to our study, Kafshdouzan et al. (2013) found that IutA, is detectable in 67.4% of APEC isolates. Rodriguez-siek et al. (2005) also reported 81.2% of *E. coli* isolates from poultry colibacillosis are positive for iutA gene. Delicato et al. (2002) identified only 12% of fecal isolates positive for iutA, compared to 63% of isolated *E. coli* from cases of colibacillosis.

In our study the tsh gene was found in 78.3% of liver and 21.7% of fecal isolates, so the importance of tsh in APEC pathogenesis is confirmed. In contrast to our results for this gene, McPeake et al. (2005) demonstrated that occurrence of tsh gene in *E. coli* isolates from healthy birds is 93.3%; But, Delicato et al. (2002) identified only 4% of fecal isolates positive for tsh compared to 39.5% of isolated *E. coli* from cases of colisepticaemia. Same as in our study, Maurer et al. (1998) detected tsh in 46% of clinical isolates and showed the absence of this gene in all commensal *E. coli*. Furthermore, Campos et al. (2005) reported that tsh gene was found in 50% of APEC strain.

In the present study frequency of fimH genes in liver and fecal *E. coli* isolates from chickens were 61.7% and 41.7% respectively.

The fimH genes that encode type 1 fimbriae, were detected in almost 50% of examined isolates, in contrast with previous data (Delicato et al., 2003; Maurer et al., 1998; Roussan et al., 2014) showing their ubiquity among commensal and clinical isolates. Ghanbarpour et al., (2011) also reported 96.4% of fecal isolates positive for fimH compared to 95% of isolated *E. coli* from cases of colibacillosis. This difference may be due to discrepancy in time and different place of studies. Such differences may also be due to the different primers used in different investigations.

In the present study the fimH+iutA+tsh+ genotype was significantly more prevalent in liver (35%) than fecal (8.3%) isolates. McPeake et al. reported that APEC plasmids possess several virulence associated genes, though some of these have been reported in *E. coli* strains isolated from apparently healthy birds (McPeake et al., 2005).

In conclusion, cases of avian colisepticaemia within Khuzestan province in the southwest of Iran could not be linked to any individual genotype of causative agent. However, these results suggest fimH+iutA+tsh+genotype may play a significant role in colisepticaemia in this area and although these virulence genes were not present in all APEC isolates their frequency is high and using the products of these genes in vaccines may be effective in protecting against infections caused by this bacterium.

Acknowledgements

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مقایسه برخی ژن‌های مرتبط با حدت در اشرشیا کلی‌های روده‌ای و خارج روده‌ای جدا شده از ماکیان

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چکیده

زمینه مطالعه: اگر چه اشرشیا کلی جزء فلور طبیعی روده موجودات خونگرم از جمله پرندگان است، اما در صورتی که طی دوره پرورش طیور، بهداشت زیر حد استاندارد باشد یا بیماریهای تنفسی و یا سرکوب کننده سیستم ایمنی رخ دهد، باعث بیماری می‌گردد. اشرشیا کلی پاتوژن پرندگان (APEC)، چندین ژن مرتبط با حدت و بیماریزایی دارد. سویه‌های APEC مسئول برخی از بیماری طیور از جمله کلی باسیلوز، سندرم سر متورم، عفونت کیسه زرده، التهاب بند ناف و کلی‌گرانولوما هستند. **هدف:** مطالعه حاضر به منظور بررسی حضور و فراوانی سه ژن مهم حدت در اشرشیا کلی‌های روده‌ای و خارج روده‌ای (کبدی) جدا شده از ماکیان استان خوزستان در جنوب غرب ایران بود. **روش‌کار:** در مجموع ۱۲۰ جدایه اشرشیا کلی (۶۰ روده‌ای و ۶۰ کبدی) جدا شده از ماکیان توسط واکنش زنجیره‌ای پلیمراز (PCR) برای حضور ژن‌های ایروباکتین (*iutA*)، هماگلوتینین حساس به حرارت (*tsh*) و فیمبریه نوع۱ (*fimH*) مورد بررسی قرار گرفتند. **نتایج:** نتایج نشان داد که *iutA*، *tsh* و *fimH* به ترتیب در ۷۸/۳٪، ۷۰٪ و ۶۱/۷٪ از جدایه‌های کبد حضور دارند در حالی که در جدایه‌های روده‌ای فراوانی این ژن‌ها به ترتیب ۲۱/۷٪، ۴۱/۷٪ و ۴۱/۷٪ بود. شایع‌ترین ژنوتیپ در جدایه‌های خارج روده‌ای و روده‌ای به ترتیب $tsh^{+}\ fimH^{+}\ iutA^{+}$ و $tsh^{-}\ fimH^{-}\ iutA^{-}$ بود. **نتیجه‌گیری نهایی:** در مجموع به نظر می‌رسد که این مجموعه از ژن‌های حدت به میزان قابل توجهی ($p<0/05$) در جدایه‌های خارج روده‌ای شایع‌تر بوده و احتمالاً این ژن‌ها نقش مهمی در پاتوژنز APEC جدا شده از جنوب غرب ایران ایفاء می‌نمایند. اگر چه این ژن‌های حدت در تمامی جدایه‌های APEC حضور نداشتند ولی شیوع آنها بالا بود و استفاده از محصولات این ژن‌ها در واکسن ممکن است در محافظت در برابر عفونت‌های ناشی از این باکتری مؤثر باشد.

واژه های کلیدی: ایروباکتین، ماکیان، اشرشیا کلی، فیمبریه نوع۱، پروتئین حساس به حرارت (tsh)

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