Molecular typing of avian *Escherichia coli* isolates by enterobacterial repetitive intergenic consensus sequences-polymerase chain reaction (ERIC-PCR)

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Abstract:

BACKGROUND: Colibacillosis is one of the most economically important diseases of poultry worldwide. **OBJECTIVES:** This study was conducted to examine the clonal relatedness and typing of 95 avian *Escherichia coli* isolates by ERIC-PCR. **METHODS:** Sixty-three *E. coli* isolates from two common manifestations of colibacillosis (yolk sac infection and colisepticemia) and 32 isolates from feces of apparently healthy broilers were provided. The PCR amplification reactions were performed in duplicate for all isolates. **RESULTS:** The molecular weight of the observed bands on gel electrophoresis ranged from 232 bp to 2690 bp. Sixty-five fingerprinting patterns were observed among 95 isolates on the basis of molecular weights and the number of bands. The numbers of 20, 22, and 23 fingerprinting patterns were found among isolates from yolk sac infection, colisepticemia, and feces, respectively. Among different fingerprinting patterns, the number of produced bands differed from 2 to 11. No identical pattern was observed among isolates of three sources. Isolates showing similar patterns in each source group belonged to a single farm. However, a few isolates that had been isolated from different farms also showed similar fingerprinting patterns. **CONCLUSIONS:** In conclusion, this study showed a high degree of polymorphism among *E. coli* isolates originated from different poultry sources when the respective bacterial genomes were analyzed by the ERIC-PCR and that no specific genotypes were responsible for different manifest-ations of colibacillosis.

Introduction

Escherichia coli is a major cause of respiratory and septicemic disease (colibacillosis) in chickens (Barnes et al., 2008). Avian pathogenic *E. coli* (APEC) that are inhaled into the respiratory tract, attach to the epithelial cells lining the trachea, then spread throughout the respiratory tract and to other systems, often resulting in airsacculitis, pericarditis and perihepatitis. The *E. coli* infection appears to be secondary to a primary respiratory condition in which other agents such as mycoplasma or viruses, or environmental factors such as unsatisfactory ventil-ation, overcrowding, and nutritional deficienc-ies

enhance the susceptibility of the respiratory tract to *E. coli* (Wary and Davies, 2002). Mortality is usually less than 5%, but morbidity can be over 50% and economic losses result from suboptimal growth rates and condemnation at the processing plant. Infections by *E. coli* are amongst the most important causes of economic loss from disease in the poultry industry (Barnes et al., 2008).

The discovery of repeated sequences such as the enterobacterial repetitive intergenic consensus (ERIC) sequence in prokaryotic genomes has expanded the molecular biology tools that are available to assess the clonal variability of many bacterial strains including *Escherichia coli* (Hulton et al., 1991; Versalovic et al., 1991; Dalla Costa et al., 1998). These molecular techniques are based on the use of primers homologous to these sequences which after PCR reaction generate a pattern of amplified bands specific for each isolate (Versalovic et al., 1991). Other molecular techniques such as ribotyping and isoenzyme profile have also been used to evaluate the clonal relatedness of avian *E. coli* (Silveira et al., 2003).

Published data on molecular epidemiology of APEC in Iranian commercial poultry flocks are limited (Ghanbarpour et al., 2010; Salehi and Ghanbarpour, 2010; Ghanbarpour et al., 2011). In this study, the clonal population structure of 95 *E. coli* isolated from septicemic poultry, cases of yolk sac infection (YSI), and feces of broilers with no apparent signs of illness were investigated using ERIC-PCR.

Materials and Methods

Sampling and bacteriological procedures: All samples were provided during summer 2010 from 23 broiler farms in the vicinity of Garmsar city in Semnan province of Iran. The number of farms sampled for cloacal swabs, yolk sac infection (YSI), and septicemic lesions included 5, 8, and 10 different broiler farms, respectively. The carcasses referred to our veterinary diagnostic clinic were sampled immediately. Isolates from septicemic cases were obtained from liver and heart blood of broiler carcasses showing the characteristic perihepatitis or pericarditis. Isolates from YSI were recovered from chicks with typical appearance of YSI. Fecal isolates were collected from cloacae region of broilers at the final stage of the flock's rearing period. All fecal samples were transported to the laboratory in cold condition within two hours. MacConkey agar (Merck, Germany) was primarily used to detect suspected lactose fermenting colonies and *E. coli* isolates were subsequently confirmed by using conventional biochemical tests (Quinn et al., 1994). Finally, one colony from each isolate was selected and processed as the representative of that sample. All recovered *E. coli* isolates were stored at -70°C in brain heart infusion (BHI) broth containing 20% glycerol for further use.

ERIC-PCR: Genomic bacterial DNA of 95 *E. coli* isolates was extracted by boiling as described by Sambrook and Russell (2001). ERIC-PCR was performed with the two primer sequences of ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') as described by Versalovic et al., 1991. The 25 µl PCR reaction mixture included 17.5 µl distilled water, 2.5 µl 10X PCR buffer, 1.25 µl MgCl2 (2.5 mM), 0.5 µl dNTP (200 µM), 1 µl from each primer (ERIC1 and ERIC2, 0.4 µM), 0.25 µl Taq DNA polymerase (1.25 U) and 1 µl template DNA. PCR amplification was performed using a TECHNE Thermal Cycler (Staffordshire ST15 0SA, UK) as follows: initial denaturation at 94 °C for 7 min, 30 cycles of denaturation at 90 °C for 30 s, annealing at 52 °C for 1 min, and extension at 72 °C for 8 min; followed by a final extension at 72 °C for 15 min (Versalovic et al., 1991; Silviera et al., 2002b). Electrophoresis was performed using 1.5% agarose gel and at 70v for two hours as described previously (Sambrook and Russell, 2001). Primers, all reagents, and size markers were purchased from CinnaGen Inc. (Tehran, Iran). Molecular weight of each observed band was calculated by SEQAID II ver. 3.5 software (Kansas State University, 1989). Reproducibility of the ERIC-PCR patterns for each *E. coli* isolate was confirmed using duplicate runs on separate occasions but on the same thermocycler.

Results

In total, 95 isolates including 63 *E. coli* isolates from two common manifestations of colibacillosis (31 YSI and 32 colisepticemia isolate) and 32 isolates

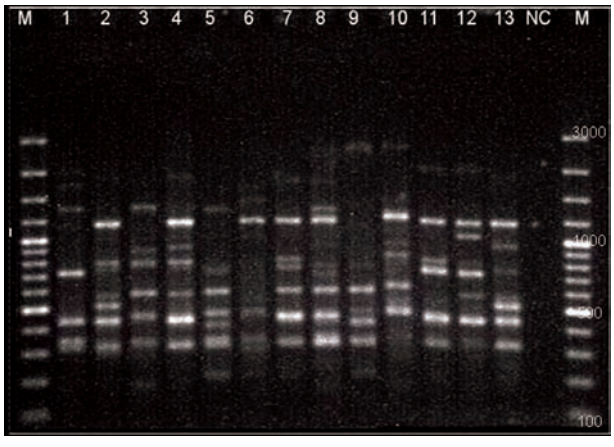

Figure 1. ERIC-PCR profiles of *Escherichia coli* isolates from Yolk Sac Infection (Lanes 1-13). Lanes M and NC indicate 100-3000 bp ladder and negative control, respectively.

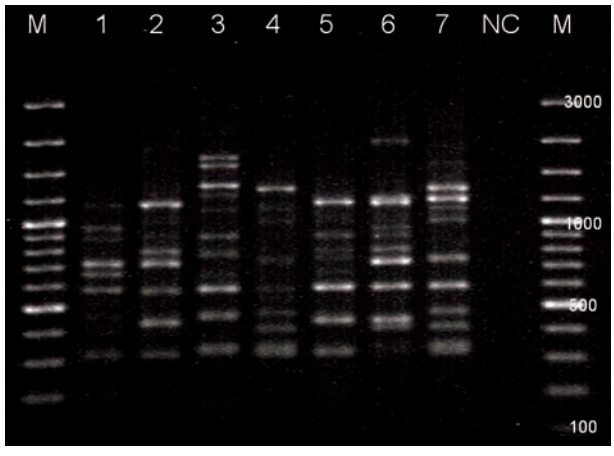

Figure 2. ERIC-PCR profiles of *Escherichia coli* isolates from Colisepticemic chickens (Lanes 1-7). Lanes M and NC indicate 100-3000 bp ladder and negative control, respectively.

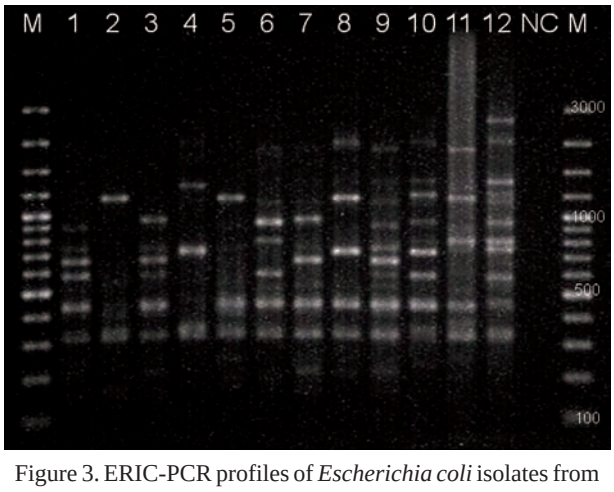

Figure 3. ERIC-PCR profiles of *Escherichia coli* isolates from feces of apparently healthy chickens (Lanes 1-12). Lanes M and NC indicate 100-3000 bp ladder and negative control, respectively.

from feces of apparently healthy broilers were obtained (Table 1). The molecular weight of the bands observed on the gels ranged from 232 bp to 2690 bp (Figures 1-3). Sixty-five fingerprinting patterns were determined among 95 isolates on the basis of molecular weights and the number of observed bands. The numbers of 20, 22, and 23 fingerprinting patterns were found among isolates from YSI, colisepticemia, and feces, respectively. Among different fingerprinting patterns the number of produced bands differed from 2 to 11. No identical pattern was observed among isolates of three sources. In a particular farm, 88.2%, 100%, and 78.5% of isolates from YSI, septicemia and fecal sources, respectively, showed similar patterns. However, a total of 10.2% of isolates in all three source groups that were isolated from different farms also showed similar fingerprinting patterns.

Discussion

This study determined the ERIC-PCR profile of *Escherichia coli* isolates recovered from diseased (*E. coli* - related infections) and apparently healthy chickens. *Escherichia coli* isolates from all three sources demonstrated variable fingerprints with no similarity. A high degree of polymorphism in the DNA sequences of *E. coli* isolates analyzed by ERIC-PCR was observed.

Bacterial typing is an important epidemiological tool to respond to issues related to the outbreaks of infectious diseases such as cross-transmission of nosocomial pathogens, determining the source of the infection, recognizing different pathotypes, and differentiation of virulent and vaccinal strains after vaccination programs (Foley et al., 2009). A variety of procedures have been used by researchers to type the bacterial species (Versalovic et al, 1991; Foley et al., 2009; Miller and Tang, 2009; Goering, 2010; Karama and Gyles, 2010). Most of the currently used molecular techniques such as random amplification of polymorphic DNA (RAPD), repetitive extragenic palindromic sequences (REP) and enterobacterial repetitive intergenic consensus (ERIC) for bacterial typing are based on electrophoretic differentiation of DNA pieces with different molecular lengths observed on agarose gels (Versalovic et al., 1991; Maurer et al., 1998; Silveira et al., 2002b; Namvar

Table 1. *Escherichia coli* isolates obtained in this study.

Site of isolation	Number of farms	Number of isolates
Infected yolk sac of 1-7 days old chicks	**8**	**31**
Pericarditis, perihepatitis from lesions of colisepticemia in broilers of 4-7 weeks old	**10**	**32**
Feces from broilers with no signs of illness	**5**	**32**

and Warriner, 2006). Due to the possible complexity of these profiles, only those methods capable of showing the straightforward interpretation can be considered as reliable and useful typing methods (Arbeit, 1995). The power of differentiation between isolates and the reproducibility of results is also important along with considerations related to a particular method's straightforward interpretation and its simplicity of use (Arbeit, 1995). The cost and time required to achieve a reliable result should also be considered when assessing the utility of a particular typing method.

Previous studies by Selander and Levin, 1980 and Achtman et al., 1983, were among the first works to describe the genetic diversity and clonal similarity of *E. coli* populations. Since then, different procedures have been applied to study bacterial clones among *E. coli* isolates (Silveira et al., 2002a; Ewers et al., 2004; Brocchi et al., 2006). Various studies have reported the use of ERIC-PCR for typing of poultry, porcine, and uropathogenic *E. coli* isolates (Ngeleka et al., 1996; De Moura et al., 2001; Silveira et al., 2002b; Warriner et al., 2002; Namvar and Warriner, 2006; Zahraei Salehi et al., 2008). ERIC-PCR has been used, particularly in discriminating between APEC and commensal *E. coli* isolates (Silveira et al., 2002b).

In this study, the 95 pathogenic and fecal avian *Escherichia coli* isolates analyzed by the ERIC-PCR technique demonstrated a high degree of polymorphism in the amplified DNA profile. Our ERIC-PCR found no similar genomic patterns among septicemic, YSI, and fecal isolates and classified those isolates in three separated groups. In contrast to findings of the previous investigation (Silveira et al., 2002b) that grouped together the YSI isolates with the fecal isolates from apparently healthy chickens and considered the YSI isolates as just opportunistic and non-pathogenic agents for chickens, in the present study, no similarity was found in genotypic patterns originated from YSI and apparently healthy chickens. Our observations also suggest that *E. coli* isolates from septicemia or yolk sac infection do not belong to clones from the same origin and similar genetic backgrounds. However, there was, as expected, a tendency of higher genetic relationship among *E. coli* isolates originated from the same farm.

Our findings are in agreement with those of de Moura et al., 2001, who used ERIC-PCR technique and suggested that no specific genotype would be responsible for colibacillosis. Interestingly, Brocchi et al., 2006, used REP-PCR for differentiating a wide variety of avian *E. coli* isolates from diseased and healthy birds and concluded that the REP-PCR was not as powerful as ERIC-PCR in discrimination between commensal and pathogenic avian isolates as shown in their previous study (Silveira et al., 2002b). They speculated that the greater molecular length and weight of ERIC against REP sequences could be the reason for the higher discriminatory power of ERIC-PCR compared to that of REP-PCR. The notion that no specific genotype is responsible for different manifestations of colibacillosis has been indicated by other researchers who used RAPD-PCR for differentiation of *E. coli* isolates from poultry sources (Chansiripornchai et al., 2001; Zahraei Salehi et al., 2008).

In conclusion, this study determined that a high degree of polymorphism exists among *E. coli* isolates originated from different poultry sources when the respective bacterial genomes are analyzed by the ERIC-PCR technique. Our observation by the ERIC-PCR technique also reinforces previous investigations that no specific genotypes are responsible for different manifestations of colibacillosis. Comparison of ERIC-PCR fingerprinting results with virulence gene profiles and more powerful fingerprinting methods such as pulse field gel electrophoresis (PFGE) can better explain the molecular epidemiology of *E. coli*- associated diseases in poultry.

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تایپینگ ملکولی جدایه‌های اشریشیا کلی طیور با به کارگیری روش ERIC-PCR

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چکیده

زمینه مطالعه: کلی باسیلوز درسرتاسردنیا به عنوان یکی از پر اهمیت ترین بیماری‌های طیور از نظر اقتصادی محسوب می‌گردد. **هدف:** این مطالعه به منظور ارزیابی سطح ارتباط کلونال و تایپینگ ملکولی ۹۵ جدایه اشریشیا کلی طیور توسط آزمون ERIC-PCR انجام گرفت. **روش‌کار:** تعداد ۶۳ جدایه اشریشیا کلی از دو تظاهر بالینی متداول کلی باسیلوز (عفونت کیسه زرده و کلی سپتیسمی) و ۳۲ جدایه از مدفوع ماکیان گوشتی ظاهرا سالم جداسازی شد. هر یک از جدایه‌های مذکور ۲ بار تحت آزمون PCR قرار گرفت. **نتایج:** وزن ملکولی باندهای مشاهده‌شده برروی ژل الکتروفورز از ۲۳۲ تا ۲۶۹۰ جفت باز متغییر بود. بر اساس تعداد و وزن ملکولی باندهای تشکیل شده ۶۵ الگوی انگشت نگاری متفاوت توسط ۹۵ جدایه مذکور نشان داده شد. تعداد ۲۰، ۲۲ و ۲۳ الگوی انگشت نگاری متفاوت به ترتیب در بین جدایه‌های حاصل از عفونت کیسه زرده، کلی سپتیسمی و مدفوع مورد شناسایی قرار گرفت. مابین الگوهای انگشت نگاری متفاوت، تعداد باندهای تشکیل شده از ۲ تا ۱۱ عدد متغییر بود. هیچ الگوی انگشت نگاری مشابهی مابین جدایه‌های متعلق به ۳ منبع ذکرشده مشاهده نگردید. جدایه‌هایی نیزکه در هر یک از گروه‌ها الگوهای انگشت نگاری مشابهی نشان می‌دادند، از یک مزرعه واحد جداسازی شده بودند. با این حال، تعداد اندکی از جدایه‌های جداسازی شده از مزارع متفاوت نیز الگوهای انگشت نگاری یکسان ایجاد نمودند. **نتیجه گیری نهایی:** در پایان، این مطالعه بیانگر حضور سطح بالایی از پلی مورفیسم مابین جدایه‌های اشریشیا کلی به دست‌آمده از منابع مختلف بوده و بر این اساس می توان بیان نمود که تظاهرات بالینی متفاوت کلی باسیلوز توسط ژنوتایپ‌های اختصاصی ایجاد نمی گردند.

واژه‌های کلیدی: کلی باسیلوز، اشریشیا کلی، ماکیان گوشتی، ERIC-PCR، ایران.

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