

Characterization of *Escherichia coli* Isolates from Commercial Layer Hens with Salpingitis

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Abstract: Problem statement: *Escherichia coli* isolates are the most common bacterial agents of salpingitis in commercial layer hens. The objectives of this study were to determine the phylogenetic groups/subgroups and antibiotic resistance of *E. coli* isolates from salpingitis cases in commercial layer hens farms in southeast of Iran. **Approach:** One hundred twenty one *E. coli* isolates from oviducts of layer hens with salpingitis were examined to determine their O-serogroup, phylogenetic group/subgroup and antibiotic resistance pattern. **Results:** O-serogroup determination test showed that 47 (38.84%) isolates were typeable and belonged to eleven different O serogroups including: O1, O2, O6, O8, O15, O20, O25, O36, O78, O86 and O111 and 71 (58.67%) isolates were O-nontypeable. Three most prevalent serogroup were O78, O2 and O1 respectively. PCR assays showed that the isolates fall into four phylogenetic groups A (41.32%), D (33.88%), B2 (14.87%) and B1 (9.91%). Ninety nine (81.81%) isolates fell into six phylogenetic subgroups including: A₀ (17.35%), A₁ (23.96%), B2₂ (5.78%), B2₃ (9.09%), D₁ (13.22%) and D₂ (20.66%). All of the *E. coli* isolates from O78 serogroup belonged to A phylo-group whereas the O2 isolates mostly fell into D group. The maximum antibiotic resistance was against tetracycline (100%) and minimum resistance was against linco-spectin (37.19%). Twenty four isolates (19.83%) were resistant to all of the examined antibiotics. Twenty three different patterns of multiple drug resistance were observed, out of which N-Te-Sxt-Fm-Nfx-C and N-Te-Sxt-Gm-Fm-Nfx-C-Ls was the two most frequent patterns respectively. The resistant isolates were found in all of the phylogenetic groups and or sub groups. **Conclusion:** There are similarities between salpingitis derived *E. coli* strains and other avian pathogenic *E. coli* isolates in phylogroups, O-serogroups and antibio-resistance patterns.

Key words: *Escherichia coli*, antibiotic, phylotyping, salpingitis

INTRODUCTION

Salpingitis is an inflammation of the oviduct. Spread of bacteria into the body cavity through the compromised oviduct wall leads to concurrent peritonitis, which is termed salpingoperitonitis (Saif *et al.*, 2003). Environmental stress has been shown to be a factor that may induce colonization of food animals by enteric pathogens, facilitate horizontal transmission of pathogens between animals, increase pathogen shedding (Soliman *et al.*, 2009). Salpingitis a complex condition of chickens associated with various bacterial agents including *Escherichia coli* (*E. coli*), *Gallibacterium anatis*, *Staphylococcus* spp., *Mannheimia haemolytica*, *Streptococcus bovis* and occasionally *Salmonella* spp. (Jordan *et al.*, 2005; Neubauer *et al.*, 2009). *E. coli* isolates are the most

common bacteria which have been reported from many countries as a frequent cause of disease in commercial laying hens (Fossum *et al.*, 2009). On the other hand *E. coli* is one of the opportunist pathogen responsible for number of disease conditions such as yolk sac infection, air sac disease, perihepatitis, enteritis, omphalitis, coligranuloma and colibacillosis (Ghanbarpour and Salehi, 2010). Avian Pathogenic *E. Coli* (APEC) serovars involved as causative agents of salpingitis in all birds (layers and broiler breeders, turkeys, ducks and geese) that are sexually mature (Pattison *et al.*, 2007). APEC strains belong predominantly to serogroups O1, O2, O5, O8, O18 and O78 (Germon *et al.*, 2005). The method of spread to the reproductive tract is unknown. Several routes of infection have been described: ascending fecal contamination from the cloaca, bacterial translocation respiratory tract (air sac and

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lungs) and bacterial translocation from the intestinal lumen (Landman and Cornelissen, 2006). However, the pathogenesis of *E. coli* isolates salpingitis and peritonitis has not been completely elucidated yet. According to the serogroups involved, pathogenicity for day-old chicks and virulence indicators, the salpingitis isolates were similar to those from cases of chronic respiratory disease (Monroy *et al.*, 2005).

Currently, there are four well-recognized phylogroups of *E. coli* strains and these have been designated A, B1, B2 and D. Genome size varies among the four phylo-groups with A and B1 strains having smaller genomes than B2 or D strains (Gordon *et al.*, 2008). However, to increase the discriminative power of phylogenetic analyses, *E. coli* strains have been segregated in seven phylogenetic subgroups (A₀, A₁, B1, B2₂, B2₃, D₁, D₂) according to presence/absence of three genetic markers *chuA*, *yjaA* and *Tspe4.C2* (Escobar-Paramo *et al.*, 2004b). *E. coli* isolates from main phylo-groups appear to differ in their ecological niches, life-history characteristics and propensity to cause disease (Walk *et al.*, 2007). The extra-intestinal pathogenic strains belong mainly to phylogenetic groups B2 and, at a lesser extend, D and possess numerous specific virulence genes that are absent in commensal strains (Escobar-Paramo *et al.*, 2006). Strains of the phylo-groups also differ in their phenotypic characteristics, such as antibiotic-resistance profiles, ability to exploit different sugars and growth rate-temperature relationships (Escobar-Paramo *et al.*, 2004a; Gordon *et al.*, 2005).

Currently APEC infections in poultry are controlled indirectly by protecting against primary respiratory pathogens. In many countries it has been common practice to administer antibiotics to suppress infection with APEC. Antimicrobials, commonly administered as feed supplements, are fundamental to treating these conditions but their persistent, frequent administration can create multi-antimicrobial resistant bacteria (Johnson *et al.*, 2003; Li *et al.*, 2010).

The objectives of this study were to determine the phylogenetic groups/subgroups and antibiotic resistance of *E. coli* isolates from salpingitis cases in commercial layer hens farms in southeast of Iran.

MATERIALS AND METHODS

Bacterial strains: A total of 121 *E. coli* isolates were obtained from oviducts of layer hens with salpingitis and/or salpingoperitonitis. Isolates were collected between 2007 and 2008 from different farms and flocks in Kerman province (south-eastern), Iran. The sampled birds were aged between 30 and 68 weeks. Standard

biochemical and bacteriological methods were used to isolate and identify the *E. coli* strains.

Several strains from the ECOR collection were used as controls for phylogenetic grouping: ECOR58 (B1 group), ECOR50 (D group) and ECOR62 (B2 group). *E. coli* strain MG1655 was used as a negative control for virulence typing and as a positive control for phylogenetic ECOR group A. The reference strains were a kind gift from Eric Oswald (Ecole National Veterinaire Toulouse, France). All the bacterial strains were routinely grown in Luria-Bertani broth at 37°C with aeration and stored at -70°C in 30% sterile glycerol until they were used. For preparation of samples for testing, isolates were cultured on Trypticase-Soy Agar (TSA) and/or Trypticase-Soy Broth (TSB) at 37°C over night.

Serogroup determination and Phylotyping assay: O serogroup of *E. coli* isolates were examined by using specific antisera provided from LREC, University of Santiago de Compostela, Lugo, Spain.

Freshly grown over night cultures of *E. coli* isolates and reference strains were used for DNA extraction by boiling. The triplex PCR method was used to assign the *E. coli* isolates (Clermont *et al.*, 2000). The specific primers (TAG Copenhagen, Denmark) used for amplification of the genes. The Clermont method has the potential to yield seven distinct phylogenetic groups and subgroups (A₀, A₁, B1, B2₂, B2₃, D₁ and D₂). The phylo-type of each isolate was determined as follows; *chuA*⁻, *YjaA*⁻, *TSPE4C2*⁻ was assigned to subgroup A₀ (group A), *ChuA*⁻, *YjaA*⁺, *TSPE4.C2*⁻ was assigned to subgroup A₁ (group A); *ChuA*⁻, *YjaA*⁻, *TSPE4C2*⁺ was assigned to group B1, *ChuA*⁺, *YjaA*⁺, *TSPE4C2*⁻ was assigned to subgroup B2₂ (group B2), *ChuA*⁺, *YjaA*⁺, *TSPE4C2*⁺ was assigned to subgroup B2₃ (group B2), *ChuA*⁺, *YjaA*⁻, *TSPE4C2*⁻ was assigned to subgroup D₁ (group D) and *ChuA*⁺, *YjaA*⁻, *TSPE4C2*⁺ was assigned to subgroup D₂ (group D) (Escobar-Paramo *et al.*, 2004a; 2004b) Isolates failing to produce any PCR products were repeated twice more using newly prepared DNA extract. After 2-3 attempts, any isolate negative for all three PCR products was confirmed to be *E. coli* by using biochemical API 20E identification kit (BioMérieux, Marcy l'Etoile, France). If the isolate was identified as *E. coli*, it was considered to be from subgroup A₀ (phylogroup A).

Antibiotic susceptibility test: Resistances against to eight antibiotics were determined by disc diffusion method according to Clinical and Laboratory Standards Institute's (CLSI) guidelines (Jorgensen and Hindler,

2007). The following antimicrobial discs (Padtan-Teb, Tehran, Iran) were used for all isolates: Chloramphenicol (C), enrofloxacin (Nfx), Flumequine (Fm), Gentamycin (Gm), linco-spectin (Ls), Neomycin (N), oxytetracycline (Te) and trimethoprim-sulfamethazole (Sxt).

RESULTS

O-serogroup determination test showed that 47 (38.84%) isolates were typeable and belonged to eleven

different O serogroups including: O1, O2, O6, O8, O15, O20, O25, O36, O78, O86 and O111 and 71 (58.67%) isolates were O-nontypeable. Nineteen isolates (15.70%) belonged to O78 serogroup, which was the most prevalent followed by O2 (8.26%) and O1 (4.95%) serogroups (Table 1).

PCR assays revealed that 121 *E. coli* isolates fall into four phylogenetic groups whereas 41.32% (50 isolates) belonged to A, 33.88% (41) to D, 14.87% (18) to B2 and 9.91% (12) to B1 groups (Table 1).

Table 1: O-serogroups of 121 *E. coli* isolates from salpingitis of hens in relation to phylo-groups

O-group	Phylogenetic group				Total No. (%)
	A	B1	B2	D	
O1	-	-	4	2	6 (4.95)
O2	-	-	3	7	10 (8.26)
O6	-	-	1	-	1 (0.82)
O8	-	-	-	1	1 (0.82)
O15	-	1	-	-	1 (0.82)
O20	-	-	-	2	2 (1.65)
O25	-	-	-	1	1 (0.82)
O36	2	-	-	-	2 (1.65)
O78	19	-	-	-	19 (15.70)
O86	-	1	-	-	1 (0.82)
O111	-	-	-	3	3 (2.47)
NT*	14	4	4	10	74 (61.15)
Total	50 (41.32%)	12 (9.91%)	18 (14.87%)	41 (33.88%)	121 (100)

*: Nontypeable

Table 2: Multiple antibiotic resistance patterns and phylogenetic groups and/or subgroups of 121 *E. coli* isolates from hens with salpingitis

Resistance pattern	Phylo-group/ subgroup							Total No.
	A ₀	A ₁	B1	B2 ₂	B2 ₃	D ₁	D ₂	
N Te Sxt Gm Fm Nfx C Ls*	4	7	2	3	1	1	6	24
N Te Sxt Gm Fm Nfx Ls					1			1
N Te Sxt Gm Fm Nfx C	2	6	1		5	4	1	19
N Te Sxt Fm Nfx C Ls		5	2	1	1	2	3	14
N Te Sxt Gm Fm C Ls							1	1
N Te Sxt Gm Fm Nfx	1	2				2		5
Te Sxt Gm Fm Nfx C							1	1
N Te Sxt Gm Fm Ls						1		1
N Te Sxt Fm Nfx C	6		4	1	2	5	7	25
N Te Sxt Gm Fm C			1	1				2
N Te Sxt Fm C Ls			1					1
N Te Sxt Fm Nfx	3	8					4	15
Te Fm Nfx C Ls							1	1
N Te Sxt Nfx C	1							1
N Te Sxt Fm C						1		1
N Te Sxt C Ls	1							1
Te Sxt Fm Nfx		1						1
N Te Sxt Fm	1			1				2
N Te Sxt Gm					1			1
N Te Fm C	1							1
N Te Sxt C							1	1
N Te Sxt			1					1
Te Fm	1							1
Total No. %	21 (17.35)	29 (23.96)	12 (9.91)	7 (5.78)	11 (9.09)	16 (13.22)	25 (20.66)	121 (100.00)

*: Chloramphenicol (C), Enrofloxacin (Nfx), Flumequine (Fm), Gentamycin (Gm), Linco-spectin (Ls), Neomycin (N), Oxytetracycline (Te) and Trimethoprim-sulfamethazole (Sxt)

Ninety nine (81.81%) isolates fell into six phylogenetic subgroups including: 21 isolates (17.35%) A₀, 29 isolates (23.96%) A₁, 7 isolates (5.78%) B₂, 11 isolates (9.09%) B₃, 16 isolates (13.22%) D₁ and 25 isolates (20.66%) D₂. Phylogenetic subgroup of A₁ was the most prevalent followed by D₂ (Table 2).

All of the *E. coli* isolates from O78 serogroup belonged to A phylo-group whereas the O2 isolates mostly fell into group D.

All of the 121 isolates showed resistance to two or more antibiotics. The maximum resistance was against tetracycline 100% (121 isolates) followed by trimethoprim-sulfamethoxazole (97.52%), neomycin (96.69%), flumequine (95.86%) and enrofloxacin (88.42%). The minimal resistance was against lincospectin (37.19%) then gentamicin (45.45%) and chloramphenicol (76.85%). Twenty three different patterns of multiple drug resistance were observed, out of which N-Te-Sxt-Fm-Nfx-C and N-Te-Sxt-Gm-Fm-Nfx-C-Ls were the two most frequent patterns respectively. The resistant isolates were found in all of the phylogenetic groups and or sub groups (Table 2).

DISCUSSION

Enterobacteriaceae form a large, heterogeneous family of medically and scientifically important, gram-negative bacteria. *Escherichia coli*, the most prevalent infecting organism in this family, is one of the prototypic bacteria studied (Carnes and Wilkins, 2005). Coliform salpingitis/salpingoperitonitis results in decreased egg production and sporadic mortality. It is one of the most common causes of mortality in commercial layers and breeder chickens (Saif *et al.*, 2003). In the present study, 47 isolates were typeable and belonged to 11 serogroups. Monroy *et al.* (2005) have reported that *E. coli* isolates from broiler breeders with salpingitis belong to serogroups O1, O2, O5, O36, O45, O53 and O78. In a study on five clinically normal chickens from three farms *E. coli* isolates from the peritoneal cavity were serogrouped as O78 and O111 (Trampel *et al.*, 2007). Variation in O-serogroups of isolates according to geographic region occur, but in most studies the common serotypes of APEC isolates have been O1, O2, O35, and O78 (Saif *et al.*, 2003). Isolates of APEC from birds with salpingitis have more similarity with *E. coli* isolates from airsacculitis. Although in a study on the molecular epidemiology of *E. coli* isolates from an infection outbreak in a layer breeder flock the epidemic clone was also found in an environmental sample, suggest have been transmitted to the flock via the environment (Timothy *et al.*, 2008). The present observation indicated that O78 is the most

prevalent serogroup in salpingitis derived isolates. This serogroup is known to include virulent strains associated with severe *E. coli* infection in poultry. It is also recognized as one of the serogroups associated with enterotoxigenic *E. coli* strains that can affect humans (Calnek *et al.*, 1997; La Ragione and Woodward, 2002).

In this study high frequency of resistant isolates against tetracycline ascribe to its frequent use as therapeutic and feed additive. On the other hand, 24 isolates (19.83%) were resistant to all of the examined antibiotics. Avian pathogenic *E. coli* are often highly resistant, especially to tetracycline, streptomycin and sulfonamides. High levels of resistance to ciprofloxacin have been reported from China. In medicine, frequency of strains resistant to ampicillin, trimethoprim-sulfamethoxazole, streptomycin, Ciproflaxin, chloramphenicol, tetracycline and cephalosporins (ceftriaxone or ceftazidime) is a cause of growing concern (Raja *et al.*, 2009). Commensal *E. coli* strains from poultry have similar patterns of resistance but at lower frequencies (Gyles, 2008). A study on prevalence and distribution of antibiotic resistance in *E. coli* isolates from pigs and poultry showed that the isolates had high rates of resistance to ampicillin (99.5%), doxycycline (95.6%), tetracycline (93.4%), trimethoprim-sulfamethoxazole (74.3%), amoxicillin (65.1%), streptomycin (54.7%) and chloramphenicol (50.2%). Resistance to cephalosporins, quinolones and aminoglycosides was also quite prevalent (Li *et al.*, 2010). Antibiotic administration is considered the most important factor promoting the emergence, selection and dissemination of antibiotic-resistant microorganisms in both veterinary and human medicine. Antibiotic usage selects for resistance not only in pathogenic bacteria but also in the endogenous flora of exposed individuals (animals and humans) or populations (Van den Bogaard *et al.*, 2001). Diarrassouba *et al.* (2007) indicated that multiple antibiotic-resistant commensal *E. coli* and *Salmonella* strains carrying virulence genes can be found on commercial broiler chicken farms and may provide a reservoir for these genes in chicken production facilities. Recent study provides clear evidence that a sequential medication of a broiler flock, with different antimicrobial classes during short periods of time for prophylactic objectives, was accompanied by a dramatic increase in both antimicrobial resistance rates and phenotype diversity of *E. coli* strains (Da Costa *et al.*, 2008). Development of resistant pathogenic *E. coli* strains pose considerable threat to public health as these strains after ingestion, are capable of transferring their resistance to other pathogens through episomal transfer of R-factor

(Johnson *et al.*, 2008). In human derived isolates, the most common resistance mechanism in Gram-negative bacteria includes production of β -lactamases that hydrolyze the antibiotic ring, thus deactivating antimicrobial activity (Kathryn *et al.*, 2005).

There are few reports on phylogenetic background of salpingitis associated *E. coli* isolates. In the current study, salpingitis derived *E. coli* isolates segregated in phylogenetic groups A (41.32%), D (33.88%), B2 (14.87%) and B1 (9.91%). Trampel *et al.* (2007) reported that *E. coli* isolates from peritonitis lesions in commercial laying hens belonged to A and D phylogenetic groups. According to virulence genotype and phylogenetic group *E. coli* isolates from the magnum and peritoneum are similar to APEC isolates. Dissanayake *et al.* (2008) showed that APEC isolates belong to A (71%), B1 (4.1%), B2 (7.9%) and D (18.65%) groups. Phylogenetic analyses of *E. coli* isolates from avian cellulitis showed that *E. coli* isolates belonged to A (51.56%), B1 (18.75%) and D (29.68%) phylogenetic groups (Ghanbarpour *et al.*, 2009). Rodriguez-Siek *et al.* (2005) found that APEC strains fell into A (38.0%), D (28.1%), B2 (18.5%) and B1 (15.5%) phylo-groups. Results of phylo-typing showed that considerable portion of APEC strains also fell into group A (46.1%) and B2 (35.1%) (Ewers *et al.*, 2009). According to the results of this study multi antibiotic resistant, isolates were found in four phylogenetic groups. Johnson *et al.* (2003) reported that nalidixic acid-resistant *E. coli* isolates from retail chicken products belonged to phylo groups B2 and D. The findings of a study on antimicrobial resistance profiles among *E. coli* isolates from human and animal wastewater led to hypothesize that abusive antibiotic use in food animal production may promote the development of resistance among the intestinal *E. coli* phylogroups (Sabate *et al.*, 2008).

CONCLUSION

In conclusion, comparison of the results of the current study with finding of other reports revealed that there are similarities in phylogroups, O-serogroups and antibio-resistance profiles between salpingitis derived *E. coli* strains and other APEC isolates. It is imperative to determine antibiotic sensitivity of *E. coli* isolates involved in different disease so that ineffective antibiotics can be avoided.

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