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: A 0 (17.35%), A 1 (23.96%), B2 2 (5.78%), B2 3 (9.09%), D 1 (13.22%) and D 2 (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 opportunistic pathogen responsible for number of disease conditions such as yolk sac infection, air sac disease, pericarditis, entropitis, 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
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 phylo-groups 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 discriminatory power of phylogenetic analyses, *E. coli* strains have been segregated in seven phylogenetic subgroups (A0, A1, B1, B2, B3, D1, D2) according to presence/absence of three genetic markers chuA, yjaA and Tsp4.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 pathogenic strains belong mainly to phylogenetic groups A, B1, B2 and, at a lesser extend, D and possess commensal strains (Escobar-Paramo et al., 2006). Strains of the phylo-groups also differ in their genomes than B2 or D strains (Gordon et al., 2008).

**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 Tryptase-Soy Agar (TSA) and/or Tryptase-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, B2, B3, D1 and D2). The phylo-type of each isolate was determined as follows; chuA, yjaA, Tsp4.C2 was assigned to subgroup A0 (group A), Chu A’, Yja A’, TSPE4 C2’ was assigned to subgroup A1 (group A); Chu A’, Yja A’, Tsp4.C2’ was assigned to group B1, Chu A’, Yja A’, TSPE4C2’ was assigned to subgroup B2 (group B2), Chu A’, Yja A’, TSPE4C2’ was assigned to subgroup B2, Chu A’, Yja A’, TSPE4C2’ was assigned to subgroup D1 (group D) and Chu A’, Yja A’, 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 (BioMe’rieux, 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,
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-sulfamethxazole (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 | A   | B1  | B2   | D   | Total No. (%) |
|---------|-----|-----|------|-----|---------------|
| 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)      |
| O25     | 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  | 12  | 18   | 41  | 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 | A0 | A1 | B1 | B2 | B2 | D1 | D2 | Total No. |
|--------------------|----|----|----|----|----|----|----|-----------|
| N Te Sxt Gn Fm Nfx C Ls* | 4  | 7  | 2  | 3  | 1  | 1  | 6  | 24        |
| N Te Sxt Gn Fm Nfx Ls | 1  |    |    |    |    |    |    | 1         |
| N Te Sxt Gn Fm Nfx C | 2  | 6  | 1  | 5  | 4  | 1  | 19 |          |
| N Te Sxt Fm Nfx C Ls | 5  | 2  | 1  | 2  | 3  | 14 |    |          |
| N Te Sxt Gn Fm C Ls | 1  |    |    |    |    |    |    | 1         |
| N Te Sxt Fm Nfx C | 1  | 2  |    |    |    |    |    | 1         |
| N Te Sxt Gn Fm C Ls | 6  | 4  | 1  | 2  | 5  | 7  | 25 |          |
| N Te Sxt Fm C Ls | 1  | 1  |    |    |    |    |    | 2         |
| N Te Sxt Fm C Ls | 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  |    |    |    |    |    | 1         |
| N Te Sxt C Ls | 1  |    |    |    |    |    |    | 1         |
| N Te Sxt C Ls | 1  |    |    |    |    |    |    | 1         |
| N Te Sxt C Ls | 1  | 1  |    |    |    |    |    | 1         |
| N Te Sxt C Ls | 1  |    |    |    |    |    |    | 1         |
| N Te Sxt C Ls | 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-sulfamethxazole (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 linco-spectin (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 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 airsaculitis. 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 -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 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 (Ghanбарpour 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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