Effects of different phylotypes of avian pathogenic Escherichia coli *isolated from broiler chickens with colibacillosis on heterophils functional activities: an* in vitro *study*

Somayyeh Ahmadi, Habib Dastmalchi Saei*, Seyed Meisam Abtahi Froush and Mohammad Zavarshani

Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

*Corresponding author at: Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
E-mail: h.dastmalchi@urmia.ac.ir.*

Veterinaria Italiana 2021, **57** (4), 305-310. doi: 10.12834/VetIt.2111.12262.1
Accepted: 28.04.2020 | Available on line: 31.12.2021

Keywords
Avian,
Colibacillosis,
Escherichia coli,
Heterophil,
PCR,
Phylogenetic groups,
Immunological activities,
Iran.

Summary
Avian pathogenic *Escherichia coli* (APEC) is a major cause of colibacillosis and is associated with economic losses to the poultry production worldwide. Heterophils are the first line of immune defense of the avian host against invasive pathogens. In this study, APEC isolates from chickens with colibacillosis were assigned to phylogenetic groups and immunological activities of heterophils against these groups were examined. A total of 92 APEC isolates was obtained from 106 samples of diverse organs collected from chickens with colibacillosis from different farms in West Azerbaijan province, Iran. Isolates were assigned to phylogenetic groups based on the Clermont triplex PCR method, and immunological activities (including phagocytosis, respiratory burst and bacterial killing) of heterophils against these groups were examined. As results, the frequency of A, B1, B2 and D groups were 35.87, 44.57, 5.43 and 14.13%, respectively. In addition, opsonized *Escherichia coli* isolates belonging to B1 group significantly enhanced the level of respiratory burst (0.52 ± 0.02%) while the killing level of them was significantly lower than the other groups (29.40 ± 5.09%). There was no significant difference in phagocytic activity of heterophils against the phylogenetic groups. In conclusion, incomplete immune responses to B1 phylogenetic group maybe a principal cause of mortality by colibacillosis caused by this group. It is suggested to study heterophilic immune reaction against *E. coli* phylogenetic group for development of effective prevention strategy.

Introduction

Escherichia coli is a facultative Gram negative bacterium belonging to the normal flora of the mammals and birds intestine (Katouli 2010). This organism is one of the most frequent causes of many common primary and secondary bacterial infections, but it is generally considered to be an opportunistic pathogen that can cause infections following the suppression of the host immune system (Hussain *et al.* 2017b). *E. coli* causes different clinical manifestations in birds among which the colibacillosis is the most common form causing major economic impact (Lutful Kabir 2010). Based on the Clermont triplex PCR method, *E. coli* strains can be classified into four phylogenetic groups (A, B1, B2 and D), of which groups A and B1 are normal flora and group B2 and, to a lesser extent, group D are extra-intestinal pathogenic strains (Clermont *et al.* 2000). In poultry, avian pathogenic *E. coli* (APEC) is the main cause of, colibacillosis characterized by fever, depression and respiratory inflammation due to primary penetration of heterophils and monoconjugate phagocytes (Ramadan *et al.* 2016). It has been reported that young animals are more susceptible than adults to this illness. This transient sensitivity of chickens and turkeys to infection during the first weeks of life is associated with impaired quality of innate and adaptive immune system (Hussain *et al.* 2017a). Heterophils, the counterparts to the mammalian neutrophils, are the first line of defense against any microbial attack and play an indispensable role in the immune response of the avian host (Genovese *et al.* 2013). These cells fight against infectious diseases by phagocytosis and respiratory burst (Kogut *et al.* 2012). The aims

of the present study were first the phylogenetic typing of *E. coli* isolated from poultry and then the evaluation phagocytosis, respiratory burst and bactericidal activity of heterophils against different phylogenetic groups.

Materials and methods

Sampling

One-hundred and six samples of different organs (liver, heart, lung and spleen) were collected between 2015 and 2016 from broiler chickens with colibacillosis on multiple flocks located in different regions (Urmia, Naqadeh, and Khoy) of West Azerbaijan province, Iran. All the samples were cultured primarily in MacConkey agar at 37 °C for 24 h (MCA/105465, Merck, Darmstadt, Germany). Pink-colored colonies which were lactose positive, suspected as *E. coli* on MCA were further streaked on Eosin-Methylene Blue agar (EMB/101347, EMB, Merck, Darmstadt, Germany) for purification. Isolates were then subjected to standard biochemical tests including IMViC (Indole, Methyl red, Voges-Proskauer and Citrate), urease production, H_2S production and various sugar fermentation tests (Li *et al.* 2010).

DNA extraction and confirmation of *E. coli* isolates

After identification of the *E. coli* isolates by conventional methods, a single colony of each isolate was cultured onto blood agar medium and incubated at 37 °C for 18-24 hours. Bacterial DNA was extracted by boiling method as described previously (Obeng *et al.* 2012). Purity of extracted DNA was determined based on measurement of OD 260/280 using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Additionally, the integrity of extracted DNA was evaluated by electrophoresis in 1% agarose gel. DNA with an OD260/OD280 ratio of ≥ 1.8 was considered to be pure and used in PCR. PCR amplification of a 662-bp DNA fragment size of 23S rRNA gene was also conducted to determine any inhibitors remained from the extraction procedure by using Eco 2083 (5'-GCT TGA CAC TGA ACA TTG AG-3') and Eco 2745 (5'- GCA CTT ATC TCT TCC GCA TT-3') primers (Riffon *et al.* 2001).

Detection of phylogenetic groups

Phylogenetic group assignment of the isolates was performed using the Clermont triplex PCR method (Clermont *et al.* 2000). Primer sequences for phylogenetic classification are shown in Table I.

Avian heterophils preparation

The isolation of heterophild, their count and their viability were carried out and assessed as previously described (Andreasen and Latimer 1989, Blattes *et al.* 2017). In brief, the blood samples from healthy chicken were transferred to the EDTA tubes and mixed with 1% methylcellulose, in calcium and magnesium-free Hank's balanced salt, at a 1:5 ratios. The samples were centrifuged for 20 min at 25 *g*. The buffy coat was isolated, washed and re-suspended. Following the procedure, the samples were centrifuged on a Ficoll-Hypaque density gradient (400 *g*, 30 min). The mononuclear cell layer and plasma were removed, and contaminant erythrocytes were deleted by hypotonic lysis. The remaining heterophils were washed twice and counted in a Neubauer chamber. The viability of the cells was checked by Trypan blue dye exclusion.

Evaluation of respiratory burst of heterophils by Nitroblue tetrazolium reduction (NBT)

The suspension of opsonized bacteria and intracellular generation of reactive oxygen species (ROS) were measured by NBT reduction as described previously (Chen and Junger 2012, Motlagh *et al.* 2015). Non-opsonized bacteria were used as control.

Phagocytosis and microbial killing activities of heterophils

Two tubes were used as test and control. Equal volume (0.5 ml) of heterophil suspension (5×10^6 cell/ml) and chicken serum were mixed in the test tube in equal volume. In the control tube, 0.5 ml of HBSS buffer was mixed with chicken serum instead of heterophil suspension. Both tubes were incubated for 5 minutes at 37 °C. Then, 0.5 ml of bacterial suspension (5×10^7 cfu/ml in HBSS) added to each tube and then at this moment, zero time, 100 µl of the test and control tubes were removed and added to the new tubes. The test and control tubes were incubated at 37 °C for 90 minutes. In this stage we had two kinds of data: zero and 90 data.

Table I. *Primer sequences used and size of PCR products.*

Primer	Primer sequence (5'→3')	Size (bp)
*chu*A	F: GACGAACCAACGGTCAGGAT	279
	R: TGCCGCCAGTACCAAAGACA	
*yja*A	F: TGAAGTGTCAGGAGACGCT	211
	R: ATGGAGAATGCGTTCCTCAAC	
TSPE4.C2	F: GAGTAATGTCGGGGCATTCA	152
	R: CGCGCCAACAAAGTATTACG	

Zero and 90 data were related to zero time (control tube: C0, testing tube: T0) and 90 min (control tube: C90, testing tube: T90) after incubation, respectively. Of note, the viability of heterophili along the experiment was checked during experimental set up. Fortunately, no significant change was found. Ten ml of sterile distilled water was added into each tube in order to lyse the heterophils. The mixture was stirred a few times slowly until the tube contents were thoroughly mixed. One hundred µl of current mixture were removed and transferred to 96 wells plate. For each dilution, five wells were considered and then 20 µl of MTT solution (5 mg/mL, Sigma - Aldrich, USA) were added to each well. Then it was incubated for 20 min at 37 °C. After incubation, 150 µl of DMSO solution were added to each well to dissolve Formasone crystals. The results of optical densities (OD) were read at 492 nm wave lenght (BioTek ELX800, USA). The bactericidal activity (percentage of killing) was calculated by the formula at the end of process (Mehrzad *et al.* 2009).

The test and the control tubes which had been incubated for 90 min were centrifuged at 300 g for 10 min. One hundred µl of supernatant (containing no phagocytize bacteria) were added to the five wells of 96-wells plate. The plate was incubated for 20 min at 37 °C after adding 20 µl of MTT to each well. One hundred fifty µl of DMSO were added to each well in the next step and then the data were read at 492 nm wave lenght. Percentage of phagocytosis was calculated using the formula (Gargan *et al.* 1993, Fijalkowski *et al.* 2012, Mehrzad *et al.* 2009).

Data statistical analysis

Data analysis was done by SPSS statistical software, version 18. The results of groups were analyzed statistically by Kruskal-Wallis test. Also, Mann-Whitney U-test was used to compare differences between two groups. P value less than 0.05 was considered significant.

Results

E. coli detection

A total of 92 *E. coli* isolates were detected from broiler chicken with colibacillosis by biochemical tests as well as amplification of the 23S rRNA gene fragment.

Phylogenetic classification of *E. coli* isolates

All *E. coli* isolates were classified to one of the four main phylogenetic groups, with A, B1, B2 and D groups were observed in 35.87, 44.57, 5.43 and 14.13% of the strains, respectively.

Evaluation of respiratory burst, phagocytic and bacterial killing activities of heterophils

The viability of heterophils was 94%. Results of Kruskal-Wallis test showed that there were statistically significant differences between different phylogenetic groups in the assessment of respiratory burst and microbial killing of heterophils (P value < 0.05), whereas no significant change in the phagocytic activity of heterophils was observed between the groups (Table II). According to our results, the mean ± standard deviation differences of the optical density in the evaluation of microbial killing activities of heterophils against B1 phylogenetic group were less than other groups (P value < 0.05). There were no significant differences between other groups. However, obtained data showed that the heterophils had the highest potential to kill the group B2 bacteria compared to bacteria in groups A and D. In contrast to microbial killing activity, the results showed that the amount of oxygen free radicals during respiratory burst was significantly higher than other groups against *E. coli* belonging to group B1 (P value < 0.05). In comparison between bacteria belonging to groups A, B1 and D, the group B2 showed the lowest potential to induce respiratory burst in the heterophils. Despite the differences between the groups, they weren't significant in evaluating the phagocytic activities of heterophils against the different phylogenetic groups of *E. coli*. Albeit, the heterophils showed more potential to uptake the group D bacteria compared to other groups (Table III).

Discussion

Avian colibacillosis caused by avian pathogenic *E. coli* (APEC) is one of the important disease of poultry in terms of economic losses throughout the world (Guabiraba and Schouler 2015). There are evidences suggesting a meaningful relationship between pathogenicity of APEC and their phylogenetic

Table II. *The details of Kruskal-Wallis test of optical density of respiratory burst, microbial killing and phagocytic activities of heterophils.*

	Respiratory burst	Microbial Killing	Phagocytosis
Chi-Square	22.55	19.64	7.56
df	3	3	3
Asymp. Sig.*	0.00	0.00	0.06

*P value < 0.05

Table III. *Comparison of optical density of respiratory burst, microbial killing and phagocytic activities of heterophils (Mean ± SD) between two groups.*

Group	Respiratory burst (Optical Density)	Microbial killing (%)	Phagocytosis (%)
A	0.41 ± 0.03	41.10 ± 6.11	58.37 ± 13.80
B[1]	0.52 ± 0.02[a]	29.40 ± 5.09[a]	78.06 ± 25.08
B[2]	0.40 ± 0.05	43.05 ± 5.84	66.36 ± 19.21
D	0.43 ± 0.03	39.26 ± 4.96	85.54 ± 27.13

[a]Significant differences ($p < 0.05$).

groups (Jaureguy *et al.* 2008, Moulin-Schouleur *et al.* 2007). In the current study, phylogenetic analysis revealed that APEC isolates segregated mainly in phylogenetic groups B1 (41/92; 44.57%) and A (33/92; 35.87%), indicating that intestinal strains are an important cause of colibacillosis in the West Azerbaijan, Iran. An high prevalence of phylogenetic groups A and B1 strains has also been reported elsewhere in Iran from colibacillosis cases (Alizade *et al.* 2017, Ghanbarpour *et al.* 2011) as well as among verotoxigenic *E. coli* (VTEC) isolates from the faeces of healthy sheep and broiler chicken in Northwestern Iran (Saei and Zavarshani 2018). Resistance to ciprofloxacin, norfloxacin, co-trimoxazole, nalidixic acid and cefoperazone/sulbactam is especially prevalent among group B1 isolates and group A. Antibiotic selection pressure may explain why most of the drug resistant strains of *E. coli* belong to the phylogenetic groups A and B1 (Obeng *et al.* 2012). Similar results have also been reported by Kazemnia and colleagues (Kazemnia *et al.* 2014). Other speculation could be the derivation of pathogenic *E. coli* strains from commensal strains by the acquisition of virulence factors (Duriez *et al.* 2001). In a study carried out on distribution of virulence genes among *E. coli* isolates from bovine mastitis in Iran, isolates belonging to the phylogenetic group B1 exhibited higher prevalence of various virulence genes in comparison to groups A and D (Ghanbarpour and Oswald 2010).

Following the identification of pathogenic agents by heterophils, immune responses are initiated and eventually the pathogen is eliminated. In this regard, it has been shown that heterophils play a significant role in early host defense against bacterial agents such as *E. coli* in poultry (Ariaans *et al.* 2008, Gomis *et al.* 2003). In the present study, we found a significant difference in functional activities of heterophils against different phylogenetic groups of APEC from broiler chicken with colibacillosis. This could be attributed to differences in genetic backgrounds, virulence factors as well as antigenicity among phylogroups of APEC. In a study carried out by Mellata and colleagues, phagocytic activities of chicken heterophils were evaluated against *E. coli*. In that study, *E. coli* isolates were categorized based on some special factors such as O, fimbriae and K antigens. Finally, they reported that the presence of fimbriae and O78 antigens were effective in protecting *E. coli* against heterophils and macrophages phagocytosis (Mellata *et al.* 2003).

As results, *E. coli* isolates belonging to group B1 were the most stimulating group in secretion of oxygen radicals by heterophils. This could be attributed to differences in bacterial cell surface antigens among studied phylogenetic groups. Study carried out by Ondrackova and colleagues showed that O149 strain of Enterotoxigenic *E. coli* (ETEC) was more efficiently associated with the neutrophils and induced a more intensive respiratory burst compared to the O147 strain of ETEC. They suggested that this difference might be due to the presence of different types of fimbriae and other factors such as flagella. Moreover, induction of higher respiratory burst by B1 phylogenetic group might be a reason for considerable economic losses due to this group which is the most common group in the studied region. It has been shown that the accumulation of inflammatory exudates in the airbag of chicken lung following the excessive release of oxygen radicals by heterophils due to much more respiratory burst is one of the main causes of chick death and as a consequence the high mortality rate of colibacillosis (Kemmett *et al.* 2014). The strong induction of respiratory burst is probably due to the strong presence of pattern recognition receptors such as Toll-like receptors (TLRs) and mannose recognition receptors in B1 group (Genovese *et al.* 2013). Interestingly, despite the strong induction of phagocytosis and respiratory burst in group B1, the bacterial death was defective by heterophils. This may be due to the presence of strong anti-free radical agents in group B1. For example *yjaA* gene encoding protein has been reported to have an important role against oxygen radicals as an anti-oxidant (Bonacorsi *et al.* 2000). In previous studies, it has been proven that APEC can cut down the antimicrobial activity of heterophils (Mellata *et al.* 2003, Qureshi 2003).

There was no significant difference in phagocytic activity of heterophils, indicating their high phagocytic ability against different phylogenetic groups of APEC likely due to the broad array of phagocytic receptors. Avian heterophils have been shown to express various combinations of pattern recognition receptors (PRR), including Toll-like receptors (TLRs), scavenger receptors, dectin-1, and mannose receptors that allow them to recognize and respond to foreign microbial invaders (Genovese *et al.* 2013). Other speculation could be the lack of difference in strategies used by tested APEC isolates to avoid phagocytic engulfment. In this regard the role for K1 capsule, O78 antigen, P fimbriae, and the

0-min region in initial avoidance of phagocytosis has been demonstrated (Mellata *et al.* 2003). Further studies on distribution of various virulence factors among different phylogenetic groups of APEC and their interaction with heterophils will provide important insight into some of the mechanisms that they employ to avoid uptake and survive elimination by avian heterophils and cause clinical disease.

This study, for the first time, indicated that there were significant differences in microbial killing and respiratory burst activities of heterophils against different phylogenetic groups of *E. coli* without any significant difference in phagocytosis. However, it should be noted that immune responses are not only influenced by bacterial genetic, but also affected by the host genetic. Therefore, the identification of the dominant phylogenetic groups of *E. coli* in a region is crucial in order to develop several strategies such as vaccination against colibacillosis. It is hoped that our results help us and other researchers to choose appropriate ways to prevent and treat colibacillosis by identifying the sensitivity of heterophils to different groups of *E. coli*.

Grant support

This work was supported by the research fund of Urmia University, Urmia, Iran.

References

Alizade H., Ghanbarpour R., Jajarami M. & Askari A. 2017. Phylogenetic typing and molecular detection of virulence factors of avian pathogenic *Escherichia coli* isolated from colibacillosis cases in Japanese quail. *Vet Res Forum*, **8**, 55-58.

Andreasen C.B. & Latimer K.S. 1989. Separation of avian heterophils from blood using Ficoll-Hypaque discontinuous gradients. *Avian Dis*, **33**, 163-167.

Ariaans M.P., Matthijs M.G., van Haarlem D., van de Haar P., van Eck J.H., Hensen E.J. & Vervelde L. 2008. The role of phagocytic cells in enhanced susceptibility of broilers to colibacillosis after Infectious Bronchitis Virus infection. *Vet Immunol Immunopathol*, **123**, 240-250.

Blattes G.B., Mestieri L.B., Bottcher D.E., Fossati A.C., Montagner F. & Grecca F.S. 2017. Cell migration, viability and tissue reaction of calcium hypochlorite based-solutions irrigants: an *in vitro* and *in vivo* study. *Arch Oral Biol*, **73**, 34-39.

Bonacorsi S.P.P., Clermont O., Tinsley C., Le Gall I., Beaudoin J.C., Elion J., Nassif X. & Bingen E. 2000. Identification of regions of the *Escherichia coli* chromosome specific for neonatal meningitis-associated strains. *Infect Immun*, **68**, 2096-2101.

Chen Y. & Junger W.G. 2012. Measurement of oxidative burst in neutrophils. *Methods Mol Biol*, **844**, 115-124.

Clermont O., Bonacorsi S. & Bingen E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol*, **66**, 4555-4558.

Duriez P., Clermont O., Bonacorsi S., Bingen E., Chaventre A., Elion J., Picard B. & Denamur E. 2001. Commensal *Escherichia coli* isolates are phylogenetically distributed among geographically distinct human populations. *Microbiology*, **147**, 1671-1676.

Fijalkowski K., Czernomysy-Furowicz D., Irwin J.A., Nawrotek P. & Pobucewicz A. 2012. Secretory virulence factors produced by *Staphylococcus aureus* isolates obtained from mastitic bovine milk effect on bovine polymorphonuclear neutrophils. *Res Vet Sci*, **93**, 82-87.

Gargan R.A., Hamilton-Miller J.M. & Brumfitt W. 1993. Effect of pH and osmolality on in vitro phagocytosis and killing by neutrophils in urine. *Infect Immun*, **61**, 8-12.

Genovese K.J., He H., Swaggerty C.L. & Kogut M.H. 2013. The avian heterophil. *Dev Comp Immunol*, **41**, 334-340.

Ghanbarpour R. & Oswald E. 2010. Phylogenetic distribution of virulence genes in *Escherichia coli* isolated from bovine mastitis in Iran. *Res Vet Sci*, **88**, 6-10.

Ghanbarpour R., Sami M., Salehi M. & Ouromiei M. 2011. Phylogenetic background and virulence genes of *Escherichia coli* isolates from colisepticemic and healthy broiler chickens in Iran. *Trop Anim Health Prod*, **43**, 153-157.

Gomis S., Babiuk L., Godson D. L., Allan B., Thrush T., Townsend H., Willson P., Waters E., Hecker R. & Potter A. 2003. Protection of chickens against *Escherichia coli* infections by DNA containing CpG motifs. *Infec immun*, **71**, 857-863.

Guabiraba R. & Schouler C. 2015. Avian colibacillosis: still many black holes. *FEMS Microbiol Lett*, **362**, 118.

Hussain A., Shaik S., Ranjan A., Nandanwar N., Tiwari S.K., Majid M., Baddam R., Qureshi I.A., Semmler T., Wieler L.H., Islam M.A., Chakravortty D. & Ahmed N. 2017a. Risk of transmission of antimicrobial resistant *Escherichia coli* from commercial broiler and free-range retail chicken in India. *Front Microbiol*, **8**, 2120.

Hussain H.I., Iqbal Z., Seleem M.N., Huang D., Sattar A., Hao H. & Yuan Z. 2017b. Virulence and transcriptome profile of multidrug-resistant *Escherichia coli* from chicken. *Sci Rep*, **7**, 8335.

Jaureguy, F., Landraud L., Passet V., Diancourt L., Frapy E., Guigon G., Carbonnelle E., Lortholary O., Clermont O., Denamur E., Picard B., Nassif X. & Brisse S. 2008. Phylogenetic and genomic diversity

of human bacteremic *Escherichia coli* strains. *BMC genomics*, **9**, 560.

Katouli M. 2010. Population structure of gut *Escherichia coli* and its role in development of extra-intestinal infections. *Iran J Microbiol*, **2**, 59-72.

Kazemnia A., Ahmadi M. & Dilmaghani M. 2014. Antibiotic resistance pattern of different *Escherichia coli* phylogenetic groups isolated from human urinary tract infection and avian colibacillosis. *Iran Biomed J*, **18**, 219-224.

Kemmett K., Williams N.J., Chaloner G., Humphrey S., Wigley P. & Humphrey T. 2014. The contribution of systemic *Escherichia coli* infection to the early mortalities of commercial broiler chickens. *Avian Pathol*, **43**, 37-42.

Kogut M.H., Genovese K.J., Nerren J.R. & He H. 2012. Effects of avian triggering receptor expressed on myeloid cells (TREM-A1) activation on heterophil functional activities. *Dev Comp Immunol*, **36**, 157-165.

Li B., Sun J.Y., Han L.Z., Huang X.H., Fu Q. & Ni Y.X. 2010. Phylogenetic groups and pathogenicity island markers in fecal *Escherichia coli* isolates from asymptomatic humans in China. *Appl Environ Microbiol*, **76**, 6698-66700.

Lutful Kabir S.M. 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and Public Health concerns. *Int J Environ Res Public Health*, **7**, 89-114.

Mehrzad J., Duchateau L. & Burvenich C. 2009. Phagocytic and bactericidal activity of blood and milk-resident neutrophils against *Staphylococcus aureus* in primiparous and multiparous cows during early lactation. *Vet Microbiol*, **134**, 106-112.

Mellata M., Dho-Moulin M., Dozois C.M., Curtiss R.3rd., Lehoux B. & Fairbrother J.M. 2003. Role of avian pathogenic *Escherichia coli* virulence factors in bacterial interaction with chicken heterophils and macrophages. *Infect immun*, **71**, 494-503.

Motlagh B.M., Ahangaran N.A. & Froushani S.M. 2015. Calcitriol modulates the effects of bone marrow-derived mesenchymal stem cells on macrophage functions. *Iran J Basic Med Sci*, **18**, 672-676.

Moulin-Schouleur M., Reperant M., Laurent S., Bree A., Mignon-grasteau S., Germon P., Rasschaert D. & Schouler C. 2007. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. *J Clin Microbiol*, **45**, 3366-3376.

Obeng A.S., Rickard H., Ndi O., Sexton M. & Barton M. 2012. Antibiotic resistance, phylogenetic grouping and virulence potential of *Escherichia coli* isolated from the faeces of intensively farmed and free range poultry. *Vet Microbiol*, **154**, 305-315.

Ondrackova P., Alexa P., Matiasovic J., Volf J. & Faldyna M. 2012. Interaction of porcine neutrophils with different strains of enterotoxigenic *Escherichia coli*. *Vet Microbiol*, **160**, 108-116.

Qureshi M.A. 2003. Avian macrophage and immune response: an overview. *Poult Sci*, **82**, 691-698.

Ramadan H., Awad A. & Ateya A. 2016. Detection of phenotypes, virulence genes and phylotypes of avian pathogenic and human diarrheagenic *Escherichia coli* in Egypt. *J Infect Dev Ctries*, **10**, 584-591.

Riffon R., Sayasith K., Khalil H., Dubreuil P., Drolet M. & Lagace J. 2001. Development of a rapid and sensitive test for identification of major pathogens in bovine mastitis by PCR. *J Clin Microbiol*, **39**, 2584-2589.

Saei H.D. & Zavarshani M. 2018. Phylogenetic grouping of Verotoxigenic *Escherichia coli* (VTEC) obtained from sheep and broiler chicken in Northwestern Iran. *Acta Vet Eurasia*, **44**, 53-58.