DR. CHENGYU LIU (Orcid ID : 0000-0002-1934-0684)

DR. HAO CHEN (Orcid ID : 0000-0001-7353-217X)

DR. YI TANG (Orcid ID : 0000-0001-5002-8253)

PROF. YOU XIANG DIAO (Orcid ID : 0000-0001-9021-0236)

Article type : Original Article

Duck viral infection escalated the incidence of avian pathogenic *Escherichia coli* in China

Running title: Avian pathogenic *E. coli* in ducks

Chengyu Liu[1,2,3], Youjiang Diao[4], Dongxue Wang[1,2,3], Hao Chen[5], Yi Tang[1,2,3,*], Youxiang Diao[1,2,3,*]

1 College of Veterinary Medicine, Shandong Agricultural University, Shandong, China;

2 Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Tai'an, Shandong, China;

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/tbed.13107

3 Shandong Provincial Engineering Technology Research Center of Animal Disease Control and Prevention, Tai'an, Shandong, China;

4 Shandong Agricultural and Engineering University, Shandong, China

5 College of life sciences, Qufu Normal University, Shandong, China

Correspondence:

Yi Tang, tyck288@163.com

Youxiang Diao, yxdiao@163.com

Summary

Avian pathogenic *Escherichia coli* (APEC) causes high mortality in poultry flocks and often is complicated with viral infections, leading to large economic losses; however, little information is available on the epidemiological characteristics of this pathogen in ducks. Therefore, a systemic epidemiological investigation was performed on 325 duck farms from 13 provinces in China during the period of 1 April 2016 until 31 March 2018, covering two years. A total of 26 APEC strains were isolated from different farms in this study, and analysis showed that all of those isolates carried multiple virulence-associated genes and drug-resistance genes, which led to high pathogenicity (15/26), strong or moderate biofilm formation (24/26) and multidrug resistant abilities (26/26). On the other hand, coinfection

with APEC, H9 avian influenza virus (AIV) and Tembusu virus (TMUV) was very common on those farms (11/26), with APEC and TMUV sharing a similar morbidity peak (from May to September) and susceptibility (60% infections occurred in ducklings); thus, we speculated that the emerging TMUV infection escalated the APEC incidence in ducks. Finally, the data presented in this report enhance the current understanding of the epidemiology of APEC and different viral infections in ducks and provide additional insight into the critical factors that determine their pathogenicity. Meanwhile, the emergence of multidrug-resistant APEC strains and their coinfection with different viruses emphasize that preventive measures against such infections on poultry farms should be implemented immediately.

Keywords: avian pathogenic *Escherichia coli*, coinfection, duck, pathogenicity, drug resistance

Introduction

Avian pathogenic *Escherichia coli* (APEC) is the major etiology for avian colibacillosis, including several diseases such as pericarditis, perihepatitis, peritonitis, airsacculitis, septicemia and other mainly extraintestinal diseases in poultry (Dho-Moulin and Fairbrother, 1999). Those diseases can induce high morbidity and mortality in ducks of different ages and threaten their long-term health (Saif et al., 2003; Wei et al., 2013). However, going from bad to worse, avian colibacillosis has emerged in an endless stream with the rapid development of the poultry industry and significant increases in stocking density, leading to large losses

(Croxen and Finlay, 2010; Kaper, 2005), especially in China (Li et al., 2015; Dou et al., 2016; Wang et al., 2016).

Initially, conventional antibacterial drugs played an important role in the control of APEC, and the extensive use of these drugs achieved some control. However, continuous indiscriminate use and abuse has led to the emergence of drug-resistant strains of *E. coli* and even to multidrug-resistant (MDR) strains (Tang et al., 2009), which decrease the medicinal effects and represent a great challenge to the poultry industry. More importantly, because the transmissibility of the drug-resistant determinants from *E. coli* to other pathogenic bacteria has the potential to create superbugs and induce incurable bacterial infections, the drug resistance of APEC should be a focus of future research (Bass et al., 1999; Wang et al., 2010; Dheilly et al., 2013).

On the other hand, the clinical and pathological symptoms of the colibacillosis in poultry are complex, and often the virus is complicated with additional viral infections (Mosleh et al., 2016). Epidemiological investigations have shown that the positive rates of H9 avian influenza virus (AIV), Tembusu virus (TMUV), Newcastle disease virus (NDV), duck parvovirus (DPV) and fowl adenovirus (FAdV) and other pathogens are universally high in different duck flocks (Niu et al., 2017a, 2017b); coinfection with those viruses, especially with emerging TMUV infections, could destroy the immune system of ducks, induce an infection secondary to APEC and produce synergy, thereby resulting in more serious clinical

signs than monoinfection, which should draw much attention (Todd, 2000). Furthermore, previous study also has shown that coinfection of APEC with H9 AIV could exacerbate the condition of either disease compared to each one alone (Mosleh et al., 2017).

Therefore, this study investigated APEC and coinfected viruses on 325 duck farms in 13 provinces of China, and the drug resistance, biofilm formation, pathogenicity, phylogenetic group, antimicrobial resistance genes and virulence genes of APEC isolated in this study were also systemically analyzed to characterize the biological properties and molecular basis of each, which can provide valuable information about the epidemiology of APEC in the field.

Materials and Methods

Ethics Statement

All animal infection experiments were approved by the Animal Care and Use Committee of Shandong Agricultural University and conducted in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China).

Sample collection

The study included 1194 clinical liver samples collected from 325 scaled duck farms (Figure S1). The period for sample collection extended over two years, from 1 April 2016 until 31 March 2018, which allowed investigation of the relationship between the infection prevalence and season.

The samples analyzed in this study were all collected from duck farms with relatively sound breeding conditions, and no obvious clinical symptoms were observed in those flocks. In addition, for welfare reasons, the ducks were euthanized using intravenous pentobarbital sodium (New Asia Pharmaceutical, China), which was followed by dissection of the liver for bacterial isolation and PCR detection.

Isolation and identification of the *E. coli*

All liver samples were collected from ducks on a super-clean bench and streaking inoculation was performed on MacConkey agar (High-Tech Park HaiBo Company, Qingdao, China) for isolation of the *E. coli*. After being cultured for 20 h at 37 °C, a monoclonal colony was picked from the culture medium for inoculation of LB agar (HaiBo), which was cultured overnight at 37 °C for further enrichment. The bacterial solution was then used for the extraction of DNA, with a Rapid DNA extraction kit (Omega, USA) being used according to the manufacturer's instructions; this DNA served as the template for PCR identification of the

16S rDNA of the isolates with the same primers and method used in the previous literature (Hu et al. 2011). Furthermore, isolates identified as *E. coli* were further culture identified on triple sugar iron agar, and additional identifications were made using glucose, lactose, maltose, mannitol and sucrose fermentation tests. Meanwhile, the phylogenetic group of those strains was determined according to a published multiplex PCR method (Clermont et al., 2000).

Pathogenicity analysis

The pathogenicity of those isolates identified as *E. coli* were further determined by systemic animal experiments. Specially, all isolates were used to inoculate LB agar (HaiBo) and cultured overnight at 37 °C, and a monoclonal colony from the culture medium was picked to inoculate LB agar (HaiBo), which was then cultured for 4 hours at 37 °C. Afterwards, 15% glycerite was used to mix all colonies in each plate, and the final concentration of the samples was determined as $1.0*10^8$ CFU/mL. Each sample was used to inoculate six one-week-old Cherry Valley ducks by left thoracic air sac with a dose of $1.0*10^7$ CFU for each duckling. After being inoculated, all ducks were monitored daily for the occurrence of clinical signs, and mortality of the ducks was recorded daily. All isolates were divided into three grades based on the follow criterion: high pathogenicity (3 or more ducks dead), middle pathogenicity (1 or 2 ducks dead) and low pathogenicity (no duck dead, but typical clinical symptoms including pericarditis and perihepatitis appeared after inoculation). Meanwhile, the main clinical signs and postmortem lesions presented by the affected ducks were recorded.

For the histological examination, samples of the livers, hearts, spleens and kidneys from the affected birds were fixed in formalin, embedded in paraffin wax and cut into sections. The sections were stained with hematoxylin and eosin and examined for lesions by light microscopy.

Biofilm formation detection

The biofilm formation detection of all APEC isolates was performed according to the previous literature (Wang et al., 2011). Specifically, after being cultured in LB agar, 100 μL of bacterial solution for each isolate was added to a well in a 96-well plate (Corning Costar, USA), with aseptic LB agar used as the negative control. After being cultured for 36 h at 37 °C, all solutions were discarded, and each well of the plate was washed three times using sterile phosphate buffered saline (PBS); the plate was then placed in a ventilation closet and allowed to dry naturally. Crystal violet (100 μL, 0.1%) was then used to stain the wells for 30 min, and each well of the plate was washed again 3 times using PBS; again, the plate was placed in a ventilation closet and allowed to dry naturally. Next, ethanol (200 μL, 95%) was added to each well of the plate, and the OD595 was determined on a SpectraMax i3X plate reader (BioTek, USA). All isolates were divided into four grades based on the method in a previous study (Stepanovic et al. 2000) (OD is the OD595 of the bacterial subject, whereas ODc is the OD595 of the negative control) as follow: OD < ODc (No biofilm-forming ability); ODc < OD < 2ODc (isolate with weak biofilm-forming ability); ODc < OD < 4ODc

(isolate with moderate biofilm-forming ability); and 4ODc < OD (isolate with strong biofilm-forming ability).

Drug susceptibility testing

In this study, 20 antimicrobial agents, including lincomycin, clindamycin, doxycycline, polymyxin B, cefotaxime, cefradine, ofloxacin, minocycline, amikacin, gentamicin, neomycin, tetracycline, streptomycin, ciprofloxacin, florfenicol, penicillin, amoxicillin, erythromycin, bacilli peptide, and kanamycin, were employed for testing the drug susceptibility of all isolates according to the Performance Standards for Antimicrobial Susceptibility Testing (Clinical and Laboratory Standards Institute (CLSI), Wayne, PA, US). Specifically, all isolates were inoculated onto LB agar plates with the susceptibility paper disks impregnated with the abovementioned 20 antimicrobial agents affixed on their surface. After being cultured for 16 h at 37 °C, the susceptibility of the isolates was determined for each drug according to the CLSI standard by measuring and comparing the diameters of the inhibition zones, which were then recorded as high susceptibility (HS), medium susceptibility (MS), low susceptibility (LS) or resistant (R) for each isolate.

PCR screening for drug resistance genes and virulence-associated genes

The DNA of each *E. coli* isolate was prepared and extracted using the abovementioned methods, with PCR primers targeting drug-resistance genes and virulence-associated genes

being designed according to published studies (Janben et al., 2001; Ragione et al., 2002; Ewers et al., 2005). Detailed information about the primers is shown in Table S1 and Table S2. All PCR amplification products were analyzed on a 1% agarose gel that was stained with ethidium bromide, purified by the Gel Band Purification Kit (Omega, Bio-Tek, Norcross, GA) and cloned into the PMD18-T vector (TaKaRa, Bio Inc., Japan), followed by sequencing in triplicate using an ABI 3730 Sanger-based genetic analyzer (Applied Biosystems, Carlsbad, CA). The DNA sequences were assembled using DNAStar (version 6.0). Multiple sequence alignment was performed using the ClustalW (BioEdit version 7.0) program, and a comparison of sequence identity was performed using MegAlign software (DNAStar). Phylogenetic analysis was performed using the MEGA 5.0 (DNAStar).

Virus detection

At first, viral nucleic acid, including DNA and RNA, was extracted from the liver samples as template using a total DNA/RNA isolation kit (Omega, USA), and the RNA was transformed into cDNA using a Reverse Transcriptase AMV kit (Takara, Japan) for the subsequent PCR assay. A triplex PCR assay was then employed to detect the H9 AIV, NDV and TMUV according to a previous study (Niu et al., 2017). At the same time, PCR for detecting DPV and FAdV was performed with primers designed according to sequences from GenBank, using the above templates. Detailed information about the primers is shown in Table S3.

Statistical analysis

The positive rate (%) of infection was calculated as follows:

$$\text{Positive rate (Farms)} = \frac{\text{number of the positive farms}}{\text{total number of farms}} \times 100\%$$

The rate of coinfection was defined as the ratio of cases with more than one infection and was calculated as follows:

$$\text{Coinfection rate} = \frac{\text{number of the positive farms with coinfeciton}}{\text{total number of farms}} \times 100\%;$$

Results

APEC identification and phylogenetic group analysis

This study analyzed 325 duck farms from across China, and APEC was isolated from 26 farms by MacConkey nutrition agar and chromogenic media and identified by 16S rDNA. Meanwhile, all isolates were further classified into two phylogenetic groups according to a previous study: group B2 (57.69%, 15/26) and group D (42.31%, 11/26).

Pathogenicity analysis and biofilm-formation ability assays

Animal experiments using 26 strains of APEC isolates verified 15 highly pathogenic isolates, 8 intermediately pathogenic isolates and 3 lowly pathogenic isolates. All APEC strains were able to induce severe perihepatitis (Figure 1a), pericarditis (Figure 1b), splenic congestion (Figure 1c), air sac turbidity (Figure 1d) and renal lesions (Figure 1e) in the experimentally

infected ducks, whereas no clinical symptoms appeared in uninfected ducks raised under the same conditions (Figure 1f-j). Histological examination revealed typical swelling and necrosis of liver and kidney cells (Figure 2a-b), cardiac particle deformation (Figure 2c), and disintegration of the splenic cells (Figure 2d) in the affected ducks. Meanwhile, this study also found that, out of 26 APEC isolates, 4 isolates (15.38%) possessed strong biofilm-forming ability, 20 isolates (76.92%) possessed moderate ability, and 2 isolates (7.69%) showed weak ability.

Drug susceptibility testing

Susceptibility testing results (Figure 3) demonstrated that all isolates had a high resistance rate to antibiotics that have been clinically applied in the poultry industry for a long time. Among this resistance, resistance rates to lincomycin, clindamycin, ofloxacin, penicillin, amoxicillin and bacilli peptide were higher than 80%, whereas low resistance was observed to doxycycline (15.40%) and amikacin (0.00%). The resistance rates to the other antibiotics (polymyxin B, cefotaxime, gentamycin, and neomycin) were less than 50%. Meanwhile, all isolates were MDR strains, whereas the resistance antibiotics ranged from 8 to 16 with corresponding proportions of 3.85%, 15.38%, 19.23%, 15.38%, 15.38%, 11.54%, 11.54%, 3.85% and 3.85%, respectively.

Detection of drug resistance genes and virulence-associated genes

Detection results for 11 virulence-associated genes of the 26 strains of APEC isolates showed that the detection rates for *iss, iucD, lusS, tsh, fimC,* and *pfs* were higher than 80%, which demonstrated that all those drug resistance genes generally exist in APEC (Figure 4). On the other hand, detection results for the drug resistance genes showed that the detection rates for *SUL3, TETa, GYRa, GYRb* and *mcr-1* were higher than 60%, whereas the detection rate of FLOR was 57.69% (Figure 5). More importantly, a phylogenetic analysis of those genes showed the detailed proportions and specific genetic relationships among them.

Positive rate of APEC and different viruses

In the study period, 325 duck farms were chosen for detection of APEC and six viral infectious diseases, as shown in Figure 7. Among the collected samples, the positive rates for APEC, H9 AIV, TMUV, DPV, NDV and FAdV were 8.00% (26/325), 39.38% (128/325), 14.76% (48/325), 13.53% (44/325), 11.38% (37/325) and 6.15% (20/325), respectively (Figure 6a). Meanwhile, in those duck farms identified as APEC-positive (n=26), the positive rates of the above viruses were 84.61% (22/26), 73.07% (19/26), 30.76% (8/26), 7.69% (2/26), 11.53% (3/26), respectively (Figure 6b).

Coinfection situation analysis

The detection data were also analyzed to show the complex coinfection situation in farms with APEC (Figure 7). The results showed that all APEC-positive farms were infected with other viruses, synchronously, and the coinfection of APEC with two viruses was common, for a rate of 62% (16/26), indicating that such coinfection is widespread. Meanwhile, an infection mix with APEC, H9 AIV and TMUV accounted for the greatest proportion, a rate of 42%, of coinfection in the field.

Association between epidemics and climates

To examine the association between prevalent diseases and the seasons, the data were analyzed in combination with cases occurring at different time of these year, as shown in Figure 8. The results demonstrated that the morbidity peak of APEC appeared from May to September (Figure 8a), whereas that of H9 AIV appeared from approximately February to September (Figure 8b) and that of TMUV appeared from April to July, which was slightly early than that of APEC (Figure 8c). Furthermore, no obvious morbidity peak was observed for the DPV (Figure 8d), whereas the positive rates of NDV and FAdV were too low for an examination of the associations between them and the seasons.

Association between epidemics and ages

As the association between epidemiological prevalence and age is not well known, the incidence rates of APEC and the different viral infections occurring in ducks at the different life stages were analyzed. The results, shown in Figure 9, suggest that infection can be observed at any stage of duck breeding. However, differences are evident with regard to the susceptible stages of particular viral infections. Both the APEC and TMUV had a tendency to decrease as the ducks matured. The results suggested that approximately 60% of APEC and TMUV infections occurred in ducklings. Meanwhile, the infection status of H9 AIV showed reverse results, which suggested that approximately 40% of the H9 AIV infections occurred in adult ducks. Additionally, the incidence rate of DPV first increased and then decreased.

Discussion

Because China is a large country of duck production customers, duck breeding accounts for a significant part of Chinese agriculture. Nevertheless, with the development of industrial-scale production and a significantly increased stocking density, contagious diseases of duck, especially APEC, H9 AIV, TMUV and DPV, have gradually become more epidemic and severe (Li et al., 2015; Dou et al., 2016; Wang et al., 2016; Lee et al., 2014; Liu et al., 2017; Sun et al., 2013; Wang et al., 2017). All these infections induce a high mortality or severe losses of productivity in ducks, whereas coinfection among them further aggravates the diseases, making the conditions worse and leading to extensive damage to the duck industry.

More importantly, APEC induced different syndromes in ducks, such as acute colibacillosis, respiratory colisepticemia, salpingitis, and yolk sac infection (Dho-Moulin and Fairbrother, 1999), which have become increasingly serious problems in ducks in China. However, reports about APEC mostly have been from chickens (Wang et al., 2016; Li et al., 2015), whereas studies on the epidemiological characteristics of it in ducks have been rare. Thus, systemic biological characteristic analysis and epidemiological investigation of APEC was performed in 325 duck farms from 13 provinces all over the country to enhance the current understanding of the epidemiology of APEC in China.

In long-term practical applications, various antibiotics have been employed to prevent and control clinical infections of pathogenic bacteria, but antibiotic use also has led to complex and varied drug resistance in those germs that post a huge threat to global public health (Glass-Kaastra et al., 2014; Mackenzie et al., 2014). As part of this trend, all APEC isolates isolated in the present study and subjected to drug resistance testing shared a high drug resistance ability, and MDR was very common. This situation not only increases the difficulty of treating animal diseases like those caused by APEC with existing antimicrobials, but it also poses a potential threat to human health in consideration of the cross exchange of drug resistance genes between animal and human pathogens (Collignon et al., 2015). Unfortunately, consistent with previous studies (Chen et al., 2014; Wang et al., 2016), the isolates in this study carried multiple drug resistance genes. Thus, to control APEC in poultry is a challenge, especially for the MDR strains, and requires further study.

On the other hand, pathogenicity analysis showed that most isolates in this study were highly or intermediately pathogenic, and phylogenetic analysis suggested they belong to the group B2 or D; B2 is mainly responsible for primary APEC infection, and D is associated with reinfection and MDR (Ejrnaes et al., 2011). Studies have demonstrated that the pathogenicity of APEC is not only determined by the serogroup, but is also determined by other important elements such as virulence genes and biofilm formation ability (Dziva and Stevens, 2008). In this study, all isolates carried multiple virulence genes, and the detection rates of *iss, iucD, lusS, tsh, fimC,* and *pfs* were greater than 80%, which might play an important part in their pathogenicity, and detection of them could provide the basis for future studies on mechanisms of pathogenicity. Furthermore, biofilms should also be blamed for the increased pathogenicity of the APEC, and the adherent microbial communities could induce a persistent infection, which is the main reason it is hard to treat bacterial infections using conventional antibiotics (Romling and Balsalobre, 2012; Sawhney and Berry, 2009; Zalewska-Piatek et al., 2009). In this study, most isolates share strong or moderate biofilm-forming abilities, which might be another reason for their high pathogenicities, and may lead to an inability to control the infection owing to its natural ability to resist antibiotics (Chen et al., 2010).

More importantly, the positive rate of APEC for the sampled duck farms was 8%, whereas the rates of the H9 AIV, TMUV, DPV, NDV and FAdV were 39.38%, 14.76%, 13.53%, 11.38% and 6.15%, respectively. In the APEC-positive duck farms, the positive rates of those viruses were 84.61%, 73.07%, 30.76%, 7.69% and 11.53%, respectively, which demonstrated that the coinfection with different pathogens was very common in APEC-positive duck

farms, leading to more severe and complex clinical diseases (Mosleh et al., 2017). Meanwhile, the triple-infection of APEC/H9 AIV/TMUV occupied approximately 42% of those APEC-positive farms, suggesting this triple infection was the most common infection in the field. A previous study indicated that *E. coli* and H9 AIV together can mutually exacerbate the condition of either disease compared to the condition of birds infected with one of the two pathogens, whereas *E. coli* infection prior to, after or concurrently with H9 AIV virus infection could exacerbate the adverse effects of the virus (Mosleh et al., 2017). In consideration of the TMUV, a newly emerged epidemic that has caused large economic losses to the aquatic breeding industry of China, causing anorexia, diarrhea, weight loss, a drop-in egg production, ovarian hemorrhage and follicle rupture in poultry (Liu et al., 2012), the effects of the emerging TMUV infection into the above coinfection of ducks are worthy of further study.

At the same time, an association between the climate and epidemics was also noted in this study. The results showed that the morbidity peaks of APEC and TMUV were very close, whereas the former was slightly earlier than the latter. Such an immunosuppressive pathogen could damage the immune system and induce secondary infections; thus, we speculated that the early infection of TMUV might be an important reason for the APEC infections. Additionally, APEC and TMUV shared a similar susceptibility to ducklings. Thus, detailed evaluation of the pathogenicity of the coinfection with APEC and TMUV also needs further study.

In conclusion, this study demonstrated that the drug resistance and pathogenicity of APEC continues to increase, and APEC, H9 AIV, TMUV, DPV, NDV and FAdV were epidemic in ducks. Meanwhile, the coinfection of APEC with viral infections was very common in the APEC-positive farms. When two or more viruses, particularly immunosuppressive pathogens such as AIV and TMUV, simultaneously or successively infect ducks, the pathogens will produce synergy, elevating the pathogenicity of the APEC and resulting in more serious clinical signs than monoinfection, which should draw much attention.

Acknowledgments

This study was funded by the National Key Research and Development Program of China (2016YFD0500107); The China Agriculture Research System (CARS-42-19)

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Bass, L., Liebert, C.A., Lee, M.D., Summers, A.O., White, D.G., Thayer, S.G., Maurer, J.J. (1999). Incidence and characterization of integrons, genetic elements mediating multipledrug resistance, in avian Escherichia coli. Antimicrob. Agents Chemother. 43, 2925–2929.

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

Collignon, P., Wegener, H.C., Braam, P., Butler, C.D. (2005). The routine use of antibiotics to promote animal growth does little to benefit protein undernutrition in the developing world. Clin. Infect. Dis. 41, 1007–1013. doi: 10.1086/433191

Croxen, M.A., Finlay, B.B., (2010). Molecular mechanisms of Escherichia coli pathogenicity. Nat. Rev. Microbiol. 8, 26–38. doi: 10.1038/nrmicro2265

Chen, C., Liao, X., Jiang, H., Zhu, H., Yue, L., Li, S., et al. (2010). Characteristics of Escherichia coli biofilm production, genetic typing, drug resistance pattern and gene expression under aminoglycoside pressures. Environ Toxicol Pharmacol. 30, 5–10. doi: 10.1016/j.etap.2010.03.004

Chen, X., Zhang, W., Yin, J., Zhang, N., Geng, S., Zhou, X., et al. (2014). Escherichia coli isolates from sick chickens in China: changes in antimicrobial resistance between 1993 and 2013. Vet J. 202, 112–115. doi: 10.1016/j.tvjl.2014.06.016

Dho-Moulin, M., Fairbrother, J.M., (1999). Avian pathogenic Escherichia coli (APEC). Vet. Res. 30, 299–316.

Dziva, F., Stevens, M.P. (2008). Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic Escherichia coli in their natural hosts. Avian Pathol. 37, 355–366. doi: 10.1080/03079450802216652

Dheilly, A., Le Devendec, L., Mourand, G., Jouy, E., Kempf, I. (2013). Antimicrobial resistance selection in avian pathogenic E.coli during treatment. Vet. Microbiol. 166, 655–658. doi: 10.1016/j.vetmic.2013.06.013

Dou, X., Gong, J., Han, X., Xu, M., Shen, H., Zhang, D., et al. (2016). Characterization of avian pathogenic Escherichia coli isolated in eastern China. Gene. 576(1), 244-8. doi: 10.1016/j.gene.2015.10.012

Ewers, C., Janssen, T., Kiessling, S., Philipp, H.C., Wieler, L.H. (2005). Rapid detection of virulence- associated genes in avian pathogenic Escherichia coli by multiplex polymerase chain reaction. Avian Diseases, 49(2), 269-273. doi: 10.1637/7293-102604R

Ejrnaes, K. (2011). Bacterial characteristics of importance for recurrent urinary tract infections caused by Escherichia coli. Dan Med Bull. 58, B4187.

Glass-Kaastra, S.K., Pearl, D. L., Reid-Smith, R., McEwen, B., Slavic, D., Fairles, J., et al. (2014). Multiple-class antimicrobial resistance surveillance in swine Escherichia coli F4, Pasteurella multocida and Streptococcus suis isolates from Ontario and the impact of the

2004–02006 Porcine Circovirus type-2 Associated Disease outbreak. Preventive veterinary medicine. 113, 159–164. doi: 10.1016/j.prevetmed.2013.11.008

Hu, Q., Tu, J., Han, X., Zhu, Y., Ding, C., Yu, S. (2011). Development of multiplex PCR assay for rapid detection of Riemerella anatipestifer, Escherichia coli, and Salmonella enterica simultaneously from ducks. J. Microbiol. Methods 87, 64–69. doi: 10.1016/j.mimet.2011.07.007

Janben, T., Schwarz, C., Preikschat, P., Voss, M., Philipp, H.C., Wieler, L.H. (2001). Virulence-associated genes in avian pathogenic Escherichia coli (APEC) isolated from internal organs of poultry having died from colibacillosis. International Journal of Medical Microbiology, 291(5), 371-378.

Kaper, J.B. (2005). Pathogenic Escherichia coli. Int. J. Med. Microbiol. 295, 355–356.

La Ragione, R.M., Woodward, M.J. (2002). Virulence factors of Escherichia coli, serotypes associated with avian colisepticaemia. Research in Veterinary Science, 73(1), 27-35.

Liu, M., Chen, S., Chen, Y., Liu, C., Chen, S., Yin, X., . . . Zhang, Y. (2012). Adapted Tembusu-like virus in chickens and geese in China. Journal of Clinical Microbiology, 50, 2807–2809. doi: 10.1128/ JCM.00655-12

Lee, D.H., Park, J.K., Yuk, S.S., Erdene-Ochir, T.O., Kwon, J.H., Lee, J.B., et al. (2014). Complete genome sequence of a natural reassortant H9N2 avian influenza virus found in bean goose (Anser fabalis): Direct evidence for virus exchange between Korea and China

via wild birds. Infection, Genetics and Evolution, 26, 250–254. doi: 10.1016/j.meegid.2014.06.007

Li, Y., Chen, L., Wu, X., Huo, S. (2015). Molecular characterization of multidrug-resistant avian pathogenic Escherichia coli isolated from septicemic broilers. Poult Sci. 94(4), 601-11. doi: 10.3382/ps/pev008

Liu, P., Zhang, J., Chen, S., Wang, M., Cheng, A. (2017). Genome Sequence of a Goose Parvovirus Strain Isolated from an Ill Goose in China. Genome Announcements, 5, e00227–17. doi: 10. 1128/genomea.00227-17

MacKenzie, M., Rae, N., Nathwani, D. (2014). Outcomes from global adult outpatient parenteral antimicrobial therapy programmes: a review of the last decade. Int J Antimicrob Agents. 43, 7–16. doi: 10.1016/j.ijantimicag.2013.09.006

Mosleh, N., Dadras, H., Asasi, K., Taebipour, M.J., Tohidifar, S.S., Farjanikish, G. (2017). Evaluation of the timing of the Escherichia coli co-infection on pathogenecity of H9N2 avian influenza virus in broiler chickens. Iran J Vet Res. 18(2), 86-91.

Niu Y, Sun Q, Zhang G, Sun W, Liu X, Xiao Y, et al. (2017). Epidemiological investigation of outbreaks of fowl adenovirus infections in commercial chickens in China. Transbound Emerg Dis. 65(1), e121-e126. doi: 10.1111/tbed.12691

Niu, X., Wang, H., Wei, L., Zhang, M., Yang, J., Chen, H., et al. (2017). Epidemiological investigation of H9 avian influenza virus, Newcastle disease virus, Tembusu virus, goose

parvovirus and goose circovirus infection of geese in China. Transbound Emerg Dis. 65(2), e304-e316. doi: 10.1111/tbed.12755

Romling, U., Balsalobre, C. (2012). Biofilm infections, their resilience to therapy and innovative treatment strategies. J Intern Med. 272, 541–561. doi: 10.1111/joim.12004

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., Svabic-Vlahovic, M. (2000). A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J. Microbiol. Methods 40, 175–179. doi: 10.1016/S0167-7012(00)00122-6

Saif, Y.M., Barnes, H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. (2003). Diseases of Poultry. Ames, IA: Iowa State University Press.

Sawhney, R., Berry, V. (2009). Bacterial biofilm formation, pathogenicity, diagnostics and control: An overview. Indian J Med Sci. 63, 313–321. doi: 10.4103/0019-5359.55113

Sun, Y., Ding, N., Ding, S. S., Yu, S., Meng, C., Chen, H., et al. (2013). Goose RIG-I functions in innate immunity against Newcastle disease virus infections. Molecular Immunology, 53, 321–327. doi: 10.1016/j.molimm.2012.08.022

Todd, D. (2000). Circoviruses: Immunosuppressive threats to avian species: A review. Avian Pathology, 29, 373–394. doi: 10.1080/030794500750047126

Tang, X., Z. Zhao, J. Hu, B. Wu, X. Cai, Q. He, Chen, H. (2009). Isolation, antimicrobial resistance, and virulence genes of Pasteurella multocida strains from swine in China. J Clin Microbiol. 47, 951–958. doi: 10.1128/JCM.02029-08

Wang, X.M., Liao, X.P., Zhang, W.J., Jiang, H.X., Sun, J., Zhang, M.J., et al. (2010). Prevalence of serogroups, virulence genotypes, antimicrobial resistance, and phylogenetic background of avian pathogenic Escherichia coli in south of China. Foodborne Pathog. Dis. 7, 1099–1106. doi: 10.1089/fpd.2010.0542

Wang, Y., Zhang,W., Wu, Z., Lu, C. (2011). Reduced virulence is an important characteristic of biofilm infection of Streptococcus suis. FEMS Microbiol. Lett. 316, 36–43. doi: 10.1111/j.1574-6968.2010.02189.x

Wei, B., Cha, S.Y., Kang, M., Park, I.J., Moon, O.K., Park, C.K., et al. (2013). Development and application of a multiplex PCR assay for rapid detection of 4 major bacterial pathogens in ducks. Poult. Sci. 92, 1164–1170. doi: 10.3382/ps.2012-02823

Wang, Y., Yi, L., Wang, Y., Wang, Y., Cai, Y., Zhao, W., et al. (2016). Isolation, phylogenetic group, drug resistance, biofilm formation, and adherence genes of Escherichia coli from poultry in central China. Poult Sci. 95(12), 2895-2901. doi: 10.3382/ps/pew252

Wang, A., Sun, L., Wang, M., Jia, R., Zhu, D., Liu, M., et al. (2017). Identification of IFITM1 and IFITM3 in Goose: Gene Structure, Expression Patterns, and Immune Reponses against Tembusu Virus nfection. BioMed Research International, 2017, 5149062. doi: 10.1155/2017/5149062

Zalewska-Piatek, B.M., Wilkanowicz, S.I., Piatek, R.J., Kur, J.W. (2009). Biofilm formation as a virulence determinant of uropathogenic Escherichia coli Dr+ strains. Pol J Microbiol. 58, 223–229.

Supplementary materials

Table S1 Primers targeting the virulence-associated genes used in this study

Table S2 Primers targeting the drug resistance genes used in this study

Table S3 Primers used in this study

Figure S1 Geographical distribution of the sites of sample collection. The provinces from which the samples were collected are indicated in color in the figure. The number of farms involved in the study are indicated for each province as follows: Jilin (11), Hebei (137), Henan (10), Shanxi (20), Shandong (168), Anhui (13), Neimenggu (12), Beijing (13), Jiangsu (15), Fujian (11), Liaoning (11), Sichuan (13) and Gansu (11).

Figure 1 Postmortem results for the liver, heart, spleen, air sac and kidney from APEC affected ducks

(a-e) liver, heart, spleen, air sac and kidney, respectively, of the affected ducks. (f-j) liver, heart, spleen, air sac and kidney, respectively, of the control. Obvious perihepatitis (a), pericarditis (b), splenic congestion (c), air sac turbidity (marked with a red arrow, d) and renal lesions (e) appeared in affected ducks.

Figure 2 Pathological changes in infected ducks

(a-d) liver, kidney, heart and spleen, respectively, of the affected ducks. Typical swelling and necrosis of liver and kidney cells (a-b), cardiac particle deformation (c), as well as disintegration of the splenic cells (d), appeared in the affected ducks.

Figure 3 Drug sensitivity assay for APEC isolates.

HS: high susceptibility; MS: middle susceptibility; LS: low susceptibility; R: resistant.

Figure 4 Phylogenetic tree based on the virulence genes of 26 APEC strains isolated in this study. Positive rate of different virulence genes, marked with different colors, following the name. The tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates using MEGA 5.0.

Figure 5 Phylogenetic tree based on the drug-resistance genes of 26 APEC strains isolated in this study. Positive rate of different drug-resistance genes, marked with different colors, follow the name. The tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates using MEGA 5.0.

Figure 6 Infection situation of the APEC and different viruses

(a), the positive rate of APEC, H9 AIV, TMUV, DPV, NDV and FAdV in 325 duck farms; (b) the positive rate of H9 AIV, TMUV, DPV, NDV and FAdV in 26 farms with APEC.

Figure 7 Coinfection situation analysis

E: APEC; A: H9 AIV; T: TMUV; D: DPV; F: FAdV; N: NDV.

Figure 8 APEV and viral infection situation among different months

(a) APEC; (b) H9 AIV; (c) TMUV; (d) DPV.

Figure 9 APEC and viral infection situation among different ages

A: ducklings (before 10 weeks age); B: young ducks (11- 20 weeks age); C: Adult ducks (after 21 weeks age)