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Research paper

Characterization of avian pathogenic *Escherichia coli* isolated in eastern China

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ABSTRACT

In order to investigate the biological characteristics of avian pathogenic *Escherichia coli* (APEC) isolated in eastern China, a total of 243 isolates were isolated from diseased poultry on different farms during the period from 2007 to 2014. These isolates were characterized for serogroups (polymerase chain reaction and agglutination), the presence of virulence-associated genes (*fimC*, *iss*, *ompA*, *fyuA*, *stx2f*, *iroC*, *iucD*, *hlyE*, *tsh*, *cvaC*, *irp2*, and *papC*) and class I integrons (polymerase chain reaction), drug susceptibilities (disk diffusion method) and the biofilm-forming abilities (semi-quantitative method). The results showed that the most predominant serogroups were O78 (87 isolates, 35.8%) and O2 (35 isolates, 14.4%). Gene profiling found that *fimC* and *ompA* were frequently distributed among the isolates and that 77.4% of the isolates were positive for class 1 integrons. Overall, isolates displayed resistance to tetracycline (97.5%), nalidixic acid (82.3%), ampicillin (81.1%), sulphafurazole (80.7%), streptomycin (79.0%), trimethoprim (78.2%) and cotrimoxazole (78.2%). Multiple-drug resistance was exhibited in 80.3% of the isolates, and the presence of class 1 integrons is associated with multidrug resistance. Finally, 151 isolates had the ability to form biofilms in vitro, and drug resistance seemed relative to biofilm-forming abilities.

1. Introduction

Avian colibacillosis is the general term for several diseases (such as pericarditis, perihepatitis, peritonitis, airsacculitis, septicemia and other mainly extraintestinal diseases) caused by Avian Pathogenic *Escherichia coli* (APEC). The antigenicity of this pathogen is complicated, and the serogroups are diverse. Currently, prevalent serogroups reported abroad are O1, O2 and O78 (Dho-Moulin and Fairbrother 1999; Ewers et al. 2003), but strains from different sources may have different serogroups. The clinical symptoms and pathological changes of the disease are complex, and often complicated with additional bacterial and viral infections. With the rapid development of an intensive poultry industry, morbidity and mortality of avian colibacillosis has also increased, leading to significant losses in the production of the poultry (Croxen and Finlay 2010; Kaper 2005). Antibacterial drugs play an important role in the control of *E. coli* infections, but continuous use leads to the emergence of drug-resistant strains of *E. coli*, including multi-drug-resistant strains. Thus, pathogenic *E. coli* drug resistance has become a focus of the international community (Bass et al. 1999; Dheilly et al. 2013; Wang et al. 2010).

Studies have shown that pathogenicity of *E. coli* is not entirely associated only with serogroups, and that virulence factors are also important in determining its pathogenicity (Dziva and Stevens 2008). APEC contains a variety of virulence factors, such as fimbriae, toxins, invasive enzymes, and outer membrane proteins (Landman and Cornelissen 2006; La Ragione and Woodward 2002; Vandemaele et al. 2002). Detection of these virulence factors could provide references for further study in bacterial pathogenicity. Integrons have the ability to capture and transfer genes responsible for drug resistance in microbes. Class I integrons are the most common form in multi-drug-resistant *E. coli*, playing an important role in the spread of resistance genes (Oosterik et al. 2014; Piccirillo et al. 2014). A bacterial biofilm (BF) is an aggregated community of single-celled organisms adhering to a solid surface, and surrounding themselves with secretions of glycoproteins, lipoproteins, fibrin and other substances. Bacterial biofilms have been shown to exist in many bacteria, including *E. coli*, *Salmonella* spp., *Streptococcus suis*, *Haemophilus* spp. and *Riemerella anatipestifer*. Studies have shown

Abbreviations: APEC, avian pathogenic *Escherichia coli*; BF, bacterial biofilm; CVCC, Chinese Medical Culture Collection; ATCC, American Type Culture Collection; LB, Luria broth; CLSI, Clinical and Laboratory Standards Institute; MDR, multi-drug resistant.

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that bacterial biofilm is associated with drug resistance, and that bacteria encapsulated in the biofilm will exhibit significantly increased resistance to drugs (Gilbert et al. 1997; Høiby et al. 2010; Lewis 2001).

In the presented study, avian pathogenic *E. coli* was isolated from poultry from eastern China for the purpose of identifying serogroups, detecting virulence-associated genes, assaying drug resistance and characterizing bacterial biofilms of these strains. Cataloging this data will provide a base reference for future studies which explore the prevalent serogroups, biological features and pathogenesis of these *E. coli* strains with the aim of developing measures to prevent and control APEC infections.

2. Materials and methods

2.1. Source of infected samples

Diseased and dead poultry (chickens, ducks and geese) were submitted from facilities in the Jiangsu, Shandong, Zhejiang, Shanghai and Anhui regions from 2007 to 2014. Animal specimens were diagnosed via the clinical presentation with lesions typical of *E. coli* infections. The livers, spleens and lungs were collected aseptically to serve as diseased tissue sources.

2.2. Reference strains

Avian pathogenic *E. coli* reference strains CVCC244 (O2), CVCC1490 (O78) and avian *Pasteurella multocida* CVCC44801 were obtained from the Chinese Veterinary Culture Collection (Beijing, China). Strains DE47 (O1) and CE66 (O18) are maintained in and were procured from our laboratory (Wang et al. 2014). *E. coli* strain ATCC25922 was obtained from ATCC (American Type Culture Collection, Washington DC, USA)

2.3. Reagents

Triple sugar iron agar, Luria broth (LB), LB agar, MacConkey agar, and Mueller-Hinton agar were purchased from the Qingdao High-Tech Park HaiBo Company. Hangzhou Tianhe Mircroorgnism Reagent Co., Ltd. supplied glucose, lactose, maltose, mannitol, sucrose, indole, methyl red, Voges–Proskauer reagents, and citrate. *E. coli* diagnosis serum was purchased from China Institute of Veterinary Drugs Control. Susceptibility disk paper was purchased from Oxoid Company, UK. Bacterial biofilm staining solution was purchased from Invitrogen Biotechnology (Shanghai) Co., Ltd. Bacterial genome Rapid DNA extraction kits were purchased from Shanghai Biological Engineering Co., Ltd. and DNAmarker and Taq DNA polymerase were purchased from TaKaRa (Dalian) Co., Ltd. Cell culture plates were purchased from Corning Corporation, US. Other conventional reagents were domestically procured analytical grade products.

2.4. Isolation and identification of avian pathogenic E. coli

Samples of the diseased tissues were used to inoculate MacConkey agar, which was subsequently cultured for 20 h at 37 °C. From these agar plates, suspect bacterial colonies were used to inoculate LB agar and cultured overnight at 37 °C. After growth, the DNA of these strains was extracted using Rapid DNA extraction kits, according to the manufacturer's instructions, for use in PCR identification of the strains. PCR primers were prepared according to the literature (Hu et al. 2011). The PCR reactions were carried out in 25 μL volumes on an Applied Biosystems 2720 Thermocycler (Applied Biosystems Life Technologies, Grand Island, NY, USA), with the following conditions: 94 °C 4 min; 94 °C 40 s, 55 °C 40 s, 72 °C 1 min, total 30 cycles; 72 °C 10 min, hold at 4 °C.

Strains which were identified by PCR as *E. coli* were culture identified on triple sugar iron agar and additional identifications were made using glucose, lactose, maltose, mannitol and sucrose fermentation tests. An IMViC test battery (indole, methyl red, Voges–Proskauer, and citrate utilization tests) was used to confirm these strains as *Enterobacteriacea*, specifically *E. coli* (Dobrowsky et al. 2014). Motility tests completed our identification efforts.

2.5. Serogroup identification

PCR and agglutination tests were used to identify the O-serogroup(s) of the isolated strains. In reference to the literature (Wang et al. 2014), PCR primers were designed for the common serogroups (O1, O2, O18 and O78) of APEC. The primers are listed in Table S1. PCR was carried out with the following reaction conditions: 94 °C 4 min; 94 °C 40 s, 55 °C 40 s, 72 °C 1 min, for 30 cycles; 72 °C 10 min, hold at 4 °C. The final determination of serogroups was made by slide agglutination tests and sterile saline was used as the negative control (Schierack et al. 2006).

2.6. Detection of virulence-associated genes and class I integron

PCR primers targeting virulence-associated genes and *int1*gene (class I integron) were designed according to the literature (La Ragione and Woodward 2002; Lévesque et al. 1995) for the detection of 12 *E. coli* virulence genes and the Class I integron, as shown in Table S1. The primers were synthesized by Suzhou Genewiz Biotechnology Co., LTD (Suzhou, China). PCR amplification was performed with the same reaction conditions as detailed above.

2.7. Drug susceptibility testing

Referring to the Performance Standards for Antimicrobial Susceptibility Testing developed by the Clinical and Laboratory Standards Institute (CLSI, Wayne, PA, US), 18 antimicrobial agents were selected for testing, specifically ampicillin, cefotaxime, ceftriaxone, ceftazidime, ertapenem, aztreonam, streptomycin, gentamicin, kanamycin, amikacin, tetracycline, ciprofloxacin, nalidixic acid, trimethoprim, sulfamethoxazole, sulfisoxazole, chloramphenicol and nitrofurantoin. Sample bacterial cultures were seeded onto MH agar plates and the susceptibility paper disks impregnated with the 18 antimicrobial agents were affixed to the surface of the solid agar medium. The plates were cultured for 16 h at 37 °C, and the diameters of the inhibition zones were measured and recorded. Referring to the CLSI standard (Clinical and Laboratory Standards Institute (CLSI) 2012), the susceptibility of the sample bacteria was determined for each drug, and the strains recorded as susceptible (S), intermediately resistant (I) or resistant (R).

2.8. Biofilm detection

Referring to methods previously described in the literature (WANG ET AL. 2011), 100 μL of bacterial culture liquid (OD600 = 0.1) was added to each well in a sterile 96-well cell culture plate (Corning Costar, Corning, NY, USA). An equal amount of LB broth was placed into negative control wells on each plate. The plates were cultured for 36 h at 37 °C. After incubation, the culture medium was discarded, and the plate washed with 3 times sterile PBS and dried naturally. Crystal violet (100 μL, 0.1%) was added into each well and allowed to stain the cells for 30 min. The plates were then washed 3 times with PBS for and dried naturally. To each well, 200 μL ethanol (95%) was added and the OD_{595} determined on a SynergyII IV/MX plate reader (BioTek Instruments, Winooski, VT, USA). The isolates were divided into four grades based on Biofilm-forming capability (Stepanovic et al. 2000) (OD is OD_{595} of the subject bacteria, while ODc is OD_{595} of the negative control):

a. OD < ODc (strain with non biofilm-forming ability)
b. ODc < OD < 2ODc (strain with weak biofilm-forming ability)