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# Detection of new antibiotic resistance gene profile in *Escherichia coli* associated with avian leukosis virus infection from broiler chickens

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**Abstract:** The *Escherichia coli* (*E. coli*) is prevailing worldwide, but the epidemiology of *E. coli* infections feature regional distribution characteristics to some extent. *E. coli*, as a zoonotic pathogen, can be transferred from animals to humans through food chain or via contact with wounds, causing a public health risk. We reported the swelling of proventriculus and tracheal bleeding following the death in two broiler chickens (*Gallus gallus domesticus*) from Beijing, China. To investigate whether a virus was involved in the infection, Madin Darby Bovine Kidney (MDCK) cells were co-cultured with supernatants of proventriculus, trachea and spleen homogenates. The avian leucosis virus was detected in the samples of proventriculus and trachea, but the avian influenza virus, the Newcastle disease virus and the avian infectious laryngotracheitis virus were not detected. *E. coli* isolates were resistant to almost all the antimicrobial as tested except for the combinations of amoxicillin/clavulanic acid and sulfamethoxazole/trimethoprim. PCR tests demonstrated the presence of antibiotic resistance genes in these *E. coli isolates and* further research revealed a novel gene profile with the presence of CTX-M-1, gyrA, gyrB, oqxA, oqxB, parC and Sul2 antibiotic resistance genes in a strain isolated from a proventriculus sample. These results demonstrated that the presence of antibiotic resistant *E. coli* would not necessarily cause outbreak of large-scale disease. However, when the bacteria carrying new antibiotic resistance genes enter the environment, it may result in the development of more virulent strains which will potentially impact human and animal health.

## Introduction

The bacterium *E. coli* can be found in many kinds of livestock such as poultry, farmed animals, other land animals and some aquatic animals, among which, pigs and chickens are the mostly susceptible of infection. *E. coli* is an important opportunistic zoonotic pathogen, belonging to the family *Enterobacteriaceae*, but it is usually considered as a commensal bacterium in humans and animals, and used as an indicator of enteric pathogens and environmental contamination by feces (Jiang *et al.*, 2014). *E. coli* is mainly found in the feces of patients with gastrointestinal infections and can be mainly transmitted among people through fecal and oral routes. Our study detected new antibiotic resistance gene profiles in *E. coli* and further research indicated avian leukosis virus infection in dead broiler chickens that were the sources of these isolates. Studies suggest that ESBL/AmpC-producing *E. coli* are isolated with increasing frequency from animals, food, environmental sources and humans (Dorado-Garcia *et al.*, 2018). To date, several different fluoroquinolone resistance mechanisms have been proposed, such as the aminoglycoside acetyltransferase AAC (6′)-Ib-cr, which acetylates several fluoroquinolones with piperazinyl substitutions; the qnr families (qnrA, qnrB,qnrS, qnrC, and qnrD) which protect DNA gyrase and topoisomerase IV from quinolone inhibition; and the active multidrug efflux pump qepA and oqxA/B, which can remove fluoroquinolones from the bacterial cells (Poirel *et al.*, 2012; Ruiz *et al.*, 2012; Wang *et al.*, 2017). Recent experiments found acquisition of mcr-1 and co-carriage of virulence genes in avian pathogenic *E. coli* isolated from municipal wastewater influents in Japan (Hayashi *et al.*, 2019). *E. coli* pathotypes have been identified to be largely responsible for moderating severe diarrhea transmitted from contaminated human or animal feces to susceptible hosts via environmental reservoirs such as hands, water and soil (Navab-Daneshmand *et al.*, 2018). The soil may represent a direct source or act as an intermediary matrix for the transmission of antibiotic resistant pathogenic *E. coli* strains, particularly in rural settings, and the presence of *E. coli* in

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soil is of public health significance (Montealegre *et al*., 2018). These studies show the significance of, and the need for urgent measures for reducing environmental contamination by bacteria carrying antibiotic resistance genes possibly resulting from contamination in the course of human activities such as random disposal of untreated water, soil and waste, etc.

The antibiotic resistant isolates and resistance genes of *E. coli* can be transferred to humans and animals through food, water, poor hygiene, improper cooking, logistics, storage and wound contact, causing potential harms to the public health (Ryu *et al*., 2012). Avian pathogenic *E. coli* is the etiologic agent of avian colibacillosis, which is the most common disease causing chicken morbidity (Mohamed *et al*., 2018). However, human activities influence the epidemiology of transmission among human, environment and animals. This study aimed at investigating the antibiotic susceptibility pattern and resistance gene types of *E. coli* through biochemical and gene sequencing methods to reveal valuable information for preventing bacterial diseases.

## Materials and Methods

*Samples collection and testing for microorganisms*

Two dead broiler chickens were taken from a chicken farm on the outskirts of Beijing, China, and transferred to the laboratory at National Research Center for Wildlife-Borne Diseases. Six isolates of *E. coli* were obtained from proventriculus and trachea of the broiler chickens. Proventriculus samples were obtained in a sterile environment by first cutting the skin and muscle tissue of the broiler with sterile scissors, and then cutting the proventriculus with another pair of sterile scissors. The inner surface of the proventriculus was sampled by using a sterile inoculation loop, and it was then removed with sterile scissors. The proventriculus was fixed in 10% neutral buffered formalin and embedded in paraffin before the tissue sections were stained with hematoxylin and eosin for histological examination. To detect the viral infections, a cell culture approach was used. Briefly, a Madin-Darby canine kidney cells (MDCK) line was cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (Gibco) and incubated for 5 days with supernatants of homogenate of proventriculus, trachea and spleen from the dead broiler chickens (Wang *et al*., 2017).

*Bacterial identification and antibiotic resistance testing*

Samples were spread onto the Mueller Hinton agar medium under aseptic conditions and incubated at 37°C for 24 h. The bacterial colonies from each sample were examined by using standard biochemical tests, and the polymerase chain reaction (PCR) amplification of 16S rDNA genes was performed with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′), 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Macrae *et al*., 2000). The BD Phoenix™ 100 (Maryland, USA) automated identification system was applied to identify the bacterial isolates. All PCR amplification products were sequenced and compared with the GenBank database by using BLAST (http://blast.ncbi.nlm.nih.gov/). The antibiotic resistance profiles of isolates were determined by using the disk diffusion test according to guidelines of the Clinical & Laboratory Standards Institute (CLSI) and the manufacturers' instructions. The antibiotics involved included amikacin (AN), ampicillin (AM), aztreonam (AZT), cefazolin (CZ), cefotaxime (CTX), cephalothin (CF), chloramphenicol (C), ciprofloxacin (CIP), gentamicin (GM), levofloxacin (LVF), nitrofurantoin (FT), ofloxacin (OFL), piperacillin (PIP), streptomycin (S), tobramycin (TM), amoxicillin/clavulanic Acid (AMX/CA) and sulfamethoxazole/trimethoprim (SXT). ATCC 25922 *E. coli* was used as a control. The degree of antibiotic resistance was measured by using the following Kirby-Bauer disc diffusion method (Biemer, 1973).

*Detection of antibiotic resistance and viral genes*

Antibiotic resistance gene identification was carried out for *AAC(3)-II*, *cmlA*, *CTX-M-l*, *gyrA*, *gyrB*, *blaKPC*, *NDM-1*, *oqxA*, *oqxB*, *OXA*, *parC*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS* and *Sul2*, as shown in Appendix Tab. 1 (Guerra *et al*., 2010; Kim *et al*., 2009; Liu *et al*., 2008; Rhee *et al*., 2010; Wang and He, 2018; Yu *et al*., 2007). To detect the presence of viruses, DNA and RNA were extracted from proventriculus samples. Then, Avian leucosis virus (ALV), avian influenza virus (AIV), Newcastle disease virus (NDV) and avian infectious laryngotracheitis virus (AILTV) targets were amplified by using specific primers by PCR with the initial denaturation of 95°C for 5 min, 35 cycles at 95°C for 30 s, 52°C for 30 s, and 72°C for 40 s; and a final incubation for 10 min at 72°C. All sequences were compared with NCBI GenBank databases by using BLAST (http://blast.ncbi.nlm.nih.gov/). Alignments of clones and reference sequences were created with Clustal X. The phylogenetic analyses were then performed by using the neighbor-joining method with 1,000 bootstraps using MEGA 6 following the kimura 2-parameter model (Tamura *et al*., 2007).

## Results

*Clinical isolates and their identification*

PCR amplified fragment was detected by 1% agarose gel electrophoresis, and the results indicated that the amplified products were 1500 bp in length with good specificity. The sequence and biochemical tests identified six isolates (E1, E2, E3, E4, E5 and E6) as *E. coli*. Our study showed that the cell morphology of the proventriculus samples was the same as that of the control (Fig. 1). Histopathological biopsy of proventriculus samples revealed cells with diffuse hemorrhage, cell gap enlargement and an increase in lymphocytes (Fig. 2). Further, evidences were found that ALV co-infection with *E. coli* was available in the proventriculus and trachea samples. BLAST analysis showed that ALV DNA sequence of the ALV subgroup E (KC610515) was present, while AIV, DNV and AILTV were not detected (Fig. 3).

*Antibiotic resistance testing of bacterial isolates*

Except for amoxicillin/clavulanic acid and sulfamethoxazole/trimethoprim, our study showed that all six isolates were multi drug resistant (MDR) to almost all antibiotics, including beta-lactams (cefazolin, cephalothin, cefotaxime, ampicillin and piperacillin), aminoglycosides (gentamicin,

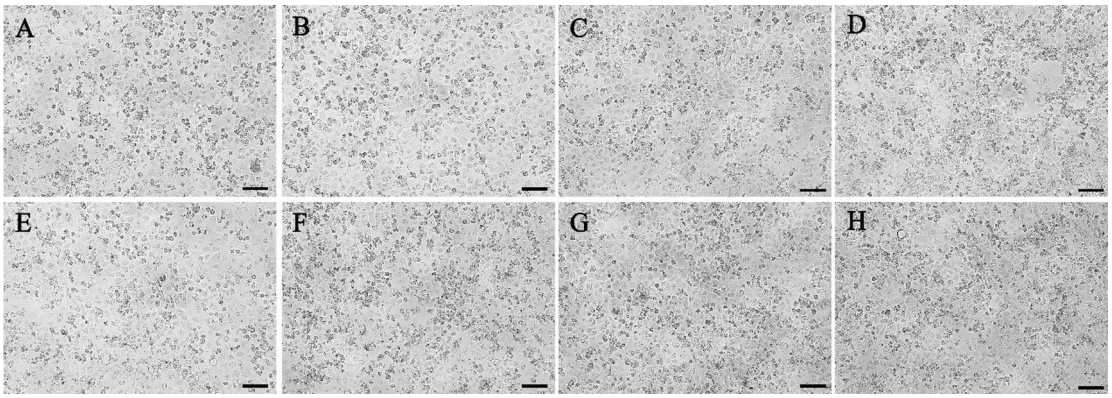


**FIGURE 1.** Homogenates of proventriculus, spleen and trachea of the broiler chickens were incubated with MDCK cells. The homogenates of proventriculus from the broiler chickens were co-cultured with MDCK cells (A-E); the homogenates of spleen were co-cultured with MDCK cells (F); the homogenates of trachea were co-cultured with MDCK cells (G); negative control (H), scale as 100 microns.

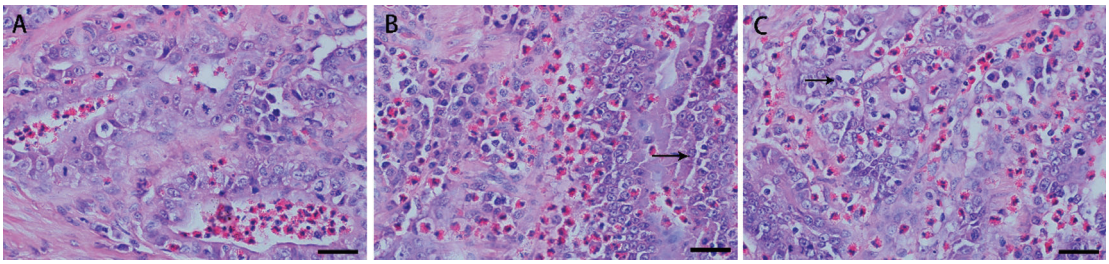


**FIGURE 2.** Histopathological observation of proventriculus of the diseased broiler chickens. Compared with the control group (A) cells diffuse hemorrhage arrowhead points to cell gap enlarged (B), and lymphocytes increase (C). Scale as 100 microns.

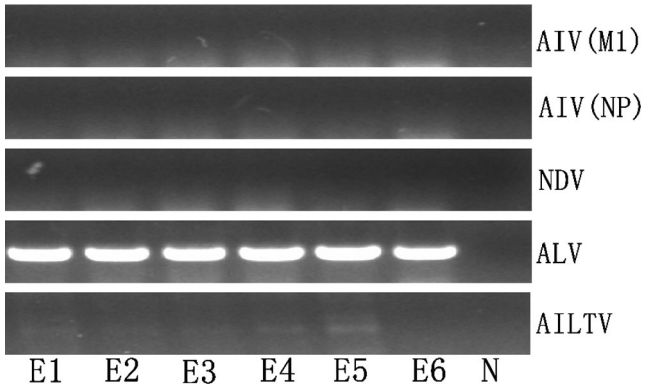


**FIGURE 3.** Detection of several viral genes from the broiler chickens. ALV was found from proventriculus and trachea, but AIV, DNV and AILTV were not detected in this study. E1-4 from proventriculus, E5 and E6 were found from trachea, N: negative control.

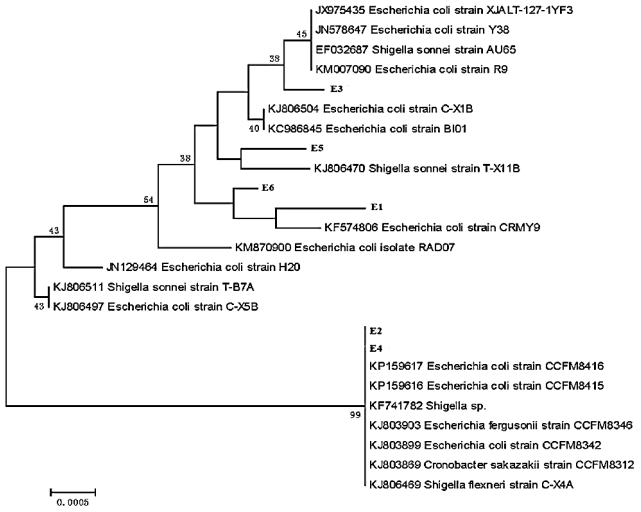


**FIGURE 4.** The phylogenetic tree based on 16SrDNA segment of isolates from the broiler chickens in Beijing, China. The phylogenetic tree was constructed by the neighbor-joining method with 1000 bootstrap replicates by MEGA 6.

tobramycin and amikacin) and quinolones (ciprofloxacin, levofloxacin and ofloxacin). Additionally, these MDR isolates were resistant to aztreonam and chloramphenicol. While the 16S rDNA gene sequences showed a closer similarity of isolates to *E. coli* (Fig. 4), several MDR isolates (E3, E4, E5 and E6) were susceptible to nitrofurantoin, although this was not found in isolates E1 and E2. The antibiotic resistance patterns of the six MDR isolates were presented in Tab. 1.

*Detection of antibiotic resistance genes*

AAC (3)-II was detected in isolates E1, E3, E5 and E6; CTX-M-1, gyrA, gyrB, parC and Sul2 were detected in all six isolates; oqxA and oqxB were found in E2; and qnrB testing produced a weak band in E2 and E3. However, cmlA, blaKPC, NDM-1, OXA, qepA, qnrA, qnrC, qnrD and qnrS antibiotic resistance genes were not amplified in any isolate (Tab. 2 and Appendix Fig. 1). The RT-PCR results revealed the novel coexistence of CTX-M-1, oqxA, oqxB, parC and Sul2 resistance genes in an isolate (E2) from a proventriculus sample that was also positive for ALV. Our study also found the combination of antibiotic resistance

**TABLE 1**

**Antibiotic resistance of *Escherichia coli* was isolated from the broiler chickens**

| Antimicrobial agents | Strains | | | | | |
|---|---|---|---|---|---|---|
| | E1 (88.2%) | E2 (82.4%) | E3 (76.5%) | E4 (76.5%) | E5 (70.6%) | E6 (82.4%) |
| Amikacin (AN) | R | R | R | R | R | R |
| Ampicillin (AM) | R | R | R | R | R | R |
| Aztreonam (AZT) | R | R | R | S | R | R |
| Cefazolin (CZ) | R | R | R | R | R | R |
| Cefotaxime (CTX) | R | R | R | R | R | R |
| Cephalothin (CF) | R | R | R | R | R | R |
| Chloramphenicol (C) | R | R | R | R | R | R |
| Ciprofloxacin (CIP) | R | R | R | R | R | R |
| Gentamicin (GM) | R | R | R | R | R | R |
| Levofloxacin (LVF) | R | R | S | R | S | R |
| Nitrofurantoin (FT) | R | S | S | S | S | S |
| Ofloxacin (OFL) | R | R | R | R | S | R |
| Piperacillin (PIP) | R | R | R | R | R | R |
| Streptomycin (S) | R | R | R | R | R | R |
| Tobramycin (TM) | R | R | R | R | R | R |
| Amoxicillin/Clavulanic Acid (AMX/CA) | S | S | S | S | S | S |
| Sulfamethoxazole/Trimethoprim (SXT) | S | S | S | S | S | S |

S = Susceptible; R = Resistant.

**TABLE 2**

**Antibiotic resistance genes profile of *Escherichia coli* isolates**

| Gene Symbol | Isolates | | | | | |
|---|---|---|---|---|---|---|
| | E1 | E2 | E3 | E4 | E5 | E6 |
| AAC (3)-II | P | N | P | N | P | P |
| cmlA | N | N | N | N | N | N |
| CTX-M-1 | P | P | P | P | P | P |
| gyrA | P | P | P | P | P | P |
| gyrB | P | P | P | P | P | P |
| blaKPC | N | N | N | N | N | N |
| NDM-1 | N | N | N | N | N | N |
| oqxA | N | P | N | N | N | N |
| oqxB | N | P | N | N | N | N |
| OXA | N | N | N | N | N | N |
| parC | P | P | P | P | P | P |
| qepA | N | N | N | N | N | N |
| qnrA | N | N | N | N | N | N |
| qnrB | N | N | N | N | N | N |
| qnrC | N | N | N | N | N | N |
| qnrD | N | N | N | N | N | N |
| qnrS | N | N | N | N | N | N |
| Sul2 | P | P | P | P | P | P |

P = positive; N = negative

genes CTX-M-1, gyrA, gyrB, parC and Sul2, whereby their presence in *E. coli* could potentially result in resistance of all single antibiotics in this study except for nitrofurantoin. Whether or not the presence of ALV will impact the distribution of antibiotic resistance genes in co-located bacteria still remain for further testing. The patterns of antibiotic resistance genes of six isolates were as follows: E1: AAC(3)-II+CTX-M-l+gyrA+gyrB +parC+Sul2; E2: CTX-M-l +gyrA+gyrB+oqxA+oqxB+parC+Sul2; E3: AAC(3)-II+CTX-M-l+gyrA+gyrB +parC+Sul2; E4: CTX-M-l+gyrA+gyrB +parC+Sul2; E5: AAC(3)-II+CTX-M-l+gyrA+gyrB +parC +Sul2; E6: AAC(3)-II+CTX-M-l+gyrA+gyrB +parC+Sul2.

## Discussion

Our results found that *E. coli* isolated from the broiler chickens showed different antibiotic resistance genes. The problem of antibiotic resistance has been concerned for many years, and the increase in antibiotic resistance is difficult to control the given population growth and the climate change. At present, the use of antibiotics is the main approach to prevent and treat diseases in the poultry industry, but the abuse of antibiotics has resulted in the emergence of antibiotic resistance in *E. coli in the* global area. Disease caused by this organism is one of the common disease agents infected by bacteria in poultry, causing vitelline peritonitis, salpingitis, septicemia, airsacculitis, perihepatitis and pericarditis (Wang *et al.*, 2013). The antibiotic resistance genes such as AAC(6')-Ib-cr and qepA have been identified in many clinical isolates with high-level antibiotic resistance (Cavaco *et al.*, 2009; Zhao *et al.*, 2010). In this study, we identified a novel isolate possessing CTX-M-1, oqxA, oqxB, parC and Sul2 resistance genes, which is, to our knowledge, the first report of such combination. Extended-spectrum β-lactamases (ESBLs), such as CTX-M-1, CTX-M-2, CTX-M-14, CTX-M-15 and CTX-M-55, have been found in many European countries and China, and are associated with *E. coli* isolates from poultry and other animals (Rao *et al.*, 2014). *E. coli* is ubiquitous in warm blooded animals and usually will not produce human diseases; but the variations within *E. coli*, especially new combinations of antibiotic resistance genes, may result in new manifestations of human diseases in the future. For example, the plasmid mediated quinolone resistance (PMQR) genes oqxA and oqxB, expressed in a novel *E. coli* isolate, may result in occurrence of a complex antibiotic resistance mechanism (Santos *et al.*, 2014).

To our knowledge, few papers have been published regarding the coexistence of PMQR and ESBL encoding genes in *E. coli* isolated from proventriculus (Ammar *et al.*, 2016; Gouvêa *et al.*, 2015). The industry is faced with a dilemma because on one hand, antibiotics are required to combat the high incidence of poultry disease; on the other hand, their use is the ultimate selection pressure that may promote widespread bacterial resistance. Our investigation showed that *E. coli* isolate E2 was not distinct in terms of antibiotic susceptibility when compared with other *E. coli* isolates although we cannot exclude the possibility that *E. coli* E2 will not have novel susceptibilities to other antibiotics. We usually only consider the presence of antibiotic resistant bacteria themselves, but our experiments showed their coexistence with ALV subgroup E in the broiler chickens. This result suggests that it will be interesting to study further whether the presence of pathogenic microorganisms other than *E. coli* can promote antibiotic resistance in bacteria.

Our study indicates that the antibiotic resistant *E. coli* is commonly seen in poultry and also reveals a novel antibiotic resistance gene pattern in *E. coli* isolated from the proventriculus. These data may help clinical veterinarians to better understand the antibiotic resistance present in relevant pathogenic *E. coli*. In addition, as an important foodborne pathogen, the antibiotic resistance gene spectrum of *E. coli* in poultry may provide an early warning of emerging antibiotic resistance patterns for the sake of human health and surveillance.

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**Conflicts of Interest:** The authors declare no conflict to interest.

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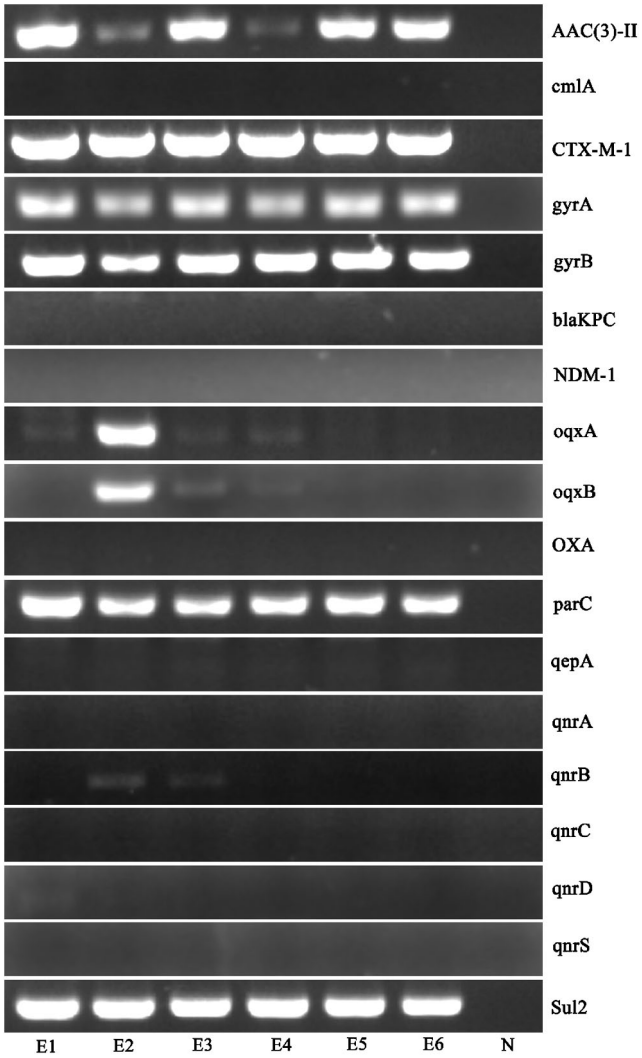
## Appendix

**APPENDIX TABLE 1**

**Resistance genes primer sequences, amplicon size and annealing temperature used in PCR assays**

| Types | Gene Symbol | Sequence (5′-3′) | Amplicon (bp) | Annealing (°C) |
|---|---|---|---|---|
| *Aminoglycosides* | *AAC(3)-II* | GGCGACTTCACCGTTTCT | 412 | 52 |
| | | GGACCGATCACCCTACGAG | | |
| *Chloramphenicol* | *cmlA* | GGGTGGCGGGCTATCTTT | 467 | 52 |
| | | GCGACACCAATACCCACTAG | | |
| *β lactams* | *CTX-M-l* | CAGCGCTTTTGCCGTCTAAG | 94 | 52 |
| | | GGCCCATGGTTAAAAAATCACTC | | |
| *Quinolones* | *gyrA* | CGATGTCGGTCATTGTTG | 496 | 52 |
| | | ACTTCCGTCAGGTTGTGC | | |
| *Quinolones* | *gyrB* | GAAATGACCCGCCGTAA | 456 | 52 |
| | | CTTGCCTTTCTTCACTTTGT | | |
| *Carbapenems* | *blaKPC* | GCTACACCTAGCTCCACCTTC | 1050 | 52 |
| | | TCAGTGCTCTACAGAAAACC | | |
| *β lactams* | *NDM-1* | ATTAGCCGCTGCATTGAT | 151 | 52 |
| | | CATGTCGAGATAGGAAGTG | | |
| *Quinolones* | *oqxA* | CTCGGCGCGATGATGCT | 393 | 52 |
| | | CCACTCTTCACGGGAGACGA | | |
| *Quinolones* | *oqxB* | TTCTCCCCCGGCGGGAAGTAC | 512 | 52 |
| | | CTCGGCCATTTTGGCGCGTA | | |
| *β lactams* | *OXA* | ACAGAAGCATGGCTCGAAAGT | 190 | 52 |
| | | TTGCTGTGAATCCTGCACCA | | |
| *Quinolones* | *ParC* | CTGAATGCCAGCGCCAAAT | 567 | 52 |
| | | GCGCATACGCACTGAAC | | |
| *Quinolones* | *qepA* | GCAGGTCCAGCAGCGGGTAG | 218 | 52 |
| | | CTTCCTGCCCGAGTATCGTG | | |
| *Quinolones* | *qnrA* | ATTTCTCACGCCAGGATTTG | 516 | 52 |
| | | GATCGGCAAAGGTTAGGTCA | | |
| *Quinolones* | *qnrB* | GATCGTGAAAGCCAGAAAGG | 476 | 52 |
| | | ATGAGCAACGATGCCTGGTA | | |
| *Quinolones* | *qnrC* | GGGTTGTACATTTATTGAATC | 447 | 52 |
| | | TCCACTTTACGAGGTTCT | | |
| *Quinolones* | *qnrD* | CGAGATCAATTTACGGGGAATA | 582 | 52 |
| | | AACAAGCTGAAGCGCCTG | | |
| *Quinolones* | *qnrS* | ACGACATTCGTCAACTGCAA | 417 | 52 |
| | | TAAATTGGCACCCTGTAGGC | | |
| *Sulfonamides* | *Su12* | GATGGCATTCCCGTCTC | 577 | 52 |
| | | TTCTTGCGGTTTCTTTCAGC | | |




**APPENDIX FIGURE 1.** Resistance genes analysis of the *E. coli*. Gel electrophoresis of AAC(3)-II, cmlA, CTX-M-1, gyrA, gyrB, blaKPC, NDM-1, oqxA, oqxB, OXA, parC, qepA, qnrA, qnrB, qnrC, qnrD, qnrS, and Sul2 resistance genes amplicon products, E1-4 were isolated from proventriculus, E5 and E6 were isolated from the trachea, N: negative controls.