Research Note: Epidemiological cutoff values and acquired resistance mechanisms of three veterinary antibiotics against *Escherichia coli* from chicken respiratory tract infections

Hui-Lin Zhang,[*] Si-Li Wu,[†] Jia-Li Fu,[†] Hong-Xia Jiang,[†] and Huan-Zhong Ding[*,1]

[*]*Guangdong Key Laboratory for Veterinary Drug Development and Safety Evaluation, South China Agricultural University, 510642 Guangzhou, China; and* [†]*National Risk Assessment Laboratory For Antimicrobial Resistance of Animal Original Bacteria, South China Agricultural University, 510642 Guangzhou, China*

ABSTRACT Florfenicol, apramycin, and danofloxacin are antibiotics approved only for veterinary use and that have good therapeutic effects on chicken respiratory infections caused by *Escherichia coli*. We established epidemiological cutoff values (**ECV**) for these antibiotics using 363 *E. coli* isolates from tracheal samples of chickens in 5 veterinary clinics in Guangdong Province, China. The minimum inhibitory concentrations (**MIC**) were determined using the agar dilution method as per Clinical and Laboratory Standards Institution guidelines. The ECV were then calculated using the statistical method and verified by normalized resistance interpretation and ECOFFinder software programs. The ECV of florfenicol, apramycin, and danofloxacin against *E. coli* were 16, 16, and 0.125 μg/mL, respectively. Susceptibility tests indicated that these isolates were resistant to florfenicol (66.7%), apramycin (22.3%), and danofloxacin (92.3%). Strains carrying *floR* were distributed in the range of MIC ≥32 μg/mL for florfenicol. Apramycin resistance was found in 77 strains (77/363, 21.1%), and isolates that carried *aac(3)-IV* were all in the range of MIC ≥512 μg/mL. Danofloxacin resistance was found in the range of MIC ≤0.125 μg/mL, but there were no mutations in the quinolone resistance–determining regions and plasmid-mediated quinolone resistance genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *aac-(6′)-Ib-cr*, *qep*, and *oqxB*. The presence of the *qnrS* gene was verified in a few of the strains with an MIC of 0.06 μg/mL. The establishment of ECV was significant for monitoring of resistance development and therapy guidance.

Key words: *Escherichia coli*, florfenicol, apramycin, danofloxacin, epidemiological cutoff value

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INTRODUCTION

Avian colibacillosis due to *Escherichia coli* can present as septicemia, granulomatosis, pericarditis, perihepatitis, aerocystitis, and enteritis. Chickens of all ages are susceptible to colibacillosis when kept under poor hygienic conditions and when improperly fed. These practices also endangere animal husbandry and cause huge economic losses. There are multiple *E. coli* serotypes that cause colibacillosis, so broadly protective vaccines against pathogenic *E. coli* are not available and antimicrobial treatments are still the best option.

Florfenicol, apramycin, and danofloxacin are approved only for veterinary use and are widely used to treat and prevent infections caused by gram-negative bacteria such as *E. coli, Salmonella*, and *Shigella* in food animals in China. Florfenicol, a structural analog of thiamphenicol with a broad antimicrobial spectrum, is approved by the US Food and Drug Administration and several Member States in the European Union for the treatment of respiratory diseases in cattle and pigs. And in China, the drug is also used in chickens to treat infections caused by *E. coli*, *Salmonella,* and *Pasteurella*. Apramycin is characterized by a wide antibacterial spectrum and low resistance and has been recommended by the US Food and Drug Administration as the drug of choice for the treatment of avian colibacillosis. Danofloxacin is a third-generation quinolone and has been approved for use in Asia, North America, and Latin American primarily for bacterial and mycoplasma diseases in cattle, pigs, and chickens.

The development of resistance in bacteria alarms that antimicrobial resistance surveillance is urgently needed to promote appropriate use of veterinary medicine.

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[1]Corresponding author: hzding@scau.edu.cn

The European Union Commission on Antimicrobial Susceptibility Testing defined wild-type (**WT**) bacterial distributions as the populations of organisms with no acquired phenotypically detectable resistance mechanism. The epidemiological cutoff value (**ECV**) was defined as the highest minimum inhibitory concentration (**MIC**) value for the WT populations when they encompass at least 95% of WT isolates (Turnidge et al., 2006). The establishment of susceptible breakpoint is a prerequisite for antimicrobial resistance surveillance.

By definition, when microorganisms are divided into the WT and non-WT population based on their ECV, the WT strain should not carry a resistance gene associated with the drug being tested. Among the 9 identified florfenicol resistance genes (*floR*, *floRv*, *floSt*, *fexA*, *fexB*, *pexA*, *cfr*, *optrA,* and *estDL136*), *floR* is the primary determinant in gram-negative bacteria causing resistance to florfenicol. The *aac(3)-IV* gene was originally discovered in an animal-derived *E. coli* and became the most prevalent apramycin resistance gene in animal and human infections. Targets gene (*gyrAB* and *parCE*) mutations in the quinolone resistance–determining regions (**QRDR**) are the primary mechanisms in *E. coli* for quinolone resistance. Plasmid-mediated quinolone resistance genes, such as *qnr*, *aac(6′)-Ib-cr, qepA,* and *oqxAB,* usually confer low-level resistance to fluoroquinolones and also can lead to an increasing quinolone resistance rate in bacteria (Kim et al., 2009).

The primary goal of the present study was to obtain information on the susceptibility to 3 commonly used veterinary drugs for *E. coli* infections and to establish the ECV for florfenicol, apramycin, and danofloxacin. We also correlated acquired resistance mechanisms and MIC distributions.

MATERIALS AND METHODS

E. coli *Isolation and Identification*

We collected 1,815 tracheal samples from chickens from 5 veterinary clinics in Guangdong province from April 2017 to December 2017. All samples were seeded onto MacConkey agar and incubated at 37°C for 18 h. Single colonies with typical *E. coli* morphology were selected from each sample and identified by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (Shimadzu Biotech, Kyoto, Japan). All isolates were stored at −80°C in Luria-Bertani broth containing 30% glycerol. This study protocol was approved by the South China Agricultural University Animal Ethics Committee.

Antimicrobial Susceptibility Testing

The MIC of florfenicol, apramycin, and danofloxacin were determined in triplicate for each bacterial strain using the agar dilution method on Mueller-Hinton agar plates as per the Clinical and Laboratory Standards Institution reference method (CLSI, 2016). *E. coli* ATCC 25922 was used as the quality control strain.

Epidemiological Cutoff Value Definition and Establishment

The ECV is used to classify bacterial populations into WT and non-WT groupings and best defines the estimated upper end of the WT population that encompasses at least 95% of the WT MIC distribution. The conventional method for MIC determination that defines the beginning of the WT MIC end point and *in vitro* resistance is a visual inspection of MIC histograms when there is a clear-cut bimodal distribution. However, in most cases, MIC distributions for WT and resistance MIC values overlap significantly. In the present study, we calculated the ECV by applying a nonlinear regression analysis to the MIC distribution data as previously described by Turnidge et al. (2006). In brief, 1) MIC histograms were transformed into $\log_2$ MIC values, and normality was assessed using SPSS software, version 23.0.0.0 (IBM, Chicago, IL); 2) the $\log_2$ mean, $\log_2$ SD, and N (sample number) were calculated by applying a nonlinear least squares regression to the multifitted $\log_2$-transformed MIC using GraphPad Prism 7.04 (GraphPad, San Diego, CA); and 3) the WT strain distribution was determined using a 95% confidence interval for $\log_2$ mean and $\log_2$ SD values using NORMINV, and then, NORMDIST in Excel (Microsoft, Redmond, WA) was used to calculate the probability that WT strains lie above the upper limit of WT strains to verify the results. The ECV was defined as the MIC value closest to the upper limit of WT strains and contained at least 95% of the WT strains. Normalized resistance interpretation (**NRI**) (http://www.bioscand.se/nri/) and ECOFFinder (ECOFFinder XL 2010, version 2.1; http://clsi.org/meetings/microbiology/ecoffinder/) were used to verify the calculated ECV.

Detection of Acquired Resistance Mechanisms

All *E. coli* isolates were screened for the presence of *aac(3)-IV*, *floR* and plasmid-mediated quinolone resistance genes and mutations in the QRDR of *gyrAB* and *parCE* using PCR and DNA sequence analysis.

RESULTS AND DISCUSSION

All 1,815 tracheal samples from chickens yielded 363 *E. coli* isolates. We analyzed the MIC distribution for our 3 tested drugs against these isolates. The MIC distribution for florfenicol ranged from 4 to 512 μg/mL, with a bimodal distribution and a maximum at 8 μg/mL. The MIC distribution for apramycin ranged from 4 to 16 μg/mL, also with an 8 μg/mL maximum that encompassed 63% (244/363) of the strains. The MIC distribution of danofloxacin was broad and ranged from 0.015 to 512 μg/mL. The first clear-cut peak

occurred from 0.015 to 0.125 μg/mL (ranged at 0.03 μg/mL), while the remaining peaks were discontinuous, indicating overlap of WT and resistant MIC values (Figure 1).

The estimated ECV for apramycin and florfenicol as determined by visual inspection were both at 16 μg/mL, whereas there was no clear-cut ECV for danofloxacin using this method. Further statistical analyses generated MIC frequency histograms for apramycin and florfenicol that appeared normally distributed when plotted logarithmically with MIC in the ranges of 2 to 16 and 2 to 32 μg/mL, respectively. The ECV were consistent when the data were plotted using the NRI and ECOFFinder programs. In contrast, for danofloxacin, both MIC frequency histograms appeared normal when plotted logarithmically in the ranges of 0.015 to 0.125 μg/mL and 0.125 to 2 μg/mL. A nonlinear least squares method was then used to simulate the cumulative frequency distribution of $\log_2$MIC in the range of MIC ≤0.125 μg/mL and MIC ≤2 μg/mL. At MIC ≤0.125 μg/mL, the estimated number of WT isolates was closest to the true total number of bacteria, and hence, the MIC ≤0.125 μg/mL was considered a tentative ECV. The NORMINV and NORMDIST functions in Microsoft Excel were then used to verify the tentative ECV of 0.125 μg/mL that covered at least 95% of MIC distributions. The ECV calculated using NRI and ECOFFinder were also 0.125 μg/mL (Table 1 and Figure 1).

The ECV for florfenicol in the present study mirrored the results of *E. coli* isolates from pigs (Lei et al., 2019).

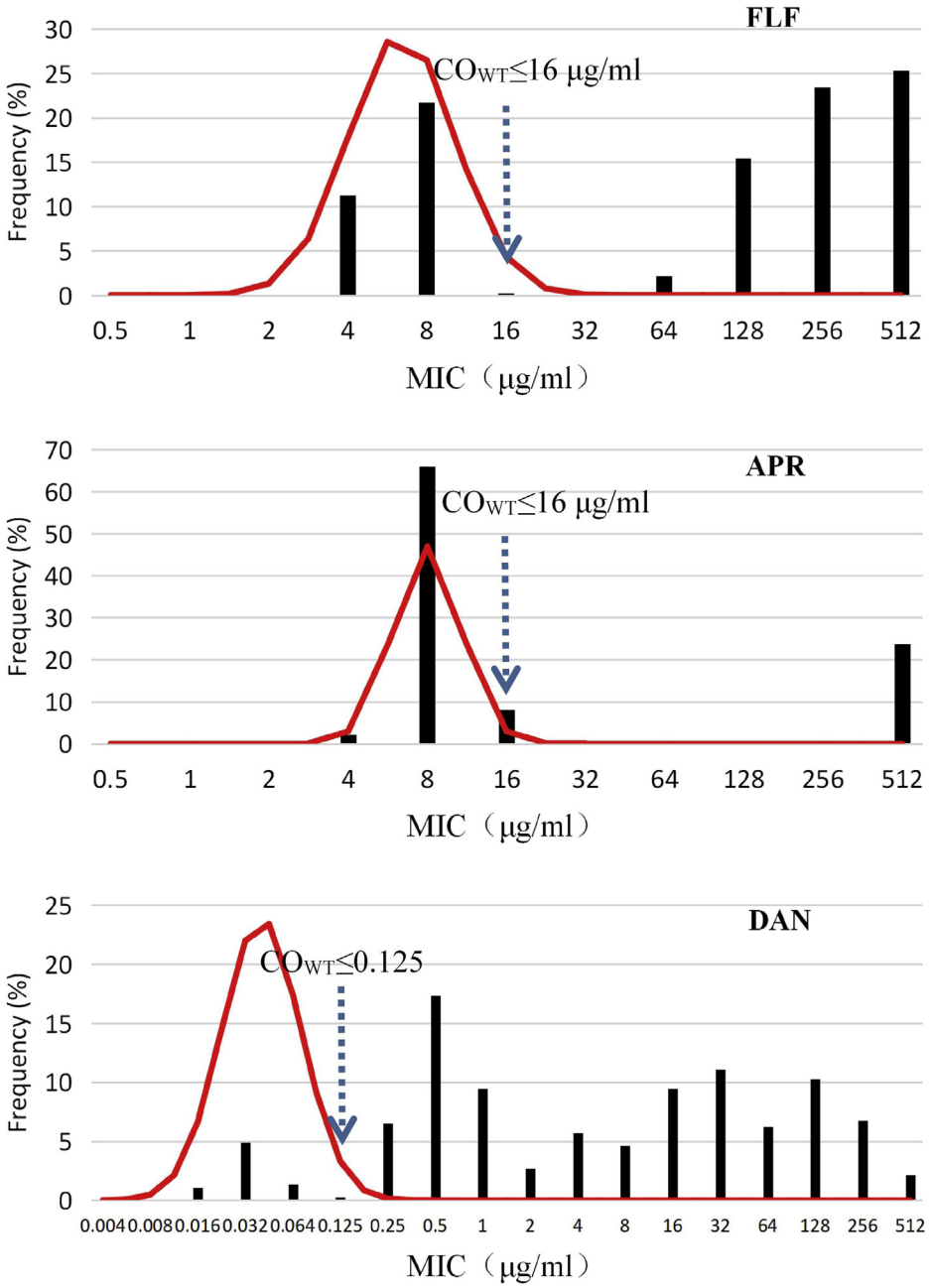

Figure 1. Distribution of MIC values and epidemiological cutoff values for florfenicol, apramycin, and danofloxacin on 363 *E. coli* isolates. Abbreviation: MIC, minimum inhibitory concentration.

Table 1. Analysis of ECV for florfenicol, apramycin, and danofloxacin against *E. coli* from chicken respiratory tract infections by statistical methods.

Test drug	Best-fit MIC range (μg/mL)	True WT number	Estimated number	Mean log_2MIC	SD log_2MIC	Upper limit (μg/mL)	Probability below the upper limit
Florfenicol	≤16	121	121	2.147	0.3549	16	99.99%
Apramycin	≤16	282	282	2.605	0.3173	16	99.94%
Danofloxacin	≤0.125	28	27.56	-5.441	0.5303	0.125	99.98%

Abbreviations: ECV, epidemiological cutoff value; MIC, minimum inhibitory concentration; WT, wild-type.

For apramycin, the breakpoint was defined as 16 to 32 μg/mL by the National Antibiotic Resistance Monitoring Study. And the ECV for apramycin was identical to a previous report for *E. coli* isolated from chicken intestinal tracts that was also calculated using a statistical method (Tian et al., 2019). A previous study of 1,233 *E. coli* isolates from pig intestinal tracts in China reported an ECV of 8 μg/mL and a pharmacokinetic/pharmacodynamic cutoff (CO_{PD}) of 0.03 μg/mL for danofloxacin (Yang et al., 2019), in which the ECV was significantly higher than the CO_{PD} value, and the CO_{PD} value was much closer to our ECV of 0.125 μg/mL. The reason for this discrepancy may be that the *E. coli* strains collected clinically were highly resistant to danofloxacin, resulting in far fewer WT strains, considering the danofloxacin resistance rate in the *E. coli* strains was 92.3%. We introduced an additional procedure and assessed the prevalence of resistance genes in our *E. coli* population. We found that strains not carrying related resistance genes, that is, WT strains, were distributed within the ECV. This indicated that the ECV we established in this study were scientific and reasonable. In addition, the freeware statistical programs NRI and ECOFFinder can be robustly applied to establish ECV levels based on MIC data obtained using the double dilution method.

A total of 363 *E. coli* isolates were screened for the presence of antibiotic resistance genes, and 121 strains possessed *floR* (33.3%); *floR* strains were distributed in the range of MIC ≥ 32 μg/mL. The apramycin resistance gene *aac(3)-IV* was carried by 77 of 363 (21.1%) strains, and all these possessed an MIC ≥512 μg/mL. A positive rate for possession of the aac(3)-IV gene in *E. coli* isolates of chicken origin was MIC value dependent (Tian et al., 2019). Plasmid-mediated quinolone resistance genes were present in 260 strains, and 71.6% of strains carried *qnrS* that were distributed in the MIC range of 0.06 to 512 μg/mL. An additional 94 strains carried *aac-(6′)-Ib-cr* (25.9%), 3 carried *qepA* (0.08%), and 49 carried *oqxB* (13.5%). Strains that possessed an MIC ≤0.125 μg/mL did not carry these resistance genes, and neither *qnrA*, *qnrB*, *qnrC*, nor *qnrD* was found in the 28 strains that possessed danofloxacin an MIC ≤0.125 μg/mL. Mutations in QRDR target genes were also not found. However, the resistance gene *qnrS* was found in strains with MIC ranging from 0.06 μg/mL to 512 μg/mL. The explanation for the results may be as follows: a high number of non-WT strains coupled with few WT strains may result in statistical errors. Moreover, bacterial quinolone resistance mechanisms are complex, and this could affect the final outcome that slightly differed from the actual situation.

Based on the ECV of 3 drugs in the present study, resistance rates of 363 isolates were 66.7% (242/363) for florfenicol, 22.3% (81/363) for apramycin, and 92.3% (335/363) for danofloxacin. Long-term use and widespread use of florfenicol have resulted in increasing emergence of *E. coli* resistance. From 2008 to 2015, the florfenicol resistance rates for chicken and pig isolates increased from 10.19 to 66.26% and 14.75 to 62.98%, respectively (Zhang et al., 2017). Studies have reported that the resistance rate to apramycin of *E. coli* of animal origin was as high as 80%, and resistance to apramycin has been detected in human Enterobacteriaceae isolates (Curcio et al., 2017). For danofloxacin, the resistance rate in *E. coli* isolates recovered from feces and viscera from chickens and turkeys was <40%, using a 0.25 μg/mL breakpoint (Vanni et al., 2014). However, we found a 92.3% resistance rate for danofloxacin using an ECV of 0.125 μg/mL, and this level is much higher than that reported in previous studies. High resistance may lead to failure of treatment. The results warn us that we should adjust dosing regimens for treatment of avian colibacillosis or avoid using drugs with high resistance rates.

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DISCLOSURES

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.

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