REPORT

Susceptibility of avian pathogenic *Escherichia coli* from Zoo birds in Indonesia to antibiotics and disinfectants

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Abstract: Antibiotic resistance in avian pathogenic *Escherichia coli* (APEC) is a common problem in the Indonesian poultry industry. Zoo birds have been postulated as sentinels, reservoirs, and potential spreaders of antibiotic resistance, although much is still unknown about the strains of zoo birds. Disinfection can reduce the infection burden. However, little is known about the presence of resistance against these products. Sixty one APEC strains were isolated from Indonesian zoo birds. The resistance to different classes of antibiotics as well as the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of five disinfectants most often used in the poultry industry was determined. Resistance to tetracycline (42.6%), sulfonamides (24.5%), ampicillin (22.9%), gentamicin (19.6), nalidixic acid (18.03%) and streptomycin (16.3%) was high, but resistance to other tested antibiotics was low and none of the isolates were resistant to extended spectrum beta-lactamase (ESBL) producers. Sixteen strains (26.2%) were found positive for multi drug resistance. The MIC of the disinfectants for the APEC strains showed normal distribution, indicating that there was no acquired resistance. MBCs were similar to MICs using the broth dilution method, showing the bactericidal effect of the disinfectants. Phenotypic resistance to commonly used disinfectants could not be found, indicating that the current use of disinfectants in the zoo and aviaries did not select for resistance. Significantly high resistance rates against commonly used antibiotics in Indonesian zoos is worrisome and indicates that widespread use of antibiotics could have negative implications for animal health and the environment. Proper use of antibiotics and surveillance programs to monitor antimicrobial resistance in pathogenic bacteria are warranted.

Keywords: Avian pathogenic *Escherichia coli*, antibiotics, disinfectants, Indonesia, Zoo birds.

INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) are specific *E. coli* strains belonging to the extra-intestinal *E. coli* group, causing colibacillosis in avian species of all ages and results in huge economic losses in the poultry industry worldwide (Barnes *et al.,* 2008; Dziva and Stevens, 2008). Antibiotics are the major weapon to reduce both the incidence and the mortality associated with avian colibacillosis (Harisberger *et al.,* 2011). However, resistance to antibiotic compounds has emerged in some pathogenic *E. coli* strains (Oosterik *et al*., 2014), which may lead to therapy failure and potential economic losses for the farmers. Antibiotic resistance among poultry *E. coli* isolates is common (Harisberger *et al*., 2011; Smet *et al.,* 2008; Vandemaele, 2002; van den Bogaard, 2001), even though restrictions were put on the use of antibiotics in most of the countries (Casewell *et al*., 2003). Zoo birds are rarely given antibiotics because infections occur less often than in broilers and layers (Harisberger *et al.,* 2011; van den Bogaard, 2001; Oosterik *et al*., 2014). During rearing, however, zoo birds do receive antibiotics, and antibiotic resistance can persist for a long time in the intestinal tract without selective pressure of antibiotics (Chaslus-Dancla, 1987). Other risk factors for bacterial resistance are crowding and poor sanitation. Sanitation, which includes regular cleaning and disinfection, is a good way to lower the bio burden and thereby the level of specific pathogens in zoo and poultry farms (Gehan *et al.,*2009).However, little is known about resistance to disinfectants and the degree of resistance. The only resistance to quaternary ammonium compounds is relatively well known which, in the case of *E. coli*, is mainly encoded by the *qacEΔ1* gene, mostly associated with class 1 integrons. This gene, together with *sul1*, which encodes resistance to sulfonamide, is found in the 39-conserved segment (39-CS) of class 1 integrons (Paulsen *et al*., 1993). Next to the 39CS and 59CS region (containing the integrase gene, an integration site, and a promoter), class 1 integrons have a variable region that can contain one or more mobile gene cassettes, normally coding for antibiotic resistance (Fluit *et al.,* 2004).

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Furthermore, multi drug efflux pumps, mainly encoded on the chromosome, can lower the sensitivity toward disinfectants and antibiotics by pumping out the antimicrobials (Poole, 2005; Oosterik *et al.,* 2014).To monitor the development of resistance and to choose the most effective antimicrobial agents for therapy, reliable data on the prevalence of resistance to specific antimicrobial compounds in bacteria isolated from humans, animals and birds are required. To our knowledge, no report has been published in scientific journals regarding to antimicrobial resistance of APEC from Indonesian zoo birds. The present study evaluated the susceptibility of APEC strains, obtained from zoo birds suffering from colibacillosis, to antibiotics and to commonly used disinfectants in the poultry industry. This report is the first study conducted on the susceptibility of APEC strains circulating in Indonesian zoo birds.

MATERIALS AND METHODS

Sample collection

A total of 310 organs from sick and dead birds were collected after necropsy from zoos and aviaries in Indonesia during January 2014 to December 2014. Samples were transferred within 2 hr to the laboratory for further investigations using aseptic measures in a cold box with ice. Among these, 61 samples were diagnosed as avian colibacillosis on the basis of necropsy findings and laboratory diagnostic methods.

Isolation and identification of E. coli

Positive samples were cultured onto 3% sheep blood agar (Oxoid, Basingstoke, UK) and incubated overnight at 37°C. Suspected *E. coli* colonies were inoculated onto MacConkey agar plates and confirmed as *E. coli* by biochemical tests. All isolates were stored in-80°C in a bacterial freeze medium (Luria broth [BD, Sparks, MD]; phosphate buffered saline containing 0.45% sodium citrate, 0.1% magnesium sulfate, 1% ammonium sulfate, and 4.4% glycerol). Bacteria were routinely grown at 37°C unless stated otherwise. The *E. coli* antisera were used to serogroup the *E. coli* strains using the microtiter agglutination test as previously described (Oosterik *et al*., 2014).

Disinfectants

Disinfectants commonly used in poultry farms were selected and the five active ingredients most prevalent in these products were selected. These ingredients were: alkyl dimethyl benzyl ammonium chloride (QAC, 50%), formaldehyde (FOR, 35% wt/vol in H_2O), glutaraldehyde (GLU, 50% wt/vol in H_2O), glyoxal (GLY, 40% wt/vol in H_2O), and hydrogen peroxide (H_2O_2, 42.1% wt/vol in H_2O; EcoClearProxH). FOR, GLU, GLY, and QAC were obtained from Sigma-Aldrich (Diegem, Belgium) and the $H_2O_2$from ABT Belgium BVBA (Affligem, Belgium).

Antibiotic susceptibility testing

Resistance to antibiotics was determined by disk diffusion on Mueller-Hinton agar (MHA) according to the European Committee on Antimicrobial Susceptibility Testing disk diffusion test guidelines (EUCAST, 2010). The isolates were cultured overnight at 37°C on Uriselect 4 plates (Bio-Rad Laboratories, Hercules, CA) in order to achieve pure cultures for antibiotic susceptibility testing. Each isolate was tested for antibiotic susceptibility using a panel of the following antimicrobial agents: amoxicillin (10µg), chloramphenicol (30µg), gentamicin (30µg), nalidixic acid (30µg), tetracycline (30µg), streptomycin (10µg), ciprofloxacin (5µg), cefuroxime (30µg), cefadroxil (30µg) and sulfonamide (25µg) in the disc form. All compounds were provided by Oxoid. An isolate of *E. coli* ATCC 25922 was used as reference strain in all tests.

Identification of ESBL production in pathogenic E. coli

Five *E. coli* isolates that showed resistance against more than six antibiotics (including β-lactam antibiotics) were cultured overnight at 37°C on chrom ID^{TM} ESBL plates (bioMerieux, Marcy L'Etoile, France) according to the manufacturer's instructions. In addition, a cefpodoxime/ cefpodoxime + clavulanic acid double-disk test (MAST Diagnostics, Bootle, UK) was performed on these isolates, and *E. coli* ATCC 25922 was used for the quality control of this test.

Disinfectant susceptibility testing

MICs of the selected disinfectants for the APEC strains were determined by the agar dilution and the broth micro dilution assay according to the Clinical and Laboratory Standards Institute (CLSI, 2008).

Agar dilution method. Fresh APEC cultures were diluted in saline to obtain a culture of approximately 1.5 x10^7 colony-forming units/ml (CFU/ml). The susceptibility test was performed on MHA (CM0337, Oxoid N.V., Erembodegem-Aalst, Belgium) to which twofold dilutions of the disinfectants were added. On every plate, the ATCC strain 25922 was inoculated as a control. Inoculation was performed with a multipoint inoculation machine (Denley Instruments, Billingshurst, U.K.) whereby approximately 2x10^4 CFU/spot of every strain was inoculated on the agar. After incubation for 24 hr the plates were examined for visible growth.

Broth microdilution assay. Two fold dilutions of the disinfectants were added to test tubes with cation-adjusted Mueller-Hinton broth (MHB; CM0405; Oxoid N.V.) after which 50µl of the disinfectant dilution was transferred to the wells of a sterile plastic 96-well micro dilution plate (Pittsburgh Corning Europe, S.A., Lasne, Belgium). Fresh, 24-hr incubated (230 rpm) APEC cultures in BHIB were diluted in saline to obtain a concentration of 1.5 x 10^6 CFU/ml. Fifty micro liters of these APEC cultures were added to the wells of the micro dilution plate,

yielding a total suspension volume of 100µl (disinfectant and APEC culture), after which the plates were incubated for 24 hr and then the MICs were read. The MIC is the lowest concentration of disinfectant where no visible growth of the APEC strains could be seen.

Minimum bactericidal concentration (MBC) determination

The MBC of the disinfectants for the different APEC strains was determined by plating the suspensions (100 µl) from the micro dilution plate where no visible growth was seen, from the broth dilution assay, onto MHA. The plates were incubated for 24 hr after which MBCs were determined. The MBC is the concentration of disinfectant where a 99.9% reduction in bacterial numbers could be seen.

STATISTICS ANALYSIS

Confidence intervals (CI) were calculated for the resistance percentages of APEC strains to the different antibiotics, based on the binominal distribution in Microsoft Excel (2010; Microsoft, Redmond, WA). The same procedure was followed for determining the number of strains being resistant to different classes of antibiotics. Differences in resistance against the tested antibiotics between sero groups was analysed by the chi square test. A value of $P<0.05$ was considered significant.

RESULTS

The prevalence of avian colibacillosis was high in the investigated zoo birds. Out of 310 investigated samples, 61 were diagnosed as avian colibacillosis through culture and biochemical tests. The APEC strains were of sero group O1 (n=5), O2 (n=16), and O78 (n=34) and O88 (n=6).

Antibiotic susceptibility

The APEC strains were tested for their susceptibility to different antibiotics. The prevalence of antibiotic resistance phenotypes of all isolates are presented in table 1. Highest resistance levels were observed against tetracycline (42.6%), followed by salfonamides (24.5%), amoxicillin (22.9%), gentamicin (19.6%), nalidixic acid (18.03%), and streptomycin (16.3%). Significant differences in resistance against the tested antibiotics among sero groups could not be found ($P>0.05$). Multi drug resistance was offered by 16 strains (26.2%) (table 2). The most common multidrug resistance was sulfonamide-tetracycline-streptomycin (four isolates), followed by tetracycline-amoxicillin-gentamicin (three isolates) and tetracycline-amoxicillin and nalidixic acid (two isolates). None of the isolates were found positive for extended-spectrum beta-lactamases (ESBLs) in this study (data not shown).

Susceptibility to disinfectants

The MICs as determined by the agar dilution and broth micro dilution assay and the MBCs of the disinfectants for the 61 tested APEC strains are given in table 3. The MIC of H_2O_2 was 64µg/ml and 64-128µg/ml for the broth micro dilution and agar dilution method, respectively. The MBC was 64µg/ml. For QAC, MICs of 32-64µg/ml and 128-256µg/ml were found via the broth and agar dilution method, respectively. The MBC was between 32-64 µg/ml. The MICs of FOR for the APEC strains using the broth micro dilution assay ranged between 80-120µg/ml and the MICs via the agar dilution assay ranged between 120-160µg/ml. The MBCs were between 80-120µg/ml. For GLU, an MIC of 1920µg/ ml was found via the broth micro dilution method and was between 1920-3840µg/ml via the agar dilution method; the MBC was 1920µg/ml. The MIC of GLY via the broth and agar dilution was between 460-1840µg/ml and 460-920µg/ml, respectively. The MBC was 920-1840µg/ml. All MICs of the disinfectants for the strains via the broth dilution method were the same as the MBCs, showing the bactericidal effect of the disinfectants.

DISCUSSION

Avian colibacillosis infection is present year-round with a high prevalence in Indonesian birds and others including zoo birds. Raising zoo birds in confined cages, with shared water troughs or feed utensils, is common practice in zoo in Indonesia and such practices may increase the probability of *E. coli* infections. A selection of 61 APEC strains was assessed; these strains were tested for susceptibility to different antibiotics and five disinfectants commonly used for disinfection in zoos. The pathogenic *E. coli* isolates from this study showed resistance phenotypes against all classes of the tested antibiotics. High resistance was found against tetracycline, sulfonamide, amoxicillin, gentamicin, nalidixic acid and streptomycin, which also are common resistance types in domesticated and wild animals in contact with human activities (Hasan *et al.,* 2011; Ewer *et al*., 2009). Interestingly, bacterial resistance to yester-generation antibiotics was high, but resistance to relatively new antibiotics, such as second and third-generation cephalosporins or quinolones, was very low (Smet et al 2008). Bacterial resistance to tetracycline, ampicillin, sulfonamides, nalidixic acid, streptomycin, chloramphenicol, and gentamicin of lower profile in present study compared to similar studies from Switzerland (Lanz *et al.,* 2003), Belgium (Oosterik *et al*., 2014), Korea (Kim *et al*., 2007), as well as to clinical, poultry, and environmental isolates from Bangladesh (Hasan *et al.*, 2011). Tetracycline has been utilized worldwide in veterinary practice for several decades, and it is a common compound in poultry feed in Indonesia. Tetracycline resistance in our isolates was quite high (42.4%), which is consistent with avian *E. coli* isolates from poultry in different countries (Salehi and Ghanbarpour, 2010; Persoons *et al*., 2012; Vandemaele *et al*., 2002; Smet *et al*., 2008). Increased occurrence of multi drug resistant bacteria in avian species is alarming

Table 1: Prevalence of antimicrobial resistance phenotypes in pathogenic *E. coli* isolated from zoo birds in Indonesia

Class of antibiotics	Antibiotics compounds	*N* tested	% prevalence (No. of resistant isolates)	(95% CI)
Tetracyclines	Tetracycline	61	42.6 (26)	(25.6–45.0)
Salfonamides	Salfonamide	61	24.5 (15)	(17.4–28.0)
Penicillin	Amoxicillin	61	22.9 (14)	(16.8–27.0)
Aminoglycosides	Gentamycin	61	19.6 (12)	(14.4–24.0)
	Streptomycin	61	16.3 (10)	(14.2–22.0)
Quinolones	Nalidixic acid	61	18.03 (11)	(9.5–25.0)
	Ciprofloxacin	61	1 (1)	(0.0–7.0)
Chloramphenicol	Chloramphenicol	61	4.9 (3)	(2.2–11.0)
Cephalosporin	Cefuroxime	61	3.2 (2)	(2.6–12.0)
	Cefadroxil	61	1(1)	(0.0–7.0)

Table 2: Number and percentage of APEC strains resistant against total number of different antibiotic classes.

N of antibiotic classes resistant	*N* of strains	% strains (95% CI)
0	25	40.9 (27.7-53.0)
1	6	9.8 (5.3-16.0)
2	3	4.9 (1.4-12.0)
3	6	9.8 (5.5-15.0)
4	4	6.5(2.2-16.0)
5	3	4.9(3.0-8.0)
6	2	3.2 (2.0-6.0)
7	1	1.0 (0.0-6.0)

Table 3: Minimum inhibitory (broth dilution and agar dilution method) and minimum bactericidal concentrations for 61 APEC strains determined for 5 different disinfectants. (A) Alkyl dimethyl benzyl ammonium chloride (QAC). (B) Formaldehyde (FOR). (C) Glutaraldehyde (GLU). (D) Glyoxal (GLY). (E) Hydrogen peroxide (H_2O_2).

Disinfectants	MIC broth	MIC agar	MBC
QAC	32–64 µg/ml	128-256 µg/ml	32–64 µg/ml.
FOR	80-120 µg/ml	120–160 µg/ml	80–120 µg/ml
GLU	1920 µg/ ml	1920–3840 µg/ml	1920 µg/ml
GLY	460–1840 µg/ml	460–920 µg/ml,	460–1840 µg/ml
H_2O_2	64 µg/ml	64–128 µg/ml	64 µg/ml

and instrumental in reducing the choice of antibiotics for treating the infection. Twenty six percent of the isolates showed multi drug resistance, and so much high rate reflects the frequent use of the co or mixed preparation of antibiotics in zoo birds in Indonesia.

Different methods are available for the determination of inhibiting concentrations of disinfectants, thus explaining differences in MICs. Previously, MICs of QAC (59 µg/ml), FOR (156µg/ml), GLU (3250µg/ml), and H_2O_2 (2505 µg/ml) were determined for *E. coli* ATCC strain 25922 via broth dilution (Mazzola *et al.,* 2009). During this study, MICs of 16µg/ml (QAC), 40µg/ml (FOR), 1920µg/ml (GLU), and 64µg/ml (H_2O_2) were found for ATCC 25922, respectively. The difference in MICs is probably due to the use of a different method for determining the MICs and the use of a different medium (trypticase soy broth (TSB) instead of MHB) and inoculum size. The higher divalent cation concentrations in TSB have an influence on certain disinfectants such as QAC, as they will react with the disinfectant and decrease the bactericidal potency **(**Chambers *et al*., 1955). Furthermore, a larger inoculum size of ATCC 25922 was used in previous studies (Mazzola *et al*., 2009; Oosterik *et al*., 2014), probably resulting in a higher MIC, thus showing the need for an optimized method for determining MICs and MBCs of disinfectants in order to be ableto compare them. Despite that fact, the relative order of activity of the disinfectants was similar (QAC>FOR >H_2O_2>GLU). It may be noted that MICs of GLU via the agar and broth dilutions are probably overestimated due to inhibition of GLU by the constituents in the media, leaving less compound available to react with the bacteria (Russell, 1994). MICs of H_2O_2 via the agar and broth dilution method were the same for most strains, namely 64µg/ml, while MICs of GLY via the broth dilution method were in general a factor 2 higher than the MICs via the agar dilution

method. MICs of FOR and GLU, on the other hand, were a factor 2 higher using the agar dilution method than via the broth dilution method, while the MICs of QAC were a factor 4 higher by using the agar dilution method. Implying that inhibitory concentrations via the two different methods are not the same, the agar dilution method showed a higher MIC in most cases. This can be due to a faster inactivation of the disinfectant in agar (due to interaction with medium components) leading to lower MICs. These results show that MICs determined for the two different methods cannot be directly compared. MBCs determined by the broth micro dilution method were, in general, the same as the MICs determined via the broth micro dilution method, showing that the disinfectants are bactericidal. The MIC and MBC of different disinfectants had a homogeneous distribution in the concentrations. This indicates that there was no acquired resistance for any of the disinfectants, similar to the previous studies (Oosterik *et al*., 2014). Little is known about the genetic background of resistance of *E. coli* to disinfectants. Only the resistance against QAC has been described. It has been shown that this bacterial resistance is encoded by the *qacE* or *qacEΔ1* gene that is present in class 1 integrons (Paulsen *et al*., 1993) or via up-regulation of the chromosomally encoded multi drug efflux pumps (Poole,2005). The presence of *qac* genes has been shown not to be associated with differences in MICs in Gram-negative bacteria (Jaglic *et al*., 2012; Kucken *et al*., 2000), although it has been shown for *Staphylococcus aureus* that the presence of the qac genes was associated with an elevated MIC to chlorhexidine and benzalkonium chloride compared to strains not having the *qac* genes (Furi *et al*., 2013), while an increased MBC for biocides containing QACs and chlorhexidine gluconate was found when *S. aureus* harboured *qac* genes (Smith *et al*., 2008). More-sensitive methods are necessary to discriminate between susceptible and resistant strains. Therefore, first of all a clear definition between reduced susceptible and resistant is necessary as well as a more-sensitive method (Maillard *et al*., 2013). Using MBCs instead of MICs might be a step in the right direction, as this reflects the concentration at which the biocide kills. Furthermore, working with molarity instead of weight/volume might provide in partially the solution because this fine tunes the weight of the disinfectant. Genetic linkage between integrons and conjugative plasmids and transposons seems to be a plausible explanation (White *et al*., 2001; Marchant *et al.,* 2013; Koczura *et al*., 2013).

CONCLUSION

The prevalence of antimicrobial resistance in APEC isolates from zoo birds was high for tetracycline, sulfonamides, amoxicillin, gentamicin, nalidixic acid and streptomycin, but low resistance percentages were detected for the other tested antibiotics. The strains showed no observable phenotypic resistance to disinfectants commonly used in the zoo, although they showed resistance to tested antibiotics. MICs determined via the broth and agar dilution method cannot be directly compared because different concentrations were found for the strains, thus showing the need for one standardized method for determining MICs and MBCs. Surveillance programs to monitor antimicrobial resistance in pathogenic bacteria are needed in Indonesia and other developing countries because in addition to animal health problems, transmission of resistant clones and resistance plasmids of *E. coli* from livestock to humans can occur. Further, in order to reduce the emergence and spread of antibiotic resistance in Indonesia, strict measures for proper sale and haphazard use of antibiotics for veterinary and human prescriptions need to be adopted in future.

ACKNOWLEDGEMENT

We thank to Dr. Jacub Terigan for helpful discussion and critical reading of the manuscript.

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