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# Comparison of the Antibiotic Resistances of the Bacteria Harboring and not Carrying Integrons

Fuying Zheng, Qiwei Chen, Xiaowei Gong and Yongsheng Liu
State Key Laboratory of Veterinary Etiological Biology, Chinese Academy of Agricultural Sciences, Lanzhou Veterinary Research Institute, No. 1 Xujiaping, Yanchangbao, 730046 Lanzhou, China

**Abstract:** Class 1-3 integrons from 87 strains of *Escherichia coli*, Salmonella, *Riemerella anatipestifer* and staphylococci isolated from pigs and ducks were screened and the variable regions of the class 1 and 2 integrons were amplified. No class 3 integrase gene fragments were amplified from any of the 87 strains; class 2 integrase gene fragments were amplified from only seven strains and no class 2 integron variable region gene cassettes were amplified. Class 1 integrase gene fragments were detected in 65 strains and variable region gene cassettes were amplified from 26 strains which were confirmed by sequencing to be the *aadA2*, *dfrA12-orfF-aadA2*, *dfrA17-aadA5*, *aacA4-catB8-aadA1* and *dfrA32-ereA-aadA2* gene cassettes. The 87 strains were tested for drug susceptibility to 10 kinds of antibiotics using the Broth Microdilution Method and minimum inhibitory concentrations. There was no significant difference in the resistance phenotypes of strains carrying gene cassettes and those not carrying gene cassettes and there was no correlation between the antibiotic resistance of a strain and whether it carried a resistance-gene cassette. The integron-mediated horizontal transfer of resistance genes might be just one resistance mechanism acquired by bacteria during their evolution and may not play a major role under the selection pressure exerted by the wide variety and high concentrations of antibiotics used at present, whereas it may play a synergistic role.

**Key words:** Integron, gene cassettes, antibiotic resistance, antibiotic susceptibility, bacteria

## INTRODUCTION

For many years, research into bacterial antibiotic resistance mechanisms has focused on: the relationship between the permeability of the bacterial cell membrane and antibiotic resistance; the relationship between the drug target site and antibiotic resistance; the relationship between bacterial deactivation enzymes or hydrolytic enzymes and antibiotic resistance; efflux pump antibiotic resistance systems; the relationship between changes in metabolic pathways and antibiotic resistance and the horizontal transfer of resistance genes.

Bacteria acquire exogenous resistance genes through horizontal gene transfer which is one reason for the accelerated emergence of drug-resistant clinical strains. The integron-gene cassette system mediates the horizontal transfer of resistance genes (Collis and Hall, 1992; Di Conza and Gutkind, 2009; Gravel *et al*., 1998; Mazel, 2006). In this study, we investigated the role played by this mechanism in mediating bacterial antibiotic resistance in clinical isolates and how important this role may be in bacteria. From November 2013 to June 2014, we isolated 26 strains of *Escherichia coli* (*E. coli*), Salmonella and Staphylococcus that carried resistance-gene cassettes from sick pigs and ducks. The types of resistance-gene cassettes carried by these strains were analyzed. At the same time, 61 strains of *E. coli*, Salmonella, *Riemerella anatipestifer* (*R. anatipestifer*) and staphylococci that carried no gene cassettes were also isolated. These 87 strains were tested for drug susceptibility according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013) with the Broth Dilution Method and 10 kinds of antibiotics were tested. We found no significant correlation between the presence of a resistance-gene cassettes and bacterial antibiotic resistance indicating that integrons may not play a major role in mediating antibiotic resistance in clinical bacterial isolates.

## MATERIALS AND METHODS

**Collection of infectious materials and bacterial isolation:** From November 2013 to January 2014, 35 samples of infectious materials including livers, spleens, hearts, lungs and kidneys from dead pigs suspected of *E. coli* or

**Corresponding Author:** Fuying Zheng, State Key Laboratory of Veterinary Etiological Biology, Chinese Academy of Agricultural Sciences, Lanzhou Veterinary Research Institute, No. 1 Xujiaping, Yanchangbao, 730046 Lanzhou, China

Salmonella infections, were collected from two pig farms in Qingyang and Lanzhou (Gansu Province), China. From April 2014 to June 2014, 67 heads of ducks suspected of *R. anatipestifer* infections were obtained from Weifang and Liaocheng (Shandong Province) and Xinyang (Henan Province). *E. coli* and Salmonella were isolated and identified as described previously (Huang *et al*., 2012; Zhang *et al*., 2012). *R. anatipestifer* was isolated and identified as described previously (Zheng *et al*., 2011). Twenty strains of *E. coli* and 12 strains of Salmonella were isolated from the infected pigs; five strains of *E. coli*, 13 strains of Salmonella and 31 strains of *R. anatipestifer* were isolated from duck brain tissues. Six strains of Staphylococcus were isolated from duck brain tissues by chance and were identified by determining their 16S rDNA sequences. The strains are listed in Table 1.

Table 1: Integrons, gene cassettes and drug susceptibility of the 87 isolates from pig and duck

| Isolates | Host | 1 | 2 | 3 | Int1 | Gene cassettes of class 1 integrons | R-phenotype[a] | S-phenotype[a] |
|---|---|---|---|---|---|---|---|---|
| E. c-1 | Pig | – | – | – | – | aadA2 | ACSGTriTetCh | NCipP |
| E. c-2 | Pig | – | – | – | – | – | ACGCipTriChP | SNTet |
| E. c-3 | Pig | – | – | – | – | – | ACSTriTet | GNCipChP |
| E. c-4 | Pig | + | – | – | + | dfrA12-orfF-aadA2 | ACSTriTetCh | GNCipP |
| E. c-5 | Pig | + | – | – | + | dfrA12-orfF-aadA2 | ACSTriTet | GNCipChP |
| E. c-6 | Pig | + | – | – | + | dfrA12-orfF-aadA2 | ACGTriTetChP | SCipN |
| E. c-7 | Pig | + | – | – | + | dfrA12-orfF-aadA2 | ACNCipTriTetCh | SGP |
| E. c-8 | Pig | + | – | – | + | – | ACSTriTetChP | GNCip |
| E. c-9 | Pig | + | – | – | + | dfrA12-orfF-aadA2 | ACNCipTriTetCh | SGP |
| E. c-10 | Pig | + | – | – | + | aadA2 | ACSCipTriTetChP | GN |
| E. c-11 | Pig | + | – | – | + | – | ACSNTriTet | GCipChP |
| E. c-12 | Pig | + | – | – | + | aacA4-catB8-aadA1 | ACNTriTetCh | SGCipP |
| E. c-13 | Pig | – | – | – | – | – | ACSGTriTetCh | NCipP |
| E. c-14 | Pig | + | – | – | + | aadA2 | ACSGCipTriChP | NTet |
| E. c-15 | Pig | + | – | – | + | dfrA17-aadA5 | ACSCipTriTetCh | GNP |
| E. c-16 | Pig | – | – | – | – | – | ACSNCipTriP | GTetCh |
| E. c-17 | Pig | – | – | – | – | – | ACSGNTriTetChP | Cip |
| E. c-18 | Pig | + | – | – | + | – | ACSP | GNCipTriTetCh |
| E. c-19 | Pig | + | – | – | + | – | ACSGCipTriChP | NTet |
| E. c-20 | Pig | + | – | – | + | dfrA17-aadA5 | ACSTriChP | GNCipTet |
| Sal-1 | Pig | + | – | – | + | – | ACCipTriCh | SGNTetP |
| Sal-2 | Pig | + | – | – | + | – | ACSNCipTriTet | GChP |
| Sal-3 | Pig | + | – | – | + | – | ACSNTriChP | GCipTet |
| Sal-4 | Pig | + | – | – | + | dfrA32-ereA-aadA2 | ACNCipTriTet | SGChP |
| Sal-5 | Pig | + | – | – | + |  | ACSNTriTet | GCipChP |
| Sal-6 | Pig | + | – | – | + | dfrA17-aadA5 | ACCipTriTetChP | SGN |
| Sala-7 | Pig | + | – | – | + | aadA2 | ACSGNTriCh | CipTetP |
| Sal-8 | Pig | + | + | – | + | – | ACGNCipTriCh | STetP |
| Sal-9 | Pig | – | – | – | – | – | ACSTriTetCh | GNCipP |
| Sal-10 | Pig | – | – | – | – | – | ACSTriChP | GNCipTet |
| Sal-11 | Pig | – | – | – | – | – | ACCipTriTet | SGNChP |
| Sal-12 | Pig | – | – | – | – | – | ACTriTetChP | SGNCip |
| E. c-21 | Duck | + | – | – | + | dfrA17-aadA5 | ACSCipTriTetCh | GNP |
| E. c-22 | Duck | + | – | – | + | – | ACSCipTri | GNTetChP |
| E. c-23 | Duck | + | – | – | + | – | ACTri | SGNCipTetChP |
| E. c-24 | Duck | + | – | – | + | dfrA17-aadA5 | ACSCipTriChP | GNTet |
| E. c-25 | Duck | + | – | – | + | – | ACTriCh | SGNCipTetP |
| Sal-13 | Duck | + | – | – | + | – | ACSCipTriCh | GNTetP |
| Sal-14 | Duck | + | – | – | + | aadA2 | ACTriTetCh | SGNCipP |
| Sal-15 | Duck | + | – | – | + | – | ACSTri | GNCipTetChP |
| Sal-16 | Duck | + | – | – | + | dfrA17-aadA5 | ACTriCh | SGNCipTetP |
| Sal-17 | Duck | – | – | – | – | – | CSCipTri | AGNTetChP |
| Sal-18 | Duck | – | – | – | – | – | CSCipTriTetCh | AGNP |
| Sal-19 | Duck | + | – | – | + | dfrA17-aadA5 | ACSTriP | SGNCipTetCh |
| Sal-20 | Duck | + | – | – | + | dfrA32-ereA-aadA2 | ACTri | SGNCipTetChP |
| Sal-21 | Duck | + | – | – | + | – | ACSCipTri | GNTetChP |
| Sal-22 | Duck | – | – | – | – | – | ACTriP | SGNCipTetCh |
| Sal-23 | Duck | + | – | – | + | aadA2 | ACCipTriTetChP | SGN |
| Sal-24 | Duck | + | – | – | + | aadA2 | ACTriTet | SGNCipChP |
| Sal-25 | Duck | + | – | – | + | – | STriTetCh | ACGNCipP |
| RA-1 | Duck | + | – | – | + | – | CCipTriTetChP | ASGN |
| RA-2 | Duck | + | – | – | + | – | ACCipTriP | SGNTetCh |
| RA-3 | Duck | + | + | – | + | – | ACTri | SGNCipTetChP |
| RA-4 | Duck | + | + | – | + | – | CSCipTriTetCh | AGNP |

Table 1: Continue

| Isolates | Host | 1 | 2 | 3 | Int1 | Gene cassettes of class 1 integrons | R-phenotype[a] | S-phenotype[a] |
|---|---|---|---|---|---|---|---|---|
| RA-5 | Duck | + | – | – | + | – | ACTri | SGNCipTetChP |
| RA-6 | Duck | + | – | – | + | – | ACCipTriTet | SGNChP |
| RA-7 | Duck | + | – | – | + | – | ACipTriTetCh | CSGNP |
| RA-8 | Duck | + | – | – | + | – | ACSTriTetCh | GNCipP |
| RA-9 | Duck | + | – | – | + | – | ATriCh | CSGNCipTetP |
| RA-10 | Duck | – | – | – | – | – | ACSCipTri | GNTetChP |
| RA-11 | Duck | – | – | – | – | – | ACTriCh | SGNCipTetP |
| RA-12 | Duck | + | – | – | + | – | ACSTri | GNCipTetChP |
| RA-13 | Duck | + | – | – | + | – | ACCipTri | SGNTetChP |
| RA-14 | Duck | + | – | – | + | – | ACCipTriCh | SGNTetP |
| RA-15 | Duck | + | – | – | + | – | ACSCipTriTetCh | GNP |
| RA-16 | Duck | – | – | – | – | – | ACCipTriChP | SGNTet |
| RA-17 | Duck | + | + | – | + | – | ACTriCh | SGNCipTetP |
| RA-18 | Duck | – | – | – | – | – | ACSCipTriChP | GNTet |
| RA-19 | Duck | + | – | – | + | – | AGTriTetCh | CSNCipP |
| RA-20 | Duck | + | – | – | + | – | ACCipTriTetChP | SGN |
| RA-21 | Duck | + | + | – | + | – | ACGTriTetChP | SNCip |
| RA-22 | Duck | + | – | – | + | – | ACSTriTetCh | GNCipP |
| RA-23 | Duck | – | + | – | – | – | ATri | CSGNCipTetChP |
| RA-24 | Duck | + | – | – | + | – | ACSCipTriCh | GNTetP |
| RA-25 | Duck | + | – | – | + | – | ACTri | SGNCipTetChP |
| RA-26 | Duck | – | – | – | – | – | ACTriCh | SGNCipTetP |
| RA-27 | Duck | – | – | – | – | – | ACSCipTriTetChP | GN |
| RA-28 | Duck | – | + | – | – | – | ACipTriTetChP | CSGN |
| RA-29 | Duck | + | – | – | + | – | ACSGTriCh | NCipTetP |
| RA-30 | Duck | + | – | – | + | – | ACSNTriTetCh | GCipP |
| RA-31 | Duck | + | – | – | + | – | ACTriTetCh | SGNCipP |
| Sta-1 | Duck | + | – | – | – | dfrA17-aadA5 | AGCipTriTetChP | CSN |
| Sta-2 | Duck | + | – | – | – | – | ACSCipTriTetCh | GNP |
| Sta-3 | Duck | + | – | – | – | dfrA17-aadA5 | ASTriTetCh | CGNCipP |
| Sta-4 | Duck | + | – | – | – | dfrA17-aadA5 | ASCipTriChP | GNTet |
| Sta-5 | Duck | – | – | – | – | dfrA17-aadA5 | ACTriTetCh | SGNCipP |
| Sta-6 | Duck | – | – | – | – | – | ACTriP | SGNCipTetCh |

E. c, Sal, RA and Sta represented E.coli, Salmonella, *Riemerella anatipestifer* and staphylococci, respectively; 1, 2 and 3 represented class 1, 2 and 3 integrons, respectively; Int1 represented the integrase gene of calss 1 integron; "+" and "–" exhibited positive and negative results respectively; [a]Abbreviations used for antimicrobial resistance phenotypes and susceptibility phenotypes; A: Ampicillin; C: Cephalexin; S: Streptomycin; G: Gentamici; N: Neomycin; Cip: Ciprofloxacin; Tri: Trimethoprim; Ch: Chloramphenicol; Tet: Tetracycline; P: Polymyxin B

**Amplification and identification of integrons and resistance-gene cassettes in the isolates:** Specific primers were designed to amplify integrase gene fragments of classes 1-3 integrons and the variable regions of the class 1 and 2 integrons from the 87 bacterial strains. The primers and amplification conditions used have been described previously (White *et al*., 2001; Wei, 2010). All PCR-amplified products from the variable regions were recovered, purified and sequenced. These sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) database with the BLAST Software to determine the classes of the resistance-gene cassettes. The complete integrase genes were amplified from those strains that were positive for class 1 integrase using primers INT-1F (5'-ATGAAAAC CGCCACTGCGCCG-3') and INT-1R (5'-CTACCTCTCA CTAGTGAGGGGCG-3').

**Antibiotic susceptibility testing:** The 87 isolated strains were tested for antibiotic susceptibility using the Broth Microdilution Method, according to CLSI standards.

The Minimum Inhibitory Concentrations (MICs) of 10 kinds of antibiotics for these strains were determined. *Escherichia coli* ATCC25922 was used as the quality control strain. LB medium was used to determine the antibiotic MICs against *E. coli* and Salmonella and nutrient broth with 5% calf serum was used to determine the antibiotic MICs against *R. anatipestifer* and Staphylococcus. The strains were cultured at 37°C for 16-18 h in a normal incubator except that *R. anatipestifer* was cultured in 5% $CO_2$ incubator. Bacterial antibiotic resistance was determined based on the measured MICs.

The 10 kinds of antibiotics tested were ampicillin, cephalexin, streptomycin, gentamicin, neomycin, ciprofloxacin, trimethoprim, tetracycline, chloramphenicol and polymyxin B. These antibiotics have different antimicrobial mechanisms. Ampicillin and cephalexin belong to the β-lactam antibiotics which inhibit the enzyme transpeptidase which synthesizes cell wall peptidoglycan thus causing defects in the bacterial cell wall, leading to cell swelling and lysis. Streptomycin, gentamicin and neomycin belong to the aminoglycoside

antibiotics which mainly inhibit bacterial protein synthesis, leading to bacterial death. Ciprofloxacin belongs to the quinolone antibiotics which inhibit DNA synthesis and replication, causing bacterial death. Trimethoprim belongs to the sulfonamide antibiotics which affect the synthesis of bacterial dihydrofolate, inhibiting bacterial growth and reproduction. Tetracycline is a tetracycline antibiotic which binds specifically to the A site of the 30S subunit of the bacterial ribosome thus preventing aminoacyl-tRNA docking at the site. This inhibits peptide growth and affects the synthesis of bacterial proteins. Chloramphenicol reacts with the 50S subunit of the bacterial ribosome, disrupting protein synthesis. Polymyxin B belongs to the polymyxin antibiotics which mainly affect the permeability of the bacterial cell membrane, resulting in the leakage of important cell components and consequent bacterial death.

## RESULTS

**Amplification and identification of integrons and resistance-gene cassettes in the isolates:** After PCR amplification, no class 3 integrase gene fragments were amplified from any of the 87 strains; class 2 integrase gene fragments were amplified from only seven strains (8.05%, 7/87) and no variable region gene cassettes of class 2 integrons were amplified. Class 1 integrase gene fragments were detected in 65 of the 87 strains (74.71%, 65/87) and among these, variable region gene cassettes were amplified from 26 strains (40%, 26/65). The gene cassettes were confirmed by sequencing to be *aadA2*, *dfrA12-orfF-aadA2*, *dfrA17-aadA5*, *aacA4-catB8-aadA1* or dfrA32-ereA-aadA2. The complete integrase genes were amplified from 59 of the 65 strains positive for class 1 integrase but were not amplified from the six staphylococci (Table 1).

**Antibiotic susceptibility testing:** All strains other than RA-23 were resistant to three or more kinds of antibiotics and were consequently multidrug-resistant strains. Ampicillin sodium and ampicillin were only effective against 13 of the strains isolated from ducks and were ineffective against the other strains isolated from pigs and ducks. All strains were highly resistant to trimethoprim. The effective drugs included ciprofloxacin, gentamicin and neomycin, at least one of which was effective against all strains except the Sal-8 strain. The less effective drugs were polymyxin B, streptomycin and tetracycline which were effective against only some strains.

## DISCUSSION

It is noteworthy that class 1 integrase gene fragments were amplified from 23 of the 31 strains of *R. anatipestifer* (74.2%) whereas class 2 integrase gene fragments were amplified from six strains of *R. anatipestifer* (19.35%). However, no variable region gene cassettes were amplified from the strains carrying class 1 integrons or class 2 integrons. These results are very different from those we reported previously (Zheng *et al*., 2012). In 2010, we isolated *R. anatipestifer* strains from material from infected ducks suspected of *R. anatipestifer* infections in Weifang (Shandong Province) and Xinyang (Henan Province). We screened 48 strains for class 1-3 integrons and variable region gene cassettes. Our results showed that the carrying rates for class 1 and 2 integrons were 97.92% (47/48) and 31.25% (15/48), respectively but no class 3 integrases were detected. The class 1 integrons carried the *aadA1*, *aadA5* and *aacA4-aadA1* gene cassettes and the carrying rate was 61.7% (29/47) whereas the class 2 integrons predominantly carried the *sat2-aadA1* gene cassette and the carrying rate was 93.33% (14/15). This indicates that the current *R. anatipestifer* epidemic strains in Shandong Province and Henan Province differ greatly from the epidemic strains present in 2010. *R. anatipestifer* strains no longer display a high rate of resistance-gene cassettes but have completely lost these genes. The cause of this change requires further investigation.

When we amplified the complete integrase genes of the class 1 integrons, we found that no target fragments could be amplified from the staphylococci with the IntF and IntR primers. We inferred that a mutation may have occurred in nucleotides corresponding to the primers. Therefore, we extended or shortened primer sequences and designed three new forward primers Int1-F1 (5'-CTAC CTCTCACTAGTGA-3'), Int1-F2 (5'-CTACCTCTCACTA GTGAGGGGCGGCAGCGC-3') and Int1-F3 (5'-CTACC TCTCACTAGTGAGGGGCGGCAGCGCATCAA-3') and three new reverse primers Int1-R1 (5'-ATGAAAACCG CCACTGC-3'), Int1-R2 (5'-ATGAAAACCGCCACTGCG CCGTTACCAC-3'), Int1-R3 (5'-ATGAAAACCGCCACT GCGCCGTTACCA CCGCTGCG-3'). Nine sets of primers were obtained by combining these three forward primers and three reverse primers. When we attempted to amplify the integrase gene from the staphylococci strains with these nine sets of primers, the results were still negative. This indicates that large nucleotide mutations have occurred at both ends of the class 1 integrase genes carried by staphylococci or that the integrase gene is incomplete because nucleotides are missing. The currently known class 1 integrase gene sequences were

predominantly obtained from class 1 integrons carried by *E. coli*, Salmonella and *Pseudomonas aeruginosa*. Many bacterial integrase genes are still unknown. The class 1 integrase genes from staphylococci were not also published in National Center for Biotechnology Information. When more bacterial integrase genes have been determined in future research, the class 1 integrase genes may not be highly conserved and may differ in different bacteria.

Integrons can mediate the horizontal transfer of resistance genes. Whether there are significant differences in the antibiotic resistance of strains carrying integrons and those not carrying integrons, no report has yet been published addressing this issue. In this study, 26 isolates from pigs and ducks carried integron resistance-gene cassettes whereas 57 did not carry a gene cassette. We determined the MICs of 10 kinds of antibiotics against these isolates to examine their resistance phenotypes. There was no significant difference in the resistance phenotypes of isolates carrying the gene cassettes and those not carrying the gene cassettes. This suggests that there is no correlation between the antibiotic resistance of these strains and the presence of resistance-gene cassettes.

The *aad* and *dfr* gene families are the most common genes found in the gene cassettes carried by class 1 integrons. The gene cassettes we identified in this study all contained the *aadA* or *dfrA* gene and the proteins encoded confer bacterial resistance to streptomycin and trimethoprim. Therefore, we specifically examined the resistance of 87 strains to streptomycin and trimethoprim. Our results showed that all strains were highly resistant to trimethoprim whereas only 45 strains were resistant to streptomycin, among which only 12 strains carried the *aadA2* or *aadA5* resistance-gene cassette and 32 strains did not. However, 14 strains carrying aadA2 or aadA5 showed sensitivity to streptomycin. Therefore, there was no significant correlation between the resistance of the strains to streptomycin or trimethoprim and whether they carried the *aadA* or *dfrA* gene.

Most bacteria carry class 1 integrons. Class 2 integrons are only found in a small number of bacteria and the class 2 integrase is defective because it cannot catalyze the integration and excision of gene cassettes (Rodriguez *et al*., 2006). So far, only the *aadA1*, *dhfr* and *sat* resistance genes have been found in class 2 integrons. Currently, class 3 integron have only been found in the plasmid of *Serratia marcescens* and the class 3 integrons contain only the carbapenem-resistance gene. Researchers have shown that the plasmid-mediated horizontal transfer of quinolone-resistance genes only confers resistance to low levels of quinolone but which is advantageous to screen of the chromosomal mutated strains those are resistant to high levels of the drug (Ajayi *et al*., 2012; Halova *et al*., 2014). Therefore, the integron-mediated horizontal transfer of resistance genes may be just one mechanism of antibiotic resistance acquired by bacteria during their evolution. However, it cannot play a major role under the selection pressure exerted by the wide variety and high concentrations of antibiotics currently in use. Probably, it plays a synergistic role.

## CONCLUSION

At present because various types of antibiotics are widely used worldwide, bacteria are constantly evolving to avoid their inhibition or destruction by antibiotics. Therefore, their resistance mechanisms are becoming very complex and multiple resistance mechanisms may act together, making bacteria multidrug resistant and even causing the evolution of "superbugs" (Kumarasamy *et al*., 2010). This is a timely warning about our use of antibiotics which must be heeded to ensure the healthy development of the livestock industry and the security of mankind itself.

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