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RESEARCH ARTICLE

Detection of Class 1 and 2 Integrons, β-Lactamase Genes and Molecular Characterization of Sulfonamide Resistance in *Escherichia coli* Isolates Recovered from Poultry in China

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ABSTRACT

This study aimed to detect integrons, β-lactamase genes and to characterize sulfonamide resistant *E. coli* isolates recovered from poultry. All the isolates (n=38) were investigated for the presence of integrons, *Sul1*, *Sul2*, *Sul3* genes by PCR. Class 1 and class 2 integron were present in 79 and 16%, respectively. Additional resistance gene cassette embedded in class 1 and 2 integrons was *aadA1, aadA5, dfrA17* and *aadA22, dfrA,* respectively. *Sul1* and *Sul2* genes were detected in 42.1 and 60.5% isolates, respectively. Both the *Sul1* and *Sul2* were present in 23% isolates. However, *Sul3* gene was not present. Co-existence of *Sul1* and *Sul2* with class 1 integrons was found in 28.9 and 60.5% of class 1 integron positive isolates, respectively. Whereas, a less percentage of isolates showed a low level of resistance to β-lactams and no *blaCTX-M, blaSHV and blaTEM* was found. The MIC results showed resistance to sulfadiazine and sulfamethoxazole-trimethoprim in 88 and 84% isolates, resistance to penicillin, ampicillin, amoxicillin was 52, 52 and 44%, respectively. Chloramphenicol, florfenicol, tetracycline and gentamycin resistance was found in 51, 5, 42 and 67% isolates, respectively. This study revealed high frequency of class 1 integrons, *Sul* genes among poultry *E. coli* isolates, therefore further spread of *Sul* genes and integrons is predictable.

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INTRODUCTION

Avian pathogenic *E. coli* (APEC) causes several infections in poultry which results in high mortality and leads to heavy economic losses to poultry industry worldwide. Antimicrobials are used for many decades as efficient and inexpensive antibacterial agent in poultry industry. Antimicrobial resistance is main reason for the treatment failure for all bacterial diseases and emergence of antimicrobial resistant pathogens in humans as well as in food producing animals is also a growing universal problem (Karczmarczyk *et al*., 2011). However, high prevalence of antimicrobial resistance genes (AMR-genes), including sulfonamide resistance genes has been reported in gram-negative bacteria of animals and humans source. Usually resistance to sulfonamides spreads extensively and rapidly by acquisition of *Sul1*, *Sul2* or *Sul3* (Trobos *et al*., 2008).

Drug resistance monitoring programs have been implemented in many countries for the purpose of protecting the health of humans as well as animals. Antimicrobial resistant *E. coli* can also be reservoirs of AMR-genes and it can further spread the resistance determinants to other zoonotic bacteria which can also cause infections in animals and humans (Aarestrup, 2004). Assessment of antimicrobial resistance at molecular level is a useful tool for understanding the contribution of genetic elements responsible for developing and dissemination of resistance in bacteria (Alekshun and Levy, 2007).

Multidrug resistant bacteria is considered a potential risk to human health through food borne infections with super bug and resistant pathogens or because integrons (Box *et al*., 2005). Horizontal gene transfer is also a main factor for the transfer of resistance genes from one bacterium to another (Warnes *et al*., 2012). It is reported that genes encoding antimicrobial resistance are often

linked integrons which are important contributors in the distribution of antimicrobial resistance among Gram-negative bacteria (Fluit and Schmitz, 2004; Cambray *et al*., 2010). Moreover, *Sul1* gene has also been detected as part of the 3' conserved segment (3'CS) of class 1 integrons, which are the most frequently detected integrons in Enterobacteriaceae family (Bean *et al*., 2009).

E. coli from poultry and livestock is exposed to a high selective pressure because most of the antimicrobials are used in food-producing animals. Consequently, antimicrobial resistance is mounting and a variety of resistance genes have been reported. This study revealed widespread of Sulfonamide resistance genes and integrons in *E. coli* isolates of poultry in Eastern China. It was found that the high level resistance to sulfonamide in poultry *E. coli* is due to presence of *Sul* genes and presence of class 1 integrons may contribute in spreading of sulfonamide resistance in other gram negative bacterial isolates or directly in the environment.

MATERIALS AND METHODS

Isolation, selection and molecular identification of *E. coli* isolates: A total of 38 *E. coli* isolates recovered from rectal/fecal samples of poultry were selected for this study. Briefly, *E. coli* were identified by standard methods, colony morphology on blood agar, Gram staining and growth on EMB agar. Molecular identification of isolates was carried out by PCR method using eubacterial primers specific for 16SrDNA gene (1520bp), all the primers used in this study are shown in Table 1.

Table 1: Primers used in this study

Target gene	Primer sequences	Length	Reference
FD1	AGAGTTTGATCCTGGCTCAG	1520	Weisburg
RD1	AGGAGGTGATCCAGCC		*et al.*(1991)
Int1-F	CCTCCCGCACGATGATC	280	Bass
Int1-R	TCCACGCATCGTCAGGC		*et al.* (1999)
Int2-F2	TTATTGCTGGGATTAGGC	250	Goldstein
Int2-R2	ACGGCTACCCTCTGTTATC		*et al.* (2001)
Int3-F	AGTGGGTGGCGAATGAGTG	484	Goldstein
Int3-R	TGTTCTTGTATCGGCAGGTG		*et al.* (2001)
5'CS	GGCATCCAAGCAGCAAG	Variable	Ahmed
3'CS	AAGCAGACTTGACCTGA		*et al.* (2008)
Hep-51	CGGGATCCCGGACGGCATGC	Variable	White
Hep-74	ACGATTTGTA GATGCCATCGCAAGTACGAG		*et al.* (2001)
Sul1 F	GTGACGGTGTTCGGCATTCT	779	Kerrn
Sul1 R	TCCGAGAAGGTGATTGCGCT		*et al.* (2002)
Sul2 F	CGGCATCGTCAACATAACCT	721	Kerrn
Sul2 R	TGTGCGGATGAAGTCAGCTC		*et al.* (2002)
Sul3 F	GAG CAAGAT TTT TGG AAT CG	750	Hammerum
Sul3 R	CTA ACC TAG GGC TTTGGA TAT		*et al.* (2006)
blaCTX-M-F	CGCTTTGCGATGTGCAG	550	Ahmed
blaCTX-M-R	ACCGCGATATCGTTGGT		*et al.* (2008)
blaSHV-F	TTATCTCCCTGTTAGCCACC	795	Ahmed
blaSHV-R	GATTTGCTGATTTCGCTCGG		*et al.* (2008)
blaTEM-F	ATAAAATTCTTGAAGACGAAA	1080	Ahmed
blaTEM-F	GACAGTTACCAATGCTTAATC		*et al.* (2008)

DNA extraction and detection of Integrons, Sulfonamide and β-lactamase genes by Simplex PCR: Genomic DNA was extracted using a commercial kit (Geneaid Biotech, Taiwan) according to the manufacturer's instructions from isolates grown in 5 ml of luria broth (Oxoid) overnight at 37°C. The PCR reaction mixture of a final volume of 50 µL and PCR conditions were followed as described previously (Soufi *et al*., 209). The PCR was done using an ABI 2720 thermal cycler (Applied biosystems, USA). All the yielded amplicons were purified using the Geneaid PCR purification kit and were sequenced (Takkara Bio, China).

PCR amplification for detecting *Sul1*, *Sul2*, *Sul3* gene and class 1, 2 and 3 integrons was carried out using specific primers shown in table 1. Gene cassettes of class 1 and 2 integrons were detected by using primers 5'cs-3'cs and hep-51 and hep-74, respectively. All the isolates which showed resistance to penicillin, ampicillin and amoxicillin were screened for the presence of *blaCTX-M*, *blaSHV* and *blaTEM* genes using primers and procedures as described previously by (Ahmed *et al.,* 2008).

The pMD19-T vector cloning: To obtain a full size fragment of the target gene, the PCR products were concentrated by ethanol precipitation before cloning into competent *E. coli* followed by TA vector cloning. A single transformant from every cloning reaction was checked for the presence of the required inserts by PCR. The pMD19-T vector (TaKaRa Bio, Shanghai, China) was used according to the manufacturer's instructions.

DNA sequencing: All amplified PCR products were sequenced (Takara Bio, shanghai, China). The obtained nucleotide sequences were blasted with National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov).

Antibacterial susceptibility determination: Minimal inhibition concentrations (MICs) of *E. coli* isolates were determined using the standard broth doubling dilution method on Muller–Hinton medium. Susceptibility to tested antimicrobial was determined by the micro broth dilution method and breakpoints were used according to Clinical Laboratory Standards Institute (CLSI), standards (CLSI, 2010). *E. coli* ATCC 25922 was used as quality control. Antimicrobials used in this study were: sulfadiazine (SUL), sulfamethaxole and trimethoprim (SUL-TRM), penicillin (PEN), ampicillin (AMP), Amoxicillin (AMO), chloromphenicol (CMP), gentamycin (GEN), tetracycline (TET) and florfenicol (FFC).

RESULTS

Antibacterial susceptibility: MIC results of studied isolates showed high level of resistance against sulfonamides, followed by tetracycline, gentamycin, streptomycin, ampicillin, amoxicillin. Whereas, only few isolates were resistant to florfenicol. The percentage of isolates resistant to tested antimicrobials is shown in Fig. 2. Multidrug resistance was found in 62% isolates.

Detection of sulfonamide resistance genes: A total of 42.1 and 60.5% of the isolates carried *Sul1* and *Sul2* genes, respectively (Table 2). Overall, *Sul2* was found in all of the class 1 integron positive isolates. The co-existence of both sulphonamide resistance genes (*Sul1* and *Sul2*) was found in 23.0% isolates. Fig. 1 shows agarose gel electrophoresis of PCR assay of *Sul1* and *Sul2* genes, respectively. However, no any tested β-lactamase gene was found in any isolate. Sequences obtained for *Sul1*, *Sul2* and integrons showed 100% similarity with sequences available at NCBI under accession numbers JN566044.1 and NC010488.1, respectively.

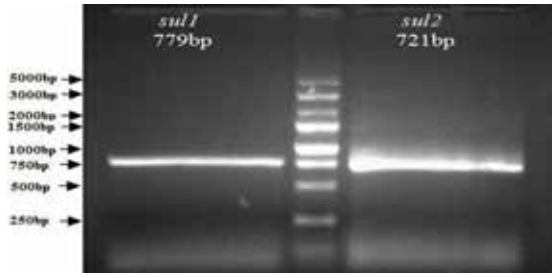

Fig. 1: Agarose gel electrophoresis of PCR assay of *Sul1* and *Sul2* genes

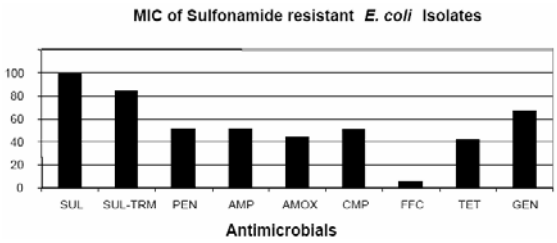

Fig. 2: MIC results (%) of sulfonamide resistant *E. coli* isolates
Antimicrobials abbreviations: SUL: sulfadiazine, SUL-TRIM sulfamethoxazole-trimethoprim, PEN: penicillin, AMP: ampicillin, CMP: chloramphenicol, AMOX: amoxicillin, FFC: florfenicol, GEN: gentamicin and TET: tetracycline.

Prevalence of class 1 and 2 integrons and their gene cassettes: The incidence of class 1 and 2 integrons and other antibiotic resistance markers among *E. coli* isolated is summarized in Table 2. PCR analysis revealed the presence of class 1 and class 2 integron in 79 and 16%, respectively. Among the 30 class 1 integron positive isolates, 28 isolates had detectable gene cassettes and the remaining isolates did not yield any PCR product. The gene cassettes embedded in class 1 and 2 integrons were *aadA1, aadA5, dfrA17* and *aadA22, dfrA5*, respectively. Moreover, 16% isolates carried both class 1 and 2 integrons. An unconditional association between class 1 integrons with *Sul2* and *Sul1* genes was pragmatic due to the co existence of *Sul2* and *Sul1* genes and class 1 integrons in the studied isolates (Table 2).

DISCUSSION

E. coli isolates are usually contaminating food of animal origin, here we selected *E. coli* isolates from poultry isolates resistant to various antibiotics but mainly to sulfonamides and this might be due to the reckless use of sulphonamides in poultry. Food producing animals can contribute in the selection and dissemination of antimicrobial resistant bacteria (Box *et al*., 2005). Moreover, it is well established that antimicrobial resistance genes can be transferred from commensal bacteria, via integrons, transposons or plasmids, into virulent bacteria present in the human intestine (Johnson *et al*., 2005). There is also a considerable difference in resistance patterns among the bacterial isolates recovered from different geographical regions and sources. Interestingly, we found that our poultry *E. coli* isolates were not ESBL producing, however recent studies from Bangladesh and Sweden has reported the poultry *E. coli* isolates were ESBL producing (Bonnedahl *et al*., 2010; Hasan *et al*., 2011). Furthermore, multidrug resistance found in 62% isolates is much greater than 22.7% previously reported (Hasan *et al*., 2012). Moreover, the Florfenicol followed by Tetracycline were found most effective antimicrobials as compared to other used antimicrobials.

The proper estimation of antimicrobial resistance in *E. coli* isolates from food producing animals is equally important to reduce the threats to health of both animal and human. In this study high frequency of *Sul2* and *Sul1* genes associated with class 1 integrons was evident. However *Sul3* gene was present in our isolates, whereas, previously *Sul3* is detected in *E. coli* isolates from pigs and human in Switzerland and Sweden, respectively (Perreten and Boerlin, 2003; Grape *et al*., 2003). High frequency of class 1 integron and *dfrA* and *aadA1* gene cassette array in class 1 integron positive *E. coli* has been reported from Spain (Machado *et al*., 2005). The high incidence of integrons is worrisome and is usually considered due to the significant association of integron-positive isolates with multi-resistance phenotypes. Integron carries antimicrobial resistance genes and recently it appears to be increasing among food *E. coli* isolates and could represent a vehicle for the gaining and distribution of antimicrobial resistance in environment. In our isolates no beta-lactamase gene was found and this is in contrast to recently reported high prevalence of beta-lactams in poultry *E. coli* isolates (Soufi *et al*., 2011). Whereas, high prevalence of class 1 integrons in poultry *E. coli* isolates is in agreement with previous study (Soufi *et al*., 2009).

Sulphonamide resistance was clearly due to presence of *Sul2* and *Sul1* genes, however some isolates did not carry *Sul1, Sul2* and *Sul3* genes but also showed mild resistance to Sulfonamides which might be due to cross resistance or presence of any unidentified *Sul* gene. Furthermore, Aminoglycosides and Trimethoprim resistance was because of high prevalence of class 1 integrons carrying gene cassettes encoding resistance to aminoglycosides and trimethoprim. High incidence of *Sul* genes, integrons and multidrug resistance in poultry *E. coli* isolates is dangerous and poses a serious threat of spreading resistance determinants to environment and direct to human through food chain.

This study for the first time reports the high prevalence of class1 integrons, *Sul* genes and their unconditional association with each other in poultry *E. coli* isolates from eastern China. Furthermore, poultry *E. coli* isolates could act as a reservoir of sulfonamide resistance genes and class 1 integrons carrying antimicrobial resistance gene cassettes. Molecular characterization of sulfonamide resistant isolates of poultry and high prevalence of integrons suggests the prudent use of sulfonamide in poultry, particularly in this region of China. This study revealed that the sulfonamide resistance is due to presence of *Sul1* and *Sul2* genes. Moreover, due to the rapid development and spread of resistance determinants among bacteria, a country wise antimicrobial resistance monitoring studies for bacterial isolates of live stock and poultry are necessary to control the situation. Further studies are needed to discover the genetic location of *Sul* genes, integrons in the bacterial genome and their transferability.

Table 2: Characterization of Sulfonamide-resistant *E. coli* isolates, presence of Integrons, their gene cassettes and an unconditional association between *Sul* genes and Integrons

Resistance genes	Positive isolates	%	With Class 1 integron	With Class 2 integron	With Class 1 & 2 integrons
Sul1	16	42.1	16/16 (100)	0/23(0)	0(0)
Sul2	23	60.5	23/23(100)	2/23(8.6)	6/23(26.0)
Sul1, Sul2	9	23	9/9(100)	4/9(44.4)	0(0)
Integrons			Gene cassettes		
Class 1 integron	30/38	79	*aadA1, aadA5, dfrA17*		
Class 2 integron	6/38	16	*aadA22, dfrA5*		
Class 1 & 2 integrons	6/38	16	*aadA5, dfrA17, aadA22, dfrA5*		

Values in parenthesis indicate percentage.

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