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Prevalence and characterisation of class 1 and 2 integrons in multi-drug resistant *Staphylococcus aureus* isolates from pig farms in Chongqing, China

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Abstract

Introduction: Integrons are mobile DNA elements that allow for acquisition and dissemination of antibiotic-resistance genes among pig farm-derived bacteria. Limited information is available on integrons of *Staphylococcus aureus* from pig farms. The aim of this study was to characterise and investigate the prevalence of class 1 and 2 integrons in multi-drug resistant (MDR) *S. aureus* isolates from pig farms. **Material and Methods:** A total of 724 swabs were collected from 12 pig farms in Chongqing, China, and examined by conventional microbial and molecular methods. **Results:** In total, 68 isolates were *S. aureus*, 57 of which were methicillin resistant (MRSA). All 68 isolates were MDR strains and carried integrons, of which 88.2% (60/68) harboured both class 1 and 2. In addition, 85.3% (58/68) of the class 2 integron-positive isolates carried the β-lactam resistance gene (bla_{TEM-1}), and 66.7% (40/60) of the class 1 integron–positive isolates carried the *aadA1c*, *aadA1* or *dfrA1* gene for respective streptomycin and spectinomycin or trimethoprim resistance. **Conclusions:** Class 1 and 2 integrons are common among the pig farm-derived *S. aureus* isolates. On account of their significance for public health, the prevalence of the integrons and their associated resistance genes in pig farm-derived *S. aureus* isolates should be paid special attention.

Keywords: pig, *Staphylococcus aureus*, antibiotic resistance, integrons, resistance gene cassettes.

Introduction

The *Staphylococcus* species are a group of Gram-positive, facultative, aerobic, and unencapsulated bacteria, which are responsible for a variety of skin and tissue infections (23). Of all the species, *Staphylococcus aureus* (*S. aureus*) has been most notable as a major human and animal pathogen responsible for a wide variety of infections, including skin infections, pneumonia, bacteraemia, and toxin-mediated food poisoning (9). Food poisoning caused by animal-derived *S. aureus* has been one of the most economically important food-borne diseases and a major threat to public health (14). In China, the production and consumption of pork has long been in great demand (20). However, with the increasing abuse of antibiotics on pig farms, multi-drug resistant (MDR) phenotypes of *S. aureus* are emerging and contaminate pork products as a result, which poses a great threat to human health (7).

The bacterial resistance mechanisms involved in the acquisition and spread of resistance genes have been illuminated in the past few years (19). These mainly resolve to one of two types: (i) mutations in pre-existing or previously acquired resistance genes, and (ii) horizontal or lateral gene transfer, which is mediated by various types of genetic elements, such as transposons, plasmids, bacteriophages, pathogenicity islands, and integrons (1, 3). Among them, integrons are an important mechanism involved in acquisition

and spreading of antibiotic resistance, which permit tandem integration and expression of various antibiotic-resistance genes (8).

Integrons contribute to horizontal gene transfer in association with transposons or plasmids. A complete integron is generally composed of three elements: the 5′ conserved segment (5′CS), an internal variable region containing one or more resistance gene cassettes, and a 3′ conserved segment (3′CS). The 5′CS contains an integrase gene (*intI*) that encodes an integrase (recombinase) responsible for site specific insertion of resistance-gene cassettes, a recombination site (*attI*), and a promoter (Pc) that is responsible for expression of the captured gene cassettes. The 3′CS contains a quaternary ammonium compound resistance gene (*qacΔE-1*), the sulphonamide resistance gene (*sul1*) and an open reading frame of unknown function (11). So far, five classes of mobile integrons have been identified and distinguished by differences in the *intI* sequences. Class 1, 2, and 3 integrons are relatively common and capture from and spread antibiotic-resistance gene cassettes to the same or other bacterial species by horizontal gene transfer (12). Class 4 and 5 integrons are relatively rare and they are involved in the development of trimethoprim resistance in *Vibrio* species (12).

Class 1 integrons are the most frequent type in clinical isolates, and most of the reported antibiotic-resistance gene cassettes are found in this class. Previously, class 1 integrons were identified in a large variety of clinical Gram-negative organisms. Recently, they were observed in a few Gram-positive bacteria as well (21). Although class 2 and 3 integrons have a similar organisation to that of class 1, they are less detected in bacteria from clinical samples and poorly studied (15). Studies have indicated that Gram-positive bacteria are also a major reservoir of integrons (6). In particular, class 1 integrons have been identified in *S. aureus* isolates from hospitals and cow's milk (11, 24). Class 2 integrons have been identified in *S. aureus* clinical isolates in Tehran, Iran (15).

Pork is a major protein source in the Chinese diet. Pigs are raised on a very large scale in China, and *S. aureus* isolates from pigs are reported to be an important source of methicillin-resistant *Staphylococcus aureus* (MRSA) (5). To investigate the prevalence of MRSA strains and *S. aureus* isolates carrying class 1 and 2 integrons from pig farms in China, we report here the characterisation of the *S. aureus* isolates from local pig farms in Chongqing.

Material and Methods

Sample collection. A total of 724 samples were collected from 12 pig farms in 6 regions of Chongqing between November 2018 and January 2019. These samples included faecal swabs (n = 426), floor swabs (n = 215), water swabs (n = 22), feed swabs (n = 20), and air swabs (n = 41), which were randomly collected by the farmers from three pig houses of each farm.

Isolation and identification of *S. aureus*. Each swab was transferred into 15 mL of buffered peptone water (Land Bridge, China) and incubated at 37°C for 16 to 18 h for recovering *S. aureus*. A loopful of the recovered *S. aureus* suspensions was sub-cultured in 10 mL of 7.5% NaCl broth (Land Bridge) at 37°C for 18 to 24 h and another loopful was streaked onto Baird-Parker agar plate (Land Bridge) and chromogenic medium (CHROMagar, France), for isolation and direct differentiation, respectively. Finally, the specific genes *femB* and *mecA* were used for identifying *S. aureus* and MRSA by PCR and the subsequent DNA sequencing (13, 18).

Antibiotic susceptibility testing. Antibiotic susceptibility of all *S. aureus* isolates against 11 subclasses of antimicrobial agents was determined by the Kirby–Bauer disk diffusion method (2) and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (4). The tested antimicrobials (Hangzhou Microbial Reagent Co, China) included penicillin (10 U), gentamicin (10 μg), tetracycline (30 μg), erythromycin (15 μg), clindamycin (2 μg), trimethoprim/sulphamethoxazole (25 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), teicoplanin (30 μg), rifampicin (5 μg), nitrofurantoin (300 μg), levofloxacin (5 μg), and oxacillin (1 μg). *Escherichia coli* (ATCC25922) was used as a quality control. The *S. aureus* isolates resistant to more than three subclasses of antimicrobial agents were defined as MDR isolates.

Extraction of *S. aureus* plasmid and genomic DNA. The plasmid DNA of *S. aureus* was extracted by using an E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek, USA) according to the manufacturer's instructions. The genomic DNA was extracted by the boiling method as previously described (24). Briefly, the overnight cultures of *S. aureus* in 7.5% NaCl broth were suspended in 100 μL of TE buffer (10 mM Tris; 1 mM EDTA, pH 8), and then boiled for 10 min, vortexed for 10 s, and finally centrifuged at 9,000 × g for 5 min. The supernatant containing genomic DNA of *S. aureus* was collected and stored at −20°C until use.

Detection of three classes of integrons. The presence of class 1, 2, and 3 integrons in MDR *S. aureus* was confirmed by PCR amplification of integrase genes *(intI1*, *intI2*, and *intI3*) with their specific primers (Table 1) as described in previous studies (16). PCR mixtures were in the volume of 25 μL and PCR amplifications were performed in a T100 thermocycler (Bio-Rad, USA). The reaction conditions for detection of *intI1* were as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Conditions for detection of *intI2* and *intI3* were: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min,

annealing at 55°C for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Furthermore, the two genes located in the 3′CS of class 1 integrons conferring resistance to *sul1* and *qacΔE-1* were also confirmed by PCR as protocolled in a previous report (25).

Detection of integron-associated gene cassettes. All integron-positive MDR *S. aureus* isolates were tested for the presence of internal gene cassettes. The PCR for detecting gene cassette arrays in class 1 integrons employed the primers *intI1-k* and *lnB* (Table 1) as previously described (24). The reaction conditions were initial denaturation at 94°C for 4 min and 10 touchdown cycles of denaturation at 94°C for 1 min, annealing at 62–53°C for 30 s (decreasing by 1°C/cycle) and extension at 72°C for 2 min, followed by 24 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 2 min, with a final extension at 72°C for 7 min. A PCR also detected gene cassette arrays in class 2 integrons. The primers *hep74* and *hep51* (Table 1) were used as previously published (25). The reaction conditions were initial denaturation at 94°C for 5 min, 12 cycles of denaturation at 94°C for 50 s, annealing at 68–57°C for 50 s (decreasing by 1°C/cycle) and extension at 72°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 4 min, with a final extension at 72°C for 5 min.

Cloning, sequencing, and analysis of amplified integron gene cassettes. To analyse the sequences of the gene cassettes, PCR amplicons of the representative cassettes that had a unique size when visualised on the gel were purified with a Gel Extraction Kit (Omega Bio-Tek) and then ligated into the pMD-19T vector (TaKaRa Biomedical Technology, China). After transformation, three positive clones carrying recombinant pMD-19T plasmids were sequenced by the Sanger method. The resulting nucleotide sequences were analysed and compared with the available sequences both in GenBank by the BLAST programme (http://www.ncbi.nlm.nih.gov/blast/) and in the Integron Database (INTEGRALL) (http://integrall.bio.ua.pt/).

Statistical analysis. Comparison of the detection rates of class 1 and 2 integrons between plasmid and genomic DNA was analysed by χ^2 test in SPSS software (IBM SPSS, USA). $P < 0.05$ was considered statistically significant.

Results

Prevalence and antibiotic susceptibility of *S. aureus* isolates. A total of 68 *S. aureus* isolates were recovered from 724 samples collected on pig farms in Chongqing, which includes 43/426 (10.1%) from faecal samples, 11/215 (5.1%) from floor samples, 3/22 (13.6%) from water samples, 6/20 (30.0%) from feed samples, and 5/41 (12.2%) from air samples (Table 2). Of these, 57 (83.8%) isolates were further confirmed as MRSA strains based on the presence of the *mecA* gene. This gene was contained in 35/43 (81.4%) isolates from faecal samples, 9/11 (81.8%) isolates from floor samples, 2/3 (66.7%) from water samples, 6/6 (100%) from feed samples, and 5/5 (100%) from air samples (Table 2). Subsequently, 13 antimicrobial agents were used to test the antibiotic susceptibility of the 68 *S. aureus* isolates. It showed that high levels of resistance to antibiotics were found in these isolates. Resistance to penicillin, tetracycline, and erythromycin were observed in all 68 *S. aureus* isolates. Additionally, 60/68 (88.2%) of these isolates were resistant to oxacillin, 46/68 (67.6%) to chloramphenicol, 15/68 (22.1%) to trimethoprim/ sulphamethoxazole, and 7/68 (10.3%) to clindamycin. Low resistance rates to rifamycin (2.9%), gentamicin (1.5%), ciprofloxacin (1.5%), levofloxacin (1.5%), nitrofurantoin (0%), and teicoplanin (0%) were noted among the isolates (Table 3).

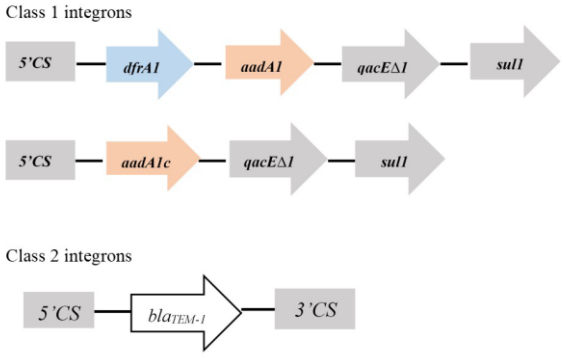

Fig. 1. Schematic representation of the various cassette arrays found in class 1 and class 2 integrons. Arrows display the open reading frames of the different genes. All *aadA1c* and *aadA1* genes are represented as orange arrows; 5′CS, 3′CS, *qacEΔ1*, and *sul1* genes as light grey; *dfrA1* as light blue; and bla_{TEM-1} genes as white

Detection and characterisation of class 1, 2, and 3 integrons. All the 68 *S. aureus* isolates were screened with a specific PCR for the presence of class 1, 2, and 3 integrons. Sixty of the 68 isolates (88.2%) were positive for class 1 integrons with an amplicon of 280 bp, while all 68 isolates had class 2 integrons with an amplicon of 233 bp. No class 3 integrons were found in any isolates (data not shown). In addition, there were significant differences in the detection rate of integrons between the bacterial chromosome and plasmid. It was found that in the *S. aureus* isolates, the positive rates for plasmids carrying class 1 and 2 integrons (88.2% and 100%, respectively) were significantly higher than those for chromosomes carrying them (36.8%, 45.6%, respectively) (Table 4). Moreover, all integrons on chromosomes were also found on the plasmids of the corresponding *S. aureus* strains (data not shown).

PCR amplification of the gene cassette indicated that antibiotic-resistance gene cassettes could be detected in 66.7% (40/60) of class 1 integrons and 85.3% (58/68) of class 2 integrons (Table 5). Sequence analysis of the representative PCR amplicons identified several gene cassettes with product sizes of about 700 bp, 1,200 bp and 2,000 bp (Table 5). In class 1 integrons, three kinds of resistance gene cassette arrays (*aadA1c*; *dfrA1* + *aadA1*; and *aadA1c, dfrA1* + *aadA1*) were detected in folate pathway inhibitors and aminoglycoside-susceptible isolates (Table 3). Of these, the gene cassette arrays *aadA1c* (aminoglycosides) and *aadA1c*, *dfrA1* + *aadA1* (aminoglycosides and folate pathway inhibitors) were found to be the most common arrays, which were carried in 25 and 14 class 1 integron-positive isolates, respectively; the *dfrA1* + *aadA1* array was only found in one class 1 integron-positive isolate. In class 2 integron-positive isolates, only one kind of cassette array (bla_{TEM-1}) related to β-lactam resistance was observed in 58 class 2 integrons of these isolates (Table 5). For better presentation, a schematic representation of the different cassette arrays found in class 1 and class 2 integrons is shown in Fig. 1.

Table 1. Primers used in this study

Primer	Target gene	Sequence (5′–3′)	Product size (bp)
intI1-F	*intI-1*	CCTCCCGCACGATGATC	280
intI1-R		TCCACGCATCGTCAGGC	
intI2-F	*intI-2*	TTATTGCTGGGATTAGGC	233
intI2-R		ACGGCTACCCTCTGTTATC	
intI3-F	*intI-3*	AGTGGGTGGCGAATGAGTG	600
intI3-R		TGTTCTTGTATCGGCAGGTG	
intI1-k	variable region 1	ACCGAAACCTTGCGCTCGT	Variable
lnB		AAGCAGACTTGACCTGAT	
hep74	variable region 2	CGGGATCCCGGACGGCATGCAC	Variable
hep51		GATGCCATCGCAAGTACGAG	

Table 2. The prevalence of pig farm-derived *S. aureus*

Sample sources	Number of samples from each source	Number of positive isolates (%)	Number of MRSA strains among the positive isolates (%)
Faeces	426	43 (10.1%)	35 (81.4%)
Floor	215	11 (5.1%)	9 (81.8%)
Water	22	3 (13.6%)	2 (66.7%)
Feed	20	6 (30.0%)	6 (100%)
Air	41	5 (12.2%)	5 (100%)
Total	724	68 (9.4%)	57 (83.8%)

Table 3. Antibiotic-resistant phenotypes and genotypes of *S. aureus* in this study

Antimicrobial subclass	Antimicrobial agent	Phenotypes (n = 68)		Genotypes (number of isolates containing different resistance gene cassettes)		
		Number of resistant isolates (%)	Number of sensitive isolates	Class 1 integron (n = 60)		Class 2 integron (n = 68)
				aadA1c or *aadA1*	*dfrA1*	bla_{TEM-1}
β-lactams	Penicillin	68 (100)	-	-	-	58
	Oxacillin	60 (88.2)	-	-	-	50
		-	8	-	-	8
Tetracyclines	Tetracycline	68 (100)	-	-	-	-
Macrolides	Erythromycin	68 (100)	-	-	-	-
Phenicols	Chloramphenicol	46 (67.6)	-	-	-	-
		-	22	-	-	-
Folate pathway inhibitors	Trimethoprim-sulfamethoxazole	15 (22.1)	-	-	2	-
		-	53	-	12	-
Lincosamides	Clindamycin	7 (10.3)	-	-	-	-
		-	61	-	-	-
Rifamycins	Rifamycin	2 (2.9)	-	-	-	-
		-	66	-	-	-
Aminoglycosides	Gentamicin	1 (1.5)	-	1	-	-
		-	67	39	-	-
Quinolones	Ciprofloxacin	1 (1.5)	-	-	-	-
		-	67	-	-	-
	Levofloxacin	1 (1.5)	-	-	-	-
		-	67	-	-	-
Nitrofurans	Nitrofurantoin	-	68	-	-	-
Glycopeptides	Teicoplanin	-	68	-	-	-

Table 4. Comparison of detection rates of class 1 and 2 integrons in plasmid and genomic DNA in pig farm-derived *S. aureus*

Types of DNA	Class 1 integrons		Class 2 integrons	
	Number of positive (negative) strains	Positive rate (%)	Number of positive (negative) strains	Positive rate (%)
Genomic DNA	25 (43)	36.8	31 (37)	45.6
Plasmid DNA	60 (8)	88.2	68 (0)	100
χ^2	38.43		50.83	
P-value	< 0.001		< 0.001	

Table 5. Different types of gene cassette amplicons among the integron-bearing *S. aureus*

Types of integrons	Number of isolates carrying different cassettes (%)	Approximate sizes of amplicon (bp)	Inserted cassette(s)
Integron 1	25 (41.7%)	1,200	*aadA1c*
	1 (1.7%)	2,000	*dfrA1* + *aadA1*
	14 (23.3%)	1,200, 2,000	*aadA1c*, *dfrA1* + *aadA1*
Integron 2	58 (85.3%)	700	bla_{TEM-1}

Discussion

The prevalence and characterisation of class 1 integrons in Gram-negative bacteria have been well documented; however, the presence of integrons in Gram-positive bacteria has been reported less so far. To our knowledge, this is the first report of the characterisation of class 1 and 2 integrons and their associated gene cassettes in MDR *S. aureus* isolates from pig farms in China. Our data indicated that MDR isolates and the class 1 and 2 integrons were widespread among pig farm–derived *S. aureus* isolates in Chongqing. The most common pattern of the isolates' resistance was insusceptibility to β-lactams, tetracyclines, and macrolides. In addition, most (83.8%) MDR isolates were also MRSA isolates.

Unexpectedly, all *S. aureus* isolates were positive for class 2 integrons, and the majority (88.2%) of these isolates carried class 1 integrons. However, in previous reports, the proportion of class 1 integrons in *S. aureus* isolates from either farms (10%–25%) or hospitals (42.5% and 53%) was apparently much lower than that reported here (17, 22, 24). The proportion of class 2 integrons (100%) in this study was also much higher than that (35.2%) in *S. aureus* isolates from hospitals in Tehran, Iran (15). This is also the first time that a report is published of class 2 integrons in *S. aureus* isolates from farm animals. The high prevalence of class 1 and 2 integrons in *S. aureus* on pig farms may reflect that the integrons have played an important role in disseminating antimicrobial resistance in bacterial populations.

The class 1 integrons have long been considered the most universal integrons and they have the capability to integrate nearly all the antibiotic-resistance genes, such as those imparting resistance to β-lactams, aminoglycosides, chloramphenicol and others (12). In our study, we found that only aminoglycoside resistance genes (*aadA1c* and *aadA1*) and folate pathway inhibitor resistance determinant (*dfrA1*) were identified in class 1 integrons. The composition of the resistance gene cassette arrays was simple and only three kinds of arrays (*aadA1c*; *dfrA1* + *aadA1*; and *aadA1c*, *dfrA1* + *aadA1*) were observed. However, previous studies have documented many more antibiotic-resistance genes among *S. aureus* isolates from farms or hospitals, such as an assortment of 12 resistance genes (*dhfrV*, *dfrA1*, *dfrA12*, *aadA1*, *aadA5*, *aadA4*, *aadA24*, *aacA4*, *aadA2*, *aadB*, *cmlA6*, and *qacH*) found in isolates from Chinese dairy farms (11) or a 10-strong variety of resistance determinants (*aadA1*, *aadA2*, *aadB*, *aacA4*, *dfrA12*, *oxa2*, *orfD*, *orfF*, *catB3*, and *cmlA6*) reported in isolates from hospitals (15, 22).

Most reported class 2 integrons carry the three specific gene cassettes *dfrA1*, *sat1*, and *aadA1*, which confer resistance to trimethoprim, streptothricin and streptomycin/spectinomycin, respectively (8, 10). In this study, only the gene for resistance to β-lactams, bla_{TEM-1}, was screened in the class 2 integrons of the isolated strains, which had not been reported previously among *S. aureus* strains. It can be seen that the diversity of gene cassettes in class 2 integrons was lower than that in class 1 integrons. The low diversity of integrated gene cassettes in class 2 integrons was probably owing to the non-functional *intl2* generated by the replacement of its internal termination codon with a codon for glutamic acid, which could not excise existing cassettes or insert novel ones (21). In addition, the gene cassettes observed in this study could not correspond to the resistance phenotypes observed (*e.g.* tetracycline, erythromycin, or chloramphenicol) among the *S. aureus* isolates (Table 3). This suggested that resistance of *S. aureus* to tetracycline, erythromycin, and chloramphenicol may not be associated with integron-mediated antimicrobial resistance. Other resistance mechanisms beyond this research's scope may be responsible for generating these resistant phenotypes. Subsequent genome-wide sequencing analysis will likely elucidate the mechanism for generation of these resistant phenotypes.

This is the first report of the presence and characterisation of class 1 and 2 integrons in

S. aureus isolates from farmed swine in Chongqing, China. The results showed that high occurrence rates (88.2% and 100%) of class 1 and 2 integrons were observed in all 68 *S. aureus* isolates. Of these, 58 isolates carrying class 2 integrons were positive for the presence of the β-lactam resistance gene bla_{TEM-1} and 40 isolates carrying class 1 integrons harboured the *aadA1c*, *aadA1* or *dfrA1* genes. Out of concerns for public health, the presence of class 1 and 2 integrons and their associated resistance gene cassettes in *S. aureus* isolates from pig farms should be paid special attention and close surveillance of that presence should be mounted.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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