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Antimicrobial Resistance, Resistance Genes and Virulence Genes in Salmonella Isolates From Chicken

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Abstract: Ninety eight Salmonella isolates (53 isolates from 2007 and 45 from 2009) from diseased chicken were examined for antimicrobial susceptibility to 15 antimicrobials, possession of resistance and virulence genes and Pulsed Field Gel Electrophoresis (PFGE) patterns. The 82 (84%), all 45 from 2009 and 37 from 2007 were resistant to 2 or more antimicrobials. A single isolate from 2009 was resistant to 13 of the 15 antimicrobials tested. The isolates from 2009 exhibited significantly greater resistance to streptomycin, florfenicol, tetracycline, doxycycline and nalidixic acid than that from 2007. Resistance genes *sul3* and *aadA1* were the most prevalent being found in 19 (36%) and 14 (31%) isolates from 2007 and 2009, respectively. All 98 isolates carried invA; in comparison with the isolates from 2007, the isolates from 2009 exhibited significantly lower rates of carrying spvC, sopE and iroB. Of the 98 isolates, 75 isolates were successfully typed, resulting in 49 different PFGE patterns with a difference of at least seven bands. This study shows that the majority of Salmonella strains from Guangdong display resistance to multiple antimicrobial compounds and carry multiple resistance genes and virulence genes.

Key words: Antimicrobial agents, resistance, resistance genes, virulence genes, salmonella

INTRODUCTION

Salmonella sp. is a gram negative, rod shaped, motile and facultative anaerobe bacterium that normally resides in the gut of wild and domestic animals (Pang *et al*., 2011). *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) is a major cause of food borne gastroenteritis in humans worldwide. Poultry and poultry products are considered the major vehicles of transmission to humans (Shah *et al*., 2011). Salmonella contamination continues to be one of the major concerns for the microbiological safety of raw poultry products. The US Department of Agriculture's Food Safety Inspection Service (USDA-FSIS) has estimated that in 2007 poultry products accounted for approximately 60% of the food borne illnesses originating from Salmonella (Benli *et al*., 2011). Salmonella was the common contamination of poultry products with the prevalence in chicken meat of 51.7% in Tunisia, 54% in China and 66% in Thailand (Abbassi-Ghozzi *et al*., 2011; Yang *et al*., 2010). *S. enterica* can easily cross contaminate other ready to eat foods exposed to these surfaces, posing a risk for foodborne illness outbreaks. The FDA recommended practice of washing kitchen implements with soap, hot water and vigorous mechanical scrubbing can remove *S. enterica* effectively and hence reduce cross contamination (Ravishankar *et al*., 2010).

The ability of chickens to carry Salmonella without displaying disease symptoms is responsible for Salmonella propagation in poultry stocks and for subsequent human contamination through the consumption of contaminated eggs or meat. The selection of animals more resistant to carrier state might be a way to decrease the propagation of Salmonella in poultry stocks and its transmission to humans (Calenge *et al*., 2009). Wisner *et al*. (2010) reported that the *S. enteritidis* SPI-2 T3SS facilitates invasion and systemic spread in chickens although alternative mechanisms for these processes appear to exist (Wisner *et al*., 2010). Pulsed Field Gel Electrophoresis (PFGE) has been successfully used for typing of *S. enteritidis*, *S. typhi* and *S. typhimurium* furthermore which has been accepted as the gold standard for Salmonella molecular typing by Pulse

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Net International, the international molecular subtyping network for foodborne disease surveillance (Chen *et al*., 2011; Hur *et al*., 2011a, b).

The objective this study was to determine the prevalence of antimicrobial resistance and their associated genes, virulence associated genes and to analyze the PFGE patterns of Salmonella strains isolated from chicken with diarrhea. The isolates were divided into 2 groups by collection period (2007 or 2009) to investigate trends over time. Researchers also analyzed the variance in strain characteristics between the 2 groups.

MATERIALS AND METHODS

Bacterial isolates: Ninety eight Salmonella were collected from diseased chicken in Guangdong province, China, in 2007 and 2009. Salmonella strains were mainly isolated from fecal swabs taken from diseased chicken with white diarrhea. Each isolate was taken from an individual animal.

Antimicrobial susceptibility testing: Antimicrobial susceptibility testing was performed on all 98 Salmonella isolates using the agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) in 2008. The following antimicrobials were used: Ampicillin (AMP), Ceftiofur (CEF), Ceftriaxone (CRO), Streptomycin (STR), Gentamicin (GEN), Apramycin (APM), Chloramphenicol (CHL), Florfenicol (FEN), Tetracycline (TET), Doxycycline (DOX), Sulfamethoxazole (SMZ), Nalidixic Acid (NA), Ciprofloxacin (CIP), Enrofloxacin (ERO), Levofloxacin (LEF). The reference strain, *E. coli* ATCC 25922 was used as a quality control strain for determining the minimum inhibitory concentrations of the 15 antimicrobial agents.

Resistance and virulence genes: All isolates were screened for 23 resistance genes and 17 virulence genes with PCR as reported earlier (Del Cerro *et al*., 2003; Karasova *et al*., 2009).

Pulsed Field Gel Electrophoresis (PFGE): PFGE was used to analyze the genomic relatedness among Salmonella isolates from diseased chicken. The method used was basically according to Chen *et al*. (2011). PFGE of chromosomal DNA digested with the restriction enzyme XbaI was carried out according to a standard protocol using a CHEF-MAPPER System (Bio-Rad Laboratories, Hercules, CA). The gels were run at 6.0 V cm^{-1} with an angle of 12°C at 14°C for 20 h and the results were interpreted according to the criteria of Tenover *et al*. (1995) Salmonella ser. Braenderup H9812 standards served as size markers.

Statistical analysis: Differences in the year by year rates of antimicrobial resistance were assessed using Fisher's exact tests (SPSS 17.0). A $p<0.05$ was considered significant.

RESULTS

Antimicrobial resistance phenotypes: The results of antimicrobial susceptibility tests were shown in Table 1. None of the Salmonella isolates were resistant to CRO and LEF, none of the Salmonella isolates collected from 2007 were resistant to GEN and ERO. In comparison with the isolates from 2007, the isolates from 2009 exhibited significantly greater resistance to STR, FEN, TET, DOX and NA ($p<0.05$ to $p<0.01$). Of the 98 isolates, 82 (84%) all 45 from 2009 and 70% of those from 2007 were resistant to 2 or more antimicrobials. A single isolate from 2009 was resistant to 13 of the 15 antimicrobials tested.

Resistance genes: Seven of the 27 resistance genes (*bla cmy-2*, *rmtB*, *tetB*, *tetC*, *sul2*, *qnrS* and *qepA*) were not detected in any of the isolates. The results of PCR identification of the other genes associated with antimicrobial resistance were shown in Table 2. Sul3 and aadA1 was the most prevalent which were found in 19 (36%) and 14 (31%) isolates from 2007 and 2009, respectively. In comparison with the isolates from 2007, the isolates from 2009 exhibited significantly higher rates of carrying aph(3')-VII, aadA1 and aadA2 ($p<0.05$ to $p<0.01$).

Virulence genes: The results of PCR identification of virulence genes were shown in Table 3. All 98 isolates carried invA; in comparison with the isolates from 2007, the isolates from 2009 exhibited significantly lower rates of carrying spvC, sopE and iroB ($p<0.05$ to $p<0.01$).

Table 1: Antimicrobial resistance rates of 98 Salmonella isolates collected from Guongdong province

	Collection period, number and percentage of resistant	
Antimicrobial	2007 (n = 53)	2009 (n = 45)
Ampicillin (AMP)	30 (57)	27 (60)
Ceftiofur (CEF)	24 (45)	20 (44)
Ceftriaxone (CRO)	0 (0)	0 (0)
Streptomycin (STR)	18 (34)	29 (64)[a]
Gentamicin (GEN)	0 (0)	4 (9)
Apramycin (APM)	1 (2)	6 (13)
Chloramphenicol (CHL)	4 (8)	9 (20)
Florfenicol (FEN)	7 (13)	30 (67)[a]
Tetracycline (TET)	4 (8)	28 (62)[a]
Doxycycline (DOX)	4 (8)	15 (33)[a]
Sulfamethoxazole (SMZ)	34 (64)	34 (76)
Nalidixic Acid (NA)	5 (9)	24 (53)[a]
Ciprofloxacin (CIP)	2 (4)	6 (13)
Enrofloxacin (ERO)	0 (0)	7 (16)
Levofloxacin(LEF)	0 (0)	0 (0)

[a]Significantly different from the rate in 2007 ($p<0.05$)

Table 2: Presence of genes associated with antimicrobial resistance in the 98 isolates

	Collection period, number and percentage of resistant	
Resistance genes	2007 (n = 53)	2009 (n = 45)
bla $_{TEM}$	11 (21)	0 (0)
bla $_{SHV}$	1 (2)	2 (4)
bla $_{DHA}$	0 (0)	3 (7)
aac (3)-IV	0 (0)	4 (9)
aph (3')-VII	3 (6)	13 (29)[a]
aadA1	6 (11)	14 (31)[a]
aadA2	3 (6)	12 (27)[a]
cat1	0 (0)	1 (2)
cat2	4 (8)	2 (4)
cmlA	1 (2)	7 (16)
cmlB	0 (0)	2 (4)
floR	7 (13)	4 (9)
tetA	5 (9)	10 (22)
sul1	8 (15)	10 (22)
sul3	19 (36)	7 (16)
qnrA	3 (6)	0 (0)
qnrB	11 (21)	1 (2)

[a]Significantly different from the rate in 2007 ($p<0.05$)

Table 3: Presence of virulence genes in the 98 isolates

	Collection period, number and percentage of resistant	
Virulence genes	2007 (n = 53)	2009 (n = 45)
spvB	7 (13)	11 (24)
spvC	12 (23)[a]	1 (2)
spvD	5 (9)	5 (11)
invA	53 (100)	45 (100)
sopE	14 (26)[a]	1 (2)
phoP/Q	47 (89)	35 (78)
stn	22 (42)	23 (51)
sodCI	24 (45)	30 (67)
sodCII	49 (92)	43 (96)
iroB	24 (45)[a]	5 (11)
hin/H2	36 (68)	29 (64)
repFIIA	6 (11)	6 (13)
slyA	50 (94)	41 (91)

[a]Significantly different from the rate in 2009 ($p<0.05$)

Genetic relatedness by PFGE: All Salmonella isolates were analyzed for their genetic relatedness by using PFGE. Of the 98 isolates, 75 isolates were successfully typed, resulting in 49 different PFGE patterns with a difference of at least seven bands. This suggests that dissemination of the Salmonella isolates might not be due to the spread of a specific clone. However, in a small number of cases, isolates from the same farms or from different farms were found to have identical PFGE patterns.

DISCUSSION

Resistance phenotypes: Salmonella colonizes the gastrointestinal tracts of a wide range of wild and domestic animals including poultry raised for food (Lestari *et al*., 2009). Antimicrobial resistance in Salmonella isolated from both food and veterinary clinical sources appears to be increasing in many countries and regions (Dutil *et al*., 2010; Iwabuchi *et al*., 2011; Yan *et al*., 2010; Yang *et al*., 2010). In the present study, there was a high rate of resistance to particular antimicrobials, notably AMP, SMZ, regardless of collection period in agreement with earlier reports (Hur *et al*., 2011b; Yang *et al*., 2010). To some antimicrobials the rate was significantly increased by year which might be the result of the widely use of the antimicrobials. In addition to most of the antimicrobials, the antimicrobial resistance rates were higher in isolates from 2009, through there was no statistical significance.

Ceftiofur is the only cephalosporin approved for systemic use in food producing animals since, 2002 in China and it is highly effective against Salmonella isolates. The rate of resistance to CEF was higher in the study than in earlier studies (Dutil *et al*., 2010; Lestari *et al*., 2009; Wang *et al*., 2010) presumably as a consequence of the increasing use of cephalosporins on animal farms. Prudent use of antimicrobials in veterinary practice is therefore fundamental to the reduction of resistance development. However, much higher rates were also reported such as 85.7% by Lu *et al*. (2011)'s report.

Fortunately, MIC assays in the present study indicated that >90% of the 98 isolates were within the susceptibility ranges of several antimicrobials including CRO, CIP, ERO and LEF. Thus, these antimicrobials are still potentially effective against Salmonella.

Resistance genes: Though many resistance genes were identified in different sources of Salmonella (Garcia-Fernandez *et al*., 2009; Kozak *et al*., 2009; Lu *et al*., 2011; Rayamajhi *et al*., 2010; Yang *et al*., 2010) however few data are available on prevalence of 27 resistance genes in Salmonella from diseased chicken origin.

Resistance to ampicillin cand cephalosporins in Gram negative bacteria is primarily mediated by β-Lactamases (BLAs) which hydrolyze the β-lactam ring and thus inactivate the antibiotic. Many different BLAs have been described such as TEM-, SHV-, CTX-M-, OXA- and CMY-type BLAs (Bradford, 2001). In the present study, researchers investigated the presence of BLAs encoding genes by using a set of primers for the conserved regions of common *BLAs* genes. PCR and DNA sequencing results showed that the gene *blaTEM-1* was identified in 11(21%) of Salmonella isolates from 2007 while none of isolates from 2009. In addition, researchers identified the *blaDHA-1* and *blaSHV-1* gene in three Salmonella isolates, respectively.

Lin *et al*. (2009) first examined the ciprofloxacin resistance level in the Salmonella strains isolated from

animal sources but researchers checked out the qnr resistance gene mediated by plasmid which can transfer to the recipient *E. coli* DH5a strain (Garcia-Fernandez *et al*., 2009)

Virulence genes: In the present study virulence associated genes were examined involved of SPIs and the chromosomally encoded stn (Salmonella enterotoxin gene), phoP/Q (two component global regulator) and iroB (Parvathi *et al*., 2011) and plasmid in Salmonella isolates from chicken. Salmonella virulence genes play important role in the pathogenicity of the organism and Salmonella pathogenesis is dictated by a group of genes responsible for colonization (Thiagarajan *et al*., 1996) while Data showed that the deletion of SPI-1 does not affect cecal colonization in 1 week old chicken but causes a milder and delayed systemic infection (Desin *et al*., 2009) in addition, *SPI-1* genes are highly expressed at early and late stages of infection in cultured epithelial cells according to Hautefort's report (Hautefort *et al*., 2008). In the present study, >90% of the 98 isolates from chicken carried the virulence genes of *sodCII* and *slyA*, >60% with *phoP/Q* and *hin/H2* and >40% with *stn* and *iroB* and all carried *invA* regardless of collection period. It is interesting that the rate of isolates carried virulence genes of *sopE* and *iroB* from 2009 was significantly lower than that from 2007 while the isolates from 2009 exhibited significantly greater resistance to some of the antimicrobials and higher rates of carrying some resistance genes. Whether the virulence is weakened with the resistance further studies are needed to examine this possibility.

PFGE: Pulsed field gel electrophoresis has been widely used to determine strain relatedness, confirm bacterial disease outbreaks and identify the sources of strains (Chen *et al*., 2011; Gaul *et al*., 2007; Lu *et al*., 2011). In this study, the PFGE results indicated a genetically diverse Salmonella population whereas several indistinguishable PFGE patterns were shared among isolates obtained from different farms. The majority of these isolates exhibited similar resistance profile. This suggests that dissemination of the Salmonella isolates might not be due to the spread of a specific clone.

CONCLUSION

The results of this study showed that the majority of Salmonella strains display resistance to multiple antimicrobial compounds and carry multiple resistance genes and virulence genes. These findings indicate that a surveillance program is needed to employ effective control measures to reduce Salmonella contaminations and the levels of antimicrobial resistant Salmonella in poultry products.

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