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Antimicrobial Resistance of *Salmonella* Serovars Isolated from Beef at Retail Markets in the North Vietnam

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ABSTRACT. Approximately 39.9% (63/158) of beef samples collected from retail markets in Hanoi from January to June 2009 were *Salmonella*-positive. Nine *Salmonella* serovars, Anatum (28.6%), Rissen (25.4%), Weltevreden (12.7%), Typhimurium (7.9%), Derby (7.9%), Lexington (7.9%), Dublin (4.6%), Newport (3.2%) and London (1.8%), were identified. Thirty-seven (58.7%) of the 63 *Salmonella* isolates were resistant to at least one antimicrobial tested, of which 29 (46%) isolates showed multidrug resistance (MDR). The isolates were commonly resistant to tetracycline (46.0%), sulphonamide (39.7%), ampicilline (31.7%), streptomycin (30.2%), trimethoprim (28.6%), kanamycin (28.6%) and chloramphenicol (22.2%). Fourteen (bla_{TEM}, bla_{OXA-1}, *aadA1*, *aadA2, sul1, tetA*, *tetB, tetG, cmlA1, floR, dfrA1, dfrA12, aac (3)-IV* and *aphA1-1AB*) out of 22 antimicrobial resistance genes were detected by PCR from the resistant isolates. The *catA1*, *Kn*, bla_{PSE-1} genes and plasmid-mediated quinolones resistance (PMQR) genes such as *qnrA*, *qnrB*, *qnrS*, *qepA* and *acc (6')-ib-cr* were not detected. Mutations in the *gyrA* gene leading to the amino acid changes *Ser83Phe* and/or *Asp87Asn* were found in 6 out of the 11 quinolone-resistant isolates. The data revealed that multidrug resistant *Salmonella* strains were widely distributed in north Vietnam via the food chain and might contain multiple genes specifying identical resistant phenotypes. Thus, continuous studies are necessary to clarify the mechanisms of MDR in *Salmonella* and its spread in the livestock market.

KEY WORDS: multidrug resistance, North Vietnam, quinolone resistance, raw beef, *Salmonella*.

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Foodborne diseases caused by non-typhoid *Salmonella* represent an important public health problem and an economic burden in many parts of the world. Non-typhoidal *Salmonella* spp. are zoonotic agents, and foods of animal origin are the main sources for their transmission.

The emergence and spread of antimicrobial-resistant *Salmonella* originating from food animals or retail meats has become a serious health hazard worldwide, especially in developing countries [34, 38]. In food animal production, antimicrobials have been used for disease treatment and disease prevention, as well as growth promotion, and are important factors in the emergence of antimicrobial-resistant bacteria. Several lines of evidence indicate that antimicrobial resistance among human *Salmonella* infections results from the use of antimicrobial agents in food animals [2].

The increasing resistance of *Salmonella* to the older generation antimicrobials has been leading to the use of quinolones in order to combat salmonellosis. The main mechanism of quinolone resistance was believed to arise from chromosomal mutations in genes encoding target enzymes, DNA gyrase (*gyrA* and *gyrB*) and/or DNA topoisomerase IV (*parC* and *parE*), or affecting drug accumulation [16, 39]. Besides mutations in chromosomes, plasmid-mediated quinolone resistance (PMQRs) has also been reported, including a *qnr*-mediated inhibition of quinolone binding to DNA, a *qepA* encoded efflux pump, and the *aac*(*6'*)-*Ib-cr* mediated fluoroquinolones by an acetyltransferase [30, 32].

In Vietnam, self-medication through retail pharmacies is a common practice, where antimicrobials for humans and animals can be freely purchased over the counter without control [28]. Farmers normally use antimicrobials to treat sick animals with high doses and using their own experience without veterinary prescription, supervision and laboratory diagnosis [35]. So far, most studies have focused on the prevalence and molecular mechanisms of antimicrobial resistance of *Salmonella* spp. isolates from human medicine, whereas similar studies on *Salmonella* originating from food animals are rare and limited [34, 37].

The aims of this study were to examine the level of *Salmonella* contamination in retail beef at markets in North Vietnam. The antimicrobial resistance of each isolate was further determined, and the antimicrobial resistance genes and mutations encoding for resistance phenotypes were identified.

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MATERIALS AND METHODS

Sampling methods: A total of 158 raw beef samples were collected from retail markets in Hanoi, Vietnam, from January to June 2009. In each meat shop, one sample from a carcass (>100 g) was collected and placed in a sterile plastic sampling bag and chilled in an ice box during transport to the laboratory at the Department of Microbiology − Infectious Disease − Pathology, Faculty of Veterinary Medicine, Hanoi University of Agriculture. All samples were analyzed on the day of arrival.

Salmonella isolation: *Salmonella* was isolated according to the Standard ISO-6579 method [19] with some modifications. For pre-enrichment of *Salmonella*, 25 g of each sample was homogenized in sterile bags with 225 m*l* of buffered peptone water (PBW) and incubated at 37°C for 18–24 hr. Next, 0.1 m*l* of the pre-enriched culture in PBW was added to 10 m*l* of Rappaport-Vassiliadis soya (RVS) broth followed by further incubation at 41.5°C for 24 hr. A loopful of culture broth was then sampled from the selective enrichment RVS broth, streaked onto xylose-lysine-tergitol 4 agar (Merck) and incubated at 37°C for 24 hr. Presumptive black colonies were selected from each plate and cultured on nutrient agar slants. The isolates were confirmed to be *Salmonella* by confirmatory biochemical tests (fermentation of glucose, lactose and sucrose; hydrogen sulphide production test; citrate test; lysine decarboxylation; and methyl red and indole tests).

Salmonella serotyping: Typical *Salmonella* isolates were serotyped on slides by the microtiter agglutination test to identify O and H antigens (Difco Laboratories, Detroit, MI, U.S.A.), according to the version of the Kauffmann and White scheme used [17] by the Department of Veterinary Hygiene, National of Institute Veterinary Research, Vietnam.

Antimicrobial susceptibility testing: The antimicrobial susceptibility of isolates was determined according to the guidelines of the Clinical and Laboratory Standards Institute [8]. Disk diffusion assays were performed on Muller-Hinton agar with disks containing 15 different antimicrobial agents (Oxoid, UK). The following antimicrobials were tested: ampicillin (A), 10 μg; amoxicillin/clavulanic acid (Ac), 20/10 μg; ceftazidime (Cf), 30 μg; chloramphenicol (Cl), 30 μg; ciprofloxacin (Ci), 5 μg; gentamicin (G), 10 μg; kanamycin (K), 30 μg; nalidixic acid (Na), 30 μg; neomycin (Ne), 10 μg; norfloxacin (No), 10 μg; streptomycin (S), 10 μg; tetracycline (T), 30 μg; trimethoprim (Tp), 5 μg; and sulphonamide (Su), 300 μg. The interpretive categories susceptible, intermediate and resistant were used according to CLSI guidelines [9], except for colistin (Co), for which the zone criteria of ≤11 mm for resistant and≥14 mm for susceptible were used [15]. *Escherichia coli* ATCC 25922 was used as the control. An isolate was defined "resistance" after confirmation of resistance to at least one agent tested, while "multiple resistance" was defined as resistance to 3 or more agents.

Detection of resistance genes: DNA templates used for PCR were prepared by boiling bacterial cultures [31]. The following genes implicated with antimicrobial resistance were detected by PCR amplification: $bla_{PSE\text{-}1}$, $bla_{OXA\text{-}1}$ and bla_{TEM} encoding β-lactam resistance; *aadA1*, *aadA2*, *aac (3)-IV*, *aphA-1AB* and *Kn* encoding aminoglycoside resistance; *catA1*, *cmlA1* and *floR* encoding chloramphenicol resistance; *sul1* encoding sulphonamide resistance; *tetA*, *tetB* and *tetG* encoding tetracycline resistance; and *dfrA1* and *dfrA12* encoding trimethoprim resistance. PMQR genes such as *qnrA*, *qnrB*, *qnrS, qepA,* and *aac (6')-ib-cr* and quinolone resistance determining regions (QRDRs) of *gyrA* were presented for quinolone resistance. The primer sets and assay conditions used for amplification were described elsewhere [4, 12, 13, 16, 18, 21, 27].

PCR amplification reactions were performed in a 25 μl volume of reaction mixture containing 12.5 μl of GoTaq® Green Master Mix, 2 × (Promega, U.S.A.), 1 μl(10 *n*g/μl) of primers, 4 μl of DNA template and nuclease-free water. The PCR program consisted of hot start cycle of 94°C for 5 min; followed by 30 cycles of 94°C for 30 sec, the corresponding temperature for each primer pair for 30 sec and 72°C for 1 min; and then a final extension step of 72°C for 5 min. The PCR products were analyzed by electrophoresis with 1.5% agarose in 1×Tris-boric acid-TBE buffer. The gels were stained with 1 μg/m*l* ethidium bromide, and visualized bands were photographed using a Polaroid camera on an ultraviolet light transilluminator. A molecular weight standard ladder was included on each gel (Toyobo, Japan).

Sequencing of gyrA: PCR amplifications of *gyrA* to detect substitutions at codon 81, 83 and 87 were carried out using the primers from a previous report [13]. Purified PCR products were sequenced and compared with the wild-type sequences (GenBank accession number X78977.1).

RESULTS

Salmonella isolation and distribution: Approximately 39.9% (63/158) of beef samples collected from retail markets in Hanoi City were *Salmonella* positive, and 9 serovars were identified (Table 1). The common serovars were Anatum (28.6%), Rissen (25.4%), Weltevreden (12.7%), Typhimurium (7.9%), Derby (7.9%) and Lexington (7.9%), and the remaining serovars were London, Newport and Dublin (ranging from 1.6 to 4.8%).

Antimicrobial susceptibility: The *Salmonella* isolates were commonly resistant to tetracycline (46.0%), sulphonamide (39.7%), ampicillin (31.7%), streptomycin (30.2%), trimethoprim (28.6%), kanamycin (28.6%) and chloramphenicol (22.2%). None of the *Salmonella* isolates showed resistance to ceftazidime and colistin (Table 1). Thirty-three (58.7%) of the 63 *Salmonella* isolates were resistant to at least one antimicrobial (Table 2). Twenty nine (46%) out of the 63 isolates showed multidrug resistance (MDR). MDR was frequently observed in serovars Anatum, Typhimurium, Rissen and Derby.

Detection of antimicrobial resistance genes: Fourteen (bla_{TEM}, $bla_{OXA\text{-}1}$, *aadA1*, *aadA2, sul1, tetA*, *tetB, tetG, cmlA1, floR, dfrA1, dfrA12, aac (3)-IV* and *aphA1-1AB*) of 17 resistance genes were detected from the resistant isolates (Table 3). Among the 29 tetracycline-resistant isolates, the

Table 1. Antimicrobial resistance of *Salmonella* serovars isolated from retail beef at retail markets

Serovars (no)	A	Ac	Cf	Co	S	G	K	C	Ne	Na	No	Ci	T	Su	Tp
Anatum (18)	7	-	-	-	6	3	8	7	3	3	-	-	8	10	8
Rissen (16)	5	-	-	-	3	1	3	-	1	2	-	-	10	4	4
Weltevreden (8)	1	-	-	-	1	-	-	-	-	-	-	-	1	1	-
Typhimurium (5)	5	-	-	-	4	4	4	4	1	4	-	2	5	5	4
Lexington (5)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Derby (5)	2	-	-	-	3	-	1	2	-	-	-	-	2	3	2
Dublin (3)	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-
Newport (2)	-	-	-	-	2	1	2	1	-	2	-	-	2	2	-
London (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Total (63)	20	0	0	0	19	9	18	14	5	11	0	2	29	25	18
% Resistance	31.7	0	0	0	30.2	14.3	28.6	22.2	7.9	17.5	0	3.2	46.0	39.7	28.6
% Intermediate	3.2	4.8	0	0	6.3	0	0	4.8	12.7	6.3	3.2	4.8	6.3	0	0
% Susceptible	65.1	95.2	100	100	63.5	85.7	71.4	73.0	79.4	76.2	96.8	92.0	47.6	60.3	71.4

Abbreviations: ampicillin, A; Amoxicillin/clavulanic acid, Ac; Colistin, Co; Chloramphenicol, C; Ceftazidime, Cf; Ciprofloxacin, Ci; Gentamicin, G; Kanamycin, K; Nalidixic acid, Na; Neomycin, Ne; Norfloxacin, No; Sulphonamide, Su; Streptomycin, S; Tetracycline, T; Trimethoprim, Tp.
- Not found

Table 2. Multidrug resistance of *Salmonella* isolates from retail beef at retail markets

No	MDR patterns	No. showing AR	Serovars (no.)	No	MDR patterns	No. showing AR	Serovars (no.)
1	A	1	Anatum (1)	15	ACSuTGK	6	Anatum (1)
2	T	1	Dublin (1), Rissen (3)	16	ASSuTKTp	6	Anatum (1), Typhimurium (1)
3	TTp	2	Anatum (1), Rissen (2)	17	CSSuTKNa	6	Newport (1)
4	ASuT	3	Rissen (3)	18	SSuTGKNa	6	Newport (1)
5	SuTTp	3	Anatum (1)	19	SSuTGKNa	6	Rissen (1)
6	ASSu	3	Derby (1)	20	SSuTKNeNa	6	Anatum (1)
7	ACSK	4	Derby (1)	21	ASTKNeTpNa	7	Rissen (1)
8	ASKTp	4	Rissen (1)	22	CSSuTKNeNa	7	Anatum (1)
9	ASSuT	4	Weltevreden (1)	23	ACSSuTGKNa	8	Typhimurium (1)
10	CSuTTp	4	Anatum (1), Derby (1)	24	ACSuTGKTpNa	8	Typhimurium (1)
11	SSuTTp	4	Derby (1)	25	ASSuTKNeTpNa	8	Anatum (1)
12	ACSGK	5	Anatum (1)	26	ACSSuTGTpNaCi	9	Typhimurium (1)
13	ACSuKTp	5	Anatum (2)	27	ACSSuTGKTpNaCi	10	Typhimurium (1)
14	CSSuGTp	5	Anatum (1)				

Abbreviation is similar to those in Table 1. AR: Antimicrobial resistance.

tetA gene was detected from 16 (55.2%), while *tetB* or *tetG* genes were detected only in one (3.4%) isolate. None of the 20 ampicillin-resistant isolates carried the bla_{PSE-1} gene; the bla_{TEM} gene was detected in 18 (90.0%) isolates, of which one contained more bla_{OXA-1} gene. The *sul1* gene was detected in 20 (80%) of the 25 sulphonamide-resistant isolates. Among the 14 chloramphenicol-resistant isolates, none of the isolates contained the *catA1* gene; *floR* and *cmlA1* genes were detected in 8 (57.1%) and 7 (50%) isolates, respectively. The *aadA1* gene was found in 15 (78.9%) of the 19 streptomycin-resistant isolates, of which one contained more *aadA2* gene. Among the 18 trimethoprim-resistant isolates, 10 *dfrA1*(55.6%) and 6 *dfrA12*(33.3%) genes were displayed. All of the 18 kanamycin-resistant isolates carried the *aphA1-1AB* gene. Eight (88.9%) of the 9 gentamicin-resistant isolates contained the *aac (3)-IV* gene.

Detection of the PMQR genes and substitutions in gyrA of the quinolone-resistant isolates: PMQR genes such as *qnrA*, *qnrB*, *qnrS*, *qepA* and *acc (6')-ib-cr* were not detected from 11 quinolone-resistant isolates. Sequence analysis of the *gyrA* revealed that 6 isolates had substitutions (Table 4). In these isolates, three *S.* Typhimurium and two *S.* Anatum isolates had single substitution at *Ser83* and one *S.* Typhimurium isolate had double substitutions at *Ser83* and *Asp87*. Substitutions were found in the codon *TCC (Ser)* at position 83 to *TTC (Phe)* and in the codon *GAC (Asp)* at position 87 to *AAC (Asn)*.

DISCUSSION

Approximately 39.9% of beef samples were contaminated with *Salmonella*. This rate was lower than that reported in similar previous studies in South Vietnam [29, 34]. The high levels of contamination indicate a potential breakdown of

Table 3. The distribution and prevalence of resistance genes among the *Salmonella* serovar isolates

AR genes (No. of isolates tested)	No. of positive isolates (%)	AR genes belonging to serovars (no. positive isolates)
Ampicillin (20)		
bla_{TEM}	18 (90.0)	Anatum (6), Derby (1), Rissen (5), Typhimurium (5), Weltevreden (1)
bla_{OXA-1}	1 (5.0)	Typhimurium (1)
bla_{TEM} + bla_{OXA-1}	1 (5.0)	Typhimurium (1)
At least one gene	18 (90.0)	
Chloramphenicol (14)		
cmlA1	7 (50.0)	Anatum (3), Typhimurium (4)
floR	8 (57.1)	Anatum (3), Derby (2), Newport (1), Typhimurium (2)
cmlA1+*floR*	2 (14.3)	Typhimurium (2)
At least one gene	14 (100)	
Gentamicin (9)		
aac (3)-IV	8 (88.9)	Anatum (3), Newport (1), Typhimurium (4)
Kanamycin (18)		
aphA1-1AB	18 (100)	Anatum (8), Derby (1), Newport (2), Rissen (3), Typhimurium (4)
Streptomycin (19) *aadA1*	15 (78.9)	Anatum (5), Derby (1), Newport (1), Rissen (3), Typhimurium (4), Weltevreden (1)
aadA2	1 (5.3)	Typhimurium (1)
aadA1+*aadA2*	1 (5.3)	Typhimurium (1)
At least one gene	15 (78.9)	
Sulphonamides (25)		
sul 1	20 (80.0)	Anatum (7), Derby (2), Newport (1), Rissen (4), Typhimurium (5), Weltevreden (1)
Tetracycline (29)		
tetA	16 (55.2)	Anatum (5), Derby (1), Newport (1), Rissen (7), Typhimurium (1), Weltevreden (1)
tetB	1 (3.4)	Derby (1)
tetG	1 (3.4)	Dublin (1)
At least one gene	18 (62.1)	
Trimethoprim (18)		
dfrA1	10 (55.6)	Anatum (5), Derby (1), Rissen (4)
dfrA12	6 (33.3)	Anatum (2), Typhimurium (4)
At least one gene	16 (88.9)	

Table 4. Mutations of gyrA in quinolone-resistant *Salmonella* isolated from retail beef

Isolation (ID)	Serovars	Diameter zones of resistance (mm)			QRDRs
		Na (30 μg)	Ci (5 μg)	No (10 μg)	gyrA
HNB30	Anatum	9	22	22	Ser83Phe
HNB65	Anatum	8	21	23	Ser83Phe
HNB 21	Anatum	10	22	20	wt
HNB88	Newport	11	24	26	wt
HNB14	Newport	10	29	25	wt
HNB81	Rissen	12	30	27	wt
HNB58	Rissen	11	30	25	wt
HNB39	Typhimurium	8	17	21	Ser83Phe
HNB33	Typhimurium	8	17	21	Ser83Phe
HNB10	Typhimurium	8	14	15	Ser83Phe
HNB112	Typhimurium	6	11	13	Ser83Phe Asp87Asn

wf: Wild type.

hygiene at various stages of the food processing and distribution chain and/or a lack of refrigeration of meat in Vietnam. Among the *Salmonella* isolates identified, *S*. Anatum was commonly detected in this study, similar to previously reports in Vietnam [36, 37]. This serovar was usually listed in the common serovars from food sources in other studies [3, 5, 7]. *S*. Rissen, one of the most commonly detected serovars from both human and nonhuman sources in Asia [3], was frequently recovered in our study. In addition, the serovars Typhimurium, Derby, Weltevreden and Newport were also

detected in this study; these serovars were previously associated with human foodborne gastroenteritis [1, 3, 6, 14, 20]. Therefore, foodborne diseases may occur in Vietnam, because non-typhoidal *Salmonella* spp. are zoonotic agents and animal products originating are the main sources of *Salmonella* spp. transmission.

In this study, 58.7% of *Salmonella* isolates were resistant to at least one antimicrobial. Resistance to tetracycline, sulphonamide, ampicillin, streptomycin, trimethoprim and chloramphenicol was commonly observed in the *Salmonella* isolates, as shown in previous reports from Vietnam [28, 34, 37] and other Asia countries [33, 38]. These antimicrobials are commonly used in animal husbandry in these countries, and the increasing and inappropriate use of antimicrobials in animal farming may be the reason for these high levels of resistance [34]. MDR was observed frequently in the *Salmonella* isolates in this study, as shown in previous studies from Vietnam [28, 34, 37], China [38] and Malaysia [33]. This might lead to human infections with foodborne antimicrobial-resistant bacteria. Therefore, it may create an enormous challenge to treatment of *Salmonella* infection in humans and animals in these countries.

All of the antimicrobial-resistant isolates were investigated for the corresponding resistance genes. High prevalence of the *tetA, bla*$_{TEM}$, *floR*, *sul1* and *dfrA1* genes was detected from the antimicrobial-resistant isolates, as shown in previous reports [24, 25]. The streptomycin resistance was mainly encoded by *aadA1* in this study, as observed in reports from Thailand [1, 10]. These resistance genes were also detected from integrons of resistant *Salmonella* isolates from foodstuffs in southern Vietnam [34, 37]. All of the kanamycin-resistant isolates contained the *aphA1-1AB* gene, similar to previous reports [13, 25]. However, it was detected at a low rate from *S.* Infantis in Japan [31]. In this study, the resistance genes were found at high rates and were widespread in different serovars from the resistant isolates, indicating that these genes play an important role in prevalence of antimicrobial resistance among the *Salmonella* isolates from retail meats in Vietnam.

The resistance to nalidixic acid (17.5%) and ciprofloxacin (3.2%) and decreased susceptibility to norfloxacin (3.2%) of the isolates in this study were similar to the results of a report in South Vietnam [37]. In addition, different levels of resistance of *Salmonella* to these antimicrobials were also described in several countries [11, 32, 33, 38]. This is a worldwide concern, because ciprofloxacin is the drug of choice for treatment of human *Salmonella* infection. Similar to other studies [11, 37], we detected substitutions at codons *Ser83* and *Asp87* of *gyrA* from six quinolone-resistant *Salmonella* isolates. Substitutions in the codon of *gyrA* such as *Gly81*, *Ser83* and *Asp87* in *Salmonella* were frequently observed when the MIC levels of nalidixic acid and/or ciprofloxacin were very high (≥128 μl/m*l* for nalidixic acid and≥0.25 μl/m*l* for ciprofloxacin) [11, 22, 23]. Thus, the absence of substitutions in *gyrA* in 5 nalidixic acid-resistant isolates in this study may be explained by other resistance mechanisms such as decreased permeability of the outer membrane, mutations in the *gyrB, parC*, *parE* genes. These will be warranted in the further our investigation. In this study, the PMQR genes were not detected in the quinolone-resistant *Salmonella* isolates. However, they were detected at a very low rate from *Salmonella* in France [4] and the U.S.A. [16]. In addition, there were several reports on *Enterobacteriaceae* harboring quinolone-resistant genes in Vietnam [26] and other countries [32]. These reports confirmed that PMQR genes in foodborne isolates may play a role in the spread of quinolone resistance through the food chain. Therefore, continuous research is needed to detect the PMQR genes in *Salmonella* spp. from food sources in Vietnam.

This study indicated a high frequency of antimicrobial resistance among the *Salmonella* isolates from beef at retail markets in the North Vietnam. Resistance genes were widespread in *Salmonella* serovars isolates. Therefore, some management strategies are needed for public health to prevent foodborne diseases caused by MDR *Salmonella* from the food supply.

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