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Detection of virulence, specific genes and antibiotic resistance of isolated *Salmonella* spp. strains from rabbits infected with salmonellosis

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Abstract

Salmonella spp. are pathogens involved in most salmonellosis in rabbits. This study examined *Salmonella* disease in rabbits raised in Thua Thien Hue, Vietnam. Two hundred and 56 rectal swabs of rabbits were taken, and a carrier rate of 33.98% was found. In addition, all the isolated *Salmonella* spp. strains were 100% motile; positive for H_2S, catalase, Voges Proskauer, coagulase, citrate, maltose, and dextrose; and negative for indole, methyl red, urease, oxidase, sucrose, and lactose. The Kirby-Bauer method showed that these *Salmonella* strains were susceptible to doxycycline (93.2%), tetracycline (84.1%), and levofloxacin (65.9%). On the other hand, they were highly resistant to streptomycin (95.5%), ampicillin (93.2%), colistin (40.9%), and gentamicin (34.1%). Furthermore, polymerase chain reaction used to screen for virulence and specific genes of *Salmonella* strains showed that all *Salmonella* strains isolated carried *InvA, fimA*, and *Stn*.

Keywords: antibiotic susceptibility; rabbits; virulence genes; specific genes

Introduction

Salmonella is a member of the *Enterobacteriaceae* family that causes disease in humans, wild animals, and domestic animals [1]. Salmonellosis is one of the most important bacterial infections causing economic losses in livestock because of high mortality and weight loss [2]. *Salmonella* has been detected in rabbits, posing potential concerns for rabbit farming [3]. Salmonellosis in rabbits has been reported previously [4–8]. The clinical signs of *Salmonella* infection in rabbits include septicemia, depression, fever, and death, often accompanied by enteritis, diarrhea, metritis, and abortion [9]. Currently, bacterial infections caused by *Salmonella* spp. in rabbits raised at Thua Thien Hue, Vietnam, remain unknown. Therefore, this study collected 256 rectal swabs of rabbits (both genders aged between 1.5–4 months) from farms and households (16 samples/farm, households) to evaluate the prevalence of *Salmonella* spp. In addition, biochemical characteristics, common virulence genes, and specific genes were assessed, and their drug resistance characteristics were analyzed to supplement the information as a basis for preventing and controlling *Salmonellosis* in rabbits in the locality.

Materials and Methods

Sample collection

Two hundred and 56 rectal swabs, including 126 healthy rabbits and 130 rabbits suspected of being infected with *Salmonella* spp., were collected from 6 households and 10 farms in Thua Thien Hue, Vietnam. Each rectal swab was placed in 5 mL of buffered peptone water (Merck, Germany) and transferred directly to the Laboratory of Immunology and Vaccines, the Institute of Biotechnology, Hue University.

Isolation and characterization of *Salmonella* spp. from rabbits

The isolation of *Salmonella* spp. was performed as follows. The samples were incubated overnight at 37°C with shaking. After incubation, 0.1 mL of an overnight culture was added to 10 mL of Rappaport-Vassiliadis, and the mixture was incubated at 42°C for 18 to 24 hours. A loop of each culture was spread onto HiCrome Salmonella Agar (Himedia, India) and incubated at 37°C for 24 hours. The colony morphology was assessed with Gram staining, and biochemical assays were performed: production (H_2S, indole, methyl red), catalase, urease activity, Voges Proskauer test, oxidase reaction, coagulase, motility, citrate, fermenting carbohydrates from maltose, sucrose, lactose and dextrose [10]. In addition, presumptive *Salmonella* spp. colonies were confirmed further by the glass slide agglutination method with multivalent O, H antisera (Denka Seiken Co., Ltd., Japan), according to Grimont and Weill [11].

Antimicrobial sensitivity test

The Kirby-Bauer method [12] was used to obtain antibiograms of all isolated *Salmonella* strains using 10 antibiotics (Namkhoa, Vietnam): ampicillin 10 μg (Am), amikacin 30 μg (Ak), ceftazidime 30 μg (Cz), chloramphenicol 30 μg (Cl), colistin 10 μg (Co), doxycycline 30 μg (Dx), gentamicin 10 μg (Ge), levofloxacin 5 μg (Lv), streptomycin 10 μg (Sm), and tetracycline 30 μg (Te). The diameters of the inhibition zones on the diffusion disk were measured using the guidelines of the Clinical and Laboratory Standards Institute [13].

Detection of the virulence and specific genes of isolated *Salmonella* spp.

The genomic DNA of isolated *Salmonella* spp. was extracted using the TopPURE genomic DNA extraction Kit according to the manufacturer's guidelines. Azenta Life Sciences synthesized the oligonucleotide primers. Polymerase chain reaction (PCR) was conducted in a total reaction volume of 25 μL (GoTaq Green Master Mix (2X) 12.5μL, DNA template 5 μL, Nuclease-Free Water 5.5 μL, each primer (20 pmol/1 μL)). Table 1 lists the PCR conditions and primer sequences [14,15]. All PCR products were resolved by electrophoresis (1.0% agarose gel in 1X TAE buffer) at 80 V. The gels were stained with GelRed stain and visualized using a UVP UV Transilluminator.

Statistical analysis

The data were processed statistically using the Minitab statistical package ver. 14.0. The results were compared using a chi-squared test, in which $p < 0.05$ was considered statistically significant.

Results

Prevalence of *Salmonella* spp. isolated from rabbits

Table 2 lists the prevalence of *Salmonella* spp. carriers in rabbits raised in Thua Thien Hue. The results revealed an overall

Table 1. Polymerase chain reaction primers and annealing temperature for detecting virulence and specific genes in *Salmonella* spp. isolates

Target gene		Oligonucleotide sequences (5′–3′)	Expected size (base pairs)	Annealing temperature (°C)	Reference
Virulence genes	*Stn*	F-CGATCCCTTTCCCGCTATC R-GGCGAATGAGACGCTTAAG	179	46.9	[14]
	fimA	F: CCT TTC TCC ATC GTC CTG AA R: TGG TGT TAT CTG CCT GAC CA	85	50.8	
Specific gene	*invA*	F: GTG AAA TTA TCG CCA CGT TCG GGC AA R: TCATCGCACCGTCAAAGGAACC	284	50.8	[15]

Table 2. Prevalence of *Salmonella* spp. infection in rabbits in Thua Thien Hue, Vietnam

Source	No. of examined samples	No. of positive samples	Prevalence of +Ve cases (%)	No. of negative samples	Prevalence of -Ve cases (%)
Healthy rabbits	130	0	0	130	100
Diseased rabbits	126	87	69.04	39	30.95
Total	256	87	33.98	169	66

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carriage rate of 33.98% (87/256). Healthy rabbits are unlikely to be infected with *Salmonella* spp. The rabbits with diarrhea were infected at a rate of 69.04% (87/126).

Characterization of *Salmonella* spp. isolates

In terms of the morphology, all *Salmonella* spp. isolated were Gram-negative and rod-shaped. The bacterial colonies appeared pink to red on HiCrome Salmonella Agar (Fig. 1). All strains were motile. They reacted positively to H_2S, catalase, Voges Proskauer, coagulase, citrate, maltose, and dextrose and negatively to indole, methyl red, urease, oxidase, sucrose, and lactose. In addition, the slide agglutination test was positive for multivalent O antisera and negative/positive for multivalent H antisera (Table 3).

Determination of antibiotic susceptibility of isolated *Salmonella* spp.

The isolated *Salmonella* spp. from rabbits were inhibited by doxycycline (93.2%), tetracycline (84.1%), levofloxacin (65.9%), chloramphenicol/ amikacin (15.9%), colistin (13.6%), ceftazidime (11.4%), and gentamicin (6.8%). On the other hand, they were resistant to streptomycin (95.5%), ampicillin (93.2%), colistin (40.9%), gentamicin (34.1%), amikacin (29.5%), chloramphenicol (11.4%), levofloxacin (4.5%), and ceftazidime (2.3%). Table 4 and Fig. 2 show the antibiotic susceptibility of bacteria.

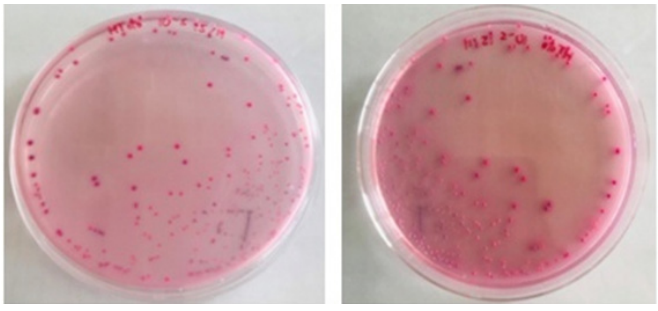

Fig. 1. *Salmonella* spp. colonies on HiCrome *Salmonella* Agar (Himedia).

Table 3. Biochemical identification of *Salmonella* spp. isolated from rabbits in Thua Thien Hue

Biochemical test	Results
H_2S	+
Indole test	-
Methyl red	-
Catalase	+
Urease activity	-
Voges Proskauer	+
Oxidase reaction	-
Coagulase	+
Motility	+
Citrate test	+
Maltose	+
Sucrose	-
Lactose	-
Dextrose	+
Polyvalent O	+
Polyvalent H	+

Detection of virulence and specific genes of *Salmonella* spp. by PCR

All the *Salmonella* spp. strains were isolated for DNA extraction, and the presence of *invA*-specific gene and *stn, fimA* virulence genes were determined by PCR. The *invA, stn,* and *fimA* genes were present in all tested isolates (Fig. 3).

Discussion

Salmonella spp. isolated on HiCrome Salmonella Agar, bacterial colonies appeared pink to red (Fig. 1). This medium is suitable for detecting *Salmonella* spp. with high specificity [16,17]. Furthermore, all isolated strains could utilize dextrose and maltose but not lactose and sucrose (Table 3), confirming they are *Salmonella* spp. [18]. The suspected *Salmonella* spp. strains were motile (Table 3). *Salmonella* motility is the basic test for detecting motile and non-motile bacteria [19]. Overall, the essential biochemical characteristics of *Salmonella* spp. in this study were similar to those of other reports [20,21].

Antibiotic resistance in humans and animals is a matter of great concern. Therefore, it is necessary to determine bacterial response to antibiotics so that the correct antibiotics can be employed promptly [22]. This study tested all isolates for suscepti-

Table 4. Susceptibility of isolated *Salmonella* spp. to 10 commonly used antibiotics

Antibiotics	Total	Sensitive	Intermediate	Resistance
Dx	44	41 (93.2)	3 (6.8)	0 (0)
Te	44	37 (84.1)	7 (15.9)	0 (0)
Lv	44	29 (65.9)	13 (29.5)	2 (4.5)
Cz	44	5 (11.4)	38 (86.4)	1 (2.3)
Cl	44	7 (15.9)	32 (72.7)	5 (11.4)
Ak	44	7 (15.9)	24 (54.5)	13 (29.5)
Co	44	6 (13.6)	20 (45.5)	18 (40.9)
Ge	44	3 (6.8)	26 (59.1)	15 (34.1)
Am	44	0 (0)	3 (6.8)	41 (93.2)
Sm	44	0 (0)	2 (4.5)	42 (95.5)

Values are presented as number (%).
Dx, doxycycline; Te, tetracycline; Lv, levofloxacin; Cz, ceftazidime; Cl, chloramphenicol; Ak, amikacin; Co, colistin; Ge, gentamicin; Am, ampicillin; Sm, streptomycin.

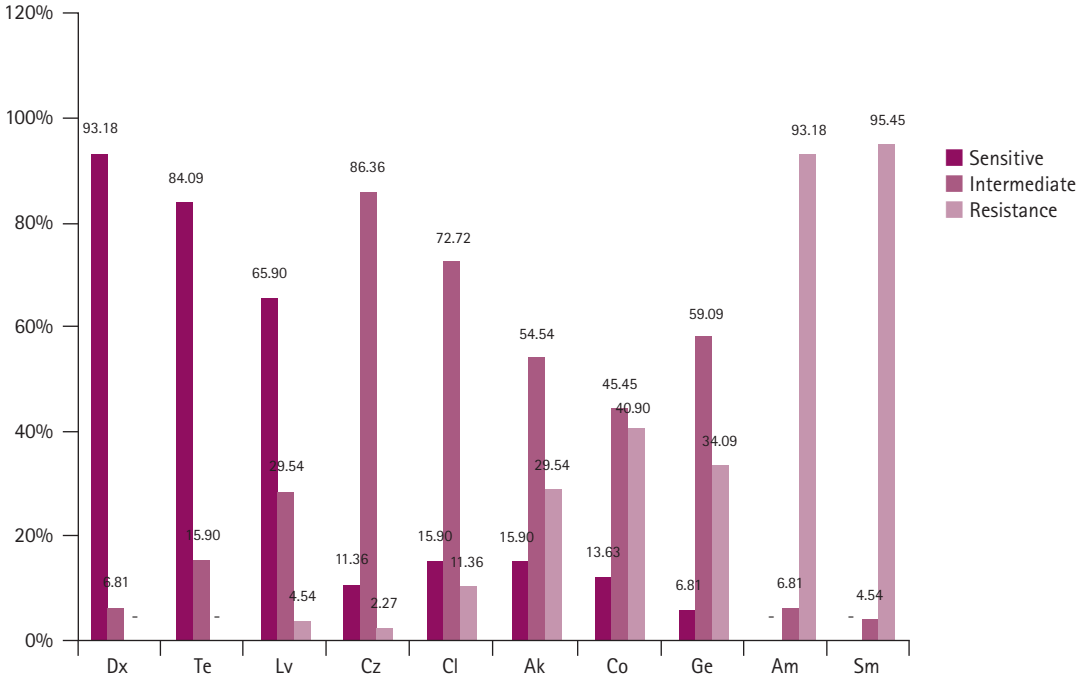

Fig. 2. Percentage of sensitivity, intermediate resistance, and resistance of *Salmonella* spp. Dx, doxycycline; Te, tetracycline; Lv, levofloxacin; Cz, ceftazidime; Cl, chloramphenicol; Ak, amikacin; Co, colistin; Ge, gentamicin; Am, ampicillin; Sm, streptomycin.

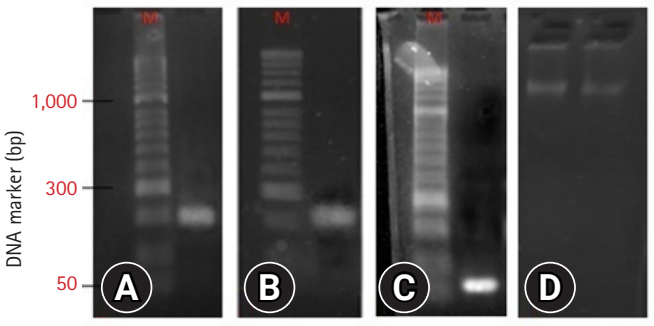

Fig. 3. Electrophoresis of polymerase chain reaction products on agarose gel. (A) *stn* gene with a size of 179 bp, (B) *invA* gene with a size of 284 bp, (C) *fimA* gene with a size of 85 bp, (D) DNA total. M, DNA Marker with a size of 50 to 2,000 bp, Bioline.

bility to 10 commonly used antibiotics in farms/households. Alarmingly, a large percentage of *Salmonella* spp. isolates displayed strong resistance to streptomycin, ampicillin, and colistin (95.5%, 93.2%, and 40.9%, respectively). On the other hand, the majority were sensitive to doxycycline, tetracycline, and levofloxacin (93.2%, 84.1%, and 65.9%, respectively).

The *invA*-specific gene has been recognized internationally as the standard for detecting the genus *Salmonella* [23,24]. *InvA* gene carries sequences unique to this genus and has been commonly used to detect *Salmonella* spp. using the PCR method [25]. This gene encodes a protein responsible for the invasion of the host epithelial cells [24,26]. Forty-four conventionally cultured *Salmonella* spp. strains were selected for DNA extraction and confirmation by PCR. All strains tested carried the *invA*-specific gene (PCR products of 284 bp in size), confirming that these isolates are *Salmonella* spp. The results were corroborated by other studies [3,27,28].

The virulence of *Salmonella* spp. is related to a combination of chromosomal and plasmid factors. There are different genes, of which the *Stn* is a vital toxin gene encoding the Stn protein that causes gastroenteritis leading to fever, abdominal cramps, nausea, vomiting, and diarrhea. The presence of toxin genes in *Salmonella* is the cause of salmonellosis and food poisoning diseases in humans [15]. In this study, all Salmonella isolates carried the *stn* gene with a predicted size of approximately 179 bp. Previously, the *stn* gene was present in all strains of *Salmonella* spp. isolated from animals [28,29]. fimA is the gene encoding fimbriae in *Salmonella* spp. Fimbria-mediated adherence to the intestinal epithelia is a crucial step in enteroaggregative bacterial pathogenesis [30]. The fimA gene was detected in all isolated strains of *Salmonella* spp., and 85pb DNA fragments were produced, which was similar to that reported by Chaudhary et al. [15] and van Asten and van Dijk [31].

In summary, the *Salmonella* spp. strains isolated from rabbit rectal swabs were confirmed by bacterial isolation, biochemical reactions, agglutination test, and PCR with primers specific for

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the *InvA* gene. Most *Salmonella* strains carried the genes encoding the enterotoxin *stn* and the adhesion factor *fimA*.

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