

Biochemical Characteristics and Antimicrobials Susceptibility of *Salmonella gallinarum* Isolated in Korea

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Abstract

Fowl typhoid (FT) reported since 1992 in Korea is a septicemic disease of domestic birds caused by *Salmonella gallinarum* (*S. gallinarum*). The purpose of this study was to investigate the biochemical characteristics and antimicrobials susceptibility of field isolates of *S. gallinarum* isolated by year in Korea.

A total of 258 isolates of *S. gallinarum* from 1995 to 2001 showed the same pattern in the majority of biochemical test such as IMViC (indole, methyl red, Voges-Proskauer and citrate utilization), carbohydrate fermentation and amino acid decarboxylation, and these results were almost in accordance with the traditional biochemical characteristics of *S. gallinarum* strain.

When the antimicrobial susceptibility test against 258 isolates of *S. gallinarum* was performed by the disk diffusion method using 12 antimicrobial agents, all isolates from 1995 appeared to be susceptible to all of the antimicrobial agents tested except for tetracycline and oxytetracycline, whereas the vast majority of isolates from 2001 showed the reduced susceptibility to ampicillin (13.0%), gentamicin (43.4%), kanamycin (69.6%), enrofloxacin (6.5%), ciprofloxacin (10.9%), norfloxacin (52.5%) and ofloxacin (82.6%). The prevalence of the prevalence of completely resisany isolates resistant isolates to one or more drugs rapidly increased from 0% in 1995 to 93.5% in 2001. The minimal concentrations range of the majority of antimicrobial agents to inhibit 50% (MIC_{50s}) against *S. gallinarum* isolates increased from 0.06~8 µg/ml in 1997 to 2~256 µg/ml in 2001. Especially, MIC_{50s} for gentamicin and fluoroquinolones of isolates from 2001 increased over 10-fold than those of isolates from 1997.

Therefore, our results indicate that sorbitol fermentation and arginine decarboxylation showed the diversity by isolates and the vast majority of isolates from 2001 showed the reduced susceptibility to antimicrobials tested.

Key words: fowl typhoid, *Salmonella gallinarum*, biochemical characteristics, antimicrobials

Introduction

Fowl typhoid (FT) is a septicemic disease of domestic birds caused by *Salmonella gallinarum* (*S. gallinarum*). The outbreak of FT is characterized by increased mortality, anorexia, greenish-yellow diarrhea, and a drop in egg production. Subacute outbreak can occur, and egg transmission may lead to increased dead or weak chickens.

FT first occurred at a chicken breeder in England, and was called 'infectious enteritidis' by Klein [10]. After that, Curtice [4] studied the disease in Rhode Island and named it "FT" in 1902. FT is rare in a modern poultry industry of the advanced countries due to extensive test and control of breeder birds. However, the disease has gained the incidence in South America and other countries throughout Africa and Asia in recent years [2, 12, 16, 18, 22].

The outbreak of FT in Korea was officially confirmed in 1992 [8]. The chickens with greenish-yellowish diarrhea, paleness and sudden death, and high mortality from several farms of different areas, such as Kimpo in Kyunggi-do, and Guechang and Chungmu in Gyeonsangnam-do, were submitted to Veterinary Research and Quarantine Service (NVRQS) in 1992. In necropsy, greenish brown and swollen liver with multiple white foci, enlarged spleen, and misshapen ova were observed. Bacterial agents isolated from these chickens were confirmed as *S. gallinarum* by biochemical and serological test. Since that time, FT has occurred all over the country and become the most serious problem in the chicken industries.

Every effort including vaccination, antimicrobial therapy, and probiotics administration, has been made for the control of the FT outbreaks in Korea. Antibiotics such as β-lactam

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penicillins, aminoglycoside, and fluoroquinolones have been widely used as antimicrobial therapy to control FT. However, these methods are now recognized as ineffective because of the acquired drug resistance of field strains against these antibiotics [7, 9, 11, 14].

Like the comparison on the pattern of antimicrobial resistance among field isolates, biological characteristics are useful in distinguishing various strains and analyzing an FT outbreak with an epidemiological basis. Especially, morphological, physiological and biochemical markers in conjunction with traditional serological methods have been widely employed for the typing of field isolates.

In this study, we present the biochemical characteristics and antimicrobials susceptibility of field isolates of *S. gallinarum* isolated in Korea for last 7 year (1995-2001).

Results

S. gallinarum field isolates

For this study, 258 isolates of *S. gallinarum* from chickens for 7 years from 1995 to 2001 were tested (Table 1). To identify *S. gallinarum*, C8-esterase spot reagent (Biolife, Milano, Italy) was used to select colonies producing a strong blue fluorescence under a wavelength of 366nm [15]. Selected colonies were confirmed by agglutination test using *Salmonella* O antiserum group D1 (Difco Laboratories, Detroit, MI), and by the biochemical test such as IMViC (indole, methyl red, Voges-Proskauer and citrate utilization), carbohydrate fermentation, amino acid decarboxylation and Jordan D-tartrate utilization [3, 5].

All isolates were stored in tryptic soy broth (TSB ; Biolife, Milano, Italy) containing 30% glycerol at -70°C prior to test, and were subcultured in 10 ml volumes of TSB for test.

Table 1. Number of *S. gallinarum* isolates used in this study by year

1995	1996	1997	1998	1999	2000	2001	Total
18	13	43	63	40	35	46	258

Antimicrobial susceptibility test

To investigate the susceptibility of *S. gallinarum* isolates against antimicrobial agents, disk susceptibility and minimal inhibitory concentrations (MICs) test were performed. The disk zone diameter interpretive standard and MIC breakpoints of antimicrobial agents were adapted in accordance with a principle in part from National Committee for Clinical Laboratory Standards (NCCLS) [13].

i) Susceptibility test by disk diffusion method: Antimicrobial susceptibilities were evaluated with disks containing ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), gentamicin (10 µg), kanamycin (30 µg), enrofloxacin (5 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), ofloxacin (5 µg), tetracycline (30 µg), oxytetracycline (30 µg), colistin (10 µg) and sulfamethoxazole/trimethoprim (25 µg) from SensiDisk™

(BBL, USA). Mueller Hinton agar (MHA; Biolife, Milano, Italy) was dispensed into plastic culture plate to yield a uniform depth of 4 mm. The density of all isolates tested was adjusted to 0.5 McFarland turbidity ($1\sim 2\times 10^8$ CFU/ml) using a spectrophotometer (Schimadzu, Japan), and inoculated by flooding over the agar surface. Antimicrobial disks were applied with a dispenser within 15 min., and the diameter of inhibition zone was measured after overnight incubation at 37°C (16).

ii) MICs test by agar dilution method: The MICs of antimicrobial agents were determined with drugs of ampicillin, gentamicin, kanamycin, norfloxacin, ofloxacin, colistin from Sigma (USA), enrofloxacin from Bayel in Korea, ciprofloxacin from Cheiljedang (Korea) and danofloxacin from Pfizer Inc. (USA). The antimicrobial agents were added to autoclaved MHA cooled to 50°C to the final concentration from 0.06 to 1,024 µg/ml by two fold dilution, and the medium were dispensed to 20 ml after gently mixing. The isolates suspension which was adjusted to 107 CFU/ml was applied to the surfaces of the agar plates containing a series of concentrations of antimicrobials with a steer's replicator device delivered about 3 µl of suspension. The MIC was defined as the lowest concentration of antimicrobials inhibiting visible growth after 18 hr incubation at 37°C, and the MIC₅₀ was determined as the concentration of the antimicrobials able to inhibit growth of more than 50% of isolates tested (16).

The biochemical properties of *S. gallinarum* isolates from 1995 to 2001 are shown in Table 2. A total of 258 isolates showed the same pattern in the majority of biochemical properties such as IMViC, carbohydrate fermentation and amino acid decarboxylation. But, dulcitol and trehalose were fermented by 95.4% and 93.7% of isolates, and lysin and ornithine were decarboxylated by 99.8% and 1.4%, respectively. Also, sorbitol fermentation (59.1%) and arginine decarboxylation (27.0%) showed the diversity by isolates, especially sorbitol fermentation increased annually from 11.1% in 1995 to 82.6% in 2001.

Antimicrobial susceptibility test against 258 isolates of *S. gallinarum* was performed by the disk diffusion method using 12 antimicrobial agents (Table 3). The majority of isolates was susceptible to amoxicillin/clavulanic acid (92.3~100%), colistin (97.1~100%) and sulfamethoxazole/trimethoprim (92.3~100%). All isolates from 1995 appeared to be susceptible to all of the antimicrobial agents tested except for tetracycline and oxytetracycline, whereas the vast majority of isolates from 2001 showed the reduced susceptibility to ampicillin (13.0%), gentamicin (43.4%), kanamycin (69.6%), enrofloxacin (6.5%), ciprofloxacin (10.9%), norfloxacin (52.5%) and ofloxacin (82.6%).

According to the resistance-criteria of NCCLS, 136 (52.7%) of 258 isolates of *S. gallinarum* were resistant to one or more drugs tested (Table 4), and twenty-nine resistance patterns were observed. The prevalence of completely resistant isolates against antimicrobial drugs increased from 0% in 1995 to 93.5% in 2001. Also, the resistant strains to more

Table 2. Biochemical properties of 258 isolates of *S. gallinarum* from 1995 to 2001

Tests	% of positive isolates							Total (n=258)
	1995 (n=18)	1996 (n=13)	1997 (n=43)	1998 (n=63)	1999 (n=40)	2000 (n=35)	2001 (n=46)	
Motility	0	0	0	0	0	0	0	0
Catalase	100	100	100	100	100	100	100	100
Oxidase	0	0	0	0	0	0	0	0
Indol	0	0	0	0	0	0	0	0
Methyl Red	100	100	100	100	100	100	100	100
Voges Proskour	0	0	0	0	0	0	0	0
Urease	0	0	0	0	0	0	0	0
Arginine dihydrolase*	33.3	7.7	32.6	11.1	77.5	25.7	1.2	27.0
Lysine decarboxylase	100	100	100	98.4	100	100	100	99.8
Ornithine decarboxylase*	5.6	0	2.3	1.6	0	0	0	1.4
d-Lactose	0	0	0	0	0	0	0	0
Dulcitol	83.3	84.6	100	100	100	100	100	95.4
Maltose	100	100	100	100	100	100	100	100
Trehalose*	55.6	100	100	100	100	100	100	93.7
D(+)Xylose*	100	100	100	100	100	100	100	100
D(+)Galatose	100	100	100	100	100	100	100	100
D(-)Sorbitol*	11.1	30.8	34.9	85.7	80.0	88.6	82.6	59.1
D(+)Glucose (gas)	100(0)	100(0)	100(0)	100(0)	100(0)	100(0)	100(0)	100(0)
Jordan's tartrate	100	100	100	100	100	100	100	100

n: Number. of isolates tested.

* Late (more than 3 days) reactions observed.

Table 3. Antimicrobials susceptibility of 258 isolates of *S. gallinarum* from 1995 to 2001

Antimicrobial agents	% of susceptible isolates by year						
	1995 (n=18)	1996 (n=13)	1997 (n=43)	1998 (n=63)	1999 (n=40)	2000 (n=35)	2001 (n=46)
Ampicillin	100	100	100	95.2	92.5	80.0	13.0
Amoxicillin/ clavulanic acid	100	92.3	97.7	96.8	97.5	94.3	93.5
Gentamicin	100	92.3	95.3	46.0	62.5	40.0	43.4
Kanamycin	100	92.3	100	87.3	87.5	65.7	69.6
Enrofloxacin	100	69.2	95.4	92.1	50.0	21.2	6.5
Ciprofloxacin	100	100	100	98.4	75.0	42.9	10.9
Norfloxacin	100	100	100	100	100	68.6	52.2
Ofloxacin	100	100	100	100	100	94.3	82.6
Tetracycline	16.7	7.7	9.3	19.0	25.0	17.4	63.0
Oxytetracycline	5.6	7.7	4.7	0	2.5	62.9	65.2
Colistin	100	100	100	100	100	97.1	100
Sufamethoxazole/ Trimethoprim	100	92.3	100	98.4	95.0	100	100

n: No. of isolates tested.

than one drugs appeared from 1998, and the strains with multiresistance to six and over drugs observed in 2001.

The minimal concentrations of drugs to inhibit 50% (MIC₅₀) of 227 isolates of *S. gallinarum* from 1997 to 2001

was determined by agar dilution method using 9 antimicrobial drugs (Table 5). The MIC₅₀s range of the majority of antimicrobial drugs tested increased from 0.06~8 µg/ml in 1997 to 2~256 µg/ml in 2001. Especially, MIC₅₀s for gen-

Table 4. Distribution of resistance patterns of 258 isolates of *S. gallinarum* from 1995 to 2001

Resistance patterns*	No. of completely resistant isolates by year							Total (%) (n=258)
	1995 (n=18)	1996 (n=13)	1997 (n=43)	1998 (n=63)	1999 (n=40)	2000 (n=35)	2001 (n=46)	
AM				1		3	13	17 (6.6)
AMC			1			1		2 (0.8)
ENO					2			2 (0.8)
GM		1	2	5	5	10	3	26 (10.1)
T		1	1	3				5 (1.9)
TE		2	3					5 (1.9)
AM, CIP							1	1 (0.4)
AM, ENO					1		2	3 (1.3)
AM, GM				1			4	5 (1.9)
AM, T						1		1 (0.4)
GM, CIP					1			1 (0.4)
GM, ENO						1		1 (0.4)
GM, KM						1	1	2 (0.8)
GM, TE				1				1 (0.4)
T, TE				4	2			6 (2.3)
AM, ENO, GM							1	1 (0.4)
AM, GM, KM				1				1 (0.4)
AM, T, TE						1		1 (0.4)
GM, T, TE				14	5	5	2	26 (10.1)
AM, AMC, GM, KM					1			1 (0.4)
AM, ENO, GM, KM							1	1 (0.4)
AM, ENO, GM, NOR							1	1 (0.4)
AM, GM, T, TE						2	7	9 (3.5)
ENO, GM, T, TE				1		1		2 (0.8)
GM, KM, T, TE				5	1	1		7 (2.7)
AM, ENO, GM, T, TE							5	5 (1.9)
AM, GM, SxT, T, TE				1				1 (0.4)
AM, CIP, ENO, GM, T, TE							1	1 (0.4)
AM, CIP, ENO, GM, NOR, T, TE							1	1 (0.4)
Total (%)	0 (0)	4 (30.8)	7 (16.3)	37 (58.7)	18 (13.2)	27 (77.1)	43 (93.5)	136 (52.7)

n: No. of isolates tested.

*: AM, ampicillin; AMC, amoxicillin; CIP, ciprofloxacin; ENO, enrofloxacin; GM, gentamicin; KM, kanamycin; NOR, norfloxacin; Sxt, sulfamethoxazole/trimethoprim; T, oxytetracyclin; TE, tetracyclin.

tamicin, fluoroquinolones and tetracycline of isolates from 2001 increased over 10-fold than those of isolates from 1997.

Discussion

The salmonella serovars are classified as either host-specific or non-host specific serovars depending on their host range. The non-host specific *Salmonella* serovars such as *Salmonella enteritidis* and *Salmonella typhimurium* are the agents of paratyphoid infections in domestic poultry and a major concern for food safety.

The two avian-adapted serovar *Salmonella gallinarum* (*S. gallinarum*) and *Salmonella pullorum* (*S. pullorum*) are causative organisms of fowl typhoid (FT) and pullorum disease, respectively. Both diseases have many similarities in terms of history, clinical signs, and control and eradication

procedures, while they have slightly different biochemical properties and age of host commonly infected [21].

The test of biochemical reaction is a traditional distinctive method between *S. gallinarum* and *S. pullorum*. In general, an isolate was considered as *S. gallinarum* if it fermented maltose and dulcitol, utilized Jordan's tartrate, not produced gas from glucose, and not decarboxylated ornithine [21]. But, Trabulsi and Edwards [23] demonstrated that occasional fermentation of dulcitol was produced by prolonged incubation of *S. gallinarum* isolates, and Christensen *et al* [3] reported that the ornithine was weakly decarboxylated in 4 of 43 isolates of *S. gallinarum*. Although there were some differences in dulcitol fermentation and ornithine decarboxylation among isolates tested in the present investigation, the results obtained were almost in accordance

Table 5. The MIC₅₀ of antimicrobial agents for 227 isolates of *S. gallinarum* from 1997 to 2001

Antimicrobial agents	MIC ₅₀ ($\mu\text{g}/\text{m}^{\ell}$)					
	1997 (n=43)	1998 (n=63)	1999 (n=40)	2000 (n=35)	2001 (n=46)	
β -Lactams	ampicillin	8	8	8	16	64
Amino-glycosides	gentamicin	2	4	16	512	256
	kanamycin	8	8	8	16	32
Fluoro-quinolones	enrofloxacin	0.13	0.13	1	2	2
	ciprofloxacin	0.06	0.06	0.5	1	2
	norfloxacin	0.5	0.5	2	4	8
	ofloxacin	0.13	0.13	1	2	2
	danofloxacin	0.13	0.13	1	1	2
Polypeptides	colistin	2	2	4	4	4

with the traditionally biochemical characteristics of *S. gallinarum* isolates. Interestingly, our results showed that sorbitol fermentation gradually increased from only 11.1% in 1995 to 82.6% in 2001. No matter what specific and combination of factors are involved in changing of biochemical characters of isolates, the fact remains that the phenotype of isolates has been continuously changed as time goes by.

Antimicrobial susceptibility patterns have been also changed for 7 years from 1995 to 2001. The results presented in this study showed that the susceptibility of *S. gallinarum* isolates tested to ampicillin, gentamicin, kanamycin, and fluoroquinolones have been gradually reduced from 100% in 1995 to 6.5~82.6% in 2001. The susceptibility to gentamicin, enrofloxacin and ciprofloxacin of the isolates from 2001 was only 13.0%, 6.5% and 10.9%, respectively. Park *et al* [17] reported all of the 84 *S. gallinarum* isolates from 1992 to 1996 in Korea were susceptible to gentamicin, ampicillin and kanamycin, and Oh *et al* [14] reported all 103 isolates of *S. gallinarum* from 1998 to 1999 were susceptible to enrofloxacin and ciprofloxacin, but only 48 isolates (46.6%) of them was susceptible to gentamicin. However, the susceptibility of the isolates to enrofloxacin and ciprofloxacin shown in this study has decreased since 1998.

One hundred and thirty eight of 258 isolates of *S. gallinarum* tested in this study showed resistance to one or more drugs, and the incidence of resistant isolates increased significantly from 0% in 1995 to 93.5% in 2001. Particularly, the isolates with resistance to more than one drugs were observed from 1998, and two isolates with multi-resistance to six and over drugs appeared in 2001. Oh *et al* [14] also reported that all of the 120 isolates of *Salmonella* spp. from chicken showed multi-resistance to more than one drugs, and the incidence of multi-resistant isolates to over 5 drugs was 68.3%.

The results of MIC₅₀ in this study showed that the minimal inhibitory concentrations of drugs to the isolates also had increased gradually. The MIC₅₀ of gentamicin and

fluoroquinolones to the isolates in 2001 increased over 10-fold, compared with those in 1997.

The administration of antimicrobial drugs is the most general method used in commercial farms for the prevention and therapy of FT. Especially, fluoroquinolones among them have been used widely due to the advantage of oral administration and high potency. These have been known to treat salmonellosis successfully, including infections caused by multi-resistant strains [1, 19, 20]. Fluoroquinolones resistance was rarely found among *Salmonella* species until Heisig [6] reported *S. typhimurium* serovar *Copenhagen* from cattle was highly resistant to ciprofloxacin. Thereafter, *Salmonella* species with high level of drug resistance have been continuously reported, and the exact location and changes of genes associated with a mutation of these species have been searched.

In the present study, the biochemical characteristics and antimicrobials susceptibility of field isolates of *S. gallinarum* isolated in Korea from 1995 to 2001 were investigated. Therefore, our results indicate that sorbitol fermentation (59.1%) and arginine decarboxylation (27.0%) showed the diversity by isolates and the vast majority of isolates from 2001 showed the reduced susceptibility to antimicrobials tested.

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