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Original Article

Change of serotype pattern of Group D non-typhoidal *Salmonella* isolated from pediatric patients in southern Taiwan

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Background and Purpose: Group D non-typhoidal *Salmonella* infection is increasing in Taiwan. This study aimed to investigate the changing serotypes and antibiotic resistance of childhood group D *Salmonella* infection.
Methods: From 1998 through 2004, children (<16 years) infected with group D *Salmonella* were retrospectively reviewed. Demographic data, clinical and laboratory features, and risk factors of bacteremia were analyzed. Enrolled patients were classified as acute gastroenteritis with bacteremia (Group I) and acute gastroenteritis without bacteremia (Group II). The minimal inhibitory concentrations were determined by agar dilution method. Genotyping was performed by use of pulsed-field gel electrophoresis (PFGE).
Results: Eighty one children (Group I, n = 15; Group II, n = 66) were enrolled with a mean (± standard deviation) age of 3.1 ± 2.6 years. Group I patients were younger and had a longer duration of fever prior to admission (≥5 days, 40% vs 9.2%; p=0.003) and total fever duration (8.3 vs 4.1 days, p<0.001) than Group II. *Salmonella enterica* serotype Enteritidis (80%) was the most common serotype, followed by *Salmonella* Panama (7%). The antibiotic resistance rates of *S.* Enteritidis were: tetracycline (36.5%), trimethoprim-sulfamethoxazole (25.4%), ampicillin (14.3%), and chloramphenicol (12.7%). *S.* Panama was associated with a higher rate of bacteremia. All strains were susceptible to quinolone and third-generation cephalosporins. PFGE study showed a single genotype of *S.* Enteritidis and diverse genotypes of *S.* Panama circulating in the area.
Conclusions: *S.* Enteritidis was the predominant serotype of group D *Salmonella* that caused pediatric infection in southern Taiwan during the study period from 1998 to 2004. *S.* Panama is associated with higher rates of bacteremia and antimicrobial resistance.

Key words: Bacteremia; Drug resistance; Pulsed-field gel electrophoresis, *Salmonella* enteritidis; Serotyping

Introduction

Salmonellosis is an important public heath issue worldwide [1-3]. Acute gastroenteritis is the most common clinical manifestation of *Salmonella* infection in children [4-6]. However, serious extraintestinal infections such as sepsis, meningitis, arthritis, and osteomyelitis have also been reported frequently [4-9]. Some virulent serotypes usually cause invasive infections rather than gastroenteritis [10,11]. Our previous study has shown that group D *Salmonella* infection was a risk factor of *Salmonella* gastroenteritis complicated with bacteremia compared with non-group D *Salmonella* [4]. Therefore, it is clinically important to investigate the serotypes and characteristics of group D *Salmonella* infection in children.

An increasing proportion of group D *Salmonella* isolates has been evident since the 1990s in Taiwan [12, 13]. *Salmonella enterica* serotype Enteritidis was one of the two most common types among the more than 2000 *Salmonella* serotypes in a large surveillance study in the United States [14]. However, our previous study

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showed that *Salmonella* Panama was the most common serotype of group D *Salmonella* from 1989 to 1996 [4]. It is of interest to study whether a change in the epidemiology of group D *Salmonella* infection has been occurring in southern Taiwan.

High rates of antimicrobial resistance among non-typhoidal *Salmonella* have been reported in the past two decades [13,15,16]. Furthermore, patients infected with multiply resistant strains have higher rates of bacteremia and case fatality than those infected with non-resistant strains [16,17]. This study aimed to investigate the clinical features and antimicrobial susceptibilities, and the evolution of serotypes and phenotypes of group D *Salmonella* isolated from children in a six-year period.

Methods

Patients and bacteria

Children (<16 years) visiting the Department of Pediatrics of National Cheng Kung University Hospital with non-typhoid group D *Salmonella* infections from 1998 through 2004 were recruited. The demographic data and clinical and laboratory findings were retrospectively reviewed. Enrolled patients were divided into two groups: acute gastroenteritis with bacteremia (Group I) and acute gastroenteritis without bacteremia (Group II). Acute gastroenteritis was defined by the presence of diarrhea and a positive stool culture for *Salmonella*. Bacteremia was defined by the isolation of *Salmonella* from a blood culture. The duration of fever prior to admission was defined as the period from the day of onset of fever to the day of admission. The total fever days was defined as the period from the day of onset of fever to the day of afebrile status.

All isolates were cultured and identified according to standard methods. In addition, 16 group D *Salmonella* strains cultured from 1989-1996 were used for comparison. All bacteria were frozen at –70°C until use.

Serotyping and genotyping

Serotyping was conducted as previously described by Brenner and McWhorter-Murlin [18], using a modified "paper-bridged" method [19]. Antisera (purchased from: S&A Reagents Lab Ltd. [Bangkok, Thailand]; Denka Seiken Co., Ltd. [Tokyo, Japan]; Statens Serum Institut [Copenhagen, Denmark]; and LTK Biolaboratories [Taoyuan, Taiwan]) were used in the experiments. Pulsed-field gel electrophoresis (PFGE) analysis was performed using the PulseNet protocol for *Salmonella* [20]. DNA fingerprints in PFGE gels were analyzed using Bio-Numeric software (Applied Maths, Kortrijk, Belgium). Clustering analysis of PFGE patterns was performed using the unweighted pair group with arithmetic averaging method and the Dice similarity coefficient.

Minimal inhibitory concentration

The tested antibiotics were ampicillin (Sigma, St. Louis, MO, USA), chloramphenicol (Sigma), tetracycline (Sigma), trimethoprim-sulfamethoxazole (TMP-SMX; Sigma), amoxicillin-clavulanic acid (SmithKline Beecham, Singapore), cephalothin (Sigma), cefotaxime (Sigma), ceftriaxone (Sigma), ciprofloxacin (Fluka, Switzerland), levofloxacin (Fluka), ofloxacin (Sigma), and imipenem-cilastatin (Merck Sharp & Dohme, Whitehouse Station, NJ, USA).

Antibiotics were diluted in Mueller-Hinton agar medium to obtain final concentrations ranging from 0.002 to 256 μg/mL. Bacteria were grown on media agar and harvested after 24 h of incubation, and then suspended in trypticase soy broth. Ten-fold dilutions of suspensions at a concentration equivalent to that of a McFarland 0.5 standard were used for susceptibility testing; these correspond to approximately 10^7 colony-forming units (CFU)/mL. One to two μL of each bacterial suspension was plated onto antibiotic-containing agar to obtain a final inoculum of 10^4 CFU/spot. Plates were incubated at 35°C and bacterial growth was evaluated after 24 h of incubation by comparison with the growth on drug-free agar. *Escherichia coli* American Type Culture Collection (ATCC) 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Breakpoint minimal inhibitory concentrations (MICs) used to determine susceptibility or resistance were those described by the National Committee for Clinical Laboratory Standards in 2005 [21].

Statistical analysis

Variables in Group I and II were compared using chi-squared test or Student's *t* test. A value of $p<0.05$ was considered significant. We used multivariate logistic regression to analyze the predictors of Group I patients.

Results

Demographic data and clinical features

Eighty one patients were enrolled in the study. The demographic data and clinical and laboratory findings

Table 1. Demographic data and clinical and laboratory features of patients with group D S*almonella* infection

Variable	Total (n = 81)	Group I (n = 15)	Group II (n = 66)	OR (95% CI)	*p*
Gender (male/female) [n]	29/52	3/12	26/40		0.236
Age (years) [mean ± SD]	3.1 ± 2.6	1.6 ± 1.2	3.4 ± 2.7		0.02
Fever (%)	90.0	93.3	89.4		1.0
Total fever days (mean ± SD)	4.9 ± 3.3	8.3 ± 3.0	4.1 ± 2.8		<0.001
Fever ≥5 days (prior to admission) [%]	15.0	40.0	9.2	6.6 (1.7-24.8)	0.003
Mucus in stool (%)	43.0	66.7	37.3	3.4 (1.0-11.0)	0.04
Blood in stool (%)	28.0	40.0	25.8	1.9 (0.6-6.2)	0.27
Vomiting (%)	44.0	20.0	51.5	0.2 (0.1-0.9)	0.04
Dehydration (%)	23.0	20.0	24.2	0.8 (0.2-3.1)	1.00
WBC ≥15,000/mm^3 (%)	15.2[a]	26.7	11.8[b]	2.8 (0.7-11.6)	0.21
I/N ratio ≥0.16 (%)	52.2[a]	60.0	51.9[b]	1.4 (0.4-4.5)	0.58
CRP ≥50 mg/L (%)	64.0[a]	54.5	66.0[b]	0.6 (0.2-2.3)	0.50

Abbreviations: OR = odds ratio; CI = confidence interval; WBC = white blood cell; I/N = immature/total neutrophil ratio; CRP = C-reactive protein

[a]n = 66.

[b]n = 51.

of the patients are shown in Table 1. The mean age was 3.1 ± 2.6 years (range, 0.3-12.4 years) and the male-to-female ratio was 1.7. Fifteen of the 81 patients (18.5%) had bacteremia. The most common clinical symptom was fever (90%), followed by vomiting (44%). Blood and mucus in stool were present in 28% and 43% of patients, respectively. None of these patients developed extraintestinal complications other than bacteremia.

Group I patients were younger (1.6 vs 3.3 years, p=0.02) and had a longer duration of fever prior to admission (≥5 days, 40% vs 9.2%; p=0.003) and total fever days (8.3 vs 4.1 days, p<0.001) than Group II patients. Mucus in stool was more frequently encountered (66.7% vs 37.3%, p=0.04) while vomiting was less common (20% vs 50.7%, p=0.04) in Group I compared with Group II patients. Fever ≥5 days independently predicted bacteremia in Group I patients (p=0.005; 95% confidence interval, 0.101-0.557). White blood cell count, immature/total neutrophil ratio and C-reactive protein were similar between the groups (p>0.05).

Serotyping and genotyping

The *Salmonella* serotypes between groups are shown in Table 2. *S.* Enteritidis (77.8%) was the most common serotype, followed by *S.* Panama (8.6%), *Salmonella* Victoria (3.7%), and *Salmonella* Seremban (2.5%). Six isolates (7.4%) could not be serotyped. Children infected by *S.* Panama were more likely to have bacteremia (57.1%). Serotyping of the additional 16 group D *Salmonella* strains isolated from 1990-1996 indicated that the majority were *S.* Panama (94%).

The dendrogram of 91 group D *Salmonella* strains revealed that *S.* Panama carried a diverse pattern of genotypes (10 patterns of 23 strains) in these two periods. However, the majority of *S.* Enteritidis belonged to the same genotype (54 of 61 strains) in 1998-2004.

Antimicrobial susceptibility and MICs

Antimicrobial resistance rate among different serotypes is shown in Table 3. Of the 81 isolates, the resistance rates of ampicillin, chloramphenicol, tetracycline, TMP-SMX, amoxicillin-clavulanic acid, and cephalothin were 19.8%, 14.8%, 37%, 19.8%, 4.9% and 8.6%, respectively (Table 3). None of the isolates were resistant to quinolones and extended-spectrum cephalosporins in this study. Multiply resistant (≥3 drugs) isolates accounted for 19.5% of strains and were more frequently isolated from Group I patients than those of Group II (35.7% vs 6.6%, p=0.009).

Table 2. Serotypes in pediatric group D *Salmonella* isolates

Serotype	Total	Group I	Group II	Bacteremia (%)[a]
Salmonella Enteritidis	63	8	55	12.7
Salmonella Panama	7	4	3	57.1
Salmonella Victoria	3	0	3	0
Salmonella Seremban	2	0	2	0
Unserotyped	6	3	3	50
Total	81	15	66	18.5

[a]Percentage associated with bacteremia.

Table 3. Resistance rates to 13 antimicrobial agents in serotypes of pediatric group D *Salmonella* isolates

Serotype (n)	Resistant isolates (%)											
	AM	CM	TE	TMP-SMX	AUG	CF	CTX	CRO	CIP	LVX	OFX	IMP
Salmonella Enteritidis (63)	14.3	12.7	36.5	25.4	6.3	11.1	0	0	0	0	0	0
Salmonella Panama (7)	85.7	42.9	85.7	0	0	0	0	0	0	0	0	0
Salmonella Victoria (3)	0	0	0	0	0	0	0	0	0	0	0	0
Salmonella Seremban (2)	0	0	0	0	0	0	0	0	0	0	0	0
Undefined (6)	16.7	16.7	16.7	0	0	0	0	0	0	0	0	0
Total (81)	19.8	14.8	37.0	19.8	4.9	8.6	0	0	0	0	0	0

Abbreviations: AM = ampicillin; CM = chloramphenicol; TE = tetracycline; TMP-SMX = trimethoprim-sulfamethoxazole; AUG = amoxicillin-clavulanic acid; CF = cephalothin; CTX = cefotaxime; CRO = ceftriaxone; CIP = ciprofloxacin; LVX = levofloxacin; OFX = ofloxacin; IMP = imipenem

Antimicrobial resistance differed among the individual serotypes. The resistance rates of ampicillin (85.7%), chloramphenicol (42.9%) and tetracycline (85.7%) to *S.* Panama were significant higher than for other serotypes. The resistance rates of antibiotics to *S.* Enteritidis were: ampicillin, 14.3% and chloramphenicol, 12.7%. None of the *S.* Panama isolated was resistant to TMP-SMX; in contrast, the resistance rate of TMP-SMX to *S.* Enteritidis was 25.4%. The MIC values at which 50% and 90% of isolates were inhibited for the tested strains are summarized in Table 4.

Discussion

Non-typhoidal group D *Salmonella* infection, particularly *S.* Enteritidis, has been increasing worldwide, including in Taiwan [12-14,22,23]. In addition, patients infected with group D *Salmonella* were at a higher risk of developing bacteremia or other serious extraintestinal infections [4,6]. Understanding of the characteristics of group D *Salmonella* infection and the risk factors of complications is needed. In this study, the mean age (3.1 ± 2.6 years) was older than in previous studies [4,24], which mainly involved infection by group B *Salmonella*.

Table 4. Minimal inhibitory concentrations (MICs) of the tested antibiotics against pediatric group D *Salmonella* isolates

	Range (μg/L)	MIC_{50} (μg/L)	MIC_{90} (μg/L)	Resistance (%)
AM	0.25->256	1	>256	19.8
CM	4->256	4	256	14.8
TE	1-256	1	256	37
TMP-SMX	<0.125-256	<0.125	256	19.8
AUG	0.25-256	0.25	4	4.9
CF	2->256	2	8	8.6
CTX	0.03-8	0.125	0.125	0
CRO	<0.125-8	<0.125	<0.125	0
CIP	<0.002-0.125	0.08	0.125	0
LVX	0.004-0.5	0.015	0.25	0
OFX	0.015-1	0.06	0.5	0
IMP	0.025-8	0.5	0.5	0

Abbreviations: MIC_{50} = minimal inhibitory concentration inhibiting 50% of isolates; MIC_{90} = minimal inhibitory concentration inhibiting 90% of isolates; AM = ampicillin; CM = chloramphenicol; TE = tetracycline; TMP-SMX = trimethoprim-sulfamethoxazole; AUG = amoxicillin-clavulanic acid; CF = cephalothin; CTX = cefotaxime; CRO = ceftriaxone; CIP = ciprofloxacin; LVX = levofloxacin; OFX = ofloxacin; IMP = imipenem

In southern Taiwan, the serotype of group D *Salmonella* changed from *S.* Panama in 1990-1996 to *S.* Enteritidis in 1998-2004 in our studies. In a previous report, the majority of group D *Salmonella* isolates from patients in 1978-1987 were *S.* Panama [25]; however, another study revealed that none of 249 patients had *S.* Panama isolates in 1991-1996 in the same area [26]. The increasing consumption of poultry after the outbreak of swine mouth-foot disease in 1997 may have contributed to the increase in group D isolates observed in the past decade. A recent study found that approximately 88% of broiler flocks and 49% of broilers were contaminated with *S.* Enteritidis [27]. *S.* Enteritidis displaced *S.* Panama as a leading serotype of group D *Salmonella* isolated from sporadic or endemic cases in the past decade [12,15,23]. These findings suggested that large-scale consumption of poultry changed the distribution of *Salmonella* serotypes that cause diseases in humans.

In a study analyzing food poisoning cases caused by *S.* Enteriditis in Taiwan from 1991 to 1997 by PFGE, most of the *S.* Enteritidis strains demonstrated very similar or highly related genotypes [23]. In this study, we also found that *S.* Enteritidis clinical isolates in 1998-2004 had almost the same genotype. It may imply that a single major genotype of *S.* Enteritidis was circulating in southern Taiwan during the study period. The source of *S.* Enteritidis may come from the same reservoir. In contrast, *S.* Panama isolates from

1989-1996 and 1998-2004 represented a diverse genotype pattern.

High antimicrobial resistance of non-typhoidal *Salmonella* from humans had been reported in Taiwan [16,25]. In comparison with western countries, a higher TMP-SMX resistance and lower ampicillin and chloramphenicol resistance in *S.* Enteritidis were found [28,29]. In addition, invasive *S.* Panama isolates resistant to TMP-SMX were not observed in this study. All of the isolates were susceptible to quinolones and extended-spectrum cephalosporins. It is suggested that the differences in antimicrobial resistance among different *Salmonella* serotypes can be ascribed to the different reservoirs of *S.* Enteritidis and *S.* Panama. This result may provide useful information for clinicians in choosing appropriate empirical antibiotics for the treatment of severe group D *Salmonella* infection in pediatric patients.

In conclusion, *S.* Enteritidis is the most prevalent serotype of group D *Salmonella* infection in the past six years. Fever ≥5 days was more likely to be associated with bacteremia. We found a major genotype of *S.* Enteritidis and diverse genotypes of *S.* Panama in children with group D *Salmonella* infection. The antimicrobial resistance rate of *S.* Panama was higher than that of *S.* Enteritidis, especially with regard to ampicillin and tetracycline. Most of the *S.* Enteritidis isolates were still susceptible to first-line antibiotics. Continued monitoring of antimicrobial resistance and deliberate use of antimicrobial agents in food animals and patients is essential.

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