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Clonal dissemination of the multi-drug resistant *Salmonella enterica* serovar Braenderup, but not the serovar Bareilly, of prevalent serogroup C1 *Salmonella* from Taiwan

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Abstract

Background: Nontyphoidal *Salmonella* is the main cause of human salmonellosis. In order to study the prevalent serogroups and serovars of clinical isolates in Taiwan, 8931 *Salmonellae* isolates were collected from 19 medical centers and district hospitals throughout the country from 2004 to 2007. The pulsed-field eletrophoresis types (PFGE) and antibiotic resistance profiles of *Salmonella enterica* serovars Bareilly (*S*. Bareilly) and Braenderup (*S*. Braenderup) were compared, and multi-drug resistance (MDR) plasmids were characterized.

Results: Over 95% of human salmonellosis in Taiwan was caused by five *Salmonella* serogroups: B, C1, C2-C3, D1, and E1. *S*. Typhymurium, *S*. Enteritidis, *S*. Stanley and *S*. Newport were the four most prevalent serovars, accounting for about 64% of isolates. While only one or two major serovars from four of the most prevalent serogroups were represented, four predominant serovars were found in serogroup C1 *Salmonellae*. The prevalence was decreasing for *S*. Choleraeuis and *S*. Braenderup, and S. Virchow and increasing for *S*. Bareilly. *S*. Braenderup mainly caused gastroenteritis in children; in contrast, *S*. Bareiley infected children and elderly people. Both serovars differed by *Xba*I-PFGE patterns. Almost all *S*. Bareilly isolates were susceptible to antibiotics of interest, while all lacked plasmids and belonged to one clone. Two distinct major clones in *S*. Braenderup were cluster A, mainly including MDR isolates with large MDR plasmid from North Taiwan, and cluster B, mainly containing susceptible isolates without R plasmid from South Taiwan. In cluster A, there were two types of conjugative R plasmids with sizes ranging from 75 to 130 kb. Type 1 plasmids consisted of replicons F1A/F1B, bla_{TEM}, IS*26*, and a class 1 integron

with the genes *dfrA12-orfF-aadA2-qacE*Δ1-*sulI*. Type 2 plasmids belonged to incompatibility group IncI, contained *tnpA-bla*$_{CMY-2}$-*blc-sugE* genetic structures and lacked both IS*26* and class 1 integrons. Although type 2 plasmids showed higher conjugation capability, type 1 plasmids were the predominant plasmid.

Conclusions: Serogroups B, C1, C2-C3, D1, and E1 of *Salmonella* caused over 95% of human salmonellosis. Two prevalent serovars within serogroup C1, *S*. Bareilly and cluster B of S. Braenderup, were clonal and drug-susceptible. However, cluster A of *S*. Braenderup was MDR and probably derived from susceptible isolates by acquiring one of two distinct conjugative R plasmids.

Background

Non-typhoidal *Salmonellae* are major zoonotic pathogens that commonly cause salmonellosis outbreaks. Globally, salmonellosis caused by non-typhoidal salmonellae generally results in about 1.3 billion cases of acute gastroenteritis and 3 million deaths annually [1]. In the United States, *Salmonellae* cause an estimated 1.4 million cases of salmonellosis and over 500 deaths annually [2]. Multidrug resistant (MDR) *Salmonella*, the global spread of which is mediated by international food trade and travel, is a global public health issue [3,4]. Often, clonal spread of MDR strains has been observed in particular serovars [4-6]. In most instances, resistance genes often associated with integrons and/or transposons are clustered within antimicrobial resistance islands that can be horizontally transferred by conjugative or mobilization plasmids [7].

In serogroup C1, *S*. Bareilly and *S*. Braenderup are closely related according to molecular analysis [8,9]. Both serovars have been highly susceptible to antimicrobials since 1971 [10,11] and are frequently isolated from feces of people with food-borne salmonellosis all over the world [12-16]. However, prevalence of both serovars differs between hosts and regions. In Denmark, *S*. Bareilly was isolated from diverse sources, including humans, animals and animal feed, while *S*. Braenderup was only found in humans [17]. In a study of a broiler-raising plant in the USA, *S*. Bareilly was often found in broilers and finished feed; however, *S*. Braenderup was only observed in hatcheries [18]. In addition, *S*. Braenderup was commonly isolated from cattle and turtles in Sweden [19], pigs [12] and chicken egg shells [20] in USA. These findings imply that animal reservoirs may be important sources of both serovars in human disease.

In this study, prevalent serogroups and serovars were determined for 8,931 *Salmonella* isolates collected from 2004 and 2007 in Taiwan. Because of the genetic similarity between *S*. Bareilly and *S*. Braenderup [8,9], the two serovars were compared with respect to antimicrobial resistance, resistance genes, PFGE and plasmid profiles. Both serovars disseminated clonally and varied in antimicrobial resistance patterns.

Results

Prevalent serogroups and serovars

Between 2004 and 2007, over 95% of 8,931 *Salmonella* isolates belonged to serogroups B, C1, C2-C3, D1 and E1 (Table 1). Prevalence differed between serogroups and across time within serogroups: prevalence decreased in serogroups B (46.9%→42.4%) and C1 (14.2%→9.1%) and increased in serogroups C2-C3 (9%→11.3%) and D1 (23.3%→30.2%) over the study period. Such changes were associated with the prevalence of major serovars in each serogroup and were due to only one or two main predominant serovars in each serogroup, except serogroup C1 with four prevalent serovars (Table 1). The top four serovars were *S*. Enteritidis (22.9-28.9%) of serogroup D1, *S*. Typhimurium (20.4-24.7%) and *S*. Stanley (8.2-11.4%) of serogroup B, and *S*. Newport of serogroup C2 (5.6 - 7.3%). In contrast to the decrease in prevalence of *S*. Typhimurium from 2005 to 2007, a gradual increase in prevalence was observed in *S*. Enteritidis.

Prevalence of serogroup C1 serovars

S. Braenderup, *S*. Choleraesuis, *S*. Bareilly and *S*. Virchow were the predominant serovars in serogroup C1 and consisted of 66 - 84% of total serogroup C1 isolates from 2004 to 2007 (Table 1). Other serovars, including *S*. Infantis, *S*. Potsdam, *S*. Mbandaka, and *S*. Montevideo, were occasionally isolated with prevalence less than 1% for each serovar. Over the study period, the prevalence of *S*. Choleraesuis declined dramatically, and *S*. Braenderup prevalence declined mildly. In contrast, the prevalence of *S*. Bareilly and other serovars gradually increased from 2004 to 2007. Since *S*. Braenderup and *S*. Bareilly were the two main serogroup C1 serovars in 2006-2007 and differed in prevalence trends, 45 *S*. Braenderup and 51 *S*. Bareilly isolates were analyzed for their antimicrobial resistance profiles and genetic characteristics.

Age distribution of patients

Patients infected with *S*. Braenderup and *S*. Bareilly were separated into four age groups. Although, both serovars were found primarily to infect children (age ≤ 4 years), *S*. Bareilly was isolated far more frequently from the elderly (age ≥ 50 years) (8.9% for *S*. Braenderup vs. 31.4% for *S*.

Table 1: Prevalence of *Salmonella* serogroups and their main serovars isolated from human from 2004 to 2007.

Serogroup/Serovar	Number of isolates					Prevalence (%)[2]				
	2004	2005	2006	2007	Total	2004	2005	2006	2007	Total
Serogroup B	1133	1045	938	854	3970	44.3	46.9	44.0	42.4	44.5
S. Typhimurium	571	551	441	412	1975	22.3[ab]	24.7[a]	20.7[b]	20.4[b]	22.1[ab]
S. Stanley	287	183	242	168	880	11.2	8.2	11.4	8.3	9.9
Serogroup C1	364	229	234	184	1101	14.2	10.3	11.0	9.1	11.3
S. Choleraesuis	111	65	30	17	223	4.3 (30.5)	2.9 (28.4)	1.41 (12.8)	0.84 (9.23)	2.50 (22.6)
S. Braenderup	96	46	66	32	240	3.8 (26.4)	2.1 (20.9)	3.1 (28.2)	1.6 (17.4)	2.7 (23.7)
S. Bareilly	54	41	47	54	196	2.1 (14.8)	1.8 (17.9)	2.2 (20.1)	2.7 (29.4)	2.2 (19.4)
S. Virchow	43	34	33	19	129	1.7 (11.8)	1.5 (14.8)	1.6 (14.1)	0.9 (10.3)	1.4 (12.8)
Other serovars[1]	60	43	58	62	223	2.3 (16.5)	1.9 (18.8)	2.7 (24.8)	3.1 (33.7)	2.5 (22.1)
Serogroup C2-C3	231	246	239	228	944	9.0	11.0	11.2	11.3	10.6
S. Newport	144	137	135	147	563	5.6	6.1	6.3	7.3	6.3
S. Albany	87	109	104	81	381	3.4	4.9	4.9	4.0	4.3
Serogroup D	597	550	583	609	2339	23.3	24.7	27.4	30.2	26.2
S. Enteritidis	586	543	567	582	2278	22.9[c]	24.4[bc]	26.6[ab]	28.9[a]	25.5
Serogroup E1	122	76	64	70	332	4.8	3.4	3.0	3.5	3.7
S. Weltevreden	94	61	556	62	273	3.7	2.7	2.6	3.1	3.1
Sum[3]	2447	2147	2058	1954	8736	95.6	96.3	96.6	96.5	96.3
Total *Salmonellae*	2,557	2,228	2,131	2,015	8,931					

[1]Other serogroup C1 serovars include are mainly *S.* Infantis, *S.* Potsdam, *S.* Mbandaka, and *S.* Montevideo.
[2]Numbers in parenthesis indicate the percentage of isolates of a C1 serovar over total serogroup C1 isolates.
[3]Sum is the total number of serogroup B, C1, C2-C3, D, and E isolates.
[abc]Different letters indicate significant difference between years.

Bareilly, $p < 0.05$) (Table 2). However, *S.* Braenderup was predominantly isolated from children (68.9% for *S.* Braenderup vs. 49% for *S.* Bareilly, $p < 0.05$).

PFGE phylogenetic analysis

The clustering analysis of *Xba*I-digested PFGE patterns demonstrated genetic differences between *S.* Braenderup and *S.* Bareilly and within each serovar (Figure 1). In *S.* Braenderup, all isolates were separated into 2 clusters (I and II) at S = 0.68. Most isolates belonged to cluster I, which was further separated into two subgroups (A and B) at S = 0.84 (Figure 1A). In cluster A, 19 isolates were separated into 9 PFGE patterns, and 78.9% (15/19) of the isolates were from northern Taiwan (Figure 1A). In cluster B, 25 isolates were grouped into 4 PFGE patterns, and 72% (18/25) of the isolates were from southern Taiwan (Figure 1A). *S.* Bareilly isolates were highly genetically homogenous and shared more than 90% pattern similarity (Figure 1B).

Table 2: Age prevalence of patient infected by *S.* Bareilly and *S.* Braenderup

Serovar	Rate (%) of each age group			
	0 ~ 4	5 ~ 12	13 ~ 50	> 50
S. Bareilly	49.0[b] (25/51)	9.8 (5/51)	9.8 (5/51)	31.4[b] (16/51)
S. Braenderup	68.9[a] (31/45)	8.9 (4/45)	13.3 (6/45)	8.9[a] (4/45)

[ab]Different letters indicate significant difference between *S.* Bareilly and *S.* Braenderup ($P_p<0.05$).

Antimicrobial resistance profiles

Among six traditional antibiotics tested, *S.* Braenderup and *S.* Bareilly isolates were almost all susceptible to chloramphenicol (CHL; 6.7% for *S.* Braenderup vs 0% for *S.* Bareilly) and kanamycin (KAN; 4.4% vs 0%) and differed significantly in resistance to ampicillin (AMP, 37.7% for *S.* Braenderup vs 0% for *S.* Bareilly), nalidixic acid (NAL; 0% vs 15.7%), streptomycin (STR, 37.7% vs 15.7%), and tetracycline (TET; 33.3% vs 0%) (Figure 1). Additionally,

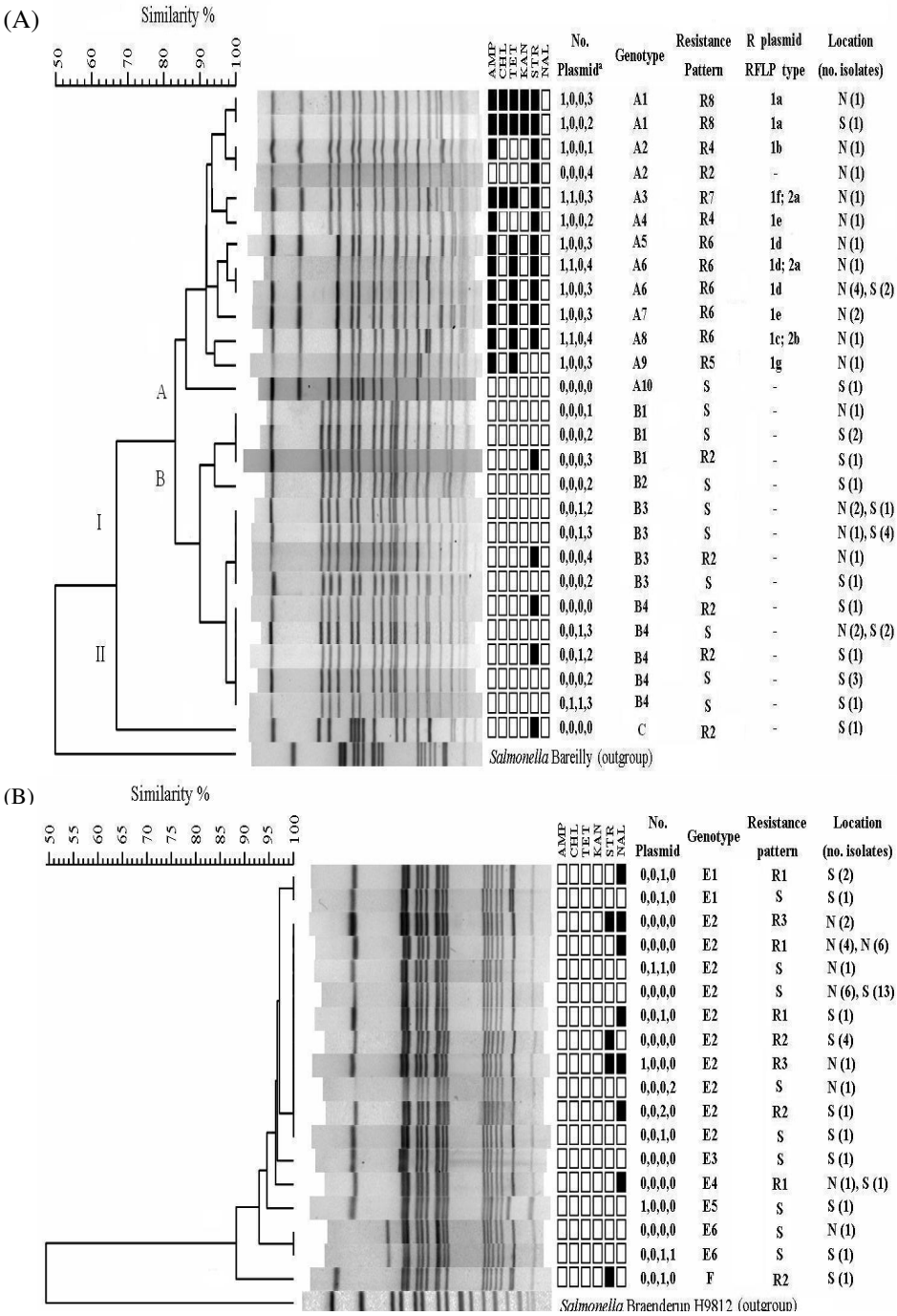

Figure 1

Dendrograms were constructed by PFGE-*Xba*I patterns to determine the genotypes for *S*. Braenderup (A) and *S*. Bareilly (B) with corresponding information including the number and size of plasmids, PFGE subtypes, antimicrobial resistance patterns and collection location of each isolate. The dendrograms were generated by the unweighted pair group method with arithmetic mean (UPGMA) using the Dice-predicted similarity value of two patterns. The BioNumerics version 4.5 statistics program was used with settings of 1.0% optimization and 0.7% tolerance. Symbols of black square and white square represent resistant and susceptible respectively. Plasmids were separated into four groups by size. Ex, 1, 1, 1, 3 indicates that this strain harbored 6 plasmids, one is >90 kb, one is from >50 to <90 kb, one is from >6.6 to <50 kb, and three are <6.6 kb.

Table 3: Characteristics of MDR plasmids from 17 *S.* Braenderup isolates.

Strains	Plasmid RFLP profile	Antibiogram[1]	Antimicrobial resistance gene			Plasmid size (kb)	Conjugation rate	Inc[3]	Class I integron	IS26	Month of isolation	Number of isolates
			aadA2	*blaTEM*	*blaCMY-2*							
S. Braenderup 2	1a	ACKTSSxt	+	+	-	137.4	4.22×10^{-6}	F1A/1B	+	ND	2004.8	2
E. coli/p2		ACKSxtT								+		
S. Braenderup 96	1a	ACKSSxtT	+	+	-	137.4	6.04×10^{-6}	F1A/1B	+	ND	2004.8	
E. coli/p96		ACKSxtT								+		
S. Braenderup 24	1b	ASSxt	+	+	-	122.6	8.25×10^{-6}	F1A/1B	+	ND	2004.8	1
E. coli/p24		ASxt								+		
S. Braenderup 87[4]	1d	ASSxtT	+	+	-	102.5	--	F1A/1B	+	ND	2004.7	7
E. coli/p30		ASxt								+		
S. Braenderup 12	1e	ASSxtT	+	+	-	99.1	-	F1A/1B	+	ND	2005.4	3
E. coli/p12		ASxt								+		
S. Braenderup 11	1g	ASxtT	-	+	-	104.4	-	F1A/1B	-	ND	2005.1	1
E. coli/p11		ASxt								+		
S. Braenderup 13		ACSSxtT	+	+	+				+	ND	2004.7	
E. coli/p13-1		A	-	-	+	75.5	8.41×10^{-2}	IncI1	-	-		1
E. coli/p13-2	1f	ACSxtT	+	+	-	127.8	-	F1A/1B	+	+		1
S. Braenderup 32		ASSxtT	+	+	+				+	ND	2005.9	
E. coli/p32-1	2a	A	-	-	+	75.5	8.66×10^{-2}	IncI1	-	-		1
E. coli/p32-2	1d	ASxt	+	+	-	102.5	ND	F1A/1B	+	+		1
S. Braenderup 36		ASSxtT	+	+	+				+	ND	2005.5	
E. coli/36-1	2b	A	-	-	+	85	1.28×10^{-1}	IncI1	-	-		1
E. coli/p36-2	1c	ASxt	+	+	-	104.8	-	F1A/1B	+	+		1

[1]Abbreviation: A, ampicillin; C, chloramphenicol; K, kanamycin; S, streptomycin; Sxt, trimethoprim-sulfamethoxazole; T, tetracycline.
[2]ND, not determined; +, conjugative; -, .non-conjugative.
[3]Inc, plasmid incompatibility group.
[4]Other 6 isolates 30 from 2005/2, 31 from 2004/10, 35 from 2005/7, 37 from 2005/3, 44 from 2004/6, and 82 from 2004/7 were not tested for conjugation.
[5]Other 2 isolates 15 from 2005/5 and 21 from 2004/9 were not tested for conjugation.

nine resistance patterns were determined, ranging from susceptibility to all antimicrobials to resistance to four antimicrobials. In *S*. Braenderup, 7 resistance patterns (S, R2, R4 to R8) were found, and significant differences were observed between cluster A (patterns R2, R4-R8) and B (patterns S and R2) for AMP (77.3% vs 0%), STR (63.6% vs 13%) and TET (54.5% vs 13%). In addition, most isolates in cluster A were MDR (73.7%) while most isolates in cluster B were susceptible (84%). In cluster A, pattern R6 (AMP, TET, and STR) was the predominant and was found in four genotypes (A3, A5, A6, and A7). In *S*. Bareilly, most isolates were either susceptible (S pattern; 52.9%) or resistant to one (pattern R1 and R2; 31.4% and 9.8%, respectively) or two (pattern R3; 5.9%) antimicrobials. NAL resistant isolates were found in *S*. Bareilly (patterns R2 and R3) but not in *S*. Braenderup. Since there were susceptible to levofloxacin (LEV) and moxifloxacin (MOX), NAL resistance may result from a mutation in the *gyrA* gene, which encodes a subunit of the enzyme DNA gyrase.

Characterization of MDR plasmids

The prevalence of plasmid profile determined by plasmid number and size differed between these two serovars. Most *S*. Braenderup isolates [93.3%, (42/45)] carried plasmids, while few *S*. Bareilly isolates [23.5 % (12/51)] did (Figure 1). Plasmids larger than ca.75 kb were only found in resistance isolates of cluster A with the R4 to R8 patterns. Cluster B *S*. Braenderup isolates and *S*. Bareilly isolates carried smaller plasmids with the size smaller than 6.6 kb or lacked plasmids. Larger plasmids were further identified as R plasmids by analysis of the antimicrobial resistance profiles of *E. coli* pir116 transformants, and assigned to type 1 and 2 based on *Hin*dIII-restriction patterns (Table 3, Figure 2). Further conjugation, antibiotic resistance and PCR characterization of incompatibility and *oriT* types, mobile element IS*26*, class 1 integron, and AMP resistance genes bla_{TEM} and bla_{CMY-2} were performed for these two plasmid types. Type 1 plasmids were separated into 7 subtypes (1a ~1g) based on differences in plasmid size ranging from 99.1 kb to 137.4 kb and restriction pattern. All plasmids carried bla_{TEM}, replicons F1A and F1B, IS*26*, and a class 1 integron (Additional files 1 and 2: Figure S1 and S2) with a gene cluster of *dfrA12-orfF-aadA2-qacE*Δ1-*sulI*, conferring resistance to trimethoprim-sulfamethoxazole (Sxt) and disappearing in plasmid 1 g (Table 3), which apparently coincides with that in the plasmid of *S*. Typhimurium (Accession number AB365868). The size of R plasmid was associated with antimicrobial resistance and conjugation capability (Table 3). Only type 1a plasmids, with a size of 137.4 kb and conferring resistance to AMP, CHL, KAN, Sxt and TET, and 1b plasmids, with a size of 122.6 kb and encoding resistance to AMP and Sxt, were capable of conjugation, with efficiencies ranging 4.22 ~ 8.25 × 10^{-6}. The other

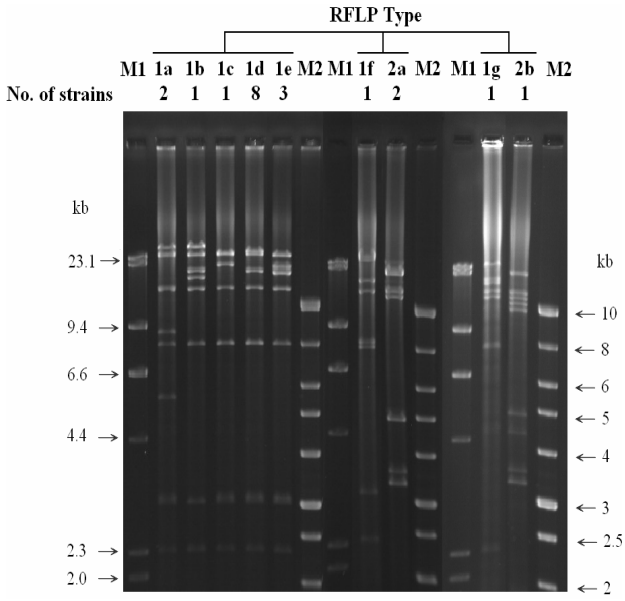

Figure 2
***Hin*dIII-digested RFLP profiles of ampicillin resistance plasmids in *S*. Braenderup isolates**. M1: *Hin*dIII-digested lambda DNA size marker. M2: 1 kb size marker.

smaller plasmids, with sizes ranging from 99.1 kb to 104.8 kb and encoding resistance to AMP and Sxt for 1c-1e and 1g, and to AMP, CHL, Sxt and TET for 1f, were not capable of conjugation. Due to differences in plasmid size and since IS*26* could be involved in plasmid transposition and recombination, we performed PCR amplification with the IS26 in primers and IS26out primers for all type 1 plasmids (Figure 3). In contrast to a 1.1-kb PCR product in the largest 1a plasmid, 1b, 1d, and 1e plasmids lacked any PCR products; 1e and 1g plasmids presented 3.1 kb PCR products; and 1c plasmid yielded two PCR products with sizes of 3.1 kb and 0.7 kb. These results suggest that the number of IS*26* and/or distance between two IS*26* elements differed among these type 1 plasmids. In contrast to type 1 plasmids, type 2 plasmids were much smaller in size (77.5 kb and 85 kb) and had higher conjugation efficiencies, ranging from 8.41 × 10^{-2} to 1.28 × 10^{-1} (Table 3). In addition, type 2 plasmids were the Inc*I1* plasmid and contained *oriT* as well as *tnpA-*bla_{CMY-2}*-blc-sugE* (Table 3, Additional files 3: Figure S3).

Discussion

Human salmonellosis was limited to five *Salmonella* serogroups: B, C1, C2-C3, D1, and E1 (Table 1). Despite the decrease in prevalence of *S*. Typhimurium and the increase in the prevalence of *S*. Enteritidis from 2005 to 2007, serogroups B and D *Salmonellae* were the major pathogens for foodborne salmonellosis in Taiwan due to

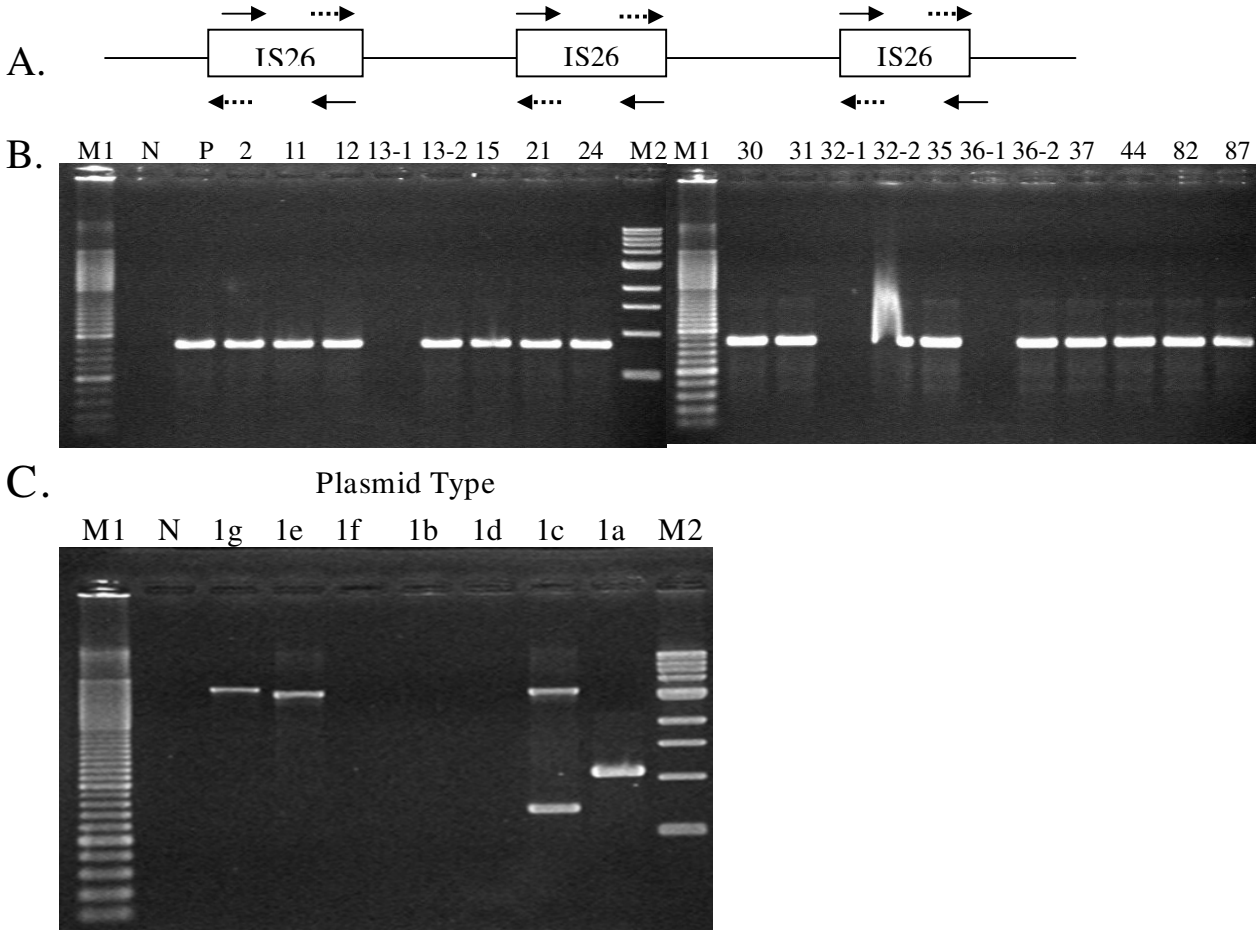

Figure 3
PCR amplification of IS26 and IS26-associated DNA fragments. (A) Primer design. Symbols of arrow and dashed arrow represent IS26in primers and IS26out primers, respectively. (B) PCR products amplified by IS26in primers. (C) PCR products amplified by IS26out primers. M1: 100-bp size marker. N: negative control. M2: 1-kb size marker.

S. Typhimurium, *S*. Stanley, and *S*. Enteritidis of serogroup D1 being the three most prevalent serovars overall. Although the prevalence of serogroups C1 and C2-C3 were similar (around 11%), 4 prevalent serovars and 2 main serovars were found in serogroup C1 and serogroup C2-C3, respectively. In the present study, a shift in prevalence was observed in these four prevalent serogroup C1 serovars: a rapidly decrease in the prevalence of *S*. Choleresuis, mainly due to enhancement of sanitation and control of swine in Taiwan, and an increase in prevalence of *S*. Bareilly and other serovars (Table 1). Compared to the 1.6% increase in the prevalence of *S*. Braenderup from 1978 to 1987 in southern Taiwan [21], the change in the prevalence of isolates in this study ranged from 1.6% to 3.8%, with a trend of decrease from 2004 to 2007, except an increase of *S*. Braenderup infection in 2006 (Table 1), suggesting possibly occurrence of outbreaks in this year.

Contrary to earlier reports that *S*. Bareilly and *S*. Braenderup are closely related genetically [8,9], resistant to 10 *Salmonella* bacteriophages [22], and infect immunocompromised patients, differences between *S*. Braenderup and *S*. Bareilly were found in the prevalence trend from 2004 to 2007 (Table 1), patients' age group (Table 2), and plasmid profile as well as antimicrobial resistance groups and *Xba*I-PFGE patterns (Figure 1A). In addition to genetic differences between these two serovars, differences in animal hosts were also observed in both serovars based on the geographic regions from which they were isolated [13,17,18,23]. In this study, we found that *S*. Bareilley isolates were highly homogeneous genetically and that *S*. Braenderup isolates were much diverse in our PFGE and plasmid analysis (Figure 1). This may explain why *S*. Braenderup, but not *S*. Bareilly, has been frequently reported [19,20,24]. To differentiate *S*. Braenderup, sev-

eral molecular methods have been developed, including phage typing [25] and plasmid analysis as performed in this study (Table 1, Figure 1 and 2).

Unlike MDR *S*. Choleraesuis isolated from pigs and humans [5,6], *S*. Braenderup and *S*. Bareilly isolated from pigs were highly susceptible to antibiotics in 1971 [10]. In addition, in a study of resistance to 11 antibiotics for *Salmonella* isolated from turtles, *S*. Bareilly was still susceptible to all antibiotics, and, in contrast, few *S*. Braenderup isolates were resistant to gentamycin (6/15), sulfisoxazole (6/15) and TET (2/15) [11]. In our study, almost all of the cluster A isolates of *S*. Braenderup were MDR and associated with large MDR plasmids (Table 3, Figure 1). Although RFLP analysis separated type 1 plasmids into 7 subtypes, based on antimicrobial resistance encoded by these plasmids, 3 subtypes were observed, conferring resistance to AMP and Sxt (1b-1e and 1g), AMP, CHL, Sxt, and TET (1f) and AMP, CHL, KAN, Sxt and TET (1a), respectively (Table 3). Apparently, the *dfrA12-orfF-aadA2-qacE*Δ1-*sulI* region of class 1 integrons, which is frequently found in MDR *Salmonella* [26-28], was located on MDR plasmid and conferred resistance to Sxt (Table 3). Insertion sequence IS*26* existed in all (Table 3) and differed from plasmids in *S*. Braenderburg found in Spain [29]. The size change in type 1 plasmids may be due to presence of multiple IS*26* elements that may be involved in plasmid rearrangement (Figure 3).

Although conjugation capability of type 2 plasmids was higher than that of type 1 plasmids, we only identified coexistence of type 1 and 2 plasmids in three *S*. Braenderup isolates, which differed in isolation day and PFGE pattern (Table 3). Isolate 13 with type 1f and 2a plasmids was collected in July of 2004 from Taipei. Isolate 32 with type 1d and 2a plasmids and isolate 36 with 1c and 2b plasmids were collected in March and May of 2005, respectively, from Taichung (Table 3). Only one isolate 44 with a type 1d plasmid was collected before those three isolates, in June of 2004 from Taichung. These results suggest possibly that isolate 32 with A6 genotype and R6 resistance pattern may be derived from isolate 44 with a type 1 plasmid, A4 genotype and R6 resistance pattern by introduction of a type 2 plasmid. Interestingly, type 2 plasmids are Inc*I1* plasmids, carrying the *tnpA-bla*$_{CMY-2}$*-blc-sugE* structure (Table 3). AmpC β-lactamases are broadly distributed among the *Enteribacteriaceae*, and plasmid-mediated AmpC β-lactamases include ACC, ACT, CFE, CMY, DHA, FOX, LAT, MIR, and MOX [30]. At least three transposase associated genetic structures for *bla*$_{CMY}$ include IS*Ecp1-bla*$_{CMY-2}$*-blc-sugE*, *ISCR1-bla*$_{CMY-9}$*-γqgF-γqgE* and *IS26-frdC-frdD-ampR-bla*$_{CMY-13}$*-blc-sugE-IS26* [30]. Recently, *bla*$_{CMY}$ has been shown to be broadly spread in *Salmonella* worldwide [29,31,32] and to be present in *S*. Braenderup [33]. In Taiwan, since we reported the *tnpA-bla*$_{CMY-2}$*-blc-sugE* structure in *S*. Choleraesuis in 2004 [34], this transposon-like element has been found in other *Salmonella* serovars and *Enterobacteriaceae* [32]. In the present study, we first reported that *S*. Braenderup harbors *tnpA-bla*$_{CMY-2}$*-blc-sugE* on a type 2 plasmid. Comparing this plasmid with the 138-kb plasmid pSC138 (accession no. NC_006856) of *S*. Choleraesuis, both are Inc*I1* plasmids with the *tnpA-bla*$_{CMY-2}$*-blc-sugE* structure. However, type 2 plasmids were conjugative and much smaller in size due to lack of a 60-kb DNA region with multiple integrons and transposons, which carry MDR genes [35-37].

Conclusions

Over 95% cases of human salmonellosis surveyed in this study were caused by 5 *Salmonella* serogroups: B, C1, C2, D1, and E1. As two prevalent serogroup C1 serovars, *S*. Braenderup and S. Bareiley differed in patients' age groups and *Xba*I-PFGE patterns. Both serovars were clonally disseminated and drug-susceptible. However, in *S*. Braenderup, cluster A MDR isolates were derived from susceptible isolates by sequential introduction of two distinct R plasmids. Type 1 plasmids carry *bla*$_{TEM}$, F1A/F1B replicons, insertion sequence IS*26*, and a class 1 integron with a gene cluster comprised of *dfrA12-orfF-aadA2-qacE*Δ1-*sulI*. In contrast, type 2 plasmids consist of Inc*I1* replicon and *tnpA-bla*$_{CMY-2}$*-blc-sugE*. Although type 2 plasmids showed higher conjugation capability, type 1 plasmids were the predominant plasmids responsible for MDR dissemination in *S*. Braenderup.

Methods

Bacterial isolates

Salmonella isolates were collected from 19 medical centers and district hospitals located throughout Taiwan from 2004 to 2007. Serotypes of the isolates were determined in the *Salmonella* Reference Laboratory of Centers for Disease Control (CDC), Department of Health, Taiwan, with antisera purchased from S&A Reagents Lab (Bangkok, Thailand), Denka Seiken (Tokyo, Japan), Statens Serum Institut (Copenhagen, Denmark), and a local biotech company, LTK Biolaboratories (Taoyuan, Taiwan). Phase induction was performed using a paper-bridged method developed by the Taiwan CDC [38]. In total, 51 *S*. Bareilly isolates and 45 *S*. Braenderup isolates collected in 2004 and 2005 were selected for further characterization. Isolates were separated into two groups based on their geographic origin: the north Taiwan group, consisting of isolates collected from north of Taichung county (including Taichung county), and the south Taiwan group, consisting of isolates collected from south of Taichung county.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion method in accordance with the guide-

lines of the CLSI standards [39] with 7 antibiotics: ampicillin (AMP, 50 μg), chloramphenicol (CHL, 20 μg), kanamycin (KAN, 30 μg), streptomycin (STR, 10 μg), tetracycline (TET, 12 μg), trimethoprim-sulfamethoxazole (Sxt, 23.75/1.25 μg), and quinolone antibiotics including nalidixic acid (NAL, 30 μg), levofloxacin (LEV, 5 μg) and moxifloxacin (MOX, 5 μg). The antimicrobials were purchased from BD (Becton Dickinson and Company, Sparks, Maryland, USA). *Escherichia coli* ATCC 25922 was used as the reference strain. An MDR isolate was defined as having resistance to three or more antibiotics belonging to different antibiotic classes.

Pulsed-field gel electrophoresis (PFGE)

The PulseNet Standardized Laboratory PFGE Protocol for Molecular Subtyping of *Echerichia coli* O157:H7, non-typhoidal *Salmonella* serotypes, and *Shigella sonnei* [40] was used for analysis of the *Salmonella* isolates: 10 U of *Xba*I were used for the restriction digestion. PFGE images were analyzed by using the fingerprint analysis software BioNumerics version 4.5 (Applied Maths). A unique PFGE pattern was defined as one or two DNA bands differing between PFGE patterns of two isolates. A dendrogram was generated by the unweighted pairgroup method with arithmetic mean (UPGMA) algorithm using the Dice-predicted similarity value of two *Xba*I-digested PFGE patterns.

Plasmid profile analysis

Plasmid profiles of each isolate were determined by the Kado and Liu method [41], and plasmid size was estimated by comparison with the plasmids of two *S*. Choleraesuis strains: OU7085 (50 kb and 6.6 kb) and OU7526 (50 kb and 90 kb).

Restriction fragment length polymorphism (RFLP) and antibiotic susceptibility analysis of the MDR-plasmid

Large plasmids (> 50 kb) of 17 AMP and STR-resistant *S*. Braenderup isolates were characterized. Plasmid DNA was purified from resistant wild-type isolates by the alkaline lysis method [42] and then transformed into the competent *E. coli* strain pir116 (STR^R), which was prepared by the $CaCl_2$ method. Transformants were selectively grown on LB agar plates supplemented with AMP (100 μg/ml) and further tested for resistance to CHL, TET, and KAN, but not for resistance to STR, since the recipient strain was inherently resistant to streptomycin. The antibiotic resistance genes bla_{TEM}, *aadA*, and bla_{CMY-2}, class 1 integron as well as the insertion sequence IS*26* and its related DNA fragments were amplified using the primers listed in Table 4. The genes bla_{SHV} and $bla_{CTX-M3\ and\ M14}$ were also detected by the multiplex method [43]. The R-plasmids of each transformant were purified by use of the Geneaid Plasmid Midi Kit (Geneaid, Taiwan) and were digested with *Hin*dIII (New England Biolabs, USA) to determine similarity. Plasmid DNA fragments were separated by electrophoresis through a 0.6 % SeaKem GTG agarose gel (Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) at 25 V for 16 h. The PCR product of class 1 integron was purified by DNA Clean/Extraction kit (GeneMark, Taiwan) and sequenced by Mission Biotech co. (Taiwan).

Table 4: The PCR primers for PCR and size of PCR products

Primer	Target	DNA sequence (5' to 3')	Product Sizesize	Note
Tem-F	bla_{TEM}	GAAGATCAGTTGGGTGCACGAGT	550 bp	This study
Tem-R		CAACTTTATCCGCCTCCATCCAGT		
STR-F1	*aadA2*	AGACGCTCCGCGCTATAGAAGT	203 bp	(46)
STR-R1		CGGACCTACCAAGGCAACGCT		
CS-F	*CS region*	GGCATCCAAGCAGCAAG	Variable	(47)
CS-R		AAGCAGACTTGACCTGA		
1.9CS-F	*Flanking region of CS region*	CTGCTGCGTAACATCGTTGCT	Variable	This study
1.9CS-R		GGCGAGATCATCAAGTCAGT		
ColE1-F	ColE1 *ori*T	CAAATGCTGTCCTTCCAGTGT	225 bp	This study
ColE1-R		CTCAGTTCGGTGTAGGTCGT		
F-F	Inc*FI ori*T	CAACAACGCGCCGACACCGT	288 bp	This study
F-R		CCCTTCCTGTCGACGCTTCT		
R100-F	Inc*F2 ori*T	CCACCAAAAGCACCACACACT	266 bp	This study
R100-R		AGACACTCCTAGCAGCGCCT		
pSC138-F	Inc*I ori*T	TGTCACGAACATCTGCCAGT	193 bp	This study
pSC138-R		GAGAGAAAGTGCCCATGGCT		
IS26in-F	*IS26*	GGCACTGTTGCAAAGTTAGC	820 bp	DQ390455.1
IS26in-R		GGCACTGTTGCAAATAGTCG		
IS26out-F	Variable	GCTAACTTTGCAACAGTGCC	Variable	DQ390455.1
IS26out-R		CGACTATTTGCAACAGTGCC		
Tn-F	Tn	ACCTAGATTCTACGTCAGTAC	Variable	(35)
AmpC-F	AmpC	CAAGTTTGATTCCTTGGACTCT		AY253913
AmpC-R		CTCATCGTCAGTTATTGCAGCT		
SugE-R	*sugE*	GCCTGATATGTCCTGGATCGT		

Plasmid conjugation and incompatibility group

Transferability of R plasmids from each RFLP group was determined by performing the conjugation test following a previously described method [44] with NAL-resistant *S*. Typhimurium LBNP4417 as the recipient strain. Briefly, 0.6 ml of overnight culture of donor strain was mixed with 1 ml of the overnight recipient strain. Then 1 ml fresh LB broth was added, and the mixture was incubated at 37°C with shaking at 100 rpm for 4 h. The bacterial solution was diluted at 10^1, 10^3, and 10^5 times with LB broth, and then 100 μl of the diluted solution was plated on MacConkey agar supplemented with AMP (100 μg/ml) and/or NAL (15 μg/ml). Conjugation efficiency was calculated by determining the number of transconjugants relative to the total number of recipients. Four primer sets were used to amplify the *oriT* regions of the ColE1, F (IncFI), R100 (IncFII), and pSC138 (Inc*I1*-like) plasmids (Table 1). In addition, replicon types of these resistant plasmids were determined as described by Carattoli et al. [45].

Statistical analysis

The difference in the antimicrobial resistance rates between two serovars was analyzed by the independent t test. P values of < 0.05 were considered significant.

List of abbreviations

AMP: ampicillin; CDC: Center for Disease Control; CHL: cloramphenicol; KAN: kanamycin; LEV: levofloxacin; MDR: multi-drug resistance; MOX: moxifloxacin; NAL: nalidixic acid; PCR: polymerase chain reaction; PFGE: pulsed-field gel electrophoresis; QRDR: quinolone resistance determining region; RFLP: restriction fragment length polymorphism; STR: streptomycin; Sxt: trimethoprim-sulfamethoxazole; TET: tetramycin; UPGMA: unweighted pairgroup method with arithmetic mean.

Authors' contributions

CC designed, instructed and supervised most aspects of this project. CSC did PFGE analysis and prepared the manuscript. JML and SWC performed the experiments and data analysis. CHC, BCW and JGT assisted in the design of the study and helped to prepare the manuscript. CLC, CHC, and CHL gave useful comments and critically read the manuscript. YFC edited and revised the manuscript. All authors read and approved the final manuscript.

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Additional material

Additional file 1

Electrophoretic pattern of 1.9 kb PCR products of CS region amplified from type 1 plasmids*. All type 1 plasmids consisted of CS region, except type 1 g and 2 plasmids.*
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Additional file 2

Electrophoretic profile of inverted PCR products of CS-flanking region amplified from type 1 plasmids*. Inversed PCR of CS flanking region amplified same PCR products from all type 1 plasmids, except those plasmid that did not show any PCR product of CS region.*
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Additional file 3

PCR amplification of plasmid-mediated **tnpA-bla$_{CMY-2}$-blc-sugE** ***genetic structure of type 2 plasmids****. All type 2 plasmids consisted of* tnpA-bla$_{CMY-2}$-blc-sugE *genetic structure.*
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[http://www.biomedcentral.com/content/supplementary/1471-2180-9-264-S3.PDF]

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