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ORIGINAL ARTICLE

Reduction of *Salmonella enterica* serovar Choleraesuis carrying large virulence plasmids after the foot and mouth disease outbreak in swine in southern Taiwan, and their independent evolution in human and pig

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Background/Purpose: *Salmonella enterica* serovar Choleraesuis (*S.* Choleraesuis) is a highly invasive zoonotic pathogen that causes bacteremia in humans and pigs. The prevalence of *S.* Choleraesuis in man has gradually decreased since the outbreak of foot and mouth disease in pigs in 1997 in southern Taiwan. The goal of this study was to investigate the change in prevalence of *S.* Choleraesuis carrying the virulence plasmid (pSCV) in human and swine isolates collected in 1995—2005 and characterize these.
Methods: 380 isolates were collected from human and swine blood samples. Large pSCVs were determined by PCR and Southern blot analysis. Antimicrobial susceptibility and resistance genes, and the phylogenetic association of these large pSCV were analyzed.
Results: The number of isolates harboring the large pSCV was significantly reduced, and their prevalence differed between human and swine isolates. These large pSCVs were a recombinant

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of original 50-kb pSCV and R plasmid. In addition, some large pSCVs lacked two pSCV-specific deletion regions from *pef* to *repC* and from *traT* to *samA*. These large pSCVs carried the resistance genes bla_{TEM}, *aadA2*, and *sulI*, as well as class I integrons of 0.65 and/or 1.9 kb in size, but were inconjugatible. Phylogenetic analysis demonstrated that the large pSCV evolves independently in human and swine isolates.
Conclusion: S. Choleraesuis with large pSCV was significantly reduced after the foot and mouth disease outbreak and may evolve in human and swine specific isolates.
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Introduction

Of more than 2500 *Salmonella enterica* serovars, only serovars Abortusovis, Abortusequi, Choleraesuis, Dublin, Enteritidis, Gallinarum or Pullorum, Sendai, and Tyhimurium a harbor virulence plasmid (pSCV), which encodes an 8-kb spv operon[1] and ranges from 50 kb in S. Choleraesuis to 285 kb in S. Sendai.[2] Functions of the pSVs are associated with genes on the plasmid and include serum resistance,[3,4] replication in spleen, proinflammatory response and engulfment of spleen, and inhibition of the lymphocyte to react with T cell mitogen.[5] In addition, the S. Typimurium antigen is involved in regulation of IL-12 p40 synthesis,[6] survival in the macrophage, and virulence.[1,7–11]

Recently, recombinant virulence plasmids have been reported in S. Choleraesuis, S. Dublin and S. Typhimurium by introduction of antimicrobial resistance genes, the replicon, and type IV secretion system[12–16]. S. Choleraesuis was the second most prevalent serovar causing salmonellosis in man in 1991—1996 in Taiwan. Since foot and mouth disease in pigs occurred in 1997, S. Choleraesuis infection in man has reduced considerably.[17,18] However, multi-drug resistant (MDR) S. Choleraesuis has emerged by gene mutation, and introduction of R plasmid and integron.[12,19–22]

Genomic analysis suggests that S. Choleraesuis evolves independently in man and pig.[23] With the emergence of S. Choleraesuis carrying large drug-resistant pSCVs with bla_{TEM} for ampicillin and *sulI* for sulfonamide resistance[3] and the occurrence of foot and mouth disease in pigs, we investigated 380 isolates collected from humans and pigs with bacteremia to understand the prevalence of S. Choleraesuis carrying large drug-resistant pSCVs between human and swine isolates, and the antimicrobial susceptibility and resistance genes, class I integron, and phylogenetic relationship of these large pSCVs.

Methods

Bacterial sources and identification

In total, 380 S. Choleraesuis isolates were investigated and included 196 human and 184 swine blood isolates kindly provided by Chiayi Chang-Gung Memorial Hospital, CDC, Animal Technology Institute Taiwan, and Animal Disease Control Center of Chiayi County. Information on most isolates was published in 2005[21,23]. All isolates were further identified in our laboratory by O- and H-antigen agglutination tests and routine culture on XDL agar and LB broth at 37°C.

Antimicrobial susceptibility and resistance genes

The disk diffusion method and the guidelines of CLSI standards were used to determine susceptibility to ampicillin (AMP, 10 μg), chloramphenicol (CHL, 30 μg), kanamtcin (KAN, 30 μg), nalidixic acid (NAL, 12 μg), gentamicin (G, 30 μg), streptomycin (STR, 10 μg), tetracycline (TET, 30 μg)[24]. *Escherichia coli* ATCC 25922 was used as the reference strain to validate the results of the antibiotic susceptibility. Disks were from Becton Dickinson (Sparks, Maryland, USA).

Primers used for detection of CS region and *aadA*, bla_{PSE}, bla_{TEM}, *floR*, *sulI*, and *tet* genes are listed in Table 1. PCR conditions and reagents were described previously[25]. Whole bacteria and purified virulence plasmid DNA were used as DNA template for PCR amplification. PCR products were separated in 2% agarose gel and visualized under UV illumination. PCR products were purified and sequenced.

Plasmid profile and characterization of virulence plasmid

Plasmid number and size were determined by the Kado-Liu method[26] using the standard plasmids of OU7085 (6.6 kb and 50 kb) and OU7526 (50 kb and 90 kb). Virulence plasmid was determined by PCR amplification of *spvC* and Southern blotting analysis with the PCR product of *spvC* as probe. Specific deletions of original 50-kb pSCV were determined by PCR amplification with the primers H4-pefF (F) and RepB3 (R) for the *pefD-repC* deletion and Esam (F) and TraT4(R) for the *traT-samA* deletion.[12]

All large virulence plasmids were purified by Geneaid Plasmid Midi Kit (Geneaid Biotech Ltd., Taipei, Taiwan) and then were transformed into *E. coli* strain pir116. Ampicillin or chloramphenicol transformants with large pSCVs were selected. These large pSCVs were purified by Geneaid Plasmid Midi Kit and digested by restriction enzyme *Hin*dIII. The digested DNA fragments were separated by 0.8% GTG agarose and transferred onto Zetaprobe membrane (BioRad, Hercules, CA, USA). The whole plasmid of the 50-kb pSCV of isolate CN36, large 90-kb pSCV of isolate CN29, and non-pSCV of isolate K12 were used as probes for Southern blotting analysis. The phylogenetic relations of the large pSCVs were constructed by Bio-Profil software (Vilber Lourmat Deutschland GmbH, Eberhardzell,

Table 1 Primers used in this study

Primers	Genes	Sequences (5′ → 3′)	Product size (bp)	Note
SPVC-1	*spvC*	GAGGCGCTGGATGTGCCTGACT′	450	Marker for virulence plasmid
SPVC-2		GAACGTCTGACTCAGGACACTGT		
H4-pefF	*pefD-repC*	CGAACAGGGTGATGAATGAGAT	859	Specific deletion region of pSCV
RepB3		CGGCGTGAAAGAGCGCATGATGAT′		
Esam	*traT-samA*	CCCGATAGCCCTGACGAAGAAT	1800	Specific deletion region of pSCV
TraT4		TGTGCTCATCGAACCACACCCT		
Pse-F	*pse*	GGCTCAATACGGTCTAGACGAGT	156	Ampicillin resistance
Pse-R		GGCAATCACACTCGATGATGCGT		
STR-F	*addA2*	AGACGCTCCGCGCTATAGAAGT	203	Streptomycin resistance
STR-R		CGGACCTACCAAGGCAACGCT		
FloR-F	*floR*	CTTTGGCTATACTGGCGATG	257	Chloramphenicol resistance
FloR-R		GATCATTACAAGCGCGACAG		
SulI-F	*sul1*	CGGATCAGACGTCGTGGATGT	355	Sulfamethoxazole resistance
SulI-R		TCGAAGAACCGCACAATCTCGT		
TETG-F	*tetG*	AGCAGCCTCAACCATTGCCGAT	391	Tetracycline resistance
TETG-R		GGTGTTCCACTGAAAACGGTCCT		
TEM-F	bla_{TEM}	GAAGATCAGTTGGGTGCACGAGT	550	Ampicillin resistance
TEM-R		CAACTTTATCCGCCTCCATCCAGT		
5′-CS	*Int*	GGCATCCAAGCAGCAAG	Variable	Integron
3′-CS		AAGCAGACTTGACCTGA		

Germany). In addition, the large pSCV of the transformants were tested for their conjugatibility based on the methods described previously.[27]

Statistical analysis

The SAS Chi-square test (2008) was used to investigate the differences in the prevalence of *S*. Choleraesuis carrying large pSCV between two different factors (hosts and periods) and Student's *t* test was performed to analyze the differences between the two hosts and two periods individually.

Results

Determination of large pSCVs

Among 380 *S*. Choleraesuis isolates, 83.7% (318/380) consisted of at least two plasmids in both human and swine isolates (Table 2). Southern blot and PCR analysis showed that 99.2% (377/380) isolates consisted of the pSCV, which was separated into two plasmid types: the original 50-kb pSCV and the >50-kb recombinant pSCV (Table 2, Fig. 1). In addition, *S*. Choleraesuis isolates with single pSCV were found to be more prevalent in human than swine samples

Table 2 The plasmid profiles of 196 human and 184 swine isolates

Plasmid profile (kb)	Sources	No. of isolates	Spv		Size of pSCV (kb)
			PCR	Southern	
Single plasmid					
50 kb	Human	14	14	14	50
	Swine	3	3	3	
>50 kb	Human	30	28	28	> 50
	Swine	14	13	13	
At least two plasmids					
50 + other plasmids	Human	147	147	147	50
	Swine	166	166	166	
>50 + other plasmids	Human	4	4	4	>50
	Swine	1	0	0	
No plasmids	Human	1	0	0	
	Swine	0	0	0	
Total	Human	196	193	193	
	Swine	184	183	183	

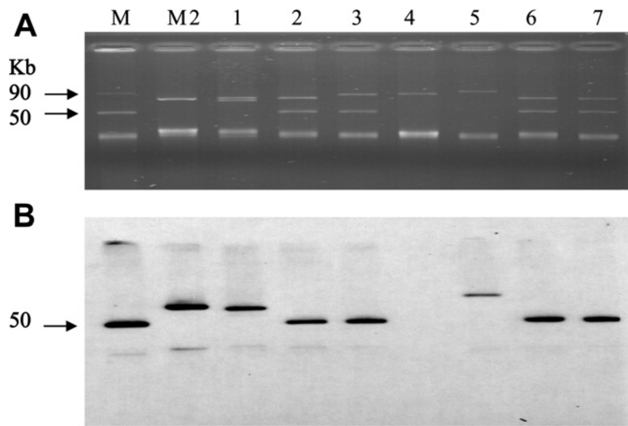

Figure 1. Plasmid profile (A) and Southern blot analysis (B) of the virulence plasmids of *S.* Choleraesuis isolates. M: *S.* Choleraesuis OU7526 containing 50-kb pSCV and 90-kb R plasmid; M2: *S.* Dublin; lanes 1–7: *S.* Choleraesuis isolates SCB103, SCB113, SCB114, SCB115, SCB117, SCB123, and SCB124.

(Table 2). In contrast to 330 isolates carrying 50-kb pSCV, only 45 isolates harbored the large pSCV, and there was a significant difference in prevalence between human and swine isolates (Table 2 and 3).

In 1997–1998, occurrence of foot and mouth disease severely damaged pig production in Taiwan. A significant decrease was found in the prevalence of isolates carrying large pSCV between the two periods for human isolates (41.67% before 1998 vs. 14.67% after 1997, $p < 0.05$) and swine isolates (34.38% before 1998 vs. 1.31% after 1997; $p < 0.05$) (Table 3). After foot and mouth disease, strains with the large pSCV were found to be significantly higher in prevalence in human than swine isolates (14.67% in human vs. 1.31% in swine isolates, $p < 0.05$).

Characterization of the large pSCV

Apart from those isolates reported in 2001,[2] 33 isolates carried large pSCVs ranging from 60 to >140 kb in size and were resistant at least to AMP, CHL, or TET (Table 4). Because competent *E. coli* pir116 is resistant to STR, presence of STR resistance on the pSCVs was determined by PCR amplification of the *aadA* gene, which was found in all but one large pSCV isolate (Table 4). Furthermore, these large pSCVs contained the antimicrobial resistance genes bla_{TEM} and *sulI*, and 0.65-kb and/or 1.9-kb CS regions, which included the *sat* and *aadA-dhfr-aadA2* genes, respectively. Previously, we determined two deletions in *pef*D-*rep*C and *traT-samA* regions in 50 kb-pSCV.[12] Here, PCR examination of these large pSCVs found that pB22, pB25, pB82, pCN28, and pCN29 lacked the *pef*D-*rep*C deletion region, pCY10 and p1030 lacked the *traT-samA* deletion region, and pMS19147 was without both deletion regions (Table 4).

After digestion with *Hin*dIII, 50-kb pSCV were separated into six major fragments: 15.9-kb H1 (*par*A-*incR-tlp*A-*spv-RAB*), 12.2-kb H2 (*rsd-rck-repFIB-pefAC*), 11.8-kb H3 (partial *repFIIA-finO-traX-traT-samA*), 3.8-kb H4 (*pef*D-*orf5-rck-*

Table 3 The prevalence of *S.* Choleraesuis harboring large virulence plasmids between human and swine isolates collected from research institutions in different periods[1]

Years*	% (prevalence) of							
	Human isolates at			Swine isolates at				Total average
	CGU	CDC	Average	CDC	Chiayi	ATIT	Average	
Before 1998	41.67% (5/12)	0% (0)	41.67%[a] (5/12)	75% (6/8)	0% (0)	20.83% (5/24)	34.38%[a] (11/32)	36.36%[x] (16/44)
After 1997	12.00% (18/150)	26.47% (9/34)	14.67%[b,x] (27/184)	0.75% (1/134)	5.56% (1/18)	0% (0)	1.31%[b,y] (2/152)	8.63%[y] (29/336)
Average	14.20% (23/162)	26.47% (9/34)	16.32%[x] (32/196)	4.93% (7/142)	5.56% (1/18)	0% (0)	7.07%[y] (13/184)	11.84% (45/380)

[a,b] means significant difference ($p < 0.05$) between two periods in same source determined by Student's *t* test.
[x,y] means significant difference ($p < 0.05$) between sources in same period determined by Student's *t* test.
[1] CGU = Chiyi Chang-Gung Hospitals; CDC = Center of Disease Control; Chiayi = Animal Disease Control Center of Chiayi County; ATIT = Animal Technology Institute Taiwan.
* Chi-square test was first performed to analyze the differences between isolates x years (2 × 2 Table).

Table 4 Characterization of the large virulence plasmids of *S.* Choleraesuis isolates

Name of pSCV	Sources	Plasmid size (kb)	Antibiogram	Deletion region[1]		bla_{TEM}	*aadA2*	*sulI*	CS region (kb)[2]	
				A	B				1.9	0.65
pB10	Human	90–140	AT	+	+	+	+	+	+	−
pB130	Human	90–140	AT	+	+	+	+	+	+	−
pB22	Human	140	ACKT	−	+	+	+	+	+	−
pB24	Human	> 140	ACKT	+	+	+	+	+	+	−
pB25	Human	140	C	−	+	+	+	−	−	+
pB30	Human	> 140	ACKT	+	+	+	+	+	+	−
pB46	Human	90–140	ACT	+	+	+	+	+	+	−
pB52	Human	90–140	ACKT	+	+	+	+	+	+	−
pB56	Human	> 140	AKT	+	+	+	+	+	+	−
pB63	Human	> 140	AKT	+	+	+	+	+	+	−
pB74	Human	140	ACKT	+	+	+	+	+	+	−
pB75	Human	90–140	AK	+	+	+	+	+	+	−
pB76	Human	90–140	AT	+	+	+	+	+	+	−
pB82	Human	90–140	ACT	−	+	+	+	+	+	−
pB85	Human	140	ACKT	+	+	+	+	−	−	+
pB97	Human	> 140	ACT	+	+	+	+	+	+	−
pB103	Human	50–90	AC	+	+	+	+	−	−	+
pC05	Human	50–90	ACT	+	+	+	+	−	−	−
pCN28	Swine	> 90	CT	−	+	+	+	−	−	+
pCN29	Swine	> 90	CT	−	+	−	+	−	−	+
pCN30	Swine	50–90	C	+	+	−	+	−	−	+
pCN31	Swine	> 90	CT	+	+	−	+	−	−	+
pCN32	Swine	> 90	C	+	+	−	+	−	−	+
pCN656	Human	> 90	T	+	+	−	+	+	−	−
pCY10	Human	90–140	CT	+	−	−	+	−	−	+
pK123	Human	50–90	AC	+	+	+	+	−	−	−
pK27	Human	> 90	CT	+	+	−	+	+	+	−
pC34	Chicken	> 90	ACKT	+	+	+	+	+	+	−
pK45	Human	> 90	ACK	+	+	+	+	+	+	−
pMS18403	Human	> 90	ACT	+	+	+	+	−	−	+
pMS19147	Human	90	CT	−	−	−	+	+	+	−
pMS22872	Human	> 90	T	+	+	−	−	−	−	−
P1030	Human	50–90	ACT	+	−	+	+	−	−	

[1] A specific deletion of pSCV in *pefD-repC* region for A and *traT-samA* region for B.
[2] 1.9 kb CS region consists of genes *aad-dhfr-aadA2*; 0.65 kb consists of *sat* encoding streptothricin acetyl-transferase.

partial repFIIA), 3.7-kb H5 (*spvCD*), and 2.6-kb H6 (*samB-parBS*).[12,28] DNA fragment profiles of *Hin*dIII-digested large pSCVs showed that changes in fragment size were mostly found in the larger H1–H3 fragments and few in smaller H4–H6 fragments (Fig. 2A). The change in the fragment size or absence of any one of these six fragments suggests the possible recombination between 50-kb pSCV and R plasmid in this fragment.

Using the 50-kb pSCV of CN36, R plasmid from isolate K12, and large pSCV of CN29 as probes, Southern blot analysis demonstrated that all large pSCVs were recombinants of the 50-kb pSCV and R plasmids. However, we did not observe any conjugatibility of these large pSCVs. The phylogenetic relations of the large pSCVs showed that the plasmid may evolve in man and pig independently because we observed that all large pSCVs of all human isolates belong to Cluster A in contrast to Cluster B for all swine isolates (Fig. 2B).

Discussion

The highly invasive and narrow-host *S.* Choleraesuis can transmit between man and pig to cause bacteremia in both hosts and appears to evolve independently in these species.[22,23] In an early study of human isolates collected in 1996–1997, we reported recombination or integration of 50-kb pSCV and R plasmid to form large recombinant pSCVs of 75–140 kb[12], which can increase the plasmid stability and host fitness under antimicrobial stress. Thereafter, an increase in isolates carrying large pSCVs could be observed. However, the prevalence of isolates harboring large pSCVs significantly decreased in human and swine isolates after the foot and mouth disease outbreak (Table 3), which led to the sacrifice of nearly 4 million pigs in 1997 in Taiwan. With reduction in infection sources from pig to human, the prevalence of *S.* Choleraesuis infection in humans decreased from 2003 to 2007.[17] However, isolates carrying

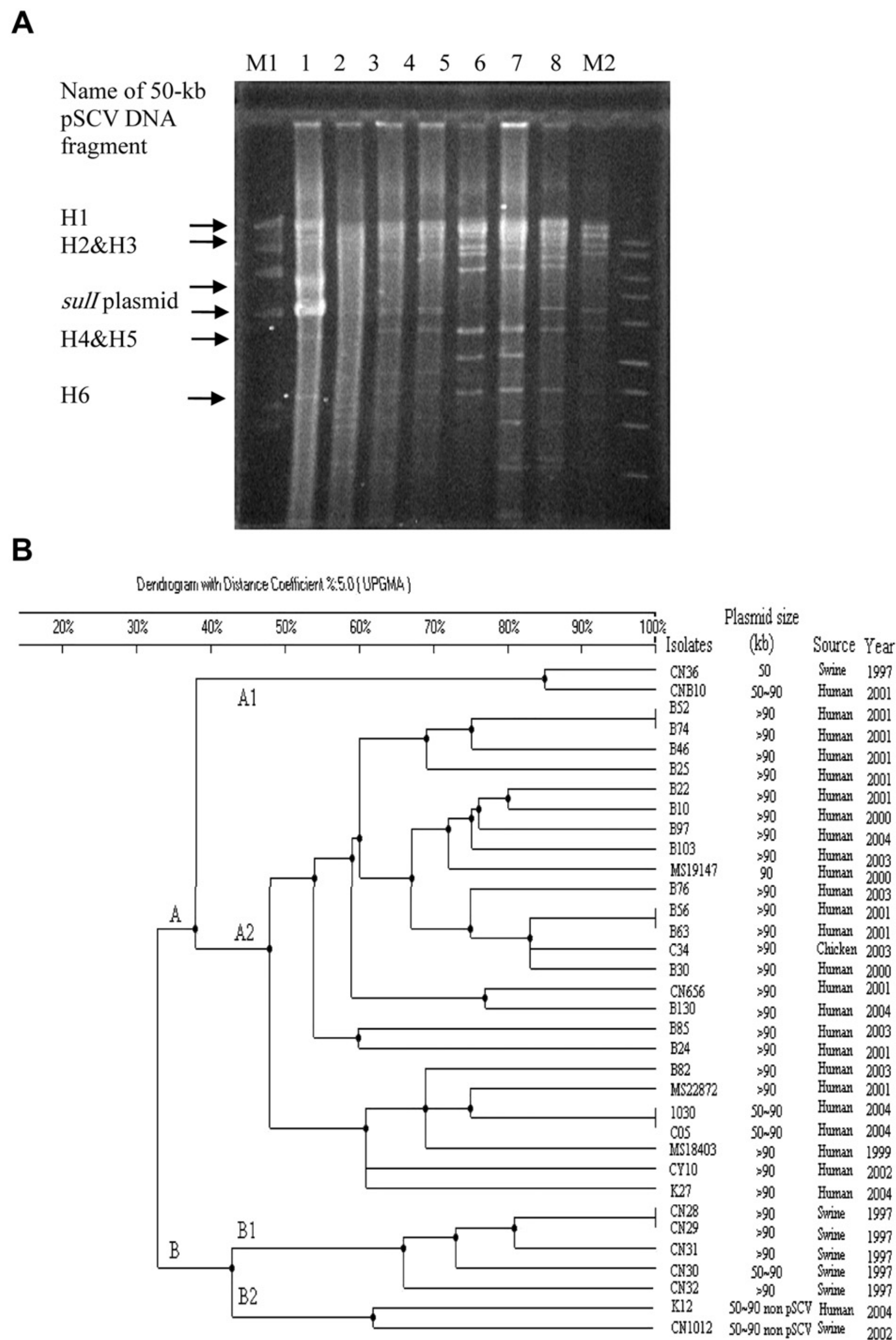

Figure 2. Restriction fragment length polymorphism profiles of representative large virulence plasmids (pSCV) of S. Choleraesuis (A) and phylogenetic tree of 31 pSCVs (B). (A) Large pSCVs were digested with restriction enzyme *Hin*dIII and then separated in 0.8% agarose at 30 V for 12 hours. M1: *Hin*dIII-digested λDNA. Lane 1: OU7085 containing 50-kb plasmid with six *Hin*dIII fragments, and one 6.6-kb *sulI* plasmid. Lanes 2–8 are isolates 10, 56, 63, CN30, CN31, 25, and KC34. M2: 1 kb size marker. (B) The phylogenetic tree of 31 large pSCV of S. Choleraesuis from different sources was constructed by Bio-Profil software. H: human isolates, S: swine isolate, C: chicken isolate as control.

the large pSCVs were more prevalent in human than swine isolates (Table 3). Phylogenetic analysis of all large pSCVs revealed independent evolutionary origins for human and swine isolates (Fig. 2B) and confirms that S. Choleraesuis evolves in man and pig independently.[23] In addition, this dramatic decrease of the isolates carrying large pSCV seems to be associated with the reduction in the prevalence of human S. Choleraesuis infection,[17] implying that large pSCV may play an important role in infection in man.

Analysis of *Hin*dIII-digested patterns of 31 large pSCV demonstrated diverse recombination sites, mostly located in the large H1, H2, and H3 fragments, which consist of *repA*/*repC*, *pef* operon, *traT*–*samB* regions, and *tlpA* and *rlgA* genes. A gene located in the 5’ of the *pef* operon and

rlgA encodes a probable site-specific recombinase.[28] The recombinant large pUO-StVR2 of *S.* Typhimurium showed common variations in IncFIB/repA2, *rck* and the *pef* operons,[29] Additionally, p9131 revealed the *rck*∼*srgC* region as a recombination hotspot.[15] In the present study, our results also demonstrated eight large pSCVs lacking either *pefD-repC* or *traT-samA* deletion regions (Table 4), suggesting that the flanking areas of these two regions may be hotspots for recombination.

In contrast to 314 isolates harboring the original 50-kb pSCV and an extra R plasmid, we only obtained 45 isolates that carried large pSCV with considerable genetic changes, and these were significantly reduced in prevalence in human and swine isolates after the foot and mouth disease outbreak (Tables 2, 3 and 4). These results imply the clonal dissemination (Fig. 2B) or plasmid conjugation of the large pSCV. However, 50-kb pSCV lacks the *oriT* and is unable to effect conjugation or mobilization[27]. It is possible that large pSCV can become conjugatible through recombination with R plasmid as has occurred in *S.* Typhimurium.[15] However, we did not find that any large pSCVs were conjugatible, indicating the R plasmids may be inconjugatible or have lost their conjugability during recombination.

Similar to the earlier report,[12] this study observed the presence of bla_{TEM} and *sul* on the large pSCV (Table 4). The gene *sulI* was located in 1.9-kb class I integron containing *aadA-dhfr-aadA2,* which was also identified in the R plasmid of *S.* Choleraesuis[25,26] and the recombinant plasmids of *S.* Typhimurium.[14,16,30] Host bacteria carrying this large multidrug-resistant pSCV can increase their fitness in an antimicrobial environment. Because of the existence of antidote system genes, the *ccdAB* operon on the pSCVs ensures stable plasmid existence in their host serovar.[28,31,32] Although antimicrobial stress frequently induces the emergence of the recombinant drug-resistant plasmids, these recombinant plasmids are not stable and evolve more quickly to cause more variations in plasmids such as deletions when the stress is removed. With similar evolutionary origins in the present study (Table 4, Fig. 2), these large pSCV changed considerably in size, CS region, and antimicrobial resistant genes indicating that recombination can recur in the R plasmid to remove the drug-resistance gene.[33,34] Because of the relatively small number before 1998, the possibility of sampling bias could not be ruled out and the representative sampling may not be good enough to indicate which *S.* Choleraesuis isolates were carrying large pSCV in that period.

In conclusion, a reduction of isolates carrying large pSCV was observed after foot and mouth disease in pig and may be associated with decrease in prevalence of *S.* Cholearesuis in humans. These large pSCVs were multi-drug resistance and differed in restriction fragment length polymorphism pattern between human and swine isolates.

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