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ORIGINAL RESEARCH

Antimicrobial Resistance and Molecular Characterization of *Salmonella* Rissen Isolated in China During 2008–2019

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Background: This study aimed to provide epidemiological features of *Salmonella enterica* serovar Rissen, determine antimicrobial susceptibility, virulence gene profiles, and describe the potential association of *S.* Rissen from different sources in China.

Methods: During 2008–2019, a total of non-repetitive 228 *S.* Rissen isolates were collected from human, animals and environment in China. The antimicrobial susceptibility test, screening of antimicrobial and virulence genes by PCR, and pulsed-field gel electrophoresis (PFGE) were performed.

Results: Among the 154 isolates from human, the majority of the cases (80.5%) occurred in summer, and *S.* Rissen was mainly detected in people aged 21–40 (37.7%) and 41–60 (28.6%) years old, and 74 non-human source *S.* Rissen strains were identified, with pork being the most common source. About 93.4% isolates were resistant to at least one of the 12 tested antimicrobial agents, and high frequencies of resistance were observed for tetracyclines (91.2%), trimethoprim-sulfamethoxazole (74.1%) and ampicillin (67.5%). A total of 171 (75%) isolates were resistant to at least three categories of antimicrobials, and the most common resistance profile was Tetracycline(s)-β-Lactams-Sulfonamides. The resistance rates to chloramphenicol, quinolones and sulfafurazole were significantly higher in strains isolated from human compared to non-human source strains. Among these isolates, the β-Lactams resistance was mainly associated with gene bla_{TEM} (54.7%), sulfonamide resistance with *sul2* (45.7%) and *sul3* (54.3%), tetracycline resistance with *tetA* (81.3%). All the isolates harbored virulence genes *hilA, sopB, sciN, stn* and *ssrB*, and most of them harbored *ssaQ* (98.7%), *mgtC* (98.7%) and *invA* (98.2%). The majority (91.7%) of *S.* Rissen isolates showed high similarity (>80%) with each other in PFGE patterns and came from human, animals and environment.

Conclusion: The high frequencies of multidrug resistance and probable clonal dissemination in this serovar call for the necessity of systematic surveillance on *S.* Rissen in China.

Keywords: *Salmonella* Rissen, antimicrobial susceptibility test, resistance genes, virulence genes, pulsed-field gel electrophoresis

Introduction

Salmonella, an important foodborne pathogen causing gastroenteritis in the human population, is responsible for about 93.8 million cases and at least 155,000 deaths worldwide each year.[1] Over 2600 serovars of *Salmonella* have been reported, among which several serovars can cause human infections.[2] *Salmonella enterica* serovar Rissen (*S.* Rissen) is considered one of the most common serovars in pork production systems and can be transmitted to humans through food or water contamination.[3,4] Apart from pork, *S.* Rissen was also isolated from other food associated poultry and seafood.[5,6] *S.* Rissen has been known to cause outbreaks and sporadic cases of human infections.[7,8]

The emergence and spread of antimicrobial resistance (AMR) in salmonella pose a serious threat to human and animal health. The profiles of antimicrobial resistance and virulence genes played a crucial role in *Salmonella*

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surveillance and clinical therapy. The antimicrobial susceptibilities of *S.* Rissen were diverse in different geographic locations. In Thailand, over 90% of isolates were resistant to ampicillin, sulfisoxazole, tetracycline and streptomycin, while in the USA, about half of the *S.* Rissen isolates were pan-sensitive.[9] In a survey concerning the multidrug resistance patterns of emerging *S.* Rissen along the Food Chain in China, most of the isolates were resistant to tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, streptomycin, sulfisoxazole, and ampicillin.[10] Limited data on the virulence profiles of *S.* Rissen are available around the world. Pulsed-field gel electrophoresis (PFGE) typing, was considered to be a "gold standard" tool to investigate outbreaks and perform epidemiological surveys and has already been used to discover the common origin of *S.* Rissen isolates across a long geographic distance.[11]

Herein, the objective of this study was to provide epidemiological features of the infections caused by *S.* Rissen, to determine antimicrobial susceptibility, virulence gene profiles, and to disclose the potential relatedness of *S.* Rissen isolated from humans, food associated with poultry and animals, and environment in China.

Materials and Methods

Collection of S. rissen Strains

A total of 228 non-repetitive strains of *S.* Rissen were isolated from six provinces (Guangxi, Guangdong, Fujian, Hubei, Hebei and Shanxi) and one municipality (Shanghai) in China during 2008–2019. The 154 isolates were obtained from anal swabs or feces of human aged from 6 months to 81 years, and the 74 isolates were from foods (animals) and river/lake. The isolation of *Salmonella* was performed following the Standard ISO-6579 and the procedure recommended by the World Health Organization.[12,13] The suspected *Salmonella* isolates were confirmed using API identification kits (bioMe´rieux, Marcy l'Etoile, France). Serotyping of the *S. Rissen* was carried out according to the White–Kauffmann–Le Minor scheme by slide agglutination with O and H antigen specific sera (Bio-Rad, Marnes-La Coquette, France; Staten Serum Institute, Copenhagen, Denmark; Sifin, Berlin, Germany) by the Shanghai Municipal Center for Disease Control (Shanghai, China).[14]

Antimicrobial Susceptibility Testing

Fourteen antimicrobials were used, including gentamicin (GEN), streptomycin (STR), ampicillin (AMP), amoxicillin-clavulanic acid (AMC), cefoxitin (FOX), ceftiofur (EFT), ceftriaxone (CRO), azithromycin (AZM), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), sulfafurazole (SIZ), trimethoprim-sulfamethoxazole (SXT) and tetracycline (TET). MICs were determined by broth microdilution using dehydrated panels CMV3AGNF (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Resistance was defined using CLSI 2023 criteria, except for STR and EFT, for which there are no clinical breakpoints. The reference strain, *E. coli* ATCC 25922 was used as a quality control strain for determining the MIC of the antimicrobial agents.

Detection of Resistance-Associated Genes and Virulence Genes

β-lactamase associated genes (bla_{TEM}, bla_{OXA}, and bla_{SHV}), sulfonamides-resistance associated genes (*sul1, sul2, sul3*, and *dfr*), tetracycline resistance-related genes (*tetA* and *tetB*), aminoglycosides-resistance associated genes (*strA, strB*, and *aadA*), chloramphenicol-resistance associated genes (*cat*), class 1 integron family (*intl1, intl1 VR(cx)* and *qacED1*), and 12 virulence genes (*invA, hilA*, ssaQ, *ssrB, mgtC, spi4D, sopB, sciN, safB, viaB, stn* and *pltA*) were screened by PCR using suitable reported primers.[15] PCR products were sequenced using a DNA analyzer (Applied Biosystem, Foster City, CA, USA). The obtained sequences were analyzed by comparison with the National Centre for Biotechnology Information (NCBI) database sequences using the BLAST program. Each run included appropriate positive and negative controls.

Pulsed-Field Gel Electrophoresis (PFGE)

The genomic DNA of *Salmonella* Rissen isolates and standard marker *Salmonella enterica* serovar Braenderup strain H9812 were embedded in 1% SeaKem Gold agarose and digested with 50 U of restriction enzyme *Xba*I (TaKaRa, Dalian, China) for 2 h. The samples of digested genomic DNA were run on CHEEF Mapper system (Bio-Rad, Hercules, CA) from 2.16 to 63.8 seconds for 19 h at 14°C. PFGE images were analyzed with BioNumerics software package 6.5 (Applied Maths, Kortrijk, Belgium) using the unweighted pair group method and an arithmetic average (UPGMA) clustering algorithm.

Results

Epidemiology of *S*. rissen in Patients

A total of 228 strains of *S*. Rissen were collected, with the largest number of strains collected in East China (Shanghai, n = 122; Fujian, n = 3), followed by South China (Guangxi, n = 80; Guangdong, n = 15), North China (Shanxi, n = 4; Hebei, n = 2), and Central China (Hubei, n = 2). 154 *S*. Rissen isolates were collected from humans, of which 51.9% were females (Table 1). The human source *S*. Rissen isolates were collected from hospitals. The prevalence of *S*. Rissen has increased in Shanghai from 2012 to 2014. Out of the cases, 47.4% showed symptoms of gastroenteritis infection, including one case that occurred in an outbreak. The majority of the cases (80.5%, 124/154) occurred from July to September. *S*. Rissen was mainly detected in people aged 21–40 (n = 58, 37.7%) and 41–60 (n = 44, 28.6%) years old. A total of 74 non-human source *S*. Rissen strains were identified (Table 1). About 74.3% of the isolates come from foods of animal origin, and 24.3% of the isolates come from environmental samples. The most common source was pork (43.2%, 32/74), followed by rivers/lakes (24.3%, 18/74).

Antimicrobial Resistance

Of the 228 *S*. Rissen isolates, 93.4% (213/228) isolates were resistant to at least one of the 12 tested antimicrobial agents. The results in Table 2, showed that *S*. Rissen isolates revealed high resistance to tetracycline (91.2%, 208/228), trimethoprim-sulfamethoxazole (74.1%, 169/228) and ampicillin (67.5%, 157/228). Likewise, the resistance to sulfafurazole was 41.2%, chloramphenicol (39.5%), ciprofloxacin (24.1%), amoxicillin-clavulanic acid (22.8%), nalidixic acid

Table 1 The Epidemiological Characteristics of Human Source and Non-Human Source *Salmonella* rissen Isolates

Category	Subcategory	No. (%)
Human source (n=154)		
Age, years	≤1	26 (16.9)
	2–5	7 (4.5)
	6–14	2 (1.3)
	15–20	5 (3.2)
	21–40	58 (37.7)
	41–60	44 (28.6)
	61–80	11 (7.1)
	≥81	1 (0.6)
Gender	Male	74 (48.1)
	Female	80 (51.9)
Month	January-March	2 (1.3)
	April-June	11 (7.1)
	July-September	124 (80.5)
	October-December	17 (11)
Symptom	Asymptomatic	81 (52.6)
	Diarrhea	73 (47.4)
Non-human source (n=74)		
Sample source	Pork	32 (43.2)
	Beef	1 (1.4)
	Mutton	1 (1.4)
	Chicken	12 (16.2)
	Duck	3 (4.1)
	Marine fish	1 (1.4)
	Shellfish	3 (4.1)
	Feed	2 (2.7)
	Snail	1 (1.4)
	Rivers/lakes	18 (24.3)

Table 2 The Antimicrobial Susceptibility Profiles of Human Source and Non-Human Source *Salmonella* rissen Isolates

Antibiotics	All Sources (n=228)			Human Source (n=154)			Non-Human Source (n=74)		
	S	I	R	S	I	R	S	I	R
β-Lactams									
Ceftriaxone	99.1%	0.0%	0.9%	99.4%	0.0%	0.6%	98.6%	0.0%	1.4%
Cefoxitin	64.9%	25.9%	9.2%	57.8%	30.5%	11.7%	79.7%	16.2%	4.1%
Ampicillin	32.0%	0.4%	67.5%	37.7%	0.6%	61.7%	20.3%	0.0%	79.7%
Amoxicillin/clavulanic acid	56.6%	20.6%	22.8%	53.2%	24.0%	22.7%	63.5%	13.5%	23.0%
Aminoglycosides									
Gentamicin	89.5%	8.3%	2.2%	88.3%	8.4%	3.2%	91.9%	8.1%	0.0%
Tetracycline(s)									
Tetracycline	7.9%	0.9%	91.2%	9.1%	0.6%	90.3%	5.4%	1.4%	93.2%
Macrolides									
Azithromycin	99.1%	-	0.9%	98.7%	-	1.3%	100.0%	-	0.0%
Chloramphenicol(s)									
Chloramphenicol	54.4%	6.1%	39.5%	43.5%	7.1%	49.4%	77.0%	4.1%	18.9%
Quinolones									
Ciprofloxacin	64.9%	11.0%	24.1%	55.2%	14.9%	29.9%	85.1%	2.7%	12.2%
Nalidixic acid	89.0%	-	11.0%	85.7%	-	14.3%	95.9%	-	4.1%
Sulfonamides									
Trimethoprim/sulfamethoxazole	25.9%	-	74.1%	28.6%	-	71.4%	20.3%	-	79.7%
Sulfafurazole	58.8%	-	41.2%	48.7%	-	51.3%	79.7%	-	20.3%

(11.0%) and 9.2% to cefoxitin. Moreover, almost all the isolates were sensitive to ceftriaxone (99.1%, 226/228) and azithromycin (99.1%, 226/228), followed by gentamicin (89.5%) and nalidixic acid (89.0%). Notably, the resistance rates to chloramphenicol, quinolones and sulfafurazole were significantly higher in strains isolated from human compared to non-human source strains, while the resistance rate to ampicillin was significantly lower in strains isolated from human than in non-human source strains ($p<0.05$) (Figure 1). The 14 tested antimicrobial agents were divided into seven

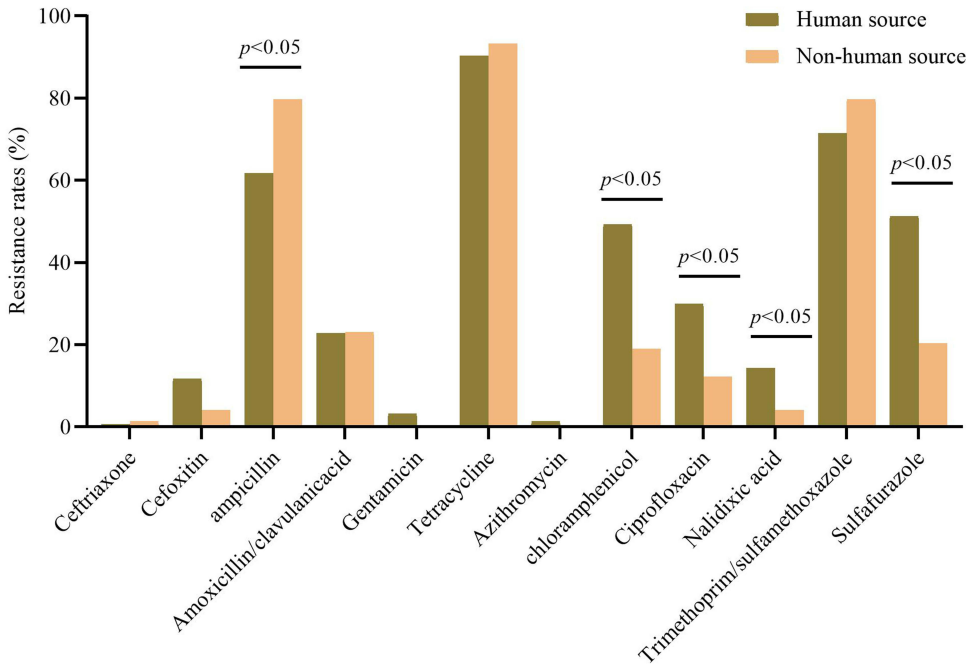

Figure 1 The resistance rates of human source and non-human source *Salmonella rissen* isolates.

categories: tetracyclines, aminoglycosides, sulfonamides, chloramphenicols, quinolones, macrolides, and β-lactams. High frequencies of resistance were observed for tetracyclines (91.2%, 208/228), sulfonamides (75.9%, 173/228), and β-lactams (69.7%, 159/228). Almost all the isolates were susceptible to macrolides (99.1%, 226/228).

A total of 171 (75%) isolates were resistant to at least three categories of antimicrobials, and the most common resistance profile was Tetracycline(s)-β-Lactams-Sulfonamides, which was represented by 35.1% (80/228). Twelve multidrug resistant (MDR) patterns were detected among these isolates, including three isolates that exhibited resistance to six categories of antimicrobials (Tetracycline(s)-β-Lactams-Aminoglycosides-Chloramphenicol(s)-Quinolones-Sulfonamides/2, Tetracycline(s)-β-Lactams-Macrolides-Chloramphenicol(s)-Quinolones-Sulfonamides/1) (Table 3).

Detection of Resistance-Associated Genes

About half of the β-lactamase-resistant isolates obtained produced amplicons specific to bla_{TEM}, but none of the β-lactamase-resistant isolates from all sources produced amplicons specific to bla_{OXA} (Table 4). Most of the TET-resistant isolates (81.3%) produced amplicons specific to *tetA*, followed by *tetB* (19.7%). Half of the SMX-resistant isolates obtained produced amplicons specific to *sul2*/*sul3*, and only one isolate from a patient produced *sul1* and *dfr* specific amplicons. Among the 5 GEN-resistant isolates from human, one isolate produced *strA* and *strB* specific amplicon gene and two isolates produced the *aadA* gene. None of chloramphenicol-resistant isolates carried the *cat* gene. We also

Table 3 The Resistance Patterns Among *Salmonella* rissen Isolates

Resistance Patterns	No. (%)
Tetracycline(s)	33 (15.5)
Sulfonamides	2 (0.9)
Quinolones	2 (0.9)
β-Lactams-Sulfonamides	1 (0.5)
Tetracycline(s)-β-Lactams	2 (0.9)
Tetracycline(s)-Sulfonamides	1 (0.5)
Tetracycline(s)-Chloramphenicol(s)	1 (0.5)
Tetracycline(s)-Chloramphenicol(s)-Sulfonamides	6 (2.8)
Tetracycline(s)-β-Lactams-Sulfonamides	80 (37.6)
Tetracycline(s)-β-Lactams-Quinolones	1 (0.5)
Tetracycline(s)-Chloramphenicol(s)-Quinolones-Sulfonamides	9 (4.2)
Tetracycline(s)-β-Lactams-Aminoglycosides-Sulfonamides	1 (0.5)
Tetracycline(s)-β-Lactams-Chloramphenicol(s)-Sulfonamides	24 (11.3)
Tetracycline(s)-β-Lactams-Aminoglycosides-Chloramphenicol(s)-Sulfonamides	1 (0.5)
Tetracycline(s)-β-Lactams-Aminoglycosides-Chloramphenicol(s)-Quinolones	1 (0.5)
Tetracycline(s)-β-Lactams-Macrolides-Chloramphenicol(s)-Sulfonamides	1 (0.5)
Tetracycline(s)-β-Lactams-Chloramphenicol(s)-Quinolones-Sulfonamides	44 (20.7)
Tetracycline(s)-β-Lactams-Aminoglycosides-Chloramphenicol(s)-Quinolones-Sulfonamides	2 (0.9)
Tetracycline(s)-β-Lactams-Macrolides-Chloramphenicol(s)-Quinolones-Sulfonamides	1 (0.5)

Table 4 The Distribution of Antimicrobial Resistance Genes in *Salmonella* Rissen Isolates from Different Sources

Sources	β-Lactamase			Chloramphenicol	Sulfonamides				Tetracycline		Aminoglycosides		
	bla_{TEM}	bla_{OXA}	bla_{SHV}	*Cat*	*Sul1*	*Sul2*	*Sul3*	*dfr*	*tetA*	*tetB*	*strA*	*strB*	*aadA*
Human source	50.0%	0.0%	15.0%	0.0%	0.9%	40.4%	50.9%	0.9%	78.4%	14.4%	20.0%	20.0%	40.0%
Non-human source	62.7%	0.0%	0.0%	0.0%	0.0%	1.7%	61.0%	0.0%	87.0%	30.4%	0.0%	0.0%	0.0%
Total	54.7%	0.0%	9.4%	0.0%	0.6%	45.7%	54.3%	0.6%	81.3%	19.7%	20.0%	20.0%	40.0%

screened genes associated with class one integron in the 228 isolates, and all strains harbored *intl1 VR (cx)*. The frequencies of carrying *intl1* and *qacED1* were 71.5% (163/228) and 46.5% (106/228), respectively.

Detection of Virulence Genes

All of the isolates were found to harbor the virulence genes *hilA, sopB, sciN, stn*, and *ssrB*. The genes *ssaQ* and *mgtC* were detected in the majority of the isolates (98.7%), followed by *invA* (98.2%, 224/228). The occurrence of genes *pltA* (5.3%, 12/228) and *safB* (2.2%, 5/228) was relatively low. None of the isolates carried the genes *spi4D* and *viaB*. There were no significant differences observed in the virulence genes between isolates obtained from human and non-human source.

Molecular Typing by PFGE

The isolates were grouped into four major clusters (A to D) and four individual distinct profiles with 80% pattern similarity (Figure S1). A total of 93 PFGE patterns were generated for the 228 isolates tested, with a similarity index ranging from 59.4% to 100%. Of which, thirty PFGE patterns containing at least two isolates that were identical. Cluster A was the largest and comprised 91.7% (209/228) of the total number of isolates, including 140 human and 69 non-human sources (one from environment and the other from animals). The second most common cluster (cluster B, n = 6) included isolates from human, chicken and river. The cluster C included five isolates, all derived from patient feces; cluster D consisted of four isolates, all derived from patient feces.

Discussion

Foodborne salmonellosis has emerged as a significant global public health concern. *S*. Rissen, a zoonotic bacterium, has been linked to a growing number of human infections.[11,16] The consumption of contaminated food or water facilitates the spread and infection of this pathogen in both humans and animals.[17]

In this study, the almost equal distribution between male and female patients (51.9% females) indicates that gender does not significantly influence the likelihood of infection. However, the high prevalence in age groups of 21–40 and 41–60 years was observed, which could be related to lifestyle, dietary habits, or higher exposure to risk factors. The majority of the cases (80.5%) occurring in summer aligns with the common understanding that *Salmonella* infections often peak in the warmer months, potentially due to increased foodborne transmission routes. This might be the reason that *Salmonella* infections in Thailand are evenly distributed throughout the four seasons.[18] The most common source of *S*. Rissen from non-human source was pork, which was consistent with findings from other studies.[6,16] The identification of *S*. Rissen in rivers and lakes (24.3%) suggests environmental reservoirs may be potential sources of contamination, emphasizing the importance of controlling the spread of *S*. Rissen in humans, animals and the environment.

The abuse of antimicrobials in human, animal husbandry, and poultry farming has led to frequent drug resistance. The majority (91.2%) of *S*. Rissen isolates from all sources in this study exhibited resistance to tetracycline and displayed limited resistance to other antimicrobials. Some studies also found 84–93% of isolates were resistant to tetracycline in Thailand, while half (48.6%) of the US isolates were resistant to TET.[9,11] Similar to findings in Thailand,[9] *S*. Rissen isolates in China exhibited showed high frequencies of resistance for several routine antimicrobials, such as tetracyclines, sulfonamides and β-lactams. The finding that 75% of isolates were resistant to at least three categories of antimicrobials and the identification of common resistance profiles (eg, Tetracyclin-β-Lactams-Sulfonamides) pointed to significant MDR challenges, which necessitates careful consideration in choosing empirical treatments and highlights the need for routine susceptibility testing in clinical practice. It is worth noting that the resistance rates to chloramphenicol, quinolones and sulfafurazole were significantly higher in strains isolated from human compared to non-human source strains. The observed differences in resistance patterns between human and non-human source strains could be indicative of different selective pressures or exposure to antimicrobial agents in clinical settings compared to environmental or food source.

Compared with the data reported by Spain in 2015, the profiles of resistance gene patterns in these two countries shared something in common and had differences as well, the common lies at for tetracycline resistance, *tetA* is the predominant genes. The difference is that *sul1* and *sul3* were the main genes mediated sulfonamide resistance in Spain but in our case were *sul2* and *sul3*.[16] Furthermore, the *cat* gene was not detected in chloramphenicol-resistant strains. It is

necessary to expand the screening range of resistance genes and conduct further research and analysis. In our study, resistance genes are widespread in *S. Rissen*, which was essential for the development of new methods to control these zoonotic diseases. Virulence genes like *hilA, ssaQ, invA, sopB, mgtC, sciN* and *ssrB* were present in majority of the study isolates as was reported in other studies.[19,20] The high detection rate of virulence genes in these isolates highlighted their pathogenic potential, which could lead to serious salmonellosis and pose a threat to public health.

It was surprising to discover that the majority (91.7%) of *S*. Rissen isolates in this study showed a high level of similarity (>80%) in PFGE patterns, for *S*. Rissen was generally considered to be a genetically diverse serovar. Moreover, indistinguishable patterns were discovered to be shared by several isolates from patients, foods and environment, including some isolates from another province. Previous reports have provided proofs that *S*. Rissen clinical cases were linked to contaminated pork meat, meat handlers or other food sources, even between different countries.[11,16,21] We also observed the formation of several sub-clusters, where isolates from patients, foods, and river water shared the same PFGE pattern and antimicrobial resistance profile. This suggested that multiple *S*. Rissen clones are circulating in China. Therefore, our data suggested clonal dissemination of *S*. Rissen might exist, probably not only in China. It was necessary to collect more epidemiological information and isolates from other provinces to support that postulation.

A limitation of this study is that we were unable to include isolates from Thailand, which has been reported as the main source of *S*. Rissen isolates.[6,18] Isolates from Denmark and the USA were found to be linked to those from Thailand, and it was suggested that traveling to Thailand and consuming pork products imported from Thailand were potential risk factors for *S*. Rissen infections.[9,11] Therefore, it was meaningful to compare the characteristics of *S*. Rissen isolates from China and Thailand.

Conclusions

We described several epidemiological, phenotypic and genotypic characterizations of *S*. Rissen in China. The high frequencies of multidrug resistance and probable clonal dissemination in this serovar highlighted the necessity of systematic surveillance on *S*. Rissen in China.

Ethical Aspects

All specimens from patients and other sources were collected as part of the surveillance on *Salmonella*, according to the national guidelines in China. Consequently, informed consent was not sought from patients, and the study was approved by Shanghai Municipal Center for Disease Control and Prevention Ethical Review Committee.

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Disclosure

The authors declare that they have no competing interests in this work.

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