

Molecular epidemiology and antimicrobial susceptibility of *Salmonella enterica* serotype Stanley isolates in Taiwan

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Background and Purpose: *Salmonella enterica* serotype Stanley became the third most common non-typhoidal *Salmonella* serotype among human isolates in 2004. The present study was conducted to gain further understanding of the epidemiology and antimicrobial susceptibility of *S. Stanley*.

Methods: A total of 20 culture-confirmed cases were retrieved from the Center for Disease Control collection and analyzed. Clinical features and demographic data of the cases were analyzed. Laboratory investigation of the isolates included antimicrobial susceptibility testing and molecular typing by pulsed-field gel electrophoresis. Ceftriaxone-non-susceptible isolates were further examined by polymerase chain reaction, sequencing, and Southern blot hybridization.

Results: The cases studied were distributed widely across Taiwan, suggesting that the infection was an island-wide problem. *S. Stanley* predominantly caused infections in patients under the age of 5 years (75%). The most common type of illness was uncomplicated enterocolitis. Molecular typing showed 1 predominant genotype with 5 subtypes among these isolates. Antimicrobial resistance to ampicillin (75%), chloramphenicol (95%), and trimethoprim-sulfamethoxazole (95%) was common. Two isolates expressed non-susceptibility to ceftriaxone, and a *bla*_{CMY-2} gene was identified on an 80-kb plasmid in both isolates.

Conclusion: The increase in *S. Stanley* infections may be associated with the spread of an epidemic clone, although this requires further epidemiological surveillance. In view of the high rate of antimicrobial resistance, especially the emergence of resistance to third-generation cephalosporins, continued surveillance of the infections caused by this bacterium should be undertaken.

Key words: beta-Lactamases; Ceftriaxone; Drug resistance; *Salmonella enterica*; Taiwan

Introduction

Salmonellae are an important cause of human infections worldwide and the spectrum of infections may include gastroenteritis, enteric fever, bacteremia or other invasive diseases as well as osteomyelitis. Non-typhoidal *Salmonella* spp., such as *Salmonella enterica* serotypes Typhimurium and Enteritidis, are frequently isolated in Taiwan according to surveillance data from the Center

for Disease Control, Taiwan. Our previous report indicated that *Salmonella Stanley* was the seventh most common *Salmonella* serotype to be isolated from human sources during 1991-1996 [1]. In 2004, however, the organism became the third most common serotype (11%), next only to *S. Enteritidis* (23%) and *S. Typhimurium* (22%), among non-typhoidal *Salmonella* isolates in Taiwan (Chiou CS; unpublished data).

S. Stanley is uncommon in western countries. The latest international outbreak of *S. Stanley* associated with dried peanuts was defined in Australia and Canada in 2001 [2]. Earlier in 1995, the clinical cases of *S. Stanley* reported in Illinois, USA, were linked to the consumption

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of alfalfa sprouts [3]. Similar to the other members of non-typhoidal salmonellae, *S. Stanley* usually causes enteric illnesses, although a high frequency of septicemia in an outbreak of *S. Stanley* infections has been reported [4]. The virulence mechanism of this organism has been rarely reported. One early report, however, has indicated that the enterotoxin of *S. Stanley* exerted dermatotoxic effects on rabbit skin, causing marked central necrosis with peripheral erythema [5].

Based on the understanding that salmonellosis is a global public health problem that may be compounded by easy transmission via the frequent international travel of people and foods, the increasing instances of *S. Stanley* infection in Taiwan has become a matter of serious concern. Thus, we carried out this study to investigate the geographic distribution, molecular epidemiology, and antimicrobial susceptibility profiles of *S. Stanley* in Taiwan.

Methods

Bacterial isolates

A total of 20 *S. Stanley* isolates were obtained from the national collection at the Center for Disease Control, Taiwan, in 2004. The number of isolates studied was limited by the availability of isolates and clinical information. All isolates were cultured and identified by standard methods [6]. Serogroups and serotypes were defined by a slide agglutination test with O antisera and a tube method with H antisera (Difco Laboratories, Detroit, MI, USA), respectively.

Antimicrobial susceptibility

Antimicrobial susceptibility was examined by the disk diffusion method. The antimicrobial agents examined included ampicillin, chloramphenicol, ciprofloxacin, trimethoprim-sulfamethoxazole, cefotaxime, and ceftriaxone. Susceptible and resistant isolates were defined according to the criteria suggested by the Clinical and Laboratory Standards Institute (CLSI) [7]. The presence of extended-spectrum beta-lactamases (ESBLs) was screened by the disk diffusion method with cefotaxime (30 µg) and ceftazidime (30 µg), and confirmed by the phenotypic confirmatory test recommended by the CLSI [7].

Genomic DNA analysis by pulsed-field gel electrophoresis

Genomic DNA was isolated and digested with *Xba*I (New England Biolabs, Beverly, MA, USA) and the

macro-fragments were analyzed by pulsed-field gel electrophoresis (PFGE), as previously described [8]. The DNA fingerprints generated were analyzed according to the criteria proposed by Tenover et al [9]. Briefly, isolates with a >4-band difference were considered to have different genotypes, which were designated arbitrarily in the alphabetical order. Isolates with identical fingerprints were considered to have the same genotypes, while those with a <4-band difference were considered to be subtypes of an existing genotype. In these cases, the subtypes were designated sequentially by Arabic numerals.

Plasmid analysis

Plasmid profiles of the isolates were determined by the Kado-Liu method [10]. The 2 laboratory strains of *Salmonella* Choleraesuis, OU7085 and OU7526, were used as size markers [11].

Investigation of ceftriaxone resistance

Ceftriaxone resistance genes were amplified by polymerase chain reaction (PCR) using consensus primer sets previously described for detecting *bla*_{TEM}, *bla*_{SHV}, *bla*_{CTX-M}, and *ampC* genes [12-16]. PCR products were purified and sequenced as described previously [17]. The nucleotide sequences obtained were compiled and analyzed using the Lasergene software (DNASTAR, Inc., Madison, WI, USA). The search for homologous sequences was done in the GenBank database using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA) through the Internet.

To locate the resistance genes, DNA-DNA hybridization with the PCR product as a probe was performed by the method of Southern [18]. Another strain of *S. Choleraesuis*, SC-B67, which carried the *bla*_{CMY-2} gene on a 140-kb plasmid was used as the positive control in these experiments [19].

Detection of other resistance genes and the virulence plasmid by multiplex PCR

A multiplex PCR was designed to characterize the resistance genes associated with the salmonella genomic island 1 (SGI1) in *S. Typhimurium* DT104 [20]. We used the method in the present study to detect the resistance genes for other antimicrobial agents. The presence or absence in *S. Stanley* isolates of the virulence gene, *spv*, was also checked by using multiplex PCR [21].

Table 1. Clinical features and laboratory findings of the 20 isolates of *Salmonella* Stanley investigated

Strain	Gender	Age (years)	Source	PFGE type	Multiplex PCR ^a			Plasmid (kb)			Antimicrobial susceptibility ^b				
					<i>str</i>	<i>sul1</i>	<i>spv</i>	<6	15	80	AM	C	CRO	CTX	SXT
C04-3308	M	31	Stool	A1	+	+	-	-	1	-	R	R	S	S	R
CA04.002	M	3	Stool	A2	+	+	-	-	-	1	S	R	S	S	R
CA04.006	M	3	Stool	A2	+	+	-	-	1	1	R	R	S	S	R
CA04.007	F	41	Stool	A2	+	+	-	-	1	1	R	R	S	S	R
CA04.009	F	2	Stool	A1	+	-	-	-	1	-	R	R	S	S	R
CA04.012	F	3	Stool	A1	+	+	-	-	1	-	R	R	S	S	R
CA04.013	M	2	Stool	A1	+	+	-	-	1	-	R	R	S	S	R
NK04.075	M	4	Stool	A1	+	-	-	-	1	-	R	R	S	S	R
NK04.076	M	3	Stool	A1	+	+	-	-	1	1	R	R	S	S	R
NK04.077	F	42	Stool	A1	+	+	-	-	1	-	R	R	S	S	R
NL04.231	F	1	Stool	A1	+	+	-	-	-	-	S	R	S	S	R
SA04.102	M	35	Stool	A1	+	+	-	-	-	1	S	R	S	S	R
SA04.115	M	2	Stool	A4	+	+	-	-	-	-	S	R	S	S	R
SA04.141	M	2	Stool	A1	+	+	-	-	1	-	R	R	S	S	R
SA04.146	M	52	Stool	A1	+	+	-	-	1	-	R	R	S	S	R
SA04.148	F	5	Stool	A1	+	+	-	-	1	-	R	R	S	S	R
SA04.160	F	2	Stool	B	-	-	-	-	-	-	S	S	S	S	S
NB04.008	M	0.9	Stool	A5	+	+	-	1	1	-	R	R	S	S	R
NB04.022	F	3	Stool	A3	+	+	-	-	-	1	R	R	I	I	R
NB04.028	F	2	Stool	A3	+	+	-	-	-	1	R	R	I	I	R

Abbreviations: PFGE = pulsed-field gel electrophoresis; PCR = polymerase chain reaction; AM = ampicillin; C = chloramphenicol; CRO = ceftriaxone; CTX = cefotaxime; SXT = trimethoprim-sulfamethoxazole; M = male; F = female; + = positive; - = negative; R = resistant; S = susceptible; I = intermediate

^aAll isolates were negative in the PCR for *pse*, *floR*, and *tetG* genes.

^bNone of the isolates was resistant to ciprofloxacin.

Results

Demographic data and regional distribution

Clinical features of the 20 cases from which *S. Stanley* isolates were derived are summarized in Table 1. The male-to-female ratio was 1.22:1.00. The isolates were all derived from stool samples and none of the patients developed bacteremia or other extraintestinal complications. These patients received treatment in the outpatient department and were mostly diagnosed with acute enterocolitis; all were treated without the use of antibiotics and recovered afterwards. The majority (75%) of the 20 cases were children aged less than 5 years (range, 11 months to 5 years). The remaining 5 cases were adults aged between 31 and 52 years (Table 1). Analysis of the geographical distribution of the 20 cases revealed that they were dispersed throughout northern (35%), central (30%), and southern (35%) Taiwan (Fig. 1).

Antimicrobial susceptibility

The antimicrobial resistance rates were high against conventional antibiotics, such as ampicillin (75%),

chloramphenicol (95%), and trimethoprim-sulfamethoxazole (95%). However, none of the isolates was resistant to ciprofloxacin. Two isolates (10%) expressed intermediate resistance to the third-generation cephalosporins; both isolates were from children who lived in the same area of Yonghe in Taipei County. Negative results of the CLSI phenotypic confirmatory test indicated that the 2 isolates may not produce ESBLs. PCR and sequencing analysis confirmed that both isolates carried a *bla*_{CMY-2} gene, but no other known ESBL genes.

Susceptibility to all antimicrobial agents tested was found in only 1 isolate, while a total of 15 isolates (75%) were resistant to more than 2 antibiotics. During the study period, the most common resistance pattern found among the isolates was multi-resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (75%) [Table 1].

Molecular epidemiology

To determine whether a particular genetic lineage was responsible for this island-wide dissemination of *S. Stanley* infections, the isolates were analyzed by

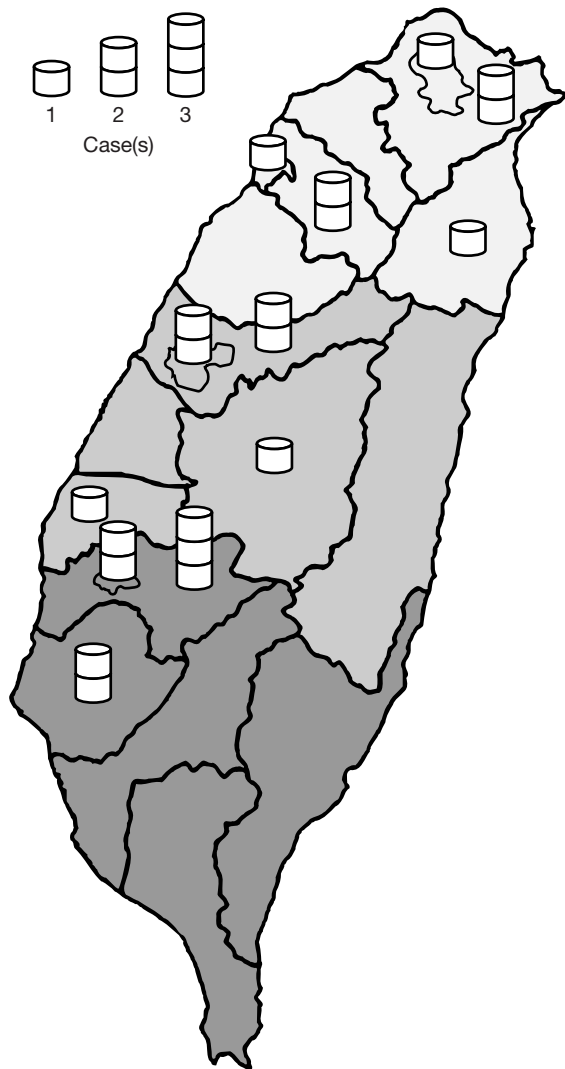


Fig. 1. Geographical distribution of the 20 cases of *Salmonella* Stanley infection investigated.

PFGE of *Xba*I-digested genomic DNA. The results of PFGE revealed similar but non-identical patterns in the majority of isolates (Fig. 2). All isolates except 1 could be categorized as genotype A and further subclassified into 5 subtypes according to the Tenover criteria [9]. A1 (n = 12) was the most predominant subtype, followed by subtypes A2 (n = 3), A3 (n = 2), A4 (n = 1), and A5 (n = 1). The 2 isolates with intermediate resistance to ceftriaxone were both subtype A3 (Table 1).

Plasmid analysis

Plasmid analysis showed that 7 isolates (35%) harbored a large 80-kb plasmid, with or without another small plasmid approximately 15 kb in size. Of the 7 isolates, 2 expressed intermediate resistance to ceftriaxone and carried a *bla*_{CMY-2} gene as described above. Southern

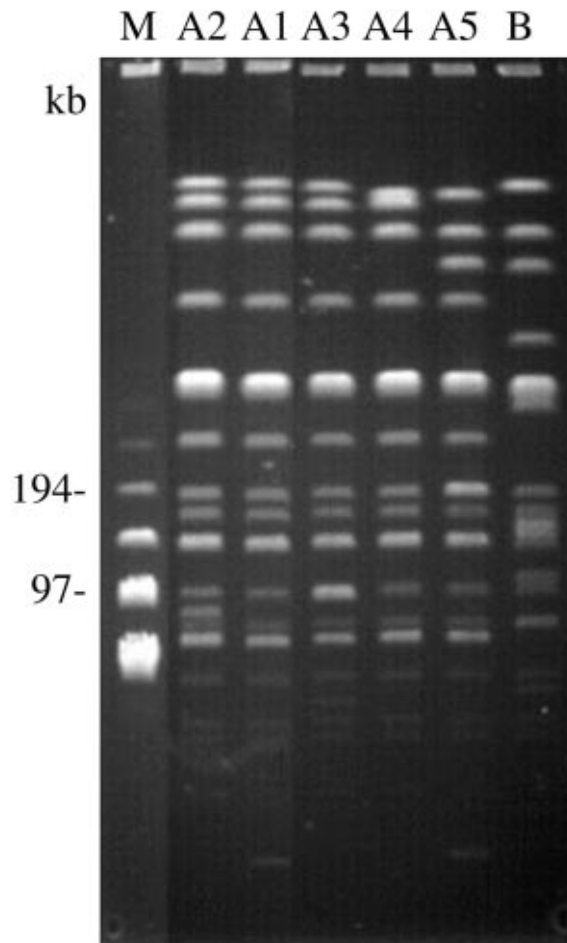


Fig. 2. Pulsed-field gel electrophoresis types and subtypes of *Salmonella* Stanley isolates. Lane M is the lambda DNA concatemer standard. The designation of each genotype is indicated at the top of the respective lane. Band sizes in kb are shown to the left.

blot hybridization confirmed that in both isolates the *bla*_{CMY-2} gene was located on the 80-kb plasmid (Fig. 3). Three isolates were contained no plasmid, while the remaining isolates carried 1 or 2 small plasmids (Table 1).

Multidrug resistance, other resistance genes and the virulence plasmid

Multidrug resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole was found in the majority of isolates. By a specific multiplex PCR assay, we found that none of the isolates had the *pse* (conferring ampicillin resistance), *floR* (conferring chloramphenicol resistance), or *tetG* (conferring tetracycline resistance) genes. On the other hand, all except 1 isolate carried the *str* (conferring streptomycin resistance) gene, and the 17 isolates that were resistant to trimethoprim-sulfamethoxazole were found to harbor

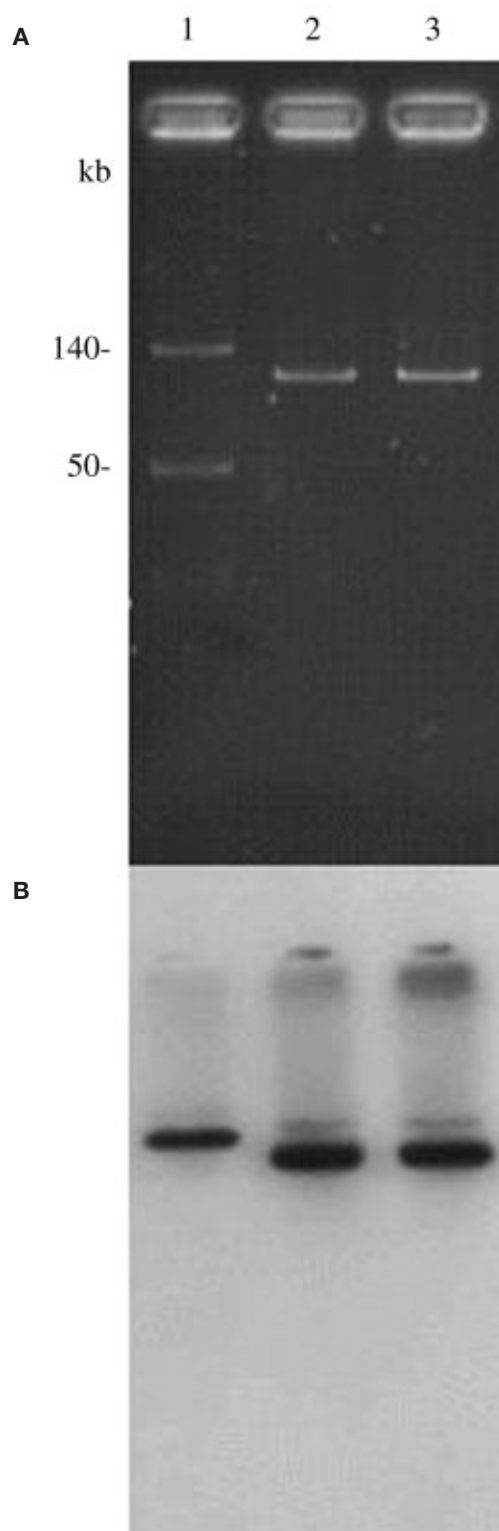


Fig. 3. Analysis of the 2 ceftriaxone-non-susceptible *Salmonella* Stanley isolates. (A) Plasmid profiles; and (B) DNA-DNA hybridization with *bla*_{CMY-2} polymerase chain reaction product as the probe. Lane 1, *Salmonella* Choleraesuis SC-B67; lane 2, *S. Stanley* NB04.022; lane 3, *S. Stanley* NB04.028. Plasmid sizes in kb are shown to the left.

the *sul1* (conferring sulphonamide resistance) gene (Table 1). To check whether or not the virulence plasmid was present among the isolates, PCR amplification of the *spv* gene was performed. None of the isolates produced positive results (Table 1).

Discussion

Non-typhoidal *Salmonella* infections have been mainly associated with foods of animal origin. Previous outbreaks of *S. Stanley* infections were related to the consumption of particular brands of imported raw peanuts or alfalfa sprouts [2,3,21]. In addition to these foodborne outbreaks, transmission of *Salmonella* can also occur from pet reptiles in preschool-aged children [22,23]. Although the present study was limited by the small number of isolates studied, our data could still provide some insights into the recent increase of *S. Stanley* infections in Taiwan. Among the 20 isolates examined, 19 belonged to the same genotype with minor variations, indicating that clonal spread of an epidemic strain might have a role in the significant increase of *S. Stanley* infections in Taiwan. However, it is also possible that *S. Stanley* organisms express high genomic conservativeness. This can be proven by further examination of isolates of *S. Stanley* collected prior to the surge. On the other hand, *S. Stanley* infections usually present as uncomplicated enterocolitis, as shown in the present study, and it is possible that outbreaks of such infections have been occurring without recognition. Epidemiological studies on a larger scale are required to confirm the transmission route and source of such infections, and the severity of the actual disease burden.

Generally, salmonellosis increases in the warmer summer seasons, a pattern which may be related to infection trends in animal hosts or food mishandling [24]. In the present study, we did not find this seasonal trend, probably due to the small number of cases studied. Most *Salmonella* infections are uncomplicated gastroenteritis, but some may result in bacteremia or focal infections, especially in a wide variety of immunocompromised hosts, elderly patients, and young infants [21,25]. In the USA, the major serotypes isolated from culture-confirmed invasive salmonellosis patients during 1996-1999 were Typhimurium, Typhi, Enteritidis, and Heidelberg [25]. In Taiwan, *S. Choleraesuis* was the predominant serotype that caused invasive non-typhoidal salmonellosis [26]. In contrast, *S. Stanley* does not cause invasive salmonellosis, as shown in the present

study. Nevertheless, Kontiainen et al reported in 1996 that *S. Stanley* infections could present a substantially high incidence of septicemia [4]. As a whole, *S. Stanley* appears to be associated with a relatively lower proportion of invasive infections, but continuous surveillance of this emerging serotype of *Salmonella* is mandatory.

In the 1980s, non-typhoidal *Salmonella* spp. were fairly “susceptible” organisms. Clinical isolates studied in the USA showed increasing resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole in the 1990s [21,27]. The resistance rate to these agents was as high as 70% in Taiwan [28]. Among non-typhoidal *Salmonella*, resistance to not only the conventional antibiotics but also the extended-spectrum cephalosporins or fluoroquinolones has been increasingly reported [12,28]. Overexpression of AmpC-type beta-lactamases by means of acquiring the resistance gene on a transferable plasmid or other mobile genetic elements constitutes one of the major extended-spectrum cephalosporin resistance mechanisms in *Salmonella* [17]. CMY-2 is currently the most common AmpC-type beta-lactamase found in *Salmonella* [17,29,30]. Several *Salmonella* serotypes, such as *S. Typhimurium*, *S. Choleraesuis*, *Salmonella* Cremieu, *S. Enteritidis*, *Salmonella* Gloucester, *Salmonella* Hadar, *Salmonella* Kimuenza, *Salmonella* Mons, *Salmonella* Newport, *Salmonella* Redba, *Salmonella* Schleissheim, and *Salmonella* Senftenberg, have been involved [13,31–35]. The emergence of ESC-resistant *S. Stanley* has been noted recently in Taiwan [36]. In the present study, we provide some further information regarding the occurrence of the *bla*_{CMY-2} gene in resistant isolates of *S. Stanley*. The *bla*_{CMY-2} was found located on an 80-kb plasmid. The 2 *bla*_{CMY-2}-harboring strains belonged to the predominant epidemic clone revealed in the present study. Further spread of the resistance clone or the resistance plasmid is possible, and this may signal yet another serious public health problem associated with *Salmonella*.

In addition to the plasmid-borne resistance mechanism described above, chromosomally integrated antibiotic resistance gene clusters in SGII as well as its variants, which are associated with multidrug-resistant *Salmonella* clones, have become a major concern in recent years [37,38]. SGII harbors genes responsible for the pentaresistance phenotype: resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline. Although in our study most of the *S. Stanley* isolates were multidrug-resistant, these isolates did not seem to carry the SGII gene clusters.

Many previous studies have suggested that resistant *Salmonella* isolates may have a selective advantage over susceptible organisms. Furthermore, they cause more severe illnesses, resulting in increased rates of hospitalization and death [39,40]. Although many patients with gastrointestinal infections recover without antimicrobial therapy, those with severe infections may require treatment. Multidrug-resistant organisms may handicap effective medication choices [39–41]. To control the public health threat that *Salmonella* poses, it is necessary that antibiotics be used judiciously at all times and this strategy be complemented by an active continuous worldwide surveillance for resistant *Salmonella* variants.

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