Prevalence of R-type ACSSuT in strains of *Salmonella* serovar Typhimurium DT193 isolated from human infections in Brazil

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ABSTRACT

Objective. *To determine the prevalence of resistance to ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines (ACSSuT) in* Salmonella *serovar Typhimurium definitive [phage] type (DT) 193 strains isolated from human sources over the last four decades.*

Methods. *From 2008 to 2010, 553 DT193 isolates out of 810 human-origin* Salmonella *ser. Typhimurium phage-typed strains isolated from the 1970s through 2008 were selected and tested for ACSSuT resistance: 91 strains isolated during the 1970s, 65 from the 1980s, 70 from the 1990s, and 327 from 2000–2008. Resistance profiles were determined using the disk diffusion method.*

Results. *An antimicrobial susceptibility assay indicated 20.9%, or 116, of all isolates tested were ACSSuT-resistant, 52.0% (287) were resistant to one or more drugs in the ACSSuT profile, and 27.1% (150) were nonresistant (susceptible to antimicrobials). Based on the assay, overall antimicrobial resistance was extremely high in the 1970s (affecting 99.0% of isolates from that period) and remained high during the 1980s, when 95.4% of isolates had some type of antimicrobial resistance and incidence of* Salmonella *ser. Typhimurium DT193 R-type ACSSuT increased to 73.8%. R-type ACSSuT dropped to 27.1% (19 isolates) during the 1990s, and to 5.2% (17) during 2000–2008, despite a substantial increase in the number of isolates tested (397 versus 204, 111, and 98, respectively, for the previous three decades).*

Conclusions. *Although prevalence of* Salmonella *ser. Typhimurium DT193 R-type ACSSuT in Brazil has decreased since the 1970s, ACSSuT resistance markers continue to circulate. Therefore, continuous surveillance should be conducted to evaluate the occurrence of* Salmonella *ser. Typhimurium DT193 and its antimicrobial resistance.*

Key words *Salmonella* infections; *Salmonella* Typhimurium; drug resistance, multiple, bacterial; Brazil.

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The *Salmonella* genus comprises more than 2 500 serotypes—most of which are considered potential human pathogens—but only a relatively small number of serovars have been associated with human infections (1). Among these, *Salmonella* serovar Typhimurium is ubiquitous, usually inducing gastroenteritis in a broad range of unrelated host species, and is one of the major serotypes causing infections in humans worldwide (1, 2), including Brazil (3).

For the purposes of epidemiological studies, phage typing of *Salmonella* can be used to subcategorize the more common *S. enterica* serotypes. It can also be

used to determine similarities and differences among isolates collected from different provenances over different time periods based on their reactions to specific sets of phages.

This study used the typing scheme described by Anderson et al. (4) in which 37 bacteriophages are used to identify more than 200 lysotypes, some of which show antimicrobial multiresistance. The importance of this technique was revealed in the characterization of a clone diffused widely worldwide: *Salmonella* ser. Typhimurium definitive type (DT) 104, the phage-type carrier of gene cassettes that demonstrates the basic resistance model (R) to ampicillin (A), chloramphenicol (C), streptomycin (S), sulphonamides (Su), and tetracyclines (T)—the profile ACSSuT—as well as trimethoprim, in some cases, and quinolones (more rarely) (5).

Salmonella ser. Typhimurium DT104 was first isolated in cattle in the United Kingdom in the 1990s and subsequently found in other countries, infecting diverse animal species, including humans. Since then, this phage type has been identified with relative frequency in the United States and Europe (2, 6–8).

Another phage type of epidemiological importance, due to its multiresistant character, is *Salmonella* ser. Typhimurium DT193. Studies have demonstrated that the acquisition of plasmids or temperate bacteriophages by several unrelated phages types of *Salmonella* ser. Typhimurium can result in their conversion to DT193 (9). From the 1970s to the present, this phage type has been one of the most prevalent in Brazil and around the world (3, 10).

In Brazil, the correlation between DT104 and the ACSSuT resistance profile seems to have limited epidemiological relevance, as it was once isolated in patients hospitalized in São Paulo (3). Nevertheless, DT193 belongs to the so-called DT104 complex, which includes other closely related phage types (11), and is of importance because it shows the ACSSuT profile and is the prevalent phage type in strains isolated from human infection (3).

Considering the importance of DT193 in Brazil, this study aimed to describe the prevalence of ACSSuT resistance among *Salmonella* ser. Typhimurium DT193 strains isolated from human sources during the last four decades.

TABLE 1. Decade distribution of 553 human-origin *Salmonella* serovar Typhimurium isolates identified as DT193[a] and selected for study on antimicrobial resistance, Rio de Janeiro, Brazil, 2008–2010

Decade of origin	Isolates phage-typed No.	Isolates identified as DT193 No.	Isolates identified as DT193 %
1970s	204	91	44.6
1980s	111	65	58.6
1990s	98	70	71.4
2000–2008	397	327	82.4

[a] Based on information from the Enterobacteria Laboratory databank of the Oswaldo Cruz Institute (FIOCRUZ), Rio de Janeiro, RJ, Brazil.

MATERIALS AND METHODS

Bacterial strains

From 2008 to 2010, 553 DT193 isolates out of 810 *Salmonella* ser. Typhimurium phage-typed strains isolated from human infections from the 1970s through 2008 were selected and tested for ACSSuT resistance. The selection of isolates was based on information from the Enterobacteria Laboratory databank of the Oswaldo Cruz Institute (FIOCRUZ) in Rio de Janeiro, Brazil. Distribution of the isolates by decade of origin is shown in Table 1. Strains from the 1970s were isolated from a unique outbreak, whereas strains collected from 1980 through 2008 were isolated from sporadic cases. Once selected, the cultures were re-isolated and characterized through biochemical and antigenic analyses using methods described by Ewing (12), and Grimont and Weill (13). Rough cultures were excluded from the study.

Antigenic confirmation (including an induction/absorption phase, when necessary) and slide agglutination serotyping (based on the Kauffmann-White scheme) were carried out for all isolates selected for the study. Strains were defined as *Salmonella enterica* ser. Typhimurium based on serology positive for the second flagellar phase (*S.* 4,[5],12:i:1,2).

Phage typing

Strains isolated from 1970 through 1998 were phage typed by the United Kingdom's Health Protection Agency (HPA) International Reference Laboratory for Enteric Phage Typing, whereas stains isolated during and after 1999 were characterized by the FIOCRUZ Enterobacteria Laboratory, using phage preparations supplied by the HPA, and following the technical procedures reported by Anderson et al. (4) and the criteria for interpretation of phage reactions described by Willshaw et al. (14).

Antimicrobial susceptibility

Antimicrobial susceptibility was tested using the standard disk diffusion method, according to the protocol of the Clinical and Laboratory Standards Institute (Wayne, PA, USA) (15). Strains were tested against the following antimicrobial agents (Oxoid Limited, Hampshire, England): ampicillin (10 µg), cefepime (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg); streptomycin (10 µg); gentamicin (10 µg); sulphonamide (300 µg); and tetracycline (30 µg).

For quality control of the antimicrobial susceptibility test, the following references strains were used: *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 25923.

Statistical analysis

The distribution of all variables, and their frequency, was also studied. Bivariate analyses of the variable categories were carried out using Fisher's exact test. Bilateral tests were employed in all analyses, using $P < 0.05$ as the level of significance.

RESULTS

The different types of antimicrobial resistance found among the 553 selected isolates of *Salmonella* ser. Typhimurium DT193 are shown in Table 2. The most prevalent resistance profile (ACSSuT) was verified in 116 isolates (20.9%), and one or more of its antibiotic resistance

TABLE 2. Antimicrobial resistance among 553 human-origin *Salmonella* serovar Typhimurium DT193 strains isolated between 1970 and 2008, Rio de Janeiro, Brazil, 2008–2010

Type of resistance[a]	Resistant isolates					
	1970s (No.)	1980s (No.)	1990s (No.)	2000–2008 (No.)	Total (No.)	%
ACSSuT	32	48	19	17	116	20.9
ACSSu	8	0	0	0	8	1.4
ACST	1	0	0	0	1	0.9
ASSuT	8	0	0	0	8	1.4
ASSu	13	0	0	0	13	2.3
ACT	NA	11	6	30	47	8.5
ACSu	NA	0	15	0	15	2.7
ASuT	0	2	1	5	8	1.4
CSuT	0	0	1	35	36	6.5
CST	6	0	0	0	6	1.0
AC	0	0	2	26	28	5.0
ASu	6	0	0	0	6	1.0
AT	5	0	0	46	51	9.2
CT	0	0	0	15	15	2.7
SSu	4	0	0	0	4	0.7
A	0	1	1	21	23	4.1
S	3	0	0	0	3	0.5
T	4	0	0	11	15	2.7
NA (susceptible to antimicrobials)	1	3	25	121	150	27.1
Total	91	65	70	327	553	100

[a] A: ampicillin; C: chloramphenicol; S: streptomycin; Su: sulphonamides; T: tetracyclines.

markers was verified in 287 isolates (52%).

A total of 99% of isolates from the 1970s had some type of antimicrobial resistance (the highest frequency of drug resistance throughout all four decades), and among those, 35.2% were R-type ACSSuT. This may be partly attributed to the fact that all strains collected during this period were isolated from a unique outbreak that occurred in the city of São Paulo.

Overall drug resistance remained high during the 1980s (at 95.4%), and an increase in R-type ACSSuT *Salmonella* ser. Typhimurium DT193 frequency (to 73.8%) was observed. In addition, the diversity of resistance profiles decreased, as did the prevalence of S and Su resistance markers.

During the 1990s 64.3% of isolated strains were antimicrobial-resistant, a reduction of 31.1% versus the 1980s, and from 2000 to 2008, 63% of the isolates were resistant, a decline of 1.3%. R-type ACSSuT detection declined over time as well, with only 19 isolates (27.1%) presenting this feature during the 1990s, dropping to 17 (5.2%) during the period 2000–2008.

Over the four decades studied, a break point between the prevalence of resistance and susceptibility was noted in the transition from the 1980s to the 1990s. Although the number of *Salmonella* ser. Typhimurium DT193 isolates has increased in recent years, the percentage of antimicrobial resistance has remained at around 60%.

On the other hand, 150 *Salmonella* ser. Typhimurium DT193 strains (27.1%) were susceptible to the antimicrobials used in the study (ampicillin, chloramphenicol, streptomycin, sulphonamide, and tetracycline), especially those isolated from 2000 to 2008 (21.9%). None of the isolates was resistant to second- or third-generation cephalosporins or fluoroquinolones.

DISCUSSION

Antimicrobial resistance that is displayed by enterobacteria, especially in the case of *Salmonella* serovars, is not something that can be identified as having developed over the last century or that has had a causal relationship to the universe of economically more developed countries. In truth, this situation depicts an ecological phenomenon, arising principally from the natural competition among microorganisms in a biocenosis of nutritional elements. While the investigations of Hughes and Datta (16) and Levy (17) that analyzed, respectively, human enterobacteria isolates from 1920 and those from African wild animals that had never received antibiotics (and were therefore free from the influence of selective anthropogenetic pressure) found low resistance to the antimicrobials, they did detect the presence of plasmids that transmit resistance factors.

As some of the most important zoonotic agents to date, *Salmonella* isolates from animals have developed antimicrobial resistance very rapidly, mainly because of the selective pressure that has arisen from the use of antimicrobials as animal food supplements. These sources of infection and their products (which are often utilized in human foodstuffs) have established propagation hubs, which account for the prevalence of certain *Salmonella* serovars in human infections, and their display of cosmopolitan and nosocomial characteristics (18). This entire evolutionary process is encountered and supported scientifically by the genetic trials of Datta (19) on a resistant strain of *Salmonella* ser. Typhimurium that has the capability of transferring its genetic resistance markers (9, 20).

In a pioneering study, Anderson (21) associated antimicrobial resistance to an established phage type of *Salmonella* ser. Typhimurium (DT29) with human enteric outbreaks, and determined that the primary source of the infection was bovine. In the 1970s, various outbreaks of *Salmonella* erupted in the United Kingdom and Europe, provoked by DT193, DT204, and DT204c, which had bovines as reservoirs (22). These phage types had their origins in DT49, and the great majority of them exhibited R-type ACKSSuT (resistance to ampicillin, chloramphenicol, neomycin-kanamycin, streptomycin, sulphonamides, and tetracyclines) (14). At present, DT104 is the dominant phage type in Europe, presenting R-type ACSSuT (23, 24).

Curiously, DT104, DT204, and DT204c, which exhibit multidrug resistance and are found in many parts of the world (9, 11, 25, 26), have not been recorded in Brazil (3, 10, 27), where prevalence of the multidrug-resistant *Salmonella* ser. Typhimurium is related to DT193, as first reported by Magalhães and Véras (28) in cases of infant enteritis in Recife (Northeast Brazil) in 1970.

During the 1970s, *Salmonella* ser. Typhimurium was prevalent in southern Brazil, especially in state hospitals. Its

rapid and continued dissemination throughout the decade could be seen as evidence of the difficulties experienced in controlling these outbreaks. During this decade, in the city of São Paulo, *Salmonella* ser. Typhimurium was the most prevalent *Salmonella* serovar, representing > 78% of all cases isolated by the central public health laboratory from diarrheal disease in the community and from nosocomial sources.

Ten years later, a new serovar without the second-phase H antigen was encountered in a chicken carcass that had been recovered in Portugal. Several years later, in 1997, it was isolated in Spain and its frequency subsequently increased rapidly. Initially, multidrug-resistant isolates with this antigenic formula were classified as DT U302 (often associated with pigs and pork products), which became the fourth most common *Salmonella* serovar identified between 1998 and 2000. Researchers realized that monophasic *Salmonella* isolates could have originated from ancestral forms that had not acquired a second-phase flagellar antigen or developed the switching mechanism during their evolution. This seroformula has therefore been referred to as a variant of *Salmonella* ser. Typhimurium or *Salmonella* ser. Lagos (23, 24).

Macro-restriction profiles obtained by pulsed-field gel electrophoresis (PFGE) led Zamperini et al. (29) to conclude that monophasic *Salmonella* 4,[5],12:i:- isolates were genotypically *Salmonella* ser. Typhimurium. Tavechio et al. (30) observed that *Salmonella* ser. Typhimurium and *Salmonella* 1,4,[5],12:i:- isolated in Brazil were distributed in highly similar PFGE clusters, also suggesting a close relationship at the serotype level.

Since 2006, R-type ASSuT (resistance to ampicillin, streptomycin, sulphonamides, and tetracyclines) monophasic *Salmonella* 4,[5],12:i:- DT193 has also been observed in outbreaks and single cases in Europe (23). In the current study, monophasic *Salmonella* 4,[5],12:i:- was not detected, and all isolates were characterized as *Salmonella* ser. Typhimurium. However, DT193 resistance to ACSSuT was confirmed. Indeed, the first isolation of *Salmonella* ser. Typhimurium DT193 R-type ACKSSuT in Brazil was identified in 1970 (28). In that study, ASSu determinants were preferentially transferred over CKT determinants to *E. coli* K12, suggesting ASSu could be encoded by a conjugative plasmid. The authors concluded that resistance to tetracycline (R-type T) was segregated from ASSu and that it may have spontaneously evolved from another plasmid originating in strains of DT193 R-type ASSuT. Hampton et al. (31) results showed that DT193 can be rapidly subdivided by antibiogram for epidemiological investigations, and that further subdivision can be achieved by molecular techniques.

The evaluation of the antimicrobial resistance profiles observed in this study is in agreement with the findings of Magalhães and Véras (28), since ASSu was detected in 61 isolates (67.8%). The detection of multidrug-resistant isolates with marker Su, present since the 1970s, show that the R-type has expanded in response to salmonellosis treatment schemes.

For years, ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol were the drugs of choice for treating severe *Salmonella* infections, but the increasing rates of resistance to these agents has decreased efficacy (32). Therefore, fluoroquinolones and extended-spectrum cephalosporins have become the usual therapies used in these cases (33, 34).

The significant decrease in R-type ACSSuT in the 1990s versus the 1980s shown in the current results may be attributed to changes in drugs of choice for salmonellosis treatment. According to Asensi and Hofer (35), second- and third-generation cephalosporins were used in human therapy in Brazil beginning in the 1990s due to the ineffectiveness of ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole.

The persistence of significant resistance to ampicillin in strains isolated during the 2000–2008 period was probably due to the drug's widespread use both within and outside of hospitals in fighting numerous bacterial infections, a practice that favors the selection of bacteria that produce β-lactamases. The antibiotic resistance to β-lactam of *Salmonella* ser. Typhimurium DT193, including multidrug resistance, has been observed in Brazil by Asensi et al. (27). This occurs in *Salmonella* spp. carriers of β-lactamase types TEM, SHV, and PSE (36, 37), forcing the use of second- or third-generation antimicrobials, which are always more expensive and often more toxic than their predecessors (38). This change in antibiotic therapy for invasive salmonellosis explains the emergence of beta-lactamase–producing *Salmonella* spp. and how it may contribute to the maintenance of ampicillin resistance.

According to the current study results, only 150 (27.1%) of the 553 selected isolates were susceptible to antimicrobials (Table 2), and 116 (20.9%) were R-type ACSSuT.[5] The prevalence of multidrug resistance observed in this study is consistent with the results of national studies, which also focused on human isolates (3, 27, 28). Other Brazilian authors had previously revealed that multidrug resistance was higher in human versus nonhuman *Salmonella* ser. Typhimurium, supporting the idea that the dissemination of human-origin strains might be attributed to a human reservoir and that antimicrobial use by humans had resulted in an increase in multidrug resistance (3, 10).

Further evidence may be seen in the progressive decrease in overall antimicrobial resistance, which dropped from 95.4% among isolates from the 1980s to 64.3% for those from the 1990s and 63% in the period 2000–2008. It is possible that employing more rigorous means of control over the use of antimicrobial agents in all areas of the food chain has had a beneficial effect, as reported by Ghilardi et al. (3) following an examination of a collection of *Salmonella* ser. Typhimurium isolated from human and nonhuman sources. Evidence of this trend was supported in the current study by the significant predominance ($P < 0.01$) of the multidrug resistance of phenotype ACSSuT in the period 1970–1980 compared with strains isolated between 2000 and 2008.

This study had several limitations, including the way in which the results were interpreted for strains isolated during the 1970s. While all isolated strains from that decade were from a unique outbreak, the general human population varies, and individuals may have had contact with several different strains, because *Salmonella* ser. Typhimurium was a common community-acquired and

[5] Since DT193 has been verified as the perennial carrier of antimicrobial susceptibility throughout the four-decade period analyzed, resistance to all five antimicrobials can be inferred even if no second- or third-generation cephalosporin resistance has been observed.

nosocomial infection in Brazil in the 1970s. In addition, no molecular assessment was conducted to determine if all analyzed strains belonged to the same clone or were multiclonal.

In conclusion, this study showed that the prevalence of human isolates of *Salmonella* ser. Typhimurium DT193 in Brazil has been increasing since the 1990s, whereas detection of R-type ACSSuT has been decreasing. These microorganisms may persist and can transfer these markers of resistance through the environment and the food chain and thus represent a potentially significant public health problem. This study also confirmed that all *Salmonella* ser. Typhimurium strains analyzed were susceptible to second- and third-generation cephalosporins and fluoroquinolones, which should therefore be considered crucial components of therapy for *Salmonella* infections in humans. Nevertheless, the factors that influence the propagation of multidrug-resistant strains in Brazil, and how they were introduced in Brazil, remains unknown. It is possible that through the control of antibiotic sales in Brazil, initiated in 2010, a reduction in or at least a slowing of the growth of antibiotic resistance can be achieved. This underscores the need for continuous surveillance to evaluate the occurrence of *Salmonella* and its antimicrobial resistance in order to avoid a threat to human therapy efficacy.

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RESUMEN

Prevalencia de resistencia de tipo ACSSuT en cepas de *Salmonella* serovariedad Typhimurium DT193 aisladas a partir de infecciones humanas en el Brasil

Objetivo. Determinar la prevalencia de resistencia a la ampicilina, el cloranfenicol, la estreptomicina, las sulfonamidas y las tetraciclinas (ACSSuT) en cepas de *Salmonella* serovariedad Typhimurium fagotipo definitivo (DT) 193 aisladas de fuentes de origen humano durante las cuatro últimas décadas.

Métodos. Entre el 2008 y el 2010 se seleccionaron 553 aislados de DT193 entre 810 cepas de *Salmonella* serovariedad Typhimurium fagotipificadas aisladas desde la década de 1970 hasta el 2008, y en ellos se analizó la resistencia a ACSSuT: se estudiaron 91 cepas aisladas durante la década de 1970, 65 aisladas durante la década de 1980, 70 aisladas durante la década de 1990, y 327 aisladas entre el 2000 y el 2008, respectivamente. Los perfiles de resistencia a los antibióticos se determinaron mediante el método de difusión en disco.

Resultados. El antibiograma indicó que 20,9% (116) de todos los aislados que se analizaron fueron resistentes a ACSSuT, 52,0% (287) fueron resistentes a uno o más antibióticos del grupo ACSSuT y 27,1% (150) no fueron resistentes (es decir, fueron sensibles a dichos antibióticos). Según el análisis, la resistencia general a los antibióticos fue muy alta en la década de 1970 (y comprendió a 99,0% de los aislados de ese período) y continuó siendo alta durante la década de 1980, cuando 95,4% de los aislados presentó algún tipo de resistencia a los antibióticos y la incidencia de *Salmonella* serovariedad Typhimurium DT193 con resistencia de tipo ACSSuT aumentó hasta 73,8%. La resistencia de tipo ACSSuT descendió a 27,1% (31 aislados) durante la década de 1990, y a 5,2% (17 aislados) entre el 2000 y el 2008, a pesar del aumento importante en el número de aislados que se evaluaron (397 frente a 204, 111 y 98 en las tres décadas anteriores, respectivamente).

Conclusiones. Aunque la prevalencia de *Salmonella* serovariedad Typhimurium DT193 con resistencia de tipo ACSSuT en el Brasil ha disminuido desde la década de 1970, los marcadores de resistencia a ACSSuT continúan en circulación. Por consiguiente, debe llevarse a cabo una vigilancia permanente para evaluar la aparición de infecciones por *Salmonella* serovariedad Typhimurium DT193 y su resistencia a los antibióticos.

Palabras clave Infecciones por *Salmonella*; *Salmonella* Typhimurium; farmacorresistencia bacteriana múltiple; Brasil.