Differences in tetracycline resistance determinant carriage among *Shigella flexneri* and *Shigella sonnei* are not related to different plasmid Inc-type carriage

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ABSTRACT

Objectives: The aim of this study was to establish the prevalence of the most common molecular mechanisms involved in tetracycline resistance as well as their relationship with plasmid incompatibility (Inc) groups in a collection of *Shigella* spp. causing traveller's diarrhoea.

Methods: Tetracycline susceptibility was established in 187 *Shigella* spp. (74 *Shigella flexneri* and 113 *Shigella sonnei*), of which 153 isolates were recovered as a confirmed cause of traveller's diarrhoea. The prevalence of the *tet*(A), *tet*(B) and *tet*(G) genes was analysed by PCR. Eighteen plasmid Inc groups was determined in a subset of 59 isolates.

Results: Among 154 tetracycline-resistant isolates, 122 (79.2%) harboured at least *tet*(A) or *tet*(B). The *tet*(B) gene was the most frequently detected, being present in 70 isolates (45.5%), whilst *tet*(A) was detected in 57 isolates (37.0%). The *tet*(G) gene was present in only 11 (7.2%) isolates. Moreover, the *tet*(A) gene was more frequent in *S. sonnei* ($P = 0.0007$), whilst the *tet*(B) gene was more frequent in *S. flexneri* ($P < 0.0001$). Plasmids belonging to Inc group B ($P < 0.05$) were significantly more frequent among *S. flexneri*, whilst those belonging to groups K, FIC and FIIA ($P < 0.05$) were preferentially detected among *S. sonnei*.

Conclusion: The prevalence of the *tet*(A) and *tet*(B) genes differed between *S. sonnei* and *S. flexneri*. Moreover, the prevalence of plasmid Inc groups in *S. flexneri* and *S. sonnei* differed. However, no relationship was found between the two phenomena.

1. Introduction

Antimicrobial resistance is continuously increasing worldwide. Mechanisms of antimicrobial resistance may be found in pristine environments such as artic soils [1]. None the less, there is a direct relationship between local human and veterinary antibiotic use patterns and specific local bacterial antimicrobial resistance profiles. However, international travel and commerce facilitate the distant spread of pathogenic, commensal and environmental micro-organisms that may carry antibiotic resistance determinants [2–5]. This dissemination of bacteria/genes in a specific geographical area may result in the detection of micro-organisms exhibiting resistance to antimicrobial agents that are not usual to that area, such as what has been described in remote Peruvian rural regions [6].

In this context, international travellers carry micro-organisms from their geographical area to that of their travel destination, and vice versa. However, this international dissemination of micro-organisms usually remains undetected since commensals do not produce disease, and this dissemination only comes to attention when a pathogenic micro-organism is acquired [5].

Traveller's diarrhoea (TD) is the most frequent illness affecting travellers to developing areas. A series of pathogens have been described underlying this illness, although bacteria are by far the most relevant aetiological cause [7]. Diarrheagenic *Escherichia coli* and *Shigella* spp. rank among the most frequent micro-organisms described in TD patients and are also of special relevance because antibacterial agents are often used due to the symptom duration or the severity of illness [8].

However, differences in the geographical prevalence of *Shigella flexneri* and *Shigella sonnei* have also been described [9,10].

Recent studies demonstrate an overall increase in the antibiotic resistance levels of *Shigella* spp. causing TD, with tetracycline resistance levels increasing from 77.1% in the period 1995–2000 to 92.5% in the period 2001–2010 [10]. Tetracycline resistance is mediated by different mechanisms (efflux pumps, ribosomal protection, enzyme inactivation, impermeability or target alterations), most of which are encoded within transferable elements, with wide dissemination within different bacteria [11].

Previous studies have shown differences in the antibiotic resistance levels of *S. flexneri* and *S. sonnei* [10,12] as well as in the prevalence of transferable antibiotic resistance determinants. Differential species-specific *bla*OXA genes have been described among *S. flexneri* and *S. sonnei* causing either TD or infections in specific areas [3,12]. Regarding tetracycline, the most relevant mechanisms of resistance in *Shigella* spp. are those related to transferable efflux pumps [11], with the *tet*(A) and *tet*(B) genes identified in 80–100% of analysed tetracycline-resistant *Shigella* isolates [12–15]. Other resistance determinants, such as *tet*(C) or *tet*(D), are rarely detected alone [12–14]. Finally, determinants such as *tet*(G) have also been detected [12–14] but remain understudied. Although local studies have shown differences between the tetracycline resistance determinants present in *S. flexneri* and *S. sonnei* [12,15], there are no data on isolates causing TD, which reflect a scenario more pertinent to human health.

Antibiotic resistance determinants, such as those involved in tetracycline resistance, are often encoded within transferable elements such as plasmids. Plasmids are classified within the so-called 'incompatibility (Inc) groups' [16]. Within each group, plasmids are mutually exclusive. Interestingly, several antibiotic resistance determinants (*bla*CTX-M-2 and *bla*CTX-M-19) have been associated with different plasmid Inc groups [17]. This association might be extended to other antibiotic resistance determinants, such as the *tet* determinants, and thus explain differences in *tet* determinant carriage among different *Shigella* spp., resulting in a possible barrier to horizontal transfer of *tet*(A) or *tet*(B) between *Shigella* spp.

Therefore, a surveillance study of different mechanisms of tetracycline resistance was performed in a subset of *Shigella* spp. associated with TD in order to investigate differences in *Shigella* spp. tetracycline resistance gene carriage, which may be related to geographical origin and/or the presence of barriers to horizontal gene transfer between *Shigella* spp.

Thus, the aim of this study was to establish the prevalence of *tet*(A), *tet*(B) and *tet*(G) genes in a collection of *Shigella* spp. recovered from 1995–2010 as a cause of TD and to determine their association with plasmid Inc groups.

2. Materials and methods

2.1. Strains

A total of 187 *Shigella* spp. isolates (74 *S. flexneri* and 113 *S. sonnei*) recovered between 1995 and 2010 at the Microbiology Service of the Hospital Clinic of

Barcelona (Barcelona, Spain) were included in this study, of which 153 isolates (60 *S. flexneri* and 93 *S. sonnei*) were isolated from patients with TD visiting the Tropical Medicine Unit. Travel history was collected from an internal database in all cases except for 34 (14 *S. flexneri* and 20 *S. sonnei*).

2.2. Antimicrobial susceptibility testing

In these travel-related isolates, antibiotic resistance patterns were established previously [10], whilst in the remaining 34 isolates tetracycline susceptibility was established by the disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. Isolates that were intermediate-resistant and resistant to tetracycline were classified together in all analyses.

2.3. Detection of tetracycline resistance determinants and plasmid incompatibility groups

Presence of the *tet*(A) and *tet*(B) genes (which are usually detected in >80% of tetracycline-resistant *Shigella* spp.) and *the tet*(G) gene (with a not well established relevance in the development of tetracycline resistance in *Shigella* spp.) was determined by PCR using primers and conditions described previously [12]. Amplified products were resolved in 2% agarose gels stained with SYBR™ Safe (Invitrogen, Carslbad, CA, USA) . Randomly selected amplified products were purified and were sent to Macrogen (Seoul, South Korea) to be sequenced in order to verify the results. Plasmid Inc groups were determined in a subset of 49 randomly chosen tetracycline-resistant isolates (23 *S. flexneri* and 26 *S. sonnei)* plus 10

tetracycline-susceptible isolates (6 *S. flexneri* and 4 *S. sonnei*) following the methodology of Carattoli et al. as described previously [16].

2.4. Statistical analysis

Micro-organisms were grouped on the basis of their geographical origins and were reported as from 'Africa', 'Latin America', 'Asia', 'not determined' or 'other' (multicontinental trips). Fisher's exact text was used to determine the presence of significant differences. A significant difference was considered at a *P*-value of <0.05, incorporating a post-hoc Holm–Bonferroni adjustment.

3. Results and discussion

Currently at least 59 tetracycline resistance determinants have been described, with 28 being present in Gram-negative micro-organisms [11]. Studies determining the presence of these genes among Enterobacteriaceae have described a great variety of genes, particularly the *tet*(A) and *tet*(B) genes [11,12]. This is also confirmed in the present study, with 154 tetracycline-resistant isolates (62 *S. flexneri* and 92 *S. sonnei*) plus 1 tetracycline-intermediate *S. sonnei* isolate being identified. Of these, 122 (79.2%) isolates presented at least one of these two genes (Table 1). In addition, although not included in the analysis, one and five tetracycline-resistant *Shigella boydii* and *Shigella dysenteriae*, respectively, were also recovered during the same period. The *tet*(B) gene was the most frequently detected tetracycline resistance determinant among the 154 tetracycline-resistant *Shigella* spp. isolates, being present in 70 isolates (45.5%), whilst the *tet*(A) gene was detected in 57 isolates (37.0%). On the other hand, the *tet*(G) gene was present in only 11 isolates

(7.1%). Interestingly, *tet*(G) was the only gene detected in three isolates, whilst in the remaining eight cases this gene was present together with *tet*(A) or *tet*(B). The *tet*(G) gene has also been previously described both in *S. flexneri* and *S. sonnei* [12] and in other Enterobacteriaceae and is typically found in *Salmonella enterica* serovar Typhimurium DT104 as a part of the Salmonella Genomic Island 1 (SGI1) [19].

No isolate harboured the three genes together. The combinations *tet*(A) + *tet*(G), *tet*(A) + *tet*(B) and *tet*(B) + *tet*(G) were found in one, five and seven isolates, respectively. The concomitant presence of genes encoding antibiotic resistance mechanisms against the same antibiotic is not new and may be related to different phenomena, including antibiotic resistance co-selection, bacterial promiscuity or potential additive bacterial benefits such as higher or expanding resistance spectrum or accelerated response to the presence of antibacterial agents.

In addition, none of the mechanisms studied were detected in 29 (18.8%) of the 154 tetracycline-resistant *Shigella* spp. isolates or in the *S. sonnei* tetracycline-intermediate isolate. No mechanism of tetracycline resistance was detected in the tetracycline-susceptible isolates.

However, on analysis by species, differences were found in the prevalence of *tet*(A) and *tet*(B). The *tet*(A) gene was statistically more frequent in *S. sonnei* [$P < 0.0001$ both when alone ($P < 0.0001$) or overall ($P = 0.0007$)]. The *tet*(B) gene was more frequently recovered from *S. flexneri* both when alone or overall ($P < 0.0001$) (Table 1). Differences, albeit not significant ($P = 0.1444$), were detected in the species

distribution of isolates in which none of the three genes studied were found (12.9% of tetracycline-resistant *S. flexneri* and 22.8% of tetracycline-resistant *S. sonnei*).

As mentioned previously, genetic divergences have been described between the two species. In a study on *Shigella* spp. causing TD, a different species distribution was observed among some ampicillin resistance determinants. OXA-1-like β-lactamases were abundant among *S. flexneri* strains, whilst *S. sonnei* mainly harboured genes encoding OXA-2 and OXA-5 [3]. In the same study, 56% of *S. flexneri* isolates were also observed to be chloramphenicol-resistant due to the production of chloramphenicol acetyltransferases, whilst all *S. sonnei* isolates were susceptible to chloramphenicol [3]. These findings have been reported in other studies developed in specific areas or using TD isolates [10,12–14]. Owing to the high diversity of geographical origins represented by micro-organisms included in aforementioned studies, these results, together with those of the present study, suggest a genetic divergence in the mechanisms of antibiotic resistance that each of these species carries.

The geographical prevalence of each determinant was also established. The different species-specific prevalence of the *tet*(A) and *tet*(B) genes was maintained, except in isolates from Latin America in which the most prevalent gene was *tet*(B) irrespective of the species analysed. This finding has also been observed in other studies in this geographical area [15]. In addition, in the isolates of *S. sonnei* in which no information about travel was recorded, the presence of the *tet*(B) gene was also slightly higher than that of the *tet*(A) gene (Table 2). The presence of other

tetracycline resistance determinants may explain the detected resistance levels in the absence of Tet(A), Tet(B) or Tet(G).

Whilst the presence of antibiotic resistance determinants has been detected in pristine environments [1], geographical differences in the carriage of specific resistance determinants may be due to different reasons, including the direct effect of human action, which may favour the selection of specific strains, as well as the presence of different bacterial clones with different characteristics. In this sense, as tetracycline and its derivatives are highly consumed worldwide both in human and veterinary settings, the present geographical differences, such as the higher presence of *tet*(B) in both species in Latin American samples, is probably related to the bacterial clones in circulation in this area, which might be selected by the specific antibiotic usage present in this area. Therefore, it is of special relevance to highlight that Tet(B) is able to confer resistance to minocycline, whereas no other efflux pump encoded in a transferable *tet* gene has this characteristic [11]. This finding might correlate with geographical differences in the use of this antimicrobial agent, or with antibiotic resistance co-selection due to the concomitant presence of *tet*(B) and other local selected antibiotic resistance determinants in the same genetic structure.

Navia et al. [3] proposed the possible association between differences in the prevalence of antibiotic resistance determinants and the carriage of specific plasmids. To evaluate this possibility, plasmids Inc groups of a subset of isolates were classified. Interestingly, it appears that the presence of the plasmid Inc groups differed between *S. flexneri* and *S. sonnei*. Thus, plasmids belonging to Inc group B ($P < 0.05$) were significantly more frequent among *S. flexneri*, whilst those belonging

to groups K, FIC and FIIA (all $P < 0.05$) were preferentially detected among *S. sonnei* isolates. No plasmid belonging to the remaining Inc groups studied (HI1, HI2, X, L and M) was detected. It is interesting to note that the number of plasmids belonging to some F types (e.g. FIIA, FIC) was higher than detected using the generic primers for F that were designed to recognise all of the plasmids belonging to the heterogeneous group of IncF plasmids. This phenomenon was previously observed by Carattoli et al. [16]. In our case, this finding might be related to the heterogeneous origin of the samples, including isolates from all continents. However, analysis of the results showed no association with either the *tet*(A) or *tet*(B) genes and any of the plasmid Inc groups studied, suggesting that these genes are not restricted to a single Inc type (Table 3). Despite this lack of association between plasmid carriage and the reported differences in the presence of *tet*(A) and *tet*(B) genes, these differences might be related to divergences described in the presence of other mechanisms of antibiotic resistance [3] or virulence [20]. Further studies are needed to determine this possible relationship.

The main limitation of this study is the sample size, which might underlie the borderline significance of some of associations analysed. None the less, this study of pathogens causing diseases in international travellers is an indirect source of sanitary information for the visited areas. Therefore, a TD collection of *Shigella* spp. isolates would be highly representative, showing a general worldwide scenario.

4. Conclusions

In summary, the present study reveals that while the *tet*(A) and *tet*(B) genes were frequently detected as a cause of tetracycline resistance in *Shigella* spp. causing TD,

the presence of *tet*(G) gene was infrequent. Although differences in the Inc plasmid groups carried by *S. sonnei* and *S. flexneri* were observed and the prevalence of the *tet*(A) and *tet*(B) genes differed between *S. sonnei* and *S. flexneri*, no specific associations between *tet* determinants and the Inc plasmid groups were observed. These species-specific differences in the *tet* determinants of *S. sonnei* and *S. flexneri* were observed in all geographical areas, except Latin America. In Latin America, the *tet*(B) gene was most frequently carried both in *S. flexneri* and *S. sonnei*. This finding suggests a different direct, or indirect, environmental pressure. The presence of barriers to horizontal gene transmission has not been shown but may not be ruled out.

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Highlights

- *tet*(B) was the most frequently detected tetracycline resistance determinant in *Shigella* isolates.

- The *tet*(A) gene was more frequent in *Shigella sonnei*.

- The *tet*(B) gene was more frequent in *Shigella flexneri*.

- Plasmid incompatibility (Inc) groups showed a species-specific distribution.

- No relationship between plasmid Inc groups and *tet* genes was found.

Table 1

Presence of tetracycline resistance determinants in *Shigella* spp. isolates from patients with traveller's diarrhoea

Shigella sp.	No. of TET^R isolates	No. of TET^R isolates with *tet* gene(s)						
		tet(A)	*tet*(B)	*tet*(G)	*tet*(A) + *tet*(B)	*tet*(A) + *tet*(G)	*tet*(B) + *tet*(G)	N/D
S. flexneri	62	9 *	36 *	1	4	0	4	8
S. sonnei	93 [a]	42 *	22 *	2	1	1	3	22 [a]
Total	155 [a]	51	58	3	5	1	7	30 [a]

TET^R, tetracycline resistant; N/D, not determined.

[a] Includes one intermediate-resistant isolate.

* The analysis of the presence of *tet*(A) and *tet*(B) alone showed a significant difference between *S. flexneri* [higher presence of *tet*(B)] and *S. sonnei* [higher presence of *tet*(A)] ($P < 0.0001$). When the overall presence of the genes was analysed *tet*(A) was more prevalent among *S. sonnei* ($P=0.0007$) and *tet*(B) among *S. flexneri* ($P<0.0001$)

Table 2

Geographical origin of the tetracycline resistance determinants detected in *Shigella* spp. isolates

Shigella sp.	*tet* gene [a]	No. of isolates with *tet* gene by geographical location				
		Africa	Latin America	Asia	N/D	Total
S. flexneri	*tet*(A)	5	4	2	2	13
	tet(B)	18	13	6	7	44
	tet(G)	2	2	0	1	5
	Total	25	19	8	10	62
S. sonnei	*tet*(A)	19	4	15	6 **	44
	tet(B)	6	12	1	7	26
	tet(G)	0	3	3	0	6
	Total	25	19	19	13	76
Total		50	38	27	23	138

N/D, not determined [cases with no travel data available plus one case (**) reporting an intercontinental journey to Asia and Oceania].

[a] Number of genes detected, irrespective of whether detected alone or in combination.

Table 3

Association between plasmid incompatibility (Inc) groups and the presence of the *tet*(A) and *tet*(B) genes in *Shigella* spp. isolates

Shigella sp.	N	No. of isolates with plasmids of Inc type:												
		A/C	B	F	FIA	FIIA	FIB	FIC	I1	K	P	T	W	Y
By resistance phenotype														
S. flexneri	R (23)	1	13	18	1	0	5	1	3	2	2	1	3	1
	S (6)	0	3	3	1	0	1	0	0	0	0	0	3	1
	Total (29)	1	16	21	2	0	6	1	3	2	2	1	6	2
S. sonnei	R (26)	9	1	9	1	13	1	12	6	19	6	3	5	12
	S (4)	1	0	2	1	1	0	3	2	3	3	0	0	3
	Total (30)	10	1	11	2	14	1	15	8	22	9	3	5	15
Total	59	11	17	32	4	14	7	16	11	24	11	4	11	17
By *tet* gene														
S. flexneri	*tet*(A) (*n* = 5)	0	2	5	0	0	1	0	0	0	1	0	0	0
	tet(B) (*n* = 14)	1	7	9	0	0	3	1	0	2	2	0	0	1
S. sonnei	*tet*(A) (*n* = 12)	7	0	4	0	7	0	6	0	10	2	0	0	6
	tet(B) (*n* = 7)	2	0	1	0	2	0	4	0	5	3	0	0	4
Total	38	10	9	19	0	9	4	11	0	17	8	0	0	11

R, resistant; S, susceptible.