VIRULENCE AND RESISTANCE PROFILES OF *SHIGELLA* STRAINS ISOLATED IN ROMANIA FROM 2016 TO 2018

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ABSTRACT

This study aimed to describe the virulence gene content and antibiotic resistance profiles as well as the genetic relatedness of Shigella strains originated from Romanian patients, collected between 2016-2018 through the National Diarrheal Disease Surveillance Program.

A total of 60 *Shigella sonnei* strains and 26 *S. flexneri* strains were tested against a set of 16 antimicrobials, screened by PCR for several genes involved in the disease pathogenesis (*ipaH*, *ipaBCD*, *ial*, *sen*, *set1A*, *set1B*, *sat* and *pic* genes), and typed by pulsed-field gel electrophoresis (PFGE) to make inferences about their genetic relatedness.

Overall, the resistance most commonly acquired by the autochthonous *Shigella* strains investigated was against sulfonamide compounds (58/86 strains), trimethoprim (46/86 strains) or combination of trimethoprim with sulfamethoxazole (44/86 strains), and ampicillin (41/86 strains). Strains with resistance to extended spectrum cephalosporins (12/86 strains) were detected, all extended spectrum beta-lactamase producers, as well as strains that displayed resistance to nalidixic acid (3/86 strains) but not to ciprofloxacin.

The virulence markers targeted ranged in prevalence from 6% (*set1A*, *set1B*, *pic*) to 100% (*ipaH*). The *S. flexneri* strains displayed a richer virulence gene content than *S. sonnei* strains. The most complex virulence genotype, represented by the association of *ipaH*, *ipaBCD*, *ial*, *sen*, *sat*, *set1A*, *set1B*, and *pic* genes was found in *S. flexneri* serotype 2a strains.

The PFGE-based molecular typing allowed the detection of several clusters of strains displaying band-based profiles with at least 85% similarity. Eight such clusters were identified among the *S. sonnei* strains and four clusters among the *S. flexneri* strains.

Continued laboratory-based surveillance is essential to generate data that help to draw the picture of the public health problem represented by shigellosis in Romania.

Keywords: *Shigella*, virulence genes, antibiotic resistance, PFGE

REZUMAT

Acest studiu a urmărit să descrie conţinutul de gene de virulenţă şi profilurile de rezistența la antibiotice, precum şi gradul de înrudire genetică al unor izolate de *Shigella* provenite de la pacienți români, colectate în perioada 2016-2018, prin programul naţional de supraveghere a bolii diareice.

Un total de 60 de tulpini de *Shigella sonnei* și 26 de *Shigella flexneri* au fost testate faţă de 16 substanţe antimicrobiene, analizate prin tehnica PCR pentru gene implicate în patogeneză (genele *ipaH*, *ipaBCD*, *ial*, *sen*, *set1A*, *set1B*, *sat* and *pic*) şi tipizate prin electroforeză în câmp pulsator, pentru a se evalua gradul lor de înrudire genetică.

Cel mai comun profil de rezistenţă la antibiotice căpătat de tulpinile autohtone de *Shigella* a fost faţă de compuşi sulfonamidici (58/86 tulpini), trimethoprim (46/86 tulpini), trimethoprim asociat cu sulfamethoxazol (44/86 tulpini) şi ampicilină (41/86 tulpini). Au fost detectate tulpini cu rezistenţă la beta-lactamine cu spectru extins (12/86 tulpini), toate producatoare de beta-lactamaze cu spectru extins, precum şi tulpini rezistente la acid nalidixic (3/86 tulpini), dar nu şi la ciprofloxacin.

Prevalenţa markerilor de virulenţă a variat între 6% (*set1A*, *set1B*, *pic*) şi 100% (ipaH). Tulpinile de *S. flexneri* au prezentat un conţinut de gene de virulenţă mai bogat decat cele de *S. sonnei.* Cel mai complex genotip de virulenţă, reprezentat de asocierea genelor *ipaH, ipaBCD, ial, sen, sat, set1A, set1B* şi *pic,* a fost găsit în tulpinile de *S. flexneri* serotip 2.

Tipizarea moleculară bazată pe electroforeza în câmp pulsator a permis detectare mai multe clustere de tulpini care prezentau profiluri de benzi cu similaritate de cel puţin 85%. Opt astfel de clustere s-au detectat printre tulpinile de *S. sonnei* şi patru printre cele de *S. flexneri.*

Supravegherea continuă bazată pe laborator este esenţială pentru a genera datele care să ajute la întelegerea problemei de sănătate publică reprezentată de shigelozele din România.

Cuvinte-cheie: *Shigella*, gene de virulenţă, rezistenţă la antibiotic, PFGE

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INTRODUCTION

Shigella species is a highly virulent enteric pathogen, reported to be an important cause of diarrhea in developing countries and highly contagious due to its low infective dose [1]. Shigellosis, the infection caused by *Shigella* spp., can progress to severe disease, depending on the virulence potential of the strain and the nutritional status of the individual [2].

Shigellosis is caused by penetration of invasive *Shigella* into the intestinal mucosa of the colon, leading to degeneration of the epithelium and a strong inflammatory response. Several virulence factors, which are the expression of arrays of virulence genes located in the chromosome or on a large virulence plasmid (pINV) are described as important for pathogenesis, being associated with colonization, invasion/penetration of the epithelium, intercellular spread and toxin-mediated disease. Among them are the following: the invasion-associated locus (ial), the invasion plasmid antigen H *(ipaH),* the invasion plasmid antigens B (ipaB), C (*ipaC)* and D (ipaD), Shigella enterotoxin 1 or ShET-1 (*set1A* and *set1B* genes), Shigella enterotoxin 2 or ShET-2 (sen gene), and the secreted auto-transporter (*sat* gene) [3].

Chromosomal genes, *set1A* and *set1B* are encoding the factors associated with the watery phase of diarrhea. ShET-1 and ShET-2, in addition to their enterotoxic activity, play an important role in the transport of electrolytes and water in the intestine.

Shigellosis is an invasive illness of the human colon that leads to varied clinical symptoms ranging from mild watery diarrhea to severe colitis. Antimicrobial therapy is required in the cases of severe dysentery to reduce the duration of clinical illness, minimize the complications, and prevent the dissemination of infectious cases, conferring significant public health benefits [4].

Fluoroquinolones, β-lactams and cotrimoxazole were considered to be the first choice for treating shigellosis. However, in the last years, the use of these drugs has been compromised by the emergence of resistant strains. *Shigella* has developed mechanisms of resistance based on different mobile genetic elements (plasmids, integrons and transposons) which play a central part in the clinical dissemination of antibiotic resistance genes and have allowed for the rapid development of multi-antibiotic resistance [5].

This study aimed to provide meaningful information about the antibiotic resistance phenotypes, virulence profiles, and the genetic relatedness of the latest *Shigella* strains reported through the Romanian national surveillance program of diarrhea disease.

MATERIAL AND METHODS

Bacterial isolates

Between 2016 and 2018 years, a total of 86 *Shigella* strains were confirmed by the Reference Laboratory for Bacterial Enteric Infections in Cantacuzino National Medico-Military Institute for Research and Development. They were serotyped as group B/*S. flexneri* (26 strains) and group D/*S. sonnei* (60 strains), respectively. The strains assigned to *S. flexneri* were distributed in the following serotypes: 1b (8 strains), 2a (5 strains), 3a (1 strain), 4a (1 strain), 5 (2 strains), 6 (9 strains). All these strains originated from patients with acute diarrheal disease residing in various Romanian counties. The laboratory confirmation was performed using conventional biochemical tests and commercially available antisera (Denka Seiken, Tokyo). All the strains were stored at -20°C in Luria Bertani broth containing 15% glycerol.

Antimicrobial susceptibility testing

All the *Shigella* strains were screened by the Kirby Bauer disk diffusion method for antibiotic susceptibility and the breakpoint values for qualitative interpretation were based on the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/ast_of_bacteria/guidance_documents). The following antimicrobial agents were tested: ampicillin (AMP, 10 μg), cefotaxime (CTX, 5μg), ceftazidime (CAZ,10 μg), cefoxitin (FOX, 30 μg), meropenem (MEM, 10 μg), imipenem (IMP, 10 μg), chloramphenicol (C, 30 μg), streptomycin (S, 10 μg), gentamicin (CN,

30μg), kanamycin (K, 30 μg), sulphonamide compounds (S3, 300 μg), tetracycline (TE, 30 μg), trimethoprim (W, 5 μg), trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 μg), nalidixic acid (NA, 30 μg) and ciprofloxacin (CIP, 5 μg). *Escherichia coli* ATCC 25922 was included as a reference strain for antimicrobial susceptibility testing quality control.

The antibiotic disks were obtained from Oxoid (Basingstoke, United Kingdom). In this study, multidrug resistance (MDR) was defined as resistance to three or more drugs from unrelated antibiotic classes. Detection of extended-spectrum beta-lactamase (ESBL) production in the isolates with resistance to third-generation cephalosporins was performed using a cefotaxime/ceftazidime/amoxicillin/clavulanate synergy test previously described [6].

PCR assays for virulence markers

All DNA templates used for PCRs were prepared by boiled cell lysis. The 4-5 colonies were suspended in 200 μl of sterile molecular-grade water and boiled for 15 minutes. The supernatant containing the DNA, obtained after centrifugation at 5 minutes at 14,000 rpm, was stored at −20°C till further use for PCR testing.

Each sample was submitted to PCR amplification of the following virulence markers: *ipaH*, *ipaBCD*, *ial*, *set1A*, *set1B*, *sen*, *sat* and *pic*, using previously described primers and protocols [7].

Pulsed-field gel electrophoresis (PFGE)

Shigella strains were analyzed by pulsed-field gel electrophoresis (PFGE) following the PulseNet standardized protocol for *Shigella* (https://www.cdc.gov/pulsenet/pdf/ecoli-shigella-salmonella-pfge-protocol-508c) and genomic DNA was digested with the restriction endonuclease *Xba*I (Roche). Genomic DNA was separated in 1% SeaKem Gold agarose (Lonza) with a CHEF Mapper system (BioRad, Hercules, USA) using a run time of 19 hours. *Salmonella enterica* serovar Braenderup H9812 strain was used as size marker. The electrophoretic profiles were analyzed using BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Banding patterns were compared based on the Dice similarity coefficient and a dendrogram was constructed by UPGMA (unweighted pair -group method with arithmetic averages) algorithm with a setting of 1.0% for optimization and position tolerance of 1.0% for band comparison. Clusters designations were assigned at the ≥85% profile similarity level (approximately 3-band difference).

RESULTS

Prevalence of virulence genes in Shigella strains

Overall, among the 86 *Shigella* strains studied, between two and eight virulence gene markers were found per strain (Table 1). While all the strains harbored *ipaH* gene only 6% of them carried *set1A*, *set1B* or *pic* genes. *S. sonnei* was represented by strains with less virulence gene markers than *S. flexneri*. Among the *S. flexneri* serotypes, serotype 2a was represented by strains exhibiting the highest number of virulence genes (virulence profiles comprising 8 virulence markers) while serotype 6 displayed the lowest number. The virulence gene distribution is shown in Table 2.

Antibiotic resistance phenotypes

Overall, ten strains were susceptible to all the antibiotics tested and 76 strains were resistant at least to one of them. The twenty-one antibiotypes defined by resistance against one to seven antimicrobial agents did not comprise resistance to meropenem, ciprofloxacin and gentamycin (Table 3). Twenty-four strains, mostly assigned to *S. flexneri* species, were MDR strains. The most commonly acquired resistance was against sulphonamide compounds (58 strains), trimethoprim alone (46 strains) or in combination with sulfamethoxazole (44 strains), and against ampicillin (41 strains), and 18 strains (21%) were tetracycline resistant. Only 3 strains (3%) displayed resistance to nalidixic acid but not to ciprofloxacin. Twelve strains (14%) were resistant to cefotaxime and/or ceftazidime and yielded a positive result in the ESBL phenotypic test.

Table 1. The virulence genotypes displayed by the 86 *Shigella* isolates

Species and serotype	Virulence patterns	Number of strains
S. flexneri		
1b	*ipaH, ipaBCD, ial, sat, sen*	4
	ipaH, ial, sat, sen	1
	ipaH, sen	1
	ipaH, sat	2
2a	*ipaH, ipaBCD, ial, sat, pic, sen, set1A, set1B*	5
3a	*ipaH, ipaBCD, ial, sat, sen*	1
4a	*ipaH, ipaBCD, ial, sat, sen*	1
5	*ipaH, ipaBCD, ial, sat, sen*	1
5	*ipaH, sen*	1
6	*ipaH, ial, sen*	9
S. sonnei	*ipaH*	27
	ipaH, ial	4
	ipaH, ial, sen	23
	ipaH, ipaBCD, ial, sen	6

Table 2. The distribution of the virulence markers among the 86 *Shigella* strains studied

Species and serotypes	No. of strains	Number (%) of strains carrying							
		ipaH	*ipaBCD*	*ial*	*sen*	*sat*	*set1A*	*set1B*	*pic*
S. flexneri	26	26 (100)	12 (46)	22 (85)	24 (92)	15 (58)	5 (19)	5 (19)	5 (19)
1b	8	8	4	5	6	7	0	0	0
2a	5	5	5	5	5	5	5	5	5
3a	1	1	1	1	1	1	0	0	0
4a	1	1	1	1	1	1	0	0	0
5	2	2	1	1	2	1	0	0	0
6	9	9	0	9	9	0	0	0	0
S. sonnei	60	60 (100)	6 (10)	33 (55)	29 (48)	0	0	0	0

According to the antibiotic resistance profiles, a total of 21 antibiotypes were distinguished among the studied isolates, defined by resistance against one to seven antimicrobial agents (Table 3).

PFGE typing in relationship with the antibiotic resistance profiles of Shigella strains

PFGE was performed in order to explore the potential dissemination of clonal groups of resistant *Shigella* across Romania. By applying the 85% similarity criterion, the genetic relatedness of the strains allowed a cluster distribution. Eight clusters were revealed among the 60 *S. sonnei* strains while among the 26 *S. flexneri* group four clusters were detected: one cluster for serotype 1b, one cluster among the serotype 6, and two clusters among serotype 2a. There was a clear correlation between the resistance profiles and the PFGE patterns. Specifically, 5 *Shigella* strains with AMP^R, W^R, $S3^R$, SXT^R resistance profile were included in the same cluster and so were also 12 other strains that had W^R, $S3^R$, SXT^R resistance profile, which suggested their genetic relatedness.

Table 3. Antibiotic resistance profiles detected among the 86 *Shigella* spp. strains

Resistance profile	No. of isolates
Susceptible	10
AMP^R	2
$S3^R$	9
W^R, NA^R	1
AMP^R, CTX^R	6
AMP^R, S^R, $S3^R$	3
W^R, $S3^R$, SXT^R	21
AMP^R, C^R, TE^R	1
AMP^R, CTX^R, CAZ^R	1
AMP^R, C^R, TE^R, S^R	5
AMP^R, W^R, $S3^R$, SXT^R	12
AMP^R, W^R, C^R, TE^R, S^R	1
AMP^R, W^R, $S3^R$, CTX^R, SXT^R	1
W^R, TE^R, S^R, $S3^R$, SXT^R	2
AMP^R, C^R, TE^R, S^R, CTX^R, CAZ^R	1
AMP^R, W^R, $S3^R$, CTX^R, CAZ^R, SXT^R	1
AMP^R, W^R, TE^R, S^R, $S3^R$, SXT^R	1
W^R, TE^R, S^R, $S3^R$, SXT^R, NA^R	2
AMP^R, W^R, C^R, S^R, $S3^R$, SXT^R	1
AMP^R, C^R, TE^R, S^R, $S3^R$, CTX^R, CAZ^R	2
AMP^R, W^R, C^R, TE^R, S^R, $S3^R$, SXT^R	3

DISCUSSION

Shigella infections are a serious health problem in the world, being responsible for 125 million diarrheal episodes each year with about 160000 deaths, a third of them among the pediatric population [2, 8]. Antibiotic therapy is recommended to diminish potential complications hence the risk of mortality, improve the clinical status and decrease transmission by eliminating *Shigella* from the gut [9].

Shigella strains harbor many virulence factors, including factors that are associated with invasion of the colonic epithelium and toxins [10]. The genetic determinants associated with invasion assist *Shigella* in penetration of epithelial cells (i.e. *ial*), are the effectors of bacterial entry into the host cell (i.e. *ipa BCD*) or facilitate cell-to-cell spread (i.e. *ipaH*). In our study, the *ipaH* gene was detected in all the isolates, while the cluster *ipaBCD* had a much lower prevalence and was more commonly found in *S. flexneri* than in *S. sonnei* strains. A possible explanation can be that *ipaH* gene is a much more stable marker with multiple copies located on both plasmids and chromosome whereas the locus which includes the *ipaBCD* genes can be lost by spontaneous deletions [11, 12]. It is worth noting that among the autochthonous strains *ipaBCD* cluster was more frequently present in *S. flexneri* strains than in *S. sonnei*.

Regarding the overall prevalence of the other virulence determinants targeted, *ial* and *sen* genes which displayed similar rates, were the second most prevalent virulence markers detected in the autochthonous strains after *ipaH*. However, when considering how many strains within each species harbored these genes, a significantly higher proportion of *S. flexneri* strains than *S. sonnei* ones possessed them (92% vs. 48%). A report resulted from an eight-year Chinese study showed a major difference between *S. sonnei* and *S. flexneri* only in terms of *sen* gene prevalence [13] and similar findings were also reported in an Indian study [14].

Across the Shigella strains investigated in this study, the genes coding for the ShET1 enterotoxin (*set1A* and *set1B*) and Pic (*pic*) were

found exclusively in *S. flexneri* 2a strains, a finding which contributed to the overall higher virulence potential assigned to this species. The studies of Noriega *et al*. [15] and Vargas *et al*. [16] also showed that *set1A* and *set1B* were present exclusively in *S. flexneri* 2a. Also, a Malaysian study confirmed that the set of *set1A* and *set1B* genes is highly conserved in this serotype [17]. The *sat* gene which codes for serine protease autotransporters of the *Enterobacteriaceae* family Sat was another marker present in *S. flexneri* (with the exception of serotype 6 members) but absent from *S. sonnei*.

Besides the description of the virulence genotypes, the study aimed to evaluate how many of the shigellosis cases diagnosed during 2016-2018 could have been treated efficiently based on the antimicrobial susceptibility expressed by the *Shigella* isolates that caused them. It is well-known that antimicrobial therapy is beneficial but microbial resistance to many of the widely used antimicrobials is leading to failures in an increasing number of cases [17]. Based on the susceptibility results, useful antibiotics such as ampicillin and the combination trimethoprim-sulfamethoxazole are no longer a choice for treating the autochthonous patients with shigellosis. Moreover, 28% of the investigated strains proved to be MDR strains.

Among them, there were strains with resistance to third-generation cephalosporins. Specifically, 14% of *Shigella* strains investigated had acquired such antibiotic resistance due to the capacity to produce extended-spectrum beta-lactamases. Based on previous reports, similar strains also circulate in other countries [19-21]. This is concerning because the third-generation cephalosporins are considered as the useful alternative for infections caused by *Shigella* strains with resistance to fluroquinolones.

Fluoroquinolones, currently recommended by the World Health Organization as first-line treatment for adults, proved to be still very efficient against the *Shigella* strains recovered from the Romanian patients, as none of them displayed resistance to ciprofloxacin. Moreover, based on the results of this study, even the nalidixic acid could be still used efficiently for treating the autochthonous patients as only 3% of the strains displayed a resistance phenotype.

By contrast, in other parts of the world, this drug is no longer used due to the high rates of resistance found, this being the reason for the switch to fluoroquinolones as an alternative [17].

In conclusion, this study provided evidence about the circulation of resistant and virulent strains of *Shigella* and underlined that continued laboratory-based surveillance is essential to generate data that help to draw the picture of the public health problem represented by shigellosis in Romania.

Conflict of interests: No conflict of interest to declare.

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