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# Prevalence of virulence markers and pHS-2-like plasmids among Shigella sonnei and Shigella flexneri isolates originating from shigellosis cases in Romania

## Prevalenţa markerilor de virulenţă şi a plasmidelor de tip pHS-2 in izolatele de Shigella sonnei şi Shigella flexneri provenite din cazurile de shigelloză din Romania

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**Abstract**

*The surveillance of shigellosis is carried out under the auspice of the European Centre for Disease Prevention and Control which requires a reliable laboratory-based surveillance at national level. To date, little information is published about the members of Shigella spp. responsible for Romanian cases of shigellosis which hinders the understanding of the current epidemiology of shigellosis. Consequently, this retrospective study aimed to assess the diversity of virulence profiles displayed by the strains circulating in our region, by using key chromosome- and plasmid-associated markers, and to document the prevalence of pHS-2-like plasmid previously proposed as a potential marker for reactive arthritis.*

*The study focused on 65 Shigella sonnei and 49 Shigella flexneri clinical isolates, originated from local patients, recovered through the national surveillance system in 2009 - 2013. PCR assays were performed for the detection of ipaH, ipaBCD, ial, sen, set1A, set1B, sat, and pic genes, and a PCR-sequencing approach on plasmid preparations was used for identifying pHS-2-specific sequences.*

*Overall, the virulence markers ranged in prevalence from 21% (set1A, set1B, pic) to 100% (ipaH). S. flexneri isolates displayed a higher content of virulence markers than S. sonnei, the most common genotype, detected exclusively in S. flexneri serotype 2a isolates, being defined by the association ipaH+ipaBCD+ial+sen+sat+set1A+set1B+pic. pHS-2-like plasmids were found in S. flexneri isolates of various serotypes suggesting the potential to trigger postinfection complications in specific subjects.*

*This study provided baseline data regarding the molecular characteristics of the Shigella strains from Romania, useful for defining the picture of shigellosis in our region.*

***Keywords**: Shigella, virulence genes, pHS-2 plasmid*

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## Rezumat

*Supravegherea shigellozei este coordonată de Centrul European pentru Prevenţia şi Controlul Bolilor, care solicită ca la nivel naţional aceasta să se realizeze printr-un sistem solid bazat pe laborator. În prezent, există puţine informaţii publicate referitoare la membrii genului Shigella implicaţi in cazurile de shigelloza din Romania, ceea ce îngreunează înţelegerea epidemiologiei actuale a acestei infecţii. Drept urmare, acest studiu retrospectiv a urmărit să evalueze diversitatea profilurilor de virulenţă la tulpinile care circulă local, folosind markeri cromozomali si plasmidici reprezentativi, precum si să aducă informaţii privitoare la prevalenţa plasmidului de tip pHS-2, propus drept marker prognostic pentru declanşarea artritei reactive.*

*Studiul s-a concentrat pe 65 de izolate clinice de Shigella sonnei si 49 de Shigella flexneri, provenite de la pacienti autohtoni si colectate în cadrul sistemului de supraveghere naţională în perioada 2009-2013. Pentru detectarea genelor ipaH, ipaBCD, ial, sen, set1A, set1B, sat şi pic s-au realizat teste PCR, iar pentru identificarea secvenţelor specifice plasmidului pHS-2 s-a aplicat un protocol care a combinat tehnica PCR cu secvenţierea ampliconului.*

*Per total, prevalenţa markerilor de virulenţă a fost între 21% (set1A, set1B, pic) şi 100% (ipaH). Izolatele de S. flexneri au prezentat un conţinut mai bogat de markeri de virulenţa prin comparaţie cu cele de S. sonnei, cel mai frecvent genotip al acestora, identificat exclusiv la S. flexneri de serotip 2a, fiind definit de asocierea ipaH+ipaBC D+ial+sen+sat+set1A+set1B+pic. Plasmide de tip pHS-2, găsite în izolatele de S. flexneri din serotipuri variate, au sugerat potenţialul acestora de a declanşa complicaţii postinfecţioase la anumite categorii de indivizi.*

*Studiul a furnizat informaţii de referinţă despre caracteristicile moleculare ale tulpinilor de Shigella circulante în Romania, utile pentru conturarea contextului epidemiologic al shigellozei în ţara noastră.*

***Cuvinte cheie**: Shigella;gene de virulenţă;plasmidul pHS-2*

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## Introduction

*Shigella* are worldwide major human-specific pathogens which cause shigellosis or bacillary dysentery, a colonic infection that may present as a benign short-lasting watery diarrhea or the aggressive acute inflammatory bowel disease characterized by mucopurulent bloody diarrhea and extraintestinal complications (1, 2). The essential features involved in the intestinal pathogenicity of the four *Shigella* species/serogroups described (i.e. *S. dysenteriae*/serogroup A, *S. flexneri*/serogroup B, *S. boydii*/serogroup C, and *S. sonnei*/serogroup D) are the bacterial ability to invade the resident macrophages and intestinal epithelium, and the potential to induce and modulate host inflammatory responses (3, 4). The mechanisms underlying these events imply the intervention of products of many genes, located on the chromosome but mostly on large single-copy (virulence) plasmids that form a closely related family, collectively termed pINV. Similar plasmids are found in the enteroinvasive *Escherichia coli* strains (5). The roles of many of these products (e.g. effectors, chaperons, components of the type III secretory system, regulatory proteins) in pathogenicity are starting to be elucidated but there is still much to learn about *Shigella* strategy of survival and infection.

In addition to the acute symptomatology and the colonic mucosa damage, a variety of rheumatologic phenomena, defined as reactive arthritis (ReA), has been associated with shigellosis (6-9). It was suggested that HLA-B27-positive individuals may have an increased susceptibility to ReA (10). A molecular mimicry between a domain of the host's HLA-B27 histocompatibility antigen and a small peptide of 5 amino acids (Ala, Gln, Thr, Asp, Arg) encoded by a 2-megadalton/3 kb plasmid (i.e. pHS-2), detected in the so-called arthritogenic strains, was proposed as a possible explanation (11, 12).

Although not endemic in Romania, shigellosis is a public health issue that needs an improved surveillance and sustained efforts to understand its epidemiologic trends. During the most recent period for which data are officially available (2010-2012) in the reports of the European Centre for Disease Prevention and Control (ECDC) (http://ecdc.europa.eu/en/publications/surveillance_reports/Pages/index.aspx?p=2), there were 1018 confirmed shigellosis cases in Romania. Since little is known about the genotypes of the strains responsible for these infections, we undertook the present study to gain an understanding of the molecular basis of virulence among a set of clinical isolates reported to the national surveillance system. Specifically, we assessed the diversity of virulence profiles using key chromosome- and plasmid-associated virulence markers (i.e. *ipa*, *ial*, *set*, *sen*, *sat*, and *pic* genes) and the presence of pHS-2-like plasmids as potential markers for ReA sequelae.

## Material and methods

### *Bacterial strains*

We analyzed a set of 114 isolates: 65 identified as *S. sonnei* and 49 as *S. flexneri*, respectively. The *S. flexneri* subset comprised isolates belonging to the following serotypes: 1a/1 isolate, 2a/26 isolates, 3a/8 isolates, 3b/1 isolate, 3c/2 isolates, 4a/5 isolates, 6/4 isolates, variant X/1 isolate, and variant Y/1 isolate. They were collected between 2009 and 2013 and originated from patients with acute diarrhoeal disease residing in various Romanian counties (i.e. Bacău, Brăila, Călăraşi, Constanţa, Covasna, Dâmboviţa, Iaşi, Mehedinţi, Neamţ, Prahova, Sălaj, Suceava). The identification at species level was performed at the National Reference Laboratory for Bacterial Enteric Infections from Cantacuzino National Institute of Research (Bucharest) using conventional biochemical tests and commercially available antisera (Denka Seiken).

### *Total DNA preparation*

A rapid boiling method was used for total DNA extraction from culture. Briefly, the 5-6 colonies were picked from overnight growth, suspended in 200 µl of sterile molecular-grade water, and boiled at 100 °C for 15 minutes. The suspension was then centrifuged at 14,000 rpm for 5 minutes and the supernatant containing the DNA was stored at −20 °C till further use for PCR testing.

### *Plasmid DNA extraction*

Plasmids were extracted from *Shigella* isolates using a commercial kit (GeneJet Plasmid Miniprep kit, Fermentas), according to the manufacturer's instructions, and individually separated by electrophoresis in 0.8% agarose gels. A supercoiled DNA ladder (Promega® Supercoiled DNA Ladder, 2-10 kb, Promega) was used as a size marker in order to detect the presence of 3 kb plasmids, corrresponding to the pHS-2 plasmid size.

### *PCR assays for virulence markers*

For virulence typing, all the isolates were screened for the following virulence markers, using previosuly described primers and protocols: *ipaH* (invasive plasmid antigen h) (13), *ipaBCD* (invasive plasmid antigens b, c, d, respectively, which were analyzed collectively) (14), *ial* (invasive associated locus) (15), *sen* (*Shigella* enterotoxin 2/ShET2) (16) located on the virulence plasmid pINV, *set1A* (*Shigella* enterotoxin 1/ ShET1 subunitA) (15), *set1B* (*Shigella* enterotoxin 1/ShET1 subunit B) (15), *sat* (secreted autotransporter toxin/Sat) (16), and *pic* (serin protease autotransporter/ Pic) (16) located on the chromosome. A virulence score was calculated for each isolate as the sum of all virulence markers for which the isolate tested as positive.

***PCR screening of Shigella spp. isolates for the presence of pHS-2-specific sequences***

All the total plasmid preparations containing plasmids of about 3 kb were subjected to a nested PCR assay, which was meant to confirm the presence of plasmid pHS-2. Additionally, four plasmid preparations that displayed various profiles of small size plasmids that lacked the 3 kb band were tested as negative controls. The two primer pairs used in the PCR assays were designed with the free Gene Runner software (http://generunner.net/) according to the published pHS-2 sequence of strain MC (GenBank accesion number M25995) (11). The outer primers (Fwd1/5'-ATTCAGCGCAGTTTGTTG-3' and Rev1/5'-CAAGACTGTTACCACGGTTC-3') were designed to amplify a larger fragment of 2039 bp from nucleotides 13 to 2051, and the inner primers (5'-TGCCTGAAATGCCCACAC–3' and 5'-AACCATTCAGCAGACATTTAAC -3') a 401 bp fragment from nucleotides 297 to 697, respectively.

***DNA sequencing of the pHS-2 amplicons***

The inner amplicons of 12 *S. flexneri* isolates (serotype 1a/1 isolate, 2a/3 isolates, 3a/3 isolates, 3b/1 isolate, 3c/2isolates, variant Y/1 isolate), arbitrarily selected, were further purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and subjected to DNA sequencing to document the presence of the HLA-B27 homologue region of the plasmid pHS-2. The amplicons were sequenced on both strands with the corresponding PCR primers and BigDye Terminator v3.1 Cycler Sequencing kit (Applied Biosystems, Foster City, CA, USA). and analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

## Results

***Prevalence of virulence genes in Shigella strains***

Among the 114 *Shigella* isolates investigated by us, the virulence markers ranged in prevalence from 21% (*set1A*, *set1B*, *pic*) to 100% (*ipaH*) (Table I). *S. sonnei* was represented by isolates with lower virulence scores than *S. flexneri* (median, 1 for *S. sonnei* vs. 5 for *S. flexneri*). The concurrent presence of all the markers sought in this study defined the most common genotype, displayed by almost half of the members of *S. flexneri* (22/49 isolates, 45%) and all assigned to serotype 2a (Table II). Of all serotypes investigated in this study, 2a exhibited the highest (median 8, range 4-8) and VI the lowest (4) virulence scores.

**Table I. Virulence markers detected in the 114 *Shigella* isolates studied**

| Isolates | No. of isolates | *ipaH* | *ipaBCD* | *ial* | *sen* | *sat* | *set1A* | *set1B* | *pic* |
|---|---|---|---|---|---|---|---|---|---|
| *Shigella* spp. | 114 | 114 | 65 | 74 | 74 | 42 | 24 | 24 | 24 |
| *S.sonnei* | 65 | 65 | 18 | 27 | 26 | 0 | 0 | 0 | 0 |
| *S. flexneri* | 49 | 49 | 47 | 47 | 48 | 42 | 24 | 24 | 24 |
| 1a | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| 2a | 26 | 26 | 24 | 24 | 25 | 24 | 24 | 24 | 24 |
| 3a | 8 | 8 | 8 | 8 | 8 | 8 | 0 | 0 | 0 |
| 3b | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| 3c | 2 | 2 | 2 | 2 | 2 | 2 | 0 | 0 | 0 |
| 4a | 5 | 5 | 5 | 5 | 5 | 4 | 0 | 0 | 0 |
| VI | 4 | 4 | 4 | 4 | 4 | 0 | 0 | 0 | 0 |
| variant X | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |
| variant Y | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |

***Detection of pHS-2-like plasmids***

The analysis of the plasmid profiles indicated that 42 (37%) of the isolates, all belonging to *S. flexneri* species, possessed plasmids with sizes that corresponded to the pHS-2 plasmid. Among them were included isolates of the following serotypes: 1a (1 isolate), 2a (23 isolates), 3a (8 isolates), 3b (1 isolate), 3c (2 isolates), 4a (5 isolates), variant X (1 isolate), and variant Y (1 isolate). All these isolates presented concordant positive results when tested in the pHS-2 specific PCRs, which resulted in amplicons of the expected sizes. The four plasmid preparations used as negative controls tested negative in the nested PCRs.

The identity of the plasmids was further confirmed for 12 isolates, DNA sequencing of the amplicons revealing the presence of the HLA-B27 signature sequence shared with HLA-B27 antigen.

## Discussion

Molecular studies on *Shigella* virulence markers are still scarce worldwide and according to our knowledge there are no published data on this subject in Romania. Consequently, we examined *Shigella* isolates collected in the last years from diverse areas of our country searching for various chromosomal and extrachromosomal determinants required for the expression of the virulence phenotypes.

Of the markers encoded by virulence plasmids, commonly found in *Shigella* and enteroinvasive *E. coli*, we focused on *ipa* and *ial*, the genes that play a major role in the invasive phenotype (17, 18), and *sen*, which encodes ShET2, an enterotoxin involved in the increased secretory activity of the intestine and possibly in inflammation (19). As expected, the *ipaH* gene was detected in all the isolates while the clus-

**Tabel II. Virulence genotypes identified among the 114 *Shigella* isolates studied**

| Species | Virulence pattern | Number of strains |
|---|---|---|
| | *ipaH*$^+$ | 37 |
| | *ipaH*$^+$ *ipaBCD*$^+$ *ial*$^+$ *sen*$^+$ | 18 |
| *S. sonnei* | *ipaH*$^+$ *ial*$^+$*sen*$^+$ | 7 |
| | *ipaH*$^+$ *ial*$^+$ | 2 |
| | *ipaH*$^+$ *sen*$^+$ | 1 |
| *S.flexneri* | | |
| 1a | *ipaH*$^+$ *ipaBCD*$^+$ *ial*$^+$ *sen*$^+$ *sat*$^+$ | 1 |
| | *ipa H*$^+$*ipaBCD*$^+$ *ial*$^+$*sen*$^+$*sat*$^+$*set 1A*$^+$*set 1B*$^+$*pic*$^+$ | 22 |
| 2a | *ipa H*$^+$*ipaBCD*$^+$ *ial*$^+$*sen*$^+$ | 2 |
| | *ipa H*$^+$*sen*$^+$*sat*$^+$*set 1A*$^+$*set 1B*$^+$*pic*$^+$ | 1 |
| | *ipa H*$^+$*sat*$^+$*set 1A*$^+$*set 1B*$^+$*pic*$^+$ | 1 |
| 3a | *ipa H*$^+$*ipaBCD*$^+$*ial*$^+$*sen*$^+$*sat*$^+$ | 8 |
| 3b | *ipa H*$^+$*ipaBCD*$^+$ *ial*$^+$*sen*$^+$ *sat*$^+$ | 1 |
| 3c | *ipa H*$^+$*ipaBCD*$^+$*ial*$^+$*sen*$^+$*sat*$^+$ | 2 |
| 4a | *ipa H*$^+$*ipaBCD*$^+$*ial*$^+$*sen*$^+$*sat*$^+$ | 4 |
| | *ipa H*$^+$*ipaBCD*$^+$ *ial*$^+$*sen*$^+$ | 1 |
| VI | *ipa H*$^+$*ipaBCD*$^+$*ial*$^+$*sen*$^+$ | 4 |
| variant Y | *ipa H*$^+$*ipaBCD*$^+$*ial*$^+$*sen*$^+$*sat*$^+$ | 1 |
| variant X | *ipa H*$^+$*ipaBCD*$^+$*ial*$^+$ *sen*$^+$ *sat*$^+$ | 1 |

ter *ipaBCD* had a much lower prevalence (57% overall) being more commonly present in *S. flexneri* than in *S. sonnei* isolates. This finding was not surprising taking into consideration that the locus that includes the *ipa*BCD genes can be lost by spontaneous deletions, while *ipaH* gene is a much more stable marker by virtue of its multiple copies on the plasmid and chromosome (20, 21). The *ial* and *sen* genes were the second most prevalent virulence markers after *ipaH* among the Romanian *Shigella* isolates, mainly due to *S. flexneri* species. Notably, most *S. flexneri* isolates possessed *ipaBCD* region as well as *ial* and *sen* genes, whereas this marker association was significantly less likely to occur among *S. sonnei* isolates (96% versus 28%). A report resulted from an eight-year Chinese study showed no major difference between *S. sonnei* and *S. flexneri* in terms of *sen* gene prevalence (22). According to our results *sen* gene prevalence was more than double in *S. flexneri* autochthonous isolates when compared to *S. sonnei* counterpart (98% versus 40%). On the other hand, an Indian study focusing on *Shigella* isolates from Andaman Islands, reported a lower prevalence of *sen* gene for *S. flexneri* (52% versus 98%) as well as for *S. sonnei* (10.5% versus 40%) (23).

Of the virulence markers encoded on the chromosome, we focused on the genes coding for the ShET1 enterotoxin (*set1A and set1B*), and the serin protease autotransporters of the *Enterobacteriaceae* family Sat (*sat*), and Pic (*pic*), respectively. They were found exclusively in *S. flexneri* isolates contributing to the higher virulence scores exhibited by these isolates when compared to *S. sonnei* isolates. A virulence advantage of serotype 2a isolates over those assigned to other serotypes was suggested by the presence of *set* and *pic* genes, detected with a rate of 92% within this serotype. This finding was the evidence of the chromosomal *she* pathogenicity island (PAI), a distinguishing feature of serotype 2a (24). Recently published reports indicated lower rates of ShET1 encoding genes in *S. flexneri* isolates collected in Peru (51%) and Gabon (66%) (25, 26).

In our study, we were also interested in finding how many of the Romanian isolates harbour the pHS-2 plasmid, given the hypothesis that it might be a marker for arthritogenicity (11, 12). We detected this plasmid largely among *S. flexneri* isolates but not in *S. sonnei* and the study results suggested that 86% of the autochthonous *S. flexneri* strains, belonging to various serotypes (e.g. serotypes 1a, 2a, 3a, 3b, 3c, 4a, variant X, and Y), could potentially trigger episodes of ReA. These findings concurred with the reduced prevalence already reported for *S. sonnei* and with the characteristic prevalence pattern of the pHS-2 plasmid observed among the *S. flexneri* serotypes (27). However, we restrain from further comments on this issue because we are aware of the limitations of our study, consisting in the absence of clinical data regarding illness severity and outcomes and the reliance on gene detection to make inferences regarding virulence information. Nevertheless, the strengths include the bacterial collection used, comprising broadly distributed isolates collected from 12 counties and spanning the predominant species and serotypes from our region and the molecular typing performed which allowed us to obtain baseline data referring to their molecular characteristics.

In conclusion, our findings include evidence that the *S. flexneri* strains involved in the local cases of shigellosis between years 2009 and 2013 possessed more virulence markers than *S. sonnei* counterpart and most of the members of serotype 2a harboured, besides a rich content of plasmid and chromosomal virulence genes, pHS-2-like plasmids.

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