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Emergence of a Novel *Shigella flexneri* Serotype 4s Strain That Evolved from a Serotype X Variant in China▽‡

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This paper describes the first isolation of a new *Shigella flexneri* serotype, designated 4s, in Beijing, China. Genotypic and phenotypic profiling suggests that this isolate is a clone of the *S. flexneri* serotype X variant reference strain. Of particular concern is the multidrug resistance exhibited by this isolate.

The *Shigella* genus contains four species, *Shigella flexneri*, *S. dysenteriae*, *S. boydii*, and *S. sonnei*, which are the causative agents of shigellosis. Shigellosis represents a significant public health burden, with more than 160 million cases and more than 1 million deaths each year, the majority of which occur in children from developing countries (6). Two surveillance studies conducted in China between 1991 and 2000 and in six Asian countries between 2000 and 2004 showed considerable disease burdens due to shigellosis in these regions (10, 11). In China, shigellosis was reported as one of the top four notifiable infectious diseases from 2005 to 2008, with nearly 500,000 cases each year (14).

S. flexneri is the major pathogenic species leading to the prevalence of shigellosis in developing countries (13). In these regions, children are often exposed to *S. flexneri* because of unclean water and poor sanitation. At present, *S. flexneri* includes at least 15 serotypes as well as other provisional serotypes identified in many parts of the world, with F2a, 3a, and 1a being the predominant serotypes in Asian countries, including China (7, 12). It is significant for the prevention and control of shigellosis to determine the emergence and epidemic trends of novel serotypes of *S. flexneri* in different regions worldwide.

During the routine surveillance of shigellosis in our laboratory, a new serotype of *S. flexneri* was identified. This strain was isolated from the stool specimens of a patient suffering from severe diarrhea on 10 June 2010 in Beijing, China. This study was approved by the ethics committee of the Academy of Military Medical Sciences (China). Written informed consent was obtained from the patient involved in this study. Bacteria were identified using conventional biochemical API 20E test strips (bioMérieux Vitek, France). Serotyping was performed by slide agglutination using a commercial *S. flexneri* antiserum kit (Denka Seiken, Tokyo, Japan) and a panel of monoclonal antibodies against *S. flexneri* (MASF; Reagensia AB, Stockholm, Sweden) specific for all *S. flexneri* type and group antigens (1). Antimicrobial susceptibility testing was performed by the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (3). The quality control organism used was *Escherichia coli* ATCC 25922.

The *set1A*, *set1B*, *sen*, *ial*, *ipaH*, and *virF* virulence genes were detected using multiplex PCR assays described previously (2, 5, 10, 11). A plasmid purification kit (Qiagen, Germany) was used to isolate the bacterial plasmids in accordance with the manufacturer's recommendations. Plasmid DNA was then separated by electrophoresis with the Chef Mapper system (Bio-Rad) on a 1% SeaKem Gold agarose gel. Lambda DNA cleaved with HindIII (Tiagen, Beijing, China) and the universal standard strain, *Salmonella braenderup* H9812 digested with XbaI, were used as electrophoresis markers. Because of the instability of large plasmids, those greater than 100 kb were not included within the scope of analysis. Genetic relatedness was studied using multilocus sequence typing (MLST), as described on the EcMLST website (http://www.shigatox.net/ecmlst), and pulsed-field gel electrophoresis (PFGE). The 15 isolates of the *S. flexneri* serotype X variant (SFxv) were used as reference strains.

The suspected isolate BJ10610 was identified as a *Shigella* species by the API 20E test, showing a biochemical profile identical to those of the reference strains of SFxv. It could ferment glucose, mannitol, melibiose, and arabinose but could not produce indole and did not utilize sorbitol or rhamnose. This isolate displayed atypical agglutination patterns, as it reacted strongly only with monovalent anti-MASF IV-type antisera but not with any group-specific antisera (Denka Seiken). After examination with the MASF antibodies, the isolate agglutinated with the serogroup B-specific antibody MASF B and the group antigen-specific antibody MASF IV-1 but not with MASF IV-2. This result indicated that it was a new subserotype of *S. flexneri* serotype 4, provisionally designated serotype 4s. Antimicrobial susceptibility testing showed that it was completely resistant to multiple drugs, including tetracycline, ampicillin, amoxicillin, ampicillin-sulbactam, chloramphenicol, trimethoprim-sulfa, nalidixic acid, norfloxacin, and ciprofloxacin, with intermediate resistance to levofloxacin. However, it was susceptible to imipenem, cefotaxime, ceftazidime, and gentamicin.

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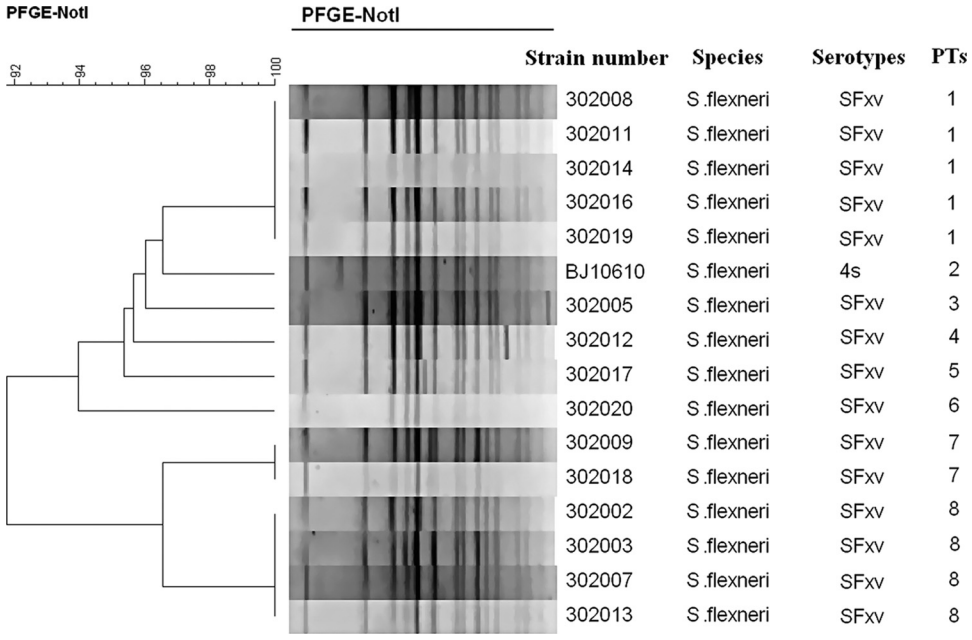

FIG. 1. Dendrogram showing the level of genetic relatedness based on the unweighted-pair group method using average linkages and the Dice coefficient for *S. flexneri* serotype 4s and SFxv strains. The strain numbers, species, serotypes, and PTs of the strains are shown.

Five of the six virulence genes, *set1A*, *set1B*, *sen*, *ipaH*, and *virF*, were present in isolate BJ10610 (see Fig. S1 in the supplemental material). This new serotype contained four plasmids similar to those of the SFxv isolates but did not contain two of the common plasmids in the SFxv isolates (see Fig. S2 in the supplemental material). Using the MLST scheme of 15 housekeeping genes, this new serotype strain was identified as a novel sequence type (ST), designated ST100, which also encompassed the isolates of SFxv. ST100 has two single-locus variants (ST18 and ST86), one double-locus variant (ST17), and two triple-locus variants (ST19 and ST92). These STs form a clonal complex. Sixteen strains in this study were subtyped into eight PFGE banding pattern types (PTs), with *S. flexneri* serotype 4s belonging to PT2 and being closely related (>96% similarity) to the SFxv reference strains in PT1 (Fig. 1).

The emergence and spread of new serotypes usually constitute a great disease burden for the prevention and control of shigellosis, for instance, the recently reported *S. flexneri* serotype 1c and SFxv. Serotype 1c was first identified in Bangladesh in 1989 (1) and was later found to be prevalent in Bangladesh, Egypt, Indonesia, and Pakistan (4, 9, 12). At present, it has been reported as the most prevalent serotype in Vietnam (8). SFxv first appeared in Henan province in 2001 and spread to several other provinces, including Shanxi, Gansu, Anhui, and Shanghai, and now it has become one of the predominant serotypes in these Chinese provinces (14). Such shifts of *S. flexneri* serotypes pose a serious threat to public health in areas where shigellosis is endemic.

We have identified a new *S. flexneri* serotype, designated 4s. Its biochemical reactions were identical to those in SFxv strains, and it contained a similar plasmid profile. To determine the phylogenetic relationship among the isolates of *S. flexneri* serotype 4s and SFxv, an extended MLST scheme of 15 genes and PFGE were used to subtype the serotype 4s and SFxv isolates. The serotype 4s and SFxv isolates were all found to belong to ST100. PFGE analysis indicated that serotype 4s was closely related to SFxv strains, with more than 96% similarity; thus, these isolates may be clonal. These findings suggest that *S. flexneri* serotype 4s may have emerged from the isolates of SFxv. SFxv was able to react with anti-7,8 group antisera, but serotype 4s could not. Two plasmids commonly detected in SFxv were absent in serotype 4s, and there was more than one band difference in PTs between serotype 4s and SFxv.

S. flexneri serotype 4s demonstrated antibiotic resistance to tetracycline, amoxicillin, nalidixic acid, ampicillin-sulbactam, ampicillin, norfloxacin, ciprofloxacin, chloramphenicol, and trimethoprim-sulfa. The last five are commonly prescribed antibiotics recommended for treating shigellosis in China; therefore, rational use of antibiotics is required in human and veterinary therapies. Improper use of antibiotics could result in the rise of superbugs, such as the NDM-1-positive bacteria recently reported in the Indian subcontinent.

In summary, a new *S. flexneri* serotype, 4s, is evident in China. Based on phenotypic and genotypic analyses, we consider that serotype 4s likely originated from SFxv and has experienced changes in its genome to adapt to altered environmental conditions. Although only one isolate has been detected, the possibility that this new serotype exhibiting multidrug resistance may become prevalent requires urgent attention. Continuous surveillance will be required to determine the distribution and resistance development of this serotype so as to understand the actual disease burden and provide guidance for the clinical treatment of shigellosis.

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