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PCR-Based Method for *Shigella flexneri* Serotyping: International Multicenter Validation

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ABSTRACT *Shigella* spp. are a leading cause of human diarrheal disease worldwide, with *Shigella flexneri* being the most frequently isolated species in developing countries. This serogroup is presently classified into 19 serotypes worldwide. We report here a multicenter validation of a multiplex-PCR-based strategy previously developed by Q. Sun, R. Lan, Y. Wang, A. Zhao, et al. (J Clin Microbiol 49:3766–3770, 2011) for molecular serotyping of *S. flexneri*. This study was performed by seven international laboratories, with a panel of 71 strains (researchers were blind to their identity) as well as 279 strains collected from each laboratory's own local culture collections. This collaborative work found a high extent of agreement among laboratories, calculated through interrater reliability (IRR) measures for the PCR test that proved its robustness. Agreement with the traditional method (serology) was also observed in all laboratories for 14 serotypes studied, while specific genetic events could be responsible for the discrepancies among methodologies in the other 5 serotypes, as determined by PCR product sequencing in most of the cases. This work provided an empirical framework that allowed the use of this molecular method to serotype *S. flexneri* and showed several advantages over the traditional method of serological typing. These advantages included overcoming the problem of availability of suitable antisera in testing laboratories as well as facilitating the analysis of multiple samples at the same time. The method is also less time-consuming for completion and easier to implement in routine laboratories. We recommend that this PCR be adopted, as it is a reliable diagnostic and characterization methodology that can be used globally for laboratory-based shigella surveillance.

KEYWORDS molecular serotyping, multicenter validation, PCR, *Shigella flexneri*, serotypes

Shigella spp. are a leading cause of human diarrheal disease worldwide, affecting about 190 million individuals and causing about 65,000 deaths each year, mainly in children under 5 years old (1). Humans are the main natural reservoir with interpersonal transmission via the fecal-oral route through the consumption of contaminated food or water, which is generally associated with poor sanitary conditions (2).

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The prevalence of the serogroups of *Shigella* can vary in different locations and over time. Nowadays, *Shigella sonnei* is the most frequently isolated species in industrialized countries, while *S. flexneri* is the most frequently isolated species in developing countries (3).

S. flexneri is presently classified into serotypes and subserotypes, and to date, 19 subserotypes have been reported in different regions (4). This classification is based on the structure and combination of antigenic determinants present in the O antigen of lipopolysaccharides (LPSs). All serotypes, except serotype 6, share a basic structure of the O antigen with a tetrasaccharide repeat composed of one *N*-acetylglucosamine (GlcNAc) and three rhamnose residues (Rha^{I} to Rha^{III}), which define the group antigenic determinant 3,4 referred to as serotype Y (5). The addition and combination of glucosyl and/or *O*-acetyl and/or phosphoethanolamine (PEtN) residues to different sugars of this basic structure confer the immune response specificity as well as the diversity of serotypes defining type (I to VII) and group antigenic determinants (6, 7, 8, and E1037) (3), (see Fig. S1 in the supplemental material). Recently, two novel *O*-acetylation groups, *viz*, an *O*-acetyl group added to Rha^{III} at position 3 or 4 (3/4-*O*-acetylation) in *S. flexneri* serotypes 1a, 1b, 2a, 5a, Y, and 6 and one added to GlcNAc at position 6 (6-*O*-acetylation) in serotypes 2a, 3a, Y, and Yv, were identified, conferring to the host a novel antigenic determinant provisionally "factor 9 and 10" (corresponding to factors 9 and 10, respectively) (6–8).

The genetic basis of this diversity is given by the acquisition and expression of genes present in bacteriophages. At present, seven of these bacteriophages have been identified (SfI, SfIC, SfII, Sf6, SfIV, SfV, and SfX) (9). Three genes arranged in a single operon are responsible for the addition of a glucosyl group at different positions of any of the four sugars in the backbone O antigen (type antigenic determinants I, II, IV, V, and VII and group antigenic determinant 7, 8). Two of them, namely, *gtrA* and *gtrB*, are highly conserved and interchangeable, while gtr_{type} is unique in each bacteriophage encoding *gtrI*, *gtrIC*, *gtrII*, *gtrIV*, *gtrV*, and *gtrX* genes, conferring serotype 1, 1c (proposed as 7), 2, 4, 5, and X, respectively. *O*-acetylation at position 2 of Rha^{I} depends on the expression of gene *oac*, which results in an acetyltransferase, giving rise to group antigenic determinant 6 in subserotypes 1b, 3a, 3b, 4b, and 7b (5, 10). The newly recognized antigenic determinant provisionally named factor 9 and 10 was found to be mediated by the genes *oacB*, *oacC*, and *oacD* (6–8, 11). These genes were not evaluated in the present work, as their inclusion in the current serotyping scheme should be defined after evaluation by various laboratories worldwide. Lastly, PEtN modification (antigen determinant E1037), at position 3 of either Rha^{III} or Rha^{II} or both, is given by a phosphoethanolamine transferase encoded by a plasmidic gene, *opt*, and confers the described serotypes of Xv, Yv, and 4av (4, 9).

Traditional identification of serotypes in *S. flexneri* is determined by serological testing based on slide agglutination of the bacterial culture against specific antisera. Polyclonal antisera are obtained by immunization of rabbits with whole heat-killed bacterial cells. To obtain monovalent antisera against a specific type or group antigenic determinant, the sera need to be absorbed with heat-killed bacteria carrying cross-reacting O-antigen epitopes (12). Nevertheless, some commercial polyclonal monovalent antisera have been shown to cross-react significantly, as is the case of type IV that also agglutinates with strains carrying the E1037 epitope (9). The use of *S. flexneri* monoclonal antibodies (MASF) overcomes this issue (12). Therefore, a complete panel of antisera is needed in order to identify all serotypes and subserotypes, and these reagents are not always available, even at reference laboratories, given their high economic costs in some regions.

Another approach for typing *S. flexneri* is through molecular (nucleic acid-based) serotyping. Diverse methods had been developed during the last decade, as is the case of those based on PCR described by Sun et al. (10).

The emergence and advances in the knowledge of new provisional serotypes of *Shigella* spp. in different geographical regions (13–17) motivated the organization of the "*Shigella* International Meeting" which was held in May 2012 in Buenos Aires,

Argentina. The purpose of the meeting was to review the epidemiologic, phenotypic, and genetic information available on new *Shigella* serotypes; assess their potential impact on vaccine development; and reach a consensus for revising their classification and nomenclature. During the meeting, a multiplex PCR method, which was developed at the National Institute for Communicable Disease Control and Prevention (CDC-China) (10), was presented. This method targets genes involved in O-antigen production or modification and identifies the recognized serotypes and subserotypes of *S. flexneri*. The increasing prevalence of new serotypes and the availability of a method for their identification lead to a proposal to revise the existing serotyping scheme and establish a working group to conduct a multicenter validation of the multiplex PCR assay. This constitutes an alternative to traditional methods used for *S. flexneri* serotyping, which are both time-consuming and require specific monovalent and monoclonal antisera, which are unavailable in many developing countries where *Shigella* spp. are endemic.

Here, we present the findings of a multicenter validation study of a multiplex PCR assay for genetically serotyping *S. flexneri* isolates.

MATERIALS AND METHODS

Laboratories. The *Shigella flexneri* Multiplex PCR Validation Working Group included the following international laboratories: (1) State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Diseases Control and Prevention, Chinese Center for Disease Control and Prevention (CDC-China), Beijing, China; (2) Enteric and Food Microbiology Laboratory, Laboratory Sciences and Services Division, International Center for Diarrheal Disease Research, Bangladesh (icddr,b); (3) Gastrointestinal Bacteria Reference Unit, Public Health England, London, England; (4) Escherichia and Shigella Reference Unit, Centers for Disease Control and Prevention (CDC-USA), Atlanta, USA; (5) School of Public Health and Primary Care, Faculty of Medicine, The Chinese University of Hong Kong SAR, China; (6) Servicio Enterobacterias, Instituto Nacional de Enfermedades Infecciosas, INEI-ANLIS "Carlos G. Malbrán," Buenos Aires, Argentina; and (7) Centro Nacional de Referencia de Bacteriología, Inciensa, Cartago, Costa Rica.

Strains. Each laboratory received the same panel of 72 coded strains (researchers were blind to their identity) in stab cultures organized in three sets that were distributed by different participant laboratories. Set A included 44 strains of serotypes 1a, 1b, 1d, 2a, 2b, 3a, 3b, 4a, 4b, 5a, 6, X, Y, 4av, Xv, and Yv from the culture collection of CDC-China previously used for the development of the PCR assay (10). A second group of 17 international reference strains (set B), including serotypes 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 4av, 5a, 5b, 6, 7a (1c), X, and Y, were provided by the Public Health England, but only 16 were included in the analysis as described in the Results. The CDC-USA (Table 1) provided a third set of 11 research strains (set C) of serotypes 5a and 5b. In addition, all participating laboratories analyzed strains from their own local culture collections, ranging from 4 to 149 additional isolates per site, bringing the total number of strains tested to 279, that included serotypes 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 4av, 5a, 5b, 6, 7a (1c), 7b, X, Xv, and Y (Table 2). Additionally, six strains of serotypes 1b, 2b, 4b, 5a, 6, and Xv were provided by CDC-China and one strain, 7a (1c), by the Public Health England to be used as control strains to cover all genes that were possibly amplified in the multiplex assay.

Traditional antibody-based serotyping. Traditional serotyping was performed by slide agglutination using the antisera available to each site (Denka Seiken, Japan; SIFIN, Germany; INPB, Argentina). MASF 1c was used to detect serotype 7a (1c), and MASF IV-1 (Reagensia AB, Sweden) or monovalent "AA479" (INPB, Argentina) (18) was used to detect the antigenic determinant E1037 for serotypes Xv, Yv, and 4av.

Preparation of DNA templates. DNA templates were obtained by the boiling method as described in Sun et al., 2011 (10).

PCR primers. Primers included in the multiplex PCR to amplify the O-antigen flippase gene *wzx*$_{1\text{-}5}$ and the *S. flexneri* serotype-specific genes *gtrI*, *gtrIC*, *gtrII*, *oac*, *gtrIV*, *gtrV*, and *gtrX* were the ones described in Sun et al., 2011 (10), with the addition of the pair of primers specific for *opt* amplification, described in Sun et al., 2013 (4). For samples for which multiplex PCR results were negative, a singleplex assay was performed to identify serotype 6 with primers described in Sun et al., 2011 (10).

PCR amplification and sequencing. Multiplex PCR, electrophoresis, and gel visualization were performed in each laboratory using the conditions described in Sun et al., 2011 (10), with locally sourced reagents and equipment, with the exception of the use of a specific brand of master mix (Qiagen, USA). In some cases, when a discordant result among traditional and molecular serotyping was found, PCR products were sequenced directly to elucidate a possibly genetic event.

Data analysis. Individual laboratory test results were reported to the coordinating laboratory (INEI-ANLIS, Argentina) in an Excel spreadsheet distributed in advance to each participating laboratory. Results were compiled as a grouping of all seven laboratories' results, by strain, and ordered by serotype to perform detailed analysis (Tables 1 and 2). Some results were not available or were treated as "missing" for various reasons, as explained in the Results.

The assessment of interrater reliability (IRR), or interrater agreement was conducted using Fleiss' kappa and Krippendorff's alpha coefficients, since for nominal data they provide the highest flexibility of the available reliability measures with respect to the number of raters and categories (19). In the case of

TABLE 1 Compiled PCR results of the shared panel of *S. flexneri* strains[a]

Expected serotype	No. of strains tested	No. of strains positive for gene with multiplex PCR									No. of strains positive for wzx_6 with Sf6 PCR	Serotype result (*n*)	Differences between serology and PCR
		$wzx_{1\text{-}5}$	*gtrI*	*gtrII*	*oac*	*gtrIV*	*gtrV*	*gtrIC*	*gtrX*	*opt*			
1a	5	5	5	0	0	0	0	0	0	0	0	1a (5)	No difference
1b	4	4	4	0	4	0	0	0	0	0	0	1b (4)	No difference
1d	4	4	4	0	0	0	0	0	4	0	0	1d (4)	No difference
2a	5	5	0	5	0	0	0	0	0	0	0	2a (5)	No difference
2b	4	4	0	4	0	0	0	0	4	0	0	2b (4)	No difference
3a	3	3	0	0	3	0	0	0	3	0	0	3a (3)	No difference
3b	4	4	1[(1)]	0	4	0	0	0	0	0	0	3b (3), 1b (1)[(1), (2)]	[(1)] 1 strain type I (−) but *gtrI* (+) in 6 lab [(2)] 1 strain group 7,8 (+) but *gtr*X (−) in 1 lab
4a	3	3	0	0	0	3	0	0	0	0	0	4a (3)	No difference
4b	2	2	0	0	2	2	0	0	0	0	0	4b (1)	No difference
4av	2	2	0	0	0	2[(3)]	0	0	0	2	0	4av (2)	[(3)] 1 strain IV-2 (−) but *gtrIV* (+) in 1 lab
5a	8[(4)]	8	0	0	7[(5)]	0	8	0	2[(6)]	0	0	5a (1), 5a *oac* (+)(7)[(5)], 5b (2)[(6)]	[(4)] 2 strains 7,8 (+) but g*trX* (−) in 1 lab [(5)] 7 strains FG6 (−) but *oac* (+) in all lab [(6)] 2 strains 7,8 (+) and *gtrX* (+) in 1 lab
5b	7	7	0	0	7[(7)]	0	7	0	7	0	0	5b oac (+) (7)[(7)]	[(7)] 7 strains FG6 (−) but *oac* (+)
6	4	0	0	0	0	0	0	0	0	0	4	6 (4)	No difference
7a (1c)	1	1	1	0	0	0	0	1	0	0	0	7a (1)	No difference
X	5	5	0	0	0	0	0	0	5	0	0	X (5)	No difference
Xv	3	3	0	0	0	0	0	0	3	3	0	Xv (3)	No difference
Y	5	5	0	0	0	0	0	0	1[(8)]	0	0	Y (4), X (1)	[(8)] 1 strain FG7,8 (−) but *gtrX* (+) in all lab
Yv	2	2	0	0	0	0	0	0	0	2	0	Yv (2)	No difference

[a](+), positive result; (−), negative result; lab, laboratory/laboratories. The superscript numbers in parentheses are defined in the far right column.

TABLE 2 Compiled PCR results of autochthonous strains of *S. flexneri*[a]

Expected serotype	No. of strains tested	No. of strains positive by gene for gene with multiplex PCR									No. of strains positive for wzx_6 with Sf6 PCR	Serotype result (*n*)	Differences between serology and PCR
		$wzx_{1\text{-}5}$	*gtrI*	*gtrII*	*oac*	*gtrIV*	*gtrV*	*gtrIC*	*gtrX*	*opt*			
1a	11	11	11	0	4(1)	0	0	0	0	0	0	1a (7)/1b (4)(1)	(1) 4 strains FG6 (−) and *oac* (+)
1b	35	35	35	0	35	0	0	0	0	0	0	1b (35)	No difference
2a	58	58	0	58	0	0	0	0	0	0	0	2a (58)	No difference
2b	25	25	0	25	0	0	0	0	25	0	0	2b (25)	No difference
3a	36	36	0	0	36	0	0	0	36	0	0	3a (36)	No difference
3b	8	8	0	0	8	0	0	0	0	0	0	3b (8)	No difference
4a	2	2	0	0	0	2	0	0	0	0	0	4a (2)	No difference
4b	2	2	0	0	0	2	2	0	0	0	0	4b (2)	No difference
4av	5	5	0	0	0	5	0	0	0	5	0	4av (5)	No difference
5a	2	2	0	0	1(2)	0	2	0	0	0	0	5a (1), untypeable (1)(2)	(2) 1 strain FG6 (−) and *oac* (+)
5b	6	6	0	0	6(3)	0	6	0	3(4)	0	0	Untypeable (6)(3)	(3) 6 strains FG6 (−) and *oac* (+); (4) 3 strains FG7,8 (+) and *gtrX* (−)
6	33	0	0	0	0	0	0	0	0	0	33	6 (33)	No difference
7a (1c)	31	31	31	0	0	0	0	31	0	0	0	7a (31)	No difference
7b	2	2	2	0	2	0	0	2	0	0	0	7b (2)	No difference
X	10	10	0	0	0	0	0	0	10	0	0	X (10)	No difference
Xv	7	7	0	0	0	0	0	0	7	7	0	Xv (7)	No difference
Y	6	6	0	2(5)	0	0	0	0	0	0	0	Y (4)/2a (2)	(5) 2 strains type II (−) and gtrII (+)

[a](+), positive result; (−), negative result. The superscript numbers in parentheses are defined in the far right column.

missing data, Krippendorff's alpha is recommended. Both measures range from −1 to 1, where 1 indicates perfect agreement, 0 indicates no agreement beyond chance, and negative values indicate inverse agreement. Fleiss' kappa and Krippendorff's alpha were calculated using R package "*irr*," by Matthias Gamer.

RESULTS

First, we analyzed the common sets of strains (sets A, B, and C) that were shared by all laboratories. One strain was excluded because in five of the seven laboratories, the culture was not viable. Table 1 shows the compiled results from 71 strains. From these strains, 35 had 7 laboratory results, but the remaining strains had only between 3 and 6 PCR results for one or more of the following reasons: cultures were not viable or the expected species or serotype was not recovered by one or more laboratories (19 strains), cultures were not received in the case of one laboratory (16 strains of set B), the laboratory did not have a complete panel of antisera to confirm the serotype and correlate with PCR results (23 strains), and the culture serotype results were rough (1 strain).

In strains of serotype 1a, 1b, 1d, 2a, 2b, 3a, 4a, 4b, 6, 7a (1c), X, Xv, and Yv, there was agreement of the PCR result with the expected serotype as obtained by serology in all strains and all laboratories (Table 1). In the remaining five serotypes assayed in these common sets of strains, some issues were observed in at least one strain and one laboratory. One of four strains of serotype 3b, negative for the type I antigen, tested positive by PCR for the *gtrI* gene in 6 out of 7 results (86% of reproducibility in the PCR result). One of these laboratories sequenced the PCR product and found a single base deletion (base 1310) in the *gtrI* gene. Also, one laboratory result of this strain tested positive for group factor 7,8 but was *gtrX* negative, suggesting there may be a problem with the traditional serotyping in this particular laboratory for this strain. In serotype 4av, one strain was identified by one laboratory as serotype Yv (as it was negative for type 4 antigen using MASF type-specific antibody IV-2), but the PCR result was for 4av as expected. In serotype 5, all laboratories found an atypical band pattern, with amplification of the *oac* gene, in 7 out of 8 strains of subserotypes 5a as well as the 7 strains of 5b. Sequencing of the PCR product in 11 out of 14 of these strains showed a 2-base deletion (bases 345 to 346), similar to that reported previously (10). Additionally, two strains expected to be 5a were confirmed by PCR as *gtrX* negative but were agglutinated by antiserum against each strain in group factor 7,8 in a different laboratory. This was likely due to problems with the specificity of the serotyping reagents used by this laboratory. Also, 1 strain expected to be 5a was typified both by serology and PCR as 5b in one laboratory. Last, all laboratories detected the *gtrX* gene in one strain of serotype Y, which lacked group factor 7,8. This appeared likely to be due to some genetic events, which would require future work to find out the exact cause(s). In summary, there were discrepancies between the traditional and PCR serotyping results for 15 strains of serotype 5, 1 strain of serotype 3b, 1 of serotype 4av, and 1 of serotype Y.

Regarding the robustness within the PCR test, overall IRR measures for the PCR test indicate a high extent of agreement among laboratories (Fleiss' kappa = 0.94 [$z = 121$, $P < 0.001$, 53 strains] and Krippendorff's alpha = 0.931).

A second analysis was obtained from results of autochthonous isolates tested in each participating laboratory; thus, in these cases, the strains had a single PCR result (Table 2). The expected serotype was in agreement with the result obtained by PCR in *S. flexneri* 1b, 2a, 2b, 3a, 3b, 4a, 4b, 4av, 6, 7a (1c), 7b, X, and Xv in all strains tested. The discordant results included four strains serotyped as 1a by serology (group 6 negative) but with a specific PCR amplification pattern of serotype 1b (both *gtrI* and *oac* positive); all were from the same laboratory. One strain of 5a and six of 5b also showed *oac* amplification as previously seen in strains of the common sets of strains. Also, three strains expected to be 5b were found negative for the *gtrX* gene; and two strains of serotype Y showed a *gtrII* positive result (Table 2).

The original method (10) did not include the *opt* gene, so we attempted to include it in the multiplex assay in this collaborative work. Our attempts to do this were not

completely successful for two reasons. First, the PCR products for the *opt* and *gtrI* genes (1,122 and 1,098 bp, respectively) were so close in size that they could not be unambiguously resolved on the 1.5% agarose gel in every laboratory. This lead to problems identifying serotypes 1d and Xv. Second, one laboratory reported having a negative result for the *opt* gene in the multiplex PCR but subsequently obtained positive results when two strains were typed as 4av in a singleplex reaction, suggesting competition among the primers in the multiplex assay. Currently, ongoing work is being conducted by some authors to redesign the *opt* pair of primers that could be included in an improved multiplex assay.

On the other hand, as was already mentioned, some challenges in determining the serotype and subserotype by performing traditional serotyping were encountered in every laboratory. These were primarily due to laboratories being unable to obtain a complete set of high-quality reagents. Without MASF IV-2 (specific for the type 4 antigen) and MASF IV-1 (specific for the group antigen E1037), it was not possible to reliably identify serotypes 4av, Yv, and Xv, making impossible to correlate an incomplete serology and the PCR result. The ability to detect the group factor 3,*4*, which impacts the identification of serotypes 1a, 1b, 3a, 3b, 4a, 4av, 5a, and Yv, also varied between laboratories and depended on which type of antibody reagent was used (MASF Y-5 or other commercial antiserum).

DISCUSSION

In this collaborative work, seven international laboratories from different countries performed a multiplex PCR assay to identify all known *S. flexneri* serotypes with the purpose of validating this method that can be applied as a molecular serotyping tool. We used a shared panel of strains, as well as selected autochthonous cultures from each participating country, to evaluate robustness (as reproducibility) as well as cover all 19 subserotypes, including strains isolated from different regions of the world.

In general, this method was successfully used by all laboratories to identify the serotypes and subserotypes included in the study; for 13 serotypes (of 19 represented among all strains tested), there was full agreement regarding PCR serotyping results and the expected serotype determined by traditional antibody-based serology in all laboratories. In the other serotypes, a specific discrepancy was found. In some cases, the gene was detected positive by the molecular method but the traditional serotyping was negative. This was the case for four strains of serotype 1a and one of serotype 3b identified as serotype 1b by PCR; three strains of serotype Y were identified as serotype X in one case and as serotype 2a in the remaining two cases. Also, an atypical amplification pattern in serotype 5 was found. In the cases of strain 3b and most of strains 5a and 5b, it was shown by PCR product sequencing that these strains carry a dysfunctional gene that did not interfere with their identification by PCR but did cause the loss of function in the specific antigen formation, as has already been reported in the case of serotypes 2 and 5 (10). Other discrepancies could be attributed to errors due to poor-quality antibody reagents, as they only occurred for limited reactions in one participating laboratory.

During the validation, we observed that laboratories were not able to reliably resolve the *opt* and *gtrI* amplification products, which differ in size by only 24 bp, using 1.5% agarose gels. Furthermore, on one occasion, the *opt* gene was only detected in a singleplex reaction but not in the multiplex assay. Both issues could be overcome by separating these primers in an additional reaction or redesigning the *opt* pair of primers, which is work that is under development. We recommend the performance of an "in-house" validation to adjust parameters using specific reagents in each laboratory.

This work highlights the difficulties of serotyping *S. flexneri* using the traditional serotyping approach, even when a complete panel of antisera was available. In many cases, it was impossible to have a complete panel of antisera that permitted achievement of a comprehensive serotyping scheme, which is one reason why a correlation between both methodologies could not be done. This was particularly so for the new serotypes described in recent years around the world. To distinguish serotype 4a from

4av, we needed not only MASF IV-1 but also MASF IV-2 that is specific for type 4. This is because of known cross-reactions of some commercial type 4 antisera (Denka Seiken, Japan) with antigen E1037 present in the serotype 4av (9). In our own study, antisera for antigen IV from Denka Seiken gave positive reactions in all strains of serotypes 4av, Xv, and Yv tested. In these cases, when no MASF IV-2 or alternative monovalent antiserum was available, the serotype could not be defined by serology. In addition, to identify Yv or Xv and 7a or 7b, it was necessary to have MASF IV-1 and MASF 1c, respectively. Globally, only a few laboratories can have their own in-house antisera production to overcome the economic and transportation issues that on occasion impede its availability and application for surveillance purposes in national reference laboratories. On the other hand, using the PCR method validated here, every serotype can be identified using a set of reagents that could be easily acquired.

We believe that the use of group factor 3,4 in the serotyping scheme is questionable, as this corresponds to the basic sugar backbone of the *O*-antigen from the LPS (Y serotype) present in all *S. flexneri* serotypes, except serotype 6. However, its presence is not always detected when the monoclonal antibody (MASF Y-5) is not used, as seen in this work. Moreover, no specific PCR amplification is performed to detect this antigenic structure; the Y serotype could be characterized only by amplification of the $wzx_{1\text{-}5}$ gene, which is usually used as a species control.

Finally, this collaborative work validated a multiplex PCR to analyze all *S. flexneri* serotypes and subserotypes. In some strains of serotypes 1a, 3b, 5a, 5b, and Y, specific genetic events could be responsible for the discrepancies between both methodologies, which should be taken into account in order to detect such events. The robustness of the method was proven, as a high extent of agreement was found among laboratories in IRR measures for PCR test (Fleiss' kappa = 0.94; $z = 121$; P value < 0.001, 53 strains; and Krippendorff's alpha = 0.931).

Furthermore, this method permitted the laboratories to overcome the antisera provision issue and was less time-consuming than traditional serotyping, as was shown by the analysis of many samples at once (depending on the capacity of the thermocycler), and could be quick and easy to implement in most reference laboratories and public health laboratories at the local hospital level. All these factors should be considered when deciding which method to use.

Given the emergence and identification of many new serotypes since the last revision and proposals for its classification (4, 20), there is a need for a new revision to reach to an international consensus regarding *S. flexneri* nomenclature, and the data presented here will inform that process.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.01592-18.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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REFERENCES

1. Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, Döpfer D, Fazil A, Fischer-Walker CL, Hald T, Hall AJ, Keddy KH, Lake RJ, Lanata CF, Torgerson PR, Havelaar AH, Angulo FJ. 2015. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. PLoS Med 12:e1001921. https://doi.org/10.1371/journal.pmed.1001921.
2. Pichel M, Brengi SP, Cooper KL, Ribot EM, Al-Busaidy S, Araya P, Fernández J, Vaz TI, Kam KM, Morcos M, Nielsen EM, Nadon C, Pimentel G, Pérez-Gutiérrez E, Gerner-Smidt P, Binsztein N, *Shigella flexneri* PulseNet PFGE Protocol Working Group. 2012. Standardization and international multicenter validation of a PulseNet pulsed-field gel electrophoresis protocol for subtyping *Shigella flexneri* isolates. Foodborne Pathog Dis 9:418–424. https://doi.org/10.1089/fpd.2011.1067.
3. Ashkenazi S, Cohen D. 2013. An update on vaccines against *Shigella*. Ther Adv Vaccines 1:113–123. https://doi.org/10.1177/2051013613500428.
4. Sun Q, Lan R, Wang J, Xia S, Wang Y, Wang Y, Jin D, Yu B, Knirel YA, Xu J. 2013. Identification and characterization of a novel *Shigella flexneri*

serotype Yv in China. PLoS One 8:e70238. https://doi.org/10.1371/journal.pone.0070238.
5. Allison GE, Verma NK. 2000. Serotype-converting bacteriophages and O-antigen modification in *Shigella flexneri*. Trends Microbiol 8:17–23. https://doi.org/10.1016/S0966-842X(99)01646-7.
6. Wang J, Lan R, Knirel YA, Luo X, Senchenkova SN, Shashkov AS, Xu J, Sun Q. 2014. Serological identification and prevalence of a novel O-antigen epitope linked to 3- and 4-*O*-acetylated rhamnose III of lipopolysaccharide in *Shigella flexneri*. J Clin Microbiol 52:2033–2038. https://doi.org/10.1128/JCM.00197-14.
7. Sun Q, Knirel YA, Wang J, Luo X, Senchenkova SN, Lan R, Shashkov AS, Xu J. 2014. Serotype-converting bacteriophage SfII encodes an acyltransferase protein that mediates 6-O-acetylation of GlcNAc in *Shigella flexneri* O-antigens, conferring on the host a novel O-antigen epitope. J Bacteriol 196:3656–3666. https://doi.org/10.1128/JB.02009-14.
8. Knirel YA, Luo X, Wang J, Senchenkova SN, Lan R, Shpirt AM, Du P, Shashkov AS, Xu J, Sun Q. 2014. Genetic and structural identification of an O-acyltransferase gene (*oacC*) responsible for the 3/4-O-acetylation on rhamnose III in *Shigella flexneri* serotype 6. BMC Microbiol 14:266. https://doi.org/10.1186/s12866-014-0266-7.
9. Sun Q, Knirel YA, Lan R, Wang J, Senchenkova SN, Jin D, Shashkov AS, Xia S, Perepelov AV, Chen Q, Wang Y, Wang H, Xu J. 2012. A novel plasmid-encoded serotype conversion mechanism through addition of phosphoethanolamine to the antigen of *Shigella flexneri*. PLoS One 7:e46095. https://doi.org/10.1371/journal.pone.0046095.
10. Sun Q, Lan R, Wang Y, Zhao A, Zhang S, Wang J, Wang Y, Xia S, Jin D, Cui Z, Zhao H, Li Z, Ye C, Zhang A, Jing H, Xu J. 2011. Development of a multiplex PCR assay targeting O-antigen modification genes for molecular serotyping of *Shigella flexneri*. J Clin Microbiol 49:3766–3770. https://doi.org/10.1128/JCM.01259-11.
11. Wang J, Knirel YA, Lan R, Senchenkova SN, Luo X, Perepelov AV, Wang Y, Shashkov AS, Xu J, Sun Q. 2014. Identification of an O-acyltransferase gene (*oacB*) that mediates 3- and 4-O-acetylation of rhamnose III in *Shigella flexneri* O antigens. J Bacteriol 196:1525–1531. https://doi.org/10.1128/JB.01393-13.
12. Carlin NI, Rahman M, Sack DA, Zaman A, Kay B, Lindberg AA. 1989. Use of monoclonal antibodies to type *Shigella flexneri* in Bangladesh. J Clin Microbiol 27:1163–1166.
13. Ansaruzzaman M, Sultana M, Talukder KA, Alam K, Matsushita S, Safa A, Khajanchi BK, Dutta DK, Islam Z, Albert MJ, Nair GB, Sack DA. 2005. Isolation and characterization of provisional serovar *Shigella boydii* E16553 from diarrhoeal patients in Bangladesh. J Med Microbiol 54:477–480. https://doi.org/10.1099/jmm.0.45889-0.
14. Grimont F, Lejay-Collin M, Talukder KA, Carle I, Issenhuth S, Le Roux K, Grimont PA. 2007. Identification of a group of shigella-like isolates as *Shigella* boydii 20. J Med Microbiol 56:749–754. https://doi.org/10.1099/jmm.0.46818-0.
15. Shmilovitz M, Kretzer B, Levi S. 1985. A new provisional serovar of *Shigella dysenteriae*. J Clin Microbiol 21:240–242.
16. Ansaruzzaman M, Kibriya AK, Rahman A, Neogi PK, Faruque AS, Rowe B, Albert MJ. 1995. Detection of provisional serovars of *Shigella dysenteriae* and designation as *S. dysenteriae* serotypes 14 and 15. J Clin Microbiol 33:1423–1425.
17. Talukder KA, Mondol AS, Islam MA, Islam Z, Dutta DK, Khajanchi BK, Azmi IJ, Hossain MA, Rahman M, Cheasty T, Cravioto A, Nair GB, Sack DA. 2007. A novel serovar of *Shigella dysenteriae* from patients with diarrhoea in Bangladesh. J Med Microbiol 56:654–658. https://doi.org/10.1099/jmm.0.46999-0.
18. van der Ploeg CA, Rogé AD, Bordagorria XL, de Urquiza MT, Viñas MR, Pichel MG, Bruno SB. 2015. AA479 antiserum: new reagent for the serotype characterization of atypical variants of *Shigella flexneri*. Rev Argent Microbiol 47:36–40. https://doi.org/10.1016/j.ram.2014.11.001.
19. Zapf A, Castell S, Morawietz L, Karch A. 2016. Measuring inter-rater reliability for nominal data—which coefficients and confidence intervals are appropriate? BMC Med Res Methodol 16:93. https://doi.org/10.1186/s12874-016-0200-9.
20. Brenner DJ. 1984. Recommendations on recent proposals for the classification of *Shigellae*. Int J Syst Bacteriol 34:87–88. https://doi.org/10.1099/00207713-34-1-87.