



BRIEF REPORT

AA479 antiserum: new reagent for the serotype characterization of atypical variants of *Shigella flexneri*

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Abstract *Shigella flexneri* is divided into 13 serotypes based on the combination of antigenic determinants present in the O-antigen. A new O-antigen modification with phosphoethanolamine has been identified. The presence of this antigenic determinant (called E1037) is recognized by monoclonal antibody MASF IV-1. Given the increasing incidence of these new variants and the difficulty in supplying the monoclonal antibody to our country, we produced a polyclonal antiserum (AA479) through immunization with a *S. flexneri* Xv strain. The antiserum specificity was assessed by slide agglutination against isolates from clinical cases and a culture collection representing all *Shigella* serotypes. The results obtained demonstrated a 100% correlation between AA479 absorbed antiserum and monoclonal antibody MASF IV-1. The availability of AA479 antiserum in every public hospital in Argentina will allow us to identify atypical *S. flexneri* isolates in order to strengthen *Shigella* surveillance in our country and to compare with global epidemiological data.

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PALABRAS CLAVE

Shigella flexneri;
Variantes atípicas;
Antisero policlonal;
Serotificación

Antisero AA479: nuevo reactivo para la serotificación de variantes atípicas de *Shigella flexneri*

Resumen *Shigella flexneri* se divide en al menos 13 serotipos sobre la base de la combinación de determinantes antigénicos presentes en el antígeno O. Se identificó una nueva modificación del antígeno O con fosfoetanolamina. La presencia de este determinante antigénico (denominado E1037) es reconocida por el anticuerpo monoclonal MASF IV-1. Teniendo en cuenta la

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incidencia creciente de estas nuevas variantes y la dificultad en la provisión del anticuerpo monoclonal para nuestro país, se elaboró un antisuero de tipo policlonal (AA479) mediante la inmunización con un cultivo de *S. flexneri* Xv. La especificidad del antisuero se evaluó por aglutinación en lámina con aislamientos clínicos y cultivos de colección, con lo que quedaron representados todos los serotipos de *Shigella*. Los resultados obtenidos demostraron una correlación del 100% entre el antisuero AA479 absorbido y el anticuerpo monoclonal MASF IV-1. La disponibilidad del antisuero AA479 en todos los hospitales públicos de Argentina permitirá identificar los aislamientos atípicos de *S. flexneri*; de esta forma se podrá fortalecer la vigilancia de *Shigella* en nuestro país y comparar con los datos epidemiológicos a nivel global.

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Shigella infections are a major health problem worldwide, mainly in developing countries. Approximately 160 million shigellosis cases have been estimated per year around the globe by the World Health Organization with the majority involving children under five years of age^{8,11}.

Shigella flexneri is the prevalent species in developing countries, particularly in Argentina where 70% of the *Shigella* infections are caused by *S. flexneri* and about 25% by *S. sonnei*⁷. In Argentina, *Shigella* spp. has been identified by the Laboratory Surveillance System (Ministry of Health), as one of the main causative bacterial agents of diarrhea².

In order to establish the diversity of the circulating serotypes and their distribution in time and space, it is necessary to determine the antigenic characteristics of the *Shigella* strains in different regions. For this reason, in Argentina, epidemiological surveillance is performed within the context of the National Network for Diarrhea and Foodborne Pathogens (Diarrhea Network) through serotyping with the antisera produced by the Servicio Antígenos y Antisueros from the Instituto Nacional de Producción de Biológicos (INPB) – ANLIS “Dr. Carlos G. Malbrán”.

The genus *Shigella* comprises four species: *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*. Except for *S. sonnei*, each species contains multiple serotypes based on the structure of the O-antigen. *S. dysenteriae* and *S. boydii* have been subdivided into 15 and 20 serotypes, respectively. In the serotyping scheme currently in use, *S. flexneri* is divided into 13 serotypes based on the combination of the type-specific antigen (I, II, III, IV, V, VI) and the presence of one or more group antigens (3,4–6–7,8)^{3,9}. All *S. flexneri* serotypes (except *S. flexneri* 6) share the backbone of the basic O-antigen repeat unit, which is a tetrasaccharide consisting of a single *N*-acetyl glucosamine and three rhamnose residues. This basic structure is referred to as serotype Y and the addition of glucosyl and/or *O*-acetyl groups to different sugars in the tetrasaccharide unit forms the basis of serotype conversion. The genes involved in the biosynthesis of the tetrasaccharide are located in the chromosome and the O-antigen modification genes known to date are encoded by temperate bacteriophages or prophages which are integrated into the conserved sites of the host *S. flexneri* genome¹.

In recent years, the emergence of atypical *S. flexneri* has been reported, such as the provisionally called *S. flexneri* Xv, *S. flexneri* Yv and *S. flexneri* 4av^{6,10–12,15}. All these strains showed agglutination with monoclonal antibody MASF IV-1,

which binds to the antigenic determinant E1037 not covered in the *S. flexneri* serotyping scheme currently in use. The MASF IV-1 positive phenotype involves the presence of a phosphoethanolamine (PEtN) group attached to at least one rhamnose of the tetrasaccharide, due to the activity of the PEtN-transferase encoded in the plasmidic gene *lpt-O*¹¹.

Considering that atypical *S. flexneri* (E1037 positive) isolates have been detected in our country since 2009 and in view of the difficulty in supplying the antibody, we have produced a polyclonal antiserum (AA479) to allow the identification of such bacterial strains and to strengthen *Shigella* surveillance in Argentina.

A randomly selected culture of *S. flexneri* Xv (#479/09 culture) was used for the production of rabbit polyclonal antiserum AA479 (anti-PEtN antiserum). The purity of the bacteria, the biochemical identification and the morphology of the colony (smoothness) were tested. In order to remove thermolabile surface antigens, the immunizing suspension was prepared by heat denatured whole cells with a concentration of approximately 5×10^9 bacteria/ml.

New Zealand White rabbits, 8 weeks old (approximately 2.5 kg), were immunized with a first intramuscular 0.5 ml dose and then a series of intravenous inoculation of 0.5, 1.0, 2.0, and two doses of 4.0 ml administered at 3–4 day interval. The rabbits were exsanguinated by cardiac puncture 7–10 days after the last injection. Then, the serum was stored at 4°C with 10% glycerin phenolized saline solution as a preservative.

Thirty-four *S. flexneri* strains (32 *S. flexneri* Xv, 1 *S. flexneri* 4av and 1 nontypeable *S. flexneri*), which showed agglutination with monoclonal antibody MASF IV-1, were used to control the AA479 antiserum. All these studied strains were received (over a one year span), between 2009 and 2010, by the National Reference Laboratory (Servicio Enterobacterias from the Instituto Nacional de Enfermedades Infecciosas (INEI) – ANLIS “Dr. Carlos G. Malbrán”) from human stool samples. Sixty-seven *Shigella* strains from the Antigens and Antisera Service Collection, including all currently recognized serotypes of the four species (15 *S. dysenteriae*, 19 *S. boydii*, 2 *S. sonnei* and 31 *S. flexneri*) and *S. flexneri* 7a^{5,14} were also used for the antiserum control. All cultures were biochemically characterized as *Shigella* spp. in accordance with the procedures previously described⁴ and kept at –70°C in tryptic soy broth with glycerol. Serotyping was performed by a slide agglutination assay using specific antisera

Table 1 Agglutination reactions of AA479 antiserum.

<i>S. flexneri</i>	No. of isolates	Titer of AA479 antiserum	
		Unabsorbed	Absorbed
1a ^a	2	10	–
1b ^a	2	–	–
2a ^a	1	–	–
2b ^a	1	40	–
3a ^a	4	40	–
3b ^a	1	10	–
4a ^a	6	10	–
4av ^b	3	40	40
4b ^a	2	10	–
5a ^a	1	40	–
5b ^a	1	40	–
6 ^a	2	–	–
7a ^c	1	–	–
X ^a	2	40	–
Xv ^d	32	40 ^e –80 ^e	40
Y ^a	3	20	–
Nontypeable ^d	1	40	40

The agglutination absence with the 1/10 diluted antiserum is considered negative (–).

The cultures corresponding to the species *S. boydii*, *S. dysenteriae* and *S. sonnei* were negative in the 1/5 dilution of AA479 antiserum without absorption.

^a Cultures belonging to the Antigens and Antisera Collection.

^b One of the isolates is from a clinical source, the other two belong to the Antigens and Antisera Collection.

^c *S. flexneri* 7a culture was kindly provided by Dr. K. Talukder (International Centre for Diarrhoeal Disease Research, Bangladesh).

^d Clinical isolates. Nontypeable is the isolate that showed no agglutination with any of the three known group factors (3,4–6–7,8) or with the type-specific antigen.

^e The titer corresponding to the antiserum without absorption was 40 or 80 according to the evaluated isolate.

for all types and group-factor antigens (INPB) and monoclonal antibody against *S. flexneri* (MASF IV-1; Reagensia AB, Sweden).

Cell suspensions were employed for the titration of AA479 antiserum. Bacterial suspensions were prepared directly from cultures grown at 37 °C for 18 h with 1.0 ml of 0.5% formalinized physiological saline solution. Twenty microliters (20 µl) of the bacterial suspension and 20 µl of serial dilutions of antiserum were placed on a glass slide and mixed with the microbiological loop or stick. The slide was tilted gently back and forth for 1 min and the appearance or non-appearance of agglutination was observed with an indirect intense light. The titer was defined as the inverse of the highest dilution giving a positive result 3+ (large and small clumps against a slightly cloudy background) or 4+ (large clumps against a clear background).

The unabsorbed antiserum showed a strong reaction (titer 40–80) with the collection cultures of *S. flexneri* 2b, 3a, 4av, 5a, 5b and X serotypes, as well as clinical isolates of *S. flexneri* 4av, Xv and nontypeable serotypes (Table 1).

AA479 antiserum was subjected to a purification process (absorption process) for the removal of heterologous agglutinins in accordance with a previously described procedure⁴. An overnight growth culture of *S. flexneri* X at 37 °C in tryptic soy agar was used as an absorbent in a ratio of 1 plate (150 mm diameter) per 4 ml of antiserum. The mixture of cells and serum was incubated for 2 h at 50 °C in a water bath; then, the absorbed antiserum was removed by centrifugation at 12,000 rpm for 30 min.

The absorbed antiserum was specific for atypical *S. flexneri* cultures, including those belonging to 4av, Xv and nontypeable serotypes (Table 1).

With regard to the correlation between AA479 and MASF IV-1, a 100% concordance between both reagent results was observed (Table 2).

Having considered that the emergence of new *S. flexneri* serotypes is not unusual^{10,12,13,15}, that rapid dissemination of these variants may occur and that multiresistant *S. flexneri* Xv strains have been detected in China¹⁵, it is necessary to keep the epidemiological surveillance of the serotypes of this species updated. Likewise, the information about the distribution of the different serotypes remains important to the proposal of the World Health Organization regarding a vaccine development to decrease the world morbidity caused by *Shigella* as well as having the knowledge that acquired immunity against this organism infection is serotype-specific⁸.

AA479 antiserum production and its immediate distribution to the public hospitals belonging to the Diarrhea Network enabled to detect in 2009, the appearance and dissemination of *S. flexneri* Xv (Viñas, van der Ploeg, *et al.*, unpublished data). This emerging serotype represented 30% of the *S. flexneri* isolates analyzed in 2010 and became the second serotype in importance after *S. flexneri* 2, the historically predominant serotype.

S. flexneri unabsorbed antisera are characterized by having cross-reactivity with all of *S. flexneri* serotype cultures and by the titer decrease of at least one dilution after

Table 2 Comparison of the results obtained with AA479 antiserum and monoclonal antibody MASF IV-1.

Serotype	No. of isolates	AA479 absorbed ^a	MASF IV-1
<i>S. dysenteriae</i> 1–15	15	–	–
<i>S. boydii</i> 1–18 and 20	19	–	–
<i>S. sonnei</i> FI and FII	2	–	–
<i>S. flexneri</i>			
1a–1b	2–2	–	–
2a–2b	1–1	–	–
3a–3b	4–1	–	–
4a–4b	6–2	–	–
5a–5b	1–1	–	–
6	2	–	–
7a	1	–	–
X	2	–	–
Y	3	–	–
<i>S. flexneri</i> 4av ^b	3	+	+
<i>S. flexneri</i> Xv ^c	32	+	+
<i>S. flexneri</i> nontypeable ^c	1	+	+

^a Absorbed AA479 antiserum was used at a dilution of 1/40, according to the titer expressed in Table 1.

^b One of these isolates is from a clinical source, the others belong to the Antigens and Antisera Service Collection.

^c Clinical isolates. Nontypeable is the isolate that showed no agglutination with any of the three known group factors (3, 4–6–7, 8) or with the type-specific antigen.

the absorption process. Nevertheless, AA479 unabsorbed antiserum showed cross-reactivity with only some of the *S. flexneri* serotypes and its titer did not decrease after absorption. These results may be due to the structural difference of the PEtN group regarding the other O-antigen determinants, mostly glucosyl and rhamnose residues.

The concordance between the results obtained with the AA479 polyclonal antiserum and monoclonal antibody MASF-IV-1 suggests that both reagents recognize the same antigenic determinant in the seroagglutination test. The serological behavior observed in the current study along with the results obtained by others researchers^{6,11} leads us to propose the epitope E1037 as a novel *S. flexneri* group factor and to highlight the need to agree on a serotyping scheme and an updated nomenclature including the new strains described.

Nowadays, monoclonal antibody MASF IV-1 is the only available reagent for the detection of *S. flexneri* E1037-antigen carrier isolates, which is neither accessible for use in our country nor in most Latin American countries. Having considered that the correlation between the antibody and the AA479 antiserum was 100%, the indistinct use of one or the other would lead to obtain comparable information worldwide. We propose AA479 antiserum to be used as a novel serotype marker for the additional characterization of *S. flexneri* isolates in Argentina as well as in Latin America, in order to cooperate with the epidemiological surveillance of *Shigella* spp. in the region.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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