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Serogroup-Specific Bacterial Engineered Glycoproteins as Novel Antigenic Targets for Diagnosis of Shiga Toxin-Producing-*Escherichia coli*-Associated Hemolytic-Uremic Syndrome

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Human infection with Shiga toxin-producing *Escherichia coli* (STEC) is a major cause of postdiarrheal hemolytic-uremic syndrome (HUS), a life-threatening condition characterized by hemolytic anemia, thrombocytopenia, and acute renal failure. *E. coli* O157:H7 is the dominant STEC serotype associated with HUS worldwide, although non-O157 STEC serogroups can cause a similar disease. The detection of anti-O157 *E. coli* lipopolysaccharide (LPS) antibodies in combination with stool culture and detection of free fecal Shiga toxin considerably improves the diagnosis of STEC infections. In the present study, we exploited a bacterial glycoengineering technology to develop recombinant glycoproteins consisting of the O157, O145, or O121 polysaccharide attached to a carrier protein as serogroup-specific antigens for the serological diagnosis of STEC-associated HUS. Our results demonstrate that using these antigens in indirect ELISAs (glyco-iELISAs), it is possible to clearly discriminate between STEC O157-, O145-, and O121-infected patients and healthy children, as well as to confirm the diagnosis in HUS patients for whom the classical diagnostic procedures failed. Interestingly, a specific IgM response was detected in almost all the analyzed samples, indicating that it is possible to detect the infection in the early stages of the disease. Additionally, in all the culture-positive HUS patients, the serotype identified by glyco-iELISAs was in accordance with the serotype of the isolated strain, indicating that these antigens are valuable not only for diagnosing HUS caused by the O157, O145, and O121 serogroups but also for serotyping and guiding the subsequent steps to confirm diagnosis.

Shiga toxin-producing *Escherichia coli* (STEC) is an important food-borne pathogen associated with sporadic cases and outbreaks of diarrhea, bloody diarrhea (BD), and hemolytic-uremic syndrome (HUS), a life-threatening condition characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure (1). Postdiarrheal HUS is caused by particular serotypes of STEC or, in regions, such as South Asia, by *Shigella dysenteriae* serotype 1 (2). STEC strains are characterized by the production of Shiga toxin 1 (Stx1) and/or Shiga toxin 2 (Stx2), and the production of these toxins is central in the pathogenesis of BD and HUS (3). *E. coli* O157:H7 is the dominant STEC serotype associated with sporadic cases and outbreaks of BD and HUS in different parts of the world (4); however, a subset of non-O157:H7 STEC serotypes (O26:H11, O103:H2, O111:NM, O121:H19, O145:NM, and O45:H2, among others) can cause a similar disease (5, 6).

STEC-associated HUS is the most common cause of acute renal failure in children worldwide and constitutes 90% of all HUS cases in this population. Five to 10 percent of children with STEC infection develop HUS, for which the only treatment is supportive care. The incidence rate of postdiarrheal HUS varies according to country, and Argentina shows the highest HUS incidence worldwide, with 12 to 14 cases per 100,000 children <5 years of age per year (6). This incidence rate is 10× higher than that in other industrialized countries (7), representing >400 new cases of HUS annually, with a mortality rate of 2 to 5% (6). In Argentina, postdiarrheal HUS is endemic and constitutes a critical health issue, since it is the leading cause of acute renal failure among children. Because 20 to 30% of children with HUS end up with long-term renal sequelae, HUS is the second cause of chronic renal failure and accounts for 20% of the renal transplants in children and adolescents (8, 9). In this country, >70% of HUS cases are associated with STEC O157 infection, followed by STEC O145 (>9%) and O121 (>2%), among those of other serogroups (6).

The diagnosis of STEC infections is based on the isolation and characterization of STEC strains, as well as the detection of free fecal Shiga toxin (FFStx). However, HUS patients without isolated STEC have frequently been reported (10, 11). It has been shown

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TABLE 1 Plasmids and strains used in this work

Plasmid or strain	Description/characteristic[a]	Reference or source
Plasmids		
pMH5	Soluble periplasmic hexa-His-tagged AcrA under the control of Tet promoter, in pACYC; CHL^r	28
pWA2	Soluble periplasmic hexa-His-tagged AcrA under the control of Tet promoter, in pBR322; AMP^r	28
pMAF10	HA-tagged PglBCj cloned in pMLBAD; TMP^r	28
pWSK129	Expression vector; KAN^r	36
pDIG5	Carries the gene encoding the O157 antigen gene cluster under the control of Plac promoter, in pLarf, TET^r	25
pLM1	Carries the gene encoding the O145 antigen gene cluster under the control of Plac promoter, in pWSK129; KAN^r	This work
pLM3	Carries the gene encoding the O121 antigen gene cluster under the control of Plac promoter, in pWSK129; KAN^r	This work
E. coli strains		
DH10B	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara*,*leu*)7697 *araD139 galU galK nupG rpsL* λ	Invitrogen
Serogroup O145:NM	FP1091/10	INEI-ANLIS
Serogroup O121:H19	FP900/07	INEI-ANLIS
CLM24	W3110, Λ*waaL*	28
CLM24 AcrA-O157	CLM24 transformed with pWA2, pMAF10, and pDIG5	25
CLM24 AcrA-O145	CLM24 transformed with pMH5, pMAF10, and pLM1	This work
CLM24 AcrA-O121	CLM24 transformed with pMH5, pMAF10, and pLM3	This work

[a] CHL, chloramphenicol; AMP, ampicillin; HA, hemagglutinin; TMP, trimethoprim; KAN, kanamycin; TET, tetracycline.

that STEC is shed rapidly during the diarrheal prodrome and often ceases before the onset of HUS, making the detection of the pathogen or its toxins difficult, if not impossible (12, 13). For these reasons, alternative laboratory diagnostic methods have been used, such as those that detect antibodies against Shiga toxins or the lipopolysaccharide (LPS) of STEC strains (14, 15). The presence of antibodies directed against the LPS in the serum of patients with HUS was initially reported by Chart, Scotland, and Rowe in 1989 (16). Since then, antibodies against this antigen have been measured using different methods, such as immunoblotting (16), enzyme-linked immunosorbent assay (ELISA) (11, 15), indirect hemagglutination assay (IHA) (10), and a microagglutination (MA) assay (17). All these assays use the whole bacteria, bacterial extracts containing high concentrations of lipopolysaccharide (LPS), or purified LPS as antigens. Consequently, these tests may result in false-positive reactions due to the presence of cross-reactive antibodies against epitopes present in the common core and lipid A moieties of LPS and other antigens shared by different STEC strains and other bacteria.

The detection of anti-LPS antibodies is a valuable and sensitive method for diagnosing STEC infections in combination with bacteriological culture and detection of FFStx (18). It has been demonstrated that bacteriological methods alone or in combination with toxin testing provide evidence of STEC involvement in only 20 to 50% of the children investigated; in contrast, the detection of anti-LPS antibodies combined with stool culture and FFStx detection improves the diagnostic performance, providing evidence of infection in >80% of HUS patients (10, 11, 15). For these reasons, and since the humoral immune response to LPS is dominated by antibodies against the O-polysaccharide section, we decided to explore the application of bacterial engineered O-polysaccharide–protein conjugates in the diagnosis of STEC infections. In the last years, bacterial glycoengineering, a field that combines the knowledge of bacterial glycobiology with genetic engineering, has emerged as a viable alternative for producing not only glycoprotein therapeutics and vaccines (19) but also recombinant glycoprotein antigens for diagnosis (20–22). The most thoroughly studied bacterial glycosylation system is the *N*-glycosylation machinery of *Campylobacter jejuni* (23–26). Previous work has demonstrated that this *N*-glycosylation system can be transferred to *E. coli* (27) and that the *C. jejuni N*-oligosaccharyltransferase (OTase) PglB, due to its relaxed specificity, can transfer an array of LPS O polysaccharides from its lipid donor to carrier proteins in a system that combines both the LPS biosynthesis pathway of Gram-negative bacteria and the *N*-glycosylation pathway of *C. jejuni* (20, 25, 28). In this system, several glycosyltransferases sequentially add the sugars required for the synthesis of the O polysaccharide to the lipid carrier undecaprenol-phosphate (Und-P) at the cytoplasmic side of the membrane. Next, the lipid-linked oligosaccharide is flipped into the periplasm, polymerized, and transferred by PglB to a carrier protein instead of transferring it to the lipid A core of LPS (28). Therefore, the resulting O-polysaccharide-protein conjugates can be produced and purified from cultures of nonpathogenic bacteria. Due to their versatility, this *in vivo* bacterial glycosylation system can be seen as a toolbox for engineering a panel of novel serotype-specific O-polysaccharide–protein conjugates. Recombinant glycoproteins produced by this method may constitute a new generation of antigens, circumventing most of the disadvantages of the conventional chemical methods used to produce glycoconjugates, significantly reducing costs and improving the reproducibility of the product.

In this work, we exploited the *C. jejuni N*-glycosylation machinery to engineer a panel of recombinant glycoproteins as antigenic targets for diagnosing STEC infections and demonstrated that these novel antigens are serogroup specific and may be of great value for diagnosing hemolytic-uremic syndrome caused by the O157, O145, and O121 serogroups.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown in 2XYP broth

(16 g/liter peptone, 10 g/liter yeast extract, and 5g/liter sodium chloride) at 37°C. Concentrations of 100 μg/ml trimethoprim, 20 μg/ml tetracycline, 20 μg/ml chloramphenicol at, 50 μg/ml kanamycin, and 100 μg/ml ampicillin were added as needed to the medium for selection. *E. coli* strain DH10B ElectroMAX (Invitrogen Life Technologies) was used for the cloning procedures.

Cloning of O145 and O121 gene clusters. The O145 gene cluster (GenBank accession no. AY647260) was amplified by PCR with the oligonucleotides NotIO145O121 (ATAGCGGCCGCATTGGTAGCTGTAAGCCAAG GGCGGTAGCG) and PstIO145 (ATACTGCAGTCACACGAACGATTTT GCAAAAGAAGGGTC) using Elongase DNA polymerase (Invitrogen Life Technologies) and genomic DNA from *E. coli* O145:NM as a template. The 15-kb PCR product was digested with NotI and PstI and ligated to the plasmid pWSK129 digested with the same enzymes. The resulting plasmid was named pLM1. The O121 gene cluster (GenBank accession no. AY208937.1) was amplified using the oligonucleotides NotIO145O121 and PstIO121 (ATAGCGGCCGCATTGGTAGCTGTAAGCCAAGGGC GGTAGCG) and genomic DNA from *E. coli* O121:H19 as a template, and cloned into the plasmid pWSK129 previously digested with NotI and PstI enzymes. The resulting plasmid was named pLM3.

E. coli CLM24 strain containing the plasmids pMAF10 (carrying the gene encoding the *C. jejuni* OTase PglB) and pMH5 (carrying the gene encoding the *C. jejuni* carrier protein AcrA fused to a histidine tag) was transformed with plasmids pLM1 (carrying the O145 gene cluster) and pLM3 (carrying the O121 gene cluster) generating the strains CLM24 serogroup O145 and CLM24 serogroup O121, respectively. The strain CLM24 serogroup O157 was previously generated by the transformation of the *E. coli* CLM24 strain with the plasmids pMAF10, pWA2 (carrying the gene encoding the *C. jejuni* carrier protein AcrA fused to a histidine tag), and pDIG5 (carrying the O157 gene cluster) (25).

Production and purification of the glycoproteins O157-AcrA, O145-AcrA, and O121-AcrA. The production and purification of O157-AcrA, O145-AcrA, and O121-AcrA were performed as previously described, with some modifications (28). The *E. coli* strains CLM24 AcrA-O157, CLM24 AcrA-O145, and CLM24 AcrA-O121 were grown overnight at 37°C. The cultures were reinoculated at a 1:100 dilution into fresh 2XYP medium, grown at 37°C for 1.5 h (optical density at 600 nm [OD_{600}], ≈0.2), and the expression of the corresponding gene cluster was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. PglB expression was induced at an OD_{600} of ≈0.5 by adding arabinose to a final concentration of 0.2% (wt/vol). Five hours after the first induction, PglB was reinduced by a second addition of arabinose to maximize the glycosylation of AcrA. AcrA was constitutively expressed in all the strains. The cells were harvested by centrifugation after 20 h of induction, and periplasmic extracts were obtained by lysozyme treatment, as described elsewhere (28). Subsequently, the periplasmic fraction was equilibrated with 1/9 volume of 10× loading buffer (0.1 M imidazole, 3 M NaCl, 0.2 M Tris-HCl [pH 8.0]) and subjected to Ni^{2+} affinity chromatography. The column was equilibrated with 10 column volumes of 1× loading buffer and loaded on a HisTrap HP column (Amersham Pharmacia Biosciences) at a flow rate of 1 ml/min. The column was washed with 25 column volumes of wash buffer (0.02 M imidazole, 0.3 M NaCl, 0.02 M Tris-HCl [pH 8.0]) and eluted from the column with elution buffer (0.25 M imidazole, 0.3 M NaCl, 0.02 M Tris-HCl [pH 8.0]).

Mass spectrometry. The purified glycoproteins O145-AcrA and O121-AcrA were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the bands corresponding to the glycoproteins were excised from the gel, transferred to 1.5-ml Eppendorf vials, and in-gel digested with trypsin (Promega) using the protocol of Shevchenko et al. (29), with some modifications. After destaining with 50 mM ammonium bicarbonate in 50% acetonitrile-water, the gel pieces were dehydrated with acetonitrile and rehydrated with 10 μl of trypsin (≈2 μg) solution. Following the addition of 50 μl of ammonium bicarbonate (50 mM) in water, the samples were incubated overnight at 37°C in an oven. The generated peptides and glycopeptides were extracted using ZipTip pipette tips (Millipore) and analyzed using a hybrid quadrupole orthogonal acceleration time of flight mass spectrometer (Waters, United Kingdom) equipped with a nanoACQUITY ultraperformance liquid chromatography system (Waters, Milford, MA). Briefly, 2 μl of the sample was injected on to a VanGuard micro precolumn C_{18} cartridge that was connected to a 75-μm inside diameter (i.d.) by 150 μm Atlantis dC_{18} column (Waters, Milford, MA). Solvent A consisted of 0.1% formic acid in water, and solvent B consisted of 0.1% formic acid in acetonitrile. After a 1-min trap wash in the precolumn with solvent A at a flow rate of 10 μl/min, peptides were separated using solvent gradient and electrosprayed to the mass spectrometer at a flow rate of 350 nl/min. The collision energy used to perform tandem mass spectrometry (MS/MS) was varied according to the mass and charge states of the eluting peptides. The instrument was calibrated every 1 min with green fluorescent protein (GFP) and LecErK using the LockSpray. For the data acquisition and analysis, MassLynx (Waters MassLynx version 4.1) was used.

Indirect enzyme-linked immunosorbent assay development and optimization. Microtiter plates (Corning polystyrene high binding microplates) were coated with 50 μl of O157-AcrA, O145-AcrA, or O121-AcrA at a concentration of 2.5 μg/ml. The antigens were diluted in 0.05 M carbonate buffer (pH 9.6) and, after incubating for 18 h at 4°C, the plates were blocked with 5% bovine skim milk in 0.02 M Tris-HCl (pH 7.6), 0.15 M NaCl, and 0.1% Tween 20 (blocking buffer) for 1 h at room temperature (RT). The serum samples were diluted in blocking buffer, added in duplicate, and incubated for 1 h at RT. Positive- and negative-control samples were included in each plate. Subsequently, bound antibodies were detected using three different horseradish peroxidase (HRP)-conjugated goat anti-human antibodies: anti-human IgM/G/A (total Ig) (Sigma), anti-human IgM (Sigma), and anti-human IgG (Sigma). In all cases, secondary antibodies were diluted in blocking buffer and incubated for 1 h at RT. Between each reaction step, the plates were washed three times with 0.02 M Tris-HCl (pH 7.6), 0.15 M NaCl, and 0.1% Tween 20 (0.1% TBST) to remove excess of reagents. After incubation with the substrate (0.36% H_2O_2, 0.1% 3,3′,5,5′-tetramethylbenzidine [TMB] in 0.1 M citric acid [pH 4.2]) for 5 min, the reaction was stopped with 2 N H_2SO_4, and the absorbance was determined at 405 nm using a plate reader (DTX 880 multimode detector; Beckman Coulter, Inc.). Optimization of the assays was established in preliminary experiments through a checkerboard titration analysis using high-positive, medium-positive, low-positive, and negative serum samples, as well as checking for strong versus low background. Based on these analyses, the optimal antigen concentration was 2.5 μg/ml (125 ng/well), the optimal sample dilution was 1:100, and the optimal dilution of conjugates was 1:2,000. These established parameters were used to test all the samples.

Serum samples. To evaluate the performances of the assays, a panel of 90 serum samples obtained from healthy individuals and patients belonging to different clinical groups were analyzed: (i) STEC-positive HUS patients ($n = 53$), who were children <8 years of age with a clinical diagnosis of HUS and a stool culture positive for STEC O157:H7 ($n = 31$), O145:NM ($n = 15$), or O121:H19 ($n = 7$); (ii) STEC-negative HUS patients ($n = 14$), who were children <8 years old with a clinical diagnosis of HUS and a stool culture negative for STEC; and (iii) healthy individuals ($n = 23$), who were children <8 years old with no clinical and epidemiological evidence of STEC infection. These samples were used as negative controls.

For the retrospective single-blind study, a set of 71 encoded serum samples (corresponding to 68 patients) was analyzed. This panel included samples obtained from patients <8 years of age with a clinical diagnosis of HUS or BD associated with STEC infection. In these patients, the STEC association of the disease was confirmed by the positive isolation of STEC from stool samples, the detection of FFStx by specific cytotoxicity on Vero cells using monoclonal antibodies for Stx1 and Stx2, and/or the detection of stx_1 and/or stx_2 genes by PCR from the confluent growth zone in a MacConkey sorbitol agar plate. Additionally, this panel included serum samples obtained from patients <8 years of age with HUS or BD but with no confirmed STEC association, either by stool culture or by FFStx or *stx*

gene detection, as well as patients of the same age group with other unrelated infectious diseases.

Ethics statement. All the samples analyzed in this study came from a previously characterized serum collection provided by the Servicio Fisiopatogenia, Instituto Nacional de Enfermedades Infecciosas (INEI)-ANLIS Dr. Carlos G. Malbrán, the national reference laboratory (NRL) for HUS and diarrhea disease associated with diarrheagenic *E. coli*. These samples were obtained from patients attended to at different hospitals in Argentina who were then sent to INEI-ANLIS during the period of 2005 to 2014. In order to ensure anonymity, the serum samples had been codified upon collection; therefore, no personally identifying information was available for the patients.

Western blotting. Nonglycosylated AcrA and the glycoproteins O157-AcrA, O145-AcrA, and O121-AcrA were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes by semidry electroblotting. Immunoblotting was performed with rabbit polyclonal anti-6xHis tag antibodies (Rockland Immunochemicals, Inc.), specific rabbit antisera against each serogroup (provided by the Instituto Nacional de Producción de Biológicos-ANLIS Dr. Carlos G. Malbrán), or with the indicated serum samples at a 1:100 dilution in blocking buffer (5% bovine skim milk in 0.1% TBST). Bound antibodies were visualized using HRP-labeled goat anti-rabbit immunoglobulin (Dako) or with HRP-labeled goat anti-human IgM/G/A (Sigma) secondary antibodies and enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate detection reagents; Pierce Chemical Co.), according to the manufacturer's instructions.

Data analysis. The glycoprotein indirect ELISA (glyco-iELISA) results were expressed as the percentage of reactivity of the mean absorbance at 450 nm (A_{450}) of the positive-control serum included in each assay run. The percentage of reactivity was calculated as follows: % reactivity = (A_{450} of the test sample/mean A_{450} of the positive control) × 100. Dot plot graphs and Mann-Whitney test analyses were performed using the GraphPad Prism software (version 5.01 for Windows; San Diego, CA).

RESULTS

Production and purification of O157-AcrA, O145-AcrA, and O121-AcrA glycoproteins. Previous reports demonstrated that *C. jejuni* PglB can transfer an array of different LPS O polysaccharides from its lipid donor to carrier proteins for the synthesis of recombinant glycoproteins (25, 28). Recently, we exploited PglB for the development of a recombinant glycoprotein consisting of an *N*-formylperosamine O polysaccharide attached to the *C. jejuni* protein AcrA for the diagnosis of human and bovine brucellosis (20–22). In the present work, we used the same strategy to generate a panel of O-polysaccharide–AcrA protein conjugates for the diagnosis of STEC-associated HUS. To generate the O157-AcrA, O145-AcrA, and O121-AcrA glycoproteins, we coexpressed PglB, AcrA-His tag, and all the enzymes required for the synthesis of the O157, O145, or O121 O polysaccharide in the nonpathogenic *E. coli* strain CLM24. This strain lacks the WaaL ligase, which transfers the O polysaccharide from the lipid carrier to the lipid A core of LPS; therefore, the introduction of PglB in this mutant favors the transfer of the corresponding O polysaccharide to the protein acceptor AcrA (28). After induction, the generated glycoproteins were purified from the periplasm by affinity chromatography and analyzed by Coomassie brilliant blue-stained SDS-PAGE (Fig. 1A) and immunoblotting with anti-histidine tag antibodies or specific polyclonal antisera against O157, O145, and O121 O polysaccharides (Fig. 1B to D). Using Coomassie blue staining, we detected a band of the same mass as that of nonglycosylated AcrA (≈38 kDa) and a ladder of bands of higher molecular weight, with two clusters centered at ≈70 and ≈100 kDa (Fig. 1A). These bands reacted with anti-His tag antibodies, indicating that they correspond to modified forms of AcrA (Fig. 1B to D). The bands of high molecular weight, but not the unmodified form of AcrA, were also detected by antisera that specifically recognize each serogroup, confirming the identity of the O polysaccharides linked to the carrier protein (Fig. 1B to D). No reactivity was observed when each glycoprotein was immunodetected with the other two specific antisera (data not shown). The bands corresponding to O157, O145, and O121 polysaccharide-linked AcrA showed extensive heterogeneity in size due to the typical variability of the chain lengths of these O polysaccharides (30).

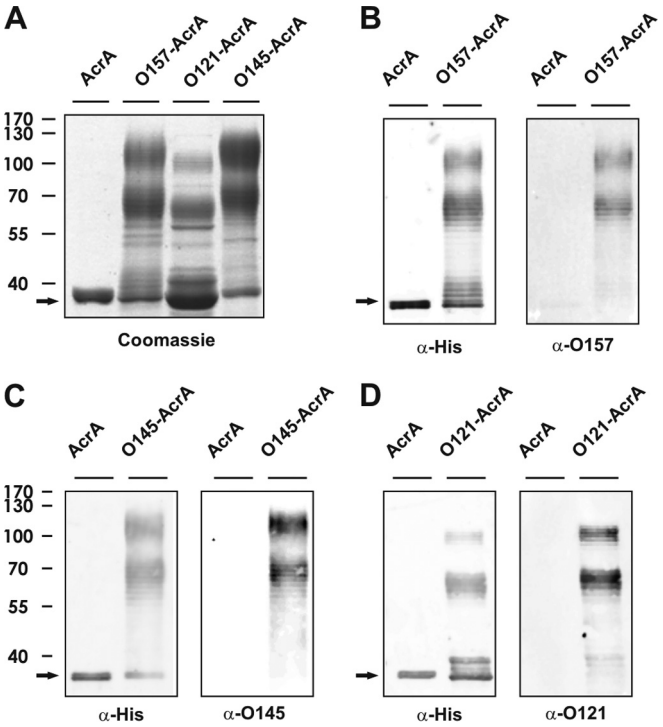

FIG 1 Coomassie brilliant blue-stained SDS-PAGE and Western blot analysis of the recombinant glycoproteins. A 10% SDS-PAGE analysis of purified O157-AcrA, O145-AcrA, O121-AcrA, and nonglycosylated AcrA by Coomassie brilliant blue staining (A), immunoblot using anti-histidine tag antibodies and specific polyclonal antisera against O157 (B), O145 (C), or O121 (D) polysaccharides. The position of the molecular mass standards (in kDa) is indicated on the left. The arrows on the left indicate the migration position of nonglycosylated AcrA.

Identification of the carbohydrates attached to AcrA by mass spectrometry. In order to fully characterize the carbohydrates attached to AcrA, mass spectrometry techniques were employed. O145-AcrA and O121-AcrA were purified from the corresponding CLM24 strain and separated by 10% SDS-PAGE. The bands of interest were excised and in-gel digested with trypsin, and the resulting peptides and glycopeptides were analyzed by electrospray ionization quadrupole time of flight (ESI-Q-TOF) MS and MS/MS.

After MS/MS analysis, the band excised from the O145-AcrA glycopeptide showed peaks corresponding to the extended glycopeptide ATFENASKDFN(glycan)R, with a glycan that matched two O145 O-antigen subunits (*m/z* 2,760.2). Further fragmentation products were observed, matching the loss of amino acids or sugar residues. The glycan can be predicted to be composed of *N*-acetylhexosamine (HexNAc), 2-acetimidoylamino-2,6-dideoxygalactose (FucNAm), and sialic acid (Neu5Ac), matching the O145 O-antigen structure (31). The spectrum is shown with full peptide (M+H; M refers to peptide, and M+H is peptide without

glycan), and fragmentation derivatives (M + glycan) or glycan fragments (Fig. 2A; see also Fig. S1 in the supplemental material). When the lower-molecular-weight region is examined closely, peaks corresponding to single sugars can be observed (see Fig. S1A in the supplemental material). The band excised from the O121-AcrA glycopeptide showed a peak corresponding to the typical DFN(glycan)R glycopeptides (20), with a glycan matching the O121 subunit (*m/z* 1,437.8) and its fragmentation derivatives (Fig. 2B). The MS/MS-derived peaks matched a glycan composed of a HexNAc, two *N*-acetyl galacturonic acid (GalNAcA), and a terminal *N*-acetylglycyl-quinovosamine (QuiNAcGly) (32); when the lower-molecular-weight region is zoomed in, single sugars can be observed (see Fig. S2 in the supplemental material).

Taken together, these results demonstrate that the structure of the oligosaccharides *N*-linked to AcrA in the O145-AcrA and O121-AcrA glycoproteins is in accordance with the published structure for these LPS O polysaccharides. O157-AcrA was previously generated and characterized (25), and the same experimental approach was used in this study to generate O157-AcrA for the detection tests.

Recombinant glycoproteins as serogroup-specific antigens for diagnosis of STEC infections. Following the production and characterization by Western blotting and MS of the O157-AcrA, O145-AcrA, and O121-AcrA glycoproteins, we evaluated if these glycoconjugates could be used as serogroup-specific antigens for the diagnosis of STEC infections in children. To test this, serum samples obtained from patients <8 years of age with a clinical diagnosis of HUS and a positive stool culture for STEC O157:H7, O145:NM, or O121:H19 were analyzed by Western blotting against the nonglycosylated and glycosylated forms of AcrA (Fig. 3). Negative-control samples obtained from healthy individuals of the same age group with no clinical and epidemiological evidence of STEC infection were also included (Fig. 3). Reactivity against the corresponding glycoprotein was observed with the samples obtained from the patients infected with STEC O157, O145, or O121 but not with the samples from the healthy individuals. None of the samples were reactive against the nonglycosylated form of AcrA, indicating that the detected antibody response is specifically directed toward the O-polysaccharide moiety of the glycoproteins. Additionally, no cross-reactivity among the three glycoproteins was observed, demonstrating that the developed antigens are specific for each STEC serogroup. The strong specific reaction toward the evaluated serogroups observed with sera from HUS patients demonstrates that these glycoproteins are potentially useful for the serodiagnosis of STEC-associated HUS, particularly in cases in which the bacterium cannot be isolated and the FFStx cannot be detected.

Development and evaluation of indirect enzyme-linked immunosorbent assays. As a proof of concept and to evaluate the usefulness of these recombinant glycoproteins for the serodiagnosis of STEC infections, we developed an indirect ELISA coupling O157-AcrA, O145-AcrA, or O121-AcrA to microtiter plates (glyco-iELISAs) (see Materials and Methods; see also Fig. S3 and S4 in the supplemental material). Bound antibodies were detected using HRP-conjugated anti-human total Ig, IgM, or IgG antibodies. A total of 76 serum samples obtained from children <8 years with a clinical diagnosis of HUS and a stool culture positive for STEC O157:H7 ($n = 31$), STEC O145:NM ($n = 15$), or STEC O121:H19 ($n = 7$), as well as control samples obtained from healthy children ($n = 23$ for O157 and $n = 19$ for O145 and O121) were analyzed, and the results were outlined in a dot plot diagram, as shown in Fig. 4. In all the assays, a significant difference in the overall reactivity values between the negative and positive populations was obtained ($P < 0.0001$), with a minimal overlap between the two sets of samples. Based on these results, a preliminary cutoff value was established for each assay as the mean plus three standard deviations of the reactivity values of negative-control samples, and the diagnostic sensitivity and specificity of the tests were calculated (see Table S1 in the supplemental material). The results indicate that using these antigens, it is possible to clearly discriminate between healthy children and patients with O157, O145, or O121 STEC-associated HUS. Interestingly, a specific IgM response against the antigens was detected in all the samples except for two samples belonging to the O157 and O145 serogroups, indicating that with the IgM glyco-iELISAs, it is possible to detect the infection even at the early stages of the disease (Fig. 4; see also Table S1). Additionally, all the samples were analyzed by the three glyco-iELISAs, and no cross-reactivity was observed, confirming that the developed glycoprotein antigens are serogroup specific (data not shown).

Finally, we analyzed by total Ig glyco-iELISAs 14 serum samples obtained from children with a clinical diagnosis of HUS in which both diagnostic criteria (culture and FFStx) were negative. As shown in Fig. 5A, specific reactivity against O157-AcrA but not toward the O145-AcrA antigen was observed in eight of the analyzed samples. In the remaining six samples, positive reactivity was observed against O145-AcrA only (Fig. 5A). In addition, none of the samples showed reactivity against the O121-AcrA glycoprotein (data not shown). As shown in Fig. 5B, the detected antibody response was specifically directed toward the O-polysaccharide moiety of the corresponding glycoprotein in all the samples (Fig. 5B and data not shown). Finally, we analyzed two consecutive serum samples obtained from a 16-month-old patient who suffered fulminant HUS and again for whom culture and FFStx were negative (see Fig. S4 in the supplemental material). In the sample obtained at 6 days after the onset of symptoms, strong specific IgM and IgG responses were detected, while at 13 days, the IgM titer dropped significantly, and the IgG titer remained high. Therefore, measuring the immunological response by glyco-iELISAs might be a valuable tool for improving diagnostic efficacy even in patients for whom the classical diagnostic procedures have failed.

Retrospective single-blind analysis. To further evaluate if the glyco-iELISAs can be useful in the diagnosis of STEC-associated HUS, a retrospective single-blind study was carried out analyzing a panel of 71 encoded serum samples (corresponding to 68 patients) sent to the NRL during the 2014 summer season. This panel included serum samples obtained from culture-positive and culture-negative HUS patients <8 years of age and from patients of the same age group with other infectious diseases (see Table S2 in the supplemental material). All the samples were analyzed blindly by the IgM, IgG, and total Ig O157, O145, and O121 glyco-iELISAs and classified as positive or negative for each serogroup, using the cutoff values previously established for these assays (see Table S1 in the supplemental material). The results were then further compared with previous laboratory and clinical data of the patients (the data and results are compiled in Table S2).

In order to facilitate the interpretation of the results, three patient categories were established (Fig. 6; see also Table S2 in the supplemental material). Category 1 included patients with other infectious diseases. Category 2 included patients with a diagnosis of HUS or BD associated with STEC infection and confirmed by STEC isolation, FFStx, or stx_1/stx_2 gene detection by PCR from the

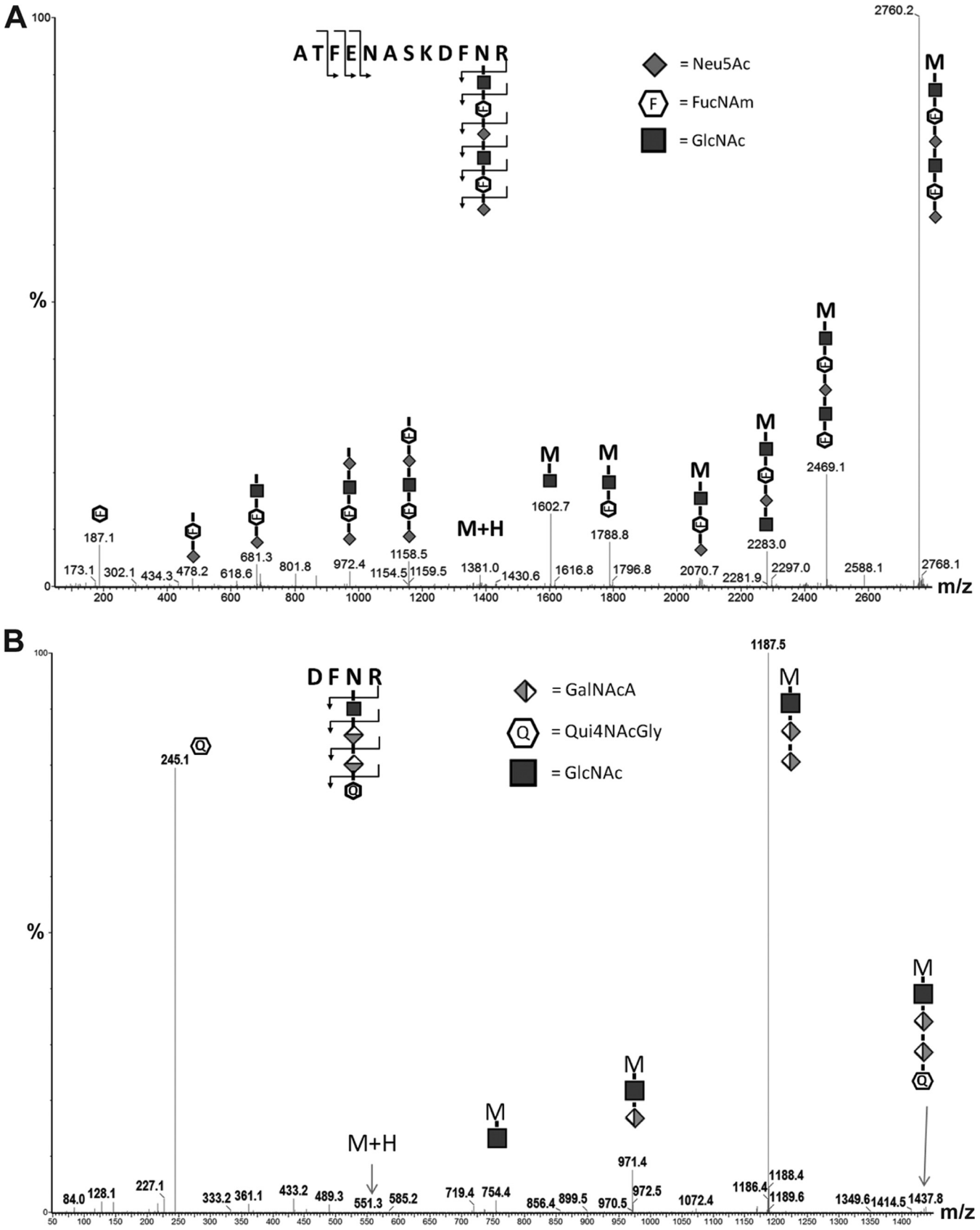

FIG 2 ESI-Q-TOF MS and MS/MS analysis of O145-AcrA and O121-AcrA glycoproteins. (A) MS/MS analysis of the O145-AcrA glycopeptide ATFENASKDFN (glycan)R. HexNAc, *N*-acetylhexosamine; FucNAm, 2-acetimidoylamino-2,6-dideoxygalactose; Neu5Ac, sialic acid. (B) MS/MS analysis of the O121-AcrA glycopeptide DFN(glycan)R. The spectra are shown with full peptide (M+H) and fragmentation derivatives (M + glycan) or glycan fragments. HexNAc, *N*-acetylhexosamine; GalNAcA, *N*-acetyl galacturonic acid; QuiNAcGly, terminal *N*-acetylglycyl-quinovosamine.

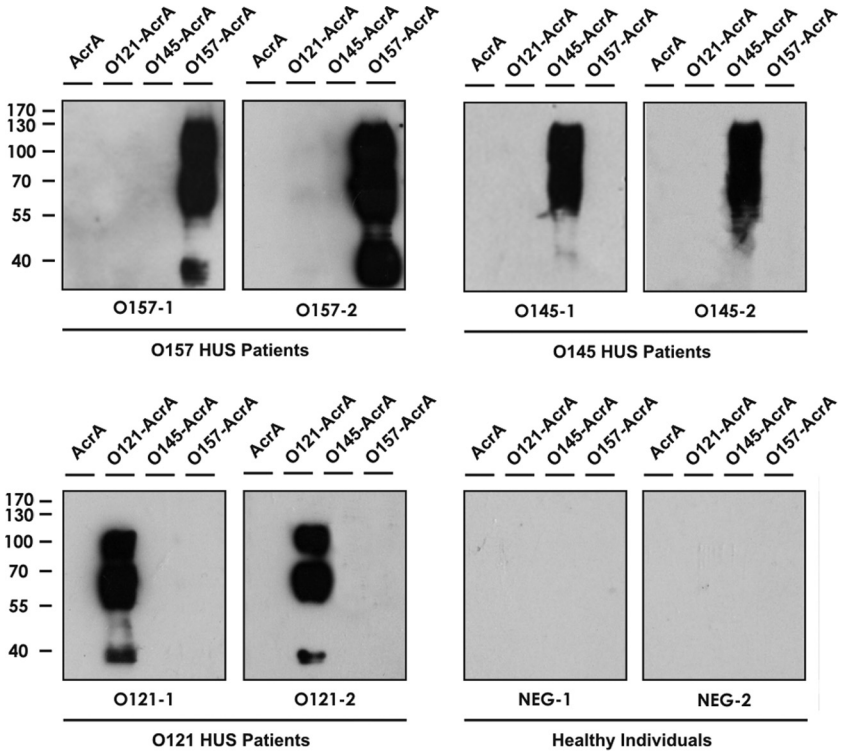

FIG 3 Western blot analysis of serum samples obtained from patients with HUS and from healthy children. We analyzed serum samples obtained from patients <8 years of age with a clinical diagnosis of HUS and a stool culture positive for STEC O157:H7, O145:NM, or O121:H19, as well as negative-control samples obtained from healthy children of the same age group. Bound antibodies were detected using HRP-conjugated goat anti-human total Ig antibodies. The samples O157-1, O145-1, O121-1, and NEG-1 were used as positive and negative controls in the glyco-iELISAs. The position of the molecular mass standards (in kDa) is indicated on the left.

confluent growth zone. Finally, category 3 included patients with a clinical diagnosis of HUS or BD without laboratory confirmation either by stool culture, FFStx, or *stx* gene detection.

All samples obtained from patients with other infectious diseases (category 1, $n = 14$), including a sample obtained from a patient with diarrhea caused by a STEC serogroup O103 strain (see Table S2 in the supplemental material, patient 1), were serologically negative by the three glyco-iELISAs, confirming that the glycoprotein antigens are highly specific (Fig. 6). An analysis of the serum samples obtained from the patients included in category 2 ($n = 13$) showed that 92.3% of the samples were serologically positive by the glyco-iELISAs (53.8% O157, 30.8% O145, and 7.7% O121) (Fig. 6). Among these patients, five had a positive result by stool culture and, in all cases, the serogroup of the isolated STEC strain matched the serogroup identified by the glyco-iELISA (see Table S2, patients 15, 20, 24, 25, and 26). Only one of the samples included in this category, which was obtained from a culture-negative HUS patient with positive detection of stx_1/stx_2 genes by PCR from the confluent growth zone, was negative by serology (see Table S2, patient 22). In category 3 ($n = 44$), 79.5% of the samples were positive by the glyco-iELISAs (Fig. 6). Of these, 56.8% were positive for O157, 15.9% for O145, and 2.3% for O121 (Fig. 6). Interestingly, in all the samples included in this single-blind study, specific IgM and IgG responses were detected, indicating a recent infection; even more, in two of these samples (see Table S2, patients 17 and 42), only a specific IgM response was detected. Additionally, two samples (4.5%) were serologically positive for O157 and O145, indicating a possible coinfection with both serotypes (see Table S2, patients 3 and 48). In categories 2 and 3, 7.7% (one sample) and 20.5% (nine samples, of which three came from the same patient) of the samples were negative by the glyco-iELISAs (Fig. 6; see also Table S2). Finally, we attempted to isolate the bacterium from stored stool samples by immunomagnetic separation (IMS) from 14 samples of categories 2 and 3 (see Table S2). IMS was performed using magnetic beads functionalized with specific antibodies against the serogroup previously identified by the glyco-iELISAs. In five of these samples (see Table S2, patients 17, 18, 19, 33, and 36), we were able to confirm the diagnosis by isolation of an STEC strain of the expected serogroup.

Taken together, these results demonstrate that the recombinant glycoprotein antigens developed in this work improve the diagnosis of HUS caused by the O157, O145, and O121 STEC serogroups.

DISCUSSION

Early and accurate diagnosis of STEC infections after the onset of diarrhea continues to challenge clinicians due to the small number of cases confirmed by bacterial isolation and/or toxin detection. However, it has been demonstrated that the detection of anti-*E. coli* O157 LPS antibodies in combination with stool culture considerably improves diagnosis (10, 11, 15, 18). For these reasons, and since one of the most immunodominant STEC antigens is the O-polysaccharide section of the LPS, we decided to explore the application of the recombinant glycoproteins O157-AcrA, O145-

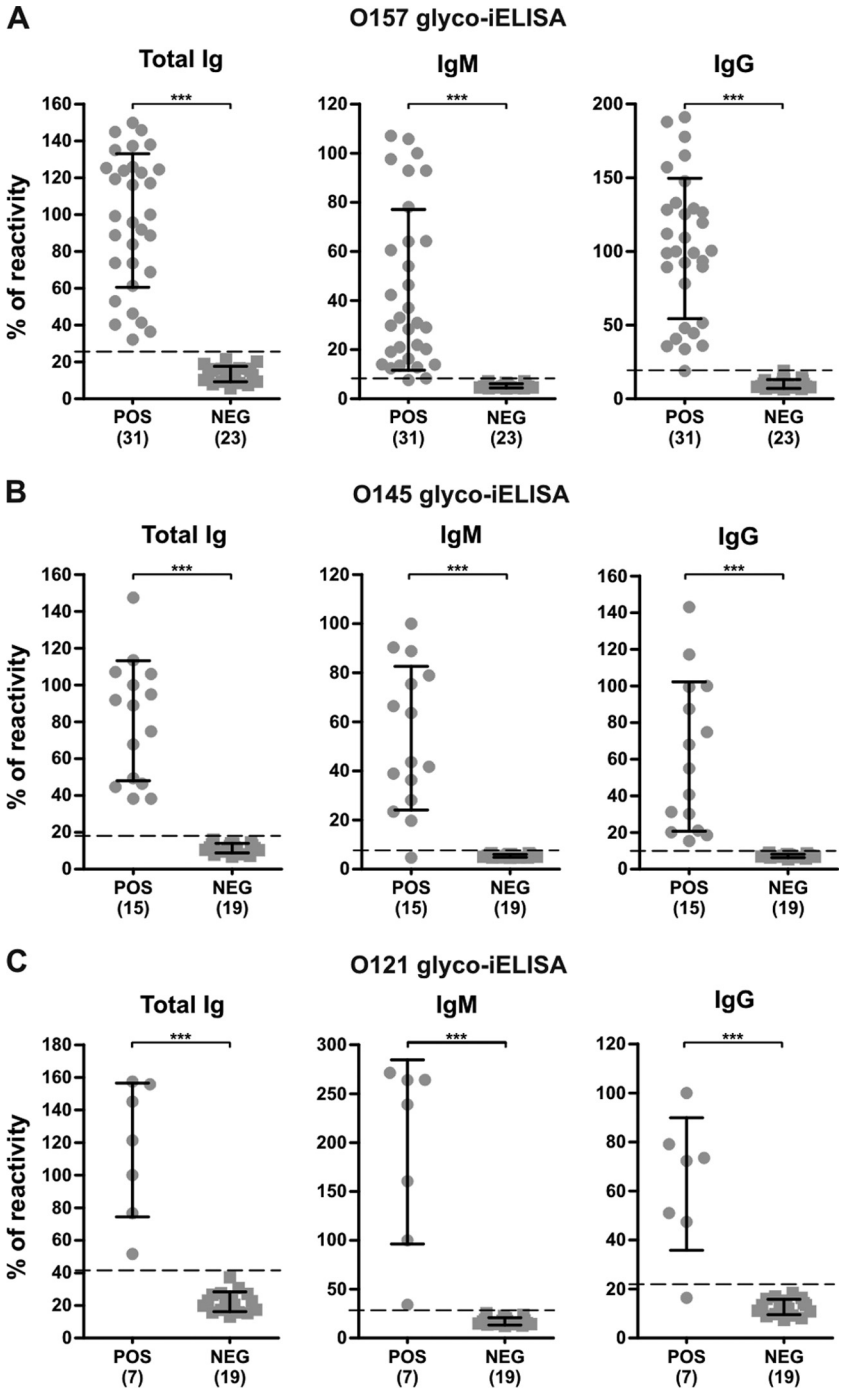

FIG 4 Dot plot analysis of glyco-iELISAs results. Microtiter plates coated with O157-AcrA, O145-AcrA, or O121-AcrA were incubated with serum samples obtained from culture-positive HUS patients (POS) for STEC O157 (A), O145 (B), and O121 (C), respectively. Serum samples from healthy children of the same age group were included as negative-control samples (NEG). Bound antibodies were detected using HRP-conjugated anti-human total Ig, IgM, or IgG antibodies. The results are expressed as the percentage of reactivity of the positive-control serum included in each assay run. The numbers in parentheses indicate the number of serum samples analyzed for each group. The mean and standard deviation for each group are indicated. The dashed lines indicate the cutoff value of each assay, calculated as the mean plus three standard deviations of the reactivity values of the negative samples. ***, $P < 0.0001$, Mann-Whitney test.

AcrA, and O121-AcrA as serogroup-specific antigens for the serological diagnosis of STEC-associated HUS.

In the present work, O157-AcrA, O145-AcrA, and O121-AcrA antigens were produced by bacterial glycoengineering technology and characterized by immunoblotting and mass spectrometry. The glycoproteins, but not the nonglycosylated form of AcrA, were recognized by specific antisera against each serogroup, and the mass spectra of O145-AcrA and O121-AcrA indicated that the structure of the sugar linked to the carrier protein in each case has the expected structure. To evaluate if these glycoproteins can be

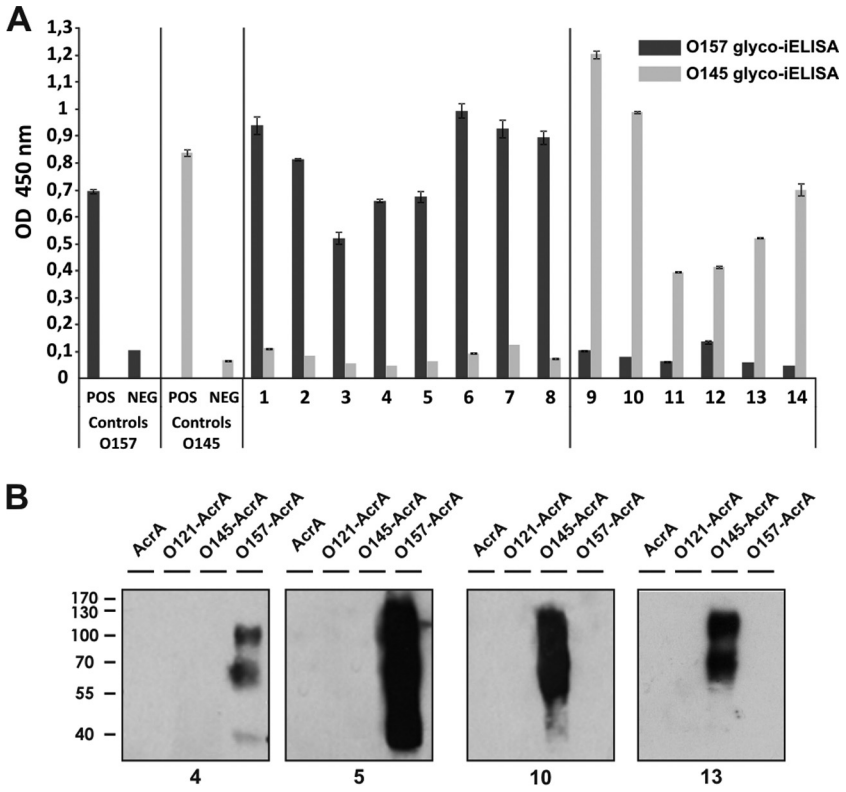

FIG 5 Analysis of serum samples obtained from culture-negative toxin-negative HUS patients representing a panel of 14 serum samples obtained from children with a clinical diagnosis of HUS in which the microbiological and Shiga toxin diagnosis were negative. (A) Glyco-iELISA analysis. Microtiter plates coated with O157-AcrA or O145-AcrA were incubated with the indicated serum samples. Bound antibodies were detected using HRP-conjugated goat anti-human total Ig antibodies. Samples O157-1 and O145-1 were used as positive controls (POS) and Neg-1 as a negative control (NEG) (see Fig. 3). Error bars indicate the standard deviations for duplicate determinations. (B) Western blot analysis of the indicated serum samples. Bound antibodies were detected using HRP-conjugated goat anti-human total Ig antibodies. The position of molecular mass standards (in kDa) is indicated on the left. Immunoblotting was performed with all the samples; only the analysis of four representative samples is shown.

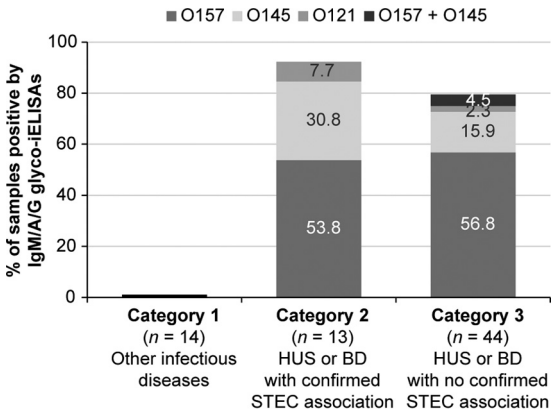

FIG 6 Retrospective single-blind study. A panel of 71 coded serum samples obtained from children under 8 years of age were analyzed blindly by IgM, IgG and total Ig glyco-iELISAs and classified as positive or negative for each serotype based on the cutoff values previously established for these assays (see Table S1). Three patient categories were considered for the analysis. Category 1, patients with other infectious diseases; category 2, patients with diagnosis of HUS or BD with confirmed STEC association by positive isolation of the bacterium from stools, FFStx detection by specific cytotoxicity on Vero cells or *stx* genes detection by PCR from the confluent zone of growth; and category 3, patients with diagnosis of HUS or BD wherein the association of the disease with STEC infection was not confirmed either by stool culture, FFStx or *stx* genes detection (see Table S2). *n*, indicates the number of patients included in each category.

used as antigens for the diagnosis of STEC infection in children, serum samples obtained from culture-positive HUS patients with STEC O157, O145, and O121 and from healthy individuals were analyzed by immunoblotting. Using this analysis, we were able to demonstrate that the detected antibody response was specifically directed toward the O-polysaccharide moiety of the glycoproteins and that the antigens are specific for each STEC serogroup. In addition, the strong reaction of the sera from HUS patients versus the low background of the negative-control samples suggested the strong potential of these antigens for diagnosis. In order to evaluate this, indirect O157-AcrA, O145-AcrA, and O121-AcrA glyco-iELISAs were developed, and a complete panel of serum samples obtained from culture-positive and culture-negative HUS patients, as well as from healthy individuals, was analyzed. Our results demonstrated that using these antigens, it was possible to clearly discriminate between infected patients with the STEC O157, O145, or O121 serogroups and healthy children, and they confirmed the serospecificities of these antigens. Interestingly, a specific IgM response was detected in almost all of the analyzed samples, indicating that is possible to detect the infection even at the early stages of the disease. To further assess if the developed glyco-iELISAs can aid in the diagnosis of STEC-associated HUS, a retrospective single-blind study was carried out, which included samples obtained from culture-positive and culture-negative patients with HUS or BD and from patients with other infectious

diseases. Using this analysis, we confirmed that the glycoprotein antigens are highly specific, even with serum samples from children with other infectious diseases, including one case of diarrhea caused by a STEC strain of the serotype O103:H2. More than 90% of the samples obtained from HUS or BD patients with confirmed STEC association were serologically positive by the glyco-iELISAs and, in all cases, the identified serotype by glyco-iELISAs was in accordance with the serotype of the isolated strain, demonstrating the value of these antigens not only for diagnosis but also for serotyping. Additionally, 79.5% of the samples obtained from patients with no confirmed association of the disease to STEC were positive by the glyco-iELISAs, indicating that these tests can improve diagnosis of the disease even in HUS patients for whom bacterial isolation and Shiga toxin detection failed. Interestingly, we were able to confirm the diagnosis in five of these patients by immunoconcentration using magnetic beads functionalized with specific antibodies against the serogroup previously identified by the glyco-iELISAs, and by plating. These results indicate that glyco-iELISAs, due to their serospecificities, are useful not only for detection of the infection but also for guiding the subsequent steps to confirm diagnosis. Finally, the negative results obtained by the glyco-iELISAs (7.7% in category 2 and 20.5% in category 3) can be explained either by low sensitivity of the glyco-iELISAs, infection with STEC serogroups different from O157, O145, and O121, or degradation of antibodies, as some of these samples were collected as early as 2005. Even more, the group of HUS patients with no confirmed STEC association (category 3) may include some cases of atypical HUS (not associated with STEC infection), explaining why isolation, Shiga toxin detection, and serology were negative. This considered, it is important to highlight that all samples from the HUS patients with confirmed STEC infection by isolation of either the O157, O145, or O121 serogroups were positive by the corresponding glyco-iELISA, indicating the high sensitivity of the method.

The recombinant glycoproteins O157-AcrA, O145-AcrA, and O121-AcrA were produced in *E. coli* coexpressing PglB, AcrA-His tag and the cluster of genes required for the synthesis of the corresponding O polysaccharide. The resulting glycoproteins were purified in one step and in large quantities from cultures of nonpathogenic *E. coli*. The glycoengineering method overcomes many of the inherent disadvantages of the chemical traditional methods used for the production of such glycoconjugates (20). With this technology, there is no need to purify the acceptor protein and LPS separately, and no chemical treatments are required for isolation of the O polysaccharide from the LPS. Additionally, no chemical cross-linking of the carbohydrate to the protein is required, which allows the production of the conjugates with a defined and reproducible sugar pattern, since the length of the O polysaccharide and the glycosylation process are controlled *in vivo*. Therefore, this system may improve the reproducibility of the assay, allowing the production of homogeneous and standardized batches of antigen. Finally, due to its versatility, this bacterial glycosylation system can be seen as a toolbox for engineering a catalogue of novel and different serotype-specific O-polysaccharide–protein conjugates. In Argentina, the dominant STEC serotype isolated from HUS patients is O157:H7 (74.8%), followed by O145:NM (6.9%), O145:HNT (2.3%), O121:H19 (2.3%), ONT:HNT (2.3%), and O20:H19, O26:H11, O91:NM, O103:HNT, O111:NM, O171:HNT, ONT:H12, ONT:H19, and ONT:NM (1.2% for each) (6). Hence, with the glycoproteins developed in this work, we are able to cover >85% of the STEC-associated HUS cases. However, due to the great versatility of our technology, it should be very simple to expand this panel of antigens to detect other serogroups currently important for the disease in our country and other parts of the world, such as the O26, O103, O111, and O45 serogroups included in the "big six" non-O157 STEC serogroups (together with O145 and O121) (5), as well as to quickly develop new antigens for emerging STEC serogroups (e.g., the O104:H4 outbreak that occurred in Germany in the year 2011), allowing a timely response in case of an outbreak and an improvement in control measures.

Currently, serological tests for the diagnosis of STEC-associated HUS use whole bacteria, bacterial extracts containing high concentrations of LPS, or purified LPS as antigens (10, 11, 15–17). Consequently, these tests may lack serospecificity due to the presence of cross-reactive antibodies against common epitopes present in the core polysaccharide and lipid A of LPS shared by different STEC serotypes. Additionally, they may result in false-positive reactions due to epitopes shared by STEC and other bacteria. Instead, the recombinant glycoproteins O157-AcrA, O145-AcrA, and O121-AcrA are serogroup specific, and no cross-reactivity among the three glycoproteins was observed by either immunoblot or glyco-iELISAs. This serospecificity may be due to the fact that only the O polysaccharide, the outer moiety of LPS, is coupled to the carrier protein. Therefore, glyco-based assays may be more specific than currently used serological tests, and due to their serospecificity, they might be valuable laboratory tools not only for diagnosis but also for studying the distribution of STEC serogroups for epidemiological purposes.

Studies of the clinical course of *E coli* O157:H7 infections in children indicate that the time between the ingestion of a contaminated vehicle and the onset of diarrhea ranges between 2 and 12 days (4, 33). Typically, *E coli* O157:H7 infections cause 1 to 3 days of nonbloody diarrhea, after which the diarrhea becomes bloody (34). The bloody diarrhea, which occurs in about 90% of cases, is generally the sign that prompts patients or their families to seek medical attention, but in some cases, the onset of HUS appears not to be preceded by bloody diarrhea (4). Independently of the occurrence of this sign, the presence of high anti-LPS antibody titers has been demonstrated to be concomitant with the beginning of the symptoms (16). In this regard, we have observed strong positive IgM and IgG responses against the corresponding O polysaccharide in samples obtained only 2 days after the onset of symptoms (see Table S2 in the supplemental material, patients 25 and 52). Furthermore, in two samples, only a specific IgM response was detected (see Table S2, patients 17 and 42). Further work will be required to evaluate the usefulness of the recombinant glycoproteins for the early diagnosis of STEC infections, especially during the diarrheal phase and before the toxin-mediated vascular injury begins (2, 35). The development of a rapid and early diagnostic technique for STEC-associated HUS could become an effective tool to avoid delays in starting a supportive or, in the case it become available, specific treatment for this disease.

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A patent has been filed regarding the diagnostic application of recombinant glycoproteins.

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