Microbial Cell Factories

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Development of a set of bacterial engineered glycoconjugates as novel serogroup-specific antigens for the serodiagnosis of *Escherichia coli* O26, O111, O103 and O45 infections associated to hemolytic uremic syndrome

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Abstract

Hemolytic uremic syndrome associated to Shiga toxin-producing *Escherichia coli* infection (STEC-HUS) is a life-threatening condition characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute kidney failure. Among STEC, *E. coli* O157:H7 is the dominant serotype related with human disease worldwide; however, a subset of STEC non-O157 serotypes -named the *"Big-Six"*- that include the *E. coli* serogroups O145, O121, O26, O111, O103 and O45 became of a great concern for their potential to cause HUS. Previously, we have demonstrated that serological tests based on bacterial engineered glycoconjugates developed by exploiting the *Campylobacter jejuni* N-glycosylation machinery, notably increases the association rate of HUS to O157, O145 and O121 STEC infections. In this work, we developed the recombinant glycoproteins O26-AcrA, O111-AcrA, O103-AcrA and O45-AcrA by co-expressing in *E. coli* the gene cluster required for the synthesis of the O polysaccharide corresponding to each serogroup, the *C. jejuni* oligosaccharyltransferase (OTase) PglB, and the carrier protein AcrA. The glycans attached to AcrA in the produced and purified glycoconjugates were characterized by mass spectrometry. The glycoconjugates were evaluated as antigens for detection of IgM antibodies against the O polysaccharide of the lipopolysaccharide of O26, O111 and O103 STEC strains in human serum samples. Our results demonstrate that O26-AcrA, O111-AcrA and O103-AcrA allow a clear discrimination between negative and positive samples obtained from patients with HUS associated to O26, O111 and O103 STEC infections. Additionally, these novel antigens are serospecific allowing *E. coli* serogroup identification which may contribute to the epidemiological surveillance of STEC-HUS patients and their contacts.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a food and waterborne pathogen that can cause human infections ranging from asymptomatic carriage or mild diarrhea to bloody diarrhea (BD) and hemolytic uremic syndrome (HUS), a life-threatening disease characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute kidney failure [1]. Although there are more than a hundred of STEC serotypes related to human illness, O157:H7 is the most prevalent in many countries and has been associated with sporadic cases as well as large outbreaks of severe illness worldwide [2]. However, in the last decades, non-O157 STEC infections became a great concern due to their potential to develop severe disease [3]. Among non-O157 serogroups, the US Food and Drug Administration (FDA) has identified six serogroups commonly associated with cases of foodborne illness across different nations, known as the "Big Six", that include STEC strains from the serogroups O26, O103, O111, O121, O145 and O45 [4]. Notably, within the European Union, O26 is the most frequently identified serogroup after O157, revealing its epidemiological relevance and contribution to infections that may lead to severe clinical outcomes [5].

HUS related to STEC infections (STEC-HUS) mainly affects children under 5 years old and represents a serious public health issue with a great social and economic impact because of the severity of the disease and long-term sequelae. Among children under the age of 5 infected with STEC, 15 to 20% develop HUS [6], although this proportion can increase to 30% during outbreaks [7]. At the acute phase, around 80% of patients with HUS require blood transfusion and 60% dialysis. Apart from kidney injury, intestinal, cardiac, and neurological complications may occur during the acute phase, with a mortality rate of 1.5 to 3%. While full recovery takes place in around 70% of the patients, renal and neurological sequelae remain in 30% and 5% of the patients, respectively [8]. In Argentina, STEC-HUS is an endemic disease with a mortality rate of 2 to 4%. It is the main cause of acute renal failure in children, an important cause of end-stage renal disease (ESRD), and for the period 1998–2021 STEC-HUS accounted for 11% of kidney transplants in children and adolescents [9].

Stool culture for STEC isolation and characterization is the gold standard test for the diagnosis of STEC infections [10]. However, this methodology can be challenging as STEC recovery rate from stool is high on the onset of diarrheal symptoms but decays on the successive days. Whereas STEC O157:H7 does not ferment sorbitol and forms colorless colonies that can be easily detected in the selective and differential culture medium SMAC (Sorbitol MacConkey Agar), non-O157 STEC strains ferment sorbitol and are phenotypically undistinguishable from non-pathogenic *E. coli* or other diarrheagenic *E. coli* (DEC); therefore, other techniques such as detection of free fecal Shiga-toxin (FFStx) by enzyme immunoassays (EIA) and *stx* genes using polymerase chain reaction are used [8, 11]. The clinical diagnosis of HUS is carried out by the identification of signs of platelet consumption, evidence of hemolysis and signs of kidney damage that generally occurs from 6 to 10 days after the onset of diarrhea [12], but the association of the disease to a STEC infection by the techniques aforementioned mostly occurs in less than 50% of HUS cases [13–16]; furthermore, the detection rate of STEC infection is even lower after the first few days of diarrhea and before the development of HUS.

In order to improve the diagnosis of STEC infections, antibodies against the lipopolysaccharide (LPS) of STEC strains associated with HUS can be detected [17–20]. Many authors reported that the detection of anti-LPS antibodies in combination with bacteriological methods and *stx*/Stx detection increase the evidence of STEC infection in more than 80% of HUS cases [13, 21–23]. However, false-positive reactions may occur because of cross-reactive antibodies against epitopes present in the core and lipid A moieties of LPS and shared by the STEC strains and other enterobacteria [24]. To circumvent this limitation, in a previous work we have developed bacterial engineered glycoconjugates in which only the O polysaccharide section of the LPS -the immunodominant portion and serogroup determinant- is covalently linked to a carrier protein generating a glycoprotein. We have demonstrated an improved performance of these molecules as antigens for the serological detection of STEC O157, O145 and O121 infections in patients with clinical diagnosis of HUS [25]. Subsequently, Wijnsma and colleagues have revealed the benefits of using O157-glycoprotein compared to O157-LPS as antigens in an indirect ELISA due to its higher specificity and better assay performance [14]. The bacterial engineered glycoconjugates were developed by exploiting the *Campylobacter jejuni* N-glycosylation machinery in which the oligosaccharyltransferase PglB transfers a lipid-linked heptasaccharide from the inner membrane to the periplasmic protein AcrA and more than 30 secreted proteins that contain a specific N-glycosylation sequon [26]. As a result of the relaxed specificity of PglB for the glycan substrate structure, this N-glycosylation machinery can be functionally reconstituted in non-pathogenic bacteria by transferring and co-expressing the genes that encode for PglB and AcrA [27, 28]. This approach has been used for the transfer of other lipid-linked oligosaccharides, such as the O polysaccharide of LPS, to a carrier protein to produce recombinant glycoproteins used as diagnostic antigens

[14, 25, 29–33] and vaccines [29, 34–41]. The use of this technology overcomes the main disadvantages of the traditional chemical coupling strategy and allows the biosafe production of glycoconjugates, reducing the process costs and increasing yields [42].

In this work, we have developed and characterized the recombinant glycoproteins O26-AcrA, O111-AcrA, O103-AcrA and O45-AcrA by expressing the *C. jejuni* N-glycosylation machinery in *E. coli*, thus completing the panel of antigens for the serological detection of the "Big-Six" STEC infections associated to HUS. We propose that these molecules can be used as novel serogroup-specific antigens for the diagnosis of *E. coli* O26, O111, O103 and O45 infections in BD and HUS cases, and to study the distribution of these serogroups for epidemiological purposes.

Materials and methods

Bacterial strains and culture conditions

The strains and plasmids used in this work are listed in Table 1. *E. coli* XL1Blue was used for cloning procedures. Cultures were grown at 37 °C, 200 rpm in 2xYP (16 g/L bactopeptone, 12 g/L yeast extract and 5 g/L sodium chloride) or TSB (17 g/L pancreatic digest of casein, 3 g/L papaic digest of soybean, 2.5 g/L K_2HPO_4, 5 g/L NaCl and 2.5 g/L dextrose) supplemented with 100 mg/L ampicillin, 50 mg/L kanamycin, 20 mg/L chloramphenicol or 100 mg/L trimethoprim for strain selection.

Cloning and expression of O103 gene cluster

The O103 gene cluster (GenBank accession number AY532664) was amplified by PCR in two fragments using Elongase DNA polymerase (Invitrogen Life Technologies) and genomic DNA from STEC O103:H2 as template. The oligonucleotides *KpnI-galF* (GGGGTACCCGCAACCTGAAAGAAGGGGCGAAGTTCCG) and *XmaI-wzx*O103 (TCCCCCCGGGTTATAGGGCCTTTTTTGTCAGATGCCAACA) were used to amplify the genes between *galF* and *wzx*, and *XmaI-wbtD*O103 (TCCCCCCGGGCAAATGATAATATCTTCAGATAACCTAAGT) and *XbaI-gnd* (GCTCTAGAAGGTTGCGCCCCATCACTGCCATACCGAC) to amplify the genes between *wbtD* and *gnd*. The 6.7-Kb *galF-wzx* PCR product was digested with *KpnI* and *XmaI* and cloned in pBBR2, resulting in pBBR2-O103A. The 5.7-Kb *wbtD-gnd* PCR product was digested with *XmaI* and *XbaI* and cloned in pBBR4, resulting in pBBR4-O103B. IPTG-dependent synthesis of O103-AcrA was confirmed by Western Blot.

Table 1 Plasmids and strains used in this work

Strain/Plasmid	Description[a]	Reference
E. coli strains		
XL1Blue MR10	*Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lacIqZΔM15 Tn10 (Tetr)]*	Stratagene
CLM24	*F- λ- rph-1 INV(rrnD, rrnE) ΔwaaL*	[27]
STEC O103:H2	STEC isolate	ANLIS-Malbrán
Serogroup O103	Non-STEC isolate CA10	ANLIS-Malbrán
Serogroup O26	Non-STEC isolate FP 1050/11	ANLIS-Malbrán
Serogroup O111	Non-STEC isolate FP 1038/11	ANLIS-Malbrán
Escherichia coli O45	Non-STEC isolate	ANLIS-Malbrán
Plasmids		
pBBR2	Cloning vector, Kan^R	[57]
pBBR4	Cloning vector, Amp^R	[57]
pACYC184	Cloning vector, Tet^R Kan^R	[58]
pMLBAD	Expression vector, Tmp^R	[59]
pMAF10	pMLBAD Ω(*pglB::HATag*), Tmp^R	[27]
pMH5	pACYC184 Ω(*acrA::6xhisTag)*, Cmp^R	[27]
pBBR2-O103A	pBBR2 Ω(O103*galF'-wzx*), Kan^R	This work
pBBR4-O103B	pBBR4 Ω(O103*wbdT-gnd'*), Amp^R	This work
pGV	pMAF10 Ω (*TETp::acrA*), Tmp^R	[43]
pACYC184-*pglB*	pACYC184 Ω(*pglB::HATag*), Cmp^R	This work
pMLBAD-*acrA*	pMLBAD Ω(*acrA::6xhisTag)*, Tmp^R	This work

[a] *Cmp* Chloramphenicol, *Amp* Ampicillin, *Tmp* Trimethoprim, *Kan* Kanamycin, *Tet* Tetracycline

Cloning and expression of PglB

The *pglB* gene fused to the DNA fragment that codify for the hemagglutinin tag was amplified by PCR with the oligonucleotides *EcoRV-pglB* Fw (AAGATATCATGTTG AAAAAAGAGTATTTAAAAAACCC) and *BamHI-pglBHA* Rv (CGGGATCCTTAAGCGTAATCTGGAAC ATC) using *Pfu* DNA polymerase and pMAF10 as template. The PCR product was digested with *EcoRV* and *BamHI* and cloned under the control of the constitutive tetracycline promoter in pACYC184, resulting in pACYC184-*pglB*. Expression of PglB fused to the HA tag (PglB-HA) was confirmed by Western blot.

Cloning and inducible expression of AcrA

The *acrA* gene fused to the DNA fragment that codify for the histidine tag was amplified by PCR with the oligonucleotides *EcoRI-acrA* Fw (CGGAATTCTGTTTA ACTTTAAGAAGGAG) and *PstI-acrA* Rv (AAACTG CAGTTCCTTTCGGGCTTTGTTAG) using *Pfu* DNA polymerase and pMH5 as template. The PCR product was digested with *EcoRI* and *PstI* and cloned in the vector pMLBAD, resulting in pMLBAD-*acrA*. Arabinose-dependent synthesis of AcrA fused to the histidine tag (AcrA-6xHis) was confirmed by Western blot.

Production and purification of the glycoproteins O103-AcrA, O111-AcrA, O26-AcrA and O45-AcrA

To produce O103-AcrA, the strain *E. coli* CLM24 containing the plasmid pGV (carrying the genes that encode for PglB and AcrA) was transformed with pBBR2-O103A and pBBR4-O103B generating the strain *E. coli* CLM24 O103. This strain was grown in 2xYP broth (16 g/L peptone, 12 g/L yeast extract and 5 g/L sodium chloride) at 37 °C and glycoprotein expression was induced as described elsewhere [25].

The production of O26-AcrA, O111-AcrA and O45-AcrA was carried out using non-toxigenic *E. coli* isolates of the serogroups O26, O111 and O45, respectively, provided by the National Reference Laboratory (NRL) for HUS and diarrhea disease [Servicio de Fisiopatogenia, Instituto Nacional de Enfermedades Infecciosas (INEI)-ANLIS Dr. Carlos G. Malbrán]. The non-toxigenic *E. coli* isolates of the serogroups O26 and O45 were transformed with pMAF10 and pMH5 that contain the genes encoding for PglB and AcrA, respectively. Cultures grown overnight at 37 °C in TSB were used for inoculation of fresh media supplemented with 1% v/v of glycerol, 20 mg/L chloramphenicol and 100 mg/L trimethoprim to an initial optical density (OD_{600nm}) of 0.1. After two hours of culture at 37 °C and 200 rpm (OD_{600nm}≈0.6), *pglB* expression was induced by the addition of L-arabinose (0.2% for O45-AcrA and 1% for O26-AcrA) and 1 mM $MnCl_2$. Five hours after the addition of the inducer, a second pulse of L-arabinose was added and cultured overnight.

To produce O111-AcrA, a non-toxigenic *E. coli* isolate of the serogroup O111 was transformed with pACYC184-*pglB* and pMLBAD-*acrA*. Cultures grown overnight at 37 °C in TSB were used for inoculation of fresh media supplemented with 1% vol/vol of glycerol, 1 mM $MnCl_2$, 20 mg/L chloramphenicol and 100 mg/L trimethoprim to an initial optical density (OD_{600nm}) of 0.1. After two hours of culture at 37 °C and 200 rpm (OD_{600nm}≈0.6), *acrA* expression was induced by the addition of 0.2% wt/vol L-arabinose and cultured overnight.

Cultures were harvested by centrifugation and periplasmic proteins were obtained from bacterial pellet by an improved two-step osmotic shock method described elsewhere [43].

Glycoproteins were purified from the periplasmic extracts by affinity chromatography using a 5 mL HisTrap High Performance (GE Healthcare) prepacked column. Briefly, column was equilibrated with 10 column volumes of binding buffer (20 mM Tris–HCl pH 8.00, 300 mM NaCl and 20 mM imidazole) and the periplasmic extract was loaded at a flow rate of 5 mL/min. Subsequently, column was washed with 10 column volumes of binding buffer to remove unbound protein. Finally, glycoprotein was eluted from the column with the elution buffer (20 mM Tris–HCl pH 8.00, 300 mM NaCl and 500 mM imidazole) and dialyzed with PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 2.7 mM KCl; pH 7.4) at 4 °C.

Western blot

Purified AcrA and the glycoproteins were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane using a semi-dry electroblotting transfer unit. After blocking with TBS-T-M (20 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.1% vol/vol Tween 20, 1% wt/vol dry skim milk), the membranes were incubated with agitation for 1 h with a mouse anti-AcrA polyclonal antibody at a 1:5.000 dilution in TBS-T-M, specific rabbit antisera against each serogroup (provided by the Instituto Nacional de Producción de Biológicos-ANLIS Dr. Carlos G. Malbrán) or the indicated human serum samples at a 1:100 dilution in TBS-T-M. Membranes were washed four times with TBS-T and incubated for 1 h with a 1:20.000 TBS-T-M dilution of anti-IgG IRDye fluorophore-labeled secondary antibodies (LI-COR, Lincoln, NE). After washing three times with TBS-T and once with TBS, the membranes were scanned using the Odyssey Imaging System (LI-COR).

Mass spectrometry

Affinity purified glycoproteins were dried and dissolved in 50 µL of 50 mM NH_4HCO_3. Samples were reduced with DTT at final concentration of 5 mM for 30 min at 37 °C and alkylated with IAA at final concentration of 15 mM for 30 min in darkness. Alkylation was quenched by incubation in final concentration of 5 mM DTT for 15 min. Trypsin was added into sample with protein-to-trypsin ratio of 100:1. Incubation was performed overnight at 37 °C. The samples were freeze-dried.

Glycopeptides enrichment was achieved by solid-phase extraction Cotton-HILIC microtips as described by Selman et al. [44]. Briefly, HILIC home-made microtips were washed and equilibrated with 85% ACN. Dried samples were resuspended in 85% ACN and load onto the stationary phase by aspirating and dispensing the protein mixture. Non-glycopeptides were washed off from the column by 85% ACN and 0.5% TFA, and the retained glycopeptides were then eluted with water. Glycopeptide fraction was freeze-dried and stored at −20 °C until mass spectrometry analysis.

Glycopeptides enriched fractions were analyzed in a nanoLC 1000 coupled to an EASYSPRAY Q Exactive Mass Spectrometer (Thermo Scientific) equipped with a HCD and an Orbitrap analyzer. An Easy Spray PepMap RSLC C18 column (50 µm × 150 mm, particle size 2.0 µm, pore size: 100 Å) at 40 °C was used for separation. Separation was achieved with a linear gradient from 5 to 35% solvent B developed in 75 min, at a flow of 300 nL/min (mobile phase A: water− 0.1% formic acid; mobile phase B: ACN− 0.1% formic acid). The injection volume was 2 µL. The mass spectrometer was operated in positive mode, with a spray voltage set at + 3.5 kV. A full-scan survey MS experiment (m/z range from 400 to 2000) was conducted, with an automatic gain control target set at 3×10^6, a maximum ion trap time (IT) of 200 ms, and a resolution of 70,000 at 400 m/z. Acquisition data method was set to the centroid mode, with a resolution of 17,500, a maximum IT 50 ms, and an automatic gain control target set at 1×10^5. To obtain the MS/MS (tandem mass spectrometry) spectra, a data-dependent mode (DDA) was used with one MS scan followed MS/MS of the top 15 peaks in each cycle. Normalized collision energy (NCE) was set to 27. The source temperature was set at 175 °C.

Indirect enzyme-linked immunosorbent assay (Glyco-iELISAs)

Microtiter plates (Corning polystyrene high binding microplates) were coated with 100 µl of 2.5 µg/ml purified O111-AcrA, O26-AcrA or O103-AcrA solution in 50 mM carbonate buffer (pH 9.6). After incubating for 18 h at 4 °C, the plates were blocked with 300 µL of 5% bovine skim milk in TBS-T (blocking buffer) for 1 h at room temperature (RT). The serum samples were diluted in blocking buffer and incubated for 1 h at RT. After four washing steps with TBS-T, 100 µL per well of horseradish peroxidase (HRP)-conjugated goat anti-human IgM antibodies (Sigma) diluted in blocking buffer were added and incubated for 1 h at RT. The plates were washed four times with TBS-T to remove excess of reagents and incubated with 100 µL of TMB substrate solution for 10 min. The reaction was stopped with 1% HCl and the absorbance was determined at 450 nm using a plate reader (Filtermax, Molecular Devices).

Optimization of the assays was established in preliminary experiments through a checkerboard titration analysis using positive and negative serum samples. Based on these analyses, the optimal antigen concentration was 2.5 µg/ml (250 ng/well), the optimal sample dilution was 1:100 for O111-AcrA and O26-AcrA and 1:400 for O103-AcrA, and the optimal dilution of the conjugate was 1:4.000. These parameters were used to test all the samples.

Serum samples

Different serum samples were analyzed in this study. I) Serum samples from children up to 6 years old with clinical diagnosis of HUS and with a stool culture positive for STEC O157:H7 (n = 5), O145:NM (n = 4) or O121:H19 (n = 3), and positive for the serological test CHEMLIS® *E. coli* O157, O145 or O121 Glyco-iELISA (Chemtest Argentina S. A.). CHEMLIS® *E. coli* O157/O145/O121 Glyco-iELISAs (Chemtest Argentina S. A.) are enzyme-linked immunosorbent assays that allow to detect exclusively and specifically anti-O157/O145/O121 polysaccharide IgM and IgG antibodies in human serum samples [25]. II) Serum samples from children up to 6 years old with clinical diagnosis of HUS and with a stool culture positive for STEC O26 (n = 1), O103 (n = 3) or O111 (n = 1). III) Serum samples from children up to 6 years old with other diseases not related to diarrhea (negative samples, NEG) (n = 30). IV) Serum samples from children up to 6 years old with clinical diagnosis of BD and a stool culture positive for an etiological agent unrelated to STEC (*Campylobacter spp.* n = 2, *Salmonella spp.* n = 2, *Shigella sonnei* n = 1, *Staphylococcus aureus* n = 1).

Ethics statement

The samples analyzed in this retrospective study came from a previously characterized serum collection of the national network for the surveillance of HUS and STEC infections and provided by the NRL (NRL-Servicio de Fisiopatogenia, Instituto Nacional de Enfermedades Infecciosas INEI-ANLIS Dr. Carlos G. Malbrán, Buenos Aires, Argentina). These samples were obtained from

patients treated at different hospitals in Argentina during the period 2015–2018 and then submitted to the NRL. To ensure anonymity, the serum samples had been de-idientified upon collection.

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: A_{450} of the test sample/mean A_{450} of the positive control × 100. Dot plots were performed using the Graph-Pad Prism software (version 5.01 for Windows; San Diego, CA).

Results

Production and purification of O103-AcrA, O26-AcrA, O111-AcrA and O45-AcrA glycoproteins

Previously, we have exploited the *C. jejuni* N-glycosylation system for the development of the recombinant glycoproteins O157-AcrA, O145-AcrA and O121-AcrA, consisting of the O157, O145 and O121 polysaccharides attached to the carrier protein AcrA, for the diagnosis of STEC-HUS [25]. In this work, we used the same glycoengineering technology to produce the O103-AcrA, O26-AcrA, O111-AcrA and O45-AcrA glycoconjugates generating a complete panel of recombinant glycoproteins for the diagnosis of the remaining non-O157 STEC infections frequently associated to BD and HUS.

To generate the O103-AcrA glycoprotein, the O103-polysaccharide biosynthesis gene cluster was co-expressed with the oligosaccharyltransferase PglB and the carrier protein AcrA fused to a 6xHis-Tag (AcrA-6xHis) in the non-pathogenic *E. coli* strain CLM24 [27]. To develop the O26-AcrA, O111-AcrA and O45-AcrA glycoproteins, PglB and AcrA-6xHis were co-expressed in non-pathogenic *E. coli* O26, O111 and O45 strains. After bacterial culture and induction, the glycoproteins were purified from periplasmic extracts by immobilized metal affinity chromatography (IMAC). Purified glycoproteins and non-glycosylated AcrA were analyzed by SDS-PAGE followed by immunoblot using anti-AcrA antibodies and specific polyclonal antisera for the O26, O45, O103 and O111 polysaccharides (Fig. 1A to D). As shown in Fig. 1, non-glycosylated AcrA was detected as a band of ≈38 kDa that reacted with anti-AcrA antibodies and its glycosylation was evidenced by a ladder of bands of higher molecular weight ranging from 40 to 100 kDa, indicating that they correspond to modified forms of AcrA (Fig. 1A to D, left panels). For O45-AcrA, a systematic shift of the signal for AcrA to a smaller size was observed probably due to a change in the electrophoretic properties because of glycosylation. The bands of molecular weight above 40 kDa, but not non-glycosylated AcrA, reacted with the specific polyclonal antisera against O26, O45, O103 and O111-polysaccharides, confirming the identity of the corresponding O polysaccharide linked to AcrA (Fig. 1A to D, right panels).

Structural characterization of glycans attached to AcrA by mass spectrometry

The glycans attached to AcrA in the O103-AcrA, O26-AcrA, O111-AcrA and O45-AcrA glycoproteins were characterized by mass spectrometry. Purified glycoproteins were digested with trypsin, the glycopeptides were enriched by micro-SPE-HILIC and analyzed by nano HPLC-ESI-Orbitrap MS and MS/MS as indicated in Material and Methods. The MS/MS data were analyzed looking for the oxonium ions as reporters of glycopeptides. The glycopeptide structures were assigned considering the previously reported structure of the corresponding O polysaccharides for each serogroup.

For O26-AcrA, the MS data analysis allowed the assignment of the glycan structure consistent with the reported structure for the O26 polysaccharide composed of N-acetylglucosamine (GlcNAc), *N*-acetylfucosamine (FucNAc) and rhamnose (Rha) [45]. The MS/MS spectrum obtained for the ion of m/z 812.3521 $^{+2}$ (Fig. 2A) could be assigned to the peptide DFNR containing two repetitive units of NAcHex-NAcdHex-Pen. The fragmentations observed were consistent with this structure.

Regarding to the O45-AcrA, the data analysis allowed the characterization of the glycopeptide with m/z 1004.4319 $^{+3}$ (Fig. 2B) as the extended ATFENASKDFNR peptide with three units of the O45 polysaccharide composed of *N*-acetylfucosamine (FucNAc), 2-O-acetyl-6-deoxy-talose (6d-Tal2Ac) and glucose (Glc) [46]. The fragmentation signals were consistent with the glycopeptide structure assignment and correspond to the structures depicted in the figure.

For the O103-AcrA (Fig. 2C) we were able to assign the signal of m/z 1433.0692 $^{+2}$ to the DFNR peptide containing three O polysaccharide subunits. In this case, the fragmentations observed were consistent with a glycan composed of two *N*-acetylglucosamines (GlcNAc), *N*-acetylgalactosamine (GalNAc) and glucose (Glc) but differs from the structure reported for the O103 polysaccharide [47] in the absence of an hydroxybutanoyl-aminofucose.

Finally, the data analysis of O111-AcrA allowed the characterization of the glycan structure present in the glycoprotein. The MS/MS spectra of the signal of m/z 669.6900 $^{+2}$ (Fig. 2D) corresponds to the DFNR peptide bearing one unit of the O111 glycan. The MS/MS fragments observed are in accordance with a glycan composed of *N*-acetylglucosamine (GlcNAc), galactose

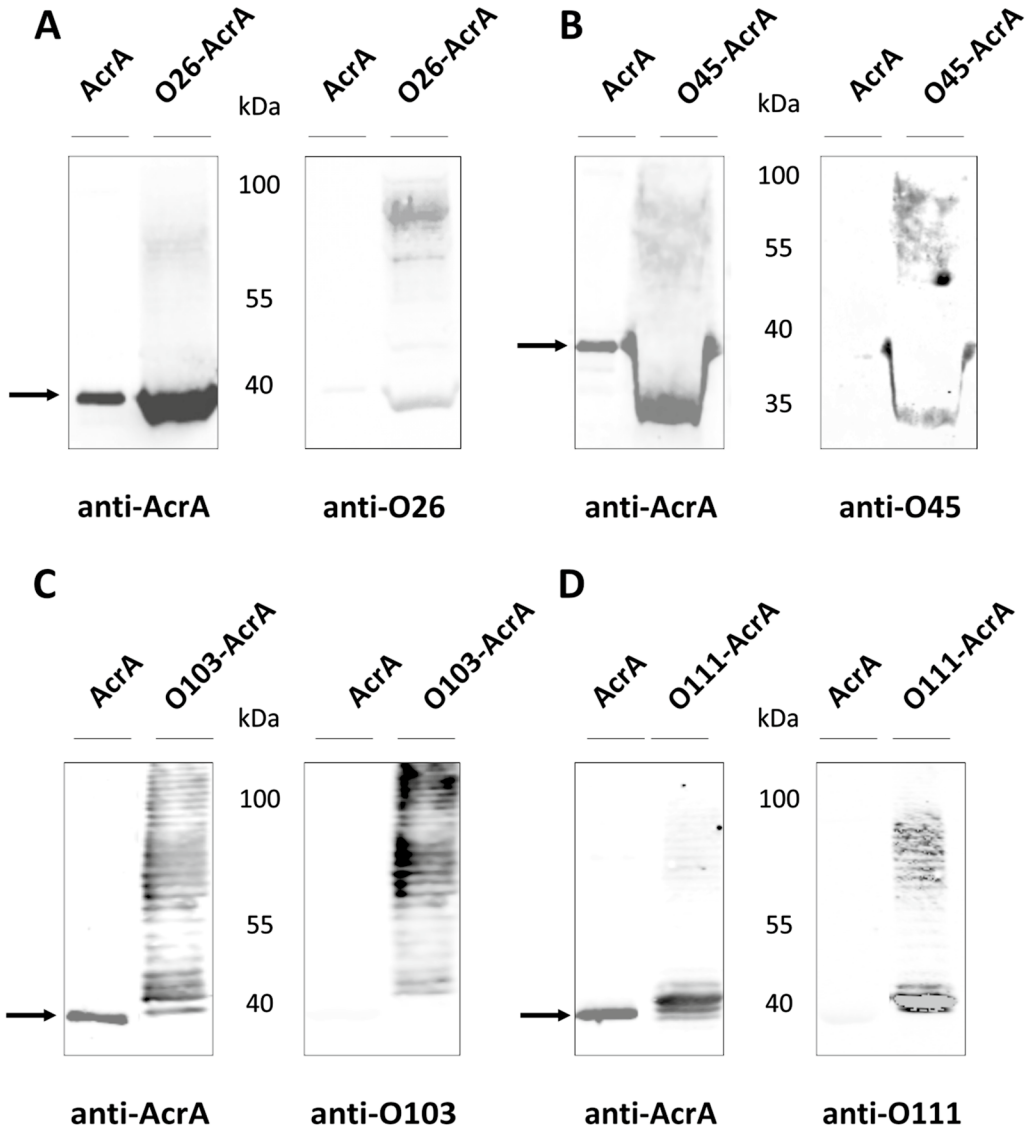


Fig. 1 Western blot analysis of the recombinant glycoproteins. Purified O26-AcrA **A** O45-AcrA **B** O103-AcrA **C** O111-AcrA **D** and non-glycosylated AcrA were subjected to 10% SDS-PAGE and analyzed by immunoblot using anti-AcrA antibodies and specific polyclonal antisera against the O26, O45, O103 or O111 polysaccharide. The arrows on the left indicate the migration position of non-glycosylated AcrA

(Gal), glucose (Glc) and colitose (Col) which is consistent with the reported structure for the O111 polysaccharide [48].

Taken together, the results demonstrate that the structures of the oligosaccharides attached to AcrA in the O26-AcrA, O45-AcrA and O111-AcrA glycoproteins are consistent with the structure of the O26, O45 and O111 polysaccharides previously reported. Instead, the structure of the oligosaccharide linked to AcrA in the O103-AcrA glycoprotein lacks a hydroxybutanoyl-aminofucose when it was compared with the reported structure of the O103 polysaccharide.

Recombinant glycoproteins as serogroup-specific antigens

To evaluate the glycoproteins as antigens for the diagnosis of *E. coli* O26, O103 and O111 infections, serum samples obtained from children with clinical diagnosis of HUS and stool culture positive for STEC O26:H11, O103:H2 or O111:NM were used as positive samples. These samples were analyzed by Western blot and indirect ELISA (iELISA) (Fig. 3). The glycoprotein O45-AcrA was not included in this analysis because no positive serum samples were available. As revealed in the Western blot analysis, specific reactivity against the glycoproteins (and not for the non-glycosylated AcrA) was observed,

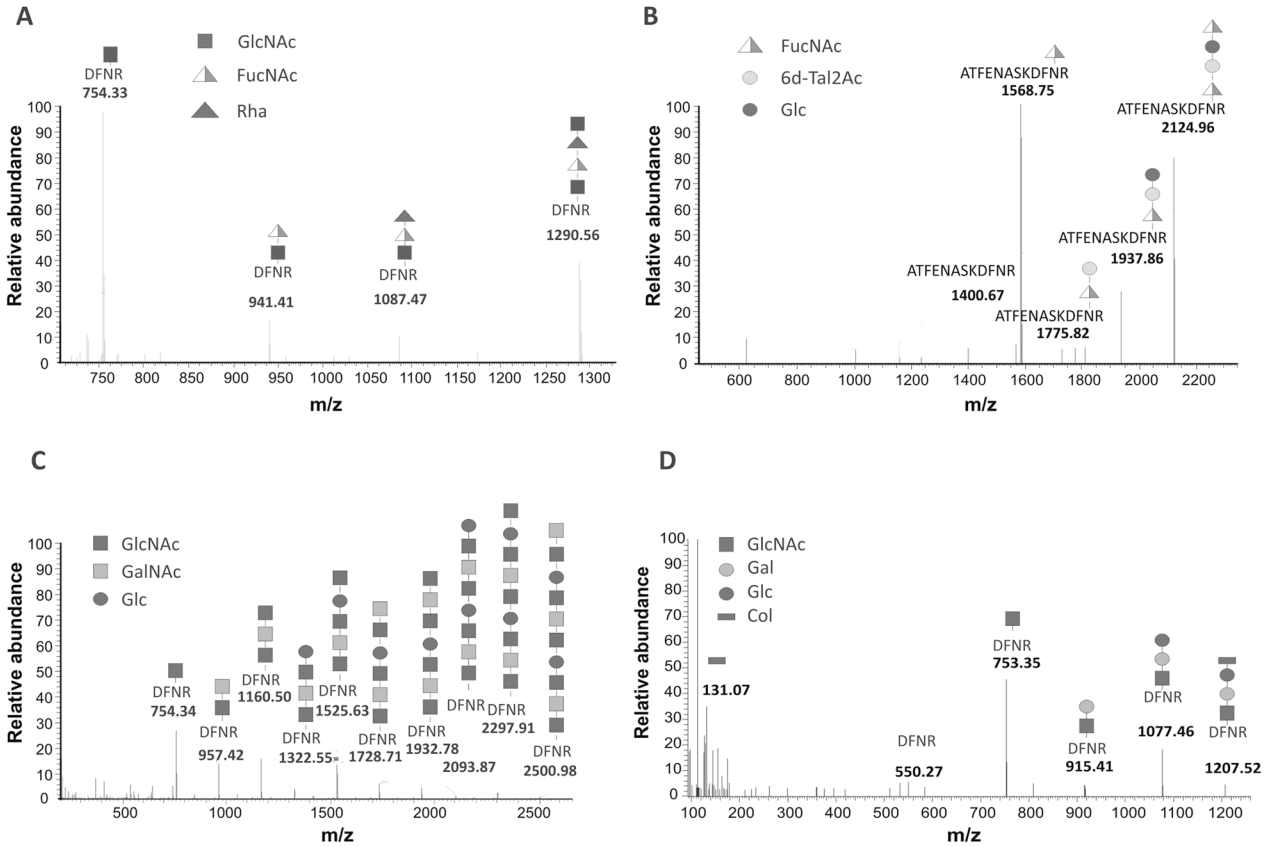


Fig. 2 Mass spectrometry analysis of glycosylated peptides of O26-AcrA, O45-AcrA, O103-AcrA and O111-AcrA. **A** MS/MS spectrum of the ion at m/z 812.3521 $^{+2}$ corresponding to the O26-AcrA glycopeptide DFN(NAcHex-NAcdHex-Pen)2R. **B** MS/MS spectrum of the ion with m/z 1004.4319 $^{+3}$ of the O45-AcrA glycopeptide with the assign structure of ATFENASKDFN(FucNAc-6d-Tal2Ac-Hex)3R. **C** MS/MS spectrum of the ion of m/z 1433.0692 $^{+2}$ corresponding to the O103-AcrA glycopeptide DFN(NAcHex3-Hex)3R. **D** MS/MS spectrum of the ion of m/z 669.6900 $^{+2}$ of the O111-AcrA glycopeptide assigned as DFN(NAcHex-Hex2-dideoxyHex)R. GlcNAc, *N*-acetylglucosamine; FucNAc, *N*-acetylfucosamine; *Rha* rhamnose, *GalNAc N*-acetylgalactosamine, *Glc* glucose, *Gal* galactose, *Col* colitose, *6d-Tal2Ac* 2-O-acetyl-6-deoxy-talose

indicating that the detected antibody response is specifically directed toward the O polysaccharide moiety of the glycoproteins (Fig. 3). Negative samples obtained from patients with other diseases not related to diarrhea of the same age group did not react against the glycoproteins by Western Blot (data not shown). The same positive and negative samples were analyzed by iELISA. As observed in Fig. 3, differential reactivity against O26-AcrA, O103-AcrA and O111-AcrA was obtained for the positive and negative-control samples at sera dilutions from 1/100 to 1/800.

To assess the serogroup specificity of the glycoproteins, serum samples obtained from children with other diseases not related to diarrhea and from pediatric patients with clinical diagnosis of HUS or BD with a stool culture positive for STEC or other enteric pathogens were analyzed by iELISA using O26-AcrA, O103-AcrA and O111-AcrA as antigens. As shown in Fig. 4, specific IgM reactivity against O26-AcrA, O103-AcrA and O111-AcrA was observed in samples obtained from patients with positive culture for STEC O26, O103 and O111, respectively, but not with the negative samples and sera obtained from patients infected with other STEC serogroups or enteric pathogens such as *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. and *Staphylococcus aureus*. Our results indicate that these novel recombinant glycoproteins are specific antigens for each *E. coli* serogroup and could be valuable tools to improve the diagnosis of STEC O26, O103 and O111 infections.

Discussion

Non-O157 STEC strains are emerging human pathogens with a worldwide distribution and an increasing incidence rate over the years in part due to the implementation of non-culture detection techniques that contribute to higher notification rates. However, early and accurate diagnosis of STEC infections is still challenging and the association of HUS to a STEC infection only occurs

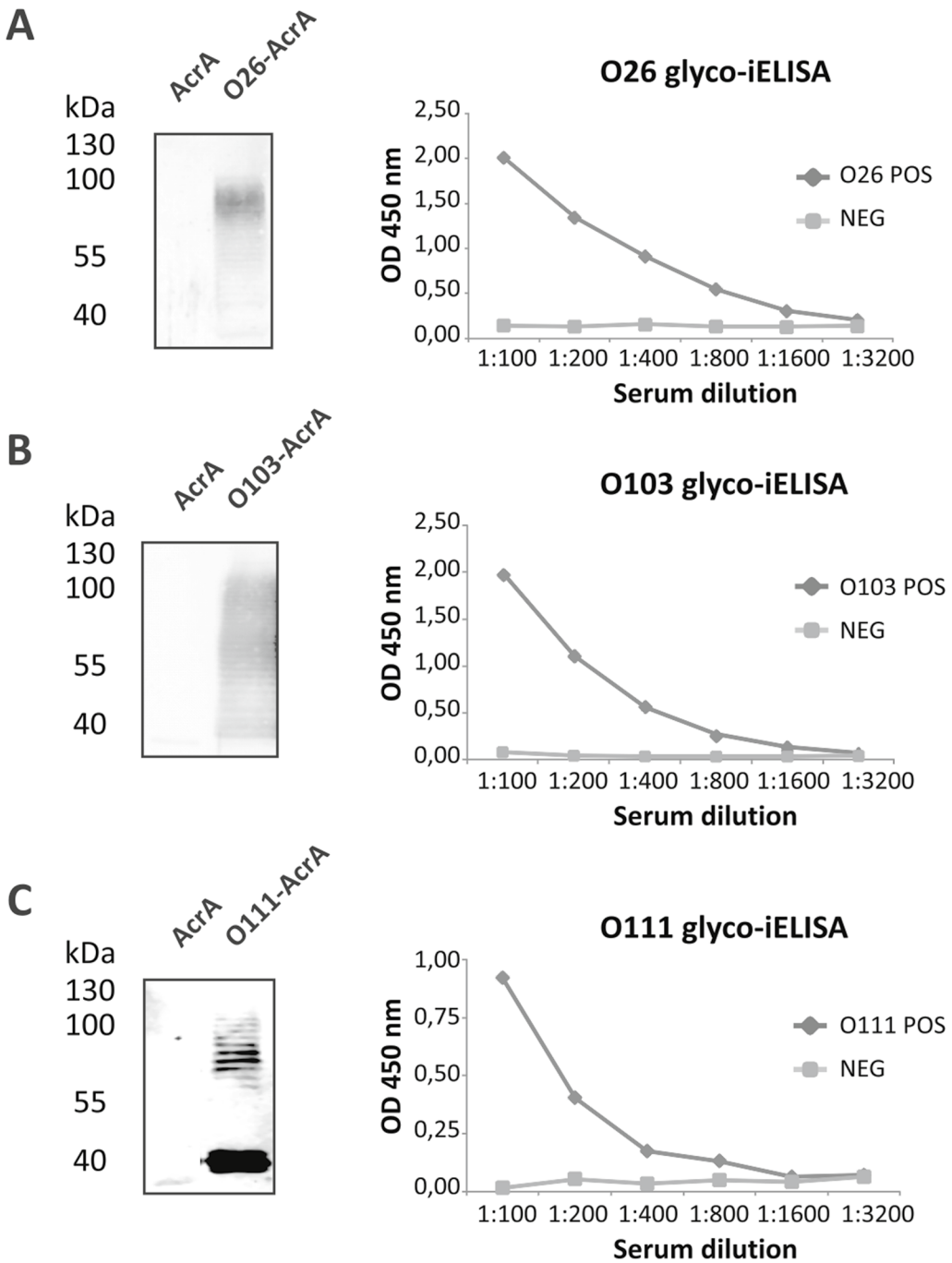
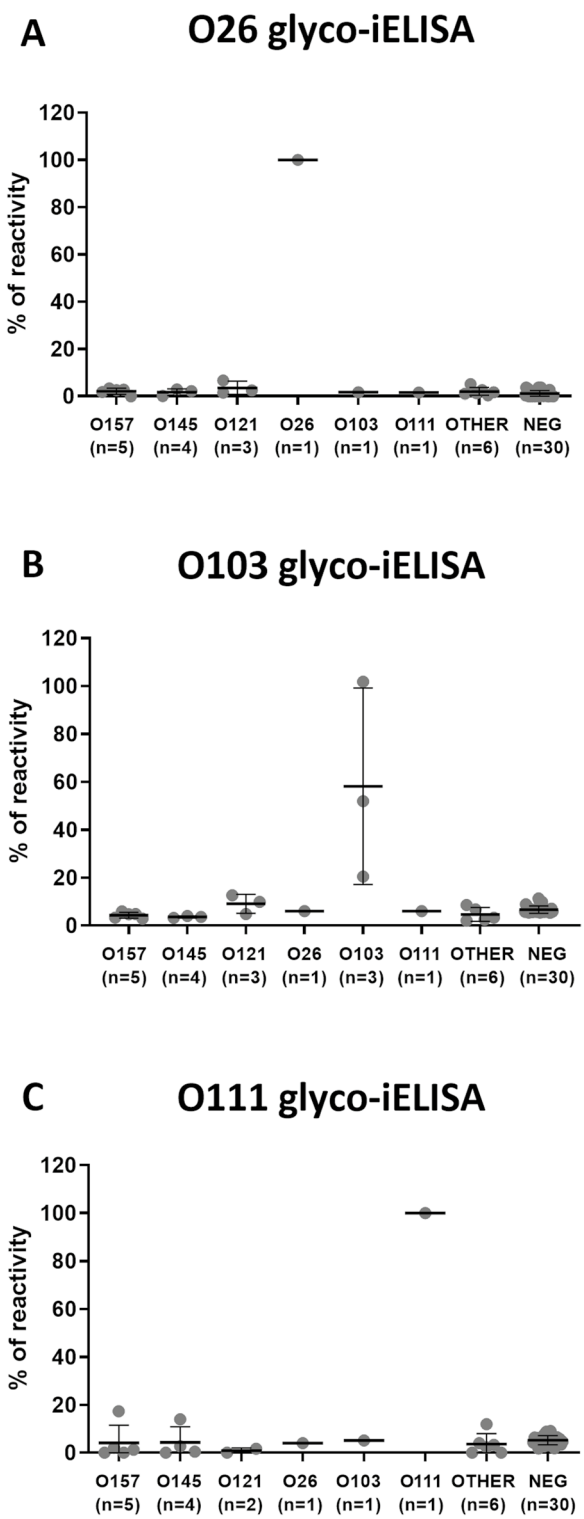


Fig. 3 Analysis of positive samples obtained from children with HUS and stool culture positive of STEC O26, O103 and O111 by Western blot and iELISA. Positive samples (POS) obtained from patients with clinical diagnosis of HUS and a stool culture positive for STEC O26:H11 **A** O103:H2 **B** or O111:NM **C** were analyzed by Western blot using the purified non-glycosylated AcrA and the glycoproteins O26-AcrA, O103-AcrA and O111-AcrA. The position of the molecular mass standards (in kDa) is indicated on the left. The same samples were analyzed by iELISA at sera dilutions from 1/100 to 1/800 (right panels). A sample obtained for a pediatric patient with other disease not related to diarrhea was analyzed as a negative control (NEG)

in less than 50% of patients. Currently, STEC diagnosis is mostly based on bacterial isolation and molecular methods for *stx* gene detection, or FFStx detection in stool samples. However, it was demonstrated that these direct methods in combination with serological assays for the detection of antibodies against the LPS of different



◄ **Fig. 4** Serogroup-specificity analysis of O26-AcrA, O103-AcrA and O111-AcrA glycoproteins by iELISA. Serum samples obtained from HUS or BD cases associated to STEC O157:H7, STEC O145:NM or STEC O121:H19, and from patients with a stool culture positive for other enteric pathogens such as *Salmonella* spp, *Shigella* spp, *Campylobacter* spp and *Staphylococcus aureus,* were analyzed by O26-AcrA **A** O103-AcrA **B** and O111-AcrA **C** glyco-iELISAs. Serum samples from pediatric patients with other diseases not related to diarrhea were included as negative samples (NEG). Bound antibodies were detected using HRP-conjugated anti-human IgM 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 is indicated

strains increase the detection of STEC infections in more than 80% of HUS patients [13, 21–23]. Nevertheless, cross-reactivity due to the presence of epitopes in the common core and lipid A moieties of the LPS shared by the different STEC strains and other enterobacteria may occur when using complete LPS as an antigen causing false-positive results. To improve the performance of the serological assays, recombinant glycoproteins consisting of the O polysaccharide, the immunodominant moiety of LPS, attached to a carrier protein have been developed by our group, as previously described. These novel molecules were used as antigens for the serodiagnosis of the infection caused by the most prevalent STEC serogroups in Argentina, i.e., O157, O145 and O121, with excellent results [15, 16, 25, 43, 49–51].

In the present work, we have developed, produced, and characterized the bacterial engineered glycoconjugates O26-AcrA, O45-AcrA, O103-AcrA and O111-AcrA for the serological detection of STEC O26, O45, O103 and O111 infections, respectively. These recombinant glycoproteins were obtained by exploiting the *C. jejuni* glycosylation machinery in which the corresponding lipid-linked O polysaccharide is transferred from the lipid carrier to the periplasmic protein AcrA by the oligosaccharyltransferase activity of PglB. Two different approaches were used for the development of the recombinant glycoproteins; one consisted in transferring the O polysaccharide biosynthesis gene cluster to *E. coli* CLM24, a non-pathogenic strain that maximizes the transference of the O polysaccharide to AcrA because it lacks the WaaL ligase which covalently attaches the O polysaccharide to the lipid A-core of the LPS. Therefore, the production of the glycoprotein in the *E. coli* CLM24 strain does not compete with the synthesis of the LPS [27]. This approach was used to generate the O103-AcrA glycoprotein with the particularity that the gene cluster for the synthesis of the O103 polysaccharide was

cloned into two different plasmids in two parts of similar size and transferred to *E. coli* CLM24 (see Materials and Methods). The other strategy, used to produce the O26-AcrA, O45-AcrA and O111-AcrA glycoproteins, exploited the expression of the endogenous O polysaccharide gene cluster of non-toxigenic isolates of *E. coli* O26, O45 and O111. Therefore, with this approach it was not necessary to transfer the gene cluster required for expression of the corresponding polysaccharide. In both strategies, the plasmids that code for the oligosaccharyltransferase PglB and the carrier protein AcrA were transferred to the producing strain. The glycoproteins obtained were antigenically characterized by Western blot using specific hyperimmune antisera against each serogroup demonstrating that the glycoproteins, but not the carrier protein AcrA, are specifically recognized by the reference sera. Once it was confirmed that the glycan moiety of each glycoprotein antigenically resembles the O polysaccharide of the corresponding LPS, they were characterized by mass spectrometry and it was demonstrated that the repetitive units of the glycans of O26-AcrA, O45-AcrA and O111-AcrA match with the reported structure for the O26, O45 and O111 polysaccharides, respectively. For O103-AcrA, the structure of the oligosaccharide differs in the absence of a hydroxybutanoyl-aminofucose residue when compared with the reported structure for the O103 polysaccharide. This difference could be explained by the potential heterogeneity of the O polysaccharide structure among different O103 isolates or by the absence of some genes required for the synthesis of the O103 polysaccharide in the gene cluster transferred to the *E. coli* CLM24 strain. However, specific reactivity of the reference anti-O103 serum against the O103-AcrA was detected indicating that the observed difference in the structure would not be affecting the antigenicity of the polysaccharide. For this reason, we proceeded with the evaluation of this glycoprotein as antigen for the serodiagnosis of STEC O103 infections.

To evaluate the potential use of the glycoproteins for the serodiagnosis of STEC O26, O103 and O111 infections, serum samples obtained from patients with diagnosis of HUS caused by STEC O26, O103 or O111, and from patients with other diseases no related to diarrhea (negative samples) were analyzed by immunoblotting and iELISA. The O45-AcrA glycoprotein was not included in this study because no serum samples from patients with HUS by STEC O45 were available. Specific reactivity against O26-AcrA, O103-AcrA and O111-AcrA was observed in samples from patients with clinical diagnosis of HUS carrying STEC O26, O103 and O111 and not in negative samples, indicating that these antigens could be useful for the detection of STEC infections from these serogroups. To evaluate the serogroup-specificity of the O26-AcrA, O103-AcrA and O111-AcrA glycoproteins, samples positive for STEC O157, non-O157 STEC and other enteric pathogens associated to bloody diarrhea, including *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. and *Staphylococcus aureus*, were analyzed by iELISA. No cross-reactivity among the different serogroups and with other enteric pathogens was observed, demonstrating that these glycoproteins are highly specific antigens that could be used for seroepidemiological purposes.

STEC-HUS is the most frequent form of HUS and constitutes 90% of the cases in children under 5 years old. However, an atypical form of this syndrome (aHUS) is associated with genetic or acquired disorders of the regulatory components of the complement system and not with bacterial infections. Despite both entities share most of the symptoms and clinical findings, the treatment and outcome of the disease depends on the HUS type: STEC-HUS has no specific treatment, and therapy is based on symptomatic and supportive care while aHUS has a specific treatment based on complement blockade using the humanized monoclonal antibody eculizumab, a life-long and expensive therapy [52]. Therefore, the early and differential diagnosis of the etiology of HUS is critical [8, 53]. Since STEC evidence in HUS patients excludes an aHUS, the early and accurate identification of STEC to confirm the infectious origin of HUS is extremely necessary for appropriate therapeutic decisions [8, 54]. In this regard, the glycoproteins developed in this work complete the panel of antigens for O157 and the "Big-Six" serogroups and could be very useful tools for the diagnosis of STEC infections at the early stages of the disease, contributing to the differential diagnosis and the correct management of the patients.

In general, the case definition of STEC-HUS occurs approximately after one week from the onset of diarrhea caused by the infection. Because seroconversion of IgM against the O polysaccharide of STEC occurs very early after the beginning of symptoms and lasts several weeks [14, 25], in this work we focused on studying IgM reactivity towards glycoproteins as an early indicator of the infection. Furthermore, Ludwig et al. reported that IgM response to LPS in children with positive culture for STEC O157 exhibited higher sensitivity and specificity when compared with IgG [55]. In our experience, IgM response against O157, O145 and O121-glycoproteins is a sensitive and specific indicator of STEC infections with these serogroups. Notably, in 2018 the Commission Implementing Decision 2018/945 of the European Union included STEC serogroup-specific antibody response as a confirmatory laboratory criterion of STEC infections in HUS patients [56]. Regarding the limitations of using LPS as antigen for the

serological detection of STEC infections, recombinant glycoproteins could be proposed as improved antigens for the detection and serogroup identification of O26, O103 and O111 STEC infections. We propose that glycoprotein-based iELISAs as well as rapid immunochromatographic tests could be used as complementary assays contributing to the early and accurate diagnosis of STEC-HUS, and for epidemiological purposes. Studies using a larger number of positive and negative samples are ongoing to complete the validation of these glycoproteins as targets for the serodiagnosis of HUS associated to O26, O103 and O111 STEC infections.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-025-02694-0.

Additional file 1.
Additional file 2.
Additional file 3.
Additional file 4.
Additional file 5.
Additional file 6.
Additional file 7.
Additional file 8.
Additional file 9.
Additional file 10.
Additional file 11.
Additional file 12.

Acknowledgements
Not applicable.

Author contributions
A.E.C., A.J.C., L.J.M., J.E.U. and D.J.C. designed research. A.J.C., L.J.M. S.M.L performed research. M.L., and A.S.C. performed mass spectrometry analysis. A.J.C., I. C. and A.E.C. analyzed data; A.J.C and A.E.C. wrote the main manuscript. All authors reviewed the manuscript.

Funding
This work was supported by grants PICT Start Up 2018/04672 from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina. S.M.L is a research fellow of Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET, Buenos Aires, Argentina. A.E.C., J.E.U., D.J.C and L.J.M are career investigators of CONICET.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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Received: 2 October 2024 Accepted: 13 March 2025
Published online: 20 May 2025

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