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CELLULAR AND MOLECULAR BIOLOGY

Development of an antibody against EtpA from enterotoxigenic *Escherichia coli* and evaluation of its use for bacterial isolation using magnetic beads

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Abstract: The enterotoxigenic *Escherichia coli* (ETEC) strain is one of the most frequent causative agents of childhood diarrhea and travelers' diarrhea in low-and middle-income countries. Among the virulence factors secreted by ETEC, the exoprotein EtpA has been described as an important. In the present study, a new detection tool for enterotoxigenic E. coli bacteria using the EtpA protein was developed. Initially, antigenic sequences of the EtpA protein were selected via in silico prediction. A chimeric recombinant protein, corresponding to the selected regions, was expressed in an E. coli host, purified and used for the immunization of mice. The specific recognition of anti-EtpA IgG antibodies generated was evaluated using flow cytometry. The tests demonstrated that the antibodiesdeveloped were able to recognize the native EtpA protein. By coupling these antibodies to magnetic beads for the capture and detection of ETEC isolates, cytometric analyses showed an increase in sensitivity, specificity and the effectiveness of the method of separation and detection of these pathogens. This is the first report of the use of this methodology for ETEC separation. Future trials may indicate their potential use for isolating these and other pathogens in clinical samples, thus accelerating the diagnosis and treatment of diseases.

Key words: diarrheagenic, detection, ETEC, magnetic beads.

INTRODUCTION

Diarrhea is a disease that is classified as an important cause of morbidity and mortality and is observed in many regions of the world. According to the World Health Organization, in 2017, diarrheal diseases were the second leading cause of death in children under five years of age, second only to pneumonia. Each year, diarrhea kills about 582,000 children in this age group (Cohen et al. 2022). In the world, there are about 1.7 billion cases of diarrheal disease recorded every year (WHO 2017, Kumar et al. 2016). Diarrhea can be defined when bowel movements occur three or more times per day, with soft or liquid stools, which are due to changes in secretion mechanisms, osmosis, or inflammatory and infection processes of intestinal cells (WHO 2017, Canani et al. 2015).

The list of enteropathogens that cause diarrhea is extensive and includes bacteria, parasites and viruses. Among bacterial pathogens, *Escherichia coli* has an important role as a causative agent, and is diagnosed in more than 49% of patients that are hospitalized

for diarrhea in low- and middle-income countries (Taniuchi et al. 2012, Walker et al. 2010). *Escherichia coli* is a non-pathogenic facultative anaerobic bacterium belonging to the Enterobacteriaceae family, which is predominant in the human gastrointestinal tract and establishes its colonization in the first hours of life (Trung et al. 2005, Nataro & Kaper 1998)

Diarrheagenic *E. coli* strains can be divided into six main categories based on distinct epidemiological and clinical characteristics, which are specific determinants of virulence that affect a wide variety of cellular processes and have an association with certain serotypes. The categories are enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (Trung et al. 2005, Taniuchi et al. 2012, Kaper et al. 2004).

The enterotoxigenic *E. coli* (ETEC) strain is considered to be one of the most frequent causative agents of pediatric diarrhea and travelers' diarrhea in low- and middle-income countries. This strain results in approximately 100 million cases of diarrhea and 60,000 deaths per year in children under the age of five (Anderson et al. 2019, Bagamian et al. 2020).

There are no appropriate methods for the identification of ETEC in developing countries. For this reason, many laboratories that conduct studies on diarrhea in these countries do not include ETEC in their routine diagnosis. New techniques for the detection of ETEC are of great relevance since they can guide epidemiological investigations, prevent unnecessary invasive procedures and serve as a tool to monitor infected patients for immediate treatment (Karch et al. 1999, Qadri et al. 2005).

The diagnosis of the pathogens that cause diarrheal diseases is usually carried out via various assays, including serotyping, plaque culture assays, microscopy, molecular biology and immunoassays. The main characteristics of an ideal test for the diagnosis of these diseases should include speed, relatively low cost, and high sensitivity and specificity (Antikainen et al. 2013, Zboromyrska et al. 2014).

In recent years, with the emergence of nanotechnology, new horizons and perspectives have been explored to achieve low limits of detection of pathogens (Zhou et al. 2012). Different shapes, sizes and compositions of nanoparticles have been used for the detection of various microorganisms, mainly aiming at increasing the amplification of the detection signal (Najafi et al. 2014, Sung et al. 2013, Yang et al. 2007). One example is the use of magnetic microparticles to improve the detection limit of conventional ELISA (Shen et al. 2014, Wang et al. 2012).

The present study proposes a method for diagnosing ETEC by detecting the EtpA protein using flow cytometry. An increase in the sensitivity and specificity of the assay was observed when magnetic beads containing the antibody were used.

MATERIALS AND METHODS

Recombinant protein design

The sequence of the gene corresponding to the EtpA protein was acquired in the GenBank database (registration number: AAX13509.2 - ncbi.nih.org). The design of the recombinant protein was performed in a chimera structure. Through *in silico* analyses, three regions of the native protein that presented higher immunogenicity were chosen. The *BepiPred* 1.0 server was used to predict immunogenic regions. The analysis of accessibility to the protein surface was performed using the NetSurfP program; prediction of protein disorder through the program IUPRED. enzim.hu and molecular weight prediction by

the Expasy-ProtParam program. The structure of the native EtpA protein was visualized in the I-TASSER program. After the *in silico* genetic analysis, the recombinant sequence was sent to IDT (Integrated DNA Technologies) for chemical synthesis of the gene sequence.

Gene cloning

After obtaining the synthetic gene, the fragment corresponding to the EtpA protein was cloned into the plasmid vector pGEM®-T Easy (Promega) using the enzyme T4 DNA ligase (Invitrogen), following the manufacturer's instructions. Subsequently, the transformation of the binding system in *E. coli* TOP 10 cells (Invitrogen) was performed, followed by plasmid extraction using the Qiaprep Spin Miniprep kit (Qiagen). The plasmid extraction product was analyzed via electrophoresis on 0.8% agarose gel in 1x TBE buffer (40 mm Tris, 40 mM boric acid and 0.5 M EDTA). Following this, the resulting colonies were selected by confirming the presence of the EtpA gene by polymerase chain reaction (PCR), using the enzyme Taq DNA polymerase (Invitrogen), using the primers R-EtpA (5` TCTAGAGAATTCTTATGCTGATCCTGCAGCA 3`) and F-EtpA (5` GGATCCGATGATGACGATAAG 3`), followed by analysis via electrophoresis on 0.8% agarose gel. Colonies that showed amplification at a size corresponding to the gene were subjected to plasmid extraction, followed by enzymatic restriction with *Bam*HI and *Eco*RI enzymes. The gene was subjected to a binding with the enzyme T4 ligase to the expression vector pRSET-A (Invitrogen) and was digested with the same enzymes. After transformation into *E. coli* Top 10, colonies presenting the target gene were subjected to plasmid extraction and subsequently transformed into *E. coli*, BL21(DE3) pLysS (Promega) for recombinant protein expression.

Expression and isolation of the chimeric recombinant EtpA (crEtpA)

A colony of *E. coli* BL21(DE3) pLysS containing the plasmid EtpA-pRSET-A was pre-inoculated into test tubes containing 3 mL of liquid LB medium with 100 µg/mL of ampicillin and 11 µg/mL of chloramphenicol. The tubes were incubated in a shaker at 37 °C for 16 hours. Then, the pre-inocula were transferred to a 2 L Erlenmeyer flask containing 1 L of liquid LB medium and containing the same antibiotics. After the transfer, the inoculum was again placed in the shaker at the same temperature until it reached a DO_{600nm} of 0.6, which was measured in a spectrophotometer. Upon reaching this level of bacterial growth, the IPTG inducer (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 1 mM was increased in the bacterial culture. Induction was performed for 4 hours, followed by centrifugation of the culture at 3,094 *xg* for 15 minutes at 4 °C. The supernatant was discarded and the bacterial pellet that was formed was stored in a freezer at -20 °C for the subsequent sonication process.

To obtain the crEtpA, initially, the post-induction bacterial pellet was lysed using sonication. This was performed using an ultrasonic processor (UP50H, Hielsher Ultrasonics) with the addition of 2.5 mL of MCAC-0 buffer (20 mM Tris pH 7.9; 0.5 M NaCl; 10% glycerol and 1 mM PMSF). Then, the sonicated content was centrifuged at 20,000 *xg* at 4 °C and the native supernatant that was formed was used for the purification of the recombinant protein. The expression confirmation was performed using the western blot technique and was developed using the Western Breeze kit (Qiagen), following the instructions provided by the manufacturer. Subsequently, purification was evaluated using 12% polyacrylamide gel electrophoresis (SDS-PAGE) as described by Maniatis et al. (1988). The purified protein was

concentrated on a Centriprep Centrifugal Filter devices YM-3 (Millipore) column, following the manufacturer's recommendations. After this process, excision of the histidine tail using the enzyme enterokinase (New England Biolabs) along with the resin Tripsin Agarose Inhibitor (Sigma Aldrich), following the manufacturer's instructions. After this step, the excision of the histidine tail was evaluated using SDS-PAGE.

Production and isolation of anti-crEtpA polyclonal antibodies

After purification and excision of the histidine tail, crEtpA was applied to ten Balb/C mice (CEUA/INPA number 019/2014). Three biweekly immunizations were performed with 50 µL of recombinant chimeric EtpA (1 µg/µL) together with 50 µL of Freund's adjuvant. After immunizations, the mice were intraperitoneally anesthetized with 90 $mg.kg^{-1}$ of ketamine (Dopalen, Vetbrands) in combination with 16 $mg.kg^{-1}$ of xylazine (Anasedan, Vetbrands) for blood collection via venipuncture of the caudal vein. At the end of the 45 days, the animals were anesthetized and euthanized for the collection of whole blood. The monitoring of the IgG humoral response against crETpA was performed using ELISA, following a protocol previously published by our team (Sousa et al. 2014). The anti-crEtpA IgG antibodies obtained were isolated from the serum of immunized mice using protein A resin (Sigma Aldrich), following the protocol provided by the manufacturer. Purified IgG antibodies were quantified using Bradford reagent (Bio-Rad).

Evaluation of the recognition of anti-crEtpA IgG antibodies to diarrheagenic bacteria

Diarrheagenic *E. coli* samples (EHEC, EAEC, EIEC, EPEC, DAEC and ETEC) belonging to the Oswaldo Cruz Foundation sample bank (ILMD-FIOCRUZ). This study was approved by the Ethics Committee of the Amazonas State University, under approval number: CEP 266/2006. The isolates were grown separately in test tubes containing liquid LB medium. After the growth of the strains (37 °C for 16 hours), the bacteria were centrifuged at 20 000 *xg* at room temperature. The pellets that were formed were washed three times with filtered PBS wash buffer (PBS 1X; 5% albumin; 1% azide pH 7.2) and resuspended with 300 µL of the same solution. Purified anti-crEtpA IgG antibodies (at a concentration of 20 µg/mL) were added to the pellets and, subsequently, the samples were incubated for 45 minutes at room temperature. After the incubation period, the samples were washed three times via centrifugation (3,622 *xg*) with PBS wash buffer. The next step was the addition of secondary anti-mouse antibody IgG Alexa Fluor™ 488 (Sigma-Aldrich) diluted 1:200 (5 µg/mL) in PBS wash buffer. After the addition of the secondary antibody, the samples were incubated for 45 minutes, washed again three times with PBS wash buffer via centrifugation and read on the flow cytometer (FACSCanto™ II, BD). The aforementioned experiment was performed three times in order to analyze the reproducibility and generate reliability in the results obtained.

The same test was subsequently performed with eleven different ETEC isolates belonging to the Oswaldo Cruz Foundation *E. coli* clinical sample bank (ILMD-FIOCRUZ). The presence of the EtpA gene in these isolates was confirmed in previous studies using PCR.

Detection of ETEC by antibodies coupled to beads

Carboxy-terminated magnetic beads (FB-101 1 µm BcMag™, Bioclone Inc) were prepared following the manufacturer's instructions. Coupling was performed using 1 µg/µL of anti-crEtpA IgG antibodies in active beads with 1-ethyl-3-(3dimethylaminopropyl) carbodiimide chloride (EDAC). In order to evaluate the degree

of recognition of the anti-crEtpA IgG antibodies, clinical isolates 144 and 178 of ETEC from the Oswaldo Cruz Foundation sample bank (Fiocruz Amazônia) were cultured in LB nutrient broth at 37 °C for 16 hours before use. After growth, the cells were centrifuged at 20,000 *xg* for 3 minutes. Then, the pellets obtained were resuspended in 50 µL of PBS buffer - ethidium bromide (0.016 mg/mL) and incubated for 30 minutes in the dark. Afterwards, the labeled bacteria were resuspended in 450 µL of PBS 1X and 2 µL of the magnetic beads coupled to the anti-crEtpA antibody were added. After adding the beads, the solution was kept under light shaking for 1 hour. Then, the samples were washed with PBS 1X using a Magjet separation rack (Thermo). This washing procedure was performed ten times with the ETEC samples and also with the tubes identified as reaction controls. After the washes, the secondary anti-mouse IgG antibody conjugated to Alexa Fluor™ 488 fluorochrome (Sigma-Aldrich) at a concentration of 5µL/mL was added and the systems were incubated for 30 minutes, then washed ten times with PBS 1X. After this step, the samples were resuspended in 200 µL of PBS and analyzed in a flow cytometer (FACSCanto™ II, BD). The specificity analysis of the experiment was performed using different isolates of diarrheagenic *E. coli* (DAEC, EPEC, EIEC, EAEC and ETEC). Different controls were used in this assay in order to avoid interpretation biases: C1 (Control 1) – only magnetic beads; C2 – blocked magnetic beads + secondary antibodies; C3 – magnetic beads + primary antibody (anti-EtpA) + secondary antibody; C4 – bromide-labeled bacteria; C5 – just bacterial strain 144; C6 – just bacterial strain 178. The analyses were performed using FACS DIVA, Flow-Jo version 10.0 software and GraphPad Prism 5.0.

Statistical analysis

Statistical evaluations were performed using Prism software (GraphPad Inc.). Comparisons between samples were performed using one way ANOVA. Significance was considered when p was ≤ 0.05.

RESULTS

Design and production of the crEtpA

The chimeric version of the EtpA protein (GenBank AAX13509) developed in this study was designed with three epitopes selected from the prediction of accessibility to the protein surface, immunogenic regions and three-dimensional structure analysis. The selected regions are located, respectively, in the ranges 25° to 107° (VGNAKATGSVEGEKSPRRGVRA M A L S L L S G M M I M A H P A M S A N L P T G G Q I V A G S G S I Q T P S G N Q M N I H Q N S Q N M V A N W N S F D I); 350° to 374°(ITATGGQGGGEVYVGGGWQGKDSNI) and from 470° to 492° (VSKTETTQSPPHTQFAPTAAGSA) of the complete amino acid sequence of the EtpA, from which the synthetic gene was designed to obtain a chimeric EtpA (Figure 1a). This was successfully cloned into vector pRSET A (Figure 1b-d).

E. coli host expression was confirmed by western blot using anti-6xHisG antibody (Figure 1e), and the crEtpA protein was isolated using Ni-NTA column chromatography (Figure 1f, channel 1). The purified crEtpA presented an electrophoretic profile with a molecular mass of 16 kDa, corresponding to what was expected from the *in silico* analysis using the Expasy-ProtParam program. After the isolation of crEtpA, excision of the histidine syrup was performed with the enzyme enterokinase (Figure 1f, channel 2), presenting a protein band with a lower molecular weight.

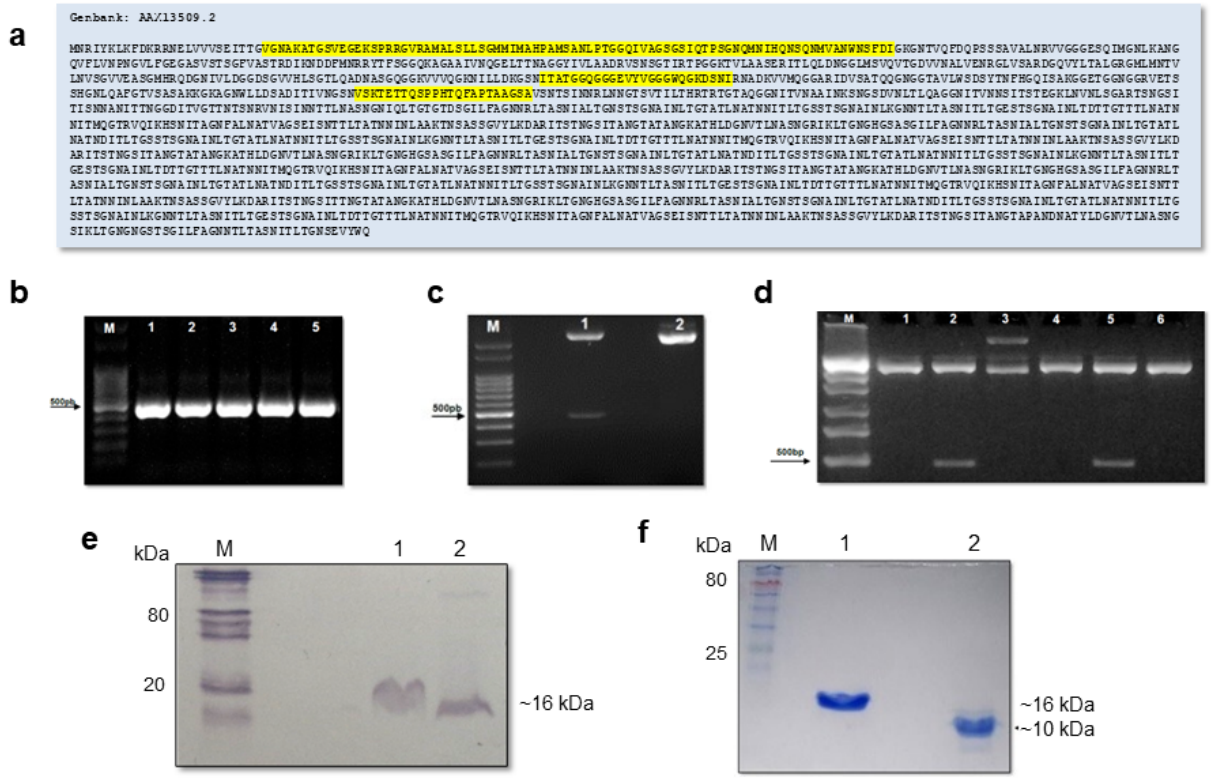

Figure 1. Selection of EtpA protein epitopes, construction and expression of the chimeric protein. (a) sequence of the gene corresponding to the EtpA protein. The sequences selected for production of the chimeric recombinant protein are highlighted in yellow; (b) 1; 2; 3; 4; 5: analysis of the PCR products of the samples that were amplified in the pGEM T-EtpA vector; M - molecular mass marker of 500 bp (Ludwig Biotec); (c) 1: analysis of the restriction profile of the recombinant plasmid with the enzymes *Eco*RI and *Bam*HI; 2: undigested pRSET A vector; M: molecular mass marker 1 Kb (Ludwig Biotec); (d) restriction analysis profile of pRSET-EtpA expression plasmid. Samples 2 and 5 confirming the correct construction of the vector with the size of the expected bands. M: molecular mass marker 1 Kb (Ludwig Biotec); (e) immunoblot against the histidine tail for the detection of EtpA protein expressed in *E. coli* BL21. M: molecular mass marker prepared with different proteins containing the histidine tail; (f) electrophoresis profile in SDS-PAGE gel of the elutions of the purified EtpA protein in an NI-NTA-Agarose column. M: molecular weight marker (Color Plus Biolabs: 10-230 kDa).

Evaluation of specific antibodies

Indirect ELISA analysis demonstrated that Balb/C mice immunized with crEtpA were able to produce anti-crEtpA IgG antibodies (Figure 2a), which were successfully purified using the affinity chromatography technique (Figure 2b).

The anti-crEtpA antibodies produced were able to recognize with specificity the native antigen EtpA present on the surface of the ETEC isolates tested, when analyzed for the presence of fluorescence using flow cytometry (Figure 3a). The samples were analyzed for their morphometric characteristics by the size (FSC-A) and complexity (SSC-A) of the bacterial population in a dotplot and the gate strategy was established (Figure 3b). The antigen-antibody recognition analysis was revealed through the positive dotplot within the gate, represented by the displacement of the events recognized by the secondary antibody to the primary anti-crEtpA antibody, in relation to the negative and positive controls (Figure 3c-d).

As shown in Figure 3e, with regards to the evaluation of the degree of specificity of

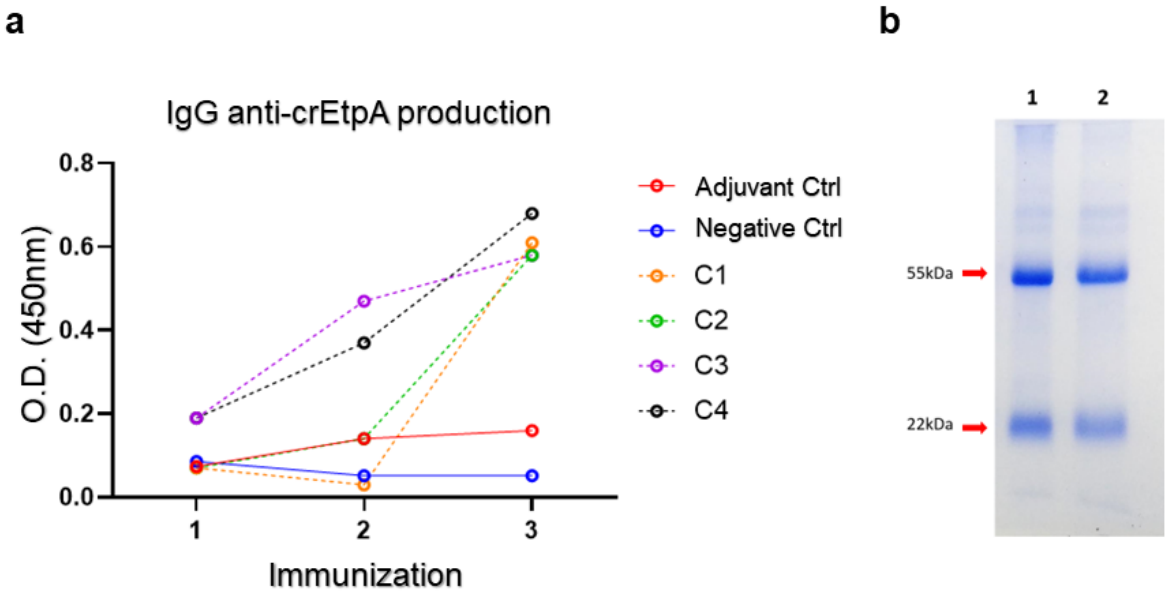

Figure 2. ELISA of the humoral response of immunized mice and SDS-PAGE analysis of purified antibodies. (a) indirect serum ELISA for humoral response analysis of mice immunized with the crEtpA protein. C1 to C4: animals immunized with purified crEtpA. Adjuvant Ctrl-: animal immunized only with the adjuvant; (b) 12% SDS-PAGE analysis of purified antibodies from mice immunized with the crEtpA protein, channels 1 and 2: electrophoretic profile of two column elutions of purified antibodies containing protein A resin (Sigma Aldrich). Ctrl: control.

anti-crEtpA recognition against EHEC, EAEC, EIEC, EPEC and DAEC strains, it is possible to observe a baseline fluorescence when the specificity of anti-EtpA antibodies of approximately 2% was evaluated. This results from the non-specific reaction of anti-mouse IgG secondary antibodies conjugated to Alexa Fluor™ 488 fluorochrome to cellular antigens. This basal shift was also observed in the control samples, to which only the secondary antibody was added in the reaction, representing the cut off of the cytometric analyses. The results showed a significant increase (p-value=0.005) of recognition percentage (about 4% more) produced for EtpA antigen recognition in ETEC, contributing to the validation of the specificity of anti-EtpA antibodies.

When evaluating the degree of sensitivity of recognition of anti-EtpA antibodies in different ETEC strains, our results, as shown in Figure 3f, show the occurrence of variation in the expression of EtpA protein. Fluorescence percentages ranged from 2 to 20%, depending on the isolate tested.

ETEC detection by the anti-EtpA bead system

A new cell-sorting method aimed at increasing the specificity of the ETEC EtpA+ bacteria detection assay using anti-crEtpA antibodies coupled to magnetic beads was proposed in this study (Figure 4a). Initially, the morphology (FSC-A) and complexity (SSC-A) of these events were observed after cell sorting (Figure 4b). Autofluorescence analysis in unlabeled bacteria (EtBr-/Alexa Fluor 488-) demonstrated no interfering reactivity (Figure 4c). The analyses for the determination of double positive events after cell sorting in ETEC EtpA+ (EtBr+/Alexa Fluor 488+) samples (Figure 4d) showed a high capacity for separation and enrichment of ETEC EtpA+ bacteria by the proposed method. This was demonstrated by the significant

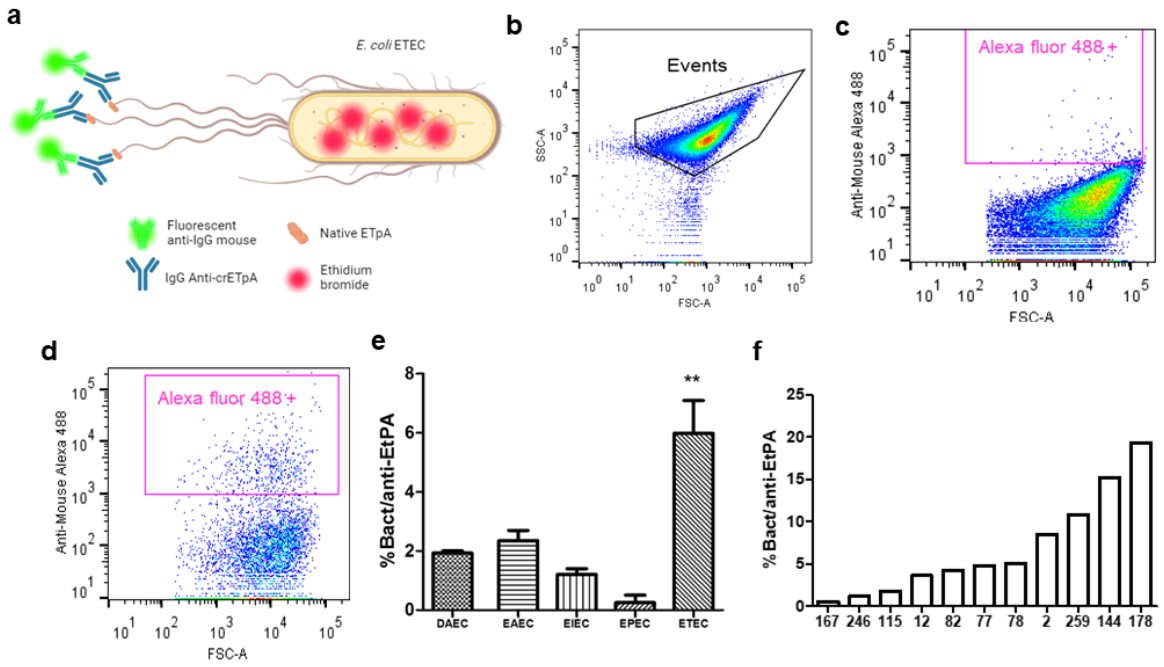

Figure 3. Morphometric analysis of *E. coli* labeled with fluorescent anti-crEtpA IgG antibody. (a) schematic representation of the immunoassay using anti-crEtpA IgG antibodies and their recognition to the native protein in ETEC (prepared using biorender.com) (b) size (FSC-A) and complexity (SSC) analysis of *E. coli*. (c) percentage of fluorescence of the negative control sample; (d) percentage of fluorescence of ETEC EtpA labeled with the fluorescent anti-crEtpA IgG antibody; (e) percentage of the fluorescence labeling of anti-crEtpA against different subtypes of *E. coli* (f) percentage of anti-crEtpA fluorescence against different clinical isolates of ETEC with PCR confirmation of the presence of the EtpA gene. Diffusely Adhering *Escherichia coli*; EAEC - Enteroaggregative *Escherichia coli*; EIEC - Enteroinvasive *Escherichia coli*; EPEC - Enteropathogenic *Escherichia coli*; ETEC - Enterotoxigenic *Escherichia coli*.
**** p-value < 0.001 in the one-way ANOVA.**

difference (p-value=0.001) of the percentage of double positive fluorescence observed in strain 178 (12.1%) compared to other isolates of diarrheagenic *E. coli* (EPEC 0.4%) Figure 4e).

DISCUSSION

This study aimed to develop a method to facilitate the detection of *Escherichia coli* ETEC, an important diarrheagenic bacterium. The EtpA adhesin used for detection was chosen as a target protein in the development of this study because it is a conserved protein within the ETEC pathovar (Luo et al. 2015, Kumar et al. 2016). However, the expression of proteins with a high number of residues can lead to the production of insoluble proteins due to the formation of inclusion bodies (Pouresmaeil & Azizi-Dargahlou 2023), which hinders their purification and limits their further use. Therefore, considering the high molecular mass of EtpA (177 kDa) (Fleckenstein et al. 2006), the linear epitope selection approach with high probability of exposure and immunogenicity was used according to the *in silico* analysis tools used. This strategy led to the production of a highly soluble chimeric protein, which facilitated its further purification via affinity chromatography.

The selection of epitopes using *in silico* analysis for the production of chimeric recombinant proteins has been shown to be an approach that reduces the risks of insolubility of the recombinant protein. In this study, the production of IgG antibodies observed

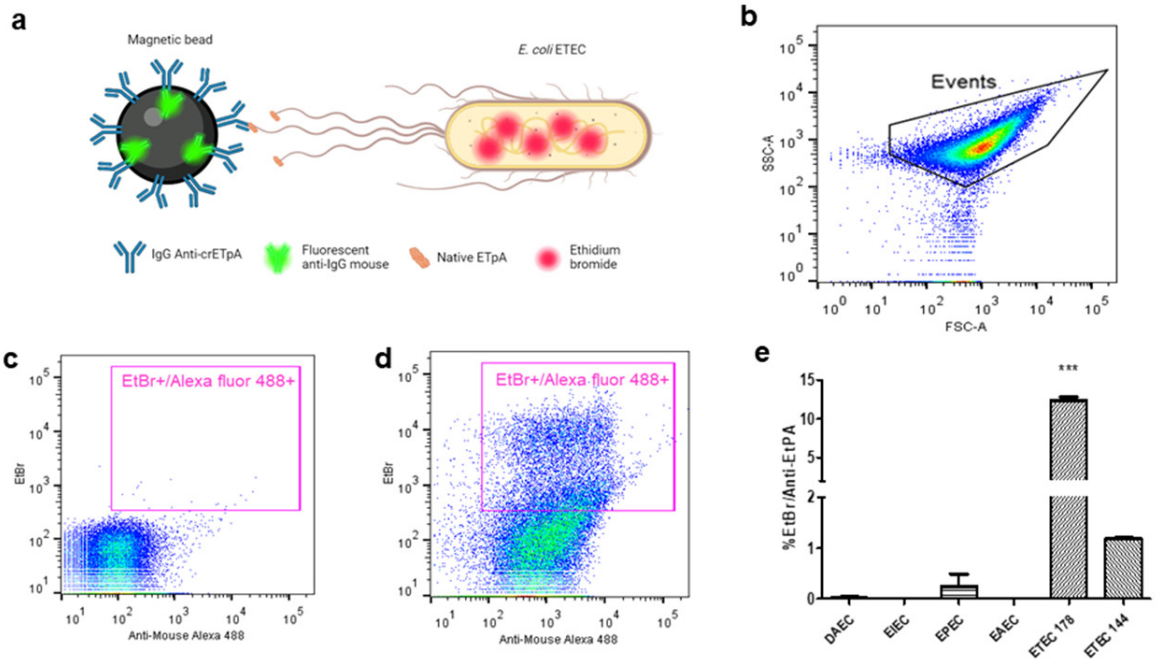

Figure 4. Morphometric analysis of *E. coli* captured using magnetic beads. (a) schematic representation of the immunoassay using anti-crEtpA magnetic beads and its recognition of the native protein in ETEC (prepared using biorender.com); (b) size analysis (FSC-A) and complexity (SSC) of *E. coli* captured using magnetic beads; (c) percentage of fluorescence of the negative control sample; (d) fluorescence percentage graph of ETEC double labeled with ethidium bromide and anti-crEtpA in magnetic beads; (e) percentage of fluorescence labeling of anti-crEtpA against different diarrheagenic *E. coli* subtypes. Diffusely Adhering *Escherichia coli*; EAEC - Enteroaggregative *Escherichia coli*; EIEC - Enteroinvasive *Escherichia coli*; EPEC - Enteropathogenic *Escherichia coli*; ETEC - Enterotoxigenic *Escherichia coli*. *** p-value < 0.0001 in the one-way ANOVA.

in the serum ELISAs of immunized animals demonstrated the immunogenic capacity of the chosen epitopes. Further studies may be carried out to identify which epitopes of the chimeric protein produced were effective for antibody production.

The strategy proposed in the present study has been addressed in other studies that aimed at the diagnosis and/or vaccine (Montero et al. 2023, Ramos et al. 2023). As an example of the use of this approach for the development of vaccines against *E. coli* ETEC and EHEC, we can cite the studies of Montero et al. (2023) and Jeshvaghani et al. (2019), who grouped different antigens into chimeric proteins and used them in mouse immunizations. After the challenge with the bacteria, it was found that antibodies against these proteins were able to confer partial protection against the pathological effects of the bacteria (Jeshvaghani et al. 2019, Montero et al. 2023).

The cytometric analyses performed indicated that the IgG anti-crEtpA antibody developed was able to recognize the native EtpA protein present in the flagella of ETEC bacteria. Despite presenting a baseline reactivity against the other subtypes analyzed, the recognition of ETEC was significantly higher. This baseline recognition was also observed in control assays using only the secondary antibody, indicating non-specific reactivity. Another important observation is the variation in reactivity among the eleven clinical ETEC isolates used, which indicates that the bioavailability of the EtpA antigen may vary among isolates, as also observed by Chakraborty et al. (2019). These

differences may be related to the different groups of colonization factors to which these strains belong (Chakraborty et al. 2019).

The use of the anti-crEtpA antibodies produced in this study, in association with the magnetic beads, caused an increase in specificity to ETEC, as well as greater reactivity when compared to assays using the free antibody. Different studies have applied magnetic beads for the detection of *E. coli* O157:H7, such as Jayamohan et al. (2015), who reported the detection of up to 3 CFU/100 mL in wastewater effluents. Shen et al. (2014) reported detection of 68 CFU mL^{-1} in PBS and 6.8×10^2 to 6.8×10^3 CFU mL^{-1} in feed samples. Wang et al. (2015) reported the detection of a concentration equal to 104.45 CFU/mL^{-1} in electrochemical sensors. The study presented here is the first to report the use of anti-crEtpA IgG antibodies coupled to magnetic beads for the detection of ETEC.

Therefore, in the present study, the sequence chosen for the construction of the chimeric protein from the *in silico* analyses allowed the production of polyclonal antibodies in mice that were capable of recognizing native enterotoxigenic *E. coli*. However, the detection levels of this protein may be even higher if the assay is applied to fresh samples, since EtpA is modulated by ETEC's contact with intestinal host cells (Roy et al. 2009)

The flow cytometry analyses allowed us to characterize the antibodies obtained, and the use of magnetic beads, in conjunction with anti-crEtpA antibodies for the isolation of ETEC in samples, shows a promising methodology that can be used to accelerate the diagnosis of this pathogen. Future research may seek to make this detection faster, such as with the use of the recently developed technique known as critical offset magnetic particle spectroscopy (COMPASS) (Vogel et al. 2022), which uses the same immunodetection principle as described here.

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REFERENCES

ANDERSON JD, BAGAMIAN KH, MUHIB F, AMAYA MP, LAYTNER LA, WIERZBA T & RHEINGANS R. 2019. Burden of enterotoxigenic Escherichia coli and shigella non-fatal diarrheal infections in 79 low-income and lower middle-income countries: a modelling analysis. Lancet Glob Heal 7: e321-e330.

ANTIKAINEN J, KANTELE A, PAKKANEN SH, LÄÄVERI T, RIUTTA J, VAARA M & KIRVESKARI J. 2013. A quantitative polymerase chain reaction assay for rapid detection of 9 pathogens directly from stools of travelers with diarrhea. Clin Gastroenterol Hepatol 11: 1300-1307.

BAGAMIAN KH, ANDERSON JD, MUHIB F, CUMMING O, LAYTNER LA, WIERZBA TF & RHEINGANS R. 2020. Heterogeneity in enterotoxigenic Escherichia coli and Shigella infections in children under 5 years of age from 11 African countries: a subnational approach quantifying risk, mortality, morbidity, and stunting. Lancet Glob Heal 8: e101-e112.

CANANI RB, CASTALDO G, BACCHETTA R, MARTÍN MG & GOULET O. 2015. Congenital diarrhoeal disorders: advances in this evolving web of inherited enteropathies. Nat Rev Gastroenterol Hepatol 12: 293-302.

CHAKRABORTY S ET AL. 2019. Interrogation of a live-attenuated enterotoxigenic Escherichia coli vaccine highlights features unique to wild-type infection. NPJ Vaccines 4: 37.

COHEN AL ET AL. 2022. Aetiology and incidence of diarrhoea requiring hospitalisation in children under 5 years of age in 28 low-income and middle-income countries: findings from the Global Pediatric Diarrhea Surveillance network. BMJ Glob Health 7: e009548.

FLECKENSTEIN JM, ROY K, FISCHER JF & BURKITT M. 2006. Identification of a Two-Partner Secretion Locus of Enterotoxigenic Escherichia coli. Infect Immun 74: 2245.

JAYAMOHAN H, GALE BK, MINSON B, LAMBERT CJ, GORDON N & SANT HJ. 2015. Highly sensitive bacteria quantification using immunomagnetic separation and electrochemical detection of guanine-labeled secondary beads. Sensors (Basel) 15: 12034-12052.

JESHVAGHANI F, AMANI J, KAZEMI R, KARIMI RAHJERDI A, JAFARI M, ABBASI S & SALMANIAN AH. 2019. Oral immunization with a plant-derived chimeric protein in mice: Toward the development of a multipotent edible vaccine against E. coli O157: H7 and ETEC. Immunobiology 224(2): 262-269.

KAPER JB, NATARO JP & MOBLEY HLT. 2004. Pathogenic Escherichia coli. Nat Rev Microbiol 2: 123-140.

KARCH H, BIELASZEWSKA M, BITZAN M & SCHMIDT H. 1999. Epidemiology and diagnosis of Shiga toxin-producing Escherichia coli infections. Diagn Microbiol Infect Dis 34: 229-243.

KUMAR P, KUHLMANN FM, BHULLAR K, YANG H, VALLANCE BA, XIA L, LUO Q & FLECKENSTEIN JM. 2016. Dynamic Interactions of a Conserved Enterotoxigenic Escherichia coli Adhesin with Intestinal Mucins Govern Epithelium Engagement and Toxin Delivery. Infect Immun 84: 3608-3617.

LUO Q, QADRI F, KANSAL R, RASKO DA, SHEIKH A & FLECKENSTEIN JM. 2015. Conservation and Immunogenicity of Novel Antigens in Diverse Isolates of Enterotoxigenic Escherichia coli. PLoS Negl Trop Dis 9: e0003446.

MANIATIS T, FRITSCH EF & SAMBROOK J. 1988. Molecular Cloning: a laboratory manual. 2nd edition, Cold Spring Harb, New York, vol. 3, 18-51 p.

MONTERO DA ET AL. 2023. A chimeric protein-based vaccine elicits a strong IgG antibody response and confers partial protection against Shiga toxin-producing Escherichia coli in mice. Front Immunol 14: 1186368.

NAJAFI R, MUKHERJEE S, HUDSON J, SHARMA A & BANERJEE P. 2014. Development of a rapid capture-cum-detection method for Escherichia coli O157 from apple juice comprising nano-immunomagnetic separation in tandem with surface enhanced Raman scattering. Int J Food Microbiol 189: 89-97.

NATARO JP & KAPER JB. 1998. Diarrheagenic Escherichia coli. Clin Microbiol Rev 11: 142-201.

POURESMAEIL M & AZIZI-DARGAHLOU S. 2023. Factors involved in heterologous expression of proteins in E. coli host. Arch Microbiol 205: 212.

QADRI F, SVENNERHOLM AM, FARUQUE ASG & SACK RB. 2005. Enterotoxigenic Escherichia coli in Developing Countries: Epidemiology, Microbiology, Clinical Features, Treatment, and Prevention. Clin Microbiol Rev 18: 465.

RAMOS FF ET AL. 2023. B-Cell Epitopes-Based Chimeric Protein from SARS-CoV-2 N and S Proteins Is Recognized by Specific Antibodies in Serum and Urine Samples from Patients. Viruses 15: 1877.

ROY K, HILLIARD GM, HAMILTON DJ, LUO J, OSTMANN MM & FLECKENSTEIN JM. 2009. Enterotoxigenic Escherichia coli EtpA mediates adhesion between flagella and host cells. Nature 457: 594-598.

SHEN Z, HOU N, JIN M, QIU Z, WANG J, ZHANG B, WANG X, WANG J, ZHOU D & LI J. 2014. A novel enzyme-linked immunosorbent assay for detection of Escherichia coli O157:H7 using immunomagnetic and beacon gold nanoparticles. Gut Pathog 6: 14.

SOUSA LP ET AL. 2014. A novel polyclonal antibody-based sandwich ELISA for detection of Plasmodium vivax developed from two lactate dehydrogenase protein segments. BMC Infect Dis 14: 1-8.

SUNG YJ, SUK HJ, SUNG HY, LI T, POO H & KIM MG. 2013. Novel antibody/gold nanoparticle/magnetic nanoparticle nanocomposites for immunomagnetic separation and rapid colorimetric detection of Staphylococcus aureus in milk. Biosens Bioelectron 43: 432-439.

TANIUCHI M, WALTERS CC, GRATZ J, MARO A, KUMBURU H, SERICHANTALERGS O, SETHABUTR O, BODHIDATTA L, KIBIKI G, TONEY DM, BERKELEY L, NATARO JP & HOUPT ER. 2012. Development of a multiplex polymerase chain reaction assay for diarrheagenic Escherichia coli and Shigella spp. and its evaluation on colonies, culture broths, and stool. Diagn Microbiol Infect Dis 73: 121-128.

TRUNG VN, PHUNG L VAN, CHINH LH, KHANH NG & WEINTRAUB A. 2005. Detection and characterization of diarrheagenic Escherichia coli from young children in Hanoi, Vietnam. J Clin Microbiol 43: 755-760.

VOGEL P, RÜCKERT MA, FRIEDRICH B, TIETZE R, LYER S, KAMPF T, HENNIG T, DÖLKEN L, ALEXIOU C & BEHR VC. 2022. Critical Offset Magnetic PArticle SpectroScopy for rapid and highly sensitive medical point-of-care diagnostics. Nat Commun 13(1): 7230. doi: 10.1038/s41467-022-34941-y. PMID: 36433976; PMCID: PMC9700695.

WALKER CLF, SACK D & BLACK RE. 2010. Etiology of diarrhea in older children, adolescents and adults: a systematic review. PLoS Negl Trop Dis 4: e768.

WANG J, MOORE J, LAULHE S, NANTZ M, ACHILEFU S & KANG KA. 2012. Fluorophore–gold nanoparticle complex for sensitive optical biosensing and imaging. Nanotechnology 23: 095501.

WANG R, LUM J, CALLAWAY Z, LIN J, BOTTJE W & LI Y. 2015. A Label-Free Impedance Immunosensor Using Screen-Printed

Interdigitated Electrodes and Magnetic Nanobeads for the Detection of E. coli O157:H7. Biosensors (Basel) 5: 791.

WHO – WORLD HEALTH ORGANIZATION. 2017. Diarrhoeal disease. Available in: https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease. Accessed in October 2023.

YANG H, QU L, WIMBROW AN, JIANG X & SUN Y. 2007. Rapid detection of Listeria monocytogenes by nanoparticle-based immunomagnetic separation and real-time PCR. Int J Food Microbiol 118: 132-138.

ZBOROMYRSKA Y, HURTADO JC, SALVADOR P, ALVAREZ-MARTÍNEZ MJ, VALLS ME, MAS J, MARCOS MA, GASCÓN J & VILA J. 2014. Aetiology of traveller's diarrhoea: evaluation of a multiplex PCR tool to detect different enteropathogens. Clin Microbiol Infect 20: O753-O759.

ZHOU F, WANG M, YUAN L, CHENG Z, WU Z & CHEN H. 2012. Sensitive sandwich ELISA based on a gold nanoparticle layer for cancer detection. Analyst 137: 1779-1784.

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PPO, LAMM, and HAA conceived the study; PPO and LAMM designed the study protocol; HAA, JCG, MEMA, JCLB, RAMA, CMG and YOC performed the cloning, expression of recombinant proteins and flow cytometry tests; HAA, LAMM, PPO, YOC and PAN performed the analysis and interpretation of the data; HAA, LAMM, YOC and JCG wrote the manuscript; HAA, LAMM, PPO, PAN, YOC, JCG and MEMA critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.