Characterization of Rabbit Polyclonal Sera against Recombinant Shiga Toxin and its Subunits for Detection of Stx-Producing E. coli

Mana Oloomi and Saeid Bouzari

Molecular Biology Unit, Pasteur Institute of Iran, Tehran, Iran

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ABSTRACT

Shiga toxin (Stx) is the principal virulence factor of Shigatoxigenic Escherichia coli (STEC), a food-born pathogen associated disease with uncomplicated diarrhea or the hemolytic-uremic syndrome.

In this study, rabbit polyclonal anti recombinant A, B subunits of Shiga toxin and holotoxin antiserum were raised and employed for immunological purpose. By immunoblotting, these antisera recognized respective subunit and the holotoxin antiserum recognized both subunits, equally. The raised antisera could also neutralize the cytotoxicity of the shiga toxin on vero cells. The neutralizing ability of the prepared sera was compared for different subunits.

The neutralization of toxicity was observed by incubation of raised sera with the rStx or Shiga toxin from wild type strains. The inhibition of cell toxicity was shown by anti-A, anti-B and anti-AB antisera, separately. It was shown that anti-A antibody, more significantly recognized Stx producing strains, comparing to anti-B antibody.

These sera from immunized rabbits were also used as specific antibodies in Enzyme-Linked Immunosorbant Assay (ELISA) for detection of Shiga toxin. It was demonstrated that the raised antibodies especially antibody against A subunit could be a useful tool for immunological diagnosis of STEC induced infection.

Key words: A and B Subunits; Polyclonal Antibodies; Shiga Toxin

INTRODUCTION

Enterohemorrhagic Escherichia coli (EHEC) strains are important human food-borne pathogens, which one of their significant virulence factors is Shiga toxin. Shiga toxins (Stx) are A-B holotoxins including one enzymatically active A subunit associated noncovalently to five identical receptor binding B subunits.

In its multimeric form the B subunit has been shown to bind to cells expressing its receptors globotriaosylceramide (Gb3/CD77), and to competitively inhibit binding and toxicity of the holotoxin. There are two major classes of Shiga toxin, designated Stx1 and Stx2, and these share approximately 60% amino acid sequence identity.
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In 1986, a mouse monoclonal antibody against B subunit of the toxin and a rabbit polyclonal antibody against the holotoxin were employed. The monoclonal antibody was used to coat wells of a microtiter plate, and the polyclonal antibody preparation was used as detecting antibody in Enzyme-Linked Immunosorbant assay (ELISA).³ It was shown that polyclonal antibodies against a synthetic peptide corresponding to residues 28 to 40 of the B subunit neutralized the cytotoxicity of Shiga toxin 1 (Stx1) towards Vero cells.³ In addition, A subunit expressed as fusion with Glutathione S-transferase (GST) and its potential for use in seroepidemiology was examined. In this experiment, no good correlation was observed in a comparison between neutralization assay and immunoblot reactivity of the serum samples with low neutralizing activity.³ There was evidence of Stx1 and Stx2 cross neutralization in vivo in the rabbit model and indicated that the in vivo cross-neutralization is a function, mainly, of antibodies directed to the Stx-A subunits.⁶

In addition, it has been shown that B subunit of Stx1 can elicits antibody responses which inhibit its binding to Gb3.⁷ Furthermore, polyclonal antibodies to GST-A subunit fusion protein exhibit specific toxin neutralizing activities.⁸ Raising antibody against Shiga toxin has been previously reported ⁹,¹⁰ while neutralization ability of raised antibody against subunits has not been compared. On the other hand, identifying all types of STEC in any kind of test sample is an important issue that can be done by detection of these toxins produced by the bacterial strain. In this study, rabbit polyclonal sera against recombinant A, B subunits and rStx as AB holotoxin were raised which could be used for diagnostic purpose.

MATERIALS AND METHODS

Bacterial Strain, Clones and Preparation of Shiga Toxin

E. coli O157 was used as a bacterial strain for constructing clones expressing A, B subunits and rStx as described previously.¹¹ The products, pBAD-A, pBAD-B and pBAD-rStx express A, B subunits and rStx as periplasmic holotoxin, respectively. The clones were produced from Stx1 as it was described earlier.¹¹ In this study, cultured E. coli O157 strain was used as standard strain producing Stx1 and Stx2. Moreover, 12 locally isolated E. coli strains detected by PCR as Stx1/Stx2 or Stx1 and Stx2 producers were also used in this study.

Expression and Purification of A, B Subunits and rStx

Pellet of the induced clones were used for purification of the expressed protein. Expressed subunits were isolated by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). A and B subunits were eluted from 12% SDS-PAGE gel by Electro-Eluter (Bio-Rad Laboratories). Furthermore, the Bacterial cells from pellet were suspended in 0.1% polymyxin B sulfate (Gibco) in Phosphate-buffered saline (PBS) (pH=7.4) as described previously.¹¹ These fractions were considered as partially purified proteins and were used for further studies. The concentration of purified protein was estimated by absorbance at 280 nm with bovine serum albumin as the control (Protein assay Kit, Bio-Rad). The expressed forms of A and B subunits were eluted from SDS-PAGE gels while for rStx the polymyxin B extract was used in the following experiments.

Immunization of Rabbits

New Zealand White rabbits were injected subcutaneously with 500 μg (0.1 ml) SDS-PAGE purified A, B subunits and rStx. The proteins were mixed with equal volumes of Freund’s complete adjuvant (Sigma Chemical Co., St. Louis, Mo.). The immunization was repeated at 4 and 6 weeks after the first injection with Freund’s incomplete adjuvant. The animals were bled one week after the third injection and sera were separated from the collected bloods.

Cytotoxicity and Neutralization Assays

The abilities of anti peptides A, B subunits and rStx anti-sera, to inhibit the cytotoxicity of rStx towards Vero cells (National Cell Bank, Pasteur Institute of Iran), were determined. Cells (1.5x10⁴) were grown in 96-well microtiter plates (Nunc) for 24 h in RPMI medium (Biosera), pH=7.4, supplemented with 5% heat inactivated fetal bovine serum (Biosera), 100 unit/ml penicillin-streptomycin (Biosera) at 37ºC in a 5% CO2 atmosphere. For cytotoxicity assay, A, B and rStx were prepared in PBS (pH=7.5) then serial dilutions of rStx and each subunit were added to Vero cells. The cell viability was measured by Neutral Red (Merck) assay.¹² Briefly after 3 h incubation with neutral red solution, viable cells were fixed with 1% CaCl₂ in 0.5%
formaldehyde for 3-5 min. After washing cells were lysed with 1% acetic acid in 50% ethanol. The absorbance was measured by ELISA reader (Awareness Technology Inc.) in 540 nm. Moreover, neutralization of the cytotoxicity was done to inhibit the toxicity (50% cytotoxic dose (CD50)). Serial dilutions of sera from immunized Rabbits (Anti-A, Anti-B and Anti-AB) were mixed with A, B subunits and rStx, and then incubated at 37°C for 30 min.

The mixtures were each dispensed to cells in a 96-well microtiter plates and the plates were incubated at 37°C for 24h. The Vero cell monolayer was fixed and stained with crystal violet as described previously.13 The color intensity of the stained cells was measured at 620 nm, and the absorbance (A) was proportional to the amount of viable cells. Percentage of neutralization was calculated using the following formula:14

\[
\left( \frac{A_{620(untreated \ cells)} - A_{620(toxin)}}{A_{620(toxin)}} \right) \times 100\%
\]

Detection of Stx and Stx Producer Strains by ELISA

Specific ELISA, designed for detection of Stx and STEC strains in the following manner; SDS-PAGE purified subunits and cultured O157 strain (Stx1 and Stx2 producer) were used as antigen to coat microtiter wells (10 μg per well) overnight. Wells were incubated with 2% (w/v) skim milk in PBS for 1h; unbound protein was removed by three times washing the wells with PBS containing 0.05% (v/v) Tween 20. The antigen coated wells were incubated with dilutions of antisera raised against recombinant A, B subunits and AB5 holotoxin at room temperature for 2h. After three times washing the wells were incubated with peroxidase conjugate anti-rabbit immunoglobulin (Sigma). Following a final three times wash, antiserum binding was detected with TMB (3, 3’, 5, 5’-tetramethylbenzidine) (Bio-Rad Laboratories) substrate. Optical density (OD) was measured at 450 nm with ELISA reader (Awareness Technology Inc.).

For detection of STEC strains, the plates were coated with 50μl polyclonal antisera raised against recombinant A, B subunits and Stx (1/10 and 1/250 diluted). Then, after three times washing, 50μl of supernatant from overnight strains culture in LB (Luria-Bertani) medium, was added to detect Stx producing strains.

Statistical Analysis

Data were subjected to ANOVA and Student’s t-test for statistical analysis, and a p value of <0.05 was considered to be significant.

RESULTS

Expression and Purification of Proteins

The E. coli clones expressing recombinant A, B subunits and rStx were established to release the protein to periplasmic space.11 On the other hand, purified A, B subunits and periplasmic extract of rStx were used to raise antisera in rabbits. The subunits were detected in 12% SDS-PAGE and confirmed by Western blotting using raised sera (Figure 1).15

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Figure 1. Polyclonal anti-AB, anti-A and anti-B antisera detection of periplasmic extracts of clones expressing A, B subunits and recombinant Stx (rStx). From left to right: 1-3; MWM, periplasmic extract of A and B subunits detected by anti-AB antiserum, 4-6; MWM, periplasmic extract of rStx and A subunit detected by anti-A antiserum, 7-9; periplasmic extract of rStx and B subunit detected by anti-B antiserum.
Neutralization of the Cytotoxicity

The biological activities of the constructed recombinant A, B and Stx were examined on Vero cells (Figure 2a). According to Figure 2a the CD50 of subunits and toxins were as follows (A=20µg/ml, B=15µg/ml and AB=10µg/ml).

Cytotoxicity was inhibited by antisera raised against each A, B subunits and rStx (Figure 2b). 50% neutralization of Vero cells was observed by anti-A (in 0.02 serum dilution), anti-B and anti-AB antisera (in 0.05 serum dilution) and it was reduced by serially sera dilutions (1/10 – 1/200). It was shown that anti-AB antiserum raised 64% neutralization while with anti-B antiserum 70% neutralization and with anti-A antiserum 80% neutralization was observed against Stx producing strains (in 0.1 serum dilution).

The neutralization abilities of the obtained sera were different. Neutralization by anti-A antiserum was shown in higher dilution, comparing to B subunit and rStx, antiserum.

Evaluation of Polyclonal Pntisera

The specificity of raised polyclonal antisera was each assessed by ELISA. The ELISA plates were coated by periplasmic extract of A, B subunit and rStx and then, different concentrations (1/10-1/1000) of anti-A, anti-B and anti-AB antisera (Figure 3a) were used. Regarding Stx production, the plates were also coated by periplasmic extract of Stx producer strain (O157) in presence of different concentrations (1/10-1/1000) of anti-A, anti-B and anti-AB antisera (Figure 3b). It was observed that the antisera against A, B subunits and rStx significantly recognized Stx and strain producing Shiga toxin.
Rabbit Polyclonal Sera and Detection of Stx-Producing E. coli

**Sandwich ELISA for Detection of Stx Producing E. coli Strains**

Stx producing strains, Stx1 and Stx2 were used to compare specificity of anti-A, anti-B and anti-AB antisera reaction. The indirect ELISA was done with, 1/10 and 1/250 serum dilutions of the anti-A, anti-B and anti-AB antisera coated the plates. 50 µl of bacterial culture producing toxin (O157) was significantly reacted with anti-A, anti-B and anti-AB antiserum (Figure 4a). The 1/10 serum dilutions of anti-A and anti-B antisera were significantly reacted with Stx2 producing E. coli strain (Figure 4b). For all eight Stx1 producing strains, anti-A antiserum reaction was significant, while regarding anti-B antibody it was not significant for all tested strains. Regarding two Stx2 producing strains, the result was significant for anti-A antibody. In two other Stx1 and Stx2 producing strains, anti-A and anti-B antibody, were both significant.

**DISCUSSION**

Detection of STEC strains producing Stx, in bacterial isolates by specific antiserum is of ultimate importance. The polyclonal antiserum detection of Stx producing strains is specific and it is an alternative usual method for the detection. ELISA-based kits have already been used for Stx detection by monoclonal antibody. It was demonstrated that rabbit polyclonal sera produced against Stx1 and Stx2, applied for detection of STEC in the immunoassay, appeared as perspective for STEC detection in developing countries. In addition, elicited specific neutralizing polyclonal antisera can also have been used for therapeutic purposes. Bacterial protein toxins have been used as therapeutic agents and production of vaccines. Another issue concerns the development of new generations of vaccines and immune adjuvant. In this regards rStx and its subunits produced and used for immunization of rabbits. The toxin subunits were detected by sera obtained from immunized rabbit and it was shown that the inhibition of cytotoxicity can be elucidated by the raised antiserum. The raised anti-A, anti-B and anti-AB antiserum recognized A, B subunits and rStx (as it was shown in Figure 3a). On the other hand, anti-A and anti-B antibody were reacted more significantly than anti-AB in Stx detection (Figure 3b). Although, anti-A and anti-B antibody specifically recognized Stx1, Stx2 producer strains (Figure 4), anti-A antibody strain detection was significant in most strains as was assessed in this study. Detection of Stx producer strains was statistically significant in 1/10 serum dilutions while in non-producer strains it was not statistically significant (Figure 4b).

It was already shown that polyclonal antibodies against produced fused A subunit neutralized Shiga toxin. In the present study, it was shown that anti-A and anti-B produced antisera, both have ability to recognize the Stx producing strains and these antisera can be useful tools for immunological detection of these strains. In our experiment, anti-A antiserum more significantly reacted against most Stx producing strains. The sequence analysis of A and B subunits, among Stx1/Stx2 producing strains, revealed similarity. Since, the A subunit is larger than the B subunit and due to the more homology of A subunit between different variants, therefore, this might be the reason for its high level of neutralizing ability in this study. Stx producing strains show a great effect in pathogenesis of...
hemolytic-uremic syndrome and therefore detection of Stx production in bacterial isolates by specific antisera is an important issue. In addition, ELISA-based kits have been developed for Stx detection by monoclonal antibody, while false-positive results have been documented. The anti-A and anti-B polyclonal specific antisera alone can also be used for immunological diagnosis. Regarding, low level of cytotoxicity of A subunit and its high production level, it can be used for immunological diagnosis of Stx producing strains (Stx1 and Stx2 producer) in developing countries where low cost detection assays are mostly needed. In this study, it was shown that the polyclonal antibodies (especially Anti-A and Anti-B antibodies) can be useful alternatives for the rapid detection and probably future therapeutic application. Low cost preparation of these antibodies in each laboratory makes them more attractive for applying especially in developing countries.

REFERENCES