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Development of an IgG/IgY Sandwich-ELISA for the Detection of Cholera Toxin Subunit B

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Abstract

Introduction: Cholera is a lethal diarrheal disease caused by *Vibrio cholerae*. Cholera toxin (CTX) is one of the major virulence factors in *V. cholerae* pathogenesis. One of the major strategies in dealing with the poisoning of the bacteria is their rapid detection. The aim of this study was to design and set up a double-sandwich ELISA diagnostic method for the direct detection of cholera toxin B (CtxB) based on chicken immunoglobulin Y (IgY) and rabbit immunoglobulin G (IgG).

Materials and Methods: Recombinant CtxB protein was expressed in *E. coli* BL21 (DE3) cells by addition of IPTG and was purified using an Ni-NTA column. Chickens and rabbits were immunized subcutaneously and the generated antibodies were purified from egg yolks by polyethylene glycol (PEG) precipitation and from the rabbits' sera by protein G column. These antibodies were used to set up the ELISA method. The sensitivity of the designed ELISA method was evaluated using serial dilutions of the protein and the specificity of this method was evaluated.

Results: Recombinant protein expression analysis showed an appropriate expression of the protein (300 µg/ml). ELISA assay results showed an increased serum antibody levels against the protein in rabbits' and chickens' sera after each injection. The yield of the purified IgY and IgG was 10 and 2.5 mg/mL, respectively. The sensitivity of the ELISA method was about 39 ng for recombinant CtxB. The results showed the high specificity of this technique.

Conclusions: Results suggest that IgY/IgG-based sandwich ELISA in this study provides a convenient preparation for the development of an immune-based method for the detection of CtxB.

Keywords: *Vibrio cholerae*, Cholera toxin, Egg yolk immunoglobulin, ELISA

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Introduction

Cholera is an infectious disease caused by *Vibrio cholerae (V. cholerae)*, which is transmitted through contaminated water and food.[1] The most important symptoms of this disease are vomiting and watery diarrhea.[1] *Vibrios* are Gram-negative bacteria that, based on their O antigen, are classified into eight serogroups. Among them, *O1* and *O139* serogroups are toxigenic and have been associated with epidemic cholera.[2] Cholera toxin is one of the major virulence factors of *V. cholerae* pathogenesis.[3] This enterotoxin contains two subunits, the A subunit (CTXA) is toxigenic, and the B subunit is responsible for the toxin binding to eukaryotic cells.[3] The CTXB is highly immunogenic and free of toxicity.[4,5] Cholera became an important global disease for the first time in 1817.[6] The epidemiology of cholera has been important in Iran since 1965 where the common serotypes were *Ogawa* and *Inaba*.[7]

The successful treatment of cholera is dependent on the rapid detection and initiation of therapy. There are many diagnostic methods for detecting the bacteria, including microscopic examination techniques, differential staining techniques, determination of growth in different diagnostic media and under different conditions, determination of life cycle, etc.[8] Also, there are precise and rapid molecular methods, such as polymerase chain reaction (PCR), for the detection of *V. cholera*e, which are usually complex, time-consuming, and expensive. Following the rapid outbreak of bacterial infections and serious problems, efforts have been made to develop a rapid, precise, and on-time detection method based on its enterotoxin to control and prevent diseases outbreaks.[9] Therefore, simple diagnostic methods with acceptable sensitivity and specificity have been provided to detect *V. cholerae* based on the cholera toxin. Among the methods, agglutination, radioimmunoassay, chromatography, and ELISA are the most important methods for the detection of cholera toxin.[10,11] ELISA method is the most used technique for the analysis of food and other samples. The double sandwich ELISA method improves the sensitivity and specificity of the ELISA method. This method is simple, highly sensitive, and specific and is used for determining particular antigens in unknown samples.[12,13]

Antibodies are typically produced by the immunization of animals, mainly mammals. However, mammalian antibodies are produced by invasive means in limited quantities, and their purification is time-consuming and expensive. As an

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alternative, the use of avian antibodies has increased spectacularly to replace mammalian antibodies. The major advantage of avian antibodies is the ease of purification from eggs instead of serum. Also, an egg-laying hen antibody productivity is much higher than that of a similar-sized mammal. Today, hens are recognized as an expedient and economical source of antibodies.[14,15]

The aim of this study was to develop a sandwich ELISA method based on rabbit IgG and chicken egg yolk IgY polyclonal antibodies for the detection of CTXB. The results of this method may be applied in studies on immunological detection of cholera toxin.

Materials and Methods

The exploited DNA construct containing the *ctxB* gene was taken from the Biology Research Center of Imam Hossein University.[16]

Expression and Purification of the Recombinant Protein

E. coli BL21 (DE3) containing pET28a-*ctxB* recombinant plasmid was grown in 5 ml of Luria-Bertani (LB) broth medium containing 80 µg/ml kanamycin (Sigma, USA) for an overnight at 37 °C. Then, 1 ml of this culture was transferred into a 50 ml LB medium containing 40 µg/ml kanamycin and was grown at 37 °C until the cells reached the mid-log phase (OD 600 nm approximately 0.5). The recombinant protein expression was induced by the addition of IPTG (Sigma, USA) with the final concentration of 1 mM, and the cells were grown on a shaker for 4 h at 120 rpm and 37 °C. The cells were collected by centrifuging for 6 min at 5000 rpm at 4 °C, and cell pellets were suspended in 4 ml of lysis buffer (8M urea, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) .The lysed cells were sonicated and centrifuged for 20 min at 13500 rpm at 4 °C. Finally, the supernatant was analyzed for the expression of protein by SDS-PAGE.[17]

Purification of the Recombinant Protein

The purification of CtxB recombinant protein was performed using an Ni-NTA column (Qiagen, USA).[18] The supernatant containing the recombinant protein was loaded onto the column, pre-equilibrated with 1 ml of lysis buffer. To wash non-specific proteins bound to the column, buffers C (8 M urea, 300 mM NaCl, 50 mM NaH_2PO_4, pH 6.2) and D (8 M urea, 300 mM NaCl, 50 mM NaH_2PO_4, pH 5.9) was applied to the column. Finally, 1 ml of elution buffer (8 M urea, 300 mM NaCl, 50 mM NaH_2PO_4, pH 4.5) was added to the column, and the flowthrough was collected. Then, the recombinant protein was eluted with 1 ml of 20, 40, and 250 mM imidazole buffers. After the purification, the purity of CtxB recombinant protein was evaluated on a 12% SDS-PAGE, and the protein concentration was determined by the Bradford method.

Immunization of Chickens

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Imam Hossein University, Iran. Also, all experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Two 25-week-old white Leghorn chickens were used in this study. The chickens were divided into test and control groups. Initial administration was performed by intramuscular injection of 150 µg of the purified recombinant CtxB protein with an equal volume of Freund's complete adjuvant (Sigma, USA). Two booster administrations were done on days 14 and 28 using incomplete Freund's adjuvant. Immunization of the control group was performed by adjuvant and PBS (phosphate-buffered saline). One week after each immunization, eggs were collected and stored at 4 °C for further analysis.[3,19]

Immunization of Rabbits

Female New Zealand white rabbits (Pasteur Institute of Iran) weighing approximately 2.25 kg were immunized on day 0 with a mixture of 150 µg of recombinant protein mixed with complete Freund's adjuvant (Sigma, USA) and boosted 3 times at two-week intervals with 100 µg, 75 µg, and 50 µg of recombinant CtxB protein mixed with incomplete Freund's adjuvant on days 14, 28, and 45, respectively. Immunization of the control group was performed by sterile PBS. One week after each administration, sera samples were collected to monitor serum antibody levels.[20]

Purification of anti-CtxB Antibodies (IgY) from Egg Yolks of Immunized Chicken

Before The purification of IgY antibodies, serum IgG antibody levels of the immunized chickens were determined by the ELISA method. Following the third and fourth administrations, the titer of antibodies in the sera of the immunized chicken reached the highest level. IgY antibodies were extracted from egg yolks by Pauly et al. method. In this method, IgY antibodies were separated from other proteins by polyethylene glycol (PEG) precipitation. Briefly, the eggshells were broken, and the egg yolks were carefully separated from the egg white and yolk membrane. PBS was added to the samples twice the volume of egg yolk. Thereafter, 3.5% w/v of PEG 6000 (Merck, Germany) was added to the mixture and vortexed and stored at room temperature for 10 min. In this step, the suspension has two phases (one phase consists of yolk solids and fatty substances and a watery phase containing IgY and other proteins) were separated by centrifugation for 20 min at 10000 rpm and at 4 °C. To remove the lipids, the collected supernatant was filtered by Whatman filter paper, and 8% w/v of PEG 6000 was added to the filtrate. Then, the filtered suspension was shaken and centrifuged as described above, and the supernatant was discarded. The pellet was dissolved carefully in 10 ml of PBS by vortexing. Then,

12% w/v of PEG 6000 was added to the solution. The mixture was shaken and centrifuged as described above, and the supernatant was discarded. The pellet was dissolved carefully in 1 ml of PBS by vortexing, and then, the final solution was dialyzed overnight in PBS (0.1 % saline) at 4 °C. Finally, the quality of the purified antibodies (IgY) were analyzed by SDS-PAGE analysis.[21]

Purification of anti-CTXB Antibodies from the Sera of the Immunized Rabbit

Before purifying IgG antibodies, serum antibody levels of the immunized rabbits were determined by the ELISA method. The rabbit IgG purification was performed using a G protein column (Abcam, USA).[22] Briefly, the protein G column was equilibrated by the addition of 100 mM and 10 mM Tris, respectively. To adjust the serum pH to 8, rabbit's serum was mixed with 1 mM Tris (with a proportion of 1:10). The rabbit serum samples were loaded onto a protein G column, and the column was washed by 100 mM and 10 mM tris. To purify IgG antibodies, 100 mM Glycine with pH 3 was loaded onto the column, and then the purified IgG antibodies were collected in tubes. Before collecting the purified antibodies, 50 µl of 1 mM Tris was added to each tube to adjust the purified antibodies' pH value to 8. The purity of the purified IgG was evaluated by SDS-PAGE analysis.

Enzyme-linked Imrnunosorbent Assays (ELISA)

Antibody titers of the immunized chickens and rabbits were determined by the indirect ELISA method.[23] 96-well plates (Nunc, Denmark) were coated with 5 µg of the CtxB diluted in 100 µl coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8) at 4 °C, overnight. Then, the plates were washed four times with PBST (0.05% phosphate-buffered saline containing Tween 20) and blocked with 100 µl of 5% dry milk in PBST for 40 min at 37 °C. Serum samples were serially diluted from 1:100 to 1:3200 in PBST and were added to each well and incubated for 40 min at 37 °C. The plates were washed four times with PBST, and 100 µl of HRP-conjugated antibody (1:1000) (Abcam, USA) was added to each well. The plate was incubated for 40 min at 37 °C and then washed four times in PBST. Then, 100 µl of the substrate solution (containing 1 mg of o-phenylene diamine (Sigma, USA), 5 ml citrate buffer, and 3 µl H_2O_2) was added to each well. Finally, the reaction was stopped with the addition of 2.5 M H_2SO_4, and the absorbance was read at 495 nm on a microplate reader (Bio-Rad, USA).

The specificity of the double sandwich ELISA was determined using the CtxB recombinant protein as positive control and the Shiga toxin (StxB) and Staphylococcal enterotoxin B (SEB) recombinant proteins as negative controls.[24] For the double sandwich ELISA, 96-well plates (Nunc, Denmark) were coated with 5 µg of the purified IgY diluted in 100 µl coating buffer (64 mM Na_2CO_3, 136 mM $NaHCO_3$, pH 9.8) at 4 °C, overnight. Then, the plates were washed four times with PBST (0.05% phosphate-buffered saline containing Tween 20) and blocked with 100 µl of 5% dry milk in PBST overnight at 4 °C. The CtxB recombinant protein was diluted from 5000 ng to 39 ng in PBST and was added to each well plate and incubated 40 min at 37 °C. 5 ug of the purified IgG was diluted in PBST and was added to each well plate and incubated for 40 min at 37 °C. The plates were washed four times with PBST, and 100 µl of 1in1000 dilution of rabbit IgG HRP-conjugated antibody was added to each well plate. The plate was incubated for 40 min at 37 °C and then washed four times in PBST. Then, 100 µl of substrate solution (containing 1 mg o-phenylene diamine (Sigma, USA), 5 ml citrate buffer, and 3 µl H_2O_2) was added to each well plate. In finally, the reaction was stopped with 2.5 M H_2SO_4, and the absorbance was read at A495 nm on a microplate reader (Bio-Rad, USA).

Statistical Analysis of Data

The normality of the data was assessed by the Kolmogorov-Smirnov test. Comparison between control and test groups was analyzed by Student's t-test. Repeated measurement tests were used to compare the effects of antigen administration number on antibody titer. For both tests, a *p*-value less than 0.05 was considered significant. Statistical analyses were carried out by SPSS 22.0.

Results

Expression and Purification of the Recombinant Protein

The recombinant protein was expressed in *E. coli* BL21 (DE3) by the addition of 1 mM IPTG. The SDS-PAGE results of the expression analysis have been demonstrated in Figure 1. As can be seen in the Figure, the molecular weight of the induced recombinant protein is 14.5 kDa. Recombinant Protein purification was performed using an Ni-NTA column. The SDS-PAGE results of the purified recombinant protein have been shown in Figure 2 with an appropriate purity. The concentration of recombinant protein estimated by the Bradford method was 300 µg/ml.

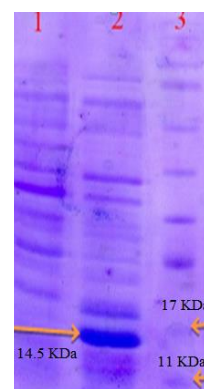

Figure 1. SDS-PAGE Analysis of Recombinant CTXB Protein Expression. Line1: Pellet uninduced by IPTG; Line 2: Sample induced by 1mM IPTG 4 h after induction; Line 3: Protein marker (10-170KDa, SinaClone, Iran).

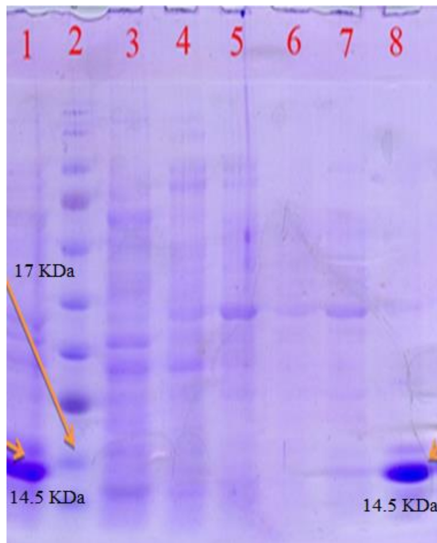

Figure 2. SDS-PAGE Analysis of the Recombinant Protein by Urea Gradient pH and Imidazole Competitive Method. Line 1: The induced cell lysate; Line 2: Protein marker; Line 3: Flow through; Line 4: Washing solution (buffer C); Line 5: Washing solution (buffer D); Line 6: Elution with 20 mM imidazole: Line 7: Elution with 40 mM imidazole; Line 8: Elution with 250 mM imidazole.

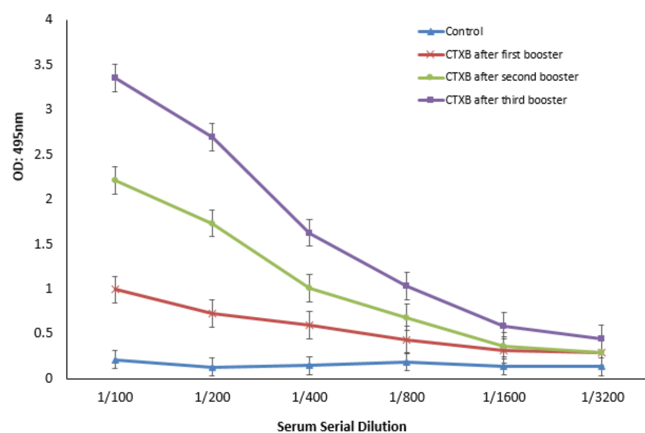

Figure 3. Antibody Titration Against CTXB in Serum of Immunized Chicken. The increase in antibody titer after each administration was significant ($p<0.05$).

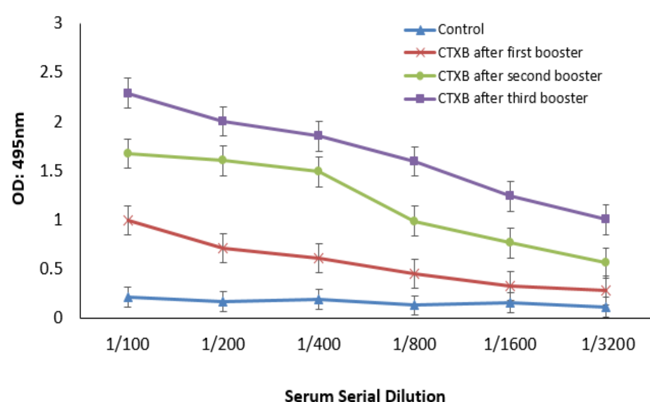

Figure 4. Antibody Titration Against CTXB in Serum of Immunized Rabbit. The increase in antibody titer after each administration was significant ($p<0.05$).

Assessment of Serum Antibody Responses Against CTXB in Immunized Chicken

Animals remained healthy and showed no signs of abnormal behavior after each administration. ELISA assay was used to determine the titer of raised IgY antibody against CTXB protein. The results of the ELISA assay have been shown in Figure 3. The results showed that the titration of antibody production in the sera from immunized chickens increased after each administration. The increase in antibody titer after each administration was significant ($p<0.05$). No increase in IgY antibody titer was observed in the control groups.

Assessment of Serum Antibody Responses Against CTXB in Immunized Rabbit

ELISA assay was used to determine the quantity of the produced IgG antibody against CTXB protein. The results of the ELISA assay have been presented in Figure 4. The results showed that the antibody production in the sera of immunized rabbits increased after each administration. The increase in antibody titer after each administration was significant ($p<0.05$). No increase in IgG antibody titer was observed in the control groups.

Evaluation of Antibody Extraction from Egg Yolk

The IgY antibodies were extracted from egg yolks by gradually increasing PEG in several stages. The SDS-PAGE results of antibody extraction have been demonstrated in Figure 4. The extracted IgY has been shown in line 2 of Figure 5 with an appropriate purity. The yield of purified IgY was 10 mg/ml.

Evaluation of Antibody Purification from Rabbit Serum

The IgG antibodies were purified from rabbit serum by protein G column. SDS-PAGE results showed that the purification had been performed successfully. The yield of the purified IgG was 2.5 mg/ml.

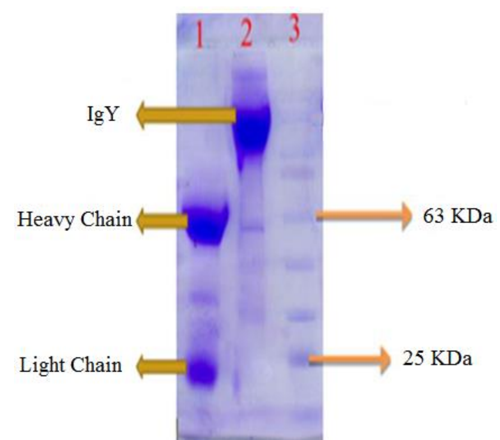

Figure 5. Antibody Extraction from Egg Yolk by PEG Precipitation. Line1: The extracted IgY with 2ME; Line 2: the extracted IgY without 2ME; Line3: protein marker.

Sensitivity of Designed Double Sandwich ELISA Method

Analytical sensitivity of the sandwich ELISA method was determined by serial dilutions of recombinant CTXB protein. The results of Analytical sensitivity showed a detection limit of about 39 ng for CTXB (Figure 6).

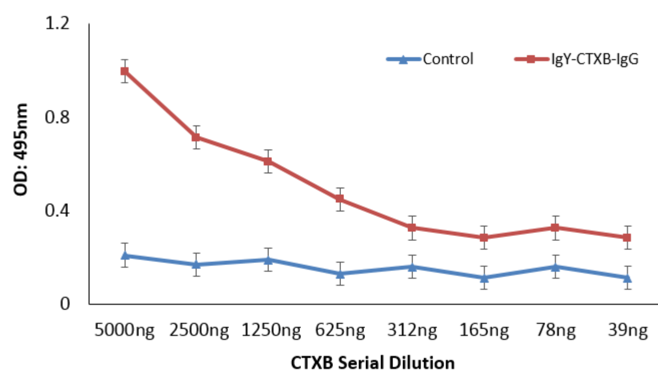

Figure 6. Sensitivity of the Double Sandwich ELISA Method for Detection of the B Subunit of the Enterotoxin (CTXB).

Discussion

Cholera is a bacterial infection caused by *V. cholera*e strains that produce cholera toxin. Cholera toxin-producing strains are the cause of endemic cholera in many countries and cause large epidemics. *V. cholerae O1* and *O139* serogroups are Cholera toxin-producing strains.[1-3] According to WHO, *V. cholerae* is a classic example of bacterial diarrhea that remains a threat to public health.[25] Effective vaccines, as well as potent and harmless drugs, have not yet been developed against cholera. The development of vaccines and rapid diagnostic methods are two essential solutions to the detection, prevention, and treatment of cholera.[26] The strategy of simple and inexpensive diagnostic methods is considered an important tool for the management of the disease. One of the main goals in these cases is the production of different antibodies against *V. cholerae* antigens and the design of ELISA diagnostic method to detect this biological agent.[27] In this study, a double sandwich ELISA method has been designed based on avian (IgY) and rabbit (IgG) antibodies. Also, in this study, recombinant CtxB protein was used for immunization of chickens and rabbits to produce antibodies for ELISA diagnostic method. The immunogenic and non-toxic protein properties of the CtxB have been investigated and confirmed in previous studies, and also, the CtxB has an important role in cholera pathogenesis.[28] These three features have made the CtxB an ideal antigen to use in diagnostic immunoassays.

A variety of animals are being used to produce the antibody,[29] including rats, goats, monkeys, rabbits, sheep, horses, camels, and chickens. Laying hens are the best choice for mass production of antibodies, but there are several limitations, including special requirements for their maintenance and lack of experience working staff.[30] According to researches, the production of antibodies from egg yolk is nearly 18 times higher than the production of antibodies in the sera of rabbits. Another advantage of this method (IgY technology) is to provide a noninvasive method for the production of antibodies.[31] In 1999, IgY technology was provided as an attractive alternative method to mammalian antibody production.[32]

Two purified antibodies are used to develop a double sandwich ELISA diagnostic method. In this research, rabbit and avian polyclonal antibodies were produced against recombinant CTXB protein of *V. Cholerae*. Avian antibodies were extracted from egg yolk using IgY technology. In this method, IgY antibodies separate from other proteins by PEG precipitation. The amounts of antibody purification yielded by this method in different studies were different. In a study conducted by Pauly et al., they could purify 28.8 and 60 mg specific antibodies per each egg yolk.[21] In 2012, in a study for the production of IgY against *Toxoplasma gondii* antigen, the researchers reported that purification yields 48 mg per egg yolk.[33] Here, the yield of purified IgY was 30 mg/egg yolk.[34] The double-sandwich ELISA diagnostic method was designed with the recombinant CtxB concentration gradient. The analytical sensitivity of this method was about 30 ng/mL. In a study to detect cholera by Tuteja et al., the analytical sensitivity of this method against CtxB was reported 60 pg.[35] In another study for detecting cholera by Bayati et al., the analytical sensitivity was reported 33 pg.[26] In comparison with similar studies, our findings confirmed that the detection range reported in the current study is lower than in other studies. There are two causes for this weakness, including the different molecular structures of avian and rabbit antibodies and the insufficient purity of these antibodies.

Conclusion

This study reported the production of avian and rabbit antibodies against recombinant CtxB protein of *V. cholera*e and optimization of the ELISA diagnostic method for the detection of this toxin. Further attention to diagnostic methods is needed in the future due to the increased mortality rate as well as the increasing natural disasters worldwide, which provide the conditions for the epidemic of diseases such as cholera.

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Authors' Contributions

ANV: Investigation, Methodology; SN: Project administration, Methodology, Data curation, Validation; DS: Methodology, Data curation; MEM: Methodology, Statistical analysis; AH: Writing and original draft preparation, Manuscript editing.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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