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PRODUCTION AND CHARACTERIZATION OF MONOCLONAL AND POLYCLONAL ANTIBODY AGAINST RECOMBINANT OUTER MEMBRANE PROTEIN

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ABSTRACT

There are many studies related to immunological and molecular methods for diagnosis of *Vibrio cholera* (*V. cholerae*). However, most assays dependent on enrichment of culture of bacteria, which need more time and involves the use of costly equipment and reagents. In this study Balb/c mice were immunized with recombinant Outer Membrane Protein (rOMPw) of *vibrio cholerae* and splenocytes of hyper immunized mice were fused with murine myeloma Sp2/0 cells. Positive hybridomas were selected by ELISA using rOMPw as coating antigen. The monoclonal antibodies from ascitic fluids were purified and its reaction with rOMPw was assessed by ELISA. Polyclonal antibodies were also produced by immunization of rabbits with the above mentioned antigen. The rabbit sera was affinity purified using Hi-Trap protein G column. The result showed that monoclonal antibody specific to rOMPw has been successfully generated. The monoclonal antibody reacted with recombinant OMPw in ELISA and immunonoblat method. Rabbit polyclonal antibody was also bound to rOMPw by ELISA. The results of agglutination test with whole bacteria also showed that both mouse monoclonal and rabbit polyclonal antibodies reacted with *whole vibrio cholera* but not other related *bacteria*. The purpose of this study was to check out if anti OMPw antibodies could use as diagnostic assay for detection of *V. cholerae*. Our results demonstrated that anti recombinant OMPw monoclonal and polyclonal antibodies are able to diagnose whole bacteria in pure culture using agglutination test but not by home made immunochromatic strip test.

Keywords: *Vibrio Cholerae*, OMPw, Monoclonal Antibody

1. INTRODUCTION

Among numerous outer membrane proteins of *Vibrio cholerae* (*V. cholerae*), which are vary in their abundance and immunogenicity, the 22 KDa Outer Membrane Protein (OMPw) has been reported to be very immunogenic. This protein which is produced in minor amounts, its function is not well known, but may use as a diagnostic indicator of *V. cholerae*. The effective and rapid method for detection of *V. cholerae* is required for control of epidemic outbreaks. Traditional identification of *V. cholerae* is often achieved through the isolation of the bacteria and time-consuming, laborious routine microbiological and biochemical analysis that require three working days before obtaining the results (Suzita *et al*., 2009; Ferdous, 2009). Among several molecular-based techniques, only PCR assay has been useful in detecting *V. cholerae* based on their gene (Vidal *et al*., 2007; Keddy *et al*., 2013). Recently, Srisuk *et al*. (2010) has reported a new Loop-mediated isothermal Amplification (LAMP) assay which target the same gene with higher sensitivity than simple PCR. It should also be mentioned that PCR assay require expensive equipment and highly skilled personnel, therefore, it is not feasible for small

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laboratories as a diagnosis method. Indeed, in control of disease outbreaks, we need a quick and easy method without sacrificing specificity and sensitivity. In this regard, it seems that immunoassay could be the simplest and easy way with enough specificity and sensitivity in recognizing different species of *V. cholerae*. Pensuk and his colleagues have demonstrated a rapid method for diagnosis of various *V. cholerae* based on specific monoclonal antibodies (Pengsuk *et al*., 2010; 2011; Prompamorn *et al*., 2013). The aim of this study was to developed mouse monoclonal and rabbit polyclonal antibodies against recombinant OMPw protein that might be useful for immunodiagnostic of *V. cholerae*.

2. MATERIALS AND METHODS

2.1. Immunization of Mice

Female Balb/c mice aged 6 to 8 weeks old (pasture Institute, Tehran, Iran) were immunized with recombinant OMPw protein.

(MKQTICGLAVLAALSSAPVFAHQEGDFIVRAG IASVVPNDSSDKVLNTQSELAVNSNTQLGLTLGY MFTDNISFEVLAATPFSHKISTSGGELGSLGDIGET KHLPPTFMVQYYFGEANSTFRPYVGAGLNYTTFF DESFNGTGTNAGLSDLKLDDSWGLAANVGFDYM LNDSWFLNASVWYANIETTATYKAGADAKSTDV EINPWVFMIAGGYKF). These recombinant proteins were mixed with equal volume of complete Freund's adjuvant or Incomplete Freund's adjuvant for the first and subsequent interaperitoneal immunization of mice respectively (50 µg protein/Immunization/mouse). One week after the last immunization, blood samples were collected by a vertical incision of the tail vein of mice for determination of antibody titers by ELISA method using HRP goat anti mouse antibody as second antibody. The last injection of 20 µg of protein (without any adjuvant) was performed intravenously three days before the cell harvesting.

2.2. Hybridoma Cell Generation

Before cell fusion, blood samples of mice were collected and its specific antibody was determined by ELISA method using pre-coated of 96 wells ELISA plates with recombinant OMPw. The mouse with the best antibody titer was selected for cell fusion and hybridoma cell generation. Briefly, Splenocytes from the immunized mice were mixed with murine myeloma Sp2/0 cell line at a ratio of 1:5 (1 Sp2/0 and 5 spleen cells). Cell fusion and selecting hybridoma cell was performed in usual manner. The reactivity of hybridoma supernatants was determined using 96-wells pre-coated ELISA plates (Nunc, Roskilde, Denmark) with 50 µL of 10 µg mL^{-1} of Phosphate-Buffered Saline (PBS) dissolved rOMPw. Finally, positive hybridoma cells were cloned 4 times by limiting dilution to select the stable hybridomas.

2.3. Ascitic Fluid Production

Briefly, 0.5 mL of 2,6,10,14-Tetramethyl pentadecane (Pristane) (Sigma-Aldrich) was injected into peritoneal cavity of Balb/c mice. After one week, 5×10^6 hybridoma cells were washed and re-suspended in 0.5 mL sterile PBS followed by inoculation into peritoneal cavity of the mice. The ascitic fluids were collected after 7-10 days.

2.4. Monoclonal Antibody Purification

Anti rOMPw monoclonal antibodies were affinity purified from ascitic fluid using Hi-Trap protein G column (GE Healthcare, Uppsala, Sweden). Briefly, the ascitic fluids were diluted in 1:5 ratio with PBS and filtered through 0.45 µm filters (Orange Scientific, Braine-1' Alleud, Belgium). Elution was performed using 0.1 M Glycine-HCl (pH 2.7). The eluted antibodies were dialyzed against PBS (pH 7.5) overnight at 4°C. The reactivity of purified antibodies was assessed by ELISA and western blot.

2.5. Polyclonal Antibody Production

Two female-white New Zealand rabbits aged 6-8 weeks old (Pasture Institute, Tehran, Iran) were immunized with recombinant OMPw. The first immunization was performed using 50µg protein emulsified in complete Freund's adjuvant and 4 booster injections were performed using 50µg protein emulsified in incomplete Freund's adjuvant. The injections were performed intramuscularly at 4 weeks intervals between the first and second injection and 2 weeks intervals between subsequent injections. Blood samples were collected and polyclonal antibody titers were determined by ELISA as previously described. The specific antibodies were affinity purified by using a column of Hi-Trap protein G column (GE Healthcare, Uppsala, Sweden).

2.6. Isotype Determination

The isotype of monoclonal antibodies were determined by isotyping kit goat anti mouse antibody for IgG1, IgG2a, IgG2b, IgG3, IgM and mouse anti goat HRP (Sigma Co.). Briefly, 100 µL of hybridoma supernatant was added to each rOMPw pre-coated 96

wells plate and incubated for 30 min at 37°C. After three usual washing, 100 µL of each of above mentioned antibodies (diluted 1/1000) was added to respective wells and incubated for further 30 min at 37°C. They washed again and 100 µL mouse anti goat HRP (diluted 1/100000) were added to each well. Plates were incubated for further 30 min at 37°C and washed again. Substrate (TMB) was added in 100 µL volume, incubated in dark room for 25 min. After that stopper solution was added and absorbance was read at 450 using Multiscan ms Labsystem.

Isostrip isotyping kit (mouse monoclonal antibody isotyping kit, Roche Diagnostics, Mannheim, Germany) was also performed for isotyping confirmation. Freshly diluted supernatant (1:10) of the growing hybridomas in 150 µL volume was added to the isotyping tubes followed by incubation at RT for 30 sec with gentle shaking. One strip was then inserted in each tube and after 5 min the appearance of a blue band indicated the monoclonal isotype.

2.7. SDS-PAGE and Western Blot Determination

Recombinant OMPw was separated using 10% SDS-PAGE gel by electrophoreses system at 50v for 3h. In order to visible the separated bands, gels were stained with coomassie brilliant blue R.250 (Sigma). For western blotting, the separated bands of SDS-PAGE were transferred onto nitrocellulose membranes for 1h at 100v or at 15v for over night using a Trans-blot apparatus (Bio Rad). After that nitrocellulose membrane were incubated with 5% BSA (sigma) for 30 min at room temperature, treated with 1/100 purified mouse monoclonal antibodies and incubated for 1h. Then, Horseradish Peroxidase-labeled (HRP) goat anti mouse IgG antibody (Sigma) was added and incubation was continued for further 1h and specific bands were visualized by respective substrate in usual manner. Low and high molecular weight markers standard were developed using coomassie brilliant blue R.250 staining.

2.8. Identification of *V. Cholerae* Using Agglutination Test

V. cholerae at 1×10^8 c.f.u/mL was treated with different dilutions (ranging between 1/20 to 1/320) of Protein G purified mouse monoclonal IgG and rabbit polyclonal antibodies respectively and incubated for two hours at 37°C. Three other related bacteria; *Salmonella*, *E.coli* and *Shigella* were also treated with above mentioned antibodies dilution looking for any cross reaction. Tubes were centrifuged at 4000×G for 5 min and agglutination was checked in usual manner.

3. RESULTS

3.1. Immune Mice

The titers of anti-OMPw antibodies in the sera of immunised mice showed that mice number 4 had higher anti-OMPw antibody (**Fig. 1**). Therefore, this mouse was chosen for further monoclonal antibody production.

3.2. Anti Recombinant OMPw Monoclonal Antibody

The screening results showed three clones of monoclonal antibodies against rOMPw. Two out of three clones, D4F10 and D8D6 produced IgG1 and clone A7C5C10 produced IgM. Clone D8D6 had higher OD value (1.80) comparing to D4F11 (1.36) therefore, in this research we followed up our work on clone D8D6 which produced IgG1 monoclonal antibody. The titer of purified antibody of ascitic is shown in **Fig. 2**. As shown in **Fig. 2** mouse monoclonal antibody showed very high absorbance values in ELISA at high dilutions between 1:250 and 1:8000 with recombinant OMPw antigen. The results of isotype identification of monoclonal antibody against rOMPw with iso strip kit showed that produced antibody is IgG2a/κ (**Fig. 3**). The specificity of monoclonal antibody to rOMPw was also determined by SDS-PAGE and immuno-blat (**Fig. 4 and 5**).

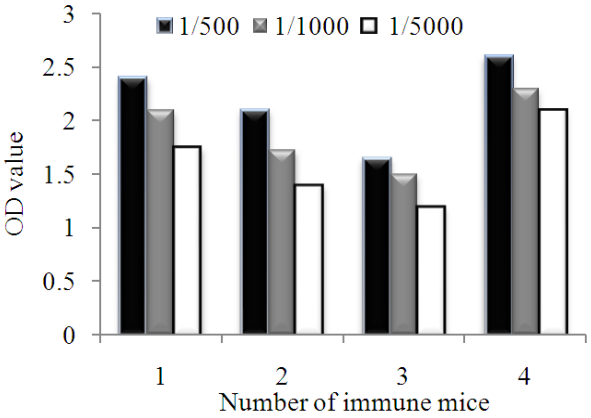

Fig. 1. Titration of specific antibodies against recombinant OMPw in sera of four Balb/c mice. A serial dilution of immunized mice serum were added to rOMPw pre-coated 96 wells plate and titration of specific antibody was assayed by ELISA method using goat anti mouse antibody conjugated to HRP as secondary antibody (Sigma Co.)

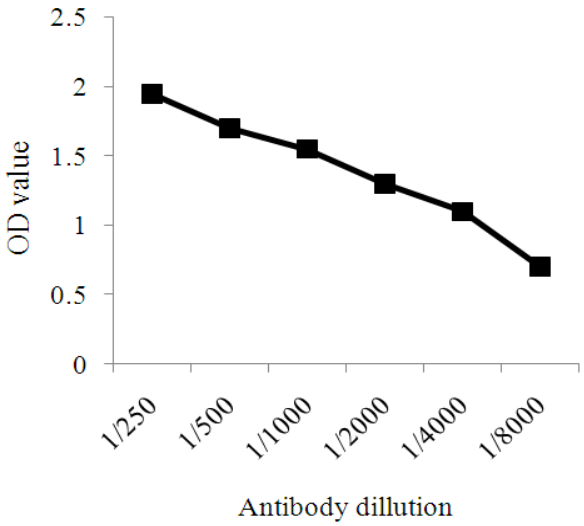

Fig. 2. Titration of specific purified mouse monoclonal antibody against recombinant OMPw. A serial dilution of purified concentrated ascitic liquids among 1/250 to 1/8000 were added to rOMPw pre-coated 96 wells plate and titration of specific antibody was assayed by ELISA method using isotyping kit goat anti mouse antibody for IgG1, IgG2a, IgG2b, IgG3, IgM and mouse anti goat HRP as secondary antibody(Sigma Co.)

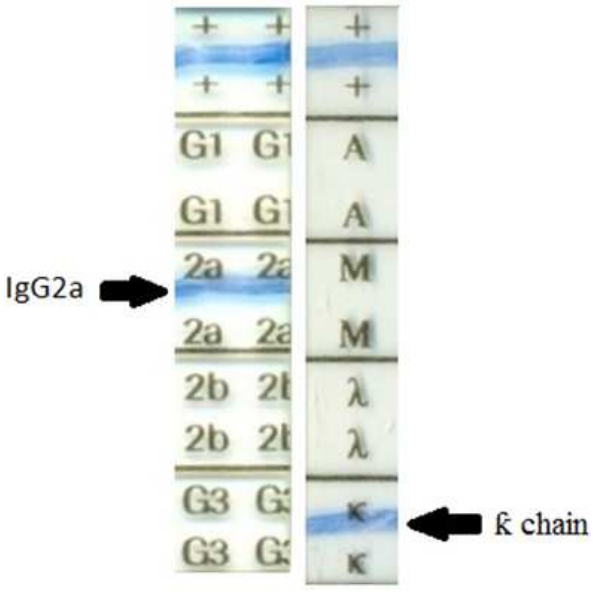

Fig. 3. Isotype identification of monoclonal antibody against rOMPw with iso strip kit (Produced antibody is IgG2a/κ)

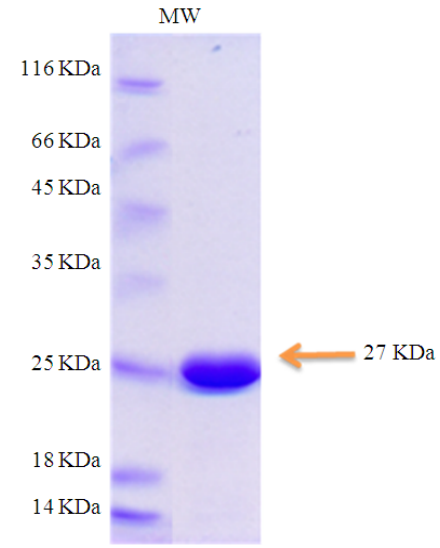

Fig. 4. SDS-PAGE determination of Ag. Recombinant OMPw was separated using 10% SDS-PAGE gel by electrophoreses system at 50v for 3h. The separated bands were stained with coomassie brilliant blue R.250 (Sigma). It showed a band corresponding to 27 KDa

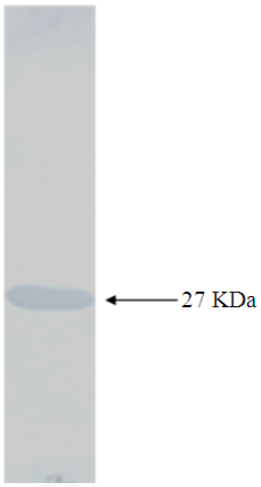

Fig. 5. Western blot determination of mouse purified monoclonal antibody. The separated bands of SDS-PAGE were transferred onto nitrocellulose membranes for 1 h at 100v or at 15v for over night using a Trans-blot apparatus (Bio Rad). After that nitrocellulose membrane were incubated with 5% BSA (sigma) for 30 min at room temperature, treated with 1/100 purified mouse monoclonal antibodies and incubated for 1h. Then, Horseradish Peroxidase-labeled (HRP) goat anti mouse IgG antibody (Sigma) was added and incubation was continued for further 1h and specific bands were visualized by respective substrate in usual manner. Low and high molecular weight markers standard were developed using coomassie brilliant blue R.250 staining

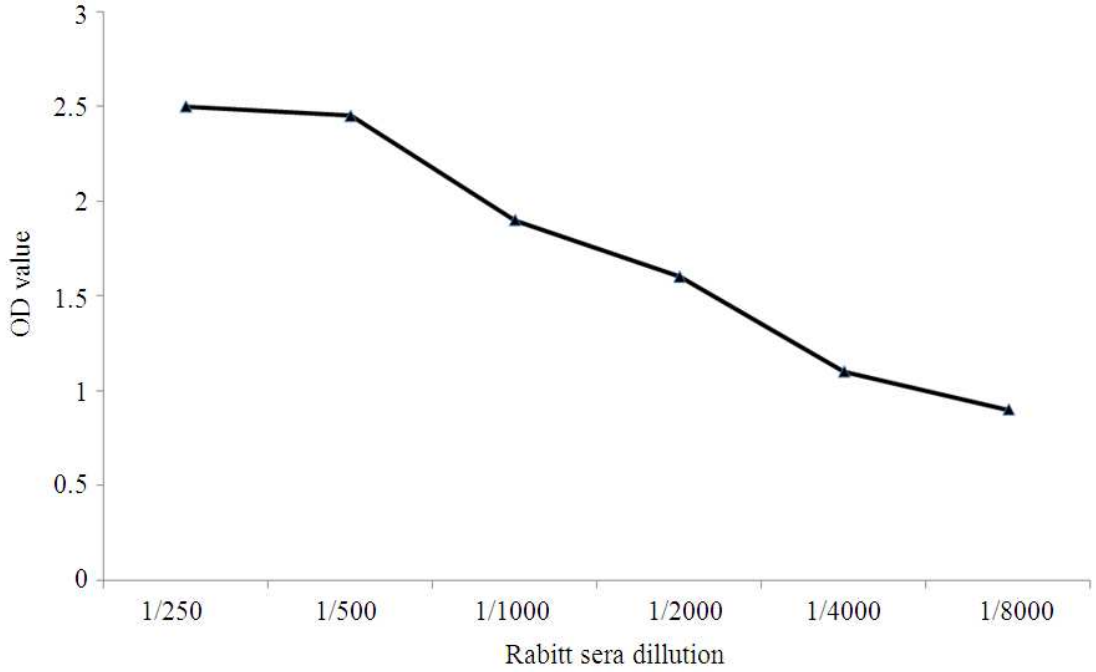

Fig. 6. A serial dilution of immunized rabbit serum were added to rOMPw pre-coated 96 wells plate and titration of specific antibody was assayed by ELISA method using goat anti rabbit antibody conjugated to HRP as secondary antibody (Sigma Co.)

3.3. Rabbit Anti Recombinant OMPw Polyclonal Antibody

As shown in **Fig. 6** rabbit polyclonal antibodies showed a high absorbance values in ELISA at high dilutions between 1:250 and 1:8000 with recombinant OMPw antigen.

3.4. Identification of *V. Cholera* and other Bacteria Using Agglutination Test

The calculation results of agglutination value of both monoclonal and polyclonal antibodies with *V. cholera* and other related bacteria showed that antibodies have been able to agglutinate specific *bacteria*. Therefore, it means that there has been a reaction between anti recombinant OMPw antibodies with relative molecule on the surface of specific bacteria.

4. DISCUSSION

The purpose of this study was to check out if anti recombinant OMPw antibodies could use as diagnostic for detection of *V.cholerae*. Our results demonstrated that antibodies recognized specific recombinant OMPw both in ELISA and immunoblat technique. Nevertheless, our antibodies reacted with whole *V.Cholerae* in agglutination test but not with relative *bacteria* demonstrating the specificity of anti rOMPw for detection of *V.Cholerae.*

While, there is similarity and differences between our work to those of others, who immunized their mice with whole *bacteria* for having hybridoma generation (Pengsuk *et al*., 2010), but all of them give the idea of possible use of monoclonal antibodies as diagnostic tools in *V.Cholerae*. They claimed to produce mouse monoclonal antibodies against *V.Cholerae*, then conjugated to gold nanoparticle and used it as immunochromatic strip test for direct detection of *V. cholerae* O139 in seafood samples. Relative to above study, Pengsuk *et al*. (2013) also developed a strip test for the detection of *V.Cholerae* O139 using monoclonal antibodies which specifically bind to the lipopolysaccharide and capsular polysaccharide of *V. cholerae* O139. While in this work we used recombinant OMPw for both immunization of mice and also screening of hybridoma cells for having monoclonal antibodies. In contrast to our work Pengsuk *et al*. (2010) immunized their mice with five isolates of *V. cholerae* (VC 1, 5, 9, 13 and 14) fixed in 4% Formaldehyde.

They also reported that the initial screening was done by dot blotting against a mixture of the five isolates of *V.cholerae* used for immunization. The interesting point is that, they screened their positive cultures by dot blotting and western immunoassay against each of the 5 isolates of *V. cholerae* that were used for the immunization of mice before cell fusion (Pengsuk *et al*., 2011).

In order to determine whether the antigen which recognized by each monoclonal antibodies in their studies, was a protein or Lipopolysaccharide (LPS), they incubated the used nitrocellulose strip in proteinase K (Roche, Basel, Switzerland) at 1 unit mL^{-1} in Tris/HCl buffer (20 mM, pH8) containing 1 mM CaCl2 for 1 h at room temperature before subjected to monoclonal antibody. In our experiment anti recombinant OMPw monoclonal and polyclonal antibodies reacted with whole *V. Cholerae* but not other related *bacteria* demonstrating the possibility of using anti rOMPw antibodies as a diagnostic tool for detection of *V. Cholerae*.

Our work is also different to those of other scientists who produced anti *V.Cholerae* monoclonal antibodies (Chaivisuthangkura *et al*., 2013; Pengsuk *et al*., 2013). They used two monoclonal antibodies specific to the lipopolysaccharides of *Vibrio cholerae* O1 Inaba and Ogawa serogroups in order to make an immunochromatographic strip test for direct detection of *Vibrio cholerae* O1.

However, in their experiments, they found that detection sensitivities is depended on the material of the other bacteria that could be involved in the growth inhibition of *V. Cholerae* during pre-enrichment step.

In contrast to antibody diagnostic, a multiplex-PCR assay has been developed by (Cherly *et al*., 2007; Hossain *et al*., 2013) for the detection of V. cholera through simultaneous amplification of genes.

5. CONCLUSION

Our results indicate that anti rOMPw antibodies showed reactivity against whole *V. cholerae* in agglutination test performed in this study. It showed no cross reaction with *Salmonella*, *E.coli* and *Shigella*. However, to further explore reactivity of these antibodies against *V. cholerae* in immunochromatic strip, more studies are suggested. This may elaborate possibility of using the anti rOMPw antibodies in immunochromatic strip test in future.

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6.1. Conflict of Interest:

The researchers have no conflicts of interest to declare.

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