

Using recombinant Chlamydia Major Outer Membrane Protein (MOMP) in ELISA diagnostic Kit

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Abstract

Chlamydia trachomatis is one of the main causes of Sexually Transmitted Diseases (STD_s) such as prostatic and epididymitis in men and cervicitis, endometriosis, vaginitis and ureogenital tract infections in women. Serological tests with sensitivities related to specific antigens are commonly used as routine laboratory tests for diagnosis of *Chlamydia*. In this research the *Chlamydia* Major Outer Membrane Protein gene was cloned in order to prepare a specific recombinant protein for use in the ELISA diagnostic kit. DNA was extracted from cultured *C. trachomatis*. PCR reaction was carried out and the resulting PCR product was cloned into the pGemex-1 expression vector and induced by IPTG (Isopropyl β-D-Thiogalactopyranoside). Recombinant protein was confirmed by gel diffusion, dot blot and western blot, using patient's serum. The use of recombinant protein for diagnosis of *Chlamydia* by ELISA is therefore recommended.

Keywords: *Chlamydia trachomatis*; recombinant MOMP protein; Expression.

INTRODUCTION

Chlamydia trachomatis is one of the major causes of sexually transmitted diseases (STDs). It has also been shown to be associated with ocular, neurological and urogenital diseases (Hausler, 1998). Trachoma is endemic in the Middle East, North Africa, and India. An estimated 400-500 million people world wide are

infected with the serovar associated with trachoma, and 7-9 million blinded as a result (Mandell, 2001). So recognition and exact identification of the infection are very important. Detection of this organism by multiplication in a wide range of animal derived cell lines or the chick embryo yolk sack takes a long time and thus it is impossible to implement them as routine diagnostic methods. So the molecular and serological tests using specific recombinant antigens are the best diagnostic procedures (Hausler, 1998; Mandell, 2001).

Chlamydia is a spherical or ovoid obligatory intracellular bacterium that undergoes a characteristic and well defined dimorphic life cycle within eukaryotic host cells. The infective form is the elementary body (EB), 200-300 nm in diameter, which survives outside the host. EB form develops within the host cell into the intracellular replicative form known as the reticulate body (RB), which is 600-1000 nm in diameter (Balous and Dwerden, 1998).

The dominant antigen at the surface of the infectious *Chlamydia* EB is the MOMP, encoded by the *omp1* gene, which was identified simultaneously by three independent groups in 1981 (Caldwell *et al.*, 1981). This antigen of approximately 40 KDa is the basis for the serological classification of *C. trachomatis* into 15 or more serotypes (Hoelzle *et al.*, 2004). In approaches involving the detection of *Chlamydia* antigens in clinical specimens, such as direct immunofluorescence staining (DFA) with fluorescein-conjugated monoclonal antibodies and enzyme-linked immunosorbent assays (ELISA), antibodies have been prepared against either the *Chlamydia* MOMP or the cell wall Lipopolysaccharide (Mandell, 2001).

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The ELISA test with a sensitivity of 80% and a specificity of 91% is the best approach for detection of *Chlamydia* as reported by Mygind *et al.* (2000). In this study a molecular genetic approach was used to generate and evaluate recombinant antigens for use in a species-specific *C. trachomatis* immunoassay. A fragment of the MOMP of *C. trachomatis* was produced as a species specific recognition antigen by ELISA.

MATERIALS AND METHODS

Sample and DNA extraction: The *C. trachomatis* cell culture was a gift from Dr. Badami, Tehran University of Medical Sciences, and was used in the following DNA extraction procedure. Briefly, the cell culture containing chlamydia trachomatis was incubated in lysis buffer (320 mM sucrose, 10 mM Tris base, 5 mM MgCl₂, 1% SDS, 40 µg/ml of proteinase K) for 4h, boiled for 15 min. and centrifuged at 10000 ×g for 10 min. The supernatant was transferred to a new micro-tube and used for the PCR reaction.

Primer design: Specific primers were designed based on the gene sequence of the *Chlamydia* MOMP (1183 bps) containing start (ATG) and termination codons. We designed *SacI* and *BamHI* restriction sites at the 5' end of forward and reverse primers respectively. Ctmof 5'-GAG CTC ATA TGA AAA AAC TCT TGA AAT CGG-3' and Ctmor 5'-GGA TCC TTA GAA GCG GAA TTG TGC ATT TA-3'.

PCR reaction: The PCR reaction was conducted as follows: 1 × PCR buffer, 0.1mM of dNTPs, 1.5 mM MgCl₂, 0.1 µg of DNA, 20 pmol each of forward and reverse primers and 1 unit of *Taq* DNA polymerase (Cinnagen, Iran). The final volume of the reaction was set to 50 µl by adding distilled water. The reaction was transferred to thermocycler machine for PCR amplification. The PCR cycling program was: denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec and extension at 72°C for 45 sec, and repeated for 30 times. Reaction was settled at 94°C and 72°C for 5 min before and after PCR cycling, respectively (Pherson *et al.*, 2000). The PCR product was electrophoresed on 1% agarose gel (Fermentas), stained by ethidium bromide and the DNA band was observed under a UV transilluminator. PCR product was confirmed by restriction analysis.

Gene cloning: PCR product (*MOMP* gene) was elec-

trophoresed on 1% low melting point (LMP) agarose gel and the DNA band was sliced under long wave UV. DNA was recovered using the DNA purification kit (Fermentas Cat. No. k0513). Recovered PCR product and the *Eco* RV blunt digested pBluescript were 3' tailed with dATP and dTTP respectively using terminal deoxy nucleotidyl transferase (Eun, 1996; Gaastra and Klemm, 1984). PCR product was ligated (Gaastra and Hansen 1984) into pBluescript via the T/A cloning method, transformed into competent cells of the *Escherichia coli* XLI-blue strain (Hanaham, 1983) and dispensed onto LB agar plates containing 100 µg/ml ampicillin. Colonies were screened on agar plates supplemented with X-gal or IPTG to discriminate between recombinant (white) and non-recombinant (blue) forms.

The recombinant plasmid was digested by *SacI* and *BamHI* restriction enzymes, which established on 5' ends of forward and reverse primers respectively, generating a 1183 bp DNA band fragment encoding the *MOMP* gene. Digested plasmid was electrophoresed on 1% LMP agarose gel and the resulting DNA fragment (*MOMP* gene) was sliced and recovered by the DNA purification kit (Fermentas). The purified DNA fragment was sub cloned in to digested PGEMEX1 (Pasteur, Iran) expression vector using *SacI* and *BamHI* restriction enzymes (Fermentas), and transformed in *E. coli* XL1-blue competent cells. Positive colonies containing the plasmid were mass cultured in LB medium. Recombinant plasmid was extracted (Felicciello and Chinali, 1993) and confirmed by restriction analysis.

Gene expression: Expression was performed as described previously (Spiro *et al.*, 1997), but with some modifications. Briefly, the *E. coli* strain JM109 was transformed with the pGemex-1-MOMP plasmid and selected on LB agar containing 50 µg/ml of ampicillin. The transformant was inoculated into 3 ml culture tube containing modified YT medium [1.2% (w/v) bacto trypton, 2.4% (w/v) yeast extract, 0.04% (v/v) glycerol, 1% (w/v) M9 salts] [M9 salts medium contains: 6.4% (w/v) Na₂H₂O₄ · 7H₂O, 1.5% (w/v) KH₂PO₄, 0.025% (w/v) NaCl, 0.05% (w/v) NH₄Cl] (Merk) and allowed to grow at 37°C in a shaker at 160 rpm, over night. The following day, the cultured bacteria were inoculated into a 50 ml flasks containing YT medium and allowed at 37°C in a shaker, at 200 rpm.

Cultures in the logarithmic phase (at OD₆₀₀ of 0.6) were induced for 6 hour with 1 mM IPTG. After induction cells were lysed in 5x sample buffer [100 mM Tris

HCl pH 8, 20% (w/v) glycerol, 4% (w/v) SDS, 2% (w/v) beta-mercaptoethanol, 0.2% (v/v) bromo phenol blue] (sigma) and analyzed on 12% (v/v) SDS-PAGE (Smith, 1984a). The resulting gel was stained with coomassie brilliant blue R-250 (Smith, 1984b). The uninduced control culture was analyzed in parallel.

Protein purification: Purification was performed as described previously (Spiro *et al.*, 1997), but with some modifications. Briefly, transformant from LB agar plates were used for preparing the pre inoculation in modified YT medium containing 50 µg/ml ampicillin. The cells were grown at 37°C to OD₆₀₀ of 0.6-0.8, followed by IPTG (1mM) induction for 6 h at 37°C. After centrifugation at 6500 rpm for 10 min. the cell pellet was suspended in 50 ml of equilibration buffer (50 mM Tris, 0.5 M NaCl) containing protease inhibitor cocktail (Sigma). The cell suspension was sonicated (2 × 30 S) on ice. The cells were harvested by centrifugation at 4000 ×g for 15 min, then resuspended in 5 ml of ice-cold buffer containing 6M urea and incubated on ice for one hour. The insoluble material was removed by centrifugation at 12000 ×g for 20 min. The supernatant was filtered through a 0.45 µm membrane before binding to the resin. The recombinant protein was purified on its N-terminal T7 Tag by affinity chromatography (Jack, 1998). The column was equilibrated with 15 ml of Bind Wash buffer (42.9 mM Na₂HPO₄, 14.7 mM KH₂PO₄, 27 mM KCl, 1.37 mM NaCl, 1% tween 20 and 0.02% (v/v) Na₃N). The filtered supernatant was dialyzed in order to remove urea and then applied to the column and allowed to bind during which the flow rate was 15 drops/min. The bound protein was eluted by Elution buffer (1M citric acid, pH 2). The eluted protein fraction was neutralized using 1.5% neutralization buffer (2M Tris base, pH 10.4). The sample was dialyzed overnight in 500 ml of bind/wash buffer that was replaced four times over a period of 24h. Dot blot analysis was carried out using T7-Tag monoclonal Ab or patients' serum and Horse Radish Peroxidase (HRP) conjugated goat anti-mouse IgG to estimate the expressed protein in *E. coli* cells collected 6 h post IPTG induction (Novagen Company Kit No. 69025-3).

Western blotting: Purified recombinant protein was electrophoresed on SDS-PAGE, transferred electrophoretically to nitrocellulose sheet and detected by colorimetric method. Briefly, nitrocellulose sheet was immersed in 3% bovine serum albumin in Tris-buffered saline, 0.1% Tween 20 at room temperature

for 1 h to block excess protein-binding sites. The nitrocellulose sheet was reacted with a human serum infected *Chlamydia* at room temperature and washed in TBS 0.1% Tween 20. Immune reactions were identified with goat anti-human immunoglobulin conjugated to horseradish peroxidase. Color development was observed by addition of 4-chloro-1-naphthol as substrate (Campbell *et al.*, 2001).

Dot blotting: Induced cells were lysed and dot blotted on nitrocellulose membrane (Shewry, 1998). Nitrocellulose membrane blot was exposed to the primary antibody (*Chlamydia* infected human serum) followed by the secondary antibody (human anti IgG peroxidase conjugated) and detected as described in previous section.

Applying of recombinant protein for ELISA: Polystyrene microtiter plates were coated with purified recombinant protein as described (Bora *et al.*, 2002). To exchange the chromatography elution buffer and to remove any urea, the purified recombinant protein was dialysed for 2h at 4°C with carbonate/bicarbonate sodium buffer (0.1 M, pH 9.5). The absorbance dialysed protein (OD=1.6) was diluted up to 20-fold to reach to OD= 0.07 (as determined by antigen serial dilution testing to obtain the optimum amount of antigen to be coated onto the Nunc Maxcisorb flat 96-well plates). After coating on the plates and incubating overnight at 4°C. ELISA procedure was carried out with OPD (Dako) +H₂O₂ as substrate. This project had 11 negative and 6 positive volunteers (confirmed by the Viro-Immun Anti- *C. trachomatis*-IgG Kit) as control which were diluted up to 1:40 and 100 µl added to each well. The OD of the reaction was measured at 492 nm (620 nm reference filter) with a Tekan ELISA reader. The mean value of the negative serum was 0.75 and all positive samples were higher than mean discriminating the positive serums from negative ones.

RESULTS

Chlamydia MOMP gene obtained from cultured *C. trachomatis* was amplified using specific primers. PCR product was tested by restriction analysis. Figure 1 illustrates the PCR product.

Recombinant plasmid (pGemex-1-momp) was transformed in JM109 *E. Coli* and was induced using 1 mM IPTG. Bacterial samples were collected before induction and at 3h intervals after induction and confirmed by SDS-PAGE, gel diffusion, dot blot and

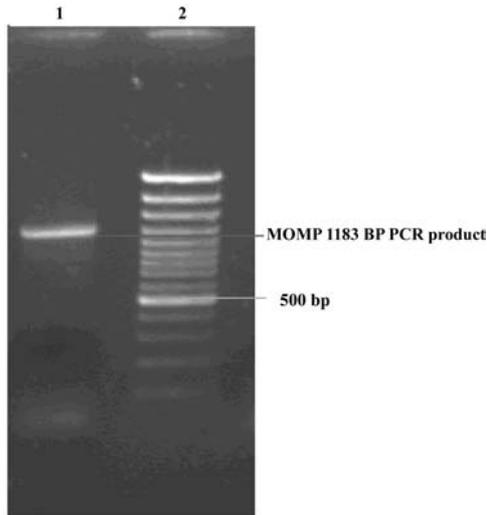


Figure 1. 1% gel electrophoresis. Lane 1: MOMP PCR product. Lane 2: 100 bp DNA ladder marker.

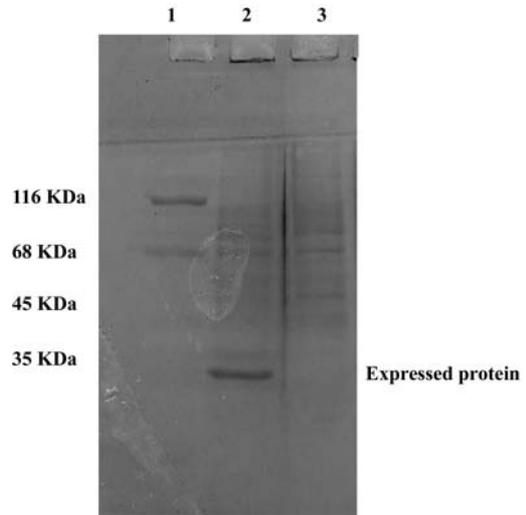


Figure 2. SDS-PAGE of induced and uninduced bacteria. Lane 1: Protein size marker. Lane 2 : Induced bacteria (expressed protein). Lane 3: Uninduced bacteria.

western blot analysis. Protein expression was optimized at 6h after induction. Figure 2 shows expressed protein on SDS-PAGE, Figures 3 and 4 show the results of dot blotting and gel diffusion confirmatory tests, respectively. Western blot analysis of purified protein is shown in Figure 5. In this study, the cloning, expression and purification conditions of the 39 KDa protein belonging to the *C. trachomatis* major outer membrane protein were accomplished. ELISA test: The purified protein was coated onto Nunc Maxcisorb flat 96-well plates and tested by ELISA. We compared 11 negative and 6 positive women sera who were confirmed by PCR and reconfirmed by Medoc kit (specific diagnostic kit based on a synthetic peptide of an immunodominant region of MOMP) at the 1/40 dilution, the results were significantly higher for positives. The PCR product of *C. trachomatis* major outer membrane protein gene was sequenced and deposited to Gene Bank under accession number: EF363779.

DISCUSSION

The organism, *C. trachomatis*, most clearly associated with non gonococcal urethritis (NGU) is an obligate intracellular parasite that causes as many as 50% of cases of NGU. Also trachoma, one of the leading causes of blindness in the world is caused by *C. trachomatis*. Urogenital tract infections are very common among sexually active people, partly due to *C. trachomatis* causing 30-50% of NGU and lymphogranuloma venereum (LGV). *C. trachomatis* is also responsible for non-genital diseases such as trachoma and keratoconjunctivitis which are major problems in societies with low hygiene.

The specific way of bacterial detection is sample inoculation into yolk sack of 7-8 day old chicken embryo (Hausler, 1998 and Mandell, 2001). *Chlamydia* can also be grown in a wide range of animal derived cell lines (McCoy, Hella 229, BAMK and SHK2), but this is not applicable since it requires

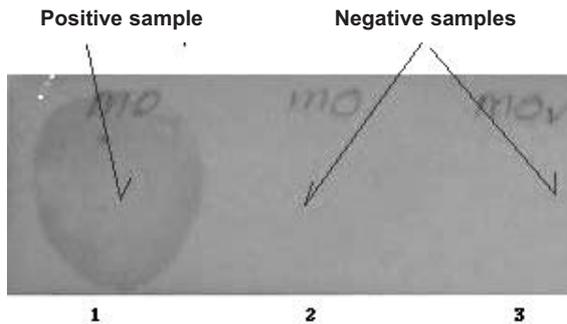


Figure 3. Dot blot analysis of recombinant MOMP protein. Dot 1: Positive sample. Dot 2 and 3: Negative sample.

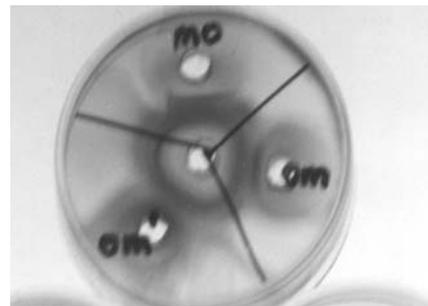


Figure 4. Agarose gel diffusion of purified protein against of patient's serum

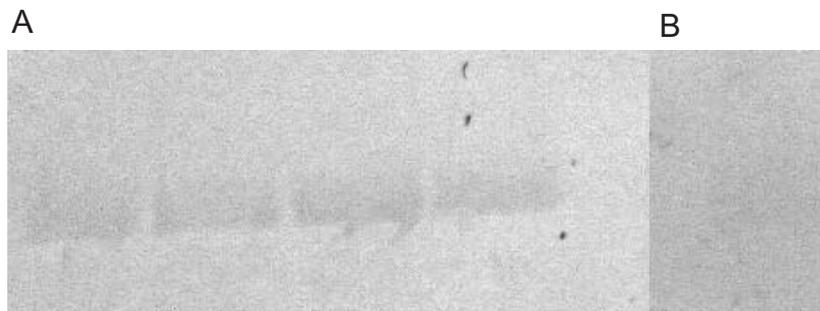


Figure 5. Western blot analysis of recombinant chlamydia MOMP protein using patient serum as primary antibody and human anti IgG conjugate as secondary antibody. A: Purified protein and B: cell lysate.

experience, talent and is very time consuming (Campbell *et al.*, 2001a; Bas *et al.*, 2001; Gdoura *et al.*, 2001; Bas *et al.*, 2001b). Immunologic tests are now of more use, taking advantage of recombinant technology to produce specific bacterial proteins.

Bas *et al.* (2001a) presented the MOMP protein as a sensitive and specific antigen to detect the antibacterial antibodies. Gdoura *et al.* (2001) used the recombinant proteins in as ELISA to detect *Chlamydia* in seminal fluid. They compared the detection results of chlamydial infection by ELISA with the results obtained by PCR and cell culture. In this study the MOMP gene of *C. trachomatis* was cloned and expressed. Because the serological methods are preferred for detection of chlamydial infection. So we designed a diagnostic kit rely on species specific antigen. This study showed that the recombinant Chlamydia MOMP protein is suitable for differentiating completely between positive and negative sera by ELISA method.

CONCLUSION

The biologically active recombinant major outer membrane protein of *Chlamydia trachomatis* expressed in *E. coli* is useful in detection of species specific antigen in immunoassays.

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References

Bas S, Muzzin P, Ninet B, Bornand JE, Scieux C, Vischer TL (2001b). Chlamydial serology: comparative diagnostic value

of immunoblotting, microimmunofluorescence test, and immunoassays using different recombinant proteins as antigens. *J Clin Microbiol.* 39: 1368-1377.

Bas S, Muzzin P, Vischer TL (2001a). *Chlamydia trachomatis* Serology: Diagnostic value of outer membrane protein 2 compared with that of other antigens. *J Clin Microbiol.* 39: 4082-4085.

Balous A, Dwerden BI (1998). *Topley and Wilson's Systematic Bacteriology*, Vol. 2. P: 1331-1334.

Bora U, Chugh L, Nahar P (2002). Covalent immobilization of proteins onto photoactivated polystyrene microtiter plates for enzyme-linked immunosorbent assay procedures. *J Immunol Method.* 268: 171- 177.

Campbell LA, Roberts S, Inoue S, Kong L, Kuo Cc CC (2001). Evaluation of Chlamydia pneumoniae 43- and 53-kilodalton recombinant proteins for serodiagnosis by Western Blot. *Clin Diagn Lab Immunol.* 8: 1231-1233.

Caldwell HD, Kromhout J, Schachter J (1981). Purification and partial characterization of the major outer membrane protein of *Chlamydia trachomatis*. *Infect Immun.* 31: 1161-1176.

Eun HM (1996). *Enzymology primer for Recombinant DNA Technology*. Academic Press. Chapter 6. DNA polymerases. P: 345-489.

Feliciello I, Chinali G (1993). A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherichia coli*. *Anal Biochem.* 212: 394-401.

Gaastra W, Klemm P (1984). Radiolabeling of DNA with 3' terminal transferase. In: *Methods In Molecular Biology*. Vol. 2. Nucleic Acids. Edited by John M Walker. Chapter 40. P: 269-271.

Gaastra W, Hansen K (1984). Ligation of DNA with T4 DNA ligase. In: *Methods In Molecular Biology*. Vol. 2. Nucleic Acids. Edited by Walker JM. Humana Press. Chapter 32. P: 225-230.

Gdoura R, Daoudi F, Bouzid F, Ben Salah F, Chaigneau C, Sueur JM, Eb F, Rekik S, Hammami A, Orfila J (2001). Detection of *Chlamydia trachomatis* in semen and urethral specimens from male members of infertile couples in Tunisia. *Eur J Contracept Reprod Health Care.* 6: 14-20.

Hanaham D (1983). Studies on transformation on *E. coli* with plasmids. *J Mol Biol.* 98: 503-517

Hausler WJ, Sussman M (1998). *Tapley and Wilson Bacterial Infection*. 9th edition, Axford University Press, Inc; New York, P: 977-991.

- Hoelzle LE, Hoelzle K, Wittenbrink MM (2004). Recombinant major outer membrane protein (MOMP) of *Chlamydomphila abortus*, *Chlamydomphila pecorum* and *Chlamydia suis* as antigens to distinguish chlamydial species-specific antibodies in animal sera. *Vet Microbiol.* 103: 85-90.
- Jack GW (1998). Affinity chromatography. In: *Molecular Biomethods Handbook*. R Rapley, J M Walker. Humana Press. P: 469-477.
- Mandell, Douglas, Bennett (2001). *Principales and practice of Infection Disease*. Section 8. P: 1424-1434.
- Mygind P, Christiansen G, person K, Birkelund S (2000). Detection of *Chlamydia trachomatis* specific antibodies in human sera by recombinant major outer-membrane protein polyantigens. *J Med Microbiol.* 49: 457-65.
- Novagen Company Catalogue (2005). T7 Tag affinity purification Kit NO. 69025-3
- Pherson Mc, Moller SG (2000). *PCR*. Bios Scientific publishers. Chapter 2. Undrestanding PCR. P: 9-21.
- Shewry PR, Fido RJ (1998). Protein blotting, principels and applications. In: *Molecular Biomethods Handbook*. Edited by Ralph Rapley, John M Walker. Humana Press. P: 435-444.
- Smith BJ (1984a). SDS polyacrylamide gel electrophoresis of proteins. In: *Methods In Molecular Biology*. Vol. 1. Proteins. Edited by John M Walker. Chapter 6. P: 41-56.
- Smith BJ (1984b). Acetic Acid-Urea polyacrylamide gel electrophoresis of proteins. In: *Methods In Molecular Biology*. Vol. 1. Proteins. Edited by John M walker. P: 63-73.
- Spiro MJ, Bhoyroo VD, Spiro RG (1997). Molecular cloning and expression of rat liver endo-alfa mannosidase, an N-linked oligosaccharide processing enzyme. *J Biol Chemistry.* 272: 29356-29363.