Sindh Univ. Res. Jour. (Sci. Ser.) Vol.45 (2) 233-238 (2013)

SINDH UNIVERSITY RESEARCH JOURNAL (SCIENCE SERIES)

Molecular and Immunological Characterization of Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH-1) of *Neisseria meningitidis* serogroup B strain MC58

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Received 27th March 2013 and Revised 16th June 2013

Abstract: The present study focuses on characterizing the putative roles of GapA-1with aim to determine the additional non glycolytic roles of the enzyme in vaccine development and pathogenesis of *N. meningitidis*. To achieve the goals, a *N. meningitidis* GapA-1 isogenic knock-out mutant constructed previously was used to serve as a negative control in characterisation experiments. Sub-cellular localisation of GapA-1 was investigated by using cell fractionation method. The DNA fragment coding for GapA1 gene was amplified from a panel of 18 meningococcal clinical isolates by PCR. Strains yielding positive results for GapA-1 gene by PCR were further investigated for the expression of GapA-1 gene by western blot analysis using rabbit polyclonal antiserum (RαGAPDH-1). Furthermore, to assess the vaccine potential of GapA-1, *in vitro* serum bactericidal assay (BSA) was conducted using polyclonal RαGAPDH-1 antiserum. The gene encoding GapA1 was present in all strains and also expressed under *in vitro* conditions tested. However, GAPDH-1 antiserum raised against denatured purified protein failed to kill meningococci *in vitro* BSA experiments. In summary, GapA-1, is well conserved, constitutively expressed protein and localized to both cytoplasm and to the cell surface of *Neisseria meningitidis* serogroup B strain MC-58.

Keywords: *N. meningitis*, GAPDH-1, Serum bactericidal assay, Surface localization.

1. INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.1.2) is a classical cytoplasmic protein and also reported to be expressed on the surface of several microbial species including bacteria and fungi, and may act as virulence factor, thus, contributing to the mechanisms of pathogenesis (Egea *et al.* 2007). The surface localisation of GAPDH was first described by two research groups simultaneously in *Streptococcus pyogenes*, a Gram-positive pathogen (Pancholi and Chhatwal 2003). Subsequently, GAPDH of Group A streptococci was reported to act as plasmin receptor protein (Winram and Lottenberg 1996), and the GAPDH of another pathogen of same family Serotype III group B streptococci was shown to be exported to the surface of the cell (Seifert *et al.* 2003). In addition, extracellular localisation of GAPDH has been first time reported on the surface of Gram-negative *E. coli*, where it has been shown to bind with human plasminogen and fibrinogen, a function other than its glycolytic role (Egea *et al.* 2007). Several fungi may also secret the GAPDH on their surface, where it has been shown to be highly immunogenic and, thus, could be a target for vaccine candidate. GAPDH of *Mycoplasma bovis* gene has also been reported to induce an immune response in infected cattle (Perez-Casal and Prysliak 2007). *N. lactamica* and *N. meningitidis* following contact with host cell may undergo surface remodelling with increase in the expression level of the *gap* gene and accumulation of GAPDH on the surface has been confirmed by flow cytometry analysis (Grifantini *et al.* 2002). Moreover, some other cytosolic proteins: Peroxiredoxin, DnaK, and Enolase, of *N. meningitidis* serogroup B, which are usually intracellular proteins, have recently been reported to be exported to the surface of bacterium and act as plasminogen receptors (Knaust *et al.* 2007). The SBA assay has been widely used to measure the functional ability of antibodies raised against surface exposed antigens to cause the killing of bacterial cells (e.g., *N. meningitidis*). Bactericidal antibodies play key role in defence against meningoccoal disease. These antibodies specific for bacterial antigens (e.g., PorA, capsular polysaccharide, and other surface-exposed immunogenic proteins) binds to surface antigens of *N. meningitidis* cells. This interaction also activates complement that leads to killing of the *N. meningitidis*. In view of the potential multifunctional roles of GAPDH-from other bacteria and fungi, the GAPDH protein found on the meningococcal surface may act as virulence factor and play a role in the pathogenesis of the meningococcal disease (Egea *et al.* 2007).

The aims of the current research study were to investigate the sub-cellular localization, to study the prevalence of GapA-1 gene in clinical isolates of known multi locus sequence type (MLST) type *N. meningitidis* strains and assessment of the vaccine potential of recombinant GapA-1 by *in vitro* serum bactericidal assay.

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2. MATERIALS AND METHODS

1.2 DNA extraction, quantification and analysis

Chromosomal DNA was prepared by a commercial DNeasy Blood & Tissue kit (QIAGEN, UK) following the manufacturer's guidelines. Purified chromosomal DNA was quantified by NanoDrop spectrophotometer (ND-1000 [Agilent Technologies, UK]). PCR products were analyzed on 1% agarose gel electrophoresis. DNA ladder of 2-Log (New England BioLabs) were loaded to estimate the size of DNA fragment in the test samples. Agarose gels were visualized using Uvitec gel documentation.

2.2 Bacterial strains, growth conditions, and culture media

All *N. meningitidis* strains, except strain MC58, were clinical isolates of different serogroups and serotypes (Table 1). Growth media used for Meningococcal strains included Brain Heart Infusion agar (BHIA), Brain Heart Infusion Broth (BHII), (Oxoid, UK) Dulbecco's Modified Eagle Medium [(DMEM) Invitrogen], where appropriate Vitox (Oxoid) and Fetal calf serum (FCS, [Invitrogen]) was added as per manufacturers recommendations . Antibiotics were added to media at the concentrations such as: Kanamycin 50µg/ml and erythromycin 5µg/ml for isolation of mutant meningococcal strains. Culture plates were incubated at 37 °C in an atmosphere of 5% CO_2.

2.3 Cell fractionation methodology of *Neisseria meningitidis*

Overnight cultures of *N. meningitidis* were used for cell fractionation. Method of Nossal and Heppel (Nossal and Heppel 1966) was used to extract periplasmic proteins. Briefly, 100 ml bacterial culture was harvested for 2 min at 13000 × *g* and resulting cell pellet was re-suspended in 1 ml of EB buffer (10 mM $MgCl_2$, 25% sucrose, 10 mM Tris-HCl pH 7.5). The cell pellet was washed twice in the same buffer and kept on ice for 10 min. The suspension was then centrifuged for 4 min at 13000 × *g*, and the resultant pellet was dissolved in 0.4 ml of ice cold water and incubated on ice for 10 min. Following incubation, the suspension was harvested at 13000 × *g* for 2 min. The supernatant consisting of periplasmic proteins was placed in a new eppendorf and stored at -20°C. After collection of the periplasmic contents, the remaining pellet containing spheroplasts was dissolved in 0.4 ml Tris-HCl (pH 7.5), mixed and sonicated to liberate the cytoplasmic contents. Suspension was then centrifugation at 5000 × *g* for 1 min to remove non-disrupted cells. The clear supernatant was put into to a new eppendorf and centrifuged at 17000 × *g* for 30 min. The supernatant representing the cytoplasmic protein was collected stored at -20°C. The remainder pellet was dissolved in 0.2 ml buffer (containing 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5 and 0.2 ml of 10 mM $MgCl_2$, 10 mM Tris-HCl pH7.5, and 4% Triton X-100 and incubated at 37°C for 30 min and then harvested at 17000 × *g* for 30 min. The supernatant representing cytoplasmic membrane contents was transferred to a new eppendorf and stored at -20°C. The final pellet (yielded after collection of the cytoplasmic membrane contents) was deemed the outer membrane protein-enriched fraction. Thus, the resulting pellet was re-suspended in 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5 by brief sonication and then 0.5 ml of 10 mM $MgCl_2$, 10 mM Tris-HCl pH7.5 and 2% Triton X-100 was added. The suspension was allowed at 37°C for 30 min and then centrifuged at 17000 × *g* for 30 min. This step was repeated to remove any remaining soluble proteins to ensure the purity of fraction. The final pellet was mixed in 0.2 ml of 10 mM Tris-HCl pH 7.5 and stored at -20°C.

2.4 PCR screening for GapA-1 sequences in clinical isolates of *N. meningitidis*

PCR was carried out essentially as described previously (Tunio *et al.* 2012d). Unless otherwise stated, all PCR reactions were carried out using a set of primers (GapA-1(F) 5'-CGCGGATCCCCCCG AAAATCTACCCAACCC-3' and GapA-1(R) 5'-CG CGGATCCGAAAACATCGGGTTTCAGATGGC-3'). The PCR conditions were set as following: initial denaturation for 3 min at 95°C, and then 30 cycles of incubation each consisting of annealing at 48°C for 1 min, extension at 68°C for 3.5 min, and 94°C for 45s and final incubation 68°C for 10 min.

2.5 Preparation of whole cell proteins for western blot analysis

Whole cell extracts of all clinical isolates of *N. meningitidis* were prepared as described previously (Tunio *et al.* 2012b). Briefly, overnight culture of *N. meningitidis* was harvested by centrifugation at 13000 × *g* for 10 and the resulting cell pellet was re-suspended in 1× SDS-PAGE sample buffer. The cell lysate was then briefly sonicated using a 10s on and 10s off pulses to achieve a homogenous suspension of the culture. All samples, before analysis on SD-PAGE, were boiled for 5 min and then mixed thoroughly by brief vortex.

2.6 SDS-PAGE and Western blot analysis

SDS-PAGE analysis and subsequent western blot analysis of whole cell extracts were carried out as described previously (Tunio *et al.* 2012a). Briefly, proteins from cell lysate following separation by SDS-PAGE were transferred onto a nitrocellulose membrane (BIO-RAD) using a Trans-Blot SD semidry transfer cell (BIO-RAD) according to directions supplied with equipment. Nitrocellulose membrane containing proteins were then probed with primary and secondary

antibodies as described in Tunio *et al.*, (Tunio *et al.* 2012c). The digital images of the membranes were taken using a GS-800 calibrated densitometer (BIO-RAD).

2.7 Serum bactericidal assay

N. meningitidis strain MC58 was grown on chocolate agar overnight. Around ten bacterial colonies transferred into 5 ml Muller Hinton Broth [(MHB), Oxoid, UK] (without supplement) and allowed to grow for two hrs at 37°C in a shaking incubator. The OD_{600} nm was measured and cells were harvested (8000 × *g* for 5 min) from a 500 µl aliquot of the culture. The cells were re-suspended in PBS/bovine serum albumin (BSA) to achieve an OD_{600} of 0.1. The assay was performed in a sterile 96-well tissue culture plate, to which the assay components (antibody, complement, and bacteria) were added sequentially. Each well contained: 20 µl of RαGAPDH-1 antiserum (or pre-immune serum taken from the same rabbit) pre-adsorbed with the cells of strain MC58Δ*gapA-1* (and de-complemented at 56°C for 30 min), 10 µl of bacteria (containing ~ 800 colony-forming-unites per well), and 10 µl of sterile baby rabbit serum (Pel-Freeze, UK) as a source of complement. Sera were used at the final dilution of 1:2, 1:4, 1:8, and 1:16. After the addition of all components to the wells, the plates were covered and incubated for 60 min at 37°C on a microplate shaker (150 rpm). Ten microliters from control wells were inoculated on to chocolate agar at time zero. After 60 min incubation serial dilutions were performed and 10 µl aliquots were inoculated onto chocolate agar. After overnight incubation, the number of colonies at each dilution of RαGAPDH-1 was counted, and the serum bactericidal titre was reported as the reciprocal of the serum dilution yielding ≥ 50% killing of the bacteria. Control wells included: a serum with known bactericidal activity (anti-meningococcal whole cell); PBS-BSA, bacteria and complement; negative control for complement contained: heat inactivated complement, RαGAPDH-1 serum, and bacteria.

Table 1. List of *N. meningitidis* clinical isolates examined for the expression of GapA-1.

Strain	Year of isolation	Disease	Serogroup	Country of origin
Z6420	1992	invasive (unspecified)	B	Greece
Z1035	1967	meningitis and septicaemia	A	Pakistan
Z6414	1994	invasive (unspecified)	C	New Zealand
Z1534	1941	invasive (unspecified)	A	UK
Z6413	1990	invasive (unspecified)	C	South Africa
Z1213	1973	invasive (unspecified)	A	Ghana
Z4678	1985	invasive (unspecified)	B	East Germany
Z4676	1962	invasive (unspecified)	B	Denmark
Z4673	1986	invasive (unspecified)	B	Netherlands
Z4667	1963	invasive (unspecified)	B	Netherlands
Z4181	1989	carrier	C	Mali
Z3842	1976	invasive (unspecified)	B	Norway
Z3771	1987	invasive (unspecified)	A	UK
Z1054	1975	invasive (unspecified)	A	Finland
Z1503	1984	invasive(unspecified)	A	China

3. RESULTS AND DISCUSSION

3.1 Sub-cellular localization of GapA-1 in *N. meningitidis*

GAPDH is a multifunctional protein displayed on the surface of variety of microbial cells. Recently, three more enzymes, which are usually intracellular proteins, from *N. meningitidis* such as Peroxiredoxin, DnaK, and Enolase, have been shown to be found on the outer membrane of *N. meningitidis* (Knaust *et al.* 2007). To investigate the sub-cellular localisation of GAPDH-1 in *N. meningitidis* MC58, a traditional method of cell fractionation was used to prepare cytoplasmic, periplasmic, cell membrane and outer membrane contents of the cell and each fraction was probed with the anti-GAPDH-1 antiserum to identify the sub-cellular localisation of GAPDH-1 in *N. meningitidis*. GAPDH-1 was predominately observed in the cytoplasmic contents and a proportion was also present in the outer membrane fraction, but was detected in the concentrated culture supernatant representing secreted protein **(Fig. 1).** A trace amount, possibly representing transient GAPDH-1 during translocation across the inner membrane, could also be observed in the cytoplasmic membrane contents. GAPDH-1 was absent in cell fractions prepared from MC58Δ*gapA-1*. These results indicate that GAPDH-1 of *N. meningitidis* is mainly a cytoplasmic protein, but is also present in outer membrane fractions.

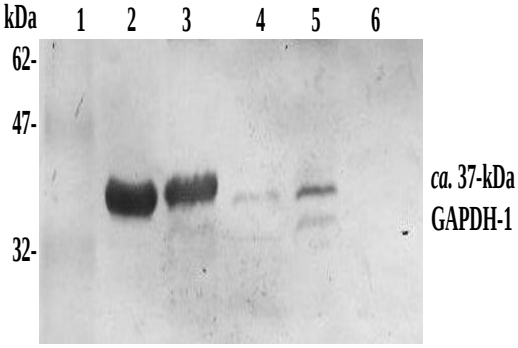

Fig. 1 Western blot analysis showing Sub-cellular localization of GAPDH-1 in *N. meningitidis* MC58-WT by cell fractionation, Lane 1, protein markers, lanes 2-6, periplasmic protein-enriched fraction, cytoplasmic membrane protein fractions, cytoplasmic membrane protein fractions, outer membrane protein fractions, and secreted proteins respectively.

3.2 Prevalence of GapA1 in clinical isolates of *N. meningitidis*

Due to immunogenic nature, surface exposure, and potential roles in pathogenesis, glycolytic enzymes are considered as candidate vaccine antigens against several bacterial and fungal infections (Kim and Dang 2005). To qualify for a vaccine, some of the prerequisites include that the relevant antigen should be present and expressed in majority of target strains. The *gapA-1* gene appears to be up-regulated following meningococcal contact with host cells suggesting that it may be involved in a critical step of pathogenesis. In present study, we examined the presence of *gapA-1* by PCR analysis and its expression using western blotting in population of *N. meningitidis*. A total of 17 strains of *Neisseria meningitidis*, including a single strain *N. gonorrhoeae*, of known MLST-type representing most of the known virulent lineages were tested for the presence of a *gapA-1* homologue by PCR. All meningococcal isolates tested by PCR screening method produced a single fragment of DNA of the expected size *ca*. 1-kb corresponding to GapA-1 **(Fig. 2 A and B).**

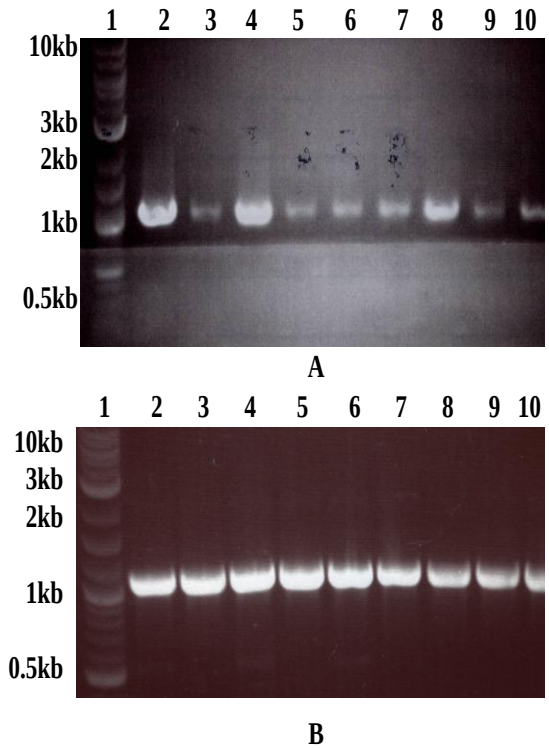

Fig. 2. Agarose gel analysis demonstrating the distribution of the *gapA-1* gene in diverse clinical isolates of *N. meningitidis* (A) Lane 1, DNA markers, lane 2, MC58-WT, lanes 3-10, Z1035, Z1054, Z1213, Z1503, Z4676, Z1534, Z3771, Z3842, respectively. (B) Lane 1, DNA markers, lane 2-10, Z4678, Z6413, Z6414, Z6420, *N. gonorrhoeae* FA1090, Z4181, Z4667, Z4673, Clone 12 (ST-11), respectively. PCR-amplified products of the expected size of *gapA-1* (~1-KB) were observed in all strains examined.

Furthermore, after confirming by PCR that GapA-1 was present across all the meningococcal strains, we extended this analysis to investigate whether the GapA-1 gene is expressed in same set of strains. Whole-cell lysate of the 17 meningococcal isolates **(Table 1)** were examined by western blot analysis using RαGAPDH-1 antiserum to detect the presence of a GAPDH-1. All isolates were found to express an immunoreactive protein band corresponding to GAPDH-1 protein, with the exception of MC58Δ*gapA-1*, confirming that the gene is intact and expressed as a full length protein in all clinical isolates of meningococci **(Fig. 3A, B and C).** In addition, single isolates of *N. gonorrhoeae* strain FA1090 was also examined that showed the protein band corresponding to the GAPDH-1. In spite of the identification of a number of cytoplasmic proteins on the microbial surface, the mechanism(s) of how they are exported to the bacterial surface is yet unclear. Further studies are needed to investigate the export mechanism (s) of such proteins to the surface of bacterial cells.

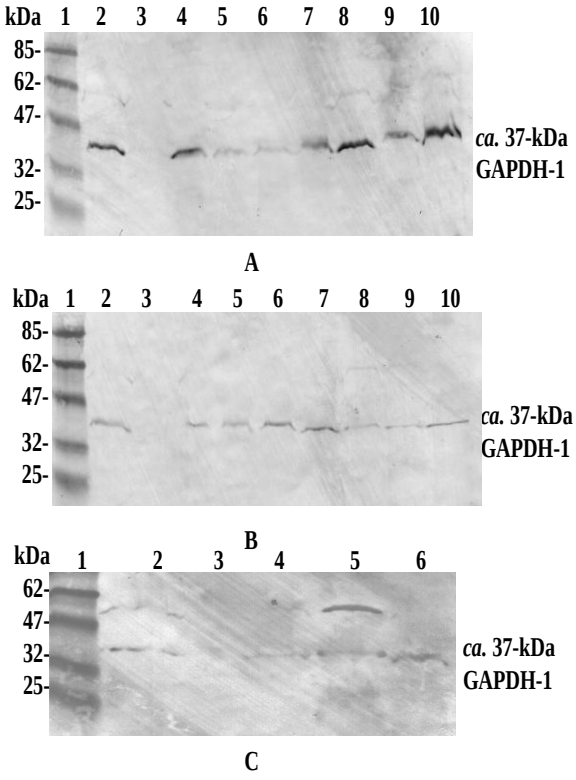

Fig. 3 Western blot analysis showing that the *gapA-1* gene is expressed across divergent clinical isolates of *N. meningitidis.* (A) Lane 1, protein markers, lane 2, MC58-WT, lane 3, MC58Δ*gapA-1*, lanes 4-10, Z1035, Z1054, Z1213, Z1503, Z4676, Z1534, Z3771, respectively (B) Lane 1, protein markers, lane 2, MC58-WT , lane 3, MC58Δ*gapA-1*, lanes 4-10 Z4678, Z6413, Z6414, Z6420, *N. gonorrhoeae* strain FA1090, Z4181, Z4667, respectively (C) Lane 1, protein markers, lane 2, MC58-WT, lane 3, MC58Δ*gapA-1*, lanes 4-6, Z4673, Z3842, Clone 12 (ST-11), respectively.

3.3 Serum bactericidal assay

Currently, the serum bactericidal assay is an important method to measure *in vitro* the functional antibodies against *N. meningitidis*. Antibodies specific to surface antigens specifically kill bacterial cells with the help of complement system which leads to the lysis of bacterium. The production of functional antibodies against recombinant proteins appears to be serious concern in evaluation and development of vaccine candidates' against *N. meningitidis*. We undertook to determine the ability of RαGAPDH-1 antiserum to kill meningococcal cells in presence of complement. Prior to using the serum in bactericidal assays, polyclonal antiserum was pre-adsorbed with MC58Δ*gapA-1* isogenic null mutant strain to remove non-specific antibodies that might result in non-specific killing. The antiserum was used at various dilutions: 1:10, 1:50, and 1:100. Unfortunately, bactericidal activity was not observed with antiserum raised against GAPDH-1. Positive control assays using anti-whole meningococcal serum consistently yielded 100% killing. The reason for absence of bactericidal activity could possibly be due to the fact that the GAPDH-1 antiserum used in the SBA was raised against denatured purified recombinant protein. Numerous studies conducted on recombinant proteins of *N. meningitidis* including PorA and PorB (Wright *et al.* 2002), have reported that generation of bactericidal antibodies depends upon refolding of the protein to exist in native conformation. Hence, antibodies generated against denatured GAPDH-1 protein, were unlikely to recognise surface-exposed, conformational epitopes, which may be important *in vivo*. It is suggested to generate antibodies against natively purified proteins for determining the bactericidal activity in SBA experiments.

4. CONCLUSION

Since last two decades, a number of studies have reported that the cytoplasmic enzymes, including GAPDH and FBA, are located on the surface of a variety of bacterial and fungal species, and can perform various non-biological functions, unconnected to their glycolytic roles (Pancholi and Chhatwal 2003). We have previously shown that FBA, a cytoplasmic enzyme of *N. meningitidis* is also a partially localized to the surface and play important role in optimal adhesion to human cells (Tunio *et al.* 2010). The distribution of GapA-1 gene among clinical isolates of *N. meningitidis* was investigated initially by PCR analysis. The data demonstrated that all isolates tested in this study, possessed full length gene. The PCR positive isolates were subjected to further analysis by western blotting using the GAPDH-1 antiserum to investigate the expression of the gene. The data showed that all isolates expressed GapA-1 demonstrating that GapA-1 is well conserved and constitutively-expressed in *N. meningitidis*. SBAs demonstrated that polyclonal antibodies raised against denatured GAPDH-1 were unable to kill *N. meningitidis* may be due to the fact that the bactericidal assays in this study were carried out by incubating the immune serum and complement with meningococcal cells grown in an enriched broth. In future, the bactericidal assays should be performed using bacteria grown under conditions that mimic *in vivo* conditions and antibodies raised against the native GAPDH-1 protein should be used to assess their serum bactericidal activity. In conclusion, meningococcal GapA-1 is a highly-conserved, constitutively-expressed, outer membrane localized protein.

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