Molecular analysis of two novel *Neisseria gonorrhoeae* virulent components: the macrophage infectivity potentiator and the outer membrane protein A

Stefania Starnino[1], Rosanna Leuzzi[2], Valeria Ghisetti[3*], Maria Antonia De Francesco[4*], Marco Cusini[5*], Giampaolo Impara[6*], Emma Galluppi[7*], Mariagrazia Pizza[2], Paola Stefanelli[1]

[1]Department of Infectious, Parasitic and Immune-mediated Diseases, Istituto Superiore di Sanità Rome-Italy;
[2]Novartis Vaccines and Diagnostics, Siena, Italy;
[3]Laboratory of Microbiology and Virology, Ospedale Amedeo di Savoia, Turin;
[4]Department of Medicina Sperimentale ed Applicata, Università degli Studi di Brescia;
[5]U.O. Dermatologia, Fondazione Policlinico Regina Elena, Milan;
[6]S. Gallicano Dermatologic Institute, IRCCS, Rome;
[7]U.O. of Microbiology, Policlinico S. Orsola-Malpighi, Bologna

**The authors equally contributed to the work*

SUMMARY

Molecular analyses of *mip* and *omp*A genes were performed on 20 *Neisseria gonorrhoeae* isolates. The genes were present with a high degree of conservation in all strains. Sera from patients with urethritis or disseminated gonococcal infections were able to recognize the purified *Neisseria gonorrhoeae* macrophage infectivity potentiator (Ng-MIP) and *Neisseria gonorrhoeae* outer membrane protein A (Ng-OmpA).

KEY WORDS: Neisseria gonorrhoeae, Ng-MIP, Ng-OmpA

Received October 14, 2009 *Accepted* February 3, 2010

Neisseria gonorrhoeae is the causative agent of gonorrhoea, a sexually transmitted disease. This microorganism is characterized by a high degree of genotypic variability particularly referred to its surface structures (Hamilton *et al*., 2006). Even if gonococcal antibodies develop during uncomplicated gonococcal infections, they are not reactive against gonococcal antigens from a different strain without immunological memory (Hedges *et al*., 1999). Moreover, gonorrhoea exhibits antimicrobial resistance to different drugs, limiting the ability to provide effective treatment and thus to control the disease (Lo *et al*., 2008; Tapsall, 2006).

Corresponding author
Dr. Paola Stefanelli
Department of Infectious
Parasitic and Immune-mediated Diseases
Istituto Superiore di Sanità
Viale Regina Elena 299 - 00161 Rome, Italy
E-mail: paola.stefanelli@iss.it

Recently, two proteins named macrophage infectivity potentiator (Ng-MIP) and outer membrane protein A (Ng-OmpA) have been identified as novel virulence factors in gonococci (Leuzzi *et al*., 2005; Serino *et al*., 2007). MIP shows high homology to MIP proteins already described in *Legionella pneumophila* (Cianciotto *et al.*, 1992), *Chlamydia trachomatis* (Lundemose *et al*., 1993) and *Trypanosoma cruzii* (Moro *et al*., 1995). It is a surface-exposed lipoprotein able to promote intracellular survival of *N. gonorrhoeae* in macrophages (Leuzzi *et al*., 2005). An isogenic *mip* knock-out strain is more sensitive to macrophage-mediated killing, suggesting that MIP has a role in the persistence of *N. gonorrhoeae* inside macrophages and, therefore, in gonococcal infection.

OmpA is an outer membrane protein able to promote adhesion and invasion of *N. gonorrhoeae* into epithelial cells, as well as entry and intracellular survival in macrophages (Serino *et al*., 2007).

The OmpA protein from different species (Jeannin *et al*., 2002) induces specific humoral (Zhang *et al*., 1987) and cytotoxic immune responses (Kim *et al*., 2000), also in the absence of an adjuvant. A recombinant OmpA from *Klebsiella pneumoniae* has been demonstrated to interact with human immune cells and particularly with antigen presenting cells via endocytic receptors on host cell surfaces (Jeannin *et al*., 2002). Expression of Ng-MIP and Ng-OmpA has been reported in a number of *N. gonorrhoeae* isolates collected in the USA, Korea and the UK (Leuzzi *et al*., 2005, Serino *et al*., 2007), but the gene sequence conservation of these genes has never been investigated.

The aim of this study was to analyze the presence and conservation of *mip* and *omp*A genes among 20 *N. gonorrhoeae* strains, collected in Italy during the period 2003-2007. The clinical picture of infected patients and microbiological features of strains were used to choose the gonococci examined in this study. The immune recognition of re-

```
G617          -MNTIFKISA LTLSAALALS ACGKKEAAPA SASEPAAASA AQGDTSSIGG 49
G627          -MNTIFKISA LTLSAALALS ACGKKEAAPA SASEPAAASA AQGDTSSIGG
G616          -MNTIFKISA LTLSAALALS ACGKKEAAPA SASEPAAASA AQGDTSSIGS
FA1090        -MNTIFKISA LTLSAALALS ACGKKEAAPA SASEPAAASA AQGDTSSIGS

G617          TMQQASYAMG VDIGRSLKQM KEQGAEIDLK VFTDAMQAVY DGKEIKMTEE 99
G627          TMQQASYAMG VDIGRSLKQM KEQGAEIDLK VFTDAMQAVY DGKEIKMTEE
G616          TMQQASYAMG VDIGRSLKQM KEQGAEIDLK VFTDAMQAVY DGKEIKMTEE
FA1090        TMQQASYAMG VDIGRSLKQM KEQGAEIDLK VFTDAMQAVY DGKEIKMTEE

G617          QAQEVMMKFL QEQQAKAVEK HKADAKANKE KGEAFLKENA AKDGVKTTAS 149
G627          QAQEVMMKFL QEQQAKAVEK HKADAKANKE KGEAFLKENA AKDGVKTTAS
G616          QAQEVMMKFL QEQQAKAVEK HKADAKANKE KGEAFLKENA AKDGVKTTAS
FA1090        QAQEVMMKFL QEQQAKAVEK HKADAKANKE KGEAFLKENA AKDGVKTTAS

                        160
G617          GLQYKITKQG EGKQPTKDDI VTVEYEGRLI DGTVFDSSKA NGGPATFPLS 199
G627          GLQYKITKQG KGKQPTKDDI VTVEYEGRLI DGTVFDSSKA NGGPATFPLS
G616          GLQYKITKQG KGKQPTKDDI VTVEYEGRLI DGTVFDSSKA NGGPATFPLS
FA1090        GLQYKITKQG EGKQPTKDDI VTVEYEGRLI DGTVFDSSKA NGGPATFPLS

G617          QVIPGWTEGV RLLKEGGEAT FYIPSNLAYR EQGAGEKIGP NATLVFDVKL 249
G627          QVIPGWTEGV RLLKEGGEAT FYIPSNLAYR EQGAGEKIGP NATLVFDVKL
G616          QVIPGWTEGV RLLKEGGEAT FYIPSNLAYR EQGAGEKIGP NATLVFDVKL
FA1090        QVIPGWTEGV RLLKEGGEAT FYIPSNLAYR EQGAGEKIGP NATLVFDVKL

G617          VKIGAPENAP AKQRY 264
G627          VKIGAPENAP AKQRY
G616          VKIGAPENAP AKQ-- 262
FA1090        VKIGAPENAP AKQRY
```

FIGURE 1 - *Alignment of N. gonorrhoeae Macrophage infectivity potentiator amino acid sequences. G617, G627 and G616 Neisseria gonorrhoeae isolates. N. gonorrhoeae FA1090 reference strain (accession number AE004969). Amino acid substitutions and their positions are indicated in bold and highlighted in grey. N. gonorrhoeae Macrophage infectivity potentiator dimerization domain, located at N-terminal region, is underlined; PPI-ase domain, located at C-terminal region, is in the box.*

combinant Ng-MIP and Ng-OmpA proteins by sera collected from 11 gonorrhoea infected patients, was also evaluated.

Primers for amplification and *mip* gene sequence analysis were previously described (Leuzzi *et al.*, 2005). The primers for *omp*A gene were: ^{1}atg act ttc ttc aaa ccc tc^{20} and ^{678}tta cat gtg ccg tgc ggc^{661} (numbering is based on gene sequence of *N. gonorrhoeae* FA1090, accession number AE004969). Amplification parameters were: 15 min at 95°C, and 1 min at 95°C, 1 min at 55°C or 60°C (for *mip* and *omp*A genes, respectively), 1 min at 72°C, for 35 cycles. For DNA sequencing, PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Germany) and subjected to sequence analysis. Generated DNA sequences of both strands were analyzed using Chromas software version 6, and aligned using DNA*Man* software version 5.2.10 (Lynnon Biosoft, Canada).

Two amino acid substitutions Glu-160-Lys and Ser-49-Gly were detected in the *mip* gene. In particular, 14 strains showed both; 4 strains (G617, G606, G651, and G520) showed only the Ser-49-Gly substitution, and 2 strains (G449 and G616), showed only the Glu-160-Lys substitution (Figure 1).

The Ser-49-Gly substitution is located in the N-terminal region of the protein, which contains the dimerization domain of the MIP protein, while the Glu-160-Lys is in the C-terminal region, which contains the catalytic site for the peptidil-prolyl *cis/trans* isomerase, PPI-ase, activity of the protein (Leuzzi *et al.*, 2005). However, the hypothetical MIP protein structure, obtained using the SwissPdb Viewer software (http://www.expacy.org/spdbe/), suggests that the two mutations do not change the protein structure and do not influence the ability of MIP to dimerize and perform its enzymatic activity.

The *omp*A genes and deduced amino acid sequences showed 100% identity in all strains examined (data not shown).

Western blot analysis was performed according to standard procedures (Laemli, 1970) using 11 sera samples from gonorrhoea infected patients and 5 sera from control patients (patients with non-gonococcus infection). Two out of the 11 were collected from patients with HIV co-infection, and 1 from a patient with disseminated gonorrhoea. Blood samples were collected in a range of 7 to 42 days from the onset of the symptoms. Recombinant Ng-OmpA and Ng-Mip proteins (kindly provided by Novartis, Siena Italy), were purified as previously described by Leuzzi 2005 and Serino 2007, respectively. Briefly, Ng-OmpA and Ng-Mip proteins with the predicted molecular weight of ~28 kDa and ~23 kDa respectively, were run on 12% acrylamide gel at 100V for 1h. After blotting the nitrocellulose membranes were incubated for 2 h at room temperature, (r. t.), washed twice with saline buffer and 0.05% of Tween 20 was added. They were then incubated for 1h at r. t. with antihuman IgG antiserum (Fc-specific)-Alkaline Phosphatase (Sigma Aldrich, USA). Peroxidase substrate (SIGMA FAST BCIP/NBT Sigma Aldrich, USA) was used for detection reaction. Molecular weight marker (PageRuler^{TM} Prestained Protein Ladder, Fermentas) was included in each gel. Polyclonal mouse antisera, raised against recombinant Ng-MIP and Ng-OmpA proteins, were used as positive controls, and sera from healthy people as negative controls. Polyclonal mouse sera were kindly provided by Novartis, Siena, Italy (Leuzzi *et al.*, 2005; Serino *et al.*, 2007). Both recombinant proteins were recognized by sera from infected patients, but not by negative sera, suggesting the two proteins are expressed during infection in vivo and are immunogenic (data not shown). Polyclonal mouse sera were able to recognize both proteins not only in a purified protein extract but also in gonococcus whole cell extract.

In conclusion, this study provides new information on the macrophage infectivity potentiator and the outer membrane protein A in *N. gonorrhoeae* clinical isolates. In particular, Ng-MIP and Ng-OmpA were always expressed and genetically conserved among the strains analyzed. The results confirmed the high rate of gene conservation of the two virulence factors that could be considered a promising starting point for a future successful application of these molecules, or some of them, for the detection and/or molecular characterization of gonorrhoea circulating strains.

The potential use of these proteins to prevent adhesion and resistance to macrophage killing could represent a new way to fight the infection since the disease is characterized by the absence of the immunological memory and by a major incidence among patients with immunological de-

ficiencies or among patients with a major risk of contracting sexually transmitted diseases (Elford, 2006 and Aral *et al.,* 2008). It is noteworthy that both the purified recombinant proteins are recognized by sera from patients affected by gonorrhoea.

Accession numbers

Submitted as representatives of *mip* and *omp*A genes the following sequences: FN552013, *mip* gene from strain G617; FN552014, *mip* gene from strain G627; FN552015, *mip* gene from strain G616; and FN552016, *omp*A gene from strain G44.

ACKNOWLEDGEMENTS

Neisseria gonorrhoeae Italian Study Group: I. Dal Conte, STI Clinic Ospedale Amedeo di Savoia Turin; A. Di Carlo, G. Palamara, G. Prignano, S. Gallicano Dermatologic Institute-IRCCS-Rome; A. D'Antuono, Centro MST Policlinico S. Orsola Malpighi Bologna; L. Scioccati, U. O. Dermatologia Fondazione Policlinico Regina Elena Milan and A. Matteelli, Università degli Studi di Brescia.

We thank Sofia Tonino for editing the manuscript.

This work was supported by the "Controllo delle infezioni nei soggetti deboli ed immunocompromessi; agenti opportunistici e nuove opportunità di controllo delle infezioni" Collaborating Project Italy-USA, 2008-2010.

REFERENCES

ARAL S.O., FENTON K.A., HOLME K.K. (2007). Sexually transmitted diseases in the U.S.A.: temporal trends. *Sex. Transm. Infect.* **83**, 257-266.

CIANCIOTTO N.P., FIELDS B.S. (1992). *Legionella pneumophila mip* gene potentiates intracellular infection of protozoa and human macrophages. *Pro. Natl. Acad. Sci. USA.* **89**, 5188-5191.

ELFORD J. (2006). Changing patterns of sexual behaviour in the era of highly active antiretroviral therapy. *Curr. Opin. Infect. Dis.* **19**, 26-32.

HAMILTON H.L., DILLARD J.P. (2006). Natural transformation of *Neisseria gonorrhoeae* from DNA donation to homologous recombination. *Mol. Microbiol.* **59**, 376-385.

HEDGES S.R., MAYO M.S., MESTECKY J., RUSSELL M.W. (1999). Limited local and systemic antibody response to *Neisseria gonorrhoeae* during uncomplicated genital infections. *Infect. Immun.* **67**, 3937-3946.

JEANNIN P., MAGISTRELLI G., GOETSCH L., HAEUW J.F., THIEBLEMONT N., BONNEFOY J.Y., DELNESTE Y. (2002). Outer membrane protein (OmpA): a new pathogenic associated pattern that interacts with antigen presenting cells impact on vaccine strategies. *Vaccine.* **20** (Suppl 4), A23-27.

KIM S.K., DEVINE L., ANGEVINE M., DEMARS R., KAVATHAS P.B. (2000). Direct detection and magnetic isolation of the *Chlamydia trachomatis* major outer membrane protein-specific CD8+ CTLs with HLA class I tetramers. *J. Immunol*. **165**, 7285-7292.

LAEMLI U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**, 680-685.

LEUZZI R., SERINO L., SCARSELLI M., SAVINO S., FONTANA M.R., MONACI E., TADDEI A., FISCHER G., RAPPUOLI R., PIZZA M.G. (2005). Ng-MIP, a surface-exposed lipoprotein of *Neisseria gonorrhoeae*, has a peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and is involved in persistence in macrophages. *Mol. Microbiol*. **58**, 669-681.

LO J.Y., HO K.M., LEUNG A.O., TIU F.S., TSANG G.K., LO A.C., TAPSALL J.W. (2008). Ceftibuten resistance and treatment failure of *Neisseria gonorrhoeae* infection. *Antimicrob. Agents Chemother*. **52**, 3564-3567.

LUNDEMOSE A.G., KAY J.E., PEARCE J.H. (1993). *Chlamydia trachomatis* Mip-like protein has peptidyl-prolyl *cis/trans* isomerase activity that is inhibited by FK506 and rapamycin and is implicated in initiation of Chlamydial infection. *Mol. Microbiol*. **7**, 777-783.

MORO A., RUIZ-CABELLO F., FERNANDEZ-CANO A., STOCK R.P., GONZALEZ A. (1995). Secretion by *Trypanosoma cruzii* of a peptidyl-prolyl *cis/trans* isomerase involved in cell infection. *EMBO J*. **14**, 2483-2490.

SERINO L., NESTA B., LEUZZI R., FONTANA M.R., MONACI E., MOCCA B.T., CARTOCCI E., MASIGNANI V., JERSE A.E., RAPPUOLI R., PIZZA M.G. (2007). Identification of a new OmpA-like protein in *Neisseria gonorrhoeae* involved in the binding to human epithelial cells and *in vivo* colonization. *Mol. Microbiol.* **64**, 1391-1403.

TAPSALL J. (2006). Antibiotic resistance in *Neisseria gonorrhoeae* is diminishing available treatment option: some possible remedies. *Expert. Rev. Anti. Infect. Ther.* **4**, 619-628.

ZHANG Y.X., STEWART S., JOSEPH T., TAYLOR H.R., CALDWELL H.D. (1987). Protective monoclonal antibodies recognize epitopes located on the outer membrane protein of *Chlamydia trachomatis*. *J. Immunol*. **138**, 575-581.