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# GONOCOCCI – PATHOGENS OF GROWING IMPORTANCE. PART 2. VIRULENCE FACTORS, ANTIMICROBIAL RESISTANCE AND VACCINE DEVELOPMENT

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*Abstract*: *Neisseria gonorrhoeae* (gonococcus) is a human pathogen, the aetiological agent of gonorrhoea, which is the second most common bacterial sexually transmitted disease (STD) in the world. The structure of *N. gonorrhoeae* cell wall is typical of Gram-negative bacteria, poses variable antigens porin B (PorB), and opacity-associated proteins (Opa proteins), lipooligosaccharide (LOS) and type IV pili (TFP) playing an essential role in pathogenesis. In addition to adhesins, gonococcus presents other virulence factors such as reducing modifiable protein (Rmp), iron transporters, membrane pumps, and IgA peptidase. The pathogen produces outer membrane vesicles (OMVs), releases peptidoglycan (PG) fragments and is well adapted to develop infection in diverse niches of the female and male reproductive tracts. The characteristic genotypic trait of *N. gonorrhoeae* is the state of natural competence, which allows DNA uptake from the environment. The antigenic and phase variability is essential to gonococcal defence against the human immune system. Because of the increasing antimicrobial resistance (AMR) of *N. gonorrhoeae* and the high incidence rate of gonococcal infections, developing an anti-gonococcal vaccine has become an urgent need. Vaccine development difficulties are mainly due to the gonococcal ability of immune evasion, the lack of an animal model, and the limited understanding of protective immune response mechanisms.

1. Introduction. 2. Genome and molecular characterization of *N. gonorrhoeae*. 3. The factors affecting the pathogenesis of gonococcal infection. 3.1. Adhesins presence. 3.2. Cytotoxic peptidoglycan fragments. 3.3. Outer membrane vesicles production. 3.4. The ability to utilize the host's iron resources. 3.5. Efflux-type membrane transporters activity. 3.6. Anaerobic metabolism. 3.7. Antimicrobial resistance. 3.8. Biofilm production. 3.9. Evasion of immune defense mechanisms. 4. *N. gonorrhoeae* vaccine development. 5. Conclusion

**Keywords:** AMR, gonorrhoea, vaccine, virulence factor

## 1. Introduction

*Neisseria gonorrhoeae* is an absolute human pathogen, the aetiological agent of gonorrhoea. Gonococci outside the body are highly sensitive to environmental factors. They die within hours at temperatures above 40°C and are sensitive to desiccation, light and low concentrations of disinfectants. Microscopically, *N. gonorrhoeae* is a Gram-negative diplococcus arranged in characteristic pairs resembling coffee beans (Janda *et al.* 2005). It is an aerobic bacterium with high nutritional requirements. The bacterium is oxidase- and catalase-positive, oxidizes only glucose to acid, and does not metabolize other carbohydrates. It requires an energy source of glucose, pyruvate or lactate, and cysteine to grow on culture media. Due to changes in metabolic pathways, some isolates show special growth requirements for amino acids, purines, and pyrimidines (Ng *et al.* 2005; Quillin *et al.* 2018). The structure of *N. gonorrhoeae* cell wall is typical of Gram-negative bacteria. There is a thin layer of PG between the inner cytoplasmic and outer membranes. The most crucial cell wall antigens are PorB (P. I) and Opa (P. II) proteins, LOS and TFP. The increasing antimicrobial resistance (AMR) of *N. gonorrhoeae* has a multifactorial basis. It is a serious threat to public health (Unemo *et al.* 2016),

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especially in the context of a high prevalence of gonorrhoea worldwide, with over 82.4 million new cases in 2020 as estimated by WHO (WHO 2021). Gonorrhoeae is a significant health risk, especially for women, due to its often asymptomatic course and the possibility of upper genital tract complications such as pelvic inflammatory disease (PID), which can result in ectopic pregnancy and infertility. Gonorrhea infection is also connected with a higher risk of HIV acquisition.

The genus *Neisseria*, family *Neisseriaceae*, includes more than 30 different species of aerobic, Gram-negative coccus or rod species found in humans and animals. Eight commensal species are part of the human microbiome, while *N. gonorrhoeae* and *N. meningitidis* are pathogenic (Humbert *et al.* 2019). Interestingly, despite presenting different pathogenicity, these two human pathogens share a high genome identity, resulting, among others, in a sequence similarity of their outer membrane proteins. These similarities can partially explain a cross-protective effect of outer membrane vesicles (OMV), meningococcal serogroup B vaccine (MenB-4C) against gonococcal infection (Hadad *et al.* 2012; Marjuki *et al.*, 2019).

## 2. Genome and molecular characterization of *N. gonorrhoeae*

*N. gonorrhoeae* chromosome has the structure of a circular, covalently closed DNA molecule of about 2.2 million bp in length. The results of genomic analysis of 14 reference *N. gonorrhoeae* strains from the WHO collection obtained by whole genome sequencing (WGS) presented in 2016 can provide a basis for representative molecular characterization of the species (Unemo *et al.* 2016; Golparian *et al.* 2021; Sánchez-Busó *et al.* 2022). The genomes of these strains were compared and showed relatively high similarity, and the differences were mainly related to the presence of the gonococcal genomic island (GGI). It is present in about 80% of *N. gonorrhoeae* isolates and encodes the type IV secretion system (T4SS) (Dillard and Seifert 2001; Hamilton and Dillard 2006). The total number of coding sequences in the genomes of the analyzed reference strains ranged from 2295 to 2450, with an average sequence length of 0.7 kb. Core genes were estimated at 1820, and auxiliary genes at 475 to 630. The coding density in the whole genome was about 87%, with an average G+C content of 52.4%. In addition, 54 tRNAs and four copies of 16S-23S-5S rRNA operons are encoded in the gonococcal genome. The most abundant type of repeats is the so-called 10–12 bp long *DNA* uptake sequence (DUS) composed of 5'-GCCGTCTGAA-3' or 5'-ATGCCGTCTGAA-3', which typically repeats every 1.1 kb and accounts for almost 1% of the genome (Frye *et al*. 2013; Spencer-Smith *et al.* 2016). An essential feature characteristic of the species *N. gonorrhoeae* is the state of natural competence, which means the constitutive ability of bacteria to take up DNA from the environment. DNA uptake from related species of the genus *Neisseria* (Spratt *et al.* 1992), as well as from unrelated bacteria, is an essential factor in the genetic variability of gonococci (Kroll *et al.* 1998). The natural transformation process in *N. gonorrhoeae* involves TFP (Chen and Dubnau 2004). DNA uptake occurs through the interaction between pili and the DNA capture sequence (Cehovin *et al.* 2020). Human commensal species of the *Neisseriaceae* family have DUS sequences identical or differing by one or two bp (Frye *et al.* 2013; Spencer-Smith *et al.* 2016). An example of chromosomal loci transfer in *N. gonorrhoeae* is the mosaic structure of antibiotic resistance-related proteins penA. The T4SS allows to secrete fragments of single-stranded DNA (ssDNA) directly into the extracellular space, which, once recognized by the recipient pilus, can be incorporated by recombination into its genome (Dillard and Seifert 2001; Hamilton and Dillard 2006). T4SS is encoded within a GGI approximately 57 kb long (Hamilton *et al.* 2005). The GGI is a mobile genome element, probably acquired by horizontal gene transfer (HGT) (Rotman and Seifert 2014). As shown experimentally, out of 66 GGI genes, only 21 are essential for the function of the T4SS system, two-thirds of which are *tra genes* homologous to T4SS system genes of *E. coli* plasmid F (Hamilton *et al.* 2005; Pachulec *et al.* 2014; Callaghan *et al.* 2017). Gonococcal T4SS exports ssDNA directly into the extracellular space independent of contact with the host or neighbouring cell. It has been shown that this unique method of DNA secretion may have an adaptive role in the pathogenesis and acquisition of antibiotic resistance (Harrison *et al.* 2016) and in biofilm formation (Zweig *et al*. 2014).

Bacterial plasmids are autonomous, extrachromosomal, replication-capable genetic elements. Unlike the chromosome, which constitutes the core genome, plasmids are not necessary for bacterial survival and are an accessory genome. Plasmids in the form of double-stranded DNA are mostly circular. Their genes, with different functions, determine various phenotypic characteristics, such as antibiotic resistance, the ability to neutralize toxic substances, and the production of bacteriocins and toxins. Plasmids are a link of HGT that promotes variability and environmental adaptation. As autonomous, mobile genetic elements, they can move from the donor bacterial cell to the recipient cell through conjugation, transformation and transduction. In gonococci, there can be three types of circular plasmids: conjugative plasmids, β-lactamase plasmids and cryptic plasmids. Most are between 4 and 9 kb in length. Only conjugation plasmids are longer,

containing 39–42 kb. Cryptic plasmids, with a size of 4.2 kb and an undefined function for the bacterial cell, are found in most (96%) strains of *N. gonorrhoeae* (Cehovin and Lewis 2017).

## 3. The factors affecting the pathogenesis of gonococcal infection

### 3.1. Adhesins presence

*N. gonorrhoeae* adhesins recognize different target sites on host cells, allowing the pathogen to interact with various tissues during gonorrheal infection. Adhesion and invasion into human cells are a crucial step in developing infection. Antigenic and phase variability of adhesins protects bacteria from effective immune defense. An important virulence factor is the glycolipid outer membrane antigen LOS, composed of endotoxin-active lipid A and a core oligosaccharide. It stimulates the release of pro-inflammatory cytokines, which promotes neutrophil recruitment to the infection site (Ng *et al.* 2005; Quillin and Seiferth 2018). The structure of LOS lacks the repeated polysaccharide chains characteristic of the analogous cell wall antigen of *Enterobacterales*, lipopolysaccharide. The branched LOS molecule has three oligosaccharide chains anchored to the cell membrane by lipid A. The variable chains are attached via a 3-deoxy-D-manno-octulosonic acid molecule to two heptose residues of lipid A: Hep1 and Hep2. The oligosaccharide core shows considerable variability, even within the same *N. gonorrhoeae* strain due to phase variation in numerous glycosyl transferase genes. This enzyme, which is crucial in the synthesis of LOS, is responsible for attaching further sugars to the oligosaccharide chain. The variability of LOS is also due to the genetic diversity of alleles encoding glycosyltransferases (Apicella *et al.* 1987; Banerjee *et al.* 1998; Shafer *et al.* 2002). LOS is involved in the adherence process to epithelial cells. Variability and modifications of LOS structure, such as attachment of exogenous sialic acid molecules, promote evasion of host immune defense mechanisms. Both sialylation of gonococcal LOS and attachment of phosphoethanolamine to lipid A protect against antibodies and reduce susceptibility to bacterial killing by neutrophils, inhibit complement activation, and increase resistance to antimicrobial peptides (Lewis *et al.* 2009; Balthazar *et al.* 2011). The importance of LOS in processes leading to oviduct damage has also been described (Gregg *et al.* 1981; Cooper *et al.* 1986).

TFPs are protein filamentous surface structures anchored to the cytoplasmic membrane, passing through the outer membrane to the outside of the cell. As adhesins, they have a crucial function in the pathogenesis of gonorrheal infection. Their presence is related to the virulence of the strain. They participate in the initial phase of bacterial adhesion to human epithelial cells and interactions with neutrophils. Pili, binding to the CD46 receptor, initiates adherence to eukaryotic cells. Based on the principle of twitching motility, they provide gonococci with a degree of motility, which promotes the spread of microorganisms across the epithelial surface and facilitates their auto-aggregation. Phase variation in TFP expression enables gonococci to defend against phagocytosis and evade the host immune response. Several genes encode different subunits of pili. The main subunit of the pilE protein shows antigenic variation due to recombination with several silent copies at the *pilS* loci. Pili are also important in the natural transformation of *N. gonorrhoeae* (Chen and Dubnau 2004).

Opa proteins are a family of 24–30 kDa outer membrane proteins. As adhesins, they are involved in gonococcal auto-aggregation and receptor-mediated adherence of bacteria to eukaryotic cells and in stimulation and modification of the immune response. Opa proteins bind to host cell CEACAM receptors (carcinoembryonic antigen family adhesion molecules). They are named after a phenomenon observed macroscopically, as they cause the lack of transparency of colonies cultured on clear agar. Depending on the expression of Opa proteins, the morphology of gonococci colonies on agar varies. Opa colonies expressing Opa + are referred to as "opaque phenotype." Based on differences in binding to two types of cell receptors, two classes of Opa proteins have been distinguished, grouping 11 different types. Opa proteins class one ($Opa_{50}$) bind to the heparan sulfate proteoglycan receptor on epithelial cells using heparan sulfate. Opa class two ($Opa_{51-60}$) binds to CD66 antigen family molecules on epithelial cells, lymphocytes and neutrophils, i.e., the receptor molecules CEACAM-1, 3, 5 and 6. The ability of bacteria to interact with different types of receptors during infection activates distinct signal transduction pathways in cells and is an essential adaptive feature (Gray-Owen *et al.* 1997; Chen *et al.* 1997). Opa proteins increase bacterial resistance to complement action. Opa protein expression is subject to antigenic and phase variation. There can be up to 11 different *opa* genes in the genome of *N. gonorrhoeae* strain, with several types being expressed simultaneously. The protein sequence of all Opa proteins is 70% identical. Conservative regions of the molecule are not exposed on the surface of the outer membrane, in contrast to the highly variable outer fragments, the second and third loops. These regions are called hypervariable domains 1 and 2 (Stern *et al.* 1986; Bhat *et al.* 1992).

PorB is the most abundant outer membrane protein of *N. gonorrhoeae*, encoded by the *porB* gene. It is essential for gonococci viability as a conserved voltage-gated ion channel-like protein. It consists of three protein

subunits. Each monomer, 32–35 kDa, has a structure of β-folded barrel, 16 trans-membrane segments, and eight characteristic extracellular loops with high variability (Chen and Seifert 2013). In gonococci, there are two different Porin B isoforms – PorB1a and PorB1b-, resulting from stable expression of one of the two alleles of the *porB* gene in a given bacterial strain. Due to this diversity, two distinct phenotypes of gonococci can be distinguished depending on the type of porin they possess: P. IA and P. IB. Strains with the P. IA phenotype are characterized by increased invasiveness, which has been found based on epidemiological data describing the more frequent involvement of this particular phenotype in generalized gonococcal infections (Bash *et al.* 2005, Guglielmino *et al.* 2022). The P. IA phenotype strains are characterized by the deletion of a fragment in loop 5, which gives them resistance to trypsin digestion and higher resistance to the complement-dependent lethal effects of human serum (Blake *et al.* 1981; Ram *et al.* 2001). P. IB phenotype gonococci are more often isolated from genitourinary tract infections and usually show sensitivity to the bactericidal effect of serum. The nucleotide sequences of both types of porins show about 80% similarity. Genetic variation within PorB distinguishes strains in epidemiological studies (Fudyk *et al.* 1999; Liao *et al.* 2009). These porins are the basis for serotyping based on reactions with monoclonal antibodies (Sandström *et al.* 1980, Tam *et al.* 1982). A fragment of the third outer loop of the PorB molecule has become, along with the TbpB fragment, the basis of NG-MAST typing (Martin *et al.* 2004), and the PorB1b gene segment encoding 30 amino acids is a well-characterized determinant of antibiotic resistance, referred to as penB (Olesky *et al.* 2002; Unemo *et al.* 2011). PorB acts as a mitogen, activates B lymphocytes and stimulates cytokine production. On the other hand, this protein influences the phagocytosis process, enabling it to block complement activation through the alternative or classical pathway and protecting bacteria from opsonization. Factor H, the main regulator of the alternative complement pathway, binds directly to PorB1a. Strains with the P. IB phenotype require LOS sialylation to bind factor H. Serum resistance in gonococci is also mediated by the C4bp protein binding to different fragments – extracellular loop 1 within PorB1a of P. IA strains and loops 5 and 7 of PorB1b (P. IB). PorB can also inhibit oxidative burst and neutrophil apoptosis, facilitating pathogens to survive infection despite the inflammatory response (Chen *et al.* 2011; Chen and Seifert 2013; Palmer and Criss 2018).

Rmp is an outer membrane protein physically associated with PorB, presenting the potential of an immunology evasion (Joiner *et al.* 1985). As a highly conserved membrane protein, it stimulates the synthesis of "blocking antibodies" specific for both LOS and PorB, inhibiting serum's bactericidal effect (Gulati *et al.* 2015). The presence of antibodies to Opa proteins and the absence of "blocking antibodies" induced by Rmp was associated with the reduction of upper reproductive tract infection in high-risk women (Plummer *et al.* 1993a, 1994b).

### 3.2. Cytotoxic peptidoglycan fragments

PG fragments that are spontaneously released during the growth and division of bacterial cells at the site of infection are important virulence factors. Recognized by human cytosolic nucleotide-binding oligomerization domains 1 and 2, pattern recognition receptors are an element that induces the inflammatory response (Mavrogiorgos *et al.* 2014). By stimulating the inflammatory process, they are responsible for, among others, damage within the fallopian tubes and destruction of the epithelial cells. The release of pro-inflammatory peptidoglycan monomers as well as dimers and free peptides, leading to remodelling of the cell wall structure of gonococci, is possible due to the activity of bacterial enzymes, mainly lytic transglycosylases (Cloud *et al.* 2002), as well as amidases and endopeptidases (Schaub *et al.* 2019).

### 3.3. Outer membrane vesicles production

OMVs by *N. gonorrhoeae* (Pettit *et al.* 1992) are characteristic of many pathogenic Gam-negative bacteria (Kulp *et al.* 2010). OMVs were first described in *Vibrio cholerae* in 1967 and recognized as artefacts of *in vitro* liquid medium culture (Chatterjee SN, Das J, 1967). Vesicles are formed by the protrusion of the bacterial outer membrane, the detachment of a fragment of this membrane and the formation of a spherical, closed structure with a diameter of 20–200 nm. Inside OMVs, components of both the bacterial periplasm and cytoplasm, such as adhesins, enzymes, and DNA fragments, can be encapsulated. Examination of gonococcal OMVs proteins concentration can suggest active sorting of proteins during natural blebbing of bacteria facilitating vesicles functions (Zielke *et al.* 2014; Deo *et al.* 2018). So far, these potent single lipid bilayer spheres, carrying plenty of outer membrane lipids, proteins, LOS, periplasmatic PG fragments and proteins, cytoplasmatic proteins and nucleic acids, have shown to be the promise vaccine antigens (van der Pol *et al.*, 2015). OMVs contain many outer membrane antigens, presented in their native form, as derived straightly from the crucial pathogen adhesion and immunogenicity surface layer of Gram-negative bacterial cell wall. Polyantigenic gonococcal OMVs can potentially overcome the challenges of high phase and antigen variation of *N. gonorrhoeae*.

### 3.4. The ability to utilize the host's iron resources

An important phenomenon in the pathogenesis of gonorrhoea is the ability to utilize the host's iron resources with the help of gonococcal membrane transporters. Unlike most bacteria, *Neisseria* species pathogenic to humans do not produce siderophores and use iron bound to human glycoproteins, mainly transferrin (West and Sparling 1985). They can also obtain iron from lactoferrin (Mickelsen and Sparling 1981; Mickelsen *et al.* 1982), hemoglobin and heme, which are available in the female reproductive tract during menstruation. Eight iron transporters dependent on TonB proteins of the inner membrane of gonococci have been described: TbpA/TbpB, LbpA/ LbpB, HpuB/ HpuA, FetA, TdfF, TdfG, TdfH, TdfJ, which enable efficient utilization of protein-bound iron (Cornelissen and Hollander 2011). Human transferrin is found in the highest concentrations in serum, cerebrospinal fluid and joint fluid. Still, it can also be detected in semen and mucous membranes, especially in inflamed tissue. Iron acquisition requires energy and direct contact between the iron transport glycoprotein and the bacterial cell surface. Increased expression of gonococcal genes (e.g., *tbp, lbp, fbp*) responsible for iron uptake from transferrin and lactoferrin has been found during gonococcal infection of the lower genital tract (McClure *et al.* 2015).

### 3.5. Efflux-type membrane transporters activity

Active efflux-type membrane transporters are an essential group of surface proteins that act as pumps that remove harmful chemicals from the bacterial cell. The expression level of such efflux transporters determines the variable level of sensitivity of the microorganism to antimicrobial substances naturally present in their environment and to antibiotics. Gonococcal membrane transporters belong to different families of bacterial pumps. The FarA-FarB transporter of the Major Facilitator System (MFS) family recognizes antibacterial long-chain fatty acids (Lee and Shafer 1999). The NorM transporter is a member of the Multidrug and Toxic Compound Extrusion (MATE) family, and its overexpression can reduce the sensitivity of gonococci to ciprofloxacin and norfloxacin (Rouquette-Loughlin *et al.* 2005). The MtrC-MtrD-MtrE system belongs to the Resistance-Nodulation-Cel Division (RND) family of pumps found in Gram-negative bacteria and depends on energy drawn from ATP. The MtrCDE pump is an active transporter of substances from the cytoplasm and periplasmic space (Maness and Sparling 1973). It can remove hydrophobic compounds, naturally occurring antimicrobial peptides, bile salts, progesterone, detergent and dye-like compounds, and antibiotics (Delahay *et al.* 1997). The mtrCDE pump comprises three protein subunits, C, D, and E, passing through the outer membrane, periplasmic space, and inner membrane. It has a typical operon, and the MtrR repressor and MtrA activator regulate its expression. The *mtrR* repressor gene is an important determinant of gonococcal chromosomal antibiotic resistance. As a result of point mutations within the *mtrR* promoter or the actual gene, or due to the mosaic structure of *mtrR* resulting from HGT derived from related species of the genus *Neisseria*, there is increased expression of a pump that removes toxic substances from the bacterial cell. Phenotypically, this increases MICs for macrolide antibiotics, tetracyclines, penicillin and cephalosporins (Zarantonelli *et al.* 2001).

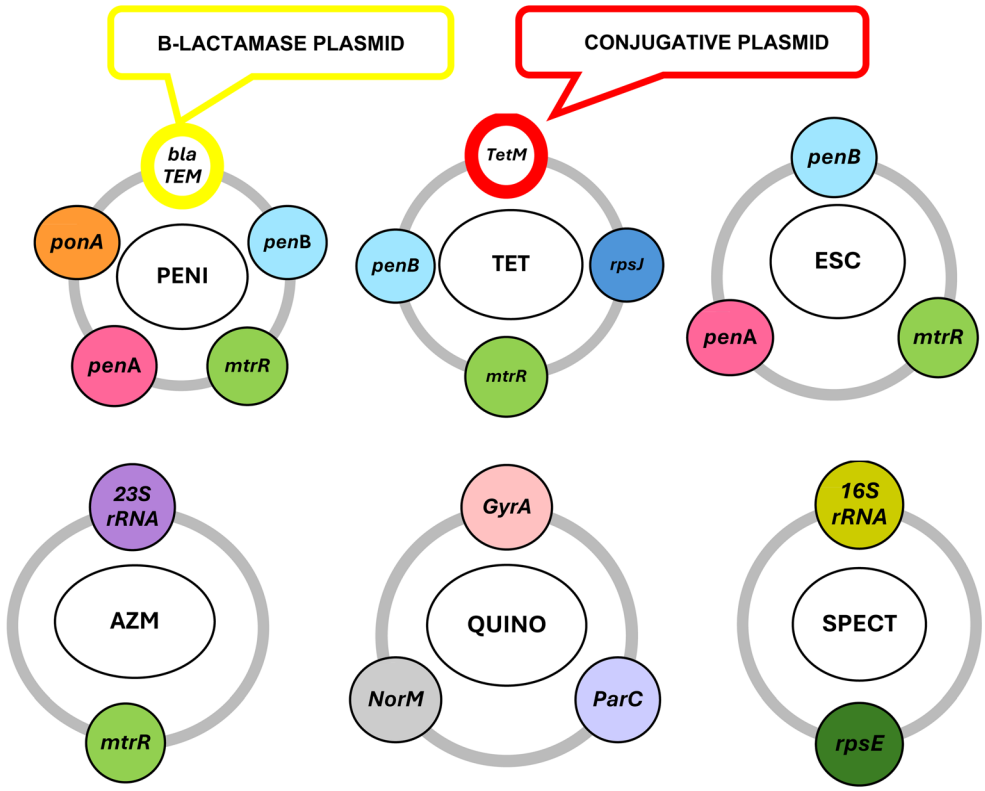
### 3.6. Anaerobic metabolism

Anaerobic metabolism in *N. gonorrhoeae* is enabled by two enzymes of the denitrification pathway: copper-containing nitrite reductase (AniA) that reduces nitrite to nitric oxide (NO) and nitric oxide reductase (NorB) (Barth *et al.* 2009). The NorB enzyme may be involved in removing toxic NO produced by macrophages. The expression of these enzymes is strictly regulated by oxygen availability, and elevated expression of AniA and NorB was found, e.g., in biofilms (Falsetta *et al.* 2009).

### 3.7. Antimicrobial resistance

The increasing antimicrobial resistance of *N. gonorrhoeae* is a global problem, and it has a complex basis, both chromosomal and plasmid-mediated (Fig. 1) (Unemo *et al.* 2016). Features of gonococci responsible for the rise of antibiotic resistance include genetic plasticity, the ability to transform naturally, high levels of antigenic and phase variation, point mutations, mosaicism of chromosomal determinants of resistance, and plasmids with resistance-determinant genes. A structurally diverse group of β-lactamase plasmids encoding blaTEM penicillinases determines penicillin resistance. In contrast, conjugative plasmids may have Tet(M) tetracycline resistance genes in their structure (Fig. 2) (Pachulec *et al.* 2014). Based on phenotypic characteristics, strains with plasmid resistance to penicillin and tetracycline, respectively, can be identified using the cephinase test for penicillinase-producing isolates (PPNG or penicillinase-producing *N. gonorrhoeae*) and a MIC value for tetracycline ≥ 16.0 mg/L, characterizing strains with high levels of tetracycline resistance – the High-Level tetracycline resistant (HLTR) phenotype (Muhammad *et al.* 2014).

Strains exhibiting low-level tetracycline resistance (LLTR), phenotypically expressed by tetracycline MIC values in the range of 1–8 mg/l, do not have the plasmid *tet*(*M*) gene, and the resistance in their case is




| MECHANISM OF AMR | PENI | ESC | TET | AZM | QUINO | SPECT |
|---|---|---|---|---|---|---|
| SYNTHESIS OF ANTIBIOTIC INACTIVATING ENZYME | *blaTEM* | | | | | |
| SYNTHESIS OF NEW PROTEIN | | | *TetM* | | | |
| CHANGES IN MEMBRANE PERMEABILITY | ***penB*** *(porB1b)* | | | | | |
| ACTIVE REMOVAL OF ANTIBIOTIC FROM THE CELL | *mtrR* | | | | *NorM* | |
| CHANGING THE TARGET SITE OF ANTIBIOTIC ACTION | *penA* | | *rpsJ* | *23SrRNA* | *GyrA* | *16SrRNA* |
| | *ponA* | | | | *ParC* | *rpsE* |

**AMR** – antimicrobial resistance, **PENI** – penicillin, **ESC** – extended spectrum cephalosporins, **TET** – tetracyclines, **AZM** – azithromycin, **QUINO** – quinolones, **SPECT** – spectinomycin

Fig. 1. Main chromosomal and plasmid-mediated determinants of *Neisseria gonorrhoeae* antimicrobial resistance and their mechanisms of action.

chromosomal and may be the result of changes in genes associated with drug susceptibility, among others, in the *mtrR gene* – encoding the membrane pump repressor MtrCDE or in the *porB1b (penB*) gene (Pitt *et al.* 2019). An example of chromosomal loci transfers in *N. gonorrhoeae,* including important determinants of antibiotic resistance, is the mosaic structure of PBP2 (*penA*) proteins associated with resistance to penicillin and cephalosporins (Muhammad *et al.* 2002; Nakayama *et al.* 2016) and the mosaic structure of *mtrR* and *mtrCDE* genes, encoding the pump gene repressor and membrane pump MtrCDE, respectively (Rouquette-Loughlin *et al.* 2018). Single nucleotide mutations that change the target site of antibiotic action in a bacterial cell cause resistance to penicillin, cephalosporins, macrolides, fluoroquinolones, and spectinomycin. Mutations within the genes of penicillin-binding protein PBP1 (*ponA*) cause an increase in the MIC for penicillin, while within the genes of PBP2 (*penA*) for penicillin and third-generation cephalosporins (Lindberg *et al.* 2007). Point mutations, e.g., adenine deletion (35Adel) in the promoter region and substitutions of the coding

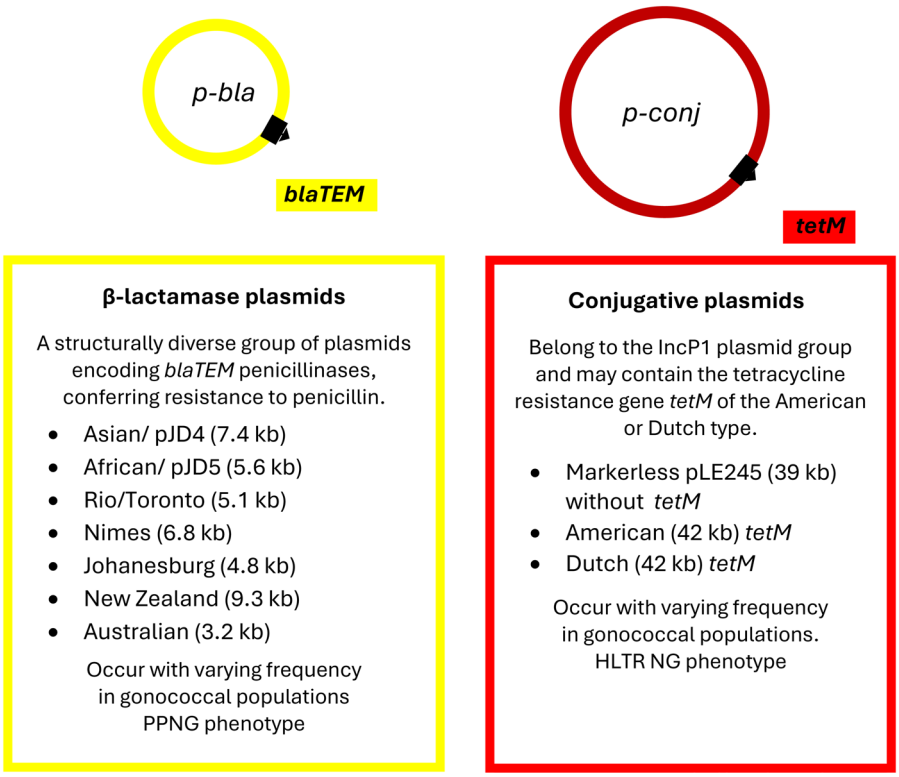



Fig. 2. Types of plasmids associated with drug resistance in *Neisseria gonorrhoeae* (according to Cehovin 2017).

region in the *mtrR* gene (A39T and G45D), can result in overexpression of the MtrCDE pump and lead to increased resistance to penicillin, tetracyclines, macrolides and cephalosporins (Zarantonelli *et al.* 2001). Mutations in the *porB1b* gene fragment, also referred to as a *penB* resistance determinant, altering the structure of the PorB1 membrane protein can result in reduced membrane permeability and impede the entry of tetracyclines, penicillin and cephalosporins into the cell. The main *PorB1b* sequence changes described so far result in amino acid substitutions of G120 and A121 (Lindberg *et al.* 2007). Resistance to azithromycin is mainly caused by mutations in the *23SrRNA* subunit allele, reducing the affinity of macrolide for the 50S ribosome unit. Mutations within the DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) genes cause resistance to fluoroquinolones. Mutations in ribosomal genes encoding the 16sRNA unit and the 5S protein, *rpsE,* inhibit spectinomycin binding to the ribosome (Unemo *et al.* 2016).

### 3.8. Biofilm production

A bacterial biofilm is understood as a spatial structure within which bacterial cells adhere to a surface, contact each other, cooperate and are surrounded by a matrix. The extracellular polymeric mainly comprises bacterial products, glycoproteins, lipids, and nucleic acids. The matrix's biochemical nature and the biofilm's structure depend on the microorganisms' properties and environmental conditions (Stoodley *et al.* 2002; Sauer *et al.* 2003). Bacterial biofilm can form on the surface of living cells and is natural for many commensal microorganisms colonizing human mucous membranes. At the same time, the biofilm phenomenon is important in terms of the pathogenicity of microorganisms. After entering the body in planktonic form, bacterial pathogens can adhere to host cells and then gradually form a biofilm. It has been found that bacterial cells residing in biofilms are characterized by high viability and increased resistance to unfavorable environmental conditions, such as physical and chemical changes in the environment (e.g., nutrient deficiency, changes in pH or oxygen concentration), and better tolerate the presence of bactericidal substances. Growth in the form of a biofilm facilitates long-term colonization of a variety of tissues and enhances bacterial resistance to natural antimicrobial peptides, other immune system factors, and antibiotics (Donlan and Costerton 2002).

The biofilm formed by *N. gonorrhoeae* was visualized in cervical biopsy preparations (Steichen *et al.* 2008). A three-dimensional 48-hour biofilm of the reference strain *N. gonorrhoeae* 1291 formed on transformed cervical epithelial cells was imaged by confocal microscopy (Steichen *et al.* 2008). The ability of gonococci to form a biofilm on the abiotic surface under conditions of continuous flow of medium and on the surface of urethral and

cervical epithelial cells in *ex vivo* cultures was described (Greiner *et al.* 2005), according to the work of Falsetta *et al*. The transcriptomes of gonococci growing in the biofilm differ from those of planktonic cells, as demonstrated using the RNA microarray technique. Biofilms showed a significant increase in the expression of genes related to the metabolism of anaerobic respiration *AniA, NorB* and the cytochrome C peroxidase (*ccp*) gene associated with oxidative stress tolerance (Falsetta 2009).

### 3.9. Evasion of immune defense mechanisms

Many mechanisms of avoiding the host immune system protection have been discussed while describing gonococci's main virulence factors. *N. gonorrhoeae* is both an extra- and intracellular pathogen able to induce a strong inflammatory response through the Th-17 pathway but a weak, insufficient adaptive response. The innate immune response is a first line of defence against gonococci. Lack of protective immunity after recovery, asymptomatic infections in women and long-term complications of gonorrhoea indicate that the pathogen possesses the mechanisms of immunological evasions (Płaczkiewicz, 2019).

The ability of gonococci to evade immune defense mechanisms, including oxidative burst and killing by neutrophil granulocytes, should be underlined. Generally, neutrophils are the first phagocytic defense line in bacterial infections and represent the major component of the inflammatory response in gonococcal infection. The influx of numerous activated polymorphonuclear leukocytes (PMNs) into the urethra following gonococcal emergence on epithelium is, in fact, typical of symptomatic infection in men (Rest and Shafer 1989). The inflammatory response in symptomatic gonorrhoea is stimulated by gonococcal cell membrane adhesins, peptidoglycan fragments released naturally during cell division, A lipid of LOS, surface lipoproteins, heptoses, which are intermediates of LOS biosynthesis, and methylated DNA fragments secreted by strains possessing a type IV secretion system (Palmer and Criss 2018). Neutrophils kill microorganisms through the activity of antimicrobial proteins and synthesize ROS (Segal *et al.* 2005). However, despite the presence of numerous neutrophils in the urethra during *N. gonorrhoeae* infection, live gonococci are cultured from the purulent secretions collected from the patient, showing that PMNs are ineffective in killing *N. gonorrhoeae* (Rest and Shafer 1989). As gonococci, during infection, are exposed to different sources of reactive oxygen species (ROS), they use many factors to protect themselves from oxidative damage: catalase, cytochrome c oxidase, methionine peptide sulfoxide reductase, cytochrome c peroxidase, bacterioferritin, manganese uptake system (Criss *et al.* 2021). The IgA protease, produced and secreted by the gonococci, capable of degrading the hinge site of IgA class immunoglobulins present on human mucous membranes, is also a virulence factor helping evasion of host epithelial immunity control (Quillin and Seiferth 2018).

Host immune response to gonococci has not been fully understood. Another interesting issue is how the reproductive tract microbiota influences human susceptibility to gonococcal infections. Due to the vaginal epithelium colonization by microorganisms, the totality of which is referred to as vaginal microbiota (VM) interaction of immune cells, epithelial cells, commensals and pathogens in this niche are complex. In a physiological state, the domination of *Lactobacillus* spp. on the vaginal mucosa, with much less amounts of other microorganisms, including *Gardnerella*, *Bifidobacterium*, *Streptococcus*, *Ureaplasma*, *Corynebacterium*, *Enterococcus etc*. are observed. *Lactobacillus* spp. is responsible for maintaining stability and a healthy vaginal environment (Ravel *et al.* 2011). *Gardnerella vaginalis* is a component of the vaginal microbiota in healthy women and also the dominant microorganism in the vagina in a state of bacterial vaginosis. The reproductive tract microbiota associations with susceptibility to infection by sexually transmitted pathogens have been shown (Brotman *et al.* 2010).

## 4. *N. gonorrhoeae* vaccine development

The constantly increasing resistance of gonococci to antibiotics, also recommended in empirical treatment ceftriaxone, contributed to the urgency for developing both new treatment options and vaccines preventing gonorrhoea. So far, no registered vaccine against gonorrhoea has been developed, as the vaccines tested have proved ineffective. The reason may be that the correlates of immune protection in humans are not fully known, and the antigenic determinants of gonococci are highly variable. Gonococci modify their epitopes through antigenic or phase variation (Boslego *et al.* 1991) and modify and even suppress the development of a protective immune response necessary to avoid reinfection in humans. More than that, the research on vaccine construction has been challenging due to the lack of proper animal gonorrhoea infection models.

The first studies on a vaccine against *N. gonorrhoeae* tested a lysed whole-cell vaccine. This vaccine had no effect compared to the placebo group (Greenberg *et al.* 1974). A whole-cell vaccine prepared from a single strain of *N. gonorrhoeae* was tested on the Aboriginal population of Inuit in northern Canada. Before vaccination, the annual prevalence of infection was 25%, while after immunization, it was 30%, showing that the vaccine was ineffective (Greenberg *et al.* 1974). Another vaccine was the pilus vaccine tested on Americans stationed in Korea. And this vaccine was not successful. The infec-

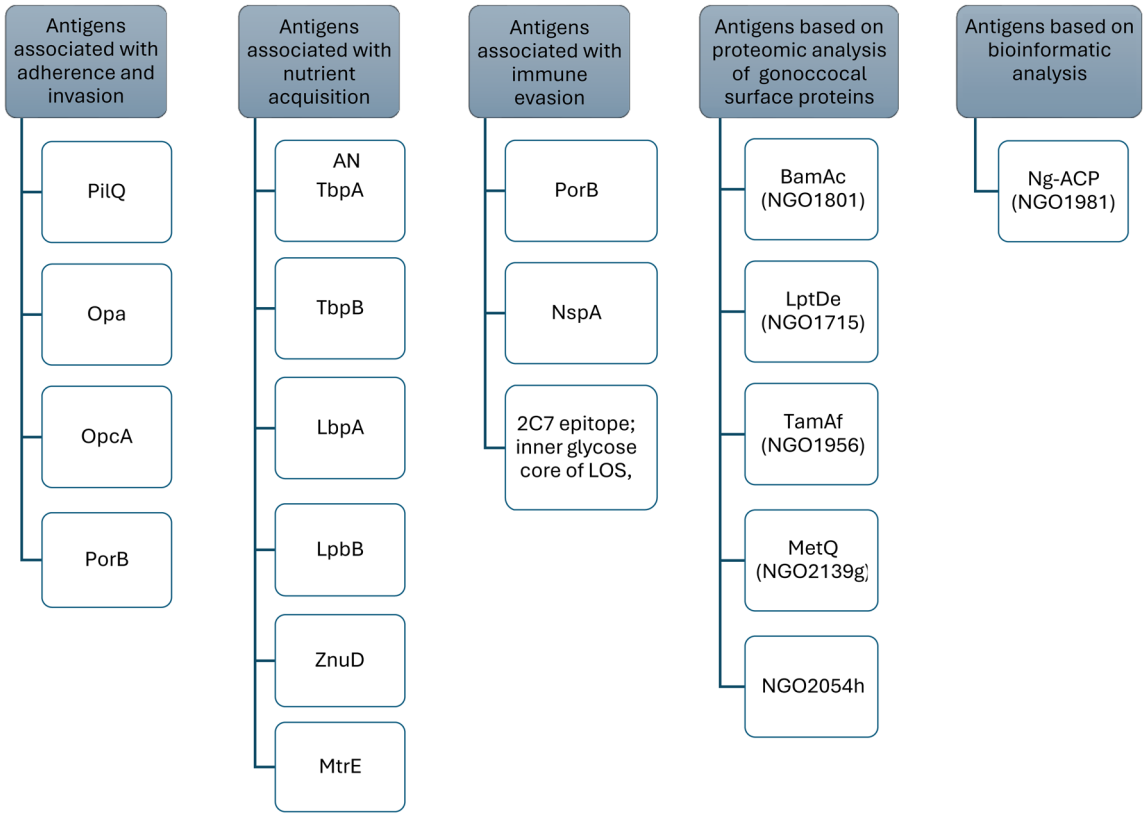



Fig. 3. Diagram of different groups of *Neisseria gonorrhoeae* vaccine candidates that induce antibodies, according to Rice et al. (2017).

tion rate was 6.9% in those receiving the vaccine and 6.5% in those receiving a placeb (Boslego *et al.* 1991).

Despite this, several promising gonococcal vaccine candidates have been identified (Fig. 3) using different research approaches. Zielke *et al.* described five potent vaccine candidates using a proteomics-driven approach, demonstrating that homologs of BamA (NGO1801), LptD (NGO1715), and TamA (NGO1956), and two uncharacterized proteins, NGO2054 and NGO2139, secreted in naturally released OMVs induce bactericidal antibodies cross-reacting with a panel of *N. gonorrhoeae* isolates (2016). In addition, the OMV vaccine against *N. meningitidis* serogroup B (MeNZB) was associated with decreased gonorrhoea rates in New Zealand. After the vaccination of over 1 million people between 2004 and 2008 with MeNZB, vaccinated individuals were significantly less likely to contract gonorrhoea compared with unvaccinated controls, with a predicted vaccine efficacy of 31% as described by Petousis-Harris *et al.* in a retrospective cohort study (Petousis-Harris *et al.* 2017). A similar prevention effect of gonorrhoea infections was observed after using the vaccine based on extracellular membrane proteins of meningococci serogroup B (Petousis-Harris *et al.* 2017). In Quebec, Canada, vaccination with 4CMenB, another anti-meningococcal vaccine, decreased the incidence of gonorrhoea (Longtin *et al.* 2017). The study conducted by Abara *et al.* again proved that MenB-4C multi-component, protein-based vaccine immunization was associated with a reduced incidence of gonorrhoea (Abara *et al.* 2022). Due to the finding that meningococcal B OMV vaccines may induce functional antibodies against gonococci (Semchenko *et al.* 2019) and the results of observational studies reporting reduced rates of gonorrhoea following the anti-meningococcal vaccination (Whelan *et al.*, 2016; Longtin *et al.*, 2017, Ochoa-Azze,) the attempts to create anti-gonococcal vaccine have been revived. Table I shows the current research on the vaccine development, approved by NIH US (National Institutes of Health USA, 2024). Further studies are needed to understand the antibody and T-cell responses to natural gonococcal infection in humans. Emerging vaccines must protect against a wide range of heterologous strains. A report suggests that the vaccine should induce a Th1 polarized response for protection (Liu *et al.* 2011; Liu *et al.* 2018; Belcher *et al.* 2023). In the future, the challenge will be the selection of specific target proteins because research shows that one of the three recombinant 4CMenB proteins is involved in the cross-protection process. However, with further discoveries regarding the pathogenesis of gonorrhoea and new vaccine antigens showing activity in preclinical studies, new studies on vaccines directly targeting *N. gonorrhoeae* will likely be designed in the future.

Table I
Research projects on gonorrhoea vaccines approved by National Institutes of Health USA
(National Institutes of Health USA, 2024.)

| Project | Organization<br>Project Leader(s) |
|---|---|
| The Gonorrhoea Vaccine Cooperative Research Center | HENRY M. JACKSON FDN FOR THE ADV MIL/MED<br>Ann E. Jerse |
| Proteomics-Driven Reverse Vaccinology for Gonorrhoea | OREGON STATE UNIVERSITY<br>Aleksandra E. Sikora |
| A Novel Sublingual Vaccine to Prevent *Neisseria gonorrhoeae* Infection | VIRTICI, LLC<br>Neil A. Fanger, |
| STI Clinical Trials Group: Phase 2 Clinical Trial to Evaluate a Vaccine Candidate for *Neisseria gonorrhoeae* | UNIVERSITY OF ALABAMA AT BIRMINGHAM<br>Edward Hook |
| Pre-clinical Vaccine Development for Emerging and Re-emerging Infectious Diseases | NIAID EXTRAMURAL ACTIVITIES<br>Wing Pui Kong |
| Starve and Kill: Engineered Antigens Targeting Nutrient Acquisition Pathways Essential for Gonococcal Infection and Disease | GEORGIA STATE UNIVERSITY<br>Cynthia N. Cornelissen |
| Development of nucleic acid-based vaccines against gonorrhoea | UNIVERSITY OF MASSACHUSETTS MED SCH WORCESTER<br>Lisa Ann Lewis, Li Li, Sanjay Ram |
| VesiVax Vaccine Formulation Against *Neisseria gonorrhoeae* | MOLECULAR EXPRESS, INC.<br>Gary Fujii |
| A novel vaccine against multidrug-resistant gonorrhoea | UNIVERSITY OF MASSACHUSETTS MED SCHOOL WORCESTER<br>Sanjay Ram, Peter A. Rice |
| Production of a Gonococcal Vaccine for Countering Antimicrobial Resistance | UNIVERSITY OF CALGARY<br>Anthony B. Schryvers, Scott D Gray-Owen, Trevor F. Moraes |
| Novel vaccine antigens against *N. gonorrhoeae* | TUFTS UNIVERSITY BOSTON<br>Paola Massari |

## 5. Conclusion

According to WHO estimations, in 2020, there were 374 million new infections in people aged 15–49 years with one of four curable STIs: chlamydia, gonorrhoea, syphilis and trichomoniasis. Unfortunately, the general knowledge of STI prevention is insufficient in many parts of the globe. As part of its mission, WHO supports countries to develop national strategic plans and guidelines, create an encouraging environment allowing them to discuss STIs, adopt safer sexual practices, and provide treatment in case of infection. Persistently increasing gonococcal antimicrobial resistance could lead to untreatable gonorrhoea in the future. For that, the WHO has awarded *N. gonorrhoeae* "superbug" status and has planned to prevent further gonococcal infections from spreading and make essential efforts to limit their incidence. Gonorrhoea prevention based on anti-gonococcal immunization is not available yet, as attempts to develop an effective vaccine have failed. Despite these, the undoubted constant progress in medicine and molecular biology, new *in vitro* and animal models, modern approaches in immunology, mathematical modelling, and especially genomics and proteomics development will hopefully succeed in combating gonorrhoea and other STDs. The step-by-step process of anti-gonococcal vaccine research should move us towards finding effective immunological protection. The global strategy of human papillomavirus vaccination proves that immunization might be both an efficient and socially approved tool for STD prevention. Currently, gonococcal infection fast diagnostic, AMR surveillance programmes, rational antibiotic therapy based on recommendations, and clinical microbiology and pharmacology knowledge are still crucial to controlling gonorrhoea. Given the presented facts, the need for further research on gonococcal biology, pathogenicity, antimicrobial resistance mechanisms, and vaccine development should be emphasized.

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The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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