Colonization efficiency of multidrug-resistant *Neisseria gonorrhoeae* in a female mouse model

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ABSTRACT

The rapid occurrence of gonococcal resistance to all classes of antibiotics could lead to untreatable gonorrhea. Thus, development of novel anti-*Neisseria gonorrhoeae* drugs is urgently needed. *N. gonorrhoeae* FA1090 is the most used in gonococcal infection mouse models because of its natural resistance to streptomycin. Streptomycin inhibits the urogenital commensal flora that permits gonococcal colonization. However, this strain is drug-susceptible and cannot be used to investigate the efficacy of novel agents against multidrug-resistant *N. gonorrhoeae.* Hence, to test the *in vivo* efficacy of new therapeutics against *N. gonorrhoeae* resistant to the frontline antibiotics, azithromycin or ceftriaxone, we constructed streptomycin-resistant mutants of *N. gonorrhoeae* CDC-181 (azithromycin-resistant) and WHO-X (ceftriaxone-resistant). We identified the inoculum size needed to successfully colonize mice. Both mutants, CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G, colonized the genital tract of mice for 14 days with 100% colonization observed for at least 7 days. CDC-181-*rpsL*A128G demonstrated better colonization of the murine genital tract compared to WHO-X-*rpsL*A128G. Lower inoculum of WHO-X-*rpsL*A128G (10^5 and 10^6 CFU) colonized mice better than higher inoculum. Overall, our results indicate that CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G can colonize the lower genital tract of mice and are suitable to be used in mouse models to investigate the efficacy of anti-gonococcal agents.

KEYWORDS

Neisseria gonorrhoeae, rpsL gene, gonococcal mouse model, antibiotic resistance, allelic exchange

1. INTRODUCTION

The Gram-negative diplococcus bacterium *Neisseria gonorrhoeae* is the causative agent of gonorrhea, which is the second most common sexually transmitted bacterial infection in the United States and the United Kingdom (Green et al. 2022; Katz et al. 2020). The United States Centers for Disease Control and Prevention (CDC) estimates that 1.6 million new cases of gonorrhea occurs every year and half of these cases are resistant to one or more antibiotics ('Centers for Disease Control and Prevention. Gonorrhea' 2022). Globally, gonorrhea is a serious public health threat associated with significant socioeconomic consequences and a high incidence rate that reached a high of 82 million cases in 2021 (Unemo and Shafer 2015; Yarwood 2022; Vashishtha, Singh, and Kundu 2022). This number may be an underestimate because gonococcal infections can be asymptomatic and there is an absence of surveillance programs to track infections in several countries (Elhassanny, Abutaleb, and Seleem 2022).

N. gonorrhoeae primarily colonizes human mucosal surfaces. Gonococcal infections are transmitted through direct contact with the mucosal membranes of the urogenital tract, oropharynx, and anal canal of an infected individual, usually during sexual intercourse (Rice et al. 2017). Birth-related transmission of *N. gonorrhoeae* is also possible, which can result in ophthalmia neonatorum and/or, rarely, disseminated infection (Młynarczyk-Bonikowska et al. 2020; Rice et al. 2017; Lin, Adamson, and Klausner 2021). If left untreated, gonorrhea can cause several complications that include infertility, pelvic inflammatory disease, and ectopic pregnancy; furthermore, gonococcal infections can enhance the acquisition and transmission of the human immunodeficiency virus (Lin, Adamson, and Klausner 2021). If the bacteria spread to the blood, the infection can lead to skin and/or joint/tendon infections and rarely, meningitis or endocarditis (Rice et al. 2017).

The increased prevalence of *N. gonorrhoeae* infections has been due to the emergence of antimicrobial-resistant strains of this bacterium. Strains of *N. gonorrhoeae* exhibiting resistance to antibiotics have been increasing worldwide. Most worrisome, *N. gonorrhoeae* has developed resistance to every class of currently available antibiotics (Lin, Adamson, and Klausner 2021). In 2019, the CDC reported a 124% increase in drug-resistant *N. gonorrhoeae* infections compared to 2013. Consequently, this bacterium was recently denoted by the World Health Organization (WHO) as a “superbug” and by the CDC as an “urgent threat” (Abutaleb, Elhassanny, and Seleem 2022; Tanwer et al. 2020). Dual therapy comprising azithromycin and ceftriaxone was the standard-of-care for treatment of gonorrhea (Lin et al. 2022; Lin, Adamson, and Klausner 2021). However, due to increasing resistance to azithromycin as well as the more potent anti-commensal activity of dual therapy, azithromycin was removed from the CDC’s guidelines. This left ceftriaxone as the last option for treatment of gonorrhea (Cyr et al. 2020; Wi et al. 2017). However, strains of *N. gonorrhoeae* that exhibit resistance to ceftriaxone have been reported in many countries (Green et al. 2022; Lin, Adamson, and Klausner 2021). These strains are also resistant to azithromycin, tetracycline, ciprofloxacin, penicillin, and tetracycline (Lin et al. 2022). Consequently, a future with untreatable *N. gonorrhoeae* infection is highly possible (Bolan, Sparling, and Wasserheit 2012).

Therefore, there is a critical and urgent need to develop new therapeutics effective against *N. gonorrhoeae*.

The use of animal models has produced essential data for the development of new medicines in human healthcare (Robinson et al. 2019). In the past, all antibiotics were tested in animal models prior to advancing to clinical trials with human participants. The United States Food and Drug Administration (FDA) evaluates the efficacy and safety of novel drug entities using information derived from animal models (Taconic ; Robinson et al. 2019). Mouse models are the most common animal models used to study diseases that impact humans. In addition to the lower costs compared to other animal models, mice are strikingly similar to humans at the genomic level and the pathophysiology of disease in mice display similarities to that of humans (Perlman 2016; Taconic ; Robinson et al. 2019). Mouse models in drug discovery also support a "fail fast" philosophy, which helps to uncover issues early before clinical trials. Potential drug candidates must demonstrate efficacy in a corresponding mouse model before being moved to clinical trials in humans (Taconic).

N. gonorrhoeae FA 1090, which was originally isolated from a female patient with a disseminated infection, is the most common strain used in mouse models of gonorrhea to test the efficacy of potential anti-gonococcal agents (Cohen et al. 1994; Connolly et al. 2019; Nachamkin, Cannon, and Mittler 1981; Hobbs et al. 2011; Cornelissen et al. 1998; Hobbs et al. 2013). This strain is used due to its natural resistance to streptomycin, the antibiotic used to repress other commensal microbes in the lower genital tract during infection to permit colonization by *N. gonorrhoeae* (Connolly et al. 2019; Butler et al. 2018). Nevertheless, this strain is sensitive to most antibiotics including the two frontline antibiotics used to treat gonorrhea, azithromycin and ceftriaxone. Therefore, the FA 1090 strain cannot be used to investigate the efficacy of new therapeutics against drug-resistant *N. gonorrhoeae* (Control and Prevention 2019). Consequently, it is important to construct streptomycin-resistant mutants of *N. gonorrhoeae* that are resistant to azithromycin and ceftriaxone in order to evaluate novel anti-gonococcal agents.

In this study, we genetically manipulated two clinical isolates, an azithromycin-resistant strain of *N. gonorrhoeae* (CDC-181) and a ceftriaxone-resistant strain of *N. gonorrhoeae* (WHO-X), to acquire streptomycin resistance which can be successfully used in a gonococcal infection mouse model. The antibacterial activity of standard antibiotics was evaluated against these two mutant strains and their colonization efficiency in a *N. gonorrhoeae* genital tract infection mouse model was also investigated.

2. MATERIALS AND METHODS

2.1. Bacterial strains, reagents, and chemicals

Clinical isolates of *N. gonorrhoeae* were obtained from the CDC and the American Type Culture Collection (ATCC) (Table 1). *N. gonorrhoeae* was grown on gonococcal agar base (GCB) (Becton,

Dickinson and Company [BD], Franklin Lakes, NJ) or GCB liquid medium (GCBL) supplemented with hemoglobin (BD) and IsoVitaleX (BD). For constructing the mutagenesis plasmids, DH5α chemically competent cells of *Escherichia coli* (Life Technologies Corporation, Rockville, MD) were used. Luria Bertani (LB; BD) agar or broth was used to maintain recombinant *E. coli* carrying the plasmids. Kanamycin (40 µg/mL) (Chem-Impex International, Inc, Wood Dale, IL) was used to maintain *E. coli.* Streptomycin (100 µg/mL) (TCI America, Portland, OR) was used to isolate streptomycin-resistant mutants of *N. gonorrhoeae.* The DNeasy Blood and Tissue Kit, for extracting genomic DNA, and Qiaprep Spin Miniprep Kit, for extracting plasmid DNA, were purchased from Qiagen (Hilden Düsseldorf, Germany). Restriction enzymes and T4 DNA ligase enzyme were purchased from New England Biolabs (NEB) (Ipswich, MA). Oligonucleotide primers were designed by the investigators and purchased from Integrated DNA Technologies, Inc (Morrisville, NC). Streptomycin, ceftriaxone, azithromycin, and trimethoprim (TCI America); gepotidacin (GlpBio, Montclair, CA); zoliflodacin (InvivoChem, Libertyville, IL); cefixime (Fisher Bioreagents, NJ); doxycycline (Alfa Aesar, Tewksbury, MA); ciprofloxacin and tetracycline (Sigma-Aldrich, Saint Louis, MO); penicillin and colistin (Cayman Chemical, Ann Arbor, MI); and vancomycin (GoldBio, St. Louis, MO) were acquired from commercial vendors. Reagents purchased commercially included yeast extract and dextrose (Fisher Bioreagents), protease peptone and nicotinamide adenine dinucleotide (NAD) (Sigma-Aldrich, MO), and hematin, Tween 80, and pyridoxal (Chem-Impex International, Inc).

2.2. Construction of streptomycin-resistant *N. gonorrhoeae* strains

All procedures involving live bacteria, DNA, and antibiotic markers were approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University. *N. gonorrhoeae* FA 1090 was grown in GCB and its genomic DNA was harvested using the DNeasy Blood and Tissue Kit. The 522-bp *rpsL* gene of *N. gonorrhoeae* FA 1090 that confers streptomycin resistance was amplified by PCR using the harvested genomic DNA. Both the forward oligonucleotide primer (5’ ATACGGGAGCTCTTCTTGTCGTTATGCTTGAC 3’) and the reverse oligonucleotide primer (5’ ATACGGCTCGAGCGGCCGTTGTTCAGCTTAGG 3’) were utilized. The *Sac*I restriction site inserted into the forward primer and the *Xho*I restriction site inserted into the reverse primer are shown underlinedwithin the primer sequences. The PCR product was digested with *Sac*I and *Xho*I and ligated into the *Sac*I + *Xho*I digested plasmid pMR32 (Ramsey et al. 2012). *E. coli* DH5α chemically competent cells were transformed with the ligation mixture, and colonies carrying the plasmids were picked from LB agar containing kanamycin (40 µg/ml). The colonies were re-streaked onto fresh plates and plasmid DNA was harvested. The presence of the *rpsL* gene in the correct orientation within the recombinant pMR32 plasmid was confirmed by test digestions of the plasmid DNA with restriction enzymes *Sac*I and *Xho*I and subsequent agarose gel electrophoresis of digested DNA. Moreover, the presence of the correct *rpsL* sequence within the recombinant plasmid was confirmed by Sanger DNA sequencing. A plasmid containing the gene *rpsL* in the correct orientation was designated as pMR32*rpsL* and was used in subsequent steps.

The recombinant plasmid pMR32*rpsL* was linearized by digesting it with *Nhe*I. *N. gonorrhoeae* WHO-X and CDC-181 parent strains were transformed with linearized DNA using the spot transformation procedure (Dillard 2011). The transformed cells were plated onto GC agar containing 100 µg/ml streptomycin. The colonies grown on plates were re-streaked on fresh plates, the *rpsL* region from cells was PCR amplified, and the PCR products were sequenced by Sanger sequencing to confirm the mutation within the gene region.The streptomycin-resistant clones from CDC-181 and WHO-X carrying the expected gene alteration were respectively designated as CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G and were used in the remaining assays described below.

2.3. Determination of the minimum inhibitory concentrations (MICs) of standard antibiotics against the constructed strains

The MICs of streptomycin, penicillin, ceftriaxone, tetracycline, doxycycline, azithromycin, and ciprofloxacin, in addition to zoliflodacin and gepotidacin (two compounds currently being evaluated in clinical trials for the treatment of gonorrhea) (Taylor, Morris, et al. 2018; Taylor, Marrazzo, et al. 2018), were determined against *N. gonorrhoeae* strains CDC-181, CDC-181-*rpsL*A128G, WHO-X, and WHO-X-*rpsL*A128G as well as FA 1090 (as a control) using the broth microdilution method as described previously (Alhashimi, Mayhoub, and Seleem 2019; Hewitt et al. 2021; Naclerio et al. 2021; Seong et al. 2020; Abutaleb et al. 2022; Giovannuzzi et al. 2022). Briefly, a 1.0 McFarland bacterial solution was prepared and diluted in brucella broth supplemented with yeast extract, dextrose, proteose-peptone, NAD, pyridoxal, hematin, and IsoVitaleX to reach an inoculum of ~1×10^6 colony forming units (CFU)/mL. Serial dilutions of test agents were incubated with bacteria at 37ºC in the presence of 5% CO_2 in a humidified incubator for 24 hours before visually recording the MICs.

2.4. Evaluation of colonization efficiency of the new mutants in a gonococcal infection mouse model

All procedures involving live mice were approved by the Institutional Animal Care and Use Committee of Virginia Polytechnic Institute and State University. *In vivo* colonization of the strains of *N. gonorrhoeae* described above in the lower genital tract of mice was assessed as described elsewhere (Huang et al. 2020; Elhassanny, Abutaleb, and Seleem 2022; Abutaleb, Elhassanny, and Seleem 2022; Raterman and Jerse 2019; Gulati et al. 2020; Gulati et al. 2015). Eight-week-old female ovariectomized BALB/c mice were subcutaneously implanted with 5-mg, 21-day controlled-release estradiol pellets (Innovative Research of America, Sarasota, FL). Mice were injected intraperitonially with 4 mg/L of vancomycin and 24 mg/L of streptomycin on days −2 through +1. The drinking water was replaced on day −2 with water containing 0.4 g/L trimethoprim. Streptomycin sulfate (5 g/L) was added to drinking water after day +1 until the end of the experiment. Antibiotic-containing water was replaced every other day for the duration of the experiment.

Two days after pellet implantation, mice were randomly allocated into groups (n=6) and were inoculated

intravaginally using three different inoculum sizes (10^7, 10^6, and 10^5 CFU/mouse) for each mutant strain. For WHO-X-*rpsL*A128G, an inoculum of 3.04×10^9 CFU/ml was prepared, and one group of mice (WHO-X-*rpsL*A128G_10^7) was infected with 15 µL of this inoculum to achieve an infectious dose of 4.56×10^7 CFU/mouse. The inoculum was also diluted 10 times and 100 times for infection of groups WHO-X-*rpsL*A128G_10^6 and WHO-X-*rpsL*A128G_10^5, respectively. Similarly, for the CDC-181-*rpsL*A128G, an inoculum of $4.58\text{x}10^9$ CFU/ml was prepared, and one group of mice (CDC-181-*rpsL*A128G_10^7) was infected with 15 µL of this inoculum to achieve 6.88×10^7 CFU/mouse. The inoculum was also diluted 10 times and 100 times for infection of groups CDC-181-*rpsL*A128G_10^6 and CDC-181-*rpsL*A128G_10^5, respectively. Additionally, one group of mice was infected with *N. gonorrhoeae* FA 1090 (3.56×10^6 CFU/mouse) as a control.

Twenty-four hours post-infection, a vaginal sample from each mouse was collected by gently inserting the entire soft tip of a swab in the vagina and rolled before suspending in 100 µL of GC broth containing 0.05% saponin (TCI America). Samples were taken daily from day 1 through day 14 post-infection. Serial dilutions of samples were performed, and samples were plated onto GCB agar plates containing vancomycin, colistin, nystatin, and trimethoprim. Plates were incubated at 37ºC with 5% CO_2 in a humidified incubator for 24 hours to determine the vaginal colony counts. To monitor the presence of commensal flora that could potentially inhibit the growth of *N. gonorrhoeae*, vaginal swabs were streaked on brain heart infusion agar (BD) and the resulting growth was Gram stained. Contaminated samples were excluded from the experiment.

2.5. Statistical analyses

GraphPad Prism version 9.0 for Windows (GraphPad Software, La Jolla, CA) was used to conduct statistical analysis on mouse vaginal CFU loads and percentage of mice infected. The data were analyzed using two-way ANOVA with post-hoc Dunnett's test for multiple comparisons ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Construction of streptomycin-resistant mutants of *N. gonorrhoeae* strains CDC-181 and WHO-X

In this study, we used strains, *N. gonorrhoeae* CDC-181 and WHO-X, that are resistant to frontline antibiotics, azithromycin and ceftriaxone. The resistance of *N. gonorrhoeae* CDC-181 to azithromycin is mediated by a mutation in the 23S rRNA gene, which is a component of the 50S subunit. This mutation can reduce the binding of azithromycin to the ribosome, making the antibiotic less effective (Unemo et al. 2016; Unemo and Shafer 2014; Unemo, Golparian, and Hellmark 2014; Ng et al. 2002). *N. gonorrhoeae* WHO-X is ceftriaxone-resistant due to a mutation in the penA gene (a mosaic penA allele) (Unemo et al. 2016; Unemo and Nicholas 2012; Unemo and Shafer 2015, 2014). According to the sequencing data, our mutagenesis procedure knocked out the native *rpsL*

gene from the parent strains of *N. gonorrhoeae* CDC-181 and WHO-X and knocked in the mutated *rpsL* gene in the place of *rpsL* in the genome. This created a single base change, A128G, within the *rpsL* gene that led to a single amino acid change (K43R) in the encoded amino acid sequence of the 30S ribosomal protein S12.

3.2. MICs of standard antibiotics and anti-gonococcal clinical molecules against CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G

We then assessed whether the genetic alteration in the *rpsL* gene of the new mutants caused any changes in their susceptibility to antibiotics. The MIC of streptomycin against the two mutant strains, CDC- 181Δ*rpsL* and WHO-X-*rpsL*A128G, was more than 32-fold greater than the parent strains, CDC-181 and WHO-X (Table 2). Unlike the streptomycin-resistant strain FA1090, our mutant CDC-181-*rpsL*A128G was resistant to both streptomycin and azithromycin. Moreover, unlike the FA 1090 strain, mutant WHO-X-*rpsL*A128G was resistant to both streptomycin and ceftriaxone. Furthermore, our mutants, CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G, still possessed the same MIC values with other antibiotics against *N. gonorrhoeae* when compared to their respective parent strains (Table 2). This indicates that the *rpsL* gene mutation in *N. gonorrhoeae* CDC-181 and WHO-X rendered the strains highly resistant to streptomycin while they retained the same MIC profile with other antibiotics when compared to their respective parent strains. Therefore, the mutation did not impact the activity of CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G other than making them resistant to streptomycin.

3.3. The *N. gonorrhoeae* mutants CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G can effectively colonize the lower genital tract of mice

Since our CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G mutants are comparable to *N. gonorrhoeae* FA 1090 in terms of resistance to streptomycin, we assessed the colonization efficiency of the two mutants in a gonococcal infection mouse model. Mice were inoculated intravaginally with 10^5, 10^6, or 10^7 CFU/mouse of mutants CDC-181-*rpsL*A128G or WHO-X-*rpsL*A128G. The vaginal colony counts were determined daily for 14 days post-infection. An additional group of mice infected with *N. gonorrhoeae* FA 1090 (10^6 CFU/mouse) served as a control. *N. gonorrhoeae* FA 1090 has been utilized extensively in an experimental urethritis model in male volunteers and the female mouse model of infection (Hobbs et al. 2011). In this study, *N. gonorrhoeae* FA 1090 was able to maintain the infection for at least 12 days at the dose of 10^6 CFU/mouse, which was in agreement with previous reports (Jerse 1999; Jerse et al. 2002).

Between 4.52 to 5.36 $\log_{10}$ CFU/mL of *N. gonorrhoeae* FA 1090 was recovered from the swab samples during the first 10 days after infection. Thereafter, the bacterial counts for this strain started to gradually decline after day 10 until it reached 3.2-$\log_{10}$ CFU/mL by day 14 (Fig. 1A). The decline in the bacterial count in this strain is attributed to a decrease in the percentage of colonized mice.

As depicted in Fig. 1B, 100% of mice were colonized with *N. gonorrhoeae* FA 1090 until day 10. On day 11, the percentage of colonized mice decreased to 66.7% and continued to decrease until it became 50% by day 14.

For the CDC-181-*rpsL*A128G mutant, over 4.5-$\log_{10}$ CFU/mL was recovered from mice inoculated with all three doses of the mutant strain. The bacterial counts of the mutant declined slightly between days 4 to 6 but increased gradually thereafter (Fig. 1A). All mice infected with 10^5 and 10^7 CFU remained colonized with the mutant until day 14 (100% colonization). On the other hand, mice infected with 10^6 CFU of CDC-181-*rpsL*A128G remained colonized with the mutant until day 13 at which point one mouse cleared the infection resulting in 83.3% of this group remaining colonized until the end of the experiment (Fig. 1B). Overall, these results suggest that the mutant CDC-181-*rpsL*A128G was capable of efficiently colonizing and proliferating in the genital tract of infected mice up to 14 days after infection (Fig. 1A and 1B).

In mice inoculated with 10^5 and 10^6 CFU of the mutant WHO-X-*rpsL*A128G strain, bacterial counts gradually decreased between days 1 and 3; the count remained steady at around 4-$\log_{10}$ CFU/mL thereafter until the end of the experiment on day 14. In contrast, in the group infected with 10^7 CFU/mouse of the WHO-X-*rpsL*A128G strain, the bacterial counts gradually declined throughout the study period until it reached ~2.5-$\log_{10}$ CFU/mL by day 12 and remained unchanged until day 14 post-infection (Fig. 2A). Mice infected with the lowest inoculum of WHO-X-*rpsL*A128G (10^5 CFU/mouse) demonstrated the highest colonization efficiency where 100% of mice were colonized until day 8. Thereafter, the percent colonization reduced to 83.3% on day 9 and remained consistent until the end of the experiment. This was followed closely by the 10^6 CFU/mouse group which showed 66.7% colonization at the end of the experiment. Conversely, mice infected with the highest inoculum of WHO-X-*rpsL*A128G (10^7 CFU/mouse) displayed the lowest colonization efficiency, which reached 33.3% colonization on day 10 and remained at that level until day 14 (Fig. 2B). These results (Figs. 2A and 2B) indicate that the lower infectious dose of the mutant WHO-X-*rpsL*A128G strain (10^5 CFU/mouse) can colonize and proliferate in the lower genital tract of mice more efficiently than the higher infectious doses (10^6 and 10^7 CFU/mouse). The bacterial CFU count in the case of WHO-X-*rpsL*A128G (10^5 and 10^6 CFU/mouse) was statistically different from that of WHO-X-*rpsL*A128G (10^7 CFU/mouse); the p-values on day 14 were 0.0014 and 0.0050, respectively.

In conclusion, to facilitate drug discovery for the treatment of gonorrhea, we developed streptomycin-resistant mutants of *N. gonorrhoeae* that are naturally resistant to frontline antibiotics, azithromycin and ceftriaxone. We also were able to identify the optimum inoculum size for each mutant in a mouse model of gonorrhea. This study determined that both mutants (CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G) can effectively colonize the lower genital tract of mice which could enhance the *in vivo* evaluation of potential anti-gonococcal agents against multidrug-resistant *N. gonorrhoeae*. Our study provides valuable tools for drug discovery and development.

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DECLARATIONS

Conflict of interest: none declared.

ETHICAL APPROVAL

All procedures related to handling and housing of experimental animals were reviewed and approved by the Virginia Tech Institutional Animal Care and Use Committee and carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

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Table 1. *Neisseria gonorrhoeae* strains used or constructed in this study.

Strain	Description
FA 1090	Obtained from the American Type Culture Collection (ATCC 700825). Naturally resistant to streptomycin. Isolated in 1983 from a patient with a disseminated gonococcal infection.
CDC-181	Obtained from the Centers for Disease Control and Prevention (CDC). Resistant to azithromycin and tetracycline.
WHO-X (H041)	Obtained from the CDC. Multidrug-resistant strain and the first high-level ceftriaxone-resistant gonococcal strain isolated from the pharynx of a female patient in Japan in 2009 following ceftriaxone treatment failure. Resistant to ceftriaxone, cefixime, penicillin, ciprofloxacin, and tetracycline (Ohnishi, Golparian, et al. 2011; Ohnishi, Saika, et al. 2011).
CDC-181-*rpsL*A128G	Generated in this study. Δ*rpsL* mutant of CDC-181 (resistant to streptomycin and azithromycin).
WHO-X-*rpsL*A128G	Generated in this study. Δ*rpsL* mutant of WHO-X (resistant to streptomycin and ceftriaxone)/

Table 2. Minimum inhibitory concentration values (µg/mL) of standard antibiotics and anti-gonococcal clinical molecules against *Neisseria gonorrhoeae* mutants CDC-181-*rpsL*A128G and WHO-X-*rpsL*A128G along with *N. gonorrhoeae* strains FA 1090, CDC-181, and WHO-X.

Test agents	**FA 1090**	**CDC-181**	**CDC-181-*rpsL*A128G**	**WHO-X**	**WHO-X-*rpsL*A128G**
Streptomycin	>1024	32	>1024	32	>1024
Penicillin	0.06	0.06	0.03	1	1
Ceftriaxone	0.002	0.0078	0.0078	1	1
Tetracycline	1	1	1	2	2
Doxycycline	0.25	2	2	4	4
Azithromycin	0.0625	1024	1024	1	1
Ciprofloxacin	0.0078	0.03	0.015	>64	>64
Zoliflodacin	0.03	0.125	0.06	0.06	0.06
Gepotidacin	0.06	0.125	0.125	0.125	0.125

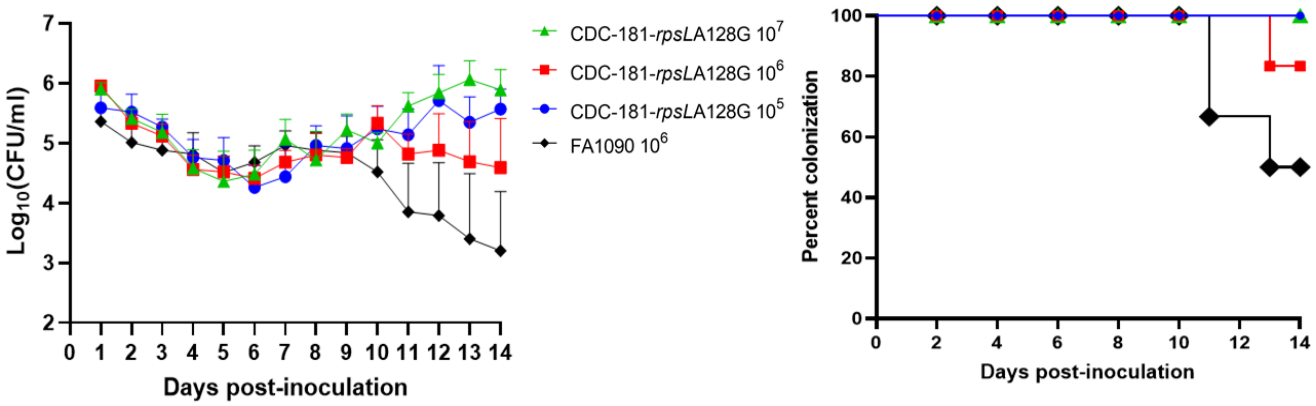

Figure 1. Colonization of mice by the streptomycin-resistant CDC-181-*rpsL*A128G strain. Groups of female ovariectomized BALB/c mice were inoculated intravaginally with 10^5, 10^6, or 10^7 CFU/mouse of *N. gonorrhoeae* CDC-181-*rpsL*A128G and vaginal bacterial counts were determined. A group of mice inoculated with 10^6 CFU/mouse of *N. gonorrhoeae* FA 1090 served as a control. (A) The bacterial counts of mice inoculated with the mutant CDC-181-*rpsL*A128G strain and the control group. (B) The percentage of mice colonized with the mutant CDC-181-*rpsL*A128G strain and the control strain. The data are shown as the average CFU from 6 mice/group.

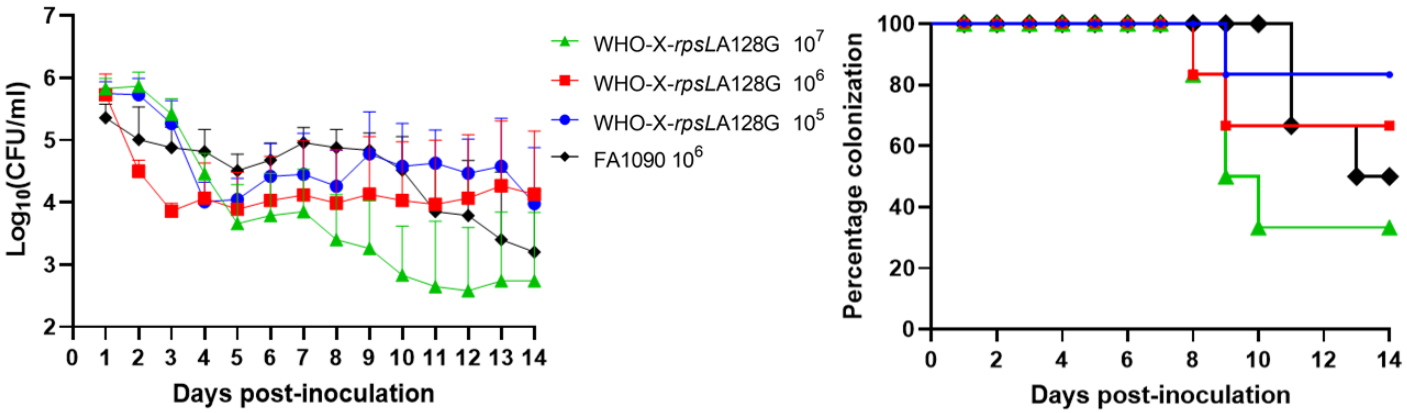

Figure 2. Colonization of mice by the streptomycin-resistant WHO-X-*rpsL*A128G strain. Groups of female ovariectomized BALB/c mice were inoculated intravaginally with 10^5, 10^6, or 10^7 CFU/mouse of *N. gonorrhoeae* WHO-X-*rpsL*A128G and vaginal bacterial counts were determined. A group of mice inoculated with 10^6 CFU/mouse of *N. gonorrhoeae* FA 1090 served as a control. (A) The bacterial counts of mice inoculated with the mutant WHO-X-*rpsL*A128G strain and the control group. (B) The percentage of mice colonized with the mutant WHO-X-*rpsL*A128G strain and the control strain. The data are shown as the average CFU from 6 mice/group.