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Experimental Human Infection with *Neisseria gonorrhoeae*

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Abstract

Experimental infection of male volunteers with *Neisseria gonorrhoeae* is safe and reproduces the clinical features of naturally acquired gonococcal urethritis. The human model is useful for testing the importance of putative gonococcal virulence factors for urethral infection in men and the model presents opportunities to examine host immune responses that may be exploited or improved in development and testing of gonococcal vaccines. In this chapter, we describe methods for production, characterization, and storage of *N. gonorrhoeae* stocks for experimental human challenge, preparation and delivery of inoculum suspensions, monitoring experimental infection, and statistical considerations for data analysis.

Keywords

Neisseria gonorrhoeae; Gonococcal urethritis; Experimental human infection

1 Introduction

The exclusive adaptation of *Neisseria gonorrhoeae* to its human host has long challenged the use of animal models to study gonorrhea and has hampered vaccine development. A murine model, in which estradiol-treated female mice are colonized in the lower reproductive tract with ascending infection in approximately 20% of infected mice, has greatly facilitated systematic vaccine testing [1] (see also Chapter 24 by Ratterman and Jerse). The relatively recent development of transgenic mice that relieve some of the host restrictions has the potential to enhance the relevance of mouse studies to human disease caused by *N. gonorrhoeae* [2–4]. Nevertheless, experimental human urethral infection in male volunteers, established decades ago as a safe and highly relevant model of natural infection in men [5], remains an important tool in gonorrhea vaccine and microbial pathogenesis research.

Experimental gonorrhea is restricted to male subjects; women are excluded due to potential reproductive complications from ascendant gonococcal infection. Inoculation is accomplished by the instillation of a bacterial suspension through a catheter inserted into the distal end of the anterior urethra. After inoculation, infection can be monitored by bacterial recovery in urogenital specimens; nucleic acid amplification-based testing (NAAT) may also prove to be a useful method to monitor infection in the model. Host responses can be

assessed by enumeration of white blood cells in urine and urethral exudates and measurement of soluble immune mediators including antibodies and cytokines or mRNA in urogenital specimens and serum. Clinical manifestations of gonococcal infection are monitored by daily interview and physical examination. Under currently approved protocols, subjects receive prompt effective antibiotic treatment when (1) they request treatment regardless of signs, symptoms or positive cultures, (2) purulent urethral discharge is observed by the examining clinician or reported by the subject, or (3) at the end of the experimental period, usually 5 days after inoculation, regardless of whether or not the subject is judged to have been infected. A negative *N. gonorrhoeae* NAAT from urine obtained 3–7 days after antibiotic treatment is required to document clearance of experimental infection. A final follow-up interview by telephone is conducted approximately 7 days after the test of cure to assess study-related adverse events.

Two *N. gonorrhoeae* strains, FA1090 and MS11mkC, have been used in the majority of experimental human infection studies [5]. In this chapter, we describe current methods for production, characterization, and storage of *N. gonorrhoeae* stocks for experimental human challenge, preparation and delivery of inoculum suspensions, and monitoring experimental infection. We note that rigorous safety and ethical review are essential for the protection of human subjects in experimental gonococcal infection studies; however, the details of these and other regulatory aspects of study design are beyond the scope of the methods described herein.

2 Materials

2.1 *Neisseria gonorrhoeae* Working Banks

Biosafety level 2 facilities are required in a research laboratory and in a location near (adjacent to, or within a 1–2 min walk, in the same building) the clinical site(s) where subjects will be inoculated and specimens to monitor experimental infection will be obtained. In addition, ready access to an accredited clinical microbiology laboratory is needed for microbial characterization of *N. gonorrhoeae* strains to be used for experimental human infection.

2.1.1 Bacterial Strains and Media

1. *Neisseria gonorrhoeae* strains FA10910, MS11mkC, or other wild-type or isogenic mutant strains, stored at -70°C in Freezer Storage Medium (FSM).
2. *Staphylococcus aureus* and *Escherichia coli* bacteria stored as frozen stocks at -70°C .
3. Freezer Storage Medium (FSM): mix 3 g of Trypticase soy broth powder (see **Note 1**) and 25 mL of glycerin in a 100 mL graduated cylinder. Add distilled, deionized (dd) H_2O to 100 mL final volume. Mix with a magnetic stirrer until all the ingredients are dissolved. Dispense 0.5 mL into sterile 2 mL freezer storage vials and cap loosely. Autoclave for 15 min at 121°C and 2.68 kg/cm^2 with slow exhaust. Remove from the autoclave and cool to room temperature. Tighten the caps and store vials at 4°C . Label racks with “FSM” and the “date of

preparation,” leaving individual tubes to be labeled with strain information as they are used.

Each batch is tested for sterility and ability to support gonococcal viability. To test for sterility, choose several vials chosen at random from the batch, and plate 100 μ L onto GCB agar plates and incubate overnight in a humidified incubator set at 37 °C with 5% (v/v) CO₂. The plates must be free of microbial growth after 24 h.

To test gonococcal viability, inoculate several vials chosen at random from the batch with gonococci grown for 18–24 h on GCB agar plates. Mix well and freeze vials at –70 °C for at least 24 h. Without allowing the contents of the vials to thaw, scrape a small amount of frozen bacterial suspension from each test vial with a sterile microbiological loop and streak a fresh GCB agar plate as shown in Fig. 1. Incubate the plates at 37 °C with 5% (v/v) CO₂ for 24 h. Plates should contain areas of heavy growth and isolated gonococcal colonies as shown.

4. GCB agar plates (see **Notes 1 and 2**): add 36 g of Difco™ GC medium base per liter of ddH₂O to a flask with a magnetic stir bar. Autoclave for 15 min at 121 °C and 2.68 kg/cm² on a slow exhaust cycle. After autoclaving, place the flask in a 56 °C water bath to cool. When cool, add 10.0 mL of Supplement I and 1.0 mL of Supplement II per liter of medium. Mix thoroughly on a magnetic stir plate to incorporate the supplements. Working on a clean, disinfected benchtop free from drafts, pour ~10 mL of medium per plastic petri plate (100 mm × 15 mm). Avoid bubbles in the medium. Invert plates when cooled and solidified. Select two plates at random from each batch of medium; streak one plate with *N. gonorrhoeae* and leave the remaining plate uninoculated. Incubate test plates overnight for GCB. Batches that support the growth of *N. gonorrhoeae* and yield no contaminant growth on the uninoculated plate are suitable for use in experimental human infection studies.
5. GCB-VCNT agar plates: aseptically reconstitute the inhibitor mixture (Remel) with sterile H₂O, mix and add 10 mL of reconstituted solution per litre of cooled GCG agar medium. The concentrations of inhibitors in the reconstituted solution are: 400 μ g/mL of vancomycin; 750 μ g/mL of colistin; 1250 units/mL of nystatin; 625 μ g/mL of trimethoprim lac-tate. Mix thoroughly on a magnetic stir plate to incorporate the supplements and inhibitors. Working on a clean, disinfected benchtop free from drafts, pour ~10 mL of medium per plastic petri plate (100 mm × 15 mm). Avoid bubbles in the medium. Invert plates when cooled and solidified. Store the inverted plates at 4 °C in plastic sleeves, dated and labeled. Prepare the plates within 2 weeks of an experimental infection start date. Test for sterility and ability to support gonococcal growth as described above for GCB agar plates, except incubate the plates for 48 h for GCB-VCNT.
6. Supplement I: add 400 g of glucose (dextrose) to a large glass beaker and add ddH₂O to approximately 900 mL volume. Carefully add a magnetic stir bar and mix on a heated stir plate until sugar is dissolved. Cool the solution completely to room temperature and add 10 g of L-glutamine and 20 mg of cocarboxylase,

and mix. When all chemicals are in solution, transfer to a graduated cylinder (reserving the stir bar) and adjust the volume to 1 L with ddH₂O. Mix thoroughly and sterilize by filtration using a 0.22 µm filter, and aseptically dispense 100 mL aliquots into sterile, labeled foil-wrapped reagent bottles. Store Supplement I at 4 °C.

7. Supplement II: add 1.25 g Fe(NO₃)₃·9H₂O into a 250 mL graduated cylinder, and add ddH₂O to the 250 mL mark. Mix with a magnetic stirrer and when Fe(NO₃)₃·9H₂O is completely dissolved, filter-sterilize the solution using a 0.22 µm filter. Aseptically dispense 25 mL aliquots into sterile labeled reagent bottles and store Supplement II at 4 °C.
8. 100 mm × 15 mm sterile plastic petri plates.
9. Humidified incubator set to 37 °C with 5% (v/v) CO₂.
10. 2 mL sterile freezer storage vials.
11. Sterile microbiological loops.
12. LB agar plates (*see Note 1*): add 20 g of LB agar powder per litre of ddH₂O to a flask with a magnetic stir bar. Autoclave for 15 min at 121 °C and 2.68 kg/cm² on slow exhaust cycle. After autoclaving, place the flask in a 56 °C water bath to cool. Working on a clean, disinfected benchtop free from drafts, pour ~10 mL medium per plastic petri plate (100 mm × 15 mm). Avoid bubbles in medium. Invert plates when cooled and solidified. Store inverted plates at 4 °C in plastic sleeves dated and labeled LB agar. Select four plates at random from each batch of medium; streak one plate each with *N. gonorrhoeae*, *S. aureus* and *E. coli*, and leave the remaining plate uninoculated. Incubate the test plates overnight in a humidified incubator set at 37 °C with 5% (v/v) CO₂. Batches that support the growth of *S. aureus* and *E. coli* but not *N. gonorrhoeae* and yield no contaminant growth on the uninoculated plate are suitable for use in experimental human infection studies.

2.1.2 Preparation of Working Banks with a Single Strain

1. *Neisseria gonorrhoeae* strains FA10910, MS11mkC, or other wild-type or isogenic mutant strains, stored at –70 °C in Freezer Storage Medium (FSM).
2. Freezer Storage Medium (FSM).
3. GCB agar plates.
4. GCB-VCNT agar plates.
5. Supplement I.
6. Supplement II.
7. 100 mm × 15 mm sterile plastic petri plates.
8. Stereomicroscope with a sub-stage light reflector.
9. Humidified incubator set to 37 °C with 5% (v/v) CO₂.

10. Sterile cotton-tipped swabs.
11. Sterile microbiological loops.
12. Sterile glass tubes that fit into chamber of spectrophotometer, with lids.
13. 2 mL sterile freezer storage vials.
14. LB agar plates.
15. 1 × sterile Dulbecco's phosphate-buffered saline (PBS), without calcium and magnesium. Commercially prepared buffer contains 0.20 g/L of KCl, 0.2 g/L of KH₂PO₄, 8.0 g/L of NaCl, and 1.15 g/L of Na₂HPO₄ (anhydrous) at a final pH of 7.4 ± 0.1 and osmolality of 276 ± 10 mOsm. It contains 0.25 endotoxin units/mL and is negative for *Mycoplasma species* as determined by culture. Store the product at 2–30 °C for up to 36 months.
16. 10 cc sterile syringe fitted with 1.2 µm filter.
17. Monoclonal antibodies and reagents for confirming piliation status (Pil), opacity protein (Opa) and lipooligosaccharide (LOS) expression.
18. Commercial whole-genome sequencing service.
19. Benchtop vortex mixer.
20. Genesys 30 spectrophotometer, or equivalent.

2.1.3 Preparation of Working Banks with Two Strains for Competitive Infections

1. All materials described in Subheadings 2.1.1 and 2.1.2.
2. GCB broth (*see Note 1*): add 15 g of proteose peptone, 5 g of NaCl, 4 g of K₂HPO₄, 1 g of KH₂PO₄ per liter of ddH₂O. Mix well and dispense into reagent bottles, autoclave for 15 min at 121 °C and 2.68 kg/cm² and store at room temperature.

2.2 Catheter Preparation

1. Sterile No. 8 paediatric French feeding catheter.
2. Scissors or scalpel.
3. Sterilization indicator strip.
4. Self-sealing pouches.
5. Low temperature ethylene oxide sterilization facility.

2.3 Preparation of Inocula for Experimental Human Infection

1. Neisseria gonorrhoeae characterized working banks.
2. 10 µL sterile microbiological loops.
3. GCB agar plates.

4. Humidified incubator set to 37 °C with 5% (v/v) CO₂.
5. Stereomicroscope with a sub-stage light reflector.
6. Oxidase reagent (BBL oxidase reagent droppers or equivalent).
7. Dead air box with a clean, decontaminated surface.
8. Sterile PBS, pH 7.4.
9. Sterile cotton-tipped swabs.
10. Sterile toothpick or wooden applicator stick.
11. Filter paper.
12. Genesys 30 spectrophotometer, or equivalent.
13. 50 mL sterile conical centrifuge tubes.
14. 2 mL sterile tubes.
15. FSM.
16. 2 mL sterile freezer storage vials.
17. LB agar plates.

2.4 Inoculation Procedure

1. Consented human male subjects (*see* **Note 3**).
2. Sterile gloves and waterproof plastic drapes.
3. 70% (v/v) isopropyl alcohol wipes, individually packaged.
4. Gas-sterilized No. 8 pediatric French catheter and hemostat clamp.
5. 0.9% (v/v) NaCl, sterile saline.
6. *Neisseria gonorrhoeae* suspension for inoculation.
7. Filtered pipette tip and P-1000 micropipettor (Pipetman or equivalent).
8. 50 mL sterile centrifuge tube.
9. Benchtop centrifuge.
10. GCB agar plates.

2.5 Monitoring Experimental Infection

1. Antibiotics.
2. Sterile urine specimen cups encircled with a marker at the 50 mL line.
3. 15 and 50 mL sterile centrifuge tubes for urine processing.
4. Sterile swabs with calcium alginate fiber tip for urethral swab collection.
5. GCB agar plates for bacterial recovery.

6. Assays for bacterial and/or host gene expression (e.g., transcriptomics, WGS).
7. Assays for host responses (e.g., for soluble immune mediators).

2.6 Statistical Considerations

1. One-sided Fisher's exact test.
2. Kaplan–Meier survival curves.
3. Competitive Indices (CI).

3 Methods

3.1 *Neisseria gonorrhoeae* Working Banks

3.1.1 Bacterial Strains and Media

1. Prepare lots of as many identical single-use vials for each *N. gonorrhoeae* strain as is practical for the intended study design. For example, working banks of wild-type gonococci for use in studies to compare experimental prevention or treatment methods, or as controls in isogenic mutant studies of gonococcal pathogenesis should contain 100 vials, to maximize comparability over time. Stocks of individual isogenic mutant strains for more limited use in individual pathogenesis studies may contain 20 or fewer vials. In addition, take into account the number of vials needed for characterization and stability testing, when determining the size of the working bank. Periodically take account of working bank inventory, and prepare a new working bank from previously characterized stock before it is depleted. All steps below should be done in a Biosafety 2 level cabinet to minimize the likelihood of contamination.

3.1.2 Preparation of Working Banks with a Single Strain

1. Use a sterile microbiological loop to transfer *N. gonorrhoeae* bacteria from a frozen stock vial of the desired strain and spread onto GCB agar plates in a pattern to yield isolated colonies. Incubate for ~24 h in a humidified atmosphere containing 5% (v/v) CO₂ at 37 °C.
2. Examine gonococcal growth through a stereo microscope with a sub-stage light reflector for the desired piliation and opacity characteristics. Historically, selected colonies are piliated (shiny, tightly domed colonies) and primarily opacity protein (Opa)-negative (transparent, as opposed to the sparkling, opaque quality of colonies formed by Opa+ gonococci).
3. Expand gonococcal cultures in a single passage from individual colonies. Pick 1–5 colonies, depending on the desired size of the working bank stock, and streak one single colony per GCB agar plate to maximize bacterial growth, of the previously characterized isolate.
4. Incubate for ~24 h in a humidified atmosphere containing 5% (v/v) CO₂ at 37 °C and visually inspect the plates carefully. Discard any plates with visible contaminating colonies.

5. Harvest 24 h bacterial growth from GCB plates containing only *N. gonorrhoeae* growth using sterile cotton-tipped swabs. Suspend material from all plates into FSM in a single, sterile tube (see **Note 4**). Use approximately 2 mL of FSM for each plate of *N. gonorrhoeae* to be harvested. Cap the tube and vortex-mix to create a uniformly dispersed suspension.
6. Aseptically transfer 0.1 mL of the gonococcal suspension to sterile freezer storage vials labeled clearly with the strain designation, date of preparation and a notation that the contents are for investigational use only. Store the vials at -70°C (see **Note 5**).
7. Confirm the purity of the frozen product by plating 20 μL from one vial onto GCB agar and streaking to obtain areas of heavy growth and areas of isolated colonies. Plate the remainder of the contents of the vial onto one LB agar plate. Incubate all agar plates for ~ 24 h in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C . If any bacterial growth is observed on LB agar, or if no growth is observed on GCB agar after incubation, discard the entire lot of vials. Submit bacterial growth on GCB agar to the clinical microbiology laboratory for biochemical identification as *N. gonorrhoeae* and antimicrobial susceptibility testing to confirm susceptibility to the antibiotics specified for treatment of experimental infection (see **Note 6**).
8. From another vial of a lot that passes the purity and identification tests, confirm the key bacterial characteristics of the frozen bacteria. Plate the contents of a test vial onto GCB to obtain isolated colonies, and repeat **step 3** to test single passage growth as needed to meet the goals of the study. Typically variable characteristics such as piliation, Opa protein and LOS expression are confirmed for each lot (see **Note 7**). For *N. gonorrhoeae* strain FA1090, monoclonal antibodies (mAbs) that recognize the Opa proteins are available [6]. Use a cocktail of these mAbs in standard immunoblots of ~ 100 colonies to estimate the proportion of the population that is Opa-negative. Confirm piliation by quantitative transformation efficiencies of 10^{-4} or higher, and identify the specific *pilE* sequence by DNA sequencing. Confirm the expression of the lacto-N-neotetraose LOS epitope that can be sialylated by western blot with mAb 3F11 of proteinase K-treated bacterial lysate separated by SDS-PAGE [7] (see **Note 8**).
9. Purify DNA from gonococcal strains for Whole Genome Sequencing (WGS, see **Note 9**), which is strongly recommended for each lot of *N. gonorrhoeae* used in experimental human infection studies. Identical sequences for strains prepared after even minimal passage from previously characterized lots are unlikely, due to the plasticity of the gonococcal genome. However, WGS data allow comparisons between strains that may help explain unexpected experimental infection outcomes that may result from unanticipated differences not recognized by strain characterization as described above.
10. The concentration of *N. gonorrhoeae* in working stock vials stored at $<-70^{\circ}\text{C}$ is not specified, but bacterial suspensions are quite dense. Bacteria grown on GCB

agar from 20 μ L of suspension should yield areas of heavy growth as well as areas of isolated colony growth consistent with typical microbiological streaking technique. Such gonococcal suspensions are stable for many years at -70°C . To document the stability of stocks for clinical use in experimental human infection studies, enumerate the cfu/mL from a single vial every 18 months after initial preparation by streaking the contents on GCB agar plates and viable counting. If the concentration of *N. gonorrhoeae* in the vial is $<10^5$ cfu/mL, a new stock should be generated. Any remaining vials from a stock that fails stability testing should be removed from product storage location (see **Note 5**) and labeled not for human use, but may be used to generate a new working stock.

11. Determine the relationship between optical density of filtered bacterial suspension in PBS and cfu/mL for each working stock of *N. gonorrhoeae* prior to use in experimental human infection (see **Note 10**). From 18–24 h bacterial growth on GCB agar, harvest gonococci with a sterile cotton-tipped swab and prepare a suspension of by swirling the bacteria into 6 mL of sterile PBS in a sterile spectrophotometer tube (see **Note 4**). The amount of bacteria collected in three swift strokes is usually sufficient. We measure absorbance at λ_{550} nm (A_{550}) in a Genesys 30 spectrophotometer. Read and record absorbance of the initial, unfiltered suspension. Attach a sterile 1.2 μ M filter to the barrel of a sterile 10 cc syringe from which the plunger has been removed (see **Note 11**). Carefully pour the gonococcal suspension from the tube into the syringe, insert the plunger and gently force the fluid gently through the filter, collecting the filtered suspension into a sterile tube. Read and record the absorbance of the filtered suspension. Prepare a series of dilutions in PBS for quantitative culture on GCB agar. Incubate the GCB agar plates in a humidified atmosphere with 5% (v/v) CO_2 at 37°C and determine the cfu/mL in the filtered suspension by viable counting. Repeat with multiple solutions across a range of optical densities.

3.1.3 Preparation of Working Banks with Two Strains for Competitive

Infections—Competitive infections with mixed gonococcal inocula allow identification of fitness differences between strains that may not be evident in noncompetitive infections with individual strains. Typically, mixed inocula contain approximately equal numbers of the two strains.

1. Prepare individual working stocks for each strain and characterize them as described in Subheading 3.1.1.
2. Plate material from a single vial from each frozen gonococcal stock onto GCB agar plates and incubate for ~ 24 h in a humidified atmosphere containing 5% (v/v) CO_2 at 37°C .
3. Prepare a suspension of each individual strain with bacteria harvested from an agar plate with a sterile cotton swab, swirled into GCB broth (see **Note 4**). Estimate the bacterial concentration from the optical density of the suspension as described in **step 11** in Subheading 3.1.2.

4. To prepare the desired mixture, combine approximately equal concentrations of the two strains, add an equal volume of 2× FSM, mix thoroughly, and dispense aliquots into clearly labeled vials and freeze as described in **step 6** of Subheading 3.1.2 (see **Note 5**). Subculture the contents of one vial of the mixture onto GCB agar, plating a dilution to yield 100–300 cfu per plate, and enumerate the cfu of each strain. Individual strains may be identified by PCR with specific primers or by subculture onto differential, selective media to determine the proportion of each strain in the mixture. Aim for a 50:50 ratio of the strains and discard any mixtures exceeding a 45:55 ratio. Characterize the mixed stocks and do periodic stability testing as described above in **steps 8** and **10** of Subheading 3.1.2.

3.2 Catheter Preparation

1. Remove sterile No. 8 pediatric French feeding catheters from their original packaging, and cut to 8 cm in length using clean scissors or scalpel (sterility is not important at this stage as catheters will be resterilized before use). Mark the catheter with a permanent laboratory marker at the 5 cm mark; this will guide insertion to the proper depth into the anterior urethra. Encircle the outside of the catheter completely (Fig. 2).
2. Insert one catheter and a sterilization indicator strip into a self-sealing pouch. Ensure that the open end of the catheter is orientated toward the end of the pouch to be opened for use. Peel off the protective strip, fold along the perforation, and press firmly to seal the pouch. Prepare batches of 10–50 individually packaged catheters and transport them to a local facility for sterilization.
3. Low temperature ethylene oxide sterilization is important to minimize shrinkage of the plastic catheters, which can occur with steam sterilization and compromise the accuracy of the marked guide for insertion into the urethra. After sterilization, label catheters with the sterilization date and store at ambient temperature, protected from light. Catheters remain sterile indefinitely, unless the package is opened or damaged. For urethral inoculation, do not use catheters more than 1 year after the sterilization date. At least 48 h before use, inspect pouches carefully. Use only catheters in undamaged pouches. If the outer pouch is opened, torn or otherwise damaged, the intact catheter can be transferred to a new pouch, resterilized and used for inoculation. Discard any catheter that is bent, twisted, crushed, or otherwise damaged.

3.3 Preparation of Inocula for Experimental Human Infection

In this protocol, Day 0 refers to the day of inoculation, Day –1 is the day before inoculation, and Days 1, 2, 3 etc. are days after inoculation.

1. On Day –1, remove a single vial of a gonococcal working stock that has passed all required lot-release tests from the freezer, and allow the contents to thaw at room temperature in a clean laminar flow hood. Thawing takes no more than a few minutes: immediately dispense the material aseptically onto five individual GCB agar plates, with approximately 20 µL per plate. Streak the surface of the

agar with a sterile microbiological loop in a pattern to yield areas of dense growth as well as areas of isolated colony growth (Fig. 1). Bacterial growth should be 18–24 h old for inoculation; determine the time that cultures should be started on Day –1, based on the expected time of inoculation of subjects on Day 0. Incubate inverted GCB agar plates overnight at 37 °C in an humidified atmosphere containing 5% (v/v) CO₂.

2. On Day 0, 1–2 h before the expected inoculation time, thoroughly examine GCB agar plates by eye and with a microscope for purity and correct colony phenotype. Discard any plates containing contaminating bacteria. Mark areas of the plates containing gonococcal growth with undesirable phenotypes (for example Opa+ or nonpiliated morphology) by circling the area with a marker on the outside of the plate. Avoid these areas when preparing the inoculum suspension.
3. From an area with isolated colony growth, remove several colonies from each plate and test with oxidase reagent. First, saturate a piece of filter paper with oxidase reagent (BBL oxidase reagent dropper or equivalent). Using a sterile toothpick or wooden applicator stick (*see Note 12*), lift each isolated colony and streak it across the wet area of the filter paper. Bacterial material must test positive, that is, turn dark purple within 30 s, in order to use growth from that plate for the inoculum suspension. Return all plates passing preinoculation testing to the incubator until transport to laboratory at facility where subjects will be inoculated (*see Note 13*).
4. When all subjects in the cohort to be inoculated with a given gonococcal suspension have been prepared, as described in Subheading 3.4, remove the GCB agar plates containing 18–24 h bacterial growth from the incubator. It is not necessary to work in a laminar flow ventilated hood at this stage, but it is helpful to work in a dead air box on a clean, decontaminated surface to minimize air flow and reduce the likelihood of contamination (*see Note 14*). Harvest the bacterial growth from the agar plates with a sterile cotton swab and prepare a suspension in 6 mL of sterile PBS in a sterile glass tube that will fit into the spectrophotometer chamber. Determine the optical density, adjust with more bacteria or more PBS as needed, and filter as described in *step 11* of Subheading 3.1.2.
5. Make a final dilution from the filtered suspension in approximately 10 mL of PBS in a sterile 50 mL conical centrifuge tube to achieve the desired inoculum concentration, based on previous work with the specific bacterial product lot. Immediately remove 1 mL from this tube to a sterile 2 mL tube. Securely cap the 50 mL tube containing the final inoculum suspension and assign a study team member to deliver it promptly to the physician who will inoculate the subjects as described in Subheading 3.4. Meanwhile, another team member should use the 1 mL of suspension that was transferred to the sterile 2 mL tube to prepare three labeled freezer vials with 0.1 mL of bacterial suspension added to 0.5 mL of

FSM in each tube, and freeze immediately at -70°C . This provides an archive of the inoculum suspension for future reference if needed.

6. Also from the 1 mL of inoculum suspension, plate 0.05–0.1 mL, undiluted onto LB agar to confirm purity. Furthermore, make serial dilutions of the suspension and plate onto GCB agar to determine the actual cfu/mL contained in the suspension by viable counting. Quantitative cultures should be plated before the first subject is inoculated and repeated with any remaining inoculum suspension that is returned to the lab after the last subject is inoculated, in order to document any potential loss of viability during the time it takes to complete the procedure.

3.4 Inoculation Procedure

1. Recline the subjects on a hospital bed, and ensure that they don sterile gloves immediately before inoculation. Cover the pelvic area with a waterproof plastic drape, and extend the penis through a hole in the drape and cleanse with 70% (v/v) isopropanol wipe. Remove a No. 8 pediatric French catheter, cut to 8 cm in length, from its sterile pouch, holding it between the cut opening and the 5 cm mark. Dip the closed end of the catheter into a container with sterile saline to moisten. This is inserted gently by the physician into the urethra until the 5 cm mark on the catheter is just visible at the opening of the urethra (*see Note 15* and Fig. 2).
2. Load 0.4 mL of gonococcal suspension prepared as described above in Subheading 3.3 into a filtered pipette tip on a Pipetman P-1000 micropipettor and immediately replace the cap on the tube. Fit the tip of the micropipettor into the open end of the end of the catheter that has already been inserted into the urethra; take care not to insert the catheter further than the 5 cm mark. Deliver the inoculum slowly into the catheter by depressing the plunger of the micropipettor. When the first stop of the micropipettor has been reached, clamp the catheter with a hemostat (Fig. 2), and then gently remove the entire assembly of pipette tip with catheter and hemostat still attached from the urethra in one smooth motion (*see Note 15*).
3. Instruct the subject to apply gentle pressure to the end of the penis and hold it upright for 30 min after inoculation to prevent leakage of the bacterial suspension. Collect the catheter, pipette tip, any liquid remaining in the catheter and any spilled drops of fluid that can be collected from the drape and deposit into an empty, sterile 50 mL centrifuge tube labeled with the subject's ID, and return these tubes with undelivered inoculum suspensions to the lab after all subjects have been inoculated.
4. Repeat **steps 1–3** for each subject in the cohort. After the last subject has been inoculated, return remaining inoculum suspension to the laboratory for postinoculation counts to be determined as described in **step 6** of Subheading 3.3.
5. Load the 50 mL tubes containing pipette tips, catheters and undelivered fluid into a benchtop centrifuge and spin briefly to collect all the fluid in the bottom of the

tube. Remove the pipette tip and catheter, and measure the volume in the tube. Subtract this volume from the original 0.4 mL volume loaded into the pipette to determine the actual volume delivered to each subject.

6. Inoculate GCB agar plates with serial dilutions of preinoculation and postinoculation cultures made in sterile PBS and incubate for 24–48 h at 37 °C in a humidified atmosphere with 5% (v/v) CO₂. Count colonies and calculate the average of the preinoculation and postinoculation counts, and adjust for the volume delivered to determine the bacterial dose delivered to each subject in the cohort, that is, cfu delivered volume delivered (mL) × cfu/mL in inoculum suspension.

3.5 Monitoring Experimental Infection

1. Physicians will perform targeted genital exams daily after inoculation and query subjects to determine signs and symptoms of infection. For pathogenesis studies, in which the timing and features of experimental infection with different strains of *N. gonorrhoeae* are being compared, subjects are housed as inpatients in a clinical research unit to facilitate consistent examination and specimen collection first thing every morning for up to 5 days after inoculation (see **Note 16**). For studies designed to test preventive treatments, in which infectivity or time to clinical disease is the main outcome, subjects may be managed as out-patients, with daily return visits to the clinical research unit scheduled as early in the day as is practical for examinations and specimen collection.
2. Administer prompt and effective antibiotic treatment to subjects when one of the following occurs: (1) subject requests treatment, (2) urethral discharge is observed by clinician or reported by subject, or (3) 5 days after inoculation, regardless of whether or not the subject was judged to have been infected. For experimental infection with *N. gonorrhoeae* FA1090, treatment is a single observed 400 mg oral dose of cefixime. If a subject cannot swallow the oral pill, treatment is a single 250 mg intramuscular dose of ceftriaxone.
3. Monitor experimental gonococcal infection by bacterial recovery from urogenital specimens including urine and urethral swabs; bacteria may also be recovered from semen if the study design dictates. After the genital exam and before antibiotic treatment, subjects in inpatient studies provide up to 50 mL of first-void urine each morning. For outpatient studies, subjects provide up to 50 mL of first-catch urine at daily clinic visits. On the day on which a subject is treated, a urethral swab is obtained. Urine and urethral swabs can be processed for gonococcal culture on GCB agar and bacterial and/or host gene expression as dictated by the study design (see **Notes 17–19**).
4. Host responses can be monitored by enumeration of white blood cells in urine and measurement of soluble immune mediators including antibodies and cytokines in urine and serum (see **Notes 17–19**).

3.6 Statistical Considerations

1. Noncompetitive infections. For experimental infections initiated with inocula containing an individual strain, the proportion of subjects that become infected is the measure of infectivity. In pathogenesis studies, the hypothesis is frequently whether the wild-type strain is more infectious than an isogenic mutant that lacks a predicted essential virulence factor. Sample size considerations for such noncompetitive infections are summarized in Table 1. Typically, we enroll subjects in groups of up to 4, which is the maximum number of inpatient rooms in our clinical research facility. Assuming the true probabilities of infection are 80% with wild-type and 4% with mutant inocula, eight men per group provides 89% power to detect a difference using a one-sided Fisher exact test and significance level of 0.025.

Compare infectivity of wild-type *N. gonorrhoeae* and an isogenic mutant using Fisher's exact test. An interim analysis may include results from up to eight subjects inoculated with the wild-type *N. gonorrhoeae* strain only and at least four subjects inoculated with the isogenic mutant only. With a clearly directional hypothesis that a mutant is less infectious than the wild-type strain, one-sided statistics are appropriate. Final analysis of wild-type vs. isogenic mutant noncompetitive infections may include comparisons of Kaplan–Meier survival curves for the two strains.

2. Competitive infections. For isogenic mutants that do not demonstrate statistically significant differences in infectivity, competitive infection studies are useful to determine whether the mutant exhibits a fitness defect compared to the wild-type strain. Competitive infections, initiated with equivalent numbers of wild-type bacteria and isogenic mutant bacteria, allow demonstration of a difference in fitness between two strains using fewer subjects. For competitive infections with mixed inocula, sample sizes are determined by the expected standard deviation of the mean proportion of strains among gonococci recovered from infected subjects. Because calculations are based on the numbers of bacteria in each cultured specimen, constituting larger sample sizes than infectious dose calculations based on the number of infected subjects, mixed infections can provide a more accurate indication of attenuation using fewer subjects than traditional comparisons of infectivity using separate, inocula with individual strains [8].

For competitive infections initiated with inocula containing a mixture of wild-type and isogenic mutant strains, the competitive index (CI) of the mutant compared to wild-type is the measure of relative fitness. The precise proportion of each strain in the inoculum is determined for each experimental cohort as described in **step 6** of Subheading 3.4. The CI is defined as the ratio of colony-forming units (cfu) of the two strains recovered from urine cultures on the day of treatment (output) compared to the ratio of strains in the inoculum (input) using the equation: $CI = \text{mutant cfu(output)}/\text{wild-type cfu(output)} \div \text{mutant cfu(input)}/\text{wild-type cfu(input)}$. If the two strains have equal fitness in experimental human

urethral infection, the CI will be close to 1. If the wild-type strain has a fitness advantage, the $CI < 1$, and if the mutant has a fitness advantage, the $CI > 1$.

4 Notes

1. Maintain certificates of origin and analysis to document use of BSE-free herds for all ingredients made from animal sources that are used to prepare and store bacteria for use in experimental human infection.
2. *N. gonorrhoeae* strains should be grown on GCB agar without added inhibitors for working stock preparation to allow visualization of potential contaminants.
3. Eligible subjects meeting all inclusion criteria and none of the exclusion criteria provide written informed consent after all study procedures are explained in detail and they have had the opportunity to ask questions. To date, we have included only subjects with no reported history of *N. gonorrhoeae* (and other sexually transmitted infections) to minimize the impact of immune responses to past pathogen exposure on experimental outcomes. Other study designs may require different inclusion/exclusion criteria (Table 2).
4. Take care not to gouge the surface of the agar when harvesting bacteria from the plates. Vigorously swirl swabs in liquid for several seconds to dislodge bacteria, and press swabs firmly against the side of the tube to wring out as much liquid as possible before discarding swabs.
5. Several lots may be created at one time to maximize the likelihood that at least one will pass lot release testing. Clearly identify each lot prepared from an independent bacterial suspension, and promptly discard any lot that fails quality control testing. After quality control testing, store selected lot(s) in a separate location in the freezer where only product that has passed all lot-release tests is kept, to avoid inadvertent use of untested material for experimental infections.
6. *N. gonorrhoeae* strain FA1090 is naturally resistant to streptomycin and susceptible to chloramphenicol, cefixime, ceftriaxone and ciprofloxacin. During isogenic mutant constructions, intermediate strains that are susceptible to streptomycin are created [9]. To document the antimicrobial susceptibility pattern in final constructs, we confirm resistance to streptomycin and susceptibility to the other four antibiotics listed for each lot of bacteria prepared for experimental human use. Other *N. gonorrhoeae* strains may require different antibiotic susceptibility testing.
7. All FA1090 stocks characterized to date have been predominantly Opa-negative, pilated, that is, expressing the Pile sequence reported by Seifert et al. [10] and expressing the 3F11 LOS epitope to maintain comparability with our previous experimental infection studies. Other phenotypic characteristics may be desired and selected based on specific goals of the research.
8. These characterization methods are not produced in detail here but can be found in the accompanying references.

9. Genomic DNA for WGS has successfully been prepared with the ArchivePure DNA purification kit Protocol 18 (5-PRIME, USA) as instructed by the manufacturer with the following modifications: 2.2 μ L of RNase A solution; 2.5 μ L of proteinase K mixed thoroughly by inverting the tube at least 25 times and incubated for 40 min at 37 °C; centrifugation time increased to 6 min for all steps, as previously described [11].
10. Do not assume that the relationship between optical density of filtered bacterial suspension in PBS and cfu/mL is the same for different strains or for different lots of a given strain of *N. gonorrhoeae*; this must be determined empirically for each lot of each strain. There may not be a strictly linear relationship between optical density and viable counts over a broad range of absorbance values; the goal is to establish an absorbance range for filtered suspensions that corresponds to a known range of concentrations, which will enable adjustment of the inoculum for the dose required just before inoculation of human subjects. Dose response data from experimental human infection studies over many years indicate that the ID₅₀ is approximately 10⁵ cfu for *N. gonorrhoeae* strain FA1090 and approximately 10³ cfu for strain MS11mkC [5]. At present, there are insufficient data from experimental human infections to estimate infectious doses of other *N. gonorrhoeae* strains.
11. Filtration through 1.2 μ M pores breaks up bacterial clumps typical of piliated gonococci without shearing pili and provides a more uniform suspension. Take care not to touch anything with the rubber tip of the plunger after it has been removed from the syringe. Work quickly with gonococcal suspensions in PBS, as viability begins to decrease within 30 min in the buffer.
12. Use only sterile wooden implements to pick colonies for oxidase testing. Do not use a sterilized metal microbiological loop, as this will inhibit the oxidase reaction.
13. Transport agar plates containing *N. gonorrhoeae* (either working bank cultures for inoculation or clinical specimens plated for gonococcal recovery) between laboratories in candle jars. We use metal canisters with tight-fitting lids. Load plates into the canister, place a votive candle on top of the stack of plates, light the candle and fit the lid onto the canister. The flame will consume oxygen until it is extinguished, approximating a 5% (v/v) CO₂ atmosphere inside the canister. Typically, a 37 °C incubator without CO₂ is used in the laboratory located at or near the clinical research unit; store plates in candle jar inside incubator until transport to the research laboratory. Transport canister(s) upright at ambient temperature. Remove plates from the candle jar and transfer to 37 °C incubator with humidified atmosphere containing 5% (v/v) CO₂ in the research laboratory as soon as possible.
14. A dead air box provides a circulation-free workspace in the laboratory near the clinical research unit. Select a box that will accommodate the Genesys 30 Spectrophotometer to be used to prepare gonococcal inoculum suspensions with

sufficient working space. Before and after use, wipe down work surfaces with 70% (v/v) ethanol and allow them to dry.

15. The inoculation process runs smoothly with a team of three people: the physician, who will place the catheter and deliver the inoculum, should don sterile gloves and avoid touching nonsterile items until the loaded pipettor is presented by an assistant wearing nonsterile laboratory gloves. Before inoculation, the assistant should open a packet containing an isopropanol wipe to be used to cleanse the penis and present it to the physician, open the package containing the sterile catheter so that the physician can remove it, open a container of sterile saline so that the physician can moisten the catheter, load the pipettor with the inoculum suspension and hand it to the physician, who will have just inserted the catheter into the anterior urethra. Once the physician has dispensed the inoculum, and before the catheter is removed from the urethra, the assistant should clamp the catheter between the urethra and the pipette tip to prevent backflow (Fig. 2). Then, in a unified movement, the physician and assistant should remove the pipettor–catheter–hemostat assembly, collecting everything in a labeled empty tube. The physician and assistant should practice all the steps before actual inoculation. The third team member should help the subject don his sterile gloves, reassure him during the procedure, note the time of inoculation and instruct the subject to apply pressure to the penis for 30 min.
16. For inpatient studies, subjects may leave the clinical research unit during the day to attend classes or go to work; they must return to the clinical research unit by 9 pm and sleep in the unit. Between 6:30 and 7:00 am, subjects are awakened, examined, and interviewed for symptoms before specimens are obtained. If none of the outcomes triggering antibiotic treatment has occurred, normally only urine is obtained. If a subject reports symptoms or the clinician observes urethral discharge and treatment is warranted, a urethral swab is obtained before the urine specimen. It is critical that specimen processing and plating for gonococcal cultures occur with minimal delay; lab space near the inpatient clinical research unit is ideal. Once plates have been inoculated, they can be transported in candle jars from the processing lab to a more distant research lab for incubation at 37 °C with 5% (v/v) CO₂. If prolonged storage in the processing lab is needed, incubate candle jars at 37 °C if a CO₂ incubator is not available.
17. Do not refrigerate urine before processing. From 50 mL of urine, decant 8–10 mL into a 15 mL centrifuge tube for white cell counts. Spin the remaining urine promptly at approximately 1500 × *g* at room temperature for 10 min. Save urine supernatant as needed, and process urine pellet for quantitative culture, plating dilutions on GCB agar with appropriate antibiotics (e.g., vancomycin, colistin, nystatin, and trimethoprim) to reduce growth of potential contaminants. Spin 15 mL tubes at approximately 400 × *g* at room temperature for 10 min, decant or save supernatant as needed, gently suspend the pellet in residual urine and count white blood cells (predominantly PMNs) in a hemacytometer. Take care to label all tubes clearly and record data for bacteriuria and pyuria.

18. To process urethral swabs for gonococcal culture, place the swab into a small volume (0.2–0.5 mL) of FSM in a sterile vial; cut the wire shaft, cap the tube, and vortex vigorously to suspend material in the medium. This suspension may be plated undiluted on GCB agar to determine whether the specimen is positive or negative for *N. gonorrhoeae*, or serial dilutions may be plated for enumeration of bacterial burden and recovery of individual colonies. We typically plate undiluted swab suspensions to determine positive culture status. Occasionally, urethral swab cultures are positive when urine cultures are negative. A subject is considered infected if urine or urethral cultures are positive on the day of antibiotic treatment. Urethral swab suspensions remaining after aliquots are removed for culture can be stored in FSM at –70 °C for future recovery and characterization of viable *N. gonorrhoeae* or further processed for analysis of bacterial or host gene expression.
19. Detailed procedures for processing urogenital specimens or blood for RNAseq analyses of bacterial and host gene expression during experimental infection are not well established and are beyond the scope of this chapter.

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Fig. 1.
Streaking pattern for areas of heavy growth and isolated bacterial colonies on agar plates

Fig. 2. Prepared catheter fitted with pipette tip shown with hemostat in the position as described after delivery of the inoculum suspension to prevent backflow

Table 1

Sample size considerations for noncompetitive infection studies

Infection status					
Wild-type		Mutant		<i>P</i> -value ^a	Number of subjects needed for analysis
Yes	No	Yes	No		
8	0	0	4	0.002	12
		1	3	0.018	
		2	2	0.091	
		2	6	0.003	
		3	5	0.013	
		4	4	0.038	
7	1	0	4	0.010	12
		1	3	0.066	
		1	7	0.005	
		2	6	0.020	
		3	5	0.059	
6	2	0	4	0.030	16
		0	8	0.003	
		1	7	0.020	
		2	6	0.066	
5	3	0	4	0.071	16
		0	8	0.012	
		1	7	0.059	

^a*P*-value is from the one-sided Fisher's exact test with $\alpha = 0.025$

Table 2

Inclusion and exclusion criteria for human challenge experiment

Inclusion criteria	<ul style="list-style-type: none">• Healthy man 18 and 36 years old• Able and willing to be located easily by providing street address and telephone number (land line and/or cell phone number)• Willingness to provide written informed consent• Able and willing to attend all study visits• Able and willing to abstain from all sexual activity until completion of study and follow-up test for gonorrhea is negative• Acceptable medical history by screening evaluation• No clinically significant abnormalities on physical exam• Urinalysis: leukocyte esterase value and white blood cell count within normal limits• Total complement (CH50) value within normal limits• Urine negative for chlamydia, gonorrhea, and Trichomonas• Negative HIV, syphilis, and HCV serologic test results• Negative HBV core and surface antibodies or results consistent with immunization (negative HBV core antibody/positive HBV surface antibody)• Denies history of sexually transmitted infections including gonorrhea, chlamydia, syphilis, HIV, HBV, and HCV• Denies history of bleeding diathesis• Denies history of seizures (due to reports of seizures with ciprofloxacin)• Denies history of cancer, except basal cell carcinoma of the skin >5 years ago• Denies history of drug abuse• Denies history of genitourinary surgery
Exclusion Criteria	<ul style="list-style-type: none">• Student or employee under the direct supervision of any of the study investigators• Any known immunodeficiencies including complement deficiency, antibody deficiency, chronic granulomatous disease, or HIV infection• Psychiatric disorders that could interfere with the integrity of the data or subject safety• Unstable depression or depression that could compromise the subject's ability to comply with protocol requirements• Heart murmur or heart disease

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- Anatomic abnormality of the urinary tract
- Any antibiotic treatment in the past 30 days, or azithromycin in the past 60 days
- Self-reported chemotherapy within the past year
- Current steroid use, except for topical application
- Allergy to penicillin, cephalosporins or ciprofloxacin or to lidocaine
- Treatment with medications in the previous month that are contraindicated with cefixime, ceftriaxone or ciprofloxacin and that cannot be withheld for the doses given in the study
- Serum creatinine level <0.7 or >1.75 mg/dL
- Serum alanine aminotransferase (ALT) level $<$ lower limit of normal or >105 U/L
- White blood cell count <2.5 or $>15.0 \times 10^9/L$
- Absolute neutrophil count (ANC) <1.5 or $>7.5 \times 10^9/L$ and deemed clinically significant by the study physician. For African Americans, ANC values as low as $1.3 \times 10^9/L$ are allowed
- Hemoglobin level <12.0 g/dL or above upper limit of normal
- Urinalysis: Qualitative protein level $>1+$ or red blood cell count >10 /high powered field