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A Natural Mouse Model for Neisseria Persistent Colonization

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Abstract

We have developed a natural mouse model to study persistent colonization by commensal *Neisseria*. The system couples the ordinary lab mouse with *Neisseria musculi* (Nmus), a commensal in the oral cavity and gut of the wild mouse, *Mus musculus*. The pairing of Nmus with its natural reservoir circumvents host restriction barriers that have impeded previous studies of *Neisseria* in vivo behavior. The model allows, for the first time, for the dissection of host and neisserial determinants of asymptomatic colonization. Inoculation procedures are noninvasive and susceptibility to Nmus colonization varies with host genetic background. In colonized mice, bacterial burdens are detectable up to 1-year post inoculation, making it an ideal model for the study of persistence. As Nmus encodes several *Neisseria gonorrhoeae* (and *Neisseria meningitidis*) host interaction factors, the system can be used to query the in vivo functions of these commonly held genes and factors. Nmus also encodes many pathogenic *Neisseria* vaccine targets including a polysaccharide capsule, making the model potentially useful for vaccine development. The ease of genetic manipulation of Nmus enhances the feasibility of such studies.

Keywords

Mouse model; Colonization and persistence; Commensal *Neisseria*; Pathogenic *Neisseria*

1. Introduction

1.1. Why Is It Important to Understand Commensalism?

Commensal organisms (microbiota, normal flora) play a critical role in physiology [1–7]. They are required for gut and immune system development, and prevent pathogen colonization. Perturbation of the composition of these microbial communities is strongly linked to chronic, non-infectious diseases such as obesity, inflammatory bowel disease, diabetes (Type I and II) and autoimmunity.

How does a commensal organism colonize and persist in the body in the presence of a robust immune system? Little is known about the mechanisms of commensal–host coadaptation. Understanding the molecular basis of these interactions will improve disease prevention and contribute to our fundamental knowledge of immunology and microbiology. Except for two species, all members of the *Neisseria* genus are commensals that inhabit a wide range of hosts, from rodents, felines, and bovines to monkeys and man [8, 9]. Animal-adapted

commensal *Neisseria* have been detected or isolated from the oral cavity and feces of their hosts, suggesting they establish niches along the alimentary tract. The niche of human-adapted commensals is generally assumed to be the upper respiratory tract, but little has been done to examine the extent of their colonization range. The two pathogenic species in the genus are *Neisseria gonorrhoeae* (Ngo) and *Neisseria meningitidis* (Nme), which only infect humans [8]. Ngo and Nme inhabit the oral cavity and urogenital tract and are generally assumed to be restricted to these sites. Whether they also colonize the gut is an open question.

Ngo and Nme exhibit a commensal-like behavior; at fairly high frequency, they colonize the oral cavity and urogenital tract without eliciting overt symptoms of disease [10, 11] (<http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.177.8777>). Asymptomatic carriage is an important facet of infection, as it is key to person-to-person transmission. The mechanisms that promote asymptomatic carriage of Ngo and Nme are little understood. As commensal *Neisseria* are closely related to Ngo and Nme, they may provide a means to understanding asymptomatic infections by these pathogens.

1.2. A Natural Mouse Model for Commensal *Neisseria* Persistent Colonization

We have developed a natural small animal model for dissecting the mechanisms of *Neisseria* colonization and persistence, from the standpoint of both host and microbe [12]. Our model pairs the lab mouse, which does not normally harbor *Neisseria*, with *Neisseria muscoli* (Nmus) a commensal of wild mice [13]. Pairing the mouse with a member of its normal flora circumvents barriers imposed by host restriction, a problem that plagues current models for studying the human-specific pathogenic *Neisseria* species [14–17] (see Note 1).

Nmus encodes many host interaction genes of the pathogenic *Neisseria*, including a complete set of genes for Type IV pilus (Tfp) biogenesis and function [12]. The Tfp fiber is generally assumed to mediate colonization [18, 19], based on in vitro studies, but in vivo function has never been tested. As final proof of principle for the validity of our model for investigating *Neisseria*-host interactions, the role of Tfp in colonization was tested using the mouse model. Nmus π ilE, which cannot produce the Tfp fiber subunit, PilE, fails to colonize CAST and B6 mice [12]. The Nmus-mouse model is therefore useful for querying the in-host function of other proteins that modulate Tfp activity, and the interaction genes/factors held in common by commensal and pathogenic *Neisseria* (see Note 2).

Finally, it should be noted that commensal *Neisseria* naturally colonize rhesus macaques (RM) [20] and persist in them for at least 6 years [21]. RM *Neisseria* are closely related to human commensal species [22] and encode many host interaction factors of the pathogenic

One oral dose of Nmus (6×10^6 CFUs), pipetted gently into the oral cavity of the mouse, will achieve lasting colonization of the oral cavity and gut. Nmus is recovered in large quantities from these sites every week, for as long as 1 year. The animals remain healthy throughout the studies. No administration of antibiotics or hormones, or invasive procedures or surgery is necessary. Since the system involves a commensal in its native host, mice do not need to be derived to introduce human markers, for example, receptor proteins for gonococcal virulence factors, to mimic human infection. Horizontal transmission between cage mates has not been detected, allowing the cohousing of naïve and inoculated mice.

Nmus is easy to grow and manipulate genetically; many reagents, genetic tools, and genome databases are available for both *Neisseria* and the lab mouse [23–25] (<http://www.sanger.ac.uk/resources/download/s/bacteria/neisseria.html>), allowing rapid dissection of microbial and host determinants of colonization, and of the elusive process of persistence.

species [21] (Weyand and So unpublished). Thus, pairing RM *Neisseria* with the macaque holds promise for understanding *Neisseria*-host interactions. However, the logistics and expense of the model, and the limited number of non-human primate facilities make the model inaccessible to most investigators. In this chapter, we provide a detailed protocol for *Nmus* colonization of mice that is readily accessible to investigators.

2. Materials

2.1. Screen for Autochthonous *Neisseria* Species

2.1.1. Sample Collection

1. Mouse (see Note 3).
2. Ear tag system or ear punch.
3. 100 μ l aliquots of sterile Dulbecco's phosphate buffered saline without magnesium and calcium (DPBS).
4. 1.5 ml Sterile Eppendorf tubes.
5. BBL CultureSwab Plus Transport system, Amies gel with charcoal.
6. Laminar flow hood: sterilize by swabbing with 70% (v/v) ethanol in distilled H₂O.
7. Sterile catch pan.

2.1.2. Oral Sample Preparation

1. Gonococcal Medium Base (GCB) agar plate (BD Difco): add 15 g/L of Proteose Peptone No. 3, 1 g/L of Cornstarch, 4 g/L of K₂HPO₄, 1 g/L of KH₂PO₄, 5 g/L of NaCl, 10 g/L of agar to 1 L of distilled H₂O. Autoclave at 121 °C and 1.05 kg/cm² for 40 min. Cool the medium to 55 °C and add 1% (v/v) Kellogg's Supplement I and 0.1% (v/v) Kellogg's Supplement II and appropriate antibiotics (see Note 4).
2. Kellogg's Supplement I: 400 mg/ml of glucose, 10 mg/ml of l-glutamine, 20 μ g/ml of thiamine pyrophosphate dissolved in distilled H₂O and sterilized by filtration (0.22 μ m filter). Store in aliquots at -20 °C.

Host genetics is a major determinant of colonization. CAST/EiJ (CAST) and A/J strains of lab mice are colonized by *Nmus* at high frequency, and C57BL/6J (B6) at intermediate frequency. All studies to date have been done with mice from 4–6 weeks of age. No sex bias has been detected. NOD/LtJ, NZO/HILtJ, PWK/PhJ, WSB/EiJ, and 129S2/SvImJ mice are resistant to colonization. A second major colonization determinant is innate immunity. B6 MyD88^{-/-} mice are exquisitely sensitive to *Nmus* colonization, unlike their parental wt B6. By contrast, B6 Rag1^{-/-} mice are no more susceptible than B6. Many genetic tools are available for manipulating the lab mouse, such as the Collaborative Cross [24], which allows for identification of mouse alleles and immune pathways that determine susceptibility/resistance to *Nmus* colonization.

The GCB agar formulation can be used for both prescreening and experimental sample propagation. The antibiotics used in each assay must be adapted as appropriate. To screen for autochthonous *Neisseria* prior to inoculation, samples must be plated on GCB agar containing 3 μ g/ml of Trimethoprim and 2 μ g/ml of Vancomycin. This medium selects for growth of *Neisseria* species and discourages growth of other commensals. The experiments described in ref. 12 were done using *Nmus* AP2365, a spontaneously arising rifampicin-resistant strain. Samples from AP2365-inoculated mice should be selected on GCB agar containing 40 μ g/ml of rifampicin, and/or the antibiotic specific to the selective marker introduced to the *Nmus* strain of interest. Samples can be stored at -80 °C indefinitely in GCBL with 20% (v/v) glycerol.

3. Kellogg's Supplement II: 500 µg/ml of Iron (III) Nitrate in distilled H₂O and sterilized by filtration (0.22 µm filter). Store in aliquots at –20 °C [23].
4. Humidified incubator set at 37 °C with 5% (v/v) CO₂.
5. Sterile plating beads or glass spreader rod.
6. GC broth (GCBL): add 15 g/L of Proteose peptone #3, 1 g/L of KH₂PO₄, 4 g/L of K₂HPO₄, 1 g/L of NaCl, and 20% (v/v) glycerol to 1 L of distilled H₂O, and adjust pH to 7.2 [23]. Autoclave at 121 °C and 1.05 kg/cm² for 40 min.
7. Wire cutters.
8. Plate spinner.
9. Bunsen burner.
10. Vortex mixer.
11. 70% (v/v) ethanol.
12. Glass spreader rod or disposable inoculating plastic loops.
13. 1.5 ml sterile Eppendorf tubes.

2.1.3. Fecal Sample Preparation

1. Same materials as described in Subheading 2.1.2.
2. Sterile P1000 pipette tips.
3. Tared balance.

2.2. Preparing the *Neisseria musculi* Inoculum

1. *Neisseria musculi* strains/isolates, stored at –80 °C in GCBL with 20% (v/v) glycerol.
2. GCB agar plates containing 40 µg/ml of rifampicin, and any additional selective antibiotics appropriate to the strain being tested.
3. Sterile CLASSIQSwabs (Copan, or equivalent).
4. Sterile plastic cell scrapers.
5. DPBS without calcium and magnesium.
6. GCBL broth.
7. Humidified incubator set at 37 °C with 5% (v/v) CO₂.
8. Dissecting microscope.
9. Spectrophotometer.
10. Disposable plastic cuvettes.
11. 1.5 ml sterile Eppendorf tubes.
12. Vortex mixer.

2.3. Inoculation of *Neisseria musculi* into the Oral Cavity

1. Prescreened mice with Identification (ID) numbers.
2. *Neisseria musculi* inoculum.
3. P200 pipet.
4. Aerosol-resistant tips.
5. Vortex mixer.
6. Sterile catch pans.

3. Methods

3.1. Screen for Autochthonous *Neisseria* Species (See Note 5)

3.1.1. Sample Collection

1. On day 1, tag or band each mouse to give it an identification (ID) number.
2. Sterilize the laminar flow hood. Collect the necessary equipment and place it in the hood, along with a sterile catch pan and the cage of naive mice.
3. Gently restrain the mouse so as to prevent the forelimbs from touching the mouth or nose.
4. Submerge a BBL culture swab into an aliquot of DPBS; remove excess liquid by gently pressing the swab along the side of the tube. Collect an oral sample by gently inserting the wetted swab into the oral cavity of the mouse, taking care that it does not reach the esophagus. Using a swirling motion, pass the swab over the palate, cheek pouches, sublingual tissue, and tongue. Return the swab to the receptacle, making sure it does not touch the side of the tube. Record the mouse ID on the side of the transport tube.
5. Use a sterile, labeled Eppendorf tube to collect a fecal sample from each mouse (see Note 6). Use the inside edge of the tube to scoop the sample into the tube, taking care not to allow the fecal pellet to contact any unsterilized surface or gloved fingers.
6. Proceed to sample preparation and plating as described in Subheadings 3.1.2 and 3.1.3.

At least 3 days prior to inoculation, prescreen all mice for the presence of endogenous *Neisseria*. This is done by plating oral cavity and fecal samples on agar that selects for *Neisseria* species (see Note 10). This will avoid complications that interfere with interpretation of colonization data. We note that we have not detected *Neisseria* in any of the lab mice we have used [12]. The sampling collection and preparation method is also used for assaying experimental animals post inoculation. If working in pairs, one person should restrain the mouse while the other collects the fecal samples and oral swabs. If working alone, the experimenter is advised to place each mouse in a sterilized container or catch basin and collect the fecal sample(s) from the animal prior to swabbing their oral cavity. This increases the efficiency of fecal sample collection, especially when working with CAST/EiJ strains.

3.1.2. Oral Sample Preparation

1. Dispense 1 ml of GCBL containing 20% (v/v) glycerol into prelabeled sterile Eppendorf tubes.
2. Dip the metal cutting edge of a pair of wire cutters in 70% (v/v) ethanol and flame to sterilize. Wait for the pliers to cool.
3. Remove the oral swab from the media receptacle in **step 4** of Subheading 3.1.1 without touching the metal shaft.
4. Insert the cotton bud of the swab into the appropriate tube with GCBL containing 20% (v/v) glycerol. Use the sterile wire cutters to clip the cotton bud from the shaft, dropping the bud into the storage media. Cap the tube firmly and vortex at high speed for 1 min.
5. Repeat **step 2** between each swab.
6. Pulse each sample on the vortex mixer for 2 s to suspend any settled material.
7. Dispense 100 μ l of each sample onto a labeled, prewarmed GCB plate with appropriate antibiotics (see Note 4).
8. Spread the liquid evenly over the agar surface using a sterilized glass rod or disposable loop. Incubate the plate for 48 h at 37 °C in an atmosphere with 5% (v/v) CO₂.
9. Check plates for growth of bacteria. When screening for endogenous *Neisseria*, exclude any mouse that is positive for either oral or fecal CFUs (see Note 7).

3.1.3. Fecal Sample Preparation

1. Weigh each fecal sample collected from **step 5** in Subheading 3.1.1 in its tube on a tared balance scale and record the weight.
2. Dispense 1 ml of GCBL containing 20% (v/v) glycerol into each tube, taking care to prevent cross contamination.
3. Crush each fecal pellet using a sterile filtered P1000 pipette tip. Vortex each sample for ~1 min at high speed until homogenous.
4. Repeat **steps 6–9** of Subheading 3.1.2.

3.2. Preparing the *Neisseria musculi* Inoculum (See Note 8)

1. Use a sterile swab to inoculate a GCB plate containing 40 μ g/ml of rifampicin with *Nmus* from a pure freezer stock, without allowing the stock to thaw, and streak for single colony isolation (see Note 9).

Thorough genetic and biochemical protocols for identifying native *Neisseria* species can be found in ref. 13. Preparation of the *Nmus* inoculum is straightforward, but good aseptic technique is crucial to prevent contamination that could confound study results. All steps should be performed in a sterile biosafety cabinet, or near a flame if working on the bench top. Do not freeze and thaw a frozen stock of *Neisseria*. To avoid this problem, multiple freezer stocks should be prepared, and *Nmus* should be taken from frozen stock by scraping the surface of the frozen culture.

2. Incubate the plate at 37 °C with an atmosphere of 5% (v/v) CO₂ for 48 h to obtain colonies large enough to visualize colony morphological type.
3. Use a dissecting microscope to view and collect ten rough colonies onto a single sterile swab. Streak a lawn onto a GCB plate containing 40 µg/ml of rifampicin with the collected colonies. Repeat five times, for a total of 50 colonies streaked to lawns on five separate plates. Repeat for each experimental strain.
4. Incubate these plates for an additional 16–18 h at 37 °C with an atmosphere of 5% (v/v) CO₂.
5. Prepare a sterile Eppendorf tube with 1 ml of sterile DPBS.
6. Harvest the plates that had been streaked to lawns in **step 3**. Collect the bacterial lawn from each plate using a sterile cell scraper, taking care not to gouge the agar. Inadvertent collection of agar with the lawn will skew absorbance readings.
7. Deposit the collected lawns into the Eppendorf tube containing 1 ml of DPBS, to create a stock suspension.
8. Vortex the suspension at maximum speed for at least 1 min to disperse any aggregates.
9. Prepare a second Eppendorf tube with 900 µl of DPBS. Dilute the stock suspension 1/10 and vortex at high speed for an additional minute.
10. Transfer the diluted bacterial suspension to a clean cuvette. Measure the optical density (OD) at $\lambda_{600\text{ nm}}$ using a spectrophotometer blanked against DPBS.
11. Calculate the OD $\lambda_{600\text{ nm}}$ of the undiluted stock suspension. Calculate the total volume needed to dose all mice with 50 µl of inoculum per mouse, leaving a minimum of 100 µl excess for long-term storage. Using these values, calculate the volume of stock suspension necessary to create an inoculum where OD $\lambda_{600\text{ nm}} = 2.0$.
12. Prepare the inoculum as calculated in **step 11**, using a new sterile Eppendorf tube and the appropriate volumes of DPBS diluent and stock suspension. Vortex the inoculum for 1 min at high speed. Move on to Subheading 3.3 immediately. Samples remain viable in DPBS for approximately 4–6 h at room temperature.

3.3. Inoculation of *Neisseria musculi* into the Oral Cavity (See Note 10)

1. Prepare the biosafety cabinet as described in **step 2** of Subheading 3.1.
2. Manually restrain each mouse as described in **step 3** of Subheading 3.1, ensuring that the animal cannot move its head or forelimbs.
3. Briefly vortex the inoculum to suspend any bacteria that may have settled to the bottom of the tube.

Inoculating the mouse oral cavity is a slow process; insufficient time in the oral cavity can result in artificially poor colonization efficiency and variability within experimental groups. To minimize potential variability arising from the inoculation process, all mice in the experiment should be inoculated by the same person. Mice should be inoculated and sampled at approximately the same time of day to further reduce variability between time points and experimental groups.

4. Withdraw 50 µl of inoculum from the tube using a sterile filtered P200 pipette. Insert the pipette tip into the oral cavity of the restrained mouse, taking care not to abrade the tissues. Slowly dispense the inoculum. Depending on the size of the mouse, and how quickly it swallows the inoculum, this may have to be done in two stages.
5. Continue to restrain the mouse until the majority of the inoculum has been swallowed. This will take up to 2–3 min. It is vital that the mouse does not move from an immobile, supine position before this time, to prevent inoculum loss and deviation between delivered burdens within the experimental group.
6. Release the mouse into the sterile catch pan and repeat for the remaining mice (see Note 11).
7. Sample mice at designated time points post inoculation following **steps 3–6** of Subheading 3.1. Prepare samples for plating as indicated in Subheadings 3.1.2 and 3.1.3 or storage (see Note 4).

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References

1. Sommer F, Backhed F (2013) The gut microbiota-masters of host development and physiology. *Nat Rev Microbiol* 11(4):227–238. 10.1038/nrmicro2974 [PubMed: 23435359]
2. Campisi L, Barbet G, Ding Y et al. (2016) Apoptosis in response to microbial infection induces autoreactive TH17 cells. *Nat Immunol* 17(9):1084–1092. 10.1038/ni.3512 [PubMed: 27455420]
3. Kugelberg E (2016) Autoimmunity: infection stimulates self-antigen presentation. *Nat Rev Immunol* 16(9):534–535. 10.1038/nri.2016.91
4. Liu X, Zeng B, Zhang J et al. (2016) Role of the gut microbiome in modulating arthritis progression in mice. *Sci Rep* 6:30594. 10.1038/srep30594
5. Linehan JL, Harrison OJ, Han SJ et al. (2018) Non-classical immunity controls microbiota impact on skin immunity and tissue repair. *Cell* 172(4):784–796 e718. 10.1016/j.cell.2017.12.033 [PubMed: 29358051]
6. Deriu E, Liu JZ, Pezeshki M et al. (2013) Probiotic bacteria reduce *Salmonella typhimurium* intestinal colonization by competing for iron. *Cell Host Microbe* 14(1):26–37. 10.1016/j.chom.2013.06.007 [PubMed: 23870311]
7. Evans CM, Pratt CB, Matheson M et al. (2011) Nasopharyngeal colonization by *Neisseria lactamica* and induction of protective immunity against *Neisseria meningitidis*. *Clin Infect Dis* 52(1):70–77. 10.1093/cid/ciq065.ciq065 [pii] [PubMed: 21148522]
8. Bennett JS, Bratcher HB, Brehony C et al. (2014) The genus *Neisseria*. The prokaryotes – alphaproteobacteria and betaproteobacteria, 4th edn Springer-Verlag, Berlin, Heidelberg
9. Liu G, Tang CM, Exley RM (2015) Non-pathogenic *Neisseria*: members of an abundant, multi-habitat, diverse genus. *Microbiology* 161(7):1297–1312. 10.1099/mic.0.000086 [PubMed: 25814039]
10. Janda W, Bohnhoff M, Morello J et al. (1980) Prevalence and site-pathogen studies of *Neisseria meningitidis* and *N. gonorrhoeae* in homosexual men. *JAMA* 244:2060–2064 [PubMed: 6776296]

If inoculating multiple strains of *Nmus*, it is vital to sterilize the hood and instruments between groups. All materials that had contact with each group of mice should be decontaminated or replaced, before moving to the next bacterial strain to prevent cross contamination.

11. Caugant DA, Maiden MC (2009) Meningococcal carriage and disease—population biology and evolution. *Vaccine* 27(Suppl 2):B64–B70. 10.1016/j.vaccine.2009.04.061.S0264-410X(09)00615-X [pii] [PubMed: 19464092]
12. Ma M, Powell DA, Weyand NJ et al. (2018) A mouse model for *Neisseria* colonization. *Infect Immun*. 10.1128/IAI.00839-17
13. Weyand NJ, Ma M, Phifer-Rixey M et al. (2016) Isolation and characterization of *Neisseria musculi* sp. nov., from the wild house mouse. *Int J Syst Evol Microbiol* 66(9):3585–3593. 10.1099/ijsem.0.001237 [PubMed: 27298306]
14. Rice PA, Shafer WM, Ram S et al. (2017) *Neisseria gonorrhoeae*: drug resistance, mouse models, and vaccine development. *Annu Rev Microbiol* 71:665–686. 10.1146/annurev-micro-090816-093530 [PubMed: 28886683]
15. Weyand NJ (2017) *Neisseria* models of infection and persistence in the upper respiratory tract. *Pathog Dis* 75(3). 10.1093/femspd/ftx031
16. Johswich KO, McCaw SE, Islam E et al. (2013) In vivo adaptation and persistence of *Neisseria meningitidis* within the nasopharyngeal mucosa. *PLoS Pathog* 9(7):e1003509. 10.1371/journal.ppat.1003509
17. Join-Lambert O, Lecuyer H, Miller F et al. (2013) Meningococcal interaction to microvasculature triggers the tissular lesions of purpura fulminans. *J Infect Dis* 208(10): 1590–1597. 10.1093/infdis/jit301 [PubMed: 23840047]
18. Merz AJ, So M (1997) Attachment of piliated, Opa– and Opc– gonococci and meningococci to epithelial cells elicits cortical actin rearrangements and clustering of tyrosine-phosphorylated proteins. *Infect Immun* 65(10):4341–4349 [PubMed: 9317047]
19. Join-lambert O, Morand PC, Carbonelle E et al. (2010) Mechanisms of meningeal invasion by a bacterial extracellular pathogen, the example of *Neisseria meningitidis*. *Prog Neurobiol* 91(2):130–139. 10.1016/j.pneurobio.2009.12.004 [PubMed: 20026234]
20. Vedros NA, Hoke C, Chun P (1983) *Neisseria macacae* sp. nov., a new *Neisseria* species isolated from the oropharynxes of rhesus monkeys (*Macaca mulatta*). *Int J Syst Bacteriol* 33(3):515–520
21. Weyand NJ, Wertheimer AM, Hobbs TR et al. (2013) *Neisseria* infection of rhesus macaques as a model to study colonization, transmission, persistence, and horizontal gene transfer. *Proc Natl Acad Sci U S A* 110(8):3059–3064. 10.1073/pnas.1217420110.1217420110 [pii] [PubMed: 23382234]
22. Bennett JS, Jolley KA, Earle SG et al. (2012) A genomic approach to bacterial taxonomy: an examination and proposed reclassification of species within the genus *Neisseria*. *Microbiology* 158(Pt 6): 1570–1580. 10.1099/mic.0.056077-0 [PubMed: 22422752]
23. Dillard JP (2011) Genetic manipulation of *Neisseria gonorrhoeae*. *Curr Protoc Microbiol*. Chapter 4:Unit4A.2. 10.1002/9780471729259.mc04a02s00
24. Aylor DL, Valdar W, Foulds-Mathes W et al. (2011) Genetic analysis of complex traits in the emerging Collaborative Cross. *Genome Res* 21(8):1213–1222. 10.1101/gr.111310.110.gr.111310.110 [pii] [PubMed: 21406540]
25. Jolley KA, Maiden MC (2010) BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 11:595 10.1186/1471-2105-11-595 [PubMed: 21143983]