**Authors:**

Adam P. Dale1**\***, Diane F. Gbesemete1,2, Robert C. Read1,2,3, Jay R. Laver1

**Author Affiliations:**

1. Clinical and Experimental Sciences, University of Southampton, Southampton, UK.

2. NIHR Clinical Research Facility, University Hospital Southampton NHS Foundation Trust, Southampton, UK.

3. NIHR Southampton Biomedical Research Centre, Southampton, UK.

**\*Corresponding author** - Email: a.p.dale@soton.ac.uk

**Running Head:** *N. lactamica* controlled human infection model

**i. Chapter Title**

*Neisseria lactamica* controlled human infection model

**ii. Summary/Abstract**

*Neisseria lactamica* is a non-pathogenic commensal of the human upper respiratory tract that has been associated with protection against *N. meningitidis* colonisation and disease. We have previously utilised the *N. lactamica* controlled human infection model to investigate the protective effect of *N. lactamica* colonisation on *N. meningitidis* colonisation, the nature of cross-reactive immune responses mounted towards *N. meningitidis* following *N. lactamica* colonisation, and the microevolution of *N. lactamica* over a 5 month colonisation period. More recently, we have assessed the possibility of utilising genetically modified strains of *N. lactamica* to enable use of the commensal as a vehicle for prolonged exposure of the nasopharynx of humans to antigens of interest, expressed in carried organisms. A controlled infection with *N. lactamica* expressing the meningococcal antigen NadA has been executed and the results demonstrate that this strategy is effective at generating immune responses to the target antigen.

Throughout this chapter we outline in a step-by-step manner the methodologies utilised when performing controlled human infection with *N. lactamica* including procedures relating to: (i) the dilution of *N. lactamica* stock vials to derive intra-nasal inocula, (ii) the delivery of intra-nasal inocula to human volunteers, (iii) the determination of *N. lactamica* colonisation status following intra-nasal inoculation using oropharyngeal swabbing and nasal wash sampling, (iv) the microbiological procedures utilised to identify *N. lactamica* colonisation amongst study volunteers, and (v) the identification of *N. lactamica* colonies as strain Y92-1009 using polymerase chain reaction.

**iii. Key Words**

*Neisseria lactamica*, *Neisseria meningitidis*, Controlled human infection model, CHIME, Human challenge

**1. Introduction**

Oropharyngeal carriage of *Neisseria meningitidis* is a prerequisite for meningococcal disease, and there is a complex biological interaction between this organism and its host ***(1)***. In most continents other than Africa, the highest carriage rates of *N.meningitidis* occur in young adults, particularly in Universities and colleges ***(2)***. Although glycoconjugate meningococcal vaccines such as the quadrivalent ‘ACWY’ vaccine have had dramatic effects on disease incidence, this is mostly due to herd protection conferred by reduced carriage and transmission ***(3)***. However, even prior to the advent of meningococcal vaccines, meningococcal disease was relatively rare other than during epidemics despite the fact that meningococcal carriage was prevalent in a significant minority of asymptomatic individuals. It is widely considered that natural suppression of widespread dissemination of carriage of disease-associated *N.meningitidis* occurs in nature by carriage of non-pathogenic Neisseriaceae. *Neisseria lactamica,* like *N. meningitidis,* is a member of the Neisseriaceae but in contrast, *N. lactamica* is non-capsulate and lacks pathogenic potential. It is a common commensal of the human nasopharynx, particularly in young children. Evidence for this includes the strong epidemiological relationship between carriage of *N. lactamica* and meningococcal disease. Age-specific rates of *N. meningitidis* carriage and disease have been shown to be inversely associated with carriage of *N. lactamica* ***(4, 5)*** and mathematical modelling suggests a period of 4-5 years of protection from meningococcal carriage following carriage of *N. lactamica* ***(6)***.

To try to determine the mechanism of this relationship we did a controlled intranasal infection study of young adults with *N. lactamica* Y92-1009 (sequence type 3493, clonal complex 613). Stocks of this strain had been manufactured at the Good Manufacturing Practices (GMP) pharmaceutical manufacturing facilities at Public Health England (PHE), Porton Down, United Kingdom, to generate the seed banks for a outer membrane vesicle vaccine, theoretically capable of generating immunity to a broad spectrum of *N. meningitidis* serogroups including serogroup B, which expresses the least immunogenic meningococcal polysaccharide. This intra-muscular vaccine had been tested in a Phase 1 study, and whilst well tolerated and capable of generating antibody to *N. lactamica*, it did not produce cross-reacting bactericidal antibodies against a bank of pathogenic *N. meningitidis* serogroup B strains ***(7)***. Using a *N. lactamica* controlled human infection model experiment (CHIME), we showed that intranasal inoculation with 104 colony forming units (CFU) of strain Y92-1000 was well tolerated and safe and caused carriage in 65 % of young adults, and this carriage persisted in most carriers for 6 months. Note that this original group included active smokers. Carriage was followed by development of humoral immunity to *N. lactamica* in those who carried the inoculated strain, but this did not induce significant cross-reactive bactericidal antibodies against *N. meningitidis* ***(8)***.

Subsequently we sought direct evidence for a carriage prevention effect by conducting controlled infection with *N. lactamica* in healthy university students, the group that has the highest rates of acquisition of meningococci. To do this, 310 non-smoking university students were inoculated with *N. lactamica* or were sham-inoculated, and carriage was monitored for 26 weeks. At baseline, natural *N. meningitidis* carriage in the control group of students was 22.4 %, which increased to 33.6 % by week 26. Two weeks after inoculation of *N. lactamica*, 33.6 % of the challenge group became colonised with *N. lactamica*. In this group, meningococcal carriage reduced significantly from 24.2 % at inoculation to 14.7 % 2 weeks after inoculation. We found that the inhibition of meningococcal carriage was only observed in volunteers who were actively colonised with *N. lactamica*, was due both to displacement of existing meningococci and to inhibition of new acquisition, and persisted over at least 16 weeks. At the end of the 26-week period we did crossover inoculation of controls with *N. lactamica* and this replicated the result in the original control group. The impact that we observed on carriage reduction of *N. meningitidis* was just as potent as that observed after glycoconjugate polysaccharide vaccination, and furthermore, genome sequencing showed that the inhibition affected multiple meningococcal sequence types ***(9)***.

A closed genome sequence of strain Y92-1000 was then defined ***(10)*** which enabled us to study microevolutionary changes of *N. lactamica* over the course of 5 months of carriage using the human cohort undergoing controlled infection. We found that most mutations are transient indels within repetitive tracts of putative phase-variable loci associated with host-microbe interactions (pgl and lgt) and iron acquisition (fetA promotor and hpuA). Recurrent polymorphisms occurred in genes associated with energy metabolism (nuoN, rssA) and the CRISPR-associated cas1. In volunteers who were naturally co-colonised with meningococci, recombination altered allelic identity in *N. lactamica* to resemble meningococcal alleles, including loci associated with metabolism, outer membrane proteins and immune response activators, but there was little evidence of recombination in the opposite direction ***(11)***.

Recently we have delineated B cell responses to *N. lactamica* colonisation and their cross-reactivity with *N. meningitidis* ***(12)*** and have investigated the possibility of genetic modification of *N. lactamica* to enable use of the commensal as a vehicle for prolonged exposure of the nasopharynx of humans to antigens of interest, expressed in carried organisms. A controlled infection with *N. lactamica* expressing the meningococcal antigen NadA has been planned and executed ***(13)*** and the results demonstrate that this strategy is effective at generating immune responses to the target antigen ***(14)***.

Throughout this chapter we outline in a step-by-step manner the methodologies utilised when performing *N. lactamica* CHIMEs including procedures relating to: (i) the dilution of *N. lactamica* stock vials to derive intra-nasal inocula, (ii) the delivery of intra-nasal inocula to human volunteers, (iii) the determination of *N. lactamica* colonisation status following intra-nasal inoculation using oropharyngeal swab and nasal wash sampling, (iv) the microbiological procedures utilised to identify *N. lactamica* colonisation amongst study volunteers, and (v) the identification of cultured *N. lactamica* colonies as the inoculum strain (Y92-1009) using polymerase chain reaction (PCR).

# 2. Materials

**2.1 Dilution of *N. lactamica* stock vials to 105 CFU/ml for intra-nasal inoculation**

1. Stock vial containing 1x108 CFU/ml *N. lactamica* Y92-1009 (ST-3493, clonal complex 613, suspended in 1 ml Franz medium) produced in the GMP-accredited facilities at PHE’s Porton Down facility, Salisbury, UK. Following transfer from PHE, stock vials of *N. lactamica* are stored in a dedicated, secure and remotely monitored -80 °C freezer.
2. Decontaminated class II microbiological safety cabinet (MSC), dedicated for *N. lactamica* inoculum preparation.
3. Decontaminated vortex mixer, dedicated for *N. lactamica* inoculum preparation.
4. Decontaminated set of calibrated Gilson-style pipettes, dedicated for *N. lactamica* inoculum preparation.
5. Incubator set at 37 °C, 5 % CO2.
6. Sterile filter pipette tips.
7. 10 μl sterile, disposable plastic microbiological loops.
8. 70 % alcohol solution (v/v).
9. Single use waste box.
10. Disposable gloves.
11. Sterile phosphate buffered saline (PBS) (1 x, autoclaved and passed through a sterile 0.2 μm filter).
12. Columbia blood agar (CBA) plates. Containing Columbia agar base with 5 % defibrinated horse blood.
13. Gonococcal (GC) agar plates with 5-bromo-4-chloro-3-indolyl-B-D galactopyranoside (X-gal) (GC-X-gal). Containing 36 g/L GC agar base (Oxoid), 100 ml/L lysed horse blood, 20 ml/L Vitox supplement (Oxoid), 4 g/L glucose, 40 mg/L X-gal, 2 mg/L vancomycin, 7.5 mg/L colistin, 3 mg/L trimethoprim and 1 mg/L amphotericin B.
14. Appropriate laboratory source document (to log all stages of *N. lactamica* inoculum preparation in real time).
15. Sterile universal containers.

**2.2 Intra-nasal inoculation with 105 CFU *N. lactamica***

1. Intra-nasal inoculum containing 105 CFU *N. lactamica* suspended in 1 ml PBS (see **Section 3.2**).
2. Dedicated room within a clinical environment, *e.g.* a clinical research facility (CRF), complete with an examination couch.
3. Disposable aprons and gloves.
4. Decontaminated and dedicated 1000 μl Gilson-style pipette.
5. Sterile pipette tips.
6. Clinical waste bin.
7. Appropriate laboratory source document (to log all stages of *N. lactamica* inoculation in real time).

**2.3 Taking and processing an oropharyngeal throat swab to determine volunteer *N. lactamica* colonisation status**

1. Sterile throat swab for microbiological culture with an appropriate transport medium (*e.g.* TS/5-17 Probact amies clear medium in tube with polystyrene viscose tip swab).
2. GC-X-gal plate.
3. Dedicated room within a clinical environment, *e.g.* a CRF, complete with an examination couch.
4. Class II MSC housed within an Advisory Committee on Dangerous Pathogens (ACDP) containment level 2 (CL2) laboratory.
5. Appropriate laboratory waste container.
6. Incubator set at 37 °C, 5 % CO2.

**2.4 Taking and processing a nasal wash sample to determine volunteer *N. lactamica* colonisation status**

1. Sterile saline (0.9 %) for irrigation or injection (20 ml) at room temperature.
2. 2 x 10ml sterile syringes.
3. Sterile plastic petri dish.
4. 2 x GC-X-gal plates.
5. Sterile PBS.
6. Sterile 50 ml centrifuge tube.
7. Set of calibrated Gilson-style pipettes and sterile tips.
8. Sterile microbiology plate spreaders.
9. Disposable gloves and aprons.
10. Tissues.
11. Transport box with ice.
12. Dedicated room within a clinical environment, *e.g.* a CRF, complete with an examination couch.
13. Class II MSC housed within an ACDP CL2 laboratory.
14. Centrifuge.
15. Appropriate laboratory waste container.
16. Incubator set at 37 °C, 5 % CO2.

**2.5 Identification of *N. lactamica* colonies**

1. Class II MSC.
2. GC-X-gal agar plate.
3. 10 μl sterile, disposable plastic microbiological loops.
4. Sterile Bacterial Storage Medium: *i.e.* 50:50 (v/v) mixture of (i) Tryptone Soya Broth supplemented with 0.2 % (w/v) yeast extract and (ii) 60:40 (v/v) mixture of glycerol and PBS. Stored in 0.5 ml aliquots in cryogenic storage vials.
5. Oxidase reagent-impregnated strips.
6. Glass microscope slides.
7. 0.5% crystal violet solution.
8. Gram’s (Lugol’s) iodine solution.
9. Carbolfuchsin or safranin solution.
10. Gram’s acetone solution.
11. Distilled H2O.
12. Heat block.
13. Blotting paper.
14. Mineral oil.
15. Microscope with 100x lens (oil immersion).
16. API NH kit (Biomerieux).
17. Genomic DNA extraction kit.
18. Set of calibrated Gilson-style pipettes.

**2.6 Identification of *N. lactamica* colonies as strain Y92-1009 using PCR**

*2.6.1 Preparation of PCR master mix*

1. Dedicated, decontaminated PCR preparation area or laminar flow cabinet.
2. Set of calibrated Gilson-style pipettes and sterile filter tips.
3. Disposable gloves and dedicated, pre-PCR laboratory coat.
4. Tissues.
5. 10 % (v/v) bleach solution in spray bottle.
6. Distilled H2O in spray bottle.
7. 70 % (v/v) alcohol solution.
8. Q5 Hot Start High Fidelity 2 x Master Mix (New England Biosciences).
9. Primer stocks: SeqA\_FOR (10 μM) and SeqA\_REV (10 μM); SeqB\_FOR (10 μM) and SeqB\_REV (10 μM); SeqC\_FOR (10 μM) and SeqC\_REV (10 μM); *lacZ*\_FOR (10 μM) & *lacZ*\_REV (10 μM). For primer sequences, see **Table 1**.
10. DNase/RNase-free, molecular biology grade H2O.
11. Thin walled, 0.2 ml PCR tubes and 0.2 ml tube racks.
12. Benchtop centrifuge.
13. Appropriate laboratory waste container.

*2.6.2 Performance of Y92-1009-specific PCR*

1. Dedicated, decontaminated PCR preparation area or laminar flow cabinet.
2. Set of calibrated Gilson-style pipettes and sterile filter tips.
3. Disposable gloves and dedicated, pre-PCR laboratory coat.
4. Tissues.
5. 10 % (v/v) bleach solution in spray bottle.
6. Distilled H2O in spray bottle.
7. Number of 24 μl aliquots of [1 x Y92-1009-specific PCR Master Mix] equal to number of isolates for identification.
8. Genomic DNA extracted from colonies positively identified as *N. lactamica* (see **Section 3.5**).
9. Genomic DNA extracted from *N. lactamica* strain Y92-1009 (@ 50 ng/μl).
10. DNAse/RNAse-free, molecular biology grade H2O.
11. Appropriate laboratory waste container.
12. Laboratory marker pen
13. Laboratory lab coat for conducting PCR.
14. Thermal cycler.

*2.6.3 Analysis of PCR products using agarose gel electrophoresis*

1. Laboratory coat and disposable gloves.
2. Set of calibrated Gilson-style pipettes and sterile tips.
3. Certified PCR agarose.
4. Tris-Acetate-EDTA (TAE) buffer (1x).
5. Non-toxic DNA intercalating agent.
6. DNA molecular weight ladder (200 bp – 1000 bp).
7. Digital gel imaging system.
8. Appropriate laboratory source document.

# 3. Methods

**3.1 Dilution of *N. lactamica* stock vials to 105 CFU/ml for intra-nasal inoculation**

*N. lactamica* inoculum preparation is performed in tandem by two trained members of the laboratory technical team (see **Note 1**).

1. Don a pair of disposable gloves and disinfect the class II MSC with 70 % alcohol.
2. Label a series of universal containers in preparation for serial dilution of the *N. lactamica* stock inoculum vial contents, as follows: 10-1, 10-2, 10-3, 10-4, 10-5 and 10-6 dilution.
3. Pipette 900 μl PBS into the universal containers labelled 10-1, 10-2, 10-4, 10-5 and 10-6 and 4.5 ml of PBS into the universal container labelled 10-3.
4. Label the CBA and GC-X-gal plates and ensure they are at room temperature prior to use.
5. Remove a stock vial containing 1x108 CFU/ml *N. lactamica* Y92-1009 from the -80 °C freezer and transport this vial immediately on ice to the class II MSC. Take the stock vial off ice and place within the class II MSC and allow it to thaw at room temperature.
6. Immediately upon thawing, vortex the stock vial for 30 seconds and then pipette 100 μl of the stock vial contents into the universal container labelled 10-1 before vortexing for 30 seconds.
7. Having applied a new filter tip, pipette 100 μl of the 10-1 dilution into the universal tube labelled 10-2 before vortexing for 30 seconds.
8. Having applied a new filter tip, pipette 500 μl of the 10-2 dilution into universal container 10-3 before vortexing for 30 seconds.
9. Having applied a new filter tip, pipette 1 ml volumes of the 10-3 dilution (containing 105 CFU *N. lactamica*/ml) into universal containers and transport to the CRF for use as intranasal inocula.
10. To verify the purity of the prepared batch of *N. lactamica* inocula, streak 10 μl volumes of the 10-3 dilution onto CBA and GC-X-gal agar plates using a microbiological loop and incubate for 48 hours at 37 °C, 5 % CO2. Following incubation, assess for the pure growth of colonies morphologically in keeping with *N. lactamica* that are grey on CBA and blue on GC-X-gal plates.
11. To enable *N. lactamica* CFU/ml of the prepared inocula to be calculated formally, utilise the 10-3 dilution to immediately perform 3 further serial dilutions in the universal containers labelled 10-4,10-5, and 10-6, ensuring that a new pipette tip is utilised between dilution steps and that each dilution is vortexed for 30 seconds.
12. Pipette 3 x 10μl drops of the 10-4, 10-5 and 10-6 dilutions onto a GC-X-gal plate and allow to dry for 5 minutes before incubating for 48 hours at 37 °C, 5 % CO2. Following incubation, identify the dilution with an easily countable number of colonies (10-50 colonies within each 10 μl drop). Use the Mean to calculate *N. lactamica* CFU/ml in the prepared inoculum taking into account the dilution factor (CFU/ml = Mean colony count x 100 x dilution factor).

**3.2 Intra-nasal inoculation with 105 CFU *N. lactamica***

Prior to enrolment onto a *N. lactamica* CHIME study, volunteers undergo extensive screening to ensure they are medically fit and that it is safe for them to participate. While the full volunteer screening protocol is beyond the scope of this chapter, key inclusion and exclusion criteria used across the *N. lactamica* CHIME studies during the screening process are outlined in **Note 2**. Once a volunteer has passed the screening process and is enrolled onto the study, the methodology outlined below is followed to ensure a standardised approach to intra-nasal inoculation. It must be noted that all intra-nasal inoculation procedures must be performed in an appropriate clinical environment, *e.g.* a CRF, by a member of the clinical research team.

1. The volunteer is asked to drink only water in the 1 hour period prior to their intra-nasal inoculation appointment.
2. Explain the procedure to the volunteer and obtain informed consent.
3. Volunteer asked to don a disposable apron and lie supine on the clinical examination couch with their head tilted back (see **Figure 1**).
4. Prepared inoculum (105 CFU *N. lactamica* suspended in 1 ml PBS) is transported from the laboratory to the CRF. The inoculum must be utilised within 30 minutes of preparation (see **Note 3**).
5. Clinical research team member washes their hands and dons a pair of gloves and a disposable apron.
6. Using a Gilson-style pipette with sterile tip, 0.5 ml of the prepared inoculum is instilled slowly in a drop-wise fashion into each nostril, one at a time. Following intra-nasal inoculation, the volunteer is asked to remain supine for 5 minutes.
7. The used pipette tip, apron and gloves are disposed in the clinical waste.
8. Following intra-nasal inoculation, the volunteer is monitored within the CRF for 30 minutes after which point a set of clinical observations is taken including pulse, blood pressure, respiratory rate and temperature (tympanic). Assuming the volunteer is well and has normal observations following intra-nasal inoculation, they are then discharged from the CRF.

**3.3 Taking and processing oropharyngeal throat swabs to determine volunteer *N. lactamica* colonisation status**

1. Explain the procedure to the volunteer and obtain informed consent.
2. Wash hands and apply a clean pair of disposable gloves and an apron.
3. While sitting upright, ask the volunteer to tilt their head back and open their mouth wide.
4. When the head of the swab has made contact with the pharyngeal wall, behind the uvula, stroke it across the pharyngeal wall 5 times.
5. Remove the swab from the oral cavity, place it into the amies transport medium, seal the swab and transfer it immediately to the class II MSC for further processing.
6. Working in the class II MSC, apply the swab contents directly to the GC-X-gal plate by streaking the swab vertically in a continuous fashion while rotating the plate and swab tip. Ensure the swab makes contact with all areas of the agar.
7. Dispose of the swab in the appropriate waste container and incubate the GC-X-gal plate immediately at 37 °C, 5 % CO2 for 48 hours.
8. Following 48 hours incubation, assess the GC-X-gal plate for growth of putative colonies of *N. lactamica* in line with the methodology outlined in **Section 3.5**.

**3.4 Taking and processing nasal wash samples to determine volunteer *N. lactamica* colonisation density**

1. Explain the procedure to the volunteer and obtain informed consent.
2. Ask the patient to don a disposable apron to protect their clothing.
3. Position the patient supine with a pillow placed under their shoulders to enable extension of the neck backwards.
4. Don an apron and a pair of gloves.
5. Draw up 10 ml of sterile 0.9 % saline into the syringe and expel any air.
6. Ask the volunteer to hold the petri dish on their chest to enable collection of the nasal wash fluid (leave the lid on the petri dish at this point).
7. Ask the volunteer to open their mouth, extend their head backwards, and position their tongue to avoid swallowing the saline. Remind them to breathe through their mouth and not to swallow.
8. Once in position and the volunteer has signalled they are ready, place the tip of the syringe in their nostril and gently instil 10 ml of saline and ask them to hold the saline in their nasopharynx for 1 minute.
9. After 1 minute, ask the volunteer to open the lid of the petri dish and to lean forward as fast as possible to allow the saline to exit the nose passively and be caught in the petri dish.
10. Withdraw the nasal wash specimen from the petri dish using the syringe and transfer it to the centrifuge tube.
11. Repeat steps 5-10 using the alternate nostril.
12. Transport the nasal wash specimen on ice immediately to the microbiology laboratory for processing.
13. Measure and record the total volume of nasal wash fluid retrieved from the volunteer.
14. Centrifuge the nasal wash fluid at 5000 x gfor 10 minutes to pellet the bacteria.
15. Carefully decant the supernatant and re-suspend the pellet in a total volume of 300 μl PBS before vortexing for 30 seconds to loosen the mucous.
16. Inoculate 2x GC-X-gal plates with 250 μl and 25 μl volumes of the resuspended pellet, respectively, and lawn over the plates using individual microbiology spreaders.
17. Incubate the GC-X-gal plates immediately at 37 °C, 5 % CO2, for 48 hours.
18. Following 48 hours incubation, assess the GC-X-gal plates for growth of putative colonies of *N. lactamica* in line with the methodology outlined in **Section 3.5**.
19. Calculate the *N. lactamica* colonisation density within the nasal wash where necessary as *N. lactamica* CFU/ml.

**3.5 Identification of *N. lactamica* colonies from throat swab and nasal wash cultures**

1. Working in a class II MSC, visually inspect the GC-X-gal agar plates inoculated with throat swab or nasal wash contents for evidence of putative *N. lactamica* colonies, *i.e.* blue colonies (beta-galactosidase positive) with morphology consistent with *Neisseria spp.*
2. Pick a well-defined, accessible blue colony and subculture it by streaking it onto GC-X-gal agar with a 10 μl bacteriological loop. Incubate at 37 °C, 5 % CO2, for 24 hours.
3. Assuming a pure subculture is obtained, produce a stock of the isolate in an aliquot of bacterial storage medium. Macerate colonies against the side of the cryogenic storage vial to produce a suspension, then incubate for 10 minutes and freeze at minus 80 °C.
4. Check the oxidase status of the organism by spreading a small amount of a single blue colony onto an oxidase strip using a 10 μl bacteriological loop. A positive oxidase test (strip turns blue/black where contact with colony is made) would be in keeping with potential *N. lactamica* and should prompt a Gram stain to be performed. If the bacteria is oxidase negative, then the isolate is not *N. lactamica* and the prepared stock (step 3, above) can be discarded.
5. To perform a Gram stain, the following steps should be followed:
* Place a drop of distilled H2O onto a clean glass microscope slide and emulsify a small amount of a single blue colony into it using a 10 μl bacteriological loop.
* Dry and fix the slide by placing on a heat block at 65 °C.
* Remove the slide from the class II MSC and flood the slide with 0.5 % crystal violet solution and leave for 30 seconds.
* Wash off the crystal violet solution with tap water and then flood the slide with Gram’s acetone and wash off with tap water rapidly.
* Flood the slide with carbolfuchsin or safranin solution and leave for 30 seconds.
* Wash off the carbolfuchsin or safranin with tap water and dry with clean blotting paper.
* View under oil immersion at 100 x magnification.
1. A Gram stain that reveals Gram negative diplococci is consistent with *N. lactamica* and should prompt speciation using a biochemical test strip, *e.g.* the API NH (Biomerieux). The API NH testing kit comes ready to use with all required reagents and clear step-by-step instructions. *N. lactamica* can be identified with the API NH test code of 5041 with the whole process taking approximately 3 hours ***(15)***.
2. Blue colonies on GC-X-gal agar that are oxidase positive, Gram negative diplococci with an API NH code of 5041 are *N. lactamica*. To confirm the cultured *N. lactamica* as strain Y92-1009, *i.e.* the inoculum strain, PCR is used (**Section 3.6**).
3. To provide template material for amplification by the Y92-1009-specific PCR: return to the frozen stock of the isolate, culture the bacteria and then isolate genomic DNA using a genomic DNA extraction kit according to the manufacturer’s instructions.

**3.6 Identification of *N. lactamica* colonies as strain Y92-1009 using PCR**

*3.6.1 Preparation of PCR master mix*

## Don pre-PCR laboratory coat and gloves.

## If not already active, ensure the laminar flow cabinet is empty, and then turn on the laminar flow cabinet. Expose inside of cabinet to UV light for 15 minutes (if available).

## Decontaminate working area by liberal application of 10 % (v/v) bleach solution.

## Wait for 10 minutes for the bleach to inactivate environmental amplicons and other potential DNA contaminants. Change gloves.

## Meanwhile: gather together the Q5 Hot-Start High Fidelity 2x Master Mix (in 500 μl aliquots), the primer stocks, the DNAse/RNAse-free molecular grade H2O and the 0.2 ml, thin walled PCR tubes and racks. Note that you will need 20 x 0.2 ml PCR tubes per 500 μl aliquot of Q5 Hot-Start High Fidelity 2x Master Mix.

## Spray working area with distilled H2O and then mop up the resulting moisture with paper towelling. Carefully dispose of the paper towelling in the waste bin. Change gloves.

## If not already deactivated, deactivate the UV light and then open the laminar flow cabinet.

## Inside the laminar flow cabinet, add the following volumes of the appropriate primers into the 500 μl aliquot of Q5 Hot-Start High Fidelity 2x Master Mix:

## 25 μl: SeqA FOR

## 25 μl: SeqA REV

## 25 μl: SeqB FOR

## 25 μl: SeqB REV

## 25 μl: SeqC FOR

## 25 μl: SeqC REV

## 25 μl: *lacZ*\_FOR

## 25 μl: *lacZ*\_REV

1. Add 30 μl of DNAse/RNAse-free molecular biology grade H2O to the 500 μl aliquot of Q5 Hot-Start High Fidelity 2x Master Mix supplemented with primers. This is the Y92-1009-specific 1x Master Mix.
2. Transfer 24 μl aliquots of the Y92-1009-specific 1x Master Mix into individual 0.2 ml, thin walled PCR tubes and stand in 0.2 ml tube racks. Make sure the tube rack is labelled as containing aliquots of the Y92-1009-specific Master Mix.
3. Place the aliquots of Y92-1009-specific 1x Master Mix into the fridge (4 oC) for use later that day, or proceed directly to **Section 3.6.2**.
4. If work in the laminar flow cabinet is completed, seal the laboratory waste container, remove all items from inside the laminar flow cabinet, spray the inside of the laminar flow cabinet liberally with 70 % (v/v) alcohol solution and then close the sash on the laminar flow cabinet.

*3.6.2 Performance of Y92-1009-specific PCR*

1. If using boiled bacterial lysates, either fresh or frozen, remove bacterial debris from each suspension by centrifugation of the lysates at 17,000 x gfor 10 minutes. Note that the Y92-1009-specific PCR will also amplify target sequences from purified genomic DNA. Solutions of extracted genomic DNA are most easy to use at a concentration of 50 μg/ml.
2. Don pre-PCR laboratory coat, safety spectacles and nitrile gloves.
3. Decontaminate working area by liberal application of 10 % (v/v) bleach solution.
4. Wait for 10 minutes for the bleach to inactivate environmental amplicons and other potential DNA contaminants. Change gloves.
5. Meanwhile: gather together the appropriate number of aliquots of the ‘Y92-1009-specific 1 x Master Mix’, such that there is one aliquot of 1x Master Mix for each sample to be analysed. Gather additional aliquots of 1 x Master Mix sufficient to include one positive and one negative control reaction for every two rows of wells that will be filled during agarose gel electrophoresis (**Section 3.6.3**). Note that each PCR control tube will provide sufficient material to load 2 wells of control material. One well containing positive control material and one well containing negative control material must be loaded in each row of wells, to allow for (i) visual comparison of sample amplicons to those generated from wild type genomic DNA extracted from *N. lactamica* strain Y92-1009, and (ii) to show that no contaminating DNA was present in the 1x Master Mix, respectively.
6. Spray working area with distilled H2O and then mop up the resulting moisture with paper towelling. Carefully dispose of the paper towelling in the waste bin. Change gloves.
7. For each sample, carefully transfer 1 μl of the lysate or purified, genomic DNA (50 ng) into a single 24 μl aliquot of ‘Y92-1009-specific 1 x Master Mix’. Appropriately label each PCR tube with the laboratory marker pen.
8. Into one of the remaining 1 x Master Mix’ aliquots, add 1 μl of *N. lactamica* gDNA derived from wild type strain Y92-1009 (50 ng), and into another add 1 μl of DNAse/RNAse-free H2O. Appropriately label each tube with the laboratory marker pen.
9. Retain all 1.5 ml microcentrifuge tubes containing the boiled lysates until a given lysate has been definitively identified. Boiled lysates can be re-frozen at -20 oC.
10. Place all PCR tubes containing complete reaction mixtures (including controls) into the Thermal Cycler. Set the Thermal Cycler to run according to the parameters shown in **Note 4**.

*3.6.3 Analysis of PCR products using agarose gel electrophoresis*

1. Don laboratory coat, safety spectacles and nitrile gloves.
2. Prepare 2 % (w/v) agarose gel using 1x TAE buffer, supplemented with appropriate concentration of DNA intercalating agent.
3. Pour agarose gel, incorporating one or more gel combs to produce wells once the gel has set.
4. When the gel sets, submerge in 1x TAE buffer in the gel tank and remove the gel combs.
5. Remove all PCR tubes from the Thermal Cycler.
6. Using filter tips, load 10 μl of DNA molecular weight ladder into the wells at either end of each row of wells. Into the second leftmost well of each row of wells, load 10 μl of a positive control reaction (*i.e.* one which has amplified target sequences from genomic DNA of wild type Y92-1009). Into the second rightmost well of each row of wells, load 10 μl of a negative control reaction (*i.e.* one which has been supplemented with only DNAse/RNAse-free H2O).
7. Using a fresh filter tip for each sample, load 10 μl of each sample into the remaining empty wells.
8. Connect the gel tank to an appropriate power pack and run the gel at 90 V for 90 minutes.
9. Remove gel from the gel tank and image using gel-imaging system.
10. Analyse the banding pattern present in each lane of the gel, with reference to **Figure 2**.
11. Record results of PCR analysis in the relevant section of the appropriate laboratory source document.

**4. Notes**

**Note 1.** Preparation of *N. lactamica* inocula from stock vials is performed by two trained members of the research team who have completed Good Clinical Laboratory Practice training. Each step of the process is verified by both technicians and logged in real time using the appropriate study-specific laboratory source document. This document then forms part of the volunteer case report form.

**Note 2**. Key volunteer inclusion and exclusion criteria utilised in *N. lactamica* CHIMEs in the United Kingdom are as follows:

Inclusion criteria:

* Healthy adults aged 18 to 45 years inclusive on the day of enrolment.
* Fully conversant in the English language.
* Able and willing (in the investigator’s opinion) to comply with all study requirements.
* Written informed consent to participate in the study.
* For females only, willingness to practice continuous effective contraception during the study and a negative pregnancy test at the screening visit.

Exclusion criteria:

* Active smokers.
* Individuals who have a current infection at the time of inoculation.
* Individuals who have been involved in other clinical studies/trials involving receipt of an investigational product over the last 12 weeks or if there is planned use of an investigational product during the study period.
* Any confirmed or suspected immunosuppressive or immunocompromised state, including HIV infection, asplenia, history of recurrent severe infections or use (more than 14 days) of immunosuppressant medication within the past 6 months (topical/inhaled steroids are allowed).
* Allergy to yeast extract.
* Any other significant disease, disorder, or finding which may significantly increase the risk to the volunteer because of participation in the study, affect the ability of the volunteer to participate in the study, or impair interpretation of the study data, for example recent surgery to the nasopharynx.
* Occupational, household or intimate contact with immunosuppressed persons.
* Pregnancy or lactation.

**Note 3**. The viability of *N. lactamica* reduces following thawing and dilution in PBS. The dilution strategy referred to within **Section 3.1** will reliably produce a 1ml volume of inoculum containing 105 CFU, with the viability being maintained up to 30 minutes following preparation. By 1 hour following inoculum preparation, the viability reduces to 5x104 CFU/ml. Thus, to ensure intra-nasal inoculation with 105 CFU, the inoculum should be utilised immediately, *i.e.* within 30 minutes of preparation.

**Note 4** – Thermal Cycling parameters for amplification of Y92-1009-specific target sequences are as follows:

STAGE A: 1 x 5 min @ 98 °C.

STAGE B: 35 x 20 seconds @ 98 °C (melting).

 15 seconds @ 63 °C (annealing).

 26 seconds @ 72 °C (extension).

STAGE C: 1 x 5 min @ 72 °C.

(*Optional*): Infinite @ 10 °C.

**Table and Figure Legends**

**Table 1** – **Nucleotide sequences of primers for Y92-1009-specific, multiplex PCR.** (Forward – FOR; Reverse – REV).

**Figure 1 - Intra-nasal inoculation procedure.** Prior to intra-nasal inoculation, the volunteer is asked to lie supine with their head tilted back.

**Figure 2 -** **Interpreting the banding pattern of reaction products from Y92-1009-specific PCR.** Possible banding patterns generated in the Y92-1009-specific PCR: **A.** PCR failure, empty lane, negative control or DNA present from species *other than N. lactamica*. **B through F.** DNA present from strains of *N. lactamica other than* Y92-1009. **G.** *N. lactamica* strain Y92-1009. Note that preliminary identification of *N. lactamica* colonies (*i.e.* oxidase test, Gram stain and API NH – see **Section 3.5**) will in most cases prevent PCR from being performed on species other than *N. lactamica*. Note that, in addition to amplification of the bands shown in this figure, strains of *N. lactamica* other than Y92-1009 may also produce additional bands of unknown and unpredictable size. Strains can only be identified as Y92-1009 if the banding pattern shown in **G** is present.

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