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TITLE:

Method to construct mutants in serotype 1 Streptococcus pneumoniae strain 519/43

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SUMMARY:

Here we describe a *S. pneumoniae* serotype 1 strain 519/43, that can be genetically modified by using its ability to naturally acquire DNA and a suicide-plasmid. As proof of principle, an isogenic

mutant in the pneumolysin (*ply*) gene was made. This protein was chosen because it has a well-known and easy to follow phenotype, the ability to lyse red blood cells.

ABSTRACT:

Streptococcus pneumoniae serotype 1 remain a huge problem in low-and-middle income countries, particularly in sub-Saharan Africa. Despite its importance, studies in this serotype have been hindered by the lack of genetic tools to modify it. In this study we describe a method to successfully modify genetically a serotype 1 clinical isolate (strain 519/43). Interestingly, this was achieved by exploiting the Pneumococcus' ability to naturally acquire DNA, however, unlike most pneumococci, the use of linear DNA was not successful, to mutate this important strain a suicide plasmid had to be used. This methodology has provided us with the means for a deeper understanding of this elusive serotype, both in terms of its biology and pathogenicity. In order to validate the method, the major known pneumococcal toxin, pneumolysin, was mutated because it has a well-known and easy to follow phenotype. We showed that the mutant, as expected, lost its ability to lyse Red Blood Cells. By being able to mutate an important gene in the serotype of interest, we were able to observe different phenotypes for loss of function mutants upon intraperitoneal and intranasal infections from the ones observed for other serotypes. In summary, this study proves that strain 519/43 (serotype 1) can be genetically modified.

INTRODUCTION:

Streptococcus pneumoniae (*S. pneumoniae*, the pneumococcus) is one of the principal causes of morbidity and mortality globally. Up until recently, close to 100 serotypes of *S. pneumoniae* have been discovered [1–7]. Yearly, invasive pneumococcal disease (IPD) claims around 700,000 deaths, of children younger than 5 years old [8]. S. *pneumoniae* is the major cause of bacterial pneumonia, otitis media, meningitis and septicaemia worldwide [9].

In the African meningitis belt, serotype 1 is responsible for meningitis outbreaks, where sequence type (ST) ST217, an extremely virulent sequence type is dominant[10–15]. Its importance in meningitis pathology has been likened to that of *Neisseria meningitidis* in the African meningitis belt [16]. Serotype 1 is often the main cause of IPD; however, it is very rarely found in carriage. In fact, in The Gambia, this serotype is accountable for 20% of all invasive disease, but it was only found in 0.5% of healthy carriers [14, 17–19]. Genetic exchange and recombination in competent pneumococci occurs generally in carriage rather than in invasive disease [20]. Furthermore, serotype 1 has been shown to have one of the shortest carriage rates described amongst pneumococci (only 9 days). Therefore, it has been proposed that this serotype might have a much lower recombination rate than others [21].

In depth studies are necessary to understand the reason behind serotype 1 strains' low rate of carriage and its importance in invasive disease in sub-Saharan Africa.

Here we report a protocol that allows genome-wide mutagenesis of a particular serotype 1 strain, 519/43. This strain can easily acquire and recombine new DNA into its genome. This method is

not yet inter-strain, but it is very efficient when done in 519/43 background (other targets have been mutated, manuscripts in preparation). By simply using 519/43 strain, and exploit its natural competence, as well as substituting the way that the exogenous DNA is provided, we were able to mutate the pneumolysin gene (*ply*) in this serotype 1 strain. This method represents an improvement on the one presented by Harvey *et al*., 2016 [22] as it is done in one-step without the need to passage the DNA through a different serotype. Nevertheless, and due to inter-strain variability, no method is yet standardized to all strains. The ability to mutate specific genes and observe its effects will allow a profound understanding of serotype 1 *S. pneumoniae* strains and it will provide answers for the role of these strains in meningitis in sub-Saharan Africa.

PROTOCOL:

1. Generation of the mutating amplicon by SOE-PCR[23] and amplification of the spectinomycin cassette

1.1. Start by performing PCR for the amplification of the homology arms (ply 5' (488bp) and ply3' (715bp) respectively) of the flanking regions of the *ply* gene from strain 519/43. Use primers plyFw1_NOTI (TTT GCGGCCGCCAGTAAATGACTTTATACTAGCTATG), ply5'R1_BamHI (CGAAATATAGACCAAAGGACGC*GGATCC*AGAACCAAACTTGACCTTGA), ply3'F1_BamHI (TCAAGGTCAAGTTTGGTTCT*GGATCC*GCGTCCTTTGGTCTATATTTCG) and plyRv2_NotI (TTTGCGGCCGCCATTTTCTACCTTATCCTCTACC).

1.2. Use the following PCR conditions denaturing at 94 ^{o}C for 60 sec, step 2: denaturing at 94 ^{o}C for 30 sec, step 3: annealing at 58 ^{o}C for 30 sec, step 4: extension at 72 ^{o}C for 30 sec, step 5: go back to step 2 and repeat for 35 cycles, step 6: final extension at 72 ^{o}C for 30 sec, for ply5', and the same PCR conditions should be used for ply3' with the exception of the extension time on step 4 and step 6 where it should be 60 sec.

1.3. Analyse the PCR products by gel electrophoresis and excise the amplicon from the gel.

1.4. Purify the PCR amplicons following the protocol described in the manufacturer's instructions (Monarch DNA Gel Extraction, NEB, UK).

1.5. Use equimolar amounts of both homology arms as templates in the SOE-PCR. Fuse the two amplicons using primers plyFw1_ NOTI (TTT GCGGCCGCCAGTAAATGACTTTATACTAGCTATG), and plyRv2_NotI (TTTGCGGCCGCCATTTTCTACCTTATCCTCTACC).

1.6. Use the following SOE-PCR conditions (step1: denaturing at 94 ^{o}C for 2 min, step2: denaturing at 94 ^{o}C for 30 sec, step3: annealing 58 ^{o}C for 30 sec, Step 4: extension at 68 ^{o}C for 60 sec, step 5: go back to step 2 and repeat for 25 cycles, step 6: final extension at 68 ^{o}C for 90 sec);

1.7. Analyse the SOE-PCR product by gel electrophoresis and excise it from the gel using a gel extraction kit and follow the instructions provided.

1.8. Amplify the spectinomycin cassette from plasmid pR412 using the following PCR conditions: step 1: denaturing at 94 ^{o}C for 60 sec, step 2: denaturing at 94 ^{o}C for 30 sec, step 3: annealing at 55 ^{o}C for 30 sec, step 4: extension at 68 ^{o}C for 60 sec, step 5: go back to step 2 and repeat for 25 cycles, step 6: final extension at 68 ^{o}C for 60 sec. Use primers BamHI_SP2F2 (GGATCC CTA GAA CTA GTG GAT CCC CC) and BamHI_SP2R2 (GGATCC AAT TCT GCA GAT TTT AC ATG ATC). Plasmid pR412 was acquired from Dr Marc PrudHomme (CNRS-Universite Paul Sabatier Toulouse France).

1.9. Analyse the PCR amplicons by gel electrophoresis and excise and purify the resulting PCR amplicon as described above.

2. Generation of plasmid pSD1 and Chemical transformation of *E. coli* Dh5α

2.1. Perform a Ligation Reaction following the pGEMT-easy system I manufacturer instructions (Promega, UK). In a microcentrifuge tube add 5 µl 2X ligation buffer, 1 µl pGEMTeasy, 2 µl of the ply_SOE product, 1 µl T4 DNA ligase and water to 20 µl total volume. Incubate overnight at 4 °C. This generates plasmid pSD1.

2.2. Transform chemically competent *E. coli* Dh5α (Invitrogen UK) with pSD1. Start by incubate 50 µl of chemically competent *E. coli* Dh5α with 3 µl pSD1 ligation reaction for 15 min on ice, then continue by exposing the cells to thermic shock (42 °C, 30 sec). Place the cells on ice for 2 min.

2.3. Remove the cells from ice and add 350 µL S.O.C media. Incubate the culture for 2 h at 37°C, 120 rpm.

2.4. Plate the transformation on Luria Bertani Agar (LBA) supplemented with 0.4mM IPTG, 0.24mg/ml X-Gal for blue/white selection and 100 µg/ml ampicillin to ensure all colonies growing in the plate have the plasmid backbone. White colonies contain pSD1.

2.5. Pick three white colonies and set up overnight growths in 10 ml LB, supplemented with 100 µg/ml ampicillin. Incubate the cultures overnight at 37 ^{o}C with shaking.

2.6. The next day centrifuge the cultures at 3082 xg and use the pellet for plasmid extraction.

3. Plasmid DNA extraction, restriction digestion of pSD1 and spectinomycin gene and assembly of pSD2

3.1. Extract the plasmid DNA following the instructions provided with the Kit (NEB, Monarch, UK).

Set up a BamHI-restriction digestion for both pSD1 plasmid and the spectinomycin cassette previously amplified and purified. Use the following conditions and quantities described in **Table 1**-Restriction digestion reaction components for pSD1 and spectinomycin cassette.

3.2. .

3.3. Incubate the restriction digestion reactions and controls at 37 °C for 3 hr.

3.4. Analyse the restriction digest by electrophoresis, excise the band and purify following the instructions provided with the Kit (Monarch, UK)**.**

3.5. Next, prepare a ligation reaction following the pGEMT-easy system I manufacturer instructions (Promega, UK) using the BamHI- digested pSD1 and spectinomycin (from 0). In a microcentrifuge tube, add the following reaction components: 5 µl 2X ligation buffer, 2 µl pSD1, 2 µl of spectinomycin cassette, 1 µl T4 DNA ligase and incubate overnight at 4 °C. This generate plasmid pSD2.

3.6. Transform plasmid pSD2 into chemically competent *E. coli* Dh5α as described in point 2.2.

3.7. Select the transformants carrying plasmid pSD2 based on their ability to grow in LBA supplemented with 100 µg/ml of spectinomycin and ampicillin.

3.8. Perform a plasmid DNA extraction (pSD2) as described above and following the manufacturer's instructions (Monarch, NEB, UK).

4. Transformation of *S. pneumoniae* strain 519/43

4.1. Prepare an overnight culture of *S. pneumoniae* 519/43 in BHI and allow it to grow statically, at 37 °C, 5% CO_2**.**

4.2. The following day dilute the cultures 1:50 and 1:100 in 10 ml of fresh BHI broth (Oxoid, UK). Incubate the cultures statically, at 37 °C until the OD_{595nm} is between 0.05 and 0.1 (optimal acquisition of DNA closer to 0.1 OD).

4.3. Once an OD of 0.1 is reached, take 860 μl and transfer into a microcentrifuge tube. In this same microcentrifuge tube add: 100 μl 100 mM NaOH, 10 μl 20% (w/v) BSA, 10 μl 100mM $CaCl_2$ and 2 μl 50 ng/ml CSP1 [24] and 500 ng of pSD2.

4.4. Incubate the reaction statically at 37°C, for 3 hours.

4.5. Plate 330 μl onto 5% blood Agar plates (BA) supplemented with 100 μg/ml spectinomycin, every hour over the 3 incubation hours.

4.6. Incubate plates overnight at 37 °C, 5% CO_2. Spectinomycin resistant colonies must be patched onto another BA plate supplemented with 100 μg/ml spectinomycin as well as onto BA plates supplemented with 100 μg/ml of ampicillin. Both sets of plates must be and incubated overnight under the conditions stated above. The ampicillin plates are to test for the presence of the plasmid backbone.

4.7.

4.8. Confirm the presence of the spectinomycin cassette by PCR using primers plyFw1_ NOTI (TTT GCGGCCGCCAGTAAATGACTTTATACTAGCTATG) and SPEC_REV (TAATTCCTCTAAGTCATAATTTCCG). Confirm the mutation by PCR using primers plySCN1 (CCAATGGAAATCGCTAGGCAAGAGATAA) and plySCN2 (ATTACTTAGTCCAACCACGGCTGAT) which attach outside of the mutated region.

4.9. Confirm the integration in the correct location of the genome by sequencing using primers plySCN1 (CCAATGGAAATCGCTAGGCAAGAGATAA), plySCN2 (ATTACTTAGTCCAACCACGGCTGAT), as well as primers with their binding site in the spectinomycin cassette sqr1 (CCTGATCCAAACATGTAAGTACC) sqf2 (CGTAGTTATCTTGGAGAGAATA) spec_sqf1 (GGTACTTACATGTTTGGATCAGG) and spec_sqr2 TATTCTCTCCAAGATAACTACG.

REPRESENTATIVE RESULTS:

The protocol described here starts by using PCR to amplify the left and right homology arms, whilst simultaneously deleting 191 bp from the middle region of the *ply* gene. While performing the PCR a BamHI site is introduced at the 3' of the left homology arm and at the 5' end of the right homology arm (**Error! Reference source not found.**A). This is followed by PCR-SOE where left and right homology arms are fused into one amplicon (Figure 1B). This SOE-PCR amplicon is then cloned into pGEMTeasy using TA cloning to generate plasmid pSD1 (Figure 1C). Successful transformation will yield white colonies that are resistant to ampicillin. Any blue colonies will be transformants containing an empty pGEMTeasy plasmid. pSD1 will then be digested at the BamHI site which was introduced at the time of the SOE-PCR (Figure 1D) and ligated to a spectinomycin cassette (also BamHI digested for compatible ends). The new plasmid is termed pSD2 (Figure 1E). Correct assembly of pSD2 is confirmed by restriction digestion (Figure 2A). pSD2, that works as a suicide plasmid since it does not contain a Gram+ compatible origin of replication, was used to transform 519/43WT (previously prepared to be competent). Positive transformants are colonies growing on 100 µg/ml spectinomycin after an overnight incubation. All colonies are patched onto newly fresh blood agar base plates supplemented with 100 µg/ml spectinomycin as well as blood agar base supplemented with 100 µg/ml of ampicillin. Any colonies that grow on the second overnight plates supplemented with spectinomycin are possible positives. Any colonies that grow on ampicillin plates will denote integration of the full plasmid or recombination of the ampicillin cassette elsewhere in the genome. So far and using strain 519/43 no colonies have grown on the plates supplemented with ampicillin. All colonies positive by PCR must be confirmed by sequencing (Figure 2C) to assess and confirm the location of the insertion. To this end, primers must have their binding site outside of the homology region (Figure 2C). The chosen target has a very marked phenotype (haemolysis of Red Blood Cells (RBC)), and therefore the mutation can also be confirmed phenotypically (Figure 2B). The mutant lost its ability to lyse RBC's.

FIGURE AND TABLE LEGENDS:

Table 1-Restriction digestion reaction components for pSD1 and spectinomycin cassette.

Figure 1-Overview of the mutagenesis strategy A: Amplification of homology arms (Ply3' and Ply5' (lanes 2 until 5); and Splicing by Overlapping Extension PCR (Lanes 6 and 7). L- hyperladder I (bioline), lane 1- negative control for the reaction, lane 2 and 3- ply5' (488 bp) and ply3' (715 bp) homology arms amplified from D39 gDNA, lanes 4 and 5- ply5' (488 bp) and ply3' (715 bp) homology arms amplified from 519/43 gDNA. Right hand side lane 6- D39 SOE PCR product, lane 7- 519/43 SOE PCR product (1235 bp). **B**-Schematic depicting the final SOE-PCR construct obtained (Ply_SOE); Indicated by arrows are the primers used to obtain the homology region between both homology arms as well as the restriction digestion chosen. **C**- Plasmid pSD1, depicting the cloning of ply_SOE; **D**- Restriction digestion of pSD1 and spectinomycin cassette. **E**- Final construct pSD2 that is then used as a suicide plasmid to transform *S. pneumoniae*.

Figure 2-A*:* Confirmation of the presence of the spectinomycin cassette in pSD2. L- hyperladder 1kb (Bioline); 1- pSD1 digested with BamHI; 2-pSD2 digested with BamHI; L hyperladder 1 kb (Bioline); 3- Spectinomycin cassette amplified from pR412, 4- Spectinomycin cassette digested with BamHI*;* **B-** Phenotypic confirmation of the pneumolysin mutation by determination of haemolytic activity for D39, 519/43WT and mutant 519/43Δply. This was compared to haemolysis of red blood cells by 0.5% saponin. Saponin-derived haemolysis is considered 100% and the rates for 519/43Wt and 519/43Δply were calculated against it. Each data point is the mean of 5 technical and 3 biological replicates. **C**-Sequencing data mapped to the mutant genome region where pneumolysin was interrupted. Primers are indicated as arrows and by name. PLYSCN1 and PLYSCN2 bind outside of the homology arms. Sequencing obtained from primers PLYSCN1 and 2 showed that there was uninterrupted sequence from the neighbouring regions outside of the homology area until the spectinomycin cassette, demonstrating the insertion in the genome.

DISCUSSION:

Streptococcus pneumoniae, in particular serotype 1, continues to be a global threat causing invasive pneumococcal disease and meningitis. Despite the introduction of various vaccines that should be protective against serotype 1, in Africa, this serotype is still capable of causing outbreaks that lead to high morbidity and mortality [13] .The ability to genetically manipulate this serotype is of critical importance because of its clinical relevance. The method described in this study allows the genetic manipulation of a representative strain within this serotype. An invasive strain 519/43 (ST5316), a clinical isolate from a meningitis patient in Denmark [25].

The methodology presented here, was successful mostly due to the chosen strain as it can acquire exogenous DNA, but also due to changes made to the traditional protocols used for *S. pneumoniae* transformation, a typical success rate to our transformation protocol is of about 70%.

With this methodology, it is paramount to use a suicide plasmid instead of the usual linear DNA. Conventionally, linear DNA[26–28] would have been used, however all attempts to use the exogenous DNA in this form were unsuccessful. Furthermore, attempts to exploit the natural competence of *S. pneumoniae* 519/43 at lower absorbance were not successful. Troubleshooting demonstrated that natural competence for strain 519/43 was higher when OD_{595} was 0.1, which is different from the data observed for other serotypes of *S. pneumoniae* where highest natural competence was observed at very low OD[24].

In order to validate the method, a pneumolysin mutant was constructed because it exhibits an easy to follow phenotype, however and to prove that the method can be applied to any gene within this strain, other genes have been successfully targeted (manuscripts in preparation). Such method, using a suicide vector that has no Gram+ compatible origin could also be used for

chromosomal complementation, overexpression of genes of interest, as well as introduction of reporter systems, all by using the neighbouring genes as homology regions.

The expansion of genetic tools to *S. pneumoniae* serotype 1, strain 519/43 is important because, we can now genetically manipulate representative strains directly. Strain 519/43 is of interest as it is genetically pliable, is pathogenic as it was isolated from a meningitis patient, and its manipulation will provide clues to better understand the development and establishment of meningitis. Previously, understanding certain determinants within the species was done by inserting the gene in question in one of the very well characterized strains of *S. pneumoniae*, such as D39 (serotype 2). Such approach was used by Paton e*t al.,* due to difficulties with the mutagenesis on serotype 1 [29]. The results reported by them on D39 carrying a less haemolytic allele of serotype 1 *ply* in comparison to 519/43 Δ*ply* differ from the ones presented by us [30] highlighting the importance of being able to mutate a gene within the original strain background. Later on, the same group was able to mutate a non-lineage A serotype 1 strain [22]. Interestingly, their protocol is quite distinct from ours, as it is a two-step approach that requires the mutation to be done first in serotype 2 strains and this is then used as a template to be transformed in their serotype 1 strain. Currently, there is one limitation in the method presented by us, for now, this method works only for the 519/43 representative strain. The same exact protocol was tried in other strains, namely clinical isolates from ST3081 and ST303 and it was not successful. Furthermore, electroporation as a method of delivery of exogenous DNA to the cell was also attempted on all three sequence types, with positive results observed only for 519/43. Expanding and standardising the methodology to all serotype 1 strains is of paramount importance as there is enormous variability throughout the group. Studies are undergoing presently to expand the applicability of the method to all strains within serotype 1.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Bentley, S.D. *et al.* Genetic Analysis of the Capsular Biosynthetic Locus from All 90 Pneumococcal Serotypes. *PLoS Genetics*. **2** (3), e31, doi: 10.1371/journal.pgen.0020031 (2006).
2. Calix, J.J., Nahm, M.H. A new pneumococcal serotype, 11E, has a variably inactivated wcjE gene. *The Journal of infectious diseases*. **202** (1), 29–38, doi: 10.1086/653123 (2010).
3. Park, I.H., Pritchard, D.G., Cartee, R., Brandao, A., Brandileone, M.C.C., Nahm, M.H. Discovery of a New Capsular Serotype (6C) within Serogroup 6 of Streptococcus pneumoniae. *Journal of Clinical Microbiology*. **45** (4), 1225–1233, doi: 10.1128/JCM.02199-06 (2007).

4. Jin, P. *et al.* First Report of Putative *Streptococcus pneumoniae* Serotype 6D among Nasopharyngeal Isolates from Fijian Children. *The Journal of Infectious Diseases*. **200** (9), 1375–1380, doi: 10.1086/606118 (2009).
5. Oliver, M.B., van der Linden, M.P.G., Küntzel, S.A., Saad, J.S., Nahm, M.H. Discovery of *Streptococcus pneumoniae* Serotype 6 Variants with Glycosyltransferases Synthesizing Two Differing Repeating Units. *Journal of Biological Chemistry*. **288** (36), 25976–25985, doi: 10.1074/jbc.M113.480152 (2013).
6. Calix, J.J. *et al.* Biochemical, Genetic, and Serological Characterization of Two Capsule Subtypes among *Streptococcus pneumoniae* Serotype 20 Strains. *Journal of Biological Chemistry*. **287** (33), 27885–27894, doi: 10.1074/jbc.M112.380451 (2012).
7. Park, I.H., Geno, K.A., Yu, J., Oliver, M.B., Kim, K.-H., Nahm, M.H. Genetic, Biochemical, and Serological Characterization of a New Pneumococcal Serotype, 6H, and Generation of a Pneumococcal Strain Producing Three Different Capsular Repeat Units. *Clinical and Vaccine Immunology*. **22** (3), 313–318, doi: 10.1128/CVI.00647-14 (2015).
8. O'Brien, K.L. *et al.* Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *The Lancet*. **374** (9693), 893–902, doi: 10.1016/S0140-6736(09)61204-6 (2009).
9. Henriques-Normark, B., Tuomanen, E.I. The Pneumococcus: Epidemiology, Microbiology, and Pathogenesis. *Cold Spring Harbor Perspectives in Medicine*. **3** (7), a010215–a010215, doi: 10.1101/cshperspect.a010215 (2013).
10. Leimkugel, J. *et al.* An Outbreak of Serotype 1 *Streptococcus pneumoniae* Meningitis in Northern Ghana with Features That Are Characteristic of *Neisseria meningitidis* Meningitis Epidemics. *The Journal of Infectious Diseases*. **192** (2), 192–199, doi: 10.1086/431151 (2005).
11. Yaro, S. *et al.* Epidemiological and Molecular Characteristics of a Highly Lethal Pneumococcal Meningitis Epidemic in Burkina Faso. *Clinical Infectious Diseases*. **43** (6), 693–700, doi: 10.1086/506940 (2006).
12. Antonio, M. *et al.* Molecular epidemiology of pneumococci obtained from Gambian children aged 2–29 months with invasive pneumococcal disease during a trial of a 9-valent pneumococcal conjugate vaccine. *BMC Infectious Diseases*. **8** (1), 81, doi: 10.1186/1471-2334-8-81 (2008).
13. Kwambana-Adams, B.A. *et al.* An outbreak of pneumococcal meningitis among older children (≥5 years) and adults after the implementation of an infant vaccination programme with the 13-valent pneumococcal conjugate vaccine in Ghana. *BMC Infectious Diseases*. **16** (1), 575, doi: 10.1186/s12879-016-1914-3 (2016).
14. Antonio, M. *et al.* Seasonality and outbreak of a predominant Streptococcus pneumoniae serotype 1 clone from The Gambia: Expansion of ST217 hypervirulent clonal complex in West Africa. *BMC Microbiology*. **8** (1), 198, doi: 10.1186/1471-2180-8-198 (2008).
15. Staples, M. *et al.* Molecular characterization of an Australian serotype 1 Streptococcus pneumoniae outbreak. *Epidemiology and Infection*. **143** (2), 325–333, doi: 10.1017/S0950268814000648 (2015).
16. Gessner, B.D., Mueller, J.E., Yaro, S. African meningitis belt pneumococcal disease epidemiology indicates a need for an effective serotype 1 containing vaccine, including for older children and adults. *BMC Infectious Diseases*. **10** (1), 22, doi: 10.1186/1471-2334-10-

22 (2010).
17. Hill, P.C. *et al.* Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian infants: a longitudinal study. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. **46** (6), 807–14, doi: 10.1086/528688 (2008).
18. Ebruke, C. *et al.* Temporal changes in nasopharyngeal carriage of *Streptococcus pneumoniae* serotype 1 genotypes in healthy Gambians before and after the 7-valent pneumococcal conjugate vaccine. *PeerJ*. **3**, e903, doi: 10.7717/peerj.903 (2015).
19. Adegbola, R.A. *et al.* Serotype and antimicrobial susceptibility patterns of isolates of Streptococcus pneumoniae causing invasive disease in The Gambia 1996–2003. *Tropical Medicine and International Health*. **11** (7), 1128–1135, doi: 10.1111/j.1365-3156.2006.01652.x (2006).
20. Marks, L.R., Reddinger, R.M., Hakansson, A.P. High Levels of Genetic Recombination during Nasopharyngeal Carriage and Biofilm Formation in Streptococcus pneumoniae. *mBio*. **3** (5), doi: 10.1128/mBio.00200-12 (2012).
21. Ritchie, N.D., Mitchell, T.J., Evans, T.J. What is different about serotype 1 pneumococci? *Future Microbiology*. **7** (1), 33–46, doi: 10.2217/fmb.11.146 (2012).
22. Harvey, R.M. *et al.* The variable region of pneumococcal pathogenicity island 1 is responsible for unusually high virulence of a serotype 1 isolate. *Infection and Immunity*. **84** (3), 822–832, doi: 10.1128/IAI.01454-15 (2016).
23. Horton, R.M., Cai, Z.L., Ho, S.N., Pease, L.R. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *BioTechniques*. **8** (5), 528–35, at <http://www.ncbi.nlm.nih.gov/pubmed/2357375> (1990).
24. Alioing, G., Granadel, C., Morrison, D.A., Claverys, J.-P. Competence pheromone, oligopeptide permease, and induction of competence in Streptococcus pneumoniae. *Molecular Microbiology*. **21** (3), 471–478, doi: 10.1111/j.1365-2958.1996.tb02556.x (1996).
25. Lund, E. *Enumeration and description of the strains belonging to the State Serum Institute, Copenhagen Denmark*. (1951).
26. Barany, F., Tomasz, A. Genetic transformation of Streptococcus pneumoniae by heterologous plasmid deoxyribonucleic acid. *Journal of bacteriology*. **144** (2), 698–709, at <http://www.ncbi.nlm.nih.gov/pubmed/6253440> (1980).
27. Terra, V.S., Homer, K.A., Rao, S.G., Andrew, P.W., Yesilkaya, H. Characterization of novel β-galactosidase activity that contributes to glycoprotein degradation and virulence in Streptococcus pneumoniae. *Infection and Immunity*. **78** (1), doi: 10.1128/IAI.00721-09 (2010).
28. Terra, V.S., Zhi, X., Kahya, H.F., Andrew, P.W., Yesilkaya, H. Pneumococcal 6-phospho-β-glucosidase (BglA3) is involved in virulence and nutrient metabolism. *Infection and Immunity*. **84** (1), doi: 10.1128/IAI.01108-15 (2015).
29. Harvey, R.M., Ogunniyi, A.D., Chen, A.Y., Paton, J.C. Pneumolysin with Low Hemolytic Activity Confers an Early Growth Advantage to Streptococcus pneumoniae in the Blood. *Infection and Immunity*. **79** (10), 4122, doi: 10.1128/IAI.05418-11 (2011).
30. Terra, V.S., Plumptre, C.D., Wall, E.C., Brown, J.S., Wren, B.W. Construction of a pneumolysin deficient mutant in streptococcus pneumoniae serotype 1 strain 519/43 and phenotypic characterisation. *Microbial pathogenesis*. **141**, 103999, doi:

10.1016/j.micpath.2020.103999 (2020).