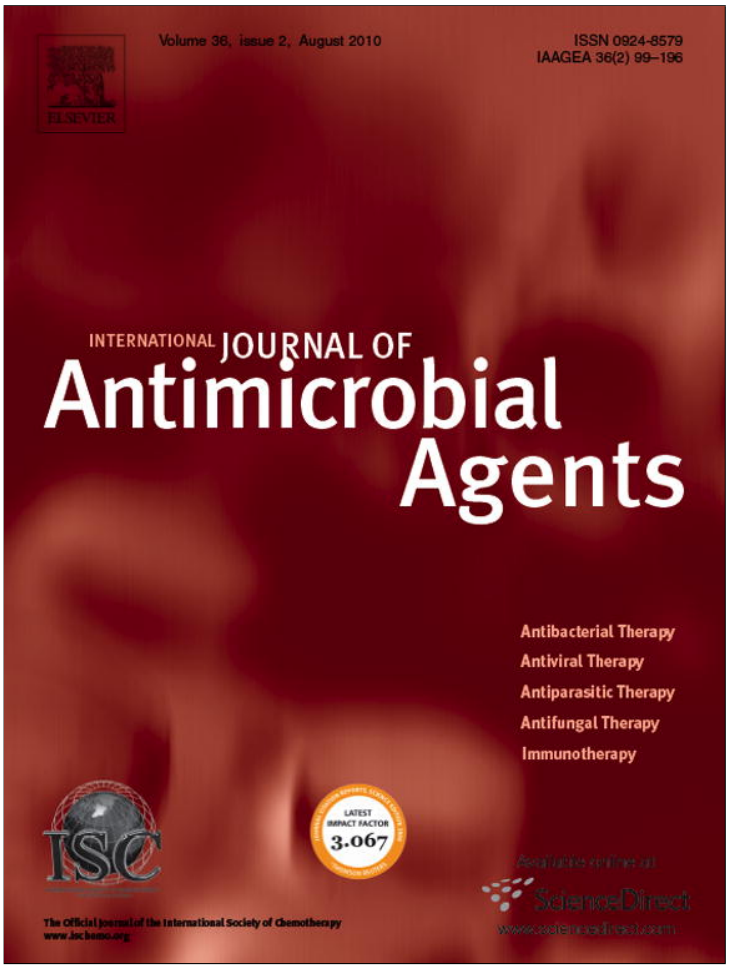

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Comparison of transformation frequencies among selected *Streptococcus pneumoniae* serotypes

Moses L. Joloba[a,*,1], Benson R. Kidenya[a,1,2], David P. Kateete[a], Fred A. Katabazi[a], Julian K. Muwanguzi[a,3], Benon B. Asiimwe[a], Simon P. Alarakol[a,4], Jessica L. Nakavuma[b], Saralee Bajaksouzian[c,d], Anne Windau[c,d], Michael R. Jacobs[c,d]

[a] *Department of Medical Microbiology, Makerere University College of Health Sciences, Upper Mulago Hill Road, P.O. Box 7072, Kampala, Uganda*
[b] *Faculty of Veterinary Medicine, Makerere University, Kampala, Uganda*
[c] *Case Western Reserve University, Cleveland, OH, USA*
[d] *University Hospitals Case Medical Center, Cleveland, OH, USA*

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ABSTRACT

Although there are over 90 serotypes of *Streptococcus pneumoniae*, antimicrobial resistance is predominantly found in a limited number of serotypes/serogroups, namely 6, 9, 14, 19 and 23. There is no compelling mechanism to account for this restriction. We aimed to determine whether serotypes commonly associated with drug resistance have higher transformation frequencies than those that are susceptible to antimicrobial agents. An in vitro investigation of the genetic transformation frequency of drug-resistant serotypes compared with that of susceptible serotypes under the influence of synthetic competence-stimulating peptides was performed. The transforming DNA was genomic DNA carrying a Tn*916*-like transposon containing the *mefE* gene that confers resistance to erythromycin. It was observed that serotypes 6, 9, 14, 19 and 23, which are highly associated with drug resistance, do not exhibit a higher degree of transformation efficiency than other serotypes. These findings suggest that the association of serotype with drug resistance is likely due to prolonged exposure to transforming DNA resulting from longer nasopharyngeal carriage and to a greater selective pressure from antimicrobials, particularly in children. This is the first study to compare the transformation frequencies of pneumococcal clinical isolates using genomic DNA that carries the composite *Tn916*-like element.

1. Introduction

Streptococcus pneumoniae is a worldwide frequent cause of potentially life-threatening infections such as pneumonia, meningitis and bacteraemia, as well as otitis media and sinusitis. It causes excess morbidity and mortality in human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) patients, young children, the elderly and debilitated patients [1,2]. Whilst the burden of pneumococcal disease is globally distributed, it is worse in developing countries where it is estimated to be responsible for greater than one million childhood deaths per year [3,4]. In sub-Saharan Africa, pneumococci are a significant cause of morbidity and mortality because of limited access to adequate health care and the high prevalence of HIV infection [5].

The number of reported antibiotic-resistant clinical isolates of *S. pneumoniae* has increased markedly over the last 30 years, and multidrug-resistant (MDR) strains have emerged [6–9]. The global emergence and spread of antimicrobial resistance in *S. pneumoniae* is a serious concern [9,10]. Many of the worldwide resistant clones have appeared with different capsular types [9,11]. Such variants of clones are thought to arise through natural transformation involving recombinational replacements, within and around the capsular biosynthesis (*cps*) locus [9]. Between 3% and 80% of pneumococcal illness is due to drug-resistant *S. pneumonia* [12]. Pneumococcal clones responsible for dispersion of resistance throughout the population locally and internationally have been identified in countries with a high prevalence of antibiotic-resistant strains [13].

Although over 90 pneumococcal serotypes exist and are assumed to be naturally competent for genetic transformation, antibiotic-resistant isolates, including those resistant to penicillin, usually belong to serotypes/serogroups 6, 9, 14, 19 or 23 and to a

* Corresponding author. Tel.: +256 41 454 1830; fax: +256 41 453 3002.
E-mail address: moses.joloba@case.edu (M.L. Joloba).
[1] These authors contributed equally to this work.
[2] Present address: Weill-Bugando University College of Health Sciences, Mwanza, Tanzania.
[3] Present address: Infectious Disease Research Institute, Queen Mary's School of Medicine, London, UK.
[4] Present address: Department of Biochemistry, Gulu University, Gulu, Uganda.

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few defined clonal groups in most parts of the world [11,14–17]. Of these, the serotypes responsible for most pneumococcal disease and drug resistance are 6B, 14, 19F and 23F [18]. This is particularly so in developing countries where the use of conjugate vaccine is still limited. The most successful clone in terms of geographical dispersion and prevalence is the MDR serotype 23F pandemic clone (Spain^{23F}-1) [9], which originated in Spain and has been found in Mexico, South Africa, South Korea, Portugal, Croatia, France and the USA. Many penicillin-resistant strains are also resistant to other drugs such as chloramphenicol, erythromycin, tetracycline and trimethoprim/sulfamethoxazole [19]. Even with the availability of antimicrobial agents, vaccines and intensive care, *S. pneumoniae* remains a frequent cause of life-threatening infections.

Hitherto, no compelling mechanism for the restriction of drug resistance to a few *S. pneumoniae* serotypes/serogroups has been proposed [9]. Induction of competence for natural genetic transformation in *S. pneumoniae* depends on pheromone-mediated cell–cell communication and a signalling pathway initiated by a competence-stimulating peptide (CSP) [20]. Two major CSP variants, CSP1 and CSP2, are found in members of this species, with individual strains responding to only one of these variants. Perhaps differences in the natural ability to develop competence for genetic transformation contribute to the development of drug resistance among various serotypes. Transformation with DNA from resistant strains of other species of streptococci is thought to be a major mechanism for the initial development of resistance in *S. pneumoniae*, since it is the major mechanism of horizontal gene transfer in this species. As such, we postulated that serotypes/serogroups commonly associated with drug resistance would have higher transformation frequencies than susceptible ones. The transformation frequencies of selected serotypes/serogroups of *S. pneumoniae* were compared and we report that serotypes/serogroups 6, 9, 14, 19 and 23, which are highly associated with drug resistance phenotypes, do not have a higher degree of transformation frequency than other serotypes/serogroups.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Streptococcus pneumoniae strains used in this study were selected from the collection of one of the authors (MRJ). Strains of serotypes 1, 3, 6B, 9V, 14, 18A/B, 19F, 23F, 29, 33, 35B and R6, all susceptible to erythromycin, were used as recipients during transformation experiments. Strain S222 of serotype 19F, provided by Dr Krzystof Trzcinski (Department of Epidemiology, Harvard School of Public Health, Boston, MA), was used as a DNA donor in the study. S222 is an erythromycin-resistant strain that carries *mefE* and *tetM* genes on a composite *Tn916*-like element. All strains except R6 were clinical isolates that were previously characterised and whose antimicrobial susceptibility profiles were determined. Strains were grown in stationary media either in tryptic soy broth (TSB) (Liofilchem, Roseto degli Abruzzi, Italy) supplemented with 10% horse serum (Sigma, St Louis, MO) or on tryptic soy blood agar base No. 2 (TSBAB) (Difco, Becton Dickinson, Sparks, MD) supplemented with 5% defibrinated sheep blood. Erythromycin (Sigma) was used at a concentration of 1 μg/mL in TSB and TSBAB for selection of resistant transformants.

2.2. Serotyping

Polysaccharide capsular types of the strains were determined using the capsular swelling reaction with factor-specific typing sera (Statens Serum Institut, Copenhagen, Denmark).

2.3. DNA extraction from the donor strain

The DNA donor strain S222 was incubated overnight at 37 °C on TSBAB/erythromycin from which five colonies were picked and inoculated in 30 mL of TSB/erythromycin supplemented with 10% horse serum. Cultures were grown without shaking at 37 °C for 6 h followed by centrifugation at 4000 × *g* for 15 min. Chromosomal DNA was extracted from the bacterial pellet using the Epicentre Master Pure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI) following the manufacturer's instructions. Extracted DNA was stored at −20 °C pending transformation experiments.

2.4. Selection and preparation of competent recipient *Streptococcus pneumoniae* strains

Serotypes of recipient strains were selected according to serotypes commonly associated with drug resistance (6B, 9V, 14, 19F and 23F), serotypes rarely associated with drug resistance (1, 3 and 18A/B) and serotypes that have recently emerged as drug-resistant (29, 33 and 35B). R6, the easily transformable laboratory pneumococcal strain, was used as a positive control for transformation efficiency. Recipient strains were incubated overnight at 37 °C on antibiotic-free TSBAB plates. Subsequently, two colonies of each strain were inoculated into 3 mL of antibiotic-free TSB medium [pH 6.8, adjusted with 1 M HCl and supplemented with 1 mM $CaCl_2$ and 0.2% bovine serum albumin (BSA) (Sigma-Aldrich, Steinheim, Germany)]. Cultures were then grown at 37 °C to an optical density at 600 nm (OD_{600}) of 0.1–0.2, after which they were stored at −80 °C in TSB/10% glycerol.

2.5. Transformation of recipient strains and selection of transformants

Competent recipient strains were thawed rapidly at room temperature just until ice had melted. Thawed recipient cells were then centrifuged at 13 000 × *g* (Eppendorf, Hamburg, Germany) for 10 min at 4 °C and the cell pellets were re-suspended in TSB (pH 8.0, adjusted with 5 N NaOH and supplemented with 1 mM $CaCl_2$, 0.2% BSA and 10% horse serum). Cells were then revived by incubating at 37 °C for 45 min without shaking. Synthetic CSP1 or CSP2 (Elim Biopharmaceuticals, Hayward, CA) was then added to a final concentration of 0.4 μg/mL, after which cells were incubated at 37 °C for 15 min. Then, chromosomal DNA extracted from the donor strain S222 was added to a final saturating concentration of 1 μg/mL. The mixture was further incubated at 37 °C for 150 min without shaking and 250 μL of the culture was plated onto TSBAB/erythromycin plates that were then incubated for 24–48 h at 37 °C. To determine total bacterial counts, 250 μL of the same culture was serially diluted (1×10^{-5}, 1×10^{-6} and 1×10^{-7}) and 250 μL was plated on antibiotic-free TSBAB plates and incubated for 24–48 h. Bacterial counts, expressed as colony-forming units per plate, were determined on plates and the transformation frequency (TF) was calculated as follows: TF = CFUE/(CFU × DF), where CFUE is the colony-forming units on the TSBAB/erythromycin plate, CFU is the colony-forming units on the antibiotic-free TSBAB plate and DF is the dilution factor.

Control transformations had water added in place of extracted chromosomal DNA. To rule out contamination, strains without added chromosomal DNA and CSP were plated on plain media and incubated under the same conditions.

2.6. Recovery and testing of transformants

Transformants obtained as described above were subcultured on TSBAB/erythromycin plates with optochin disks to verify that they were pneumococci and that they had acquired the

erythromycin resistance phenotype of the S222 DNA donor strain. Acquisition of the *mefE* gene was determined by polymerase chain reaction (PCR) amplification of chromosomal DNA extracts from the recipient strains before and after transformation. Chromosomal DNA was extracted from transformants using the Epicentre Master Pure DNA Purification Kit.

Chromosomal DNA from the donor strain S222 was used as a positive control in the PCR amplifications. The PCR reaction included 20 pmol of forward and reverse primers (mefE-fwd, 5′-AGTATCATTATTCACTAGTGC-3′ and mefE-rev, 5′-TCCTCCTGGTACTAAAAGTGG-3′ that amplify a 346-bp fragment of the *mefE* gene), 1.5 U of *Taq* polymerase, Custom PCR Master Mix (Thermo Scientific, Epsom, UK), template DNA and nuclease-free water in a total volume of 10 μL. The reaction was carried out in a Peltier thermocycler (MJ Research, Watertown, MA) at the following conditions: initial denaturation at 96 °C for 3 min; 30 cycles each consisting of 95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 5 min. Amplicons were separated by electrophoresis on a 2% agarose gel in TBE [Tris base–boric acid–ethylene diamine tetra-acetic acid (EDTA)]. For each strain, transformation and PCR were repeated at least three times for confirmation.

2.7. Data analysis

Transformation frequency raw data were converted into logarithms of transformation frequency. Differences in transformation frequencies among various serotypes were analysed by statistical analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, IL).

3. Results

3.1. Transformation frequency of various Streptococcus pneumoniae serotypes

Of the 12 strains studied, only 8 (R6, 6B, 14, 19F, 3, 18A/B, 29 and 23F) resulted in growth of pneumococci on TSBAB/erythromycin following transformation. Of these eight strains recovered, seven had acquired phenotypic erythromycin and tetracycline resistance by disk testing and were shown by PCR to have acquired the erythromycin resistance-conferring gene *mefE* by showing an amplicon of 346 bp (Fig. 1). The remaining strain (serotype 23F) that acquired phenotypic erythromycin resistance without tetracycline resistance did not show any *mefE* band upon PCR and was presumed to have developed mutational erythromycin resistance (Fig. 1). The seven pneumococcal strains transformed were R6, 6B, 14, 19F, 3, 18A/B and 29, with transformation stimulated by CSP1 for five strains (R6, 19F, 3, 18A/B and 29) and CSP2 for two strains (6B and 14). Transformation frequency results are shown in Table 1. Serotype 3 had the highest transformation efficiency (2.7×10^{-5}) and serotype 18A/B had the lowest (7.9×10^{-8}). Mean log transformation frequencies of the transformant strains were similar ($P = 0.679$) (Table 1). None of the strains was transformed in parallel cultures (negative controls) without the addition of chromosomal DNA. The five strains that did not show transformation with CSP1 or CSP2 were 1, 9V, 23F, 33 and 35B.

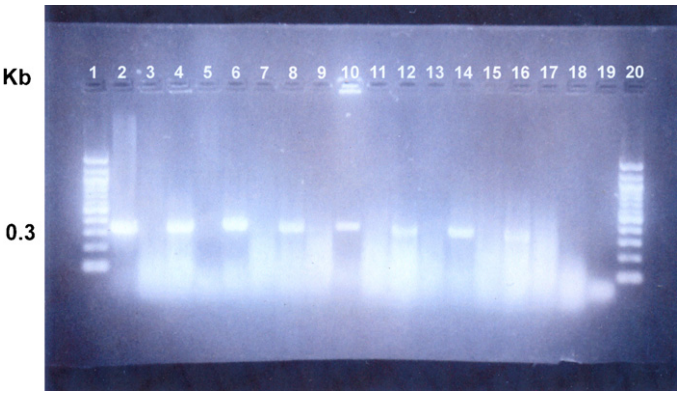

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction (PCR) amplification of a 346-bp fragment of the *mefE* gene in transformed or mutant strains. A 346-bp amplicon was detected on PCR in the transformants of R6, 3, 6B, 14, 18A/B, 19F and 29 (lanes 4, 6, 8, 10, 12, 14 and 16, respectively) but not in the 23F mutant (lane 18) or in any of the parent strains R6, 3, 6B, 14, 18A/B, 19F, 23F and 29 (lanes 3, 5, 7, 9, 11, 13, 15 and 17, respectively). Depicted in lanes 1 and 20 is a 100-bp DNA ladder; lane 2, positive control S222 donor; and lane 19, negative control (no DNA added).

4. Discussion

Natural competence for genetic transformation in *S. pneumoniae* is mediated by a quorum-sensing-regulated mechanism in which CSP functions as an autoinducer [21,22]. The ability of synthetic CSP to induce competence in clinical encapsulated isolates [23] and the possibility that transformation can constitute a means for entry for transposons, which provide flanking homologous sequences that mediate homology-dependent recombination events [24], rendered transformation successful in this experimental system. Few studies have been performed so far to compare the competence and transformation frequencies of various clinical isolates of *S. pneumoniae*. To the best of our knowledge, this is the first study to compare the transformation frequencies of various clinical isolates of *S. pneumoniae* using whole genomic DNA that carries the composite *Tn916*-like element.

We investigated whether serotypes commonly associated with drug resistance have higher transformation frequencies than susceptible ones. All successful transformants were resistant to erythromycin as well as tetracycline, indicating that the entire *Tn916*-like transposon carrying *mefE* and *tetM* genes had been incorporated into the recipient genome. Six of eleven clinical serotypes were transformed, in which serotype 3 had the highest transformation frequency of 2.7×10^{-5}. A higher transformation frequency of 4×10^{-3} was observed in a previous study for this

Table 1
Mean transformation frequency and mean log transformation frequencies of *Streptococcus pneumoniae* serotypes[a].

Serotype	CSP stimulating transformation	Mean transformation frequency	Mean ± S.D. log transformation frequency
R6[b]	CSP1	3.0455×10^{-6}	−5.5186 ± 0.06442
3	CSP1	2.6799×10^{-5}	−4.6269 ± 0.31565
6B	CSP2	2.7550×10^{-6}	−5.5873 ± 0.22048
14	CSP2	2.4525×10^{-7}	−6.5336 ± 0.70463
18A/B	CSP1	7.8911×10^{-8}	−7.0540 ± 0.17876
19F	CSP1	1.0293×10^{-7}	−7.0030 ± 0.16469
29	CSP1	1.4136×10^{-6}	−5.9101 ± 0.33128

CSP, competence-stimulating peptide; S.D., standard deviation.
[a] Transformation was not detected with serotypes 1, 9V, 23F, 33 or 35B.
[b] Positive control for transformation.

serotype, which was transformed with genomic DNA carrying an erythromycin resistance gene [25]. Differences in reported transformation frequencies could be due to the use of different competence media for transformation. In contrast, Hsieh et al. [26] assessed the competency of serotypes using plasmid DNA in which a very low transformation frequency of 7.8×10^{-9} was reported for serotype 3. The use of plasmid DNA as the transforming DNA could account for this difference. Furthermore, serotype 3 has a relatively rich amount of capsular polysaccharide, which apparently does not block uptake of exogenous DNA, making this serotype easily transformable at high frequency. These findings contradict earlier reports that large amounts of capsular polysaccharide have an inhibitory effect on transformation in *S. pneumoniae* [27].

Serotype 18 had the lowest transformation frequency of 7.9×10^{-8}, similar to that observed in a previous study [26]. Similarly, serotype 6B had a higher transformation frequency than serotypes 14, 19F and 18, and the profile of transformation frequencies for serotypes 6B, 14 and 19F were similar to those reported by Hsieh et al. [26]. These similarities are intriguing since the mechanisms involved in the uptake and establishment of plasmid DNA are different compared with the uptake and homologous recombination events involved in processing genomic DNA [28]. Although transforming DNA was extracted from serotype 19F, recipient strains of this serotype did not exhibit a higher transformation frequency than the majority of serotypes. This implies that capsule homology does not favour transformation frequency of the Tn*916*-like element, as previously observed [29].

Serotypes/serogroups 6, 9, 14, 19 and 23, which are commonly associated with drug resistance phenotypes [14–17], did not show higher transformation frequencies than serotypes 1, 3 and 18 that are commonly susceptible to antimicrobials. The reason why serotypes/serogroups 6, 9, 14, 19 and 23 are commonly associated with drug resistance phenotypes is still elusive. The reason could be that these serotypes are carried for longer periods in the nasopharynx of children who are exposed to antimicrobials more frequently than adults [29]. In humans, the nasopharynx is one of the environments most colonised by microorganisms; it offers the availability of exogenous transforming DNA from bacteria co-colonising with pneumococci. Whilst in the nasopharynx *S. pneumoniae* serotypes come into contact with other commensal bacteria, such as viridans group streptococci, *Gemella* spp., *Streptococcus pyogenes* and other closely related oral streptococci. These commensal bacteria may none the less carry a number of drug resistance genes and ultimately exchange genetic material with pneumococci sharing their habitat owing to the fact that pneumococci are naturally competent for genetic transformation. The acquired genes will subsequently increase the pneumococcal overall fitness. Many genes that confer drug resistance, virulence and colonisation have been acquired in this manner [4,30,31]. Recent studies have shown that *S. pneumoniae* can also be transformed in vivo with whole genomic DNA from different species of viridans group streptococci and from *Gemella* spp. containing the *mefE* gene at a transformation frequency as high as 10^{-6} [30]. Transfer of the *mefE* gene by transformation clearly indicates that gene transfer can occur between commensal bacteria and *S. pneumoniae*, as has already occurred for genes encoding penicillin-binding proteins [30,32]. This further supports the fact that *S. pneumoniae* in the nasopharynx can take up exogenous DNA that may contain drug resistance genes from commensal microorganisms.

Five serotypes were not transformed, probably due to the low homology that could have blocked homologous recombination processes. Integration of genetic markers into the chromosome in naturally competent cells requires DNA processing during entry. Lefrancois et al. [33] reported a number of possible reasons that can result in failure to obtain chromosomal transformants, which can probably be extrapolated to the inability to obtain transformants for these serotypes. The inability to detect transformants if they are very rare, the absence of induced competence or the absence of requirements of the complex process at the entry stages during natural competence to integrate the recipient chromosome were reported as possible reasons for failed natural transformation in pneumococci [33]. Alternatively, the homologous recombination machinery of the non-transformed cells could have failed to process the large size of the composite transposon [34]. Failure to transform encapsulated pneumococci with whole genomic DNA is common, especially with larger markers [23]. Among the strains that did not transform was serotype 23F, where a presumed resistant mutant at a mutation frequency of 8.7×10^{-8} was detected. This strain was also tested with primers that amplify the *ermB* gene, but this was negative (results not shown). We therefore speculated that this strain probably has a mutation in 23S ribosomal RNA and/or ribosomal protein L4 and/or L22; a few examples of clinical isolates with mutational resistance have been described [35–37].

The shortcoming of this study is that the Tn*916*-like transposon element used as a marker may not be representative enough for generalisation of transformation frequencies among various *S. pneumoniae* serotypes. Therefore, to establish the role of natural genetic transformation in relation to drug resistance among *S. pneumoniae* serotypes/serogroups, we recommend further studies involving a large number of serotypes/serogroups using several genetic markers in the transformation procedures. Furthermore, since this was an in vitro study, an in vivo study in mice aimed at assessing transformation frequency of the various *S. pneumoniae* serotypes is also necessary.

In addition, more studies on the role of CSP in genetic competence and transformation of *S. pneumoniae* are necessary since CSP is known to stimulate a variety of genes that drive virulence, pathogenesis, recombination and colonisation. Knowledge from such studies will be invaluable for drug and vaccine discovery since the genes are involved in important cellular processes of the pneumococcus. Currently, information from sequenced genomes of several pneumococcal serotypes is a boon to these studies.

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Ethical approval: Not required.

References

[1] Bogaert D, Hermans PW, Adrian PV, Rumke HC, de Groot R. Pneumococcal vaccines: an update on current strategies. Vaccine 2004;22:2209–20.
[2] Reinert RR, Reinert S, van der Linden M, Cil MY, Al-Lahham A, Appelbaum P. Antimicrobial susceptibility of *Streptococcus pneumoniae* in eight European countries from 2001 to 2003. Antimicrob Agents Chemother 2005;49:2903–13.
[3] Hausdorff WP, Siber G, Paradiso PR. Geographical differences in invasive pneumococcal disease rates and serotype frequency in young children. Lancet 2001;357:950–2.
[4] Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rev Microbiol 2008;6:288–301.
[5] Blossom DB, Namayanja-Kaye G, Nankya-Mutyoba J, Mukasa JB, Bakka H, Rwambuya S, et al. Oropharyngeal colonization by *Streptococcus pneumoniae* among HIV-infected adults in Uganda: assessing prevalence and antimicrobial susceptibility. Int J Infect Dis 2006;10:458–64.

[6] Appelbaum PC, Bhamjee A, Scragg JN, Hallett AF, Bowen AJ, Cooper RC. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol. Lancet 1977;2:995–7.
[7] Jacobs MR, Koornhof HJ, Robins-Browne RM, Stevenson CM, Vermaak ZA, Freiman I, et al. Emergence of multiply resistant pneumococci. N Engl J Med 1978;299:735–40.
[8] Mera RM, Miller LA, Daniels JJ, Weil JG, White AR. Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States over a 10-year period: Alexander Project. Diagn Microbiol Infect Dis 2005;51: 195–200.
[9] Trzcinski K, Thompson CM, Lipsitch M. Single-step capsular transformation and acquisition of penicillin resistance in *Streptococcus pneumoniae*. J Bacteriol 2004;186:3447–52.
[10] Felmingham D, Farrell DJ, Reinert RR, Morrissey I. Antibacterial resistance among children with community-acquired respiratory tract infections (PROTEKT 1999–2000). J Infect 2004;48:39–55.
[11] Pantosti A, Gherardi G, Conte M, Faella F, Dicuonzo G, Beall B. A novel, multiple drug-resistant, serotype 24F strain of *Streptococcus pneumoniae* that caused meningitis in patients in Naples, Italy. Clin Infect Dis 2002;35:205–8.
[12] Rantala M, Huikko S, Huovinen P, Jalava J. Prevalence and molecular genetics of macrolide resistance among *Streptococcus pneumoniae* isolates collected in Finland in 2002. Antimicrob Agents Chemother 2005;49:4180–4.
[13] Camargos P, Fischer GB, Mocelin H, Dias C, Ruvinsky R. Penicillin resistance and serotyping of *Streptococcus pneumoniae* in Latin America. Paediatr Respir Rev 2006;7:209–14.
[14] Clarke SC, Scott KJ, McChlery SM. Erythromycin resistance in invasive serotype 14 pneumococci is highly related to clonal type. J Med Microbiol 2004;53:1101–3.
[15] Garcia-Suarez Mdel M, Villaverde R, Caldevilla AF, Mendez FJ, Vazquez F. Serotype distribution and antimicrobial resistance of invasive and non-invasive pneumococcal isolates in Asturias, Spain. Jpn J Infect Dis 2006;59: 299–305.
[16] Jacobs MR, Good CE, Beall B, Bajaksouzian S, Windau AR, Whitney CG. Changes in serotypes and antimicrobial susceptibility of invasive *Streptococcus pneumoniae* strains in Cleveland: a quarter century of experience. J Clin Microbiol 2008;46:982–90.
[17] Imöhl M, Reinert RR, Ocklenburg C, van der Linden M. Association of serotypes of *Streptococcus pneumoniae* with age in invasive pneumococcal disease. J Clin Microbiol 2010;48:1291–6.
[18] Mokaddas EM, Rotimi VO, Albert MJ. Implications of *Streptococcus pneumoniae* penicillin resistance and serotype distribution in Kuwait for disease treatment and prevention. Clin Vaccine Immunol 2008;15:203–7.
[19] Tomasz A. Multiple-antibiotic-resistant pathogenic bacteria. A report on the Rockefeller University Workshop. N Engl J Med 1994;330:1247–51.
[20] Johnsborg O, Kristiansen PE, Blomqvist T, Havarstein LS. A hydrophobic patch in the competence-stimulating peptide, a pneumococcal competence pheromone, is essential for specificity and biological activity. J Bacteriol 2006;188:1744–9.
[21] Claverys JP, Dintilhac A, Mortier-Barriere I, Martin B, Alloing G. Regulation of competence for genetic transformation in *Streptococcus pneumoniae*. Soc Appl Bacteriol Symp Ser 1997;26:32S–41S.
[22] Morrison DA. Streptococcal competence for genetic transformation: regulation by peptide pheromones. Microb Drug Resist 1997;3:27–37.
[23] Pozzi G, Masala L, Iannelli F, Manganelli R, Havarstein LS, Piccoli L, et al. Competence for genetic transformation in encapsulated strains of *Streptococcus pneumoniae*: two allelic variants of the peptide pheromone. J Bacteriol 1996;178:6087–90.
[24] Mortier-Barriere I, Humbert O, Martin B, Prudhomme M, Claverys JP. Control of recombination rate during transformation of *Streptococcus pneumoniae*: an overview. In: Tomasz A, editor. *Streptococcus pneumoniae*: molecular biology and mechanism of disease. New Rochelle, NY: Mary Ann Liebert, Inc.; 2000. p. 57–66.
[25] Lau GW, Haataja S, Lonetto M, Kensit SE, Marra A, Bryant AP, et al. A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. Mol Microbiol 2001;40:555–71.
[26] Hsieh YC, Wang JT, Lee WS, Hsueh PR, Shao PL, Chang LY, et al. Serotype competence and penicillin resistance in *Streptococcus pneumoniae*. Emerg Infect Dis 2006;12:1709–14.
[27] Weiser JN, Kapoor M. Effect of intrastrain variation in the amount of capsular polysaccharide on genetic transformation of *Streptococcus pneumoniae*: implications for virulence studies of encapsulated strains. Infect Immun 1999;67:3690–2.
[28] Kamerling JP. Pneumococcal polysaccharides: a chemical view. In: Tomasz A, editor. *Streptococcus pneumoniae*: molecular biology and mechanisms of disease. New Rochelle, NY: Mary Ann Liebert, Inc.; 2000. p. 81–114.
[29] Trzcinski K, MacNeil A, Klugman KP, Lipsitch M. Capsule homology does not increase the frequency of transformation of linked penicillin binding proteins PBP 1a and PBP 2x in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 2005;49:1591–2.
[30] Cerda Zolezzi P, Laplana LM, Calvo CR, Cepero PG, Erazo MC, Gomez-Lus R. Molecular basis of resistance to macrolides and other antibiotics in commensal viridans group streptococci and *Gemella* spp. and transfer of resistance genes to *Streptococcus pneumoniae*. Antimicrob Agents Chemother 2004;48:3462–7.
[31] Dowson CG, Coffey TJ, Spratt BG. Origin and molecular epidemiology of penicillin-binding-protein-mediated resistance to β-lactam antibiotics. Trends Microbiol 1994;2:361–6.
[32] Potgieter E, Chalkley LJ. Reciprocal transfer of penicillin resistance genes between *Streptococcus pneumoniae*, *Streptococcus mitior* and *Streptococcus sanguis*. J Antimicrob Chemother 1991;28:463–5.
[33] Lefrancois J, Samrakandi MM, Sicard AM. Electrotransformation and natural transformation of *Streptococcus pneumoniae*: requirement of DNA processing for recombination. Microbiology 1998;144:3061–8.
[34] Del Grosso M, Camilli R, Libisch B, Fuzi M, Pantosti A. New composite genetic element of the Tn*916* family with dual macrolide resistance genes in a *Streptococcus pneumoniae* isolate belonging to clonal complex 271. Antimicrob Agents Chemother 2009;53:1293–4.
[35] Doktor SZ, Shortridge VD, Beyer JM, Flamm RK. Epidemiology of macrolide and/or lincosamide resistant *Streptococcus pneumoniae* clinical isolates with ribosomal mutations. Diagn Microbiol Infect Dis 2004;49:47–52.
[36] Musher DM, Dowell ME, Shortridge VD, Flamm RK, Jorgensen JH, Le Magueres P, et al. Emergence of macrolide resistance during treatment of pneumococcal pneumonia. N Engl J Med 2002;346:630–1.
[37] Tait-Kamradt A, Davies T, Appelbaum PC, Depardieu F, Courvalin P, Petitpas J, et al. Two new mechanisms of macrolide resistance in clinical strains of *Streptococcus pneumoniae* from Eastern Europe and North America. Antimicrob Agents Chemother 2000;44:3395–401.