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Contribution of Serotype and Genetic Background to Virulence of Serotype 3 and Serogroup 11 Pneumococcal Isolates

Lauren J. McAllister,[1] Abiodun D. Ogunniyi,[1] Uwe H. Stroeher,[1†] Amanda J. Leach,[2] and James C. Paton[1]*

Research Centre for Infectious Diseases, School of Molecular and Biomedical Science, University of Adelaide, Adelaide 5005, South Australia, Australia[1], Menzies School of Health Research, Charles Darwin University, Darwin, 0811, Northern Territory, Australia[2].

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† Current address, School of Biological Sciences, Flinders University, Bedford Park 5042, South Australia, Australia.

*Corresponding author. Mailing address: School of Molecular and Biomedical Science, University of Adelaide, Adelaide, S.A., 5005, Australia. Phone: + 61-8-83035929. Fax: + 61-8-83033262. E-mail: james.paton@adelaide.edu.au.

ABSTRACT

Capsular serotype has long been associated with the virulence of *Streptococcus pneumoniae*. Here, we present an in-depth study of phenotypic and genetic differences between serotype 3 and serogroup 11 *S. pneumoniae* clinical isolates from both the general and indigenous populations of Australia. Both serotypes/groups included clonally unrelated strains with differences in well-known polymorphic virulence genes, such as *nanA* and *pspA*, as demonstrated by multilocus sequence typing and Western blot analysis, respectively. Nonetheless, the serotype 3 strains were consistently and significantly more virulent in mice than the serogroup 11 strains. Despite extensive genomic analysis, non-capsular genes common to one serotype/group but not the other were not identified. Nevertheless, following the conversion of a serotype 11A isolate to serotype 3 and subsequent analysis in an intranasal infection model, it was evident that both capsular and non-capsular factors determine virulence phenotype in mice. However, it appears that these non-capsular factors vary from strain to strain.

INTRODUCTION

Streptococcus pneumoniae (the pneumococcus) is a formidable pathogen, responsible for a broad spectrum of diseases including pneumonia, meningitis, bacteremia and otitis media (OM), and accounts for more deaths worldwide than any other single pathogen (48). In Australia, the overall rate of invasive pneumococcal disease (IPD) in the indigenous population is over 4 times the rate seen in non-indigenous Australians (54). Furthermore, chronic suppurative OM, which is normally rare in developed countries, is present in remote Aboriginal communities at 10 times the rate defined as a major public health concern by the World Health Organization (9). Children living in these communities not only suffer the highest rates of tympanic membrane perforation in the world, but 80% are estimated to suffer from some degree of OM-related hearing impairment (45).

Significant research has already been undertaken on the pneumococcus, but there are still many unanswered questions due to the complex nature of its pathogenicity. The polysaccharide capsule that surrounds the pneumococcus and determines serotype, of which there are more than 90, is the most well-established virulence factor. Certain serotypes have been shown to have a higher association with disease or carriage than others (7, 19, 20, 60). In addition, a number of conserved virulence proteins have been widely characterized, including neuraminidase A (NanA), pneumolysin (Ply), autolysin (LytA) and pneumococcal surface proteins A and C (PspA and PspC), and shown to be critical in pathogenesis (29, 50). However, these are only a subset of potential virulence factors. Like other pathogens, such as *Haemophilus influenzae*, another common cause of invasive disease and OM, *S. pneumoniae* has a pangenome (23, 24). Core genes, which are conserved across pneumococcal strains, only represent 70-80% of the genome and a gene pool of over 5000 orthologous clusters is estimated to be available to this naturally transformable organism from other pneumococcal strains and related bacterial species (23). An understanding of the role in virulence of these noncore genes, which are generally found in regions of the genome known as regions of diversity or accessory regions (ARs), is only just beginning to emerge. The majority of studies on pneumococcal ARs to date have focused on IPD

(5, 13, 59). In addition, although there have been numerous epidemiological studies highlighting the problem of OM in remote Australian Aboriginal communities (33, 38, 45), very limited molecular analysis of isolates from these communities has been performed. Accordingly, this study aimed to characterize a range of isolates from geographically and temporally diverse Australian Aboriginal communities and the general population using a variety of molecular techniques with a view to identifying common genes or ARs potentially associated with but not necessarily limited to OM.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. Pneumococci were routinely grown in THY (Todd-Hewitt broth supplemented with 1% Bacto™ yeast extract), serum broth (SB; 10% [v/v] donor horse serum in nutrient broth), brain heart infusion (BHI) broth, or on blood agar (BA) plates at 37°C in 95% air and 5% CO_2. Gentamicin and optochin were added at concentrations of 5 μg/ml where appropriate. For storage, *S. pneumoniae* were grown in SB supplemented with glycerol to 30% and stored at -80°C. Pneumococcal opacity phase morphology was determined on THY-catalase plates, as described previously (65). Strains were confirmed as *S. pneumoniae* by optochin sensitivity, while serotype-specific capsule production was confirmed by Quellung reaction using diagnostic antisera obtained from Statens Serum Institut, Copenhagen, Denmark. The level of capsule expression was determined by the uronic acid assay (44). *Escherichia coli* One Shot® TOP10 chemically competent cells (Invitrogen, Victoria, Australia) were grown in Luria-Bertani (LB) broth or on LB agar plates, in the presence of ampicillin (Amp) at 50 μg/ml where appropriate.

Bacterial transformation. Pneumococci were transformed using complete transformation medium (CTM) (17, 40). For cloning and transformation of *E. coli*, the TOPO TA cloning® kit (Invitrogen) with pCR®2.1-TOPO® and One Shot® TOP10 chemically competent cells was used, according to the manufacturer's instructions. LB-Amp plates containing 1.6 mg of X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside) were used for selection.

DNA manipulations. All oligonucleotide primer sequences are available on request. Chromosomal DNA for genome sequencing and PCR was extracted and purified as described previously (55). PCR reactions were carried out in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using a final volume of either 25 or 50 μl. Standard reactions were performed using *Taq* DNA polymerase (Roche Diagnostics, Basel Switzerland), according to the manufacturer's instruction. The Expand™ Long Template or High Fidelity PCR Systems (Roche Diagnostics) were used when high fidelity PCR was required. DNA sequencing reactions were

carried out using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA).

Multi-locus sequence typing (MLST). *S. pneumoniae* isolates were subjected to MLST using the method and primer sequences described previously (15) and classified according to the pneumococcal MLST database (http://spneumoniae.mlst.net/).

DNA Microarray analysis. DNA microarray experiments were performed on whole genome *S. pneumoniae* PCR arrays based on TIGR4 and R6 annotations (26, 61). Array slides were obtained from the Bacterial Microarray Group at St George's, University of London. The array design is available in BμG@Sbase (Accession No. A-BUGS-14; http://bugs.sgul.ac.uk/A-BUGS-14) and also ArrayExpress (Accession No. A-BUGS-14). Briefly, *S. pneumoniae* DNA (10.5 μg in 100 μl) was digested with *Sau*3A1 or *Rsa*I (New England Biolabs [NEB], Massachusetts, USA), and cleaned using a Qiagen MinElute® PCR Purification Kit. Thereafter, 20 μl of purified digest was labeled for each dye (Alexa Fluor 555 and Alexa Fluor 647) per slide, using the Genisphere Array 900 DNA™ DNA Labeling Kit for Microarrays (Genisphere, Pennsylvania, USA) according to the manufacturer's instructions. Slides were incubated overnight in a dark, humidified chamber at 65 ^{o}C, washed in a 3-step process (15 min with 2× SSC, 0.03% (v/v) SDS, at 65 ^{o}C; 15 min with 1× SSC at room temperature [RT]; 15 min with 0.2× SSC at RT) and dried. Slides were scanned using a GenePix® 4000B scanner (Molecular Devices) and images were acquired using GenePix® Pro 6.0 software (Axon). Fully annotated microarray data have been deposited in BμG@Sbase and also ArrayExpress databases (accession number E-BUGS-126).

PCR-based subtractive hybridization. PCR-based subtractive hybridization (3) was performed using the Clontech PCR-Select™ Bacterial Genome Subtraction Kit (Clontech, California, USA), with the exception that the primary hybridization incubation time was increased to 8 h. Ligations were performed following the manufacturer's instructions using T4 DNA Ligase and buffer (NEB). PCR reactions were performed with a hot start, using *Taq*

polymerase and 1.6× reaction buffer as outlined in the kit manual. Secondary PCR products were used for cloning.

Next generation sequencing. Sequencing and genome assembly were performed by Geneworks (Thebarton, Adelaide, Australia) using chromosomal DNA prepared as described above, using an Illumina Genome Analyzer *II* (California, USA) and Lasergene® 8 software (DNASTAR Inc, Wisconsin, USA). Sequencing data have been deposited in the NCBI Sequence Read Archive (Accession number SRX026162).

Bioinformatics. The pneumococcal genomes and BLAST programs available through the Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg/) and National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) were used in the analysis of sequencing data. The ClustalW program available through the website of the European Bioinformatics Institute (EBI, http://ebi.ac.uk/Tools) was used to align multiple sequences.

Protein analysis. Bacteria were lysed and subjected to SDS-PAGE, as described previously (41). Separated proteins were electro-blotted onto nitrocellulose (Protran, Melbourne Australia), as described previously (41). After transfer, the membrane was probed with specific polyclonal mouse or rabbit antisera at a dilution of 1/3,000 and then reacted with blotting grade goat anti-mouse or goat anti-rabbit IgG-alkaline phosphatase (AP) conjugates (BioRad Laboratories, USA). The hemolytic activity of pneumococcal lysates was determined as described previously (51), using PBS-washed human erythrocytes.

Virulence and pathogenesis studies. All animal experiments used outbred 5-6 week old CD-1 (Swiss) mice and were approved by the Animal Ethics Committee of the University of Adelaide. For intraperitoneal (i.p.) and pneumonia/sepsis models, opaque variants were cultured in SB to approximately 1×10^8 CFU/ml and diluted in sterile SB to the appropriate challenge dose. The actual challenge doses were determined retrospectively by plating on BA with or without antibiotic selection, as appropriate. Mice in the pneumonia/sepsis study were anesthetized with Nembutal (pentobarbitone sodium, Rhone-Merieux) and challenged intranasally (i.n.), as previously described (36, 39). For survival studies, mice were monitored

closely over a 3-4 week period following challenge for signs of disease and the survival time of each mouse was recorded. For the capsule switch study, mice were sacrificed by CO_2 asphyxiation and nasal wash, ear wash, nasal tissue, lung, blood and brain samples were taken. The nasal wash and blood perfusion techniques have been described previously (36). The ear wash was performed by puncturing both tympanic membranes with a pipette tip and washing the ear cavities with a combined 100µl of sterile trypsin buffer (0.5% trypsin in phosphate buffered saline [PBS]). The nasal tissue, lungs and brain were removed and homogenized in 1 ml PBS using a Precellys 24 homogenizer (Bertin Technologies, Pessac, France) according to manufacturer's instructions. Enumeration of bacteria in the various samples was performed as previously described (36, 39). For i.n. colonization studies, transparent phase bacteria were grown in THY to mid-logarithmic phase. I.n. challenge without anesthesia, sample processing and enumeration of bacteria was performed as previously described (41).

RESULTS

Strain selection. For the purposes of this study, a number of clinical *S. pneumoniae* isolates belonging to serotypes 3 and 11A were obtained from the Menzies School of Health Research (Menzies), Darwin, Australia, and the Women's and Children's Hospital (WCH), North Adelaide, Australia (Table 1). The Menzies isolates were derived from a variety of indigenous communities in temporally and geographically diverse locations. Serotype 3 is frequently isolated from the ear discharge of indigenous children with acute OM with perforation of the tympanic membrane, yet is less frequently isolated from nasal swabs in asymptomatic members of the same population (34). In addition, this serotype has a high association with OM worldwide (20, 21, 58) and isolation of serotype 3 strains from OM cases has reportedly risen following the introduction of 7-valent pneumococcal conjugate vaccine (PCV7) (42). Serotype 3 is a less frequent cause of IPD, but nevertheless, cases of type 3 IPD have a high fatality rate (19, 22, 60). In Europe, Japan and North America, ST180 is the major clone within serotype 3 (7, 28, 42, 60), whereas in South Africa and Ghana, ST458 is more dominant (35, 46). The second serotype examined, 11A, is a common nasopharyngeal isolate in Aboriginal children but is rarely isolated from the middle ear (34). All serotype 11A isolates used in this study are of nasopharyngeal origin (Table 1). Serotype 11A has been associated with OM in other communities, including in Australia (18, 64), but is generally not as common as serotype 3 (20, 21, 53). Even though it also has a low association with IPD (19), serotype 11A clinical isolates of ST62 have been shown to have a high case-fatality rate in patients with underlying medical conditions (60).

In addition to the Menzies isolates, the serotype 3 and serogroup 11 isolates obtained from WCH are from the general population and have a well-documented clinical history (Table 1). We could not determine whether the WCH serogroup 11 isolates were serotype 11A because the available antiserum used in this study is only serogroup 11-specific. The serotype 2 strain

D39 (4), the serotype 4 strain TIGR4 (1), and the serotype 3 strain WU2 (6), which have been used in numerous published studies, were also included in some experiments (Table 1).

Preliminary characterization of serotype 3 and serogroup 11 clinical isolates. The serotype 3 and serogroup 11 clinical isolates from Menzies and WCH had hitherto been uncharacterized and so it was important to determine their pathogenicity profiles in order to establish any correlation between human disease and our mouse models. To this end, we first compared the expression profiles of well-characterized virulence proteins PspA, PspC, Ply, LytA and NanA with that in the highly virulent serotype 2 *S. pneumoniae* strain D39 by Western blotting, using polyclonal antisera (see Fig. S1 in the supplemental material). Western blot analysis showed no apparent difference in size or intensity using anti-LytA and anti-Ply polyclonal sera for all serotype 3 and serogroup 11 strains. Furthermore, a Ply-specific hemolysis assay using human erythrocytes showed there were no differences in specific hemolytic activity between strains of the same serotype (data not shown). The differences observed in the Western blots for PspA, PspC and NanA were further investigated in selected strains by PspA family typing (63), PCR or sequencing of *pspA*, *pspC* and *nanA* genes, with results summarized in Fig. 1. The alleles identified for these three genes have been previously described by Hollingshead et al. (24), Iannelli et al. (26) and King et al. (25, 27, 32), respectively. Interestingly, the serogroup 11 strains revealed more variable results than the serotype 3 strains, although some strains, such as Menzies1 and Menzies3 exhibited identical protein profiles. The serotype 3 strains could be divided into two distinct groups (one comprising WCH206 and WCH207, and the other the remainder) based on their Western blot profiles.

Selected isolates were subsequently characterized by multi-locus sequence typing (MLST) (14) based on their Western blot profiles. Serotype 3 strains that exhibited identical protein profiles in the Western blot analysis were found to have identical STs; WCH206 and WCH207 were ST180, whereas WCH208, Menzies11, Menzies12, Menzies14 and Menzies17 were ST458. WU2 was typed as ST378 (as found by Poolman et al. (52). As predicted from the divergent protein profiles, the serogroup 11 strains WCH211, WCH213, Menzies1 and Menzies5

all belong to unrelated STs (ST3020, ST62, ST3021 and ST662, respectively). WCH211 and Menzies1 are the first reported isolates of their respective STs.

The colonies of the ST180 strains WCH206 and WCH207 were larger and more homogenous in size compared to the other serotype 3 strains following overnight growth on BA. No differences in colony morphology were evident between serogroup 11 strains. Capsular polysaccharide production was compared between WCH206, WCH207, Menzies11, Menzies17 and WU2 using the uronic acid assay (44). There was no significant difference in capsule production between isolates of the same ST. However, WCH206 and WCH207 produced statistically significantly less capsular polysaccharide than Menzies11 and Menzies17 (Fig. 2). WU2 produced significantly less capsule than Menzies17 but not Menzies11.

We subsequently evaluated selected serotype 3 and serogroup 11 strains for their virulence characteristics in mouse pneumonia/sepsis and intraperitoneal (i.p.) (sepsis) models (Fig. 3A and 3B). Statistical analysis of survival data is shown in Table 2. Serotype 3 strains were highly virulent in the pneumonia/sepsis model with the exception of WU2, which killed less than 50% of the mice and was significantly less virulent than all of the other serotype 3 strains. Menzies17 was also more virulent than all the other serotype 3 strains, including Menzies 11, which belongs to the same ST (Fig. 3A and Table 2). In contrast, serogroup 11 strains were essentially avirulent in the pneumonia/sepsis model and survival times were significantly different from all the serotype 3 strains except WU2. In the i.p. model, they killed less than 50% of mice when injected at very high doses (1×10^7 CFU/mouse), and survival times for three of the strains were significantly greater than those for mice given only 5×10^2 CFU of the serotype 3 strains (Fig. 3B and Table 2). Strains were also compared in an intranasal (i.n.) colonization model (Fig. 3C). Interestingly, serogroup 11 strains were isolated more frequently and (for most strains) in significantly greater numbers from the nasopharynx of mice on days 1 and 2 post i.n. challenge than the serotype 3 strains (Fig. 3C and Table 3). Longer term colonization was also explored by performing nasopharyngeal washes on surviving mice in the pneumonia-sepsis study

(presented in Fig. 3A) 4-weeks post-inoculation. Both serotypes/groups were found to be capable of longer term colonization of the nasopharynx (Fig. 3D).

Microarray analysis and PCR-based subtractive hybridization of ST180 and ST458 strains. The existence of two clonally unrelated groups within the serotype 3 clinical isolates presented an attractive opportunity for assessment of commonality and/or differences in the overall genetic composition between these strains. First, we compared the genomes of WCH208 (ST458), Menzies12 (ST458), Menzies13 and Menzies15, which displayed identical protein profiles to one another, by DNA microarray analysis using slides representing the genomes of TIGR4 and R6 (a rough derivative of D39). No differences were detectable, consistent with the protein profiles and ST data. Similarly, microarray and genome sequence analysis of strains from the ST180 lineage have shown them to exhibit few genetic differences, even when isolated from different continents (11, 23). Next, we compared the genomes of selected ST180 (WCH206) and ST458 (Menzies17) strains. The results showed that 3 ORFs were present in WCH206, but absent in Menzies17, while 15 ORFs were detectable in Menzies17 but absent in strain WCH206 (see Table S1 in the supplemental material). Similarly, genomic comparisons between serogroup 11 strains were made, and several genes were detected to be either present or absent in these comparisons (Table S1). We subsequently carried out genomic comparisons of the serotype/groups 3 and 11 strains to detect any genes that were unique to one serotype. However, no non-capsular gene which was present in all isolates of one serotype but absent in every isolate of the other was identified.

One of the limitations of DNA microarray analysis is that it gives no information on genes which may be present in the strains tested, but absent on the array. In order to overcome this, a PCR-based subtractive hybridization method (3) was adapted to detect genes present in both ST180 and ST458, but absent in R6 and TIGR4. Using this procedure, we identified serotype 3 capsule genes (effectively a positive control), and the IIA and IIB components of a putative cellobiose phosphotransferase system (PTS1), which was first sequenced by Shen et al. in a study identifying pneumococcal ORFs absent in the TIGR4 and R6 genomes (57).

Interestingly, the pneumococcus has a second cellobiose PTS (PTS2), which is also part of a larger mobile genetic element, and its components were identified during a signature-tagged mutagenesis virulence gene screen using a chinchilla middle ear infection model, suggesting a role in OM (8).

Forbes et al. reported that PTS1 belongs to a mobile genetic element consisting of 10 genes (16). A putative choline sulfatase was also reported in this region in ATCC 700669 (Spain 23F ST81 lineage) by Croucher et al. (10). We compared the genomic organization with those annotated for G54 (12) and Hungary19A-6 (J. Craig Venter Institute). The PTS was found to be associated with a genomic island harboring a ROK (regulator, ORF of unknown function, kinase) family protein and a putative sulfatase with an associated modifying factor, as well as hypothetical proteins (Fig. 4). The ROK family protein does not have the DNA binding motif characteristic of repressors in this family. We agree with the observation of Forbes et al. that the organization of this island is well conserved (16), but G54 has a deletion of about 2.5 kb, including the putative sulfatase gene, as well as parts of the sulfatase modifying factor and the last gene of the putative cellobiose PTS. The same deletion is apparent in the serotype 3 strains Menzies11 and Menzies17 (isolates of ST458), but WCH206 (an isolate of ST180) carries the full genomic island. The sequence of G54 suggests it also has two insertions in two genes of the PTS operon, resulting in frameshifts and premature stop codons, but these insertions are not present in the island sequences of Menzies11 or Menzies17. The absence of the sulfatase in the ST458 isolates explains why genes downstream of the cellobiose PTS were not detected by PCR-based subtractive hybridization. The gene of the ROK family protein is located on a restriction fragment over 3.6 kb, which is well outside the optimal size for PCR-based subtractive hybridization (2).

Distribution of the genomic island carrying a putative cellobiose PTS. WU2 and the serogroup 11 isolates were analyzed by PCR to determine whether they carried PTS1 or not (see Table S2 in supplemental material). Interestingly, WU2 did not, but all four serogroup 11 strains did. We also examined the distribution of PTS1, as well as the sulfatase and PTS2, by both

bioinformatic analysis utilizing publically available pneumococcal genomes and PCR analysis of clinical isolates with a range of serotypes and virulence potential (Table S2). Only one strain appeared to possess neither PTS1 nor PTS2, with the majority of strains possessing both. Only 3 out of the 4 serogroup 11 isolates possessed PTS2. Most isolates carrying PTS1 also carried the sulfatase.

Genome sequence comparison. In order to determine whether there were additional ARs missed by PCR-based subtractive hybridization, the genome of Menzies17 was sequenced and aligned against the published genome of OXC141 (serotype 3, ST180, ftp://ftp.sanger.ac.uk/pub/pathogens/spn/). The average coverage depth was 14.68 and sequence gaps over 100 bp in the alignment represented approximately 6.3% of the OXC141 genome. Although serotype 3 ST180 isolates are known to carry additional genes apart from the PTS1 AR which are absent in TIGR4 and D39, none common to Menzies17 were detected (23, 57). This indicates that the PCR-based subtractive hybridization methodology enabled efficient identification and isolation of major genetic differences between isolates.

Impact of capsular serotype and genetic background on pathogenesis. Previous studies have indicated the importance of additional genetic factors to serotype in pneumococcal disease (31, 43, 56, 59). Indeed the difference in survival between WU2 and the other serotype 3 isolates in the pneumonia-sepsis model (Fig. 3A) is indicative of the importance of genotype. However, it has also been demonstrated that serotype influences the behavior of genotype in mouse in vivo models (30, 47, 62). As the isolates of the two groups in this study have distinct behavior in our mouse models, and there are no additional factors to distinguish the two apart from the capsule loci, the role of serotype for our particular isolates was further explored. For this purpose, one of the serotype 11A strains from a remote Aboriginal community (Menzies5) was capsule-switched with a serotype 3 capsule by transformation with a PCR product comprising the capsule locus from WCH206. This strain was designated Menzies5^{3} and type 3 capsule production was confirmed by the Quellung reaction. In addition, loss of reactivity to serogroup 11 antiserum was observed. There was no significant difference in serotype 3 capsular

polysaccharide production between Menzies5[3] and the donor strain WCH206 as assessed by the uronic acid assay. The mutant also exhibited an identical growth profile to Menzies5 and WCH206 (data not shown). Colonization of various tissues by the three strains in the pneumonia/sepsis model over a 3-day period was investigated (Fig. 5). Acquisition of the serotype 3 capsule by Menzies5[3] reduced nasopharyngeal colonization significantly on all three days, but did not increase bacterial numbers recovered in any other niche (ears, lungs, blood or brain) relative to Menzies5 at any time point. WCH206 was isolated at significantly higher numbers than Menzies5[3] from the ears (day 3), lungs (day 2), blood (day 2), and brain (day 2 but not day 1). Therefore, although serotype clearly influences the ability of the ST662 isolate to colonize the nasopharynx, the ST180 genotype is fitter than the ST662 genotype in various niches of the pneumonia-sepsis model for serotype 3.

DISCUSSION

Serotype 3 *S. pneumoniae* is an important OM pathogen worldwide, including remote Australian Aboriginal communities (20, 34, 52). Despite the burden of pneumococcal OM in these communities, there has been very little molecular characterization of isolates. In this study we have analyzed isolates from remote communities using a variety of techniques, including Western blot analysis, a hemolysis assay, capsular polysaccharide assay, MLST and murine virulence and colonization models. Although the isolates were acquired from diverse locations, the serotype 3 isolates were found to be from the ST458 clonal cluster, which is unrelated to the ST180 clone that normally dominates in developed countries (7, 28, 42, 60). Interestingly, this ST has also been seen to dominate over ST180 in South Africa and Ghana, which are also socioeconomically disadvantaged (35, 46). Both ST458 and ST180 isolates are highly virulent in our mouse models, in contrast to the serogroup 11 isolates. However, despite observed differences in the epidemiology of serogroup 11 isolates and behavior in our mouse model, the capsule switch experiment performed in this study further underscores the importance of non-capsular factors in disease. This does not mean that serotype is unimportant. In the capsule switch study, Menzies5^{3} was clearly attenuated in the nasopharynx in comparison to Menzies5. Kadioglu et al. also found their serotype 3 capsule switch variant of D39 and A66 (serotype 3 capsule donor) colonized the nasopharynx at lower numbers over a 24 h period in comparison to wild-type D39 (30). Furthermore, although extensive analyses were conducted using DNA microarray, PCR-based subtractive hybridization and Illumina sequencing, we could not identify a specific factor common to the serotype 3 ST180 and ST458 isolates, but absent in the 4 unrelated serogroup 11 isolates. In addition, host factors are likely to influence genotype. The prevalence of the ST458 clone over ST180 suggests it may be better adapted for the micro-environmental niches encountered in remote Aboriginal communities.

In this study we have shown that PCR-based subtractive hybridization can be adapted to complement DNA microarray hybridization by identifying genes absent on the microarray slide. A previously discovered but uncharacterized putative cellobiose PTS (PTS1) common to ST180

364 and ST458 was identified through this technique, which constitutes part of a genomic island
365 harboring a putative ROK family protein and a putative sulfatase with an associated modifying
366 factor. The putative cellobiose utilization AR had initially been noted to be more common in
367 invasive isolates (16), but as with other pneumococcal ARs (5), when a larger number of isolates
368 were characterized, this relationship could not be confirmed. However, the wide distribution of
369 the AR and the putative role of its genes both suggest potential importance. Furthermore, the
370 apparent involvement of a second pneumococcal cellobiose PTS (PTS2) in OM (8) also suggests
371 that a role for PTS1 in OM is worthy of exploration. It is also interesting to note that the ST662
372 isolate Menzies5, which was less fit than WCH206 (ST180) in various niches of the pneumonia-
373 sepsis model in a serotype 3 background, and WU2 (ST378), which was less virulent than ST180
374 and ST458 isolates in the same model, only possess one of the PTSs, while ST180 and ST458
375 isolates possess both.
376 In this study, despite extensive molecular analyses, we could not identify any ARs that
377 are unique to OM isolates. It is likely that different ARs contribute to virulence in unrelated
378 strains, and that virulence of a given strain is a function of multiple ARs. The impact of any
379 combination of ARs could be influenced by other factors, including redundancy of ARs, and
380 capsular type, as well as host factors. Overall, there appears to be no simple answer to why one
381 strain is more prevalent in disease or carriage than another, and the ability of an isolate to cause
382 disease is likely to reflect a complex mix of serotype, genotype and host factors.
383

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628 **Table 1. Pneumococcal strains used in this study.**
629

Strain	Description	Source (Reference)
D39	serotype 2	(4)
D39-ABΔe5	D39 Δ*cpsAB ery*^{r}	(44)
TIGR4	serotype 4	Tim Mitchell, University of Glasgow, Scotland (1)
WU2	serotype 3	David Briles, University of Alabama, USA (6)
WCH206	serotype 3 (ST180) OM isolate	Women's & Children's Hospital, South Australia (WCH)
WCH207	serotype 3 (ST180) OM isolate	WCH
WCH208	serotype 3 (ST458) OM isolate	WCH
WCH209	serogroup 11 sinusitis isolate	WCH
WCH210	serogroup 11 sinusitis isolate	WCH
WCH211	serogroup 11 (ST3020) sinusitis isolate	WCH
WCH212	serogroup 11 sinusitis isolate	WCH
WCH213	serogroup 11 (ST62) OM isolate	WCH
Menzies1	serotype 11A (ST3021) nasopharyngeal isolate	Menzies School of Health Research, Darwin, Australia)
Menzies2	serotype 11A nasopharyngeal isolate	Menzies
Menzies 3	serotype 11A nasopharyngeal isolate	Menzies
Menzies 4	serotype 11A nasopharyngeal isolate	Menzies
Menzies 5	serotype 11A (ST662) nasopharyngeal isolate	Menzies
Menzies 6	serotype 11A nasopharyngeal isolate	Menzies
Menzies 7	serotype 11A nasopharyngeal isolate	Menzies
Menzies 8	serotype 11A nasopharyngeal isolate	Menzies
Menzies 9	serotype 11A nasopharyngeal isolate	Menzies
Menzies 10	serotype 11A nasopharyngeal isolate	Menzies
Menzies 11	serotype 3 (ST458) nasopharyngeal isolate	Menzies
Menzies 12	serotype 3 (ST458) OM isolate	Menzies
Menzies 13	serotype 3 nasopharyngeal isolate	Menzies
Menzies 14	serotype 3 (ST458) OM isolate	Menzies
Menzies 15	serotype 3 nasopharyngeal isolate	Menzies
Menzies 16	serotype 3 nasopharyngeal isolate	Menzies
Menzies 17	serotype 3 (ST458) OM isolate	Menzies
WCH43	serotype 4 (ST205) blood isolate	WCH
2663	serotype 11A (ST3019) lung isolate	Michael Watson, Path West,

		Perth, Western Australia
3518	serotype 11A (ST62)	Path West
1	serotype 1 (ST304) nasopharyngeal isolate	MSHR
4	serotype 1 (ST227) nasopharyngeal isolate	MSHR
1861	serotype 1 (ST3079) blood isolate	MSHR
4496	serotype 1 (ST3018) blood isolate	WCH
3773	serotype 15B (ST199)	Path West
4104	serotype 19A (ST199)	Path West
WCH16	serotype 6A	WCH
WCH18	serotype 6B	WCH
WCH50	serotype 6	WCH
WCH33	serotype 8	WCH
WCH36	serotype 14	WCH
WCH39	serotype 14	WCH
WCH60	serotype 14	WCH
WCH61	serotype 14	WCH
WCH38	serogroup 19	WCH
WCH64	serogroup 19	WCH
WCH65	serogroup 19	WCH
EF3030	serotype 19F	University of Alabama
WCH67	serogroup 23	WCH
WCH68	serogroup 23	WCH
WCH69	serogroup 23	WCH
WCH101	serogroup 23	WCH

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631

Table 2. Statistical analysis of survival data.

Strain	WU2	WCH206	WCH207	Menzies11	Menzies17
Pneumonia Sepsis Model					
WU2	-	0.0007	0.0172	0.0185	0.0003
WCH206	0.0007	-	NS	NS	0.0355
WCH207	0.0172	NS[a]	-	NS	0.003
MSHR11	0.0185	NS	NS	-	0.0039
MSHR17	0.0003	0.0355	0.003	0.0039	-
WCH211	NS	0.0004	0.0023	0.0049	0.0004
WCH213	NS	0.0004	0.0023	0.0049	0.0004
Menzies1	NS	0.0004	0.0023	0.0049	0.0004
Menzies5	NS	0.0004	0.0023	0.0049	0.0004
I.P. Model					
WU2	-	NS	0.0355	NS	0.0015
WCH206	NS	-	0.0147	NS	0.0147
WCH207	0.0355	0.0147	-	NS	NS
MSHR11	NS	NS	NS	-	NS
MSHR17	0.0015	0.0147	NS	NS	-
WCH211	0.0298	0.0302	0.0114	0.0412	0.0123
WCH213	0.0002	0.0004	0.0001	0.0008	0.0002
Menzies1	NS	NS	NS	NS	0.0168
Menzies5	0.0021	0.0028	0.0025	0.0078	0.0027

Survival time data were analyzed using the Mann Whitney U-test (two tailed).

[a] NS indicates not significant ($P > 0.05$).

640
641 **Table 3. Statistical analysis of nasopharyngeal colonization data.**
642

Serogroup 11 Strains	WU2	WCH206	WCH207	Menzies11	Menzies17
			DAY 1		
WCH211	0.0345	NS[a]	NS	0.0097	NS
WCH213	0.0112	0.0200	0.0159	0.0097	0.0200
Menzies1	NS	NS	NS	0.0259	NS
Menzies5	NS	NS	NS	0.0259	NS
			DAY 2		
WCH211	NS	NS	NS	NS	NS
WCH213	0.0112	0.0112	0.0200	0.0112	0.0317
Menzies1	0.0112	0.0112	0.0112	0.0112	0.0079
Menzies5	0.0112	0.0112	0.0112	0.0112	0.0079
			DAY 3		
WCH211	NS	0.0311	NS	NS	NS
WCH213	NS	0.0178	NS	NS	NS
Menzies1	NS	0.0097	NS	NS	NS
Menzies5	0.0317	0.0097	NS	NS	NS
			DAY 4		
WCH211	NS	NS	NS	NS	NS
WCH213	NS	NS	NS	NS	NS
Menzies1	NS	NS	NS	NS	NS
Menzies5	NS	NS	NS	NS	NS

643
644 Survival time data were analyzed using the Mann Whitney U-test (two tailed).
645 [a] NS indicates not significant ($P > 0.05$).
646

FIGURE LEGENDS

FIG. 1. Alleles of *nanA*, *pspA* and *pspC* detected in serogroup 3 and 11 strains. Alleles were identified for *nanA*, *pspA* and *pspC* by sequencing or PspA family typing, in accordance with King et al. (32), Hollingshead et al. (25) and Iannelli et al. (27), respectively. Proteins are shown in proportion to their approximate size. NanA is represented in lilac. The NanA identified in WCH206 is identical to the NanA present in D39. The other *nanA* alleles have a 15 bp insertion (indicated by a dark purple box) and a 180 bp deletion (indicated by a black line), which have been previously described in the *nanA* alleles of other pneumococcal strains (32). In addition, one of the alleles, which is found in both serotype 3 and serogroup 11, has a 500 bp region of diversity at the 5'-terminus, which is indicated by pink and has also been previously described (32). PspA and PspC proteins are categorized as described by Hollingshead et al. (25) and Iannelli et al. (27), respectively. The colour scheme of Iannelli et al. has been used. Homologous sequences are represented by boxes of the same colour. Dark grey/black = signal peptide, other shades of grey = random coil, green = proline rich region, blue = anchor, other colours = α-helix. The clade determining regions of PspA (25) are indicated by white dots. Lines indicate absence of DNA.

FIG. 2. Capsular polysaccharide production by type 3 isolates. Capsular polysaccharide produced by the indicated strains was quantitated by uronic acid assay, as described in the Materials and Methods. Data are the mean ± SD of five independent experiments; *, $P < 0.05$; **, $P < 0.01$, unpaired *t*-test).

FIG. 3. Virulence and nasopharyngeal colonization of selected serotype 3 and serogroup 11 isolates. (A) Survival of CD-1 mice in the pneumonia/sepsis model. Groups of 10 mice were inoculated with $1.5 – 4 \times 10^7$ CFU of the indicated strains. The median survival time for each

group is indicated by a horizontal bar. (B) Survival of CD-1 mice following i.p. injection of groups of 10 mice with either 5×10^2 CFU (serotype 3) or 1×10^7 CFU (serogroup 11) of bacteria. The median survival time for each group is indicated by a horizontal bar. (C) The number of bacteria in the nasopharynx (nasopharyngeal tissue and nasal wash) over a 7 day period following intranasal inoculation with 1-2 $\times 10^7$ CFU/mouse. Groups of 20 mice were inoculated without anaesthesia and 5 mice were euthanased per timepoint, survival permitting. The median number of bacteria in the nasopharynx for each strain is represented by a horizontal bar. The broken line represents the limit of detection (40 CFU). (D). Long term colonization after intranasal challenge was determined by plating nasopharyngeal washes from the mice that were still alive 4 weeks after intranasal challenge in (A) above.

FIG. 4. The genomic islands in *S. pneumoniae* Hungary^{19A}-6 and G54 containing the putative cellobiose PTS system. The figure is based on the genome maps of Hungary^{19A}-6 (nt 1784704-1794633, Refseq NC_010380) and G54 (nt 1630647-1637715, Refseq NC_011072). The indicated ORFs are annotated as follows: sph1921/spg1693, hypothetical protein; sph1922/spg1693, hypothetical; sph1923, sulfatase-modifying factor 1 (spg1694, hypothetical); sph1924, sulfatase; sph1925, PTS system cellobiose-specific IIC component (spg1995, hypothetical; spg1696, hypothetical); sph1926/spg1697, lichenan-specific phosphotransferase enzyme IIA component; sph1927/spg1698, lichenan-specific phosphotransferase enzyme IIB component; sph1928, hypothetical protein (spg1699, hypothetical; spg1700, hypothetical); sph1929/spg1701, lichenan permease IIC component; sph1930/spg1702, putative ROK family protein. Genes were shaded according to their KEGG pathway function with black indicating membrane transport and white indicating an unassigned pathway. WCH206 possesses the same island as Hungary19A-6. Menzies17 and Menzies11 have the same deletion as G54, but do not have the two single bp insertions causing frame-shifts and premature stop codons.

FIG. 5. Ability of the capsule switch mutant to colonize various niches of the mouse. Groups of mice were anesthetized and inoculated i.n. with approximately 10^7 CFU/mouse of WCH206, Menzies5[3] or Menzies5. Numbers of pneumococci in various host niches were determined for 5 mice from each group after 1, 2 or 3 days, as described in the Materials and Methods. The median number of bacteria is indicated by a black, horizontal bar, and the limit of detection is indicated by a broken line, which is 40 CFU in the nasopharynx, lungs, blood and brain, and 4 CFU in the ears. The Mann Whitney *U* test (two tailed) was used to analyse data. *, $P < 0.05$; **, $P < 0.01$.

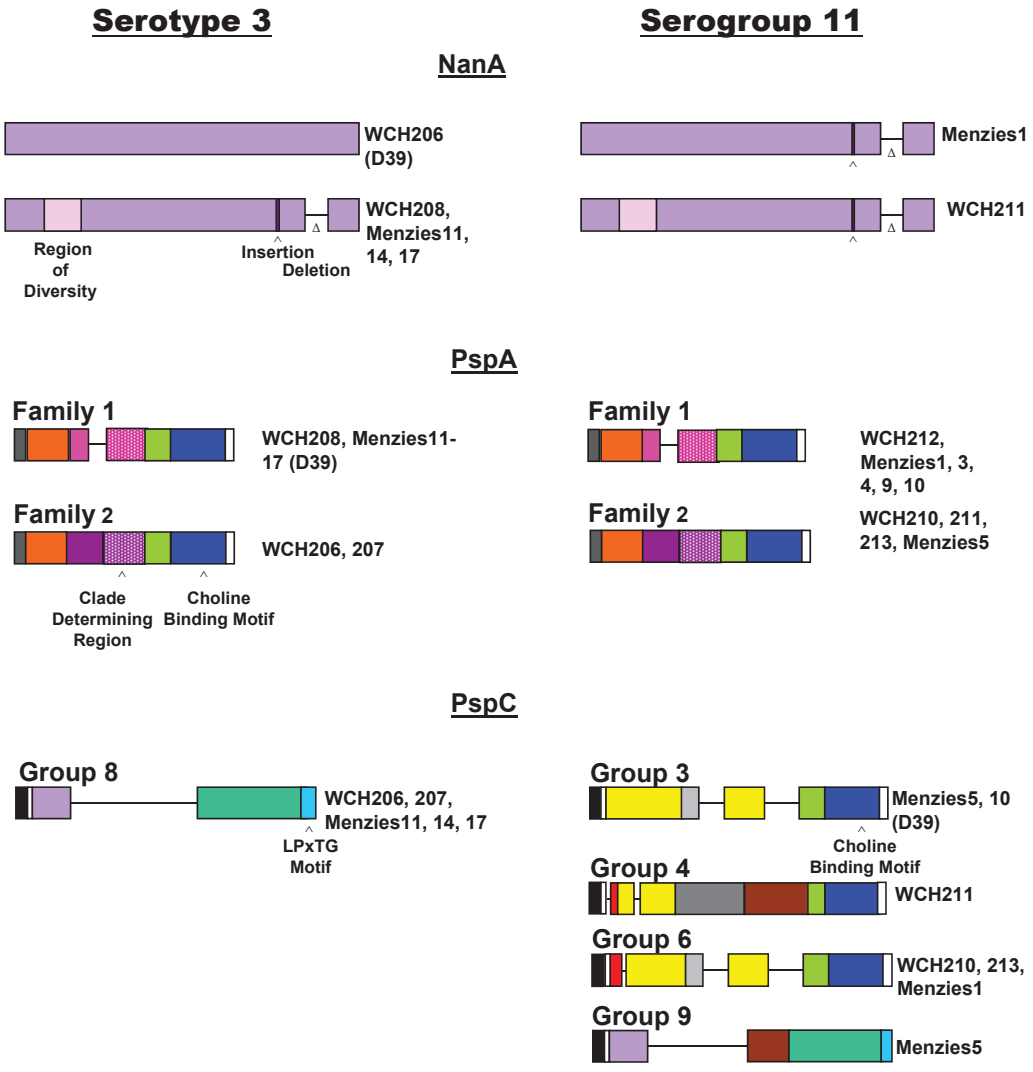

Fig. 1

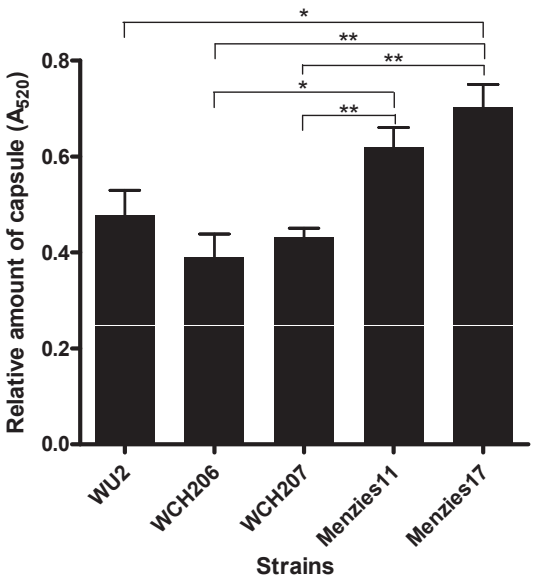

Fig. 2

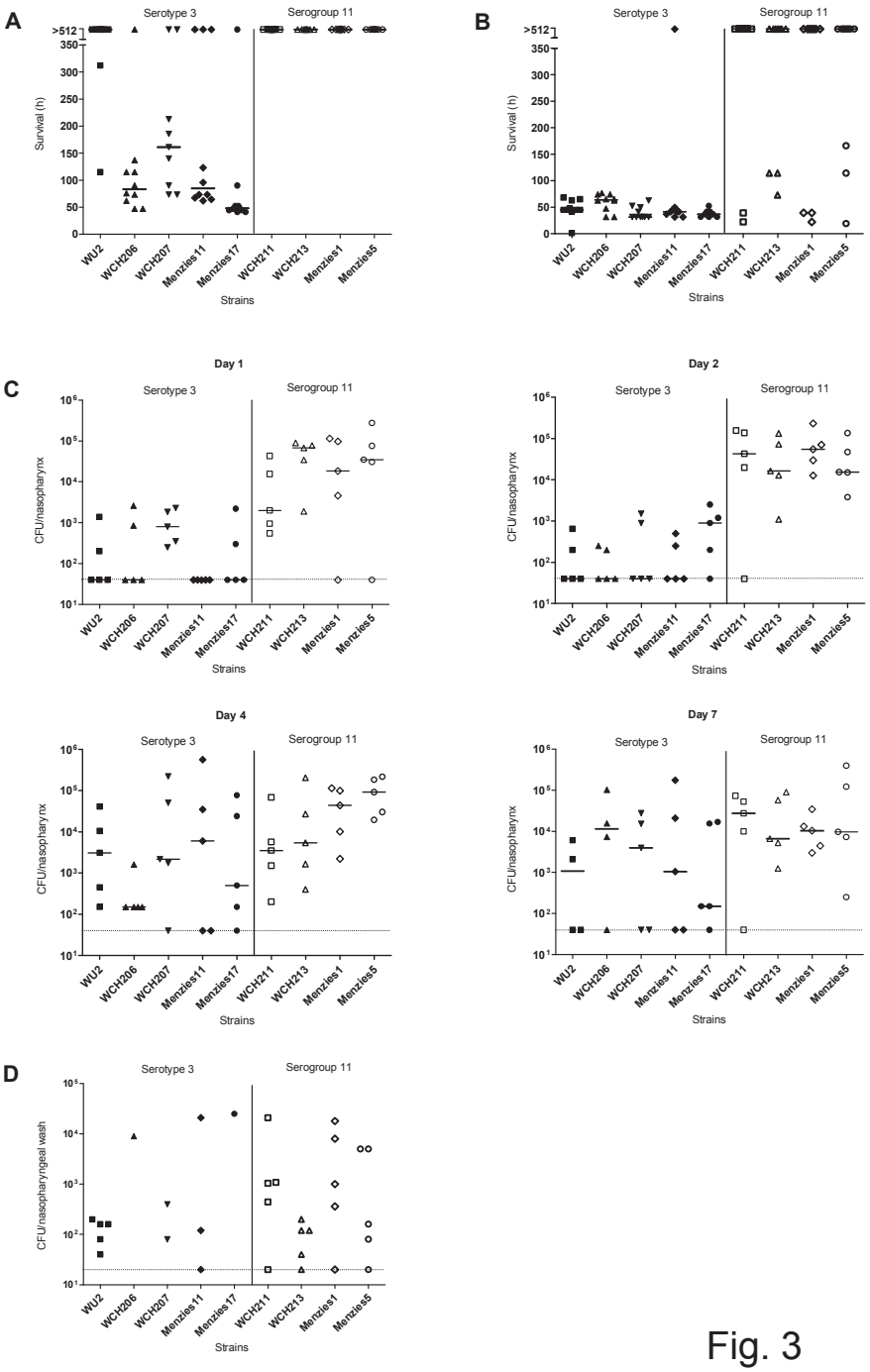

Fig. 3

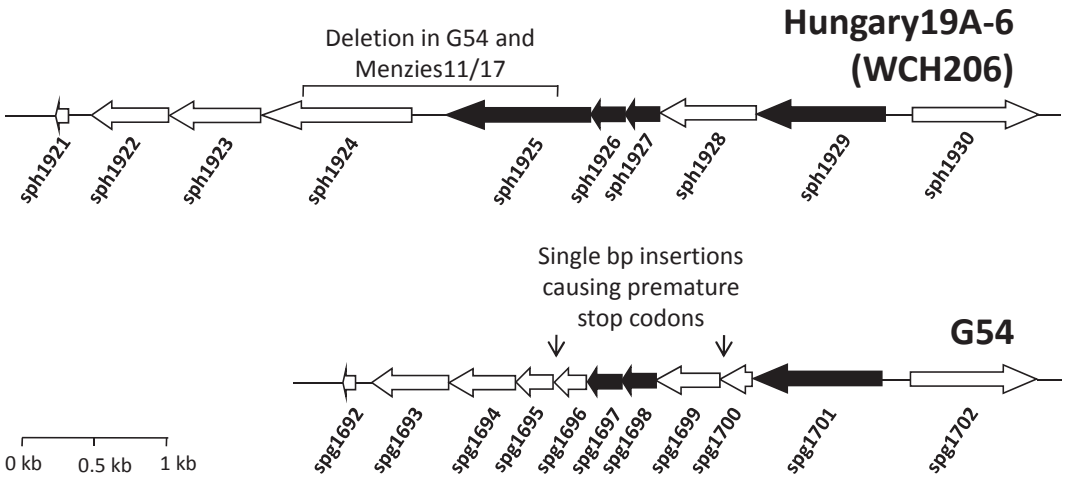

Fig. 4

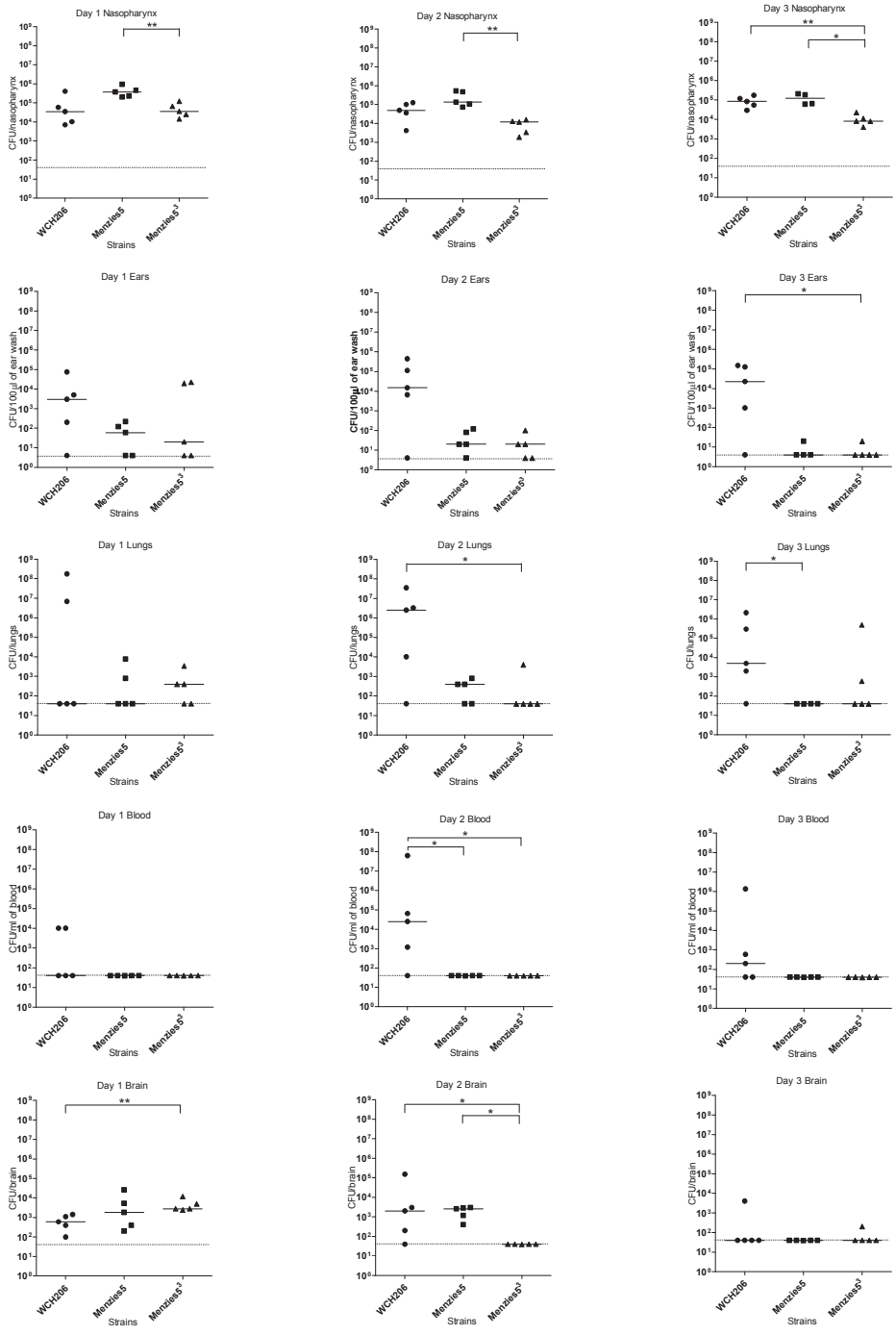

Fig. 5