Veterinärmedizinische Universität Wien

Aus dem Department für Pathobiologie
der Veterinärmedizinischen Universität Wien
(Departmentsprecher: Univ.Prof. Dr.rer.nat. Armin Saalmüller)

Institut für Mikrobiologie
(Leiterin: Univ.-Prof. Dipl.-Ing. Dr.rer.nat. Monika Ehling-Schulz)

Characterization of *Streptococcus pneumoniae* Isolates from Austrian Companion Animals and Horses

Diplomarbeit
zur Erlangung der Würde des
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Maximilian Ginders

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Betreuer:

Dr.med.vet. Igor Loncaric
Igor.Loncaric@vetmeduni.ac.at

Priv.-Doz. Dr.med.vet. Joachim Spergser
Joachim.Spergser@vetmeduni.ac.at

Department für Pathobiologie
Institut für Mikrobiologie, Arbeitsgruppe für Klinische Mikrobiologie
(vormals Institut für Bakteriologie, Mykologe und Hygiene, Abteilung für Klinische Mikrobiologie und Infektionsbiologie)
Veterinärmedizinische Universität Wien
Veterinärplatz 1, 1210 Wien Österreich

Begutachterin:

O. Univ.-Prof. Dr.rer.nat. Dr.med.vet.habil. Renate Rosengarten
Renate.rosengarten@vetmeduni.ac.at

Ordentliche Universitätsprofessorin für Bakteriologie und Hygiene
Vorm. Vorstand des Instituts für Bakteriologie, Mykologie und Hygiene
Veterinärmedizinische Universität Wien

Anmerkung

Diese Arbeit wurde zeitgleich als Manuskript unter dem Titel „Characterization of *Streptococcus pneumoniae* isolates from Austrian companion animals and horses“ zur Publikation in *Acta Veterinaria Scandinavica* eingereicht.

Im Folgenden die Auflistung der Namen der Autoren und ihr jeweiliger Beitrag:

Michael Leschnik, Frank Künzel und Doris Kampner (Department/Universitätsklinik für Kleintiere und Pferde, Veterinärmedizinische Universität Wien) partizipierten bei der Studienplanung, der Datenerhebung und der Probensammlung.

Claudia Mikula und Georg Steindl (Nationale Referenzzentrale für Pneumokokken, AGES – IMED Graz, Zentrum für lebensmittelbedingte Infektionskrankheiten) führten die Serotypisierung durch.

Inga Eichhorn, Andrea T. Feßler und Stefan Schwarz (Institut für Mikrobiologie und Tierseuchen, Fachbereich Veterinärmedizin, Freie Universität Berlin) unterstützten bei der Studienplanung, Methodik und führten die MHK (Minimale Hemm-Konzentration) durch.

Joachim Spergser (Institut für Mikrobiologie, Department für Pathobiologie, Veterinärmedizinische Universität Wien) war an der Planung des Studiendesigns beteiligt und unterstützte bei der Durchführung der Studie.

Igor Loncaric (Institut für Mikrobiologie, Department für Pathobiologie, Veterinärmedizinische Universität Wien) hatte die Idee der Studie, führte die mikrobiologischen und molekulargenetischen Untersuchungen durch und unterstützte bei der Verfassung des Manuskripts.

Maximilian Ginders war an den mikrobiologischen Untersuchungen beteiligt, führte die PCR-Untersuchungen durch und verfasste das Manuskript.

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1. Introduction

The aim of the present study was to investigate the genetic relatedness and the antimicrobial resistance profiles of a collection of Austrian *Streptococcus* (*S.*) *pneumoniae* isolates from companion animals and horses. A total of 12 non-repetitive isolates presumptively identified as *S. pneumoniae* were obtained during routine diagnostic investigations between March 2009 and January 2017.

S. pneumoniae is a major human pathogen that colonizes the upper respiratory tract and causes both life-threatening diseases such as pneumonia, sepsis and meningitis but also sinusitis and otitis in both children and adults (Gillespie, 1989). *S. pneumoniae* is responsible for community-acquired respiratory tract infections in infants. *S. pneumoniae* infections of pregnant women may be associated with stillbirth and fetal death (Adriani et al., 2012). Several animal models have been used to study *Pneumococcus*-associated pneumonia, meningoencephalitis and otitis (Chiavolini et al., 2008; Moxon, 1981). *S. pneumoniae* is assumed to be a human pathogen only. Nevertheless, there are established mouse and rat models for various *S. pneumoniae*-caused diseases (Chiavolini et al., 2008). Zooanthropogenic transmission of other streptococcal species is well-documented, in particular for *S. equi* subsp. *zooepidemicus*, *S. canis*, *S. suis*, *S. porcinus* and *S. phocae* (Duarte et al., 2005; Kuusi et al., 2006; Michaud et al., 1996; Higgins, 2000; Whatmore et al., 2001). Pneumococci can easily exchange DNA in their natural habitat, the human mouth and throat. This environment is populated by several streptococcal species, which form a 'gene pool' out of which the pneumococci can recruit resistance genes. Gene transfer and mosaic genes have been intensively reported for *S. pneumoniae* and other streptococci (Hakenbeck et al., 2001). While comprehensive data on human *S. pneumoniae* infections exists, there is still a lack of information on infections, carriage and zoonotic potential of this particular pathogen in pet and companion animals. Therefore, the aim of the present study was to investigate the genetic relatedness and the antimicrobial resistance pattern of a collection of Austrian *S. pneumoniae* isolates from infections of pet or companion animals as well as horses.

2. Material and Methods

A total of 12 non-repetitive isolates, presumptively identified as *S. pneumoniae,* were obtained between 2009 and 2017 during diagnostic examinations at the Institute of Microbiology (formerly Institute of Bacteriology, Mycology and Hygiene) of the University of Veterinary Medicine Vienna, Austria. The isolates were identified as *S. pneumoniae* using classical bacteriological methods and originated from guinea pigs (n=6), horses (n=3), a dog (n=1) and pet rats (n=2). The annual isolation frequency of *S. pneumoniae* was inconstant. All isolates were stored in glycerol stocks at -80°C. For the present study, isolates were re-grown on Mueller Hinton Agar with 5% sheep blood and on Improved II agar (Becton Dickinson, Heidelberg, Germany). They were confirmed as *S. pneumoniae* by bile solubility (Acharya, 2013) and optochin susceptibility, MALDI-TOF mass spectrometry (MS) (Bruker Daltonik), as well as sequence analyses of a part of the *recA* (Zbinden et al., 2011) and 16S rRNA genes (Loncaric et al., 2013). Isolates were further characterized by pneumolysin PCR (Toikka et al., 1999) and genotyped by multilocus sequence typing (MLST). MLST was carried out by PCR amplification and sequencing of 7 housekeeping genes (*aroE, gdh, gki, recP, spi, xpt, ddl*) as described previously (Adamiak et al., 2014). Allelic profiles and sequence types (ST) were assigned using the MLST database hosted at http://pubmlst.org/spneumoniae/ Antimicrobial susceptibility testing was performed by agar disc diffusion according to the Clinical and Laboratory Standards Institute (CLSI 2015, 2017) including oxacillin, tetracycline, doxycycline, erythromycin, clindamycin, chloramphenicol, vancomycin, trimethoprim-sulfamethoxazole, enrofloxacin, marbofloxacin and linezolid (all from Becton Dickinson, Heidelberg). With respect to the resistance phenotype, the presence of resistance genes was confirmed by previously described PCR analyses applying specific primers for *tet*(M), *erm*(B), *mef*(A), *mef*(E), *msr*(E), cat_{pC194}, cat_{pC221} and cat_{pC223} (Ambrose et al., 2005; Maskell et al., 2001; Schnellmann et al., 2006; Shiojima et al., 2005). The presence of mutations within fragments of the genes encoding either a dihydropteroate synthase (DHPS) that plays a role in conferring resistance to sulfamethoxazole or a dihydrofolate reductase (DHFR) conferring resistance to trimethoprim in *S. pneumoniae* was examined by PCR followed by DNA sequence analysis (Padayachee and Klugman, 1999).

Furthermore, PCR analysis for the detection of two *int-Tn* genes encoding the integrases of the conjugative transposons Tn*1545* (conferring resistance to tetracycline, kanamycin and macrolides) and Tn*5252* (chloramphenicol resistance) was performed (Shiojima et al., 2005). Amplicons of *int*-Tn*1545* as well as *int*-Tn*5252* were sequenced. Finally, capsular serotyping was conducted by the capsular reaction test, the quellung reaction (Sørensen, 1993) employing sera obtained from the Statens Serum Institute, Copenhagen, Denmark. Diagnostic antisera included 12 serum pools for serogrouping and various type-specific antisera. Serotyping was performed according to the manufacturer's recommendations.

3. Results

All 12 isolates examined were bile-soluble, optochin-susceptible and positive for the pneumolysin gene. MALDI-TOF MS identified all strains as *S. pneumoniae*. Sequence analyses of the *recA* and 16S rDNA genes showed that all isolates were closely related to the type strain of *S. pneumoniae.* MLST revealed that all 6 guinea pig isolates belonged to the sequence type (ST) 6937 (allelic profile: 2-5-4-5-27-20-5), all three equine isolates belonged to ST6934 (allelic profile: 10-9-4-12-287-426-470), and the dog isolate and the two rat isolates were assigned to ST36 (allelic profile: 1-8-4-1-1- 4-6) and ST3546 (allelic profile 1-5-41-5-10-28-8), respectively. Susceptibility testing of the isolates showed that all but two isolates were susceptible to all antimicrobial agents tested. The two isolates 2946 and 880, both obtained from rats, exhibited resistance to tetracycline, erythromycin, clindamycin, chloramphenicol and trimethoprim-sulfamethoxazole and were positive for *tet*(M), *erm*(B), *cat*pC194, and *int-Tn1545* as well as *int-Tn5252*. Moreover, they displayed mutations in the genes *sulA* and *dfr*, i.e. an insertion of 6 bp within *sulA*, the gene encoding DHPS, resulting in the duplication of amino acids Arg58 and Pro59, as well as mutations within the *dfr* gene that resulted in amino acid exchanges at the position 92 (Asp-92-Arg) and 100 (Ile-100-Leu) of the dihydrofolate reductase. These alterations have previously been described to be associated with sulfamethoxazole and trimethoprim resistance (Cornick et al., 2014). Serotyping identified all 6 guinea pig isolates as serotype 19F, the three horse isolates as serotype 3, the dog isolate as serotype 23F and the two rat isolates as serotype 19A (Table 1).

Table 1. Characteristics of *Streptococcus pneumoniae* isolates recovered from different animal species.

Isolate	Year of isolation	Host species	Site of isolation	Symptoms	ST	Resistance phenotype	Resistance genotype	Serotype
649	2009	guinea pig	lung	respiratory	6937	-	-	19F
2704	2009	horse	diverticulum tubae auditivae	respiratory	6934	-	-	3
2902	2009	horse	trachea	respiratory	6934	-	-	3
2946	2009	rat	lung	respiratory	3546	TET, ERY, CLI, CHL, SXT	*tet*(M), *erm*(B), cat_{PC194}, *dfr*	19A
747	2010	horse	trachea	respiratory	6934	-	-	3
1166	2010	guinea pig	lung	central nervous, respiratory	6937	-	-	19F
1409	2010	guinea pig	lung	respiratory	6937	-	-	19F
864	2011	guinea pig	lung	respiratory	6937	-	-	19F
2994	2012	guinea pig	pleural punction	respiratory	6937	-	-	19F
1537	2014	dog	cerebrospinal fluid	central nervous	36	-	-	23F
271	2017	guinea pig	ear	central nervous	6937	-	-	19F
880	2017	rat	lung	respiratory	3546	TET, ERY, CLI, CHL, SXT	*tet*(M), *erm*(B), cat_{PC194}, *dfr*	19A

ST, sequence type; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole

4. Discussion

In the present study a total of 12 non-repetitive *S. pneumoniae* isolates, originated from pet and companion animals and horses were analysed. The comparison of ST/serotype combination with the MLST Database (http://pubmlst.org/spneumoniae/) revealed the following results. All 6 guinea pig isolates displayed a ST6937 and serotype 19F combination. Serotype 19F is a common serotype encountered in human isolates; it is – according to the MLST database (contains both ST and serotypes) - associated with a large number of different STs and is often linked with invasive pneumococcal disease as well as *S. pneumoniae* carriage. This ST/serotype combination has currently 21 entries in the database, including 19 isolates from guinea pigs in Germany, the Netherlands, France and Peru. Another two isolates were found in a nasal and wound swab from infants in Germany and the Netherlands. It seems to be a typical ST/serotype combination among guinea pigs and this observation might suggest that guinea pigs seem to represent a reservoir for *S. pneumoniae* of this specific sequence type (Linden et al., 2009) with a still not clearly defined relevance in human hosts. Nevertheless, the close contact between children and their pets could be a risk factor for transmission. The origin of the 6 guinea pig isolates in connection with clinical symptoms suggests that this ST/serotype combination might be pathogenic for guinea pigs (Table 1).

The three equine isolates examined in the present study, belong to ST6934 and serotype 3. Serotype 3 is also a serotype commonly found in humans, but in the combination with ST6934, only 5 more entries were identified in the MLST database. All these 5 isolates originated from horses in Germany and the UK. Whether this particular serotype plays a role as a zoonotic pathogen remains unclear. Whatmore et al. (1999) compared a collection of equine and human pneumococcal isolates of serotype 3 using restriction fragment length polymorphism (RFLP) analysis of housekeeping genes. RFLP revealed that equine pneumococci were indistinguishable from each other but also very closely related to human isolates.

Strain 1537/14 was isolated from a dog suffering from encephalitis, belonging to ST36 and serotype 23F. To the authors' best knowledge this ST/serotype combination has never been isolated from a dog or another animal before. In the *S. pneumoniae* MLST database, *S. pneumoniae* ST36/23F is represented by 92 records, which all originate from human hosts. Serotype 23F is one of the most prevalent serotypes involved in IPD (Johnson et al., 2010).

The two multidrug-resistant isolates 2946 and 880 originated from rats, kept as pet animals, that suffered from pneumonia. They belonged to ST3546 and serotype 19A. The MLST database revealed 5 records with this combination. Four these 5 isolates with this ST/serotype combination were isolated from humans in Germany, Norway and Czech Republic. One isolate was also obtained from a rat in Austria in 2007 with a similar multi-drug resistance profile. Van der Linden et al. (2009) also found two pet rats with the same ST/serotype combination. Rats are often kept by teenagers in close physical contact and may suffer from pneumonia resistant to therapy (Weisbroth et al., 2006). It is therefore recommended to examine those pets for the carriage of bacterial pathogens and – if positive – also by antimicrobial susceptibility testing more frequently than it is currently done. Serotype 19A is a common serotype found in humans suffering from invasive pneumococcal disease (IPD) (Riva et al., 2012). The epidemiology of this serotype is constantly changing. Possible reasons for this are the introduction of pneumococcal conjugate vaccination, increased use of antibiotics, import of multidrug-resistant isolates and increased reporting. The prevalence of serotype 19A, for example in Germany, has increased significantly between 2007 and 2011 (van der Linden et al., 2013).

Serogroup 19 (12.8 %) as well as serogroup 3 (8.6 %) are some of the most prevalent serogroups in humans in Europe. Among the most commonly reported serogroups, dual non-susceptibility to penicillin and macrolides was mainly observed in serogroup 19. Serotype associated in the present study with all 6 isolates from guinea pigs and serotype 23F associated with the dog isolate are known as common 'classic' resistant serotypes (European Centre for Disease Prevention and Control, 2015).

Whether the isolates found in dogs and rats can be correlated with human pneumococcal isolates, but further research is needed to improve the knowledge of the possible zoonotic potential of these bacteria. All pet and companion animals from which the isolates investigated in this study came from, suffered from severe respiratory or central nervous symptoms (Table 1). This strongly suggests that pneumococci are able to cause diseases in different animal species.

However, the results of the present study have limitations, because only 12 isolates have been included. Nevertheless, the frequency of *S. pneumoniae* isolation on which this study is based is similar to that described by van der Linden et al. (2009) who identified 41 strains during 22 years from pets and zoo animals.

Due to the close contact between pet and companion animals and humans, isolation of strains from diseased companion animals representing human-associated clones deserves special consideration, particularly if those isolates display multidrug resistant pheno- and genotypes. The results of the present study therefore underline that a closer collaboration between human medicine and veterinary medicine is needed.

5. Summary

A total of 12 non-repetitive isolates presumptively identified as *Streptococcus pneumoniae* were obtained between 2009 and 2017 during diagnostic examinations at the Institute of Microbiology (formerly Institute of Bacteriology, Mycology and Hygiene) of the University of Veterinary Medicine, Vienna. The isolates originated from guinea pigs (n=6), horses (n=3), a dog (n=1) and a pet rats (n=2), all with symptoms of clinical disease.

All isolates were identified as *S. pneumoniae* by bile solubility and optochin susceptibility testing, MALDI-TOF MS, as well as sequence analyses of the *recA* and 16S rRNA genes. Isolates were further characterized by pneumolysin PCR and genotyped by MLST based on 7 housekeeping genes (*aroE, gdh, gki, recP, spi, xpt, ddl*). Allelic profiles and sequence types (ST) were assigned using the MLST database hosted at http:// pubmlst.org/spneumoniae/. Antimicrobial susceptibility testing was performed by agar disc diffusion. In addition to resistance phenotype determination, the presence of resistance genes and transposons was examined. Serotyping of the isolates was conducted by the capsular reaction test, the quellung reaction.

All 12 isolates were bile-soluble, optochin-susceptible, and positive for the pneumolysin gene. MALDI-TOF MS identified all strains as *S. pneumoniae*. Sequence analyses of the *recA* and 16S rDNA genes showed that all isolates were most closely related to the type strain of *S. pneumonia*. MLST revealed that all 6 guinea pig isolates belonged to the sequence type (ST) 6937, all three equine isolates belonged to ST 6934, the dog isolate was assigned to ST 36 and both rat isolates were defined as ST 3546. Susceptibility testing of the isolates showed that all but two isolates from pet rats were susceptible to all antibiotics tested. The two isolates from pet rats, exhibited resistance to tetracycline, erythromycin, chloramphenicol, clindamycin and trimethoprim-sulfamethoxazole, and were positive for *tet*(M), *erm*(B), cat_{pC194} and *Int*-Tn*1545* as well as *Int*-Tn*5252*. Moreover, they displayed the same mutations in the genes *sulA* and *dfr*, i.e. an insertion of 6 bp within *sulA*, the gene encoding DHPS, resulting in the duplication of amino acids Arg58 and Pro59, as well as mutations within the *dfr* gene that resulted in amino acid exchanges at the position 92 (Asp-92-Arg) and 100 (Ile-100-Leu) of the dihydrofolate reductase. These alterations have previously been described to be associated with sulfamethoxazole and trimethoprim resistance (Cornick et al., 2014).

Serotyping identified all 6 guinea pig isolates as serotype 19F, the three horse isolates as serotype 3, the dog isolate as serotype 23F and the two rat isolates as serotype 19A.

The present study represents the first comprehensive investigation on characteristics of *S. pneumoniae* isolates recovered from Austrian pet and companion animals and horses. The results indicate that common human sero- and sequence types may circulate in pet and companion animals and horses. Moreover, *S. pneumoniae* strains representing common human-associated clones implicated in causing invasive pneumococcal disease (IPD) were present in pet and companion animals (a dog and two pet rats) suffering from encephalitis or pneumonia. It is unclear whether this is a rare phenomenon or may be an emerging problem in the keeping of companion animals.

6. Zusammenfassung

Am Institut für Mikrobiologie bzw. dem vormaligen Institut für Bakteriologie, Mykologie und Hygiene der Veterinärmedizinischen Universität Wien, wurden in einem Zeitraum von 8 Jahren (2009-2017), im Rahmen der Routinediagnostik 12 nicht-repetitive Isolate von *Streptococcus pneumoniae* gewonnen. Diese Isolate stammten von Meerschweinchen (n=6), Pferden (n=3), einem Hund (n=1) und zwei Hausratten (n=2), alle mit Symptomen klinischer Erkrankung.

In der vorliegenden Arbeit wurden die Isolate mittels klassischer bakteriologischer Methoden, MALDI-TOF Massenspektrometrie (MS) und molekulargenetischer Analysen identifiziert und charakterisiert. Des Weiteren wurde eine Genotypisierung durch Multilocus Sequence Typing (MLST) durchgeführt, und alle Isolate wurden auf Antibiotikaresistenz geprüft. Auf der Basis der Ergebnisse des Agardiffusionstestes wurden die Isolate auf das Vorkommen von Resistenzgenen untersucht. Darüber hinaus wurde eine Kapsel-Serotypisierung durch Quellungsreaktion vorgenommen. Alle 12 Isolate zeigten Gallelöslichkeit, Optochin-Sensibilität, und das Pneumolysin-Gen war bei allen Isolaten mittels PCR nachweisbar. MALDI-TOF MS identifizierte alle Isolate als *S. pneumoniae*, und Sequenzanalysen des recA-Gens und der 16S rDNA ließen ebenfalls einer enge Verwandtschaft aller Isolate zu *S. pneumoniae* erkennen.

Mittels MLST-Analyse konnten alle 6 Meerschweinchen-Isolate dem Sequenztyp ST 6937, die drei Pferde-Isolate ST 6934, das Hunde-Isolat ST 36 und die zwei Hausratten-Isolate ST 3546 zugeordnet werden. Mit Ausnahme der zwei Hausratten-Isolate waren alle Isolate sensibel auf die getesteten Antibiotika. Die zwei Isolate von Hausratten zeigten einen multiresistenten Phänotyp, mit Resistenzen gegen Tetracylin, Erythromycin, Chloramphenicol, Clindamycin und Trimothoprim-Sulfamethoxazol. Des Weiteren konnten die Resistenz-Gene *tet*(M), *erm*(B), *cat*pC194 und die Integrase-Gene *Int*-Tn*1545* und *Int*-Tn*5252* der zugehörigen Trasposons nachgewiesen werden. Auβerdem konnten Mutationen in den Genen *sulA* und *dfr* gezeigt werden, welche mit einer Sulfamethoxazol- und Trimethoprim-Resistenz assoziiert sind. Die Serotypisierung identifizierte alle 6 Meerschweinchen-Isolate als Serotyp 19F, die drei Pferde-Isolate als Serotyp 3, das Hunde-Isolat als Serotyp 23F und die zwei Ratten-Isolate als Serotyp 19A.

Die hier durchgeführte Studie beschreibt erstmalig die Charakterisierung von *S. pneumoniae*-Isolaten, welche von Haus- und Begleittieren sowie Pferden in Österreich gewonnen wurden. Die Ergebnisse deuten darauf hin, dass humane Sero- und Sequenztypen von *S. pneumoniae* in Haus- und Begleittieren und Pferden zirkulieren. Dabei schienen sämtliche 12 Isolate pathogen für die jeweilige Tierart zu sein, auch wenn die genetischen Analysen der Meerschweinchen- und Pferdeisolate darauf hindeuten, dass es sich hier voraussichtlich um an diese Tierarten angepasste Stämme handelt. Dennoch ist es gerade vor dem Hintergrund des engen Kontaktes von Haus- und Begleittieren zu ihren Besitzern und Besitzerinnen und des nicht eindeutig geklärten zoonotischen Potentials von *S. pneumoniae* alarmierend, dass klassische, invasive Humanstämme in Haus-und Begleittieren, d.h. in der vorliegenden Studie in einem Hund und zwei Hausratten nachgewiesen werden konnten. Außerdem ist die Isolierung von zwei multiresistenten *S. pneumoniae*-Stämmen aus Hausratten beunruhigend, zumal Hausratten häufig von jugendlichen Besitzern und Besitzerinnen in engem Kontakt gehalten werden. Aufgrund mangelnder Beprobung ist die Prävalenz solcher Keime jedoch nicht abzuschätzen. Es ist daher zum gegenwärtigen Zeitpunkt noch unklar, ob es sich hierbei um ein seltenes Phänomen oder um ein alarmierendes Problem in der Haltung von Haustieren handelt.

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8. List of Abbreviations

Arg	arginine
aroE	*S. pneumoniae* housekeeping gene
Asp	aspartarte
bp	base pairs
*cat*pC194	gene coding for cloramphenicol resistance
*cat*pC221	gene coding for cloramphenicol resistance
*cat*pC223	gene coding for cloramphenicol resistance
CHL	chloramphenicol
CIP	ciprofloxacin
CLI	clindamycin
CLSI	Clinical and Laboratory Standards Institute
ddl	*S. pneumoniae* housekeeping gene
dfr	gene coding for dihydroflavono-4-reductase
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
erm(B)	gene coding for resistance against macrolide, lincosamide and streptogramin b
ERY	erythromycin
gdh	*S. pneumoniae* housekeeping gene
gki	*S. pneumoniae* housekeeping gene
Ile	isoleucine
int-Tn	gene coding for integrase
IPD	invasive pneumococcal disease
Leu	leucine
mef(A)	gene coding for macrolide efflux
mef(E)	gene coding for macrolide efflux
msr(E)	gene coding for macrolide transporter
MLST	multilocus sequence typing

PCR	polymerase chain reaction
Pro	proline
recA	*S. pneumoniae* housekeeping gene
recP	*S. pneumoniae* housekeeping gene
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
S.	*Streptococcus*
spi	*S. pneumoniae* housekeeping gene
ST	sequence type
sulA	gene coding for SOS cell division inhibitor
SXT	trimethoprim/sulfamethoxazole
TET	tetracycline
tet(M)	gene coding for resistance against tetracycline
Tn*1545*	transposon conferring resistance to tetracycline, kanamycin and macrolides
Tn*5252*	transposon conferring resistance to chloramphenicol
UK	United Kingdom
xpt	*S. pneumoniae* housekeeping gene

9. List of Tables