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Streptococcus pneumoniae as an agent of urinary tract infections – a laboratory experience from 2010 to 2014 and further characterization of strains

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ABSTRACT

Streptococcus pneumoniae is a rare cause of urinary tract infection. Between January 2010 and December 2014, 26 urine samples from 18 different patients contained *S. pneumoniae* at the Department for Infectious Diseases, University Hospital of Heidelberg. Patient age varied between three and 72 years. 13 patients were male and five were female. Past medical histories of 16 patients were available. Eight patients had a past medical history of renal transplant and four patients had other renal dysfunctions. Further analyses of the isolates revealed that the aspect of colonies is more resembling *S. mitis* than invasive isolates of *S. pneumoniae*. Optochin disk diameters tend to be 14 mm or smaller. Identification using MALDI-TOF or VITEK2 identification cards was accurate. Only 2 isolates showed a decreased susceptibility towards penicillin (MIC = 0.5 mg/L). Eight different serotypes were identified using a PCR approach as well Neufeld-Quellungs reaction.

1. Introduction

The main agents for urinary tract infections (UTI) are gram-negative bacteria with *Escherichia coli* causing the majority of cases (Gupta et al., 2011; Hooton et al., 2010). The same is true for asymptomatic bacteriuria (Nicolle et al., 2005). In general *Streptococcus pneumoniae* is not considered to be an agent of UTI. In the current Infectious Diseases Society of America (IDSA) guidelines on UTI and bacteriuria *S. pneumoniae* is not mentioned as a possible agent for UTI (Gupta et al., 2011; Hooton et al., 2010; Nicolle et al., 2005). The same is true for the current German guideline for the diagnosis of urinary tract infections (German Society for Microbiology and Hygiene) (Gatermann et al., 2005).

However, in 2007 we realized for the first time that in some urine samples we could cultivate *S. pneumoniae* in high numbers (≥10^4 colony forming units (cfu)). Looking into patient records we found that all patients had symptoms of a UTI. Additionally all patients were children (<12 years) with malformations of the urinary tract (Burckhardt and Zimmermann, 2011).

As a consequence of our findings we altered our culture conditions for the blood agar plates inoculated with urine. Because the main agents for UTI grow very well without additional CO_2 no guideline recommends incubation of plates with additional CO_2. However, *S. pneumoniae* grows much better in the presence of elevated CO_2 levels compared to growth in ambient air (Austrian and Collins, 1966). In 2010 we changed our standard operating procedure accordingly. Since then all blood agar plates inoculated with urine have been incubated in the presence of 5% CO_2. Additionally we instructed our technicians to perform optochin testing on all alpha-hemolytic streptococci grown in pure culture or with a bacterial count >=10^4 cfu in the presence of additional bacteria. All isolates identified as *S. pneumoniae* during our routine work-flow were stored for future work-up.

In this report we want to summarize our experience with this altered work-flow (2010-2014) and describe the characteristics of the isolated *S. pneumoniae* strains.

2. Materials and methods

2.1. Routine workflow

Each urine sample was inoculated onto a Columbia agar plate with 5% sheep blood (BD, Heidelberg, Germany) and a chromID™ CPS Agar plate (bioMerieux, Nürtingen, Germany). Each plate was inoculated with 10 µL using a PREVI Isola (bioMerieux, Nürtingen, Germany). Blood agar plates were incubated at 36°C, 5% CO_2 and chromID CPS agar plates were incubated at 36 °C, ambient air. Plates were read after overnight incubation.

During routine reading of plates identification of alpha-hemolytic streptococci was performed using optochin disks (5 µg, BD, Heidelberg, Germany). Inhibition zones of ≥14 mm were indicative of *S. pneumoniae*. In case of ambiguous results biochemistry (VITEK2, identification card

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Table 1
Data of samples (and corresponding isolates) identified during routine workflow including year of sample, age of patient, sex, colony counts of alpha-hemolytic colonies and additional bacteria in the respective sample.

Patient No	Sample/isolate No	year of sample	Age (y)	Sex	Optochin disk test (routine workflow)	cfu alpha-hemolytic streptococci	Additional bacteria	Isolate available/viable
1	**1**	2010	**3**	F	Susceptible	10 5	10 4 enterococci, 10 2 enterobacteriaceae	No
2	**2**	2010	13	**M**	Susceptible	10 5	10 4 *P. mirabilis*	Yes
3	**3***	2010	14	**M**	Susceptible	10 5	No	Yes
4	**4**	2010	15	F	Susceptible	10 2	10 4 group B streptococci, 10 2 S. aureus	Yes
5	**5**	2010	5	**M**	Susceptible	10 3	No	No
6	**6**	2011	12	**M**	Susceptible	10 5	No	Yes (from 2009)
7	**7**	2011	56	**M**	Susceptible	10 3	No	Yes
8	**8**	2011	50	F	Susceptible	10 4	No	Yes
9	**9**	2011	66	**M**	Susceptible	10 5	10 4 *E. coli*	Yes
10	**10**	2011	**72**	F	Susceptible	10 4	10 3 enterobacteriaceae	Yes
3	**11***	2011	14	**M**	Susceptible	10 5	No	No
3	**12***	2011	14	**M**	Susceptible	10 5	No	No
11	**13****	2011	12	**M**	Susceptible	10 5	No	Yes
11	**14****	2011	12	**M**	Susceptible	10 5	No	No
12	**15*****	2011	19	**M**	Susceptible	10 5	No	Yes
12	**16*****	2012	19	**M**	Susceptible	10 5	No	No
13	**17******	2012	6	**M**	Susceptible	10 5	No	Yes
14	**18**	2013	17	**M**	Susceptible	10 5	10 5 *S. aureus*	Yes
15	**19*******	2013	11	**M**	Susceptible	10 5	No	Yes
15	**20*******	2013	11	**M**	Susceptible	10 5	10 4 *P. aeruginosa*	No
16	**21**	2014	14	F	Susceptible	10 5	10 5 Prov. rettgeri	No
17	**22**	2014	46	**M**	Susceptible	10 5	10 5 *K. pneumoniae*	Yes
18	**23**	2014	48	**M**	Susceptible	10 3	No	Yes
13	**24********	2014	8	**M**	Susceptible	10 5	No	Yes
13	**25********	2014	8	**M**	Susceptible	10 5	No	Yes
13	**26********	2014	8	**M**	Susceptible	10 3	10 2 enterococci	Yes

cfu = colony forming units; asteriks indicate samples from the same patient; from 5 different patients more than one sample was available (*–*****).

for gram-positive cocci (GP)) or MALDI-TOF (microflex, Bruker, Bremen, Germany) were used for identification.

All strains identified as *S. pneumoniae* were stored at −80 °C.

2.2. Follow-up analyses

Stored strains were re-cultured on Columbia agar plates with 5% sheep blood (BD, Heidelberg, Germany), 36 °C, 5% CO_2. In general, fresh overnight cultures were used for further tests. The following tests were performed on well growing cultures: optochin-sensitivity (as described above) using MH-F agar plates (Müller-Hinton fastidious agar, bioMerieux, Nürtingen, Germany) and Columbia agar plates with 5% sheep blood (BD, Heidelberg, Germany), agglutination with Slidex pneumo-kit (bioMerieux, Nürtingen, Germany) and agglutination with Immulex *S. pneumoniae* omni-serum (Statens Serum Institute, Copenhagen, Denmark). Both agglutination reactions were performed as recommended by the manufacturer. Additionally all strains were identified using MALDI-TOF (microflex, Bruker, Bremen, Germany) using the latest database (5627 entries). Biochemical identification was performed using the identification card for gram-positive bacteria for VITEK2 (GP-Card, bioMerieux, Nürtingen, Germany). Biochemical reactions are summarized as a bio-number. For susceptibility testing two different methods were used. First, disk diffusion tests with oxacillin disks (1 μg, BD, Heidelberg, Germany) were done following the latest EUCAST guidelines (www.eucast.org). Second, susceptibility testing using AST-P576 for VITEK2 (bioMerieux, Nürtingen, Germany) was performed. Results were interpreted according to the latest EUCAST breakpoints. For serotyping two different methods were used. First, all strains underwent serotyping via PCR following a published protocol (Burckhardt et al., 2014) and additional primers as described in (Pai et al., 2006). This protocol includes a PCR for cpsA as a control. Second, all strains were sent to the National Reference Center for Streptococci, University Aachen, Germany for serotyping using the Neufeld-Quellungs reaction.

3. Results

From January 2010 until December 2014 the Department for Infectious Diseases at the University of Heidelberg, Germany received roughly 110.000 urine specimens. During that time we identified 26 urine samples that contained alpha-hemolytic streptococci with a recorded susceptible optochin disk test (i.e. ≥14 mm). Accordingly these strains were reported as *S. pneumoniae*. The samples originated from 18 different patients. Ten out of 26 patient samples contained additional species (*Escherichia coli*, *Proteus mirabilis*, *Providencia rettgeri, Klebsiella pneumoniae, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae,* enterococci, and enterobacteriaceae (not further identified)). Sixteen samples contained *S. pneumoniae* in pure culture. Bacterial counts varied between 10^2 (one sample) and 10^5 (19 samples) (for details see Table 1). Age at the time of sampling varied between three and 72 years. 13 patients were male, five patients were female. From the stored samples 18 strains from 15 different patients could be grown. For one patient (No 6) the isolate from 2011 could not be re-cultivated. However, an *S. pneumoniae* strain from 2009 was available and included into the study instead. Additionally we included the three strains from our previous findings from 2008 and 2009 into the study (Burckhardt and Zimmermann, 2011). Therefore the in-depth analysis of strains was done with 21 strains from 18 different patients.

Optochin disk test results (including mm) are shown in Table 2a. Agglutination with Slidex pneumo-kit was positive for 17 isolates. Agglutination with the Immulex omni-serum from the Statens Serum Institute was positive for 16 isolates. Identification with MALDI-TOF (first hits) resulted in *S. pneumoniae* with 14 isolates and *S. mitis* with seven isolates. Scores varied between 1.97 and 2.55. Identification with biochemical reactions using the GP-Card for VITEK2 resulted in *S. pneumoniae* with 14 isolates and in *S. mitis/S. oralis* with seven isolates. VITEK results, biochemical reactions (bio-number) and probability of ID are shown in Table 2a.

Probability of identification varied between 87% and 99%. Susceptibility testing for oxacillin was performed using disk diffusion. Inhibition zones and interpretation (EUCAST, 2015) are shown in Table 2b (for

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Table 2a
Characterization of isolates; optochin diameters >6 mm and <14 mm are marked in orange; non-*S. pneumoniae* isolates are in italics.

Isolate	Origin of strain	Optochin disk test		Slidex Pneumokit	Omni Serum	Maldi-TOF	score	Vitek 2	bio-number	probability of ID
see table 1		Columbia agar 5% sheep blood (mm)	MH-F agar (mm)							
2	this paper	15	14	pos	pos	S. pneumoniae	2,55	S. pneumoniae	061032364303030	95%
3	*this paper*	*6*	*6*	*pos*	*neg*	*S. mitis*	*2,21*	*S. mitis/ S. oralis*	*001410364305110*	*96%*
4	*this paper*	*6*	*6*	*neg*	*neg*	*S. mitis*	*2,31*	*S. mitis/ S. oralis*	*021010364305411*	*95%*
6	this paper	13	12	pos	pos	S. pneumoniae	2,44	S. pneumoniae	071133364303530	94%
7	*this paper*	*6*	*6*	*pos*	*neg*	*S. mitis*	*2,29*	*S. mitis/ S. oralis*	*001010364305011*	*99%*
8	*this paper*	*6*	*6*	*neg*	*pos*	*S. mitis*	*2,43*	*S. mitis/ S. oralis*	*001010364305011*	*99%*
9	*this paper*	*6*	*6*	*pos*	*pos*	*S. mitis*	*2,47*	*S. mitis/ S. oralis*	*001010364305010*	*99%*
10	*this paper*	*6*	*6*	*neg*	*neg*	*S. mitis*	*2,41*	*S. mitis/ S. oralis*	*001010364305010*	*99%*
13	*this paper*	*6*	*6*	*pos*	*neg*	*S. mitis*	*2,39*	*S. mitis/ S. oralis*	*001010344305011*	*99%*
15	this paper	13	17	pos	pos	S. pneumoniae	2,52	S. pneumoniae	061113364301131	95%
17	this paper	12	14	pos	pos	S. pneumoniae	2,44	S. pneumoniae	071113364305130	94%
18	this paper	14	11	pos	pos	S. pneumoniae	2,47	S. pneumoniae	071113364301130	96%
19	this paper	13	13	neg	pos	S. pneumoniae	2,23	S. pneumoniae	061113364301030	98%
22	this paper	15	15	pos	pos	S. pneumoniae	2,3	S. pneumoniae	141013364303510	91%
23	this paper	13	16	pos	pos	S. pneumoniae	2,08	S. pneumoniae	147032360307530	87%
24	this paper	13	14	pos	pos	S. pneumoniae	2,1	S. pneumoniae	145032360305530	94%
25	this paper	13	14	pos	pos	S. pneumoniae	1,97	S. pneumoniae	161112364305130	94%
26	this paper	14	14	pos	pos	S. pneumoniae	2,3	S. pneumoniae	171112364305470	88%
27	previous paper (5)	13	13	pos	pos	S. pneumoniae	2,38	S. pneumoniae	061133364301130	99%
28	previous paper	12	13	pos	pos	S. pneumoniae	2,46	S. pneumoniae	061132364303030	98%
29	previous paper	12	12	pos	pos	S. pneumoniae	2,41	S. pneumoniae	163133364307130	90%

optochin >6 mm isolates only). Two *S. pneumoniae* isolates were resistant according to disk diffusion (OXA-1).

Additionally all isolates (optochin >6 mm) were submitted to susceptibility testing using VITEK2 AST-P576. The two resistant isolates according to agar diffusion had a MIC of 0.5 mg/L for penicillin. One isolate, which was susceptible in the agar diffusion test showed a non-susceptible MIC (0.25 mg/L; isolate 22; Table 2b).

Serotyping was attempted using a PCR protocol. 13 isolates showed a positive result in the cpsA PCR.

These strains were genetically serotyped as serotype 3, 6A, 6C, 10A, 15B (2×), 19A (4× (same patient), 19F, and 34 (2×). Serotyping using the Neufeld-Quellungs reaction was done for 10 isolates and confirmed 9 serotypes determined by PCR. Isolate 18 determined as 15B with PCR was typed as 15C with the Quellungs reaction. Isolate 22, identified as *S. pneumoniae* by MALDI-TOF, VITEK and optochin disk test, was negative in the cpsA PCR, non-typable in the genetic serotyping assay and non-typable in the Quellungs reaction. SodA sequencing revealed *S. pseudopneumoniae*.

We were able to obtain a diagnosis for 16 of 18 patients using our laboratory information system.

Eight patients had a kidney transplant due to final renal failure. The remaining eight patients had one of the following diagnoses: renal failure, hydronephrosis, vesico-uretral reflux, interstitial nephritis, carcinoma of the prostatic gland, transversal spinal cord syndrome, gastro-intestinal bleeding or amino acid transport deficiency. For four patients blood cultures were sent in addition to the urine sample (patients 6, 11, 14, 16). Only the culture from patient 14 was positive with pneumococci. All other blood cultures remained negative throughout their routine incubation time of 5 days.

4. Discussion

Reports on *S. pneumoniae* as an agent for urinary tract infection (UTI) are scarce. In 1988 Nguyen and co-workers published a paper on pneumococcosuria in adults (Nguyen and Penn, 1988). From 1982–1985 their laboratory had processed 22.744 urine samples. Of these, 42 urine cultures grew *S. pneumoniae* (0.18%). The 42 urine samples originated from 38 different patients. 25 urine cultures grew pneumococci in pure culture. Bacterial counts varied between $<10^4$ (3 cases) to $>10^5$ (2 cases). For 31 patients records were available. All of these patients were men. 13 had underlying genitourinary disorders, 7 patients had urinary symptoms. In 1989 Miller and co-workers published a similar report on pneumococcosuria in children (Miller et al., 1989). From 1981 to 1985 their lab had processed 53.499 urine samples from children (<18 y). Forty-three samples were positive for *S. pneumoniae*. The positive samples originated from 40 different children. Nine samples grew pneumococci in pure culture. Bacterial counts varied between $\leq 10^3$ (10 cases) and $\geq 10^5$ (2 cases). Hospital charts were available for 28 patients. Twenty-seven of these 28 patients were female, 7 of 28 patients had genitourinary symptoms. Three children had genitourinary abnormalities.

In 2004 Dufke and colleagues published a case report on an 82-year-old male patient with pyelonephritis and urosepsis caused by *S. pneumoniae* (Dufke et al., 2004). His underlying disease was chronic lymphatic leukemia. There is no information on any malformations of the urinary tract.

In 2012 and 2013 two additional case series were published by Krishna and co-workers (Krishna et al., 2012) and Choi and co-workers, respectively (Choi et al., 2013). Again all patients had a pre-existing disease or malformation of the urinary tract.

In our case series all samples had been sent because of clinical signs of urinary tract infection. From a microbiological view at least the cases with a pure culture of 10 5 *S. pneumoniae* undoubtedly fulfill the criteria for a UTI. Looking into the respective diagnoses revealed that of the 16 patients for whom we could find a diagnosis eight had a kidney transplant and another four had chronic problems with their kidneys. This confirms our first impression that there is a strong association of chronic

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Table 2b
Susceptibility testing and serotypes of isolates; *One patient; NT = not typable; # sodA sequencing revealed: *S. pseudopneumoniae*; nd = not done (isolates died during transport); non-susceptible *S. pneumoniae* isolates are marked in orange.

isolate	origin of strain	oxacillin (disk diffusion)	interpretation	MIC (mg/L) penicillin	cps-PCR	serotype	serotype
see table 1		MH-F agar	EUCAST (2015)	AST-P576		PCR	Quellungs reaction
2	this paper	20	S	<=0,06	pos	34	34
3	this paper	20	nd	nd	neg	/	no S. pneu.
4	this paper	11	nd	nd	neg	/	no S. pneu.
6	this paper	23	S	<=0,06	pos	6A	6A
7	this paper	12	nd	nd	neg	/	no S. pneu.
8	this paper	16	nd	nd	neg	/	no S. pneu.
9	this paper	11	nd	nd	neg	/	no S. pneu.
10	this paper	16	nd	nd	neg	/	no S. pneu.
13	this paper	14	nd	nd	neg	/	no S. pneu.
15	this paper	31	S	<=0,06	pos	6C	6C
17 *	this paper	32	S	<=0,06	pos	19A	19A
18	this paper	6	R	0,5	pos	15B	15C
19	this paper	26	S	<=0,06	pos	3	3
22	this paper	20	S	0,25	neg	NT	NT#
23	this paper	6	R	0,5	pos	10A	nd
24 *	this paper	27	S	<=0,06	pos	19A	19A
25 *	this paper	27	S	<=0,06	pos	19A	nd
26 *	this paper	27	S	<=0,06	pos	19A	nd
27	previous paper	35	S	<=0,06	pos	15B	15B
28	previous paper	36	S	<=0,06	pos	34	34
29	previous paper	28	S	<=0,06	pos	19F	19F

kidney problems and the detection of *S. pneumoniae* in the respective patient samples. Only for four patients additional blood cultures were sent. From this we deduce that systemic disease is a rather rare event. However, we are well aware that our case number is low and for in depth analysis one would have to review patient charts. Unfortunately they are already closed for privacy protection and cannot be assessed for further investigations.

Identification of *S. pneumoniae* in urine samples is a rare event. Additionally the strains have some peculiar characteristics, which make identification even harder. First, colonies tend to be rather small. They do not show the characteristic morphology of the central depression or checker piece appearance that is common to invasive isolates (data not shown). Second, the optochin disk diffusion diameters tend to be rather small, too. In our lab we perform the optochin disk test on Mueller-Hinton-fastidious agar and not on TSA 5% sheep blood as the manufacturer recommends it. This is for two reasons. A) TSA 5% sheep blood agar is not used and in the last 20 years was not used in Germany. B) In the laboratory routine it is very convenient to perform the test for optochin susceptibility and beta-lactam susceptibility (oxacillin 1 mg, EUCAST) on one day on one plate in one step. Out of interest we performed the optochin disk test using the other commonly used blood-containing agar, i.e. Columbia 5% sheep blood agar. The sizes of the inhibition zones tended to be even smaller.

Initially isolates 3, 4, 7, 8, 9, 10, and 13 apparently showed an inhibition zone of ≥14 mm in the disc diffusion test. Why this could not be repeated with the frozen isolate we cannot explain. Most probably there was a problem during freezing and recultivation of samples. Indeed there is a report that strains can lose their optochin susceptibility during freezing (Robson et al., 2007). However, this does not explain results from MALDI-TOF (*S. mitis*) or VITEK2 (*S. mitis/S. oralis*)

The phenomenon of *S. pneumoniae* in some urine samples apparently is not caused by a single strain or serotype. On the contrary, the diversity in terms of serotypes is remarkable.

Finally, from a laboratory perspective one has to admit that identification of these *S. pneumoniae* strains is rather difficult. Morphology of colonies is not typical, optochin disk test diameters tend to be rather small and agglutination has its problems (at least in our hands). Neither the Slidex Pneumokit nor the Omni Serum from the Statens Serum Institute was able to reliably distinguish between *S. mitis* and *S. pneumoniae*. MALDI-TOF or biochemical identification using VITEK2 proved to be accurate with the exception of isolate 22 where a sodA sequencing was needed for final identification. Of course cpsA PCR or serotyping is even more accurate. However, time needed and cost for the necessary PCRs is much too high to recommend it as routine diagnostic procedure.

What we learn from this study is that initial optochin disk diffusion is good initial test for identifying *S. pneumoniae* from urine; however, we will introduce a compulsory further identification step into our work-flow, either using MALDI-TOF or VITEK2.

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