Molecular biological characterization of non-typeable (NT) *Streptococcus pneumoniae* isolates

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OBJECTIVES

The GNRCS has been carrying out surveillance of invasive pneumococcal disease since 1992. Of a total of 37,000 pneumococcal isolates, 353 were classified as non-typeable. NT-isolates are not covered by any of the current vaccines and may have the intrinsic capacity of being replacement serotypes. Additionally, they form the pool in which 'new serotypes' might be detected. Therefore, in this study we (re-) analyzed these isolates using modern molecular techniques.. .

METHODS

Identification of isolates was re-assessed using our current expertise with the Quellung method, PCRs of 16S rDNA and of the *lytA*-gene, both followed by restriction analyses, and sequencing of the *sodA*-gene. MLST, multiplex-PCR, microarray and genome sequencing were used for identification and characterization of the NT-isolates.

RESULTS

A total of 353 NT-isolates were analysed. Of these, 130 isolates (36.8%) were typeable using the Quellung method, 97 (27.5%) were identified as other viridans streptococci and 126 isolates (35.7%) were genuine non-typeable *S. pneumoniae* (**Fig. 1**).

Most of the typeable isolates were identified with with rare serotypes (27, 29, 31, 34, 37, 38; **Fig.2**) These isolates were all bile soluble, reacted positively with omni-serum and were pneumococcus specific in the LytA PCR-restriction. Al serotype 38 isolates were cpsA negative (as described in the literature).

Non-pneumococcal isolates were identified as *S.pseudopneumoniae, S.mitis*, *S.oralis* and *S.parasanguinis* (**Fig. 3**). Several of these isolates were cpsA positive, optochin susceptible or showed a pneumococcaus specific 16S PCR-restriction (**Table 1**).

Among the genuine non-typeable S. pneumoniae isolates, 13 were optochin resistant, 67 were cpsA negative, 3 showed non-pneumo 16S n and 7 non-pneumo lytA. All were omniserum negative and bile soluble (**Table 2**).

Microarray analysis showed that 47 NTs carry serotype genes (**Table 3**), and these isolates have MLSTs which are normally reported with serotypes (**Table 4**). The other NTs have MLSTs which are reported for well known uncapsulated clones (**Table 5**).

Three of the NT-isolates were positive with multiplex PCR serotyping (**Table 6**). Genomic sequencing of these isolates showed point-mutations in capsular genes (**Table 7**).

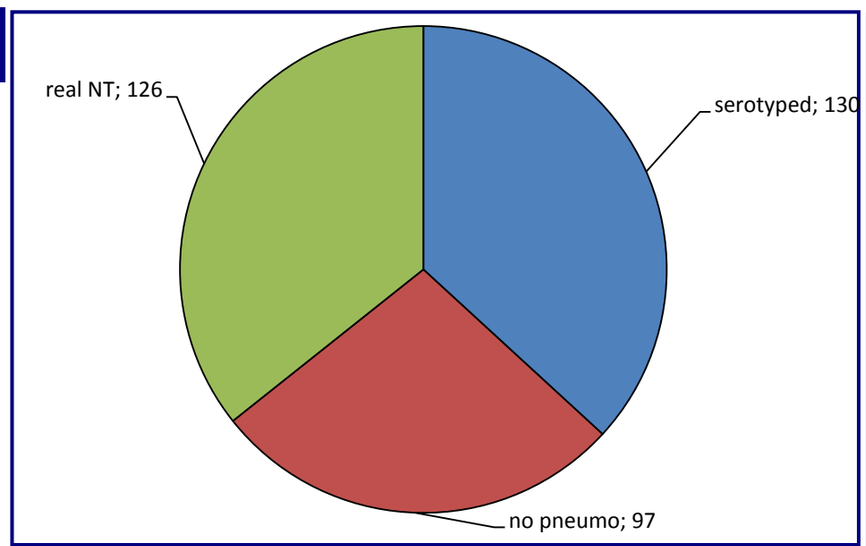
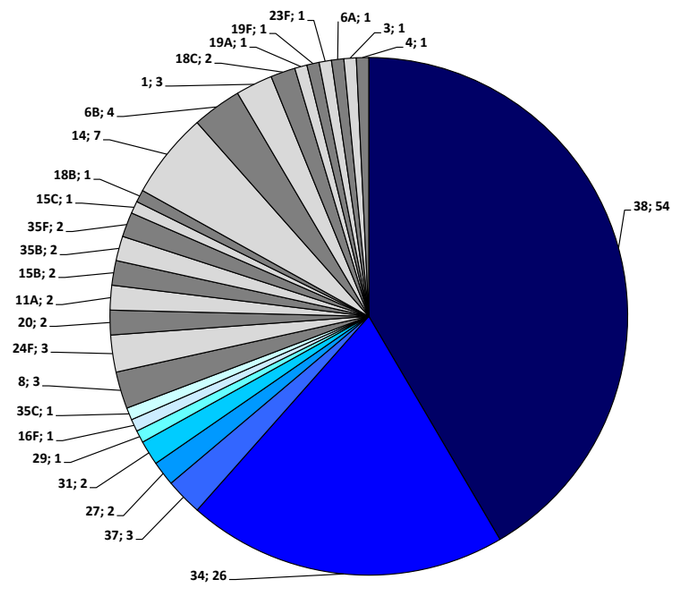


Fig. 1: Results from re-analysis of isolates depicted as non-typeable *S. pneumoniae* in the isolate collection of the GNRCS.



	S	%	R	%
Optochin	123	94,6	7	5,4
	positive	**%**	**negative**	**%**
Omniserum	130	100,0	0	0,0
Bile	130	100,0	0	0,0
LytA PCR-restriction	130	100,0	0	0,0
cpsA PCR	76	58,5	54	41,5

Fig. 2: Analysis results for 130 serotypeable isolates, previously identified as non-typeable. Blue: rare serotypes, grey: common serotypes in Germany.

Table 4: MLST data of NT isolates with serotype microarray data

MLST	aroE	gdh	gki	recP	Spi	xpt	ddl	Array Serotype	Quellung Serotype
15	1	5	4	5	5	3	8	14	NT
42	1	8	9	9	6	4	6	23A	NT
53	2	5	1	11	16	3	14	8	NT
62	2	5	29	12	16	3	14	11A/D/E[11D]	NT
62	2	5	29	12	16	3	14	11A/D/E[11D](80%) + 19A(20%)	NT
66	2	8	2	4	6	1	1	8	NT
66	2	8	2	4	6	1	1	9N/L[9N]	NT
81	4	4	2	4	4	1	1	23F	NT
102	5	13	11	4	15	12	19	18B/C[18C]	NT
143	7	5	10	18	6	8	1	14	NT
156	7	11	10	1	6	8	1	14	NT
156	7	11	10	1	6	8	1	9A/V[9V]	NT
176	7	13	8	6	10	6	14	6A/B[6B]	NT
191	8	9	2	1	6	1	17	7A/F[7F]	NT
199	8	13	14	4	17	4	14	15B/C[15B]	NT
199	8	13	14	4	17	4	14	19A	NT
205	10	5	4	5	13	10	18	4	NT
230	12	19	2	17	6	22	14	24B/F[24F]	NT
383	1	10	62	10	15	1	6	16F	NT
416	1	13	14	4	17	51	14	19A	NT
423	1	5	4	12	5	3	8	19F-like	NT
433	1	1	4	1	18	58	17	22F	NT
447	29	33	19	1	36	22	31	37	NT
1171	1	14	40	12	1	1	14	19F	NT
1381	10	11	4	16	15	1	145	18B/C[18C]	NT
1480	7	9	15	11	93	1	70	8	NT
1551	5	7	4	2	10	1	6	10A	NT
2100	2	5	36	12	8	21	14	19F	NT
2705	5	12	29	16	9	39	1	33A/F[33A]	NT
8437	2	89	9	38	6	1	18	14	NT
8653	7	13	1	1	10	6	1	6C/D[6C]	NT
8947	10	17	4	38	6	3	6	35A/35C/42[35A]	NT
9199	7	13	1	6	10	6	525	6A/B[6B]	NT
9730	5	5	4	10	9	562	615	14-like	NT
11480	7	25	8	4	15	14	18	6A/B[6B]	NT
new1	5	94%184	96%392	10	9	94%63	93%356	10C/21-like*(87%) + 14-like(13%)	NT
new2	2	99%5	99%9	1	17	21	14	14-like	NT
new3	7	98%371	264	47	17	3	9	15B/C[15C]	NT
new4	8	5	166	29	9	562	615	14-like	NT

Table 6: Multiplex PCR analysis of the serotype genes of three truly non-typeable *S. pneumoniae* isolates.

NT-Nr	MLST	Serotype in mlst.net	Serotypes tested with multiplex PCR	Serotype/serogroup found with multiplex PCR
61	2100	19F	19F	19F
116	DLV 176	6A, 6B, 6C, 19A	6A, 6B, 6C, 19A	6
178	15	14, 19F, 19A, 22F	14, 19F, 19A, 22F	14

Table 7: Genomic-sequencing of three truly non-typeable *S. pneumoniae* isolates.

NT-Nr	MLST	Serotype found on Bµg@s array	Genome Sequence
61	2100	19F	Divergence *wchA*, premature stop *wze*
116	DLV 176	6B	Mutation promoter *wzg*
178	15	14	deletion in *wciY*, rearrangements in *whaH*, frameshift mutation *wze*

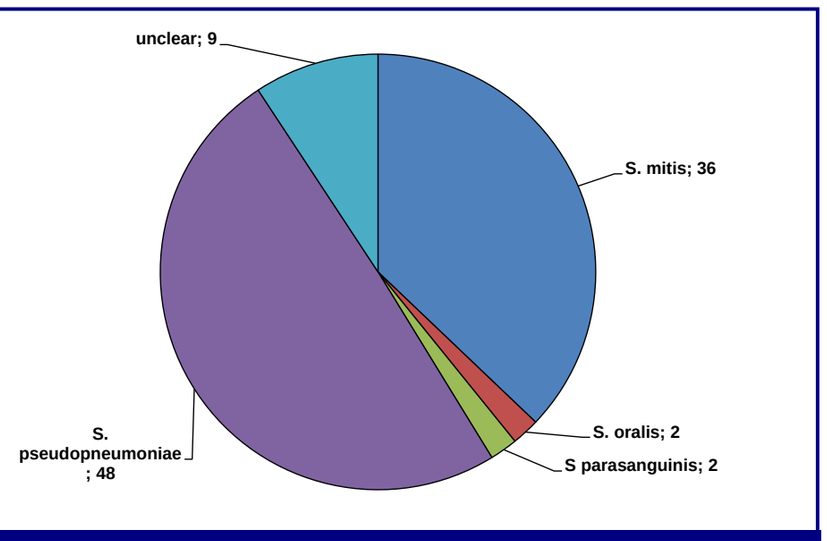


Fig. 3: Strain characterization of 97 non-pneumococcal isolates previously identified as non-typeable *S. pneumoniae*.

Table1: Characterization of 97 non- pneumococcal isolates

Isolate	n	cpsA	16S PCR restr.	lytA	omni	Optochin	Bile	Path ID
		positive	negative	negative	negative	R	positive	
S. mitis	36	10	36	36	36	21	0	SP-3/5
S. oralis	2	1	2	2	2	2	0	SP-3/5
S parasanguinis	2	0	1	1	2	2	0	SP-1/5, SP-3/5
S. pseudopneumoniae	48	10	44	48	48	29	12	SP-4/5

Table2: Characterization of 126 genuine NT pneumococcal isolates

	S	%	R	%
Optochin	113	89,7	13	10,3
	positive	**%**	**negative**	**%**
Omniserum	0	0,0	126	100,0
Bile	126	100,0	0	0,0
LytA PCR-restriction	119	94,4	7	5,6
cpsA PCR	59	46,8	67	53,2
16S PCR-restriction	123	97,6	3	2,4

Table3: Microarray data of 126 real NT pneumococcal isolates

n=	ArraySero	PathID		n=	ArraySero	PathID
2	4	SP-5/5		5	NT1	SP-5/5
3	8	SP-5/5		2	NT2	SP-5/5
6	14	SP-5/5		1	NT2(53%) +	SP-5/5
1	37	SP-5/5		1	NT2(54%) +	SP-5/5
1	10A	SP-5/5		1	NT3a	SP-5/5
1	10C/21-like*(87%) + 14-like(13%)	SP-5/5		45	NT3b	SP-5/5
2	11A/D/E[11D]	SP-5/5		1	NT3b(51%)	SP-5/5
1	11A/D/E[11D](80%) + 19A(20%)	SP-5/5		1	NT3b(53%)	SP-5/5
3	14-like	SP-5/5		4	NT3b(55%)	SP-5/5
1	15B/C[15B]	SP-5/5		1	NT3b(56%)	SP-5/5
2	15B/C[15C]	SP-5/5		1	NT3b(58%)	SP-5/5
1	16F	SP-5/5		1	NT3b(58%)	SP-5/5
3	18B/C[18C]	SP-5/5		4	NT3b(60%)	SP-5/5
2	19A	SP-5/5		1	NT3b-like	SP-5/5
2	19F	SP-5/5		4	NT4a	SP-5/5
1	19F-like	SP-5/5		1	NT4b	SP-5/5
2	22F	SP-5/5		1	NT4b(50%)	SP-5/5
1	23A	SP-5/5		1	NT4b(52%)	SP-5/5
1	23F	SP-5/5		1	NT4b(56%)	SP-5/5
1	24B/F[24F]	SP-5/5		1	NT4b(59%)	SP-5/5
1	33A/F[33A]	SP-5/5				
1	35A/35C/42[35A]	SP-5/5				
4	6A/B[6B]	SP-5/5				
1	6C/D[6C]	SP-5/5				
1	7A/F[7F]	SP-5/5				
1	9A/V[9V]	SP-5/5				
1	9N/L[9N]	SP-5/5				

Table 5: MLST data of NT isolates with known NT MLSTs

MLST	aroE	gdh	gki	recP	Spi	xpt	ddl	Array Serotype	Quellung Serotype
8406	2	5	2	1	6	1	17	NT1	NT
new	2	5	15	12	99	99%21	96%447	NT3b	NT
9727	2	5	36	12	17	47	14	NT2(54%) + NT3b(46%)	NT
8965	2	5	327	4	17	21	565	NT2	NT
SLV3036	2	5	97%51	12	181	21	31	NT3b	NT
DLV1153	2	13	2	29	98	19	71	NT3b	NT
9732	2	33	36	261	17	21	41	NT4a	NT
4144	2	37	2	65	2	19	14	NT2	NT
8443	2	98	9	65	2	47	14	NT3b	NT
105	5	15	4	1	6	1	6	NT3b	NT
new	7	11	10	1	6	1	18	NT1	NT
9731	7	13	8	6	6	267	8	NT1	NT
448	8	5	2	27	2	11	71	NT3b(55%) + NT2(45%)	NT
448	8	5	2	27	2	11	71	NT3b(56%) + NT2(44%)	NT
448	8	5	2	27	2	11	71	NT3b(58%) + NT2(42%)	NT
448	8	5	2	27	2	11	71	NT3b(60%) + NT2(40%)	NT
448	8	5	2	27	2	11	71	NT3b(60%) + NT2(40%)	NT
8940	8	5	244	206	6	277	59	NT3b(53%) + NT2(47%)	NT
7599	8	8	22	1	13	1	484	NT3b	NT
942	8	10	15	27	2	28	4	NT4b(50%) + NT2(50%)	NT
942	8	10	15	27	2	28	4	NT4b(56%) + NT2(44%)	NT
942	8	10	15	27	2	28	4	NT4b(59%) + NT2(41%)	NT
941	8	10	15	27	2	28	71	NT3b(55%) + NT2(45%)	NT
941	8	10	15	27	2	28	71	NT4b(52%) + NT2(48%)	NT
7604	8	10	246	70	10	407	5	NT3b	NT
SLV6557	8	10	246	70	2	99%407	5	NT3b	NT
9401	8	12	9	29	2	12	53	NT3b	NT
11481	8	13	34	29	9	1	14	NT3b	NT
new	8	14	4	16	99	4	18	NT1	NT
3137	8	37	9	29	2	12	5	NT3b	NT
1619	8	37	9	29	2	12	14	NT3b	NT
344	8	37	9	29	2	12	53	NT3b	NT
SLV344	8	37	9	29	2	12	98%5	NT3b	NT
SLV344	8	37	9	29	2	12	99%5	NT3b	NT
SLV344	8	37	9	29	2	12	97%38	NT3b	NT
SLV344	8	37	9	29	2	12	99%38	NT3b	NT
SLV334	8	37	9	29	2	12	97%53	NT3b	NT
SLV344	8	37	9	29	2	12	98%53	NT3b	NT
SLV344	8	37	9	29	2	12	99%53	NT3b	NT
SLV344	8	37	9	29	2	12	98%257	NT3b	NT
SLV344	8	37	9	29	2	12	98%257	NT3b(51%) + NT2(49%)	NT
SLV344	8	37	9	29	2	12	97%257	NT3b	NT
SLV344	8	37	9	29	2	12	99%282	NT3b	NT
DLV344	8	37	9	29	96	12	98%220	NT4a	NT
DLV344	8	5	9	29	2	12	99%257	NT3b	NT
449	8	37	9	29	2	47	5	NT3b	NT
1054	8	37	9	29	2	47	59	NT3b	NT
SLV1054	8	37	9	29	2	47	99%59	NT3b	NT
SLV3097	8	178	9	29	2	12	98%38	NT3b	NT
new	8	505	508	70	505	678	59	NT3b(60%) + NT2(40%)	NT
new	8	505	508	327	491	103	59	NT3b	NT
new	8	505	508	327	491	103	59	NT4b	NT
393	10	43	41	18	13	49	6	NT2(53%) + 38(47%)	NT
9728	12	43	413	1	181	49	31	NT3a	NT
9728	12	43	413	1	181	49	31	NT3b	NT
508	13	8	65	1	60	16	6	NT3b(60%)+NT2(40%)	NT
new	51	8	4	10	10	170	14	NT1	NT
new	99%	8	4	12	11	99%49	435	NT3b-like	NT

CONCLUSIONS

- About one third of the isolates previously depicted as non-typeable were serotypeable, another one third were not *S. pneumoniae.*
- Bile-solubility, Optochin-susceptibility and presence of the cpsA-gene are not good markers for *S. pneumoniae*.
- Combination of *lytA*-PCR + restriction, 16S-PCR + restriction and *sodA* sequencing works to differentiate *S. pneumoniae*, *S. mitis* and *S. pseudopneumoniae*.
- Real non-typeable isolates were either of the known NT-MLSTs (344, 448, 941, 942, 3137), or had MLSTs found among serotypeable isolates.
- Three NTs were found to have point mutation changes in their capsular genes. One of these isolates was from invasive disease.

IMPLICATIONS

- PCR serotyping will mistype NTs with point-mutations as their corresponding serotypes.
- The label 'NT' should mean: Using Quellung with the full set of antisera a serotype could not be determined.