JOURNAL OF BACTERIOLOGY, Aug. 2007, p. 6085–6088 Vol. 189, No. 16
0021-9193/07/$08.00+0 doi:10.1128/JB.00468-07

New *Streptococcus pneumoniae* Clones in Deceased Wild Chimpanzees▽

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Received 21 March 2007/Accepted 11 June 2007

In wild chimpanzees in the Taï National Park, Côte d'Ivoire, sudden deaths which were preceded by respiratory problems had been observed since 1999. Two new clones of *Streptococcus pneumoniae* were identified in deceased apes on the basis of multilocus sequence typing analysis and *ply*, *lytA*, and *pbp2x* sequences. The findings suggest that virulent *S. pneumoniae* occurs in populations of wild chimpanzees with the potential to cause infections similar to those observed in humans.

Bacterial human pathogens are important not only for therapeutic and socioeconomic reasons but also in respect to the evolution of infectious diseases. *Streptococcus pneumoniae* is a major human pathogen causing a variety of diseases, including meningitis, sepsis, sinusitis, otitis media, and pneumonia. Pneumococci can colonize the nasopharynx and cause respiratory disease in several animal species, including rodents, racing horses (25), equine species (1), rhesus monkeys (5, 6), and chimpanzees (22). However, these cases occurred in animals that were held in human captivity, and it had been suggested that the animals had acquired the organisms from human contacts rather than being their natural hosts.

Wild chimpanzees in the Taï National Park, Côte d'Ivoire, have been closely monitored since the early 1980s (2). The three ape communities investigated here (North, South, and East) inhabit specific territories that overlap slightly with neighboring territories. The human observers permanently follow their behavior and health, but direct contact with the apes is not allowed and strict hygienic measures were progressively implemented when it became evident that diseases were a mortality factor (4). Such measures included, e.g., maintaining a distance of at least 7 m during daily follows and recently the wearing of a chirurgical mask when the animals are in sight (18) (F. Leendertz et al., submitted).

Since 1999, clusters of sudden deaths have been observed in three ape communities (North, East, and South communities), affecting animals that had been in good health. Necropsies were performed on 14 chimpanzees that died between 1999 and 2006. Samples were taken from lung tissue and all other organs and preserved in liquid nitrogen. In order to identify the organisms responsible for the animal infection, PCR analyses were performed on lung tissue samples from deceased individuals of these ape communities. In addition to virus diagnostics, PCR-based screens for bacteria were performed. In several cases, a new *Bacillus anthracis*-like species was detected and identified as the likely cause of the sudden deaths (14, 16). In the samples taken from the eight chimpanzees that showed symptoms and pathology of respiratory disease, subsequent experiments revealed rRNA genes from *S. pneumoniae*, and in none was *B. anthracis* detected.

In order to verify the presence of *S. pneumoniae* in the samples (Table 1), primers specific for pneumococcal virulence genes were tested in PCRs. Three *S. pneumoniae*-specific genes were investigated by DNA sequence comparison of PCR products covering internal gene fragments: *ply*, encoding the cytolysin pneumolysin (8); *lytA*, encoding the autolysin highly conserved in this species (12); and the *pbp2x* gene, encoding the penicillin target protein PBP2x, involved in beta-lactam resistance (7).

DNA was isolated from ape lung tissue using the DNeasy tissue kit or a viral RNA kit (QIAGEN, Hilden, Germany). From human pharyngeal swabs, DNA was isolated using the QIAmp DNA blood minikit. 16S rRNA sequences were am-

TABLE 1. Demographic data on chimpanzee samples

Chimpanzee name	Date of finding dead chimpanzee	Age (yr/mo)	Community	ST[b]
Loukoum	05/10/1999	27[a]	North	2308
Lefkas	05/14/1999	7/7	North	2308
Candy	02/16/2006	Adult[a]	East	2308
Vasco	02/09/2006	Adult[a]	East	2308
Orest	03/10/2004	5/10	South	2309
Virunga	03/19/2004	39[a]	South	2309
Ophelia	03/10/2004	1/4	South	2309
Ishas Baby	02/09/2006	0/2	South	2309

[a] Age estimated or unknown.
[b] *S. pneumoniae* ST.

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▽ Published ahead of print on 22 June 2007.

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                1111111111111111111111111111
                4444444445555555555555555777
                7778889990001112223334445554 44
                1231231231231231231231231231 23
R6              CACGTTGACCCTTATCCATATCTTGCTATT
N/E             --T--G--T--A--C--T--CT-G--AC--
R6 (aa)         H  V  D  P  Y  P  Y  L  A  I
N/E(aa)         -  -  -  -  -  -  -  -  -  L
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FIG. 1. Sequence of the *lytA* gene obtained from the chimpanzee *S. pneumoniae* North/East clone. The *S. pneumoniae* R6 *lytA* sequence is used for comparison; N/E refers to the *S. pneumoniae* North/East clone. The vertical numbers indicate the codon, with the fourth digit representing the position within the codon. The amino acid sequence is indicated below (aa). Only nucleotides and amino acids that differ from those of the R6 sequence are shown.

plified using bacterium-specific primers. The following oligonucleotides were used for PCR analysis of *S. pneumoniae* genes (3′→5′): *ply*, GAGGGTAATCAGCTACCC and GACCAAA GGACGCTCTGC; *lytA*, GCACATTGTTGGGAACGG and CCAGCCTGTCTTCATGGC; and *pbp2x*, GAAAGAAATTG CCAATTCACACG and GGATAAAGGCCGGAATTCC. PCR was performed under standard conditions with 30 cycles of 96°C for 30 s, 50°C for 30 s, and 72°C for 1 min (for *pbp2x*, 2 min), followed by a 10-min period at 72°C.

PCR fragments were obtained in all cases, and their DNA sequences were highly related to those of the laboratory strain *S. pneumoniae* R6, used as a representative of antibiotic-sensitive human isolates (9), confirming the presence of *S. pneumoniae* in the animal samples. PCR products were sequenced using the corresponding PCR primers and in the case of *pbp2x* a series of primers designed according to the published sequence (9). The PBP2x genes were identical in all cases but differed from *pbp2x* of the susceptible *S. pneumoniae* R6 strain by seven base pair changes that were scattered throughout the gene, resulting in one amino acid change, Leu565Val. No mosaic block common to *pbp2x* from penicillin-resistant strains was found, indicating that the sequences obtained from the chimpanzee samples are derived from penicillin-susceptible *S. pneumoniae* strains (7, 15). The *ply* sequences were identical in all samples except for 1 nucleotide change in the North group and differed from the R6 sequences by 6 bp (South) or 7 bp (North group). The four *lytA* sequences obtained from the North and East samples were also identical but were distinct from those obtained from the South community samples: *lytA* contained an unusual highly altered region between codons 147 and 155, with 10 base pair changes that did not result in amino acid changes (Fig. 1), while bearing all typical pneumococcal signatures (20, 24). This mosaic block has not been observed in *S. pneumoniae lytA* previously. In addition, a single base pair change resulted in an amino acid mutation, Ile174 to Leu. The *lytA* sequence obtained from the South group was identical to that of the R6 gene. The amino acid mutation found in LytA of the South clone was located between the catalytic domain and the choline binding repeat domain of LytA at a position that presumably does not affect the function of the protein (3).

The autolysin LytA is required for release of the pneumolysin Ply, a cytolysin which results in severe damage of the host cells (12). Thus, the presence of *ply* and *lytA*, both of which represent pathogenicity factors, in all samples strongly suggests that the chimpanzees contained *S. pneumoniae* strains with the potential to be virulent.

In order to determine the clonal relatedness of the ape pneumococci, multilocus sequence typing (MLST) analyses were performed and PCR fragments of all seven housekeeping genes were obtained from eight samples. Standard primers (http://spneumoniae.mlst.net/misc/info.asp) were used, except for *gdh* (CCCTCCGTGACATGGTCC and GTCATGAAGT GGGGCACC), *gki* (CTTGGATTGGCAGCAGCG and GAT GTGCGTACTTGTGGG), and *spi* (ACGCTTAGAAAGGT AAGTTATG and GGTTTCTTAAAATGTTCCGATAC). Again, the sequences from samples of the North and East communities were identical (Table 2). A new *spi* allele was identified, and a new sequence type (ST), 2308, was assigned to this virtual *S. pneumoniae* clone. The MLST data obtained from the ape samples of the South community were distinct, including a new *gdh* allele, resulting in the new ST 2309. Phylogenetic analysis was performed with the concatenated sequences of the MLST genes used for *S. pneumoniae* (www.mlst.net) with the SplitsTree4 program (10). A neighbor-joining tree was constructed using uncorrected parameters, and the tree was tested by bootstrap analysis using 1,000 replicates. A dendrogram of the genetic relationships between the concatenated gene sequences of the MLST genes from the chim-

TABLE 2. Allelic profile of seven housekeeping genes and ST of new *S. pneumoniae* clones from chimpanzees and closest human isolates

S. pneumoniae isolate	Allele no. for gene[a]:							ST
	aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	
"North/East clone"	8	134	4	4	132*	142	74	2308
Closest human isolates[b]								
2061 (19A/Spain)	8	13	4	4	6	4	14	2109
6A-12 (6A/United States)	8	13	4	4	17	4	14	2154
3852 (4/Poland)	8	70	4	4	6	116	6	2190
"South clone"	8	138*	74	10	6	4	161	2309
Closest human isolates								
M16 (23A/United Kingdom)	8	8	9	9	6	4	6	190
263/99 (23F/Norway)	2	10	4	10	6	4	65	322
2828-02 (13/Kenya)	1	11	74	10	6	14	18	701

[a] *, New allele.
[b] Closest human isolates identified by comparison of the allelic profiles listed at http://spneumoniae.mlst.net/. The strain number is indicated in addition to the serotype and the country of isolation.

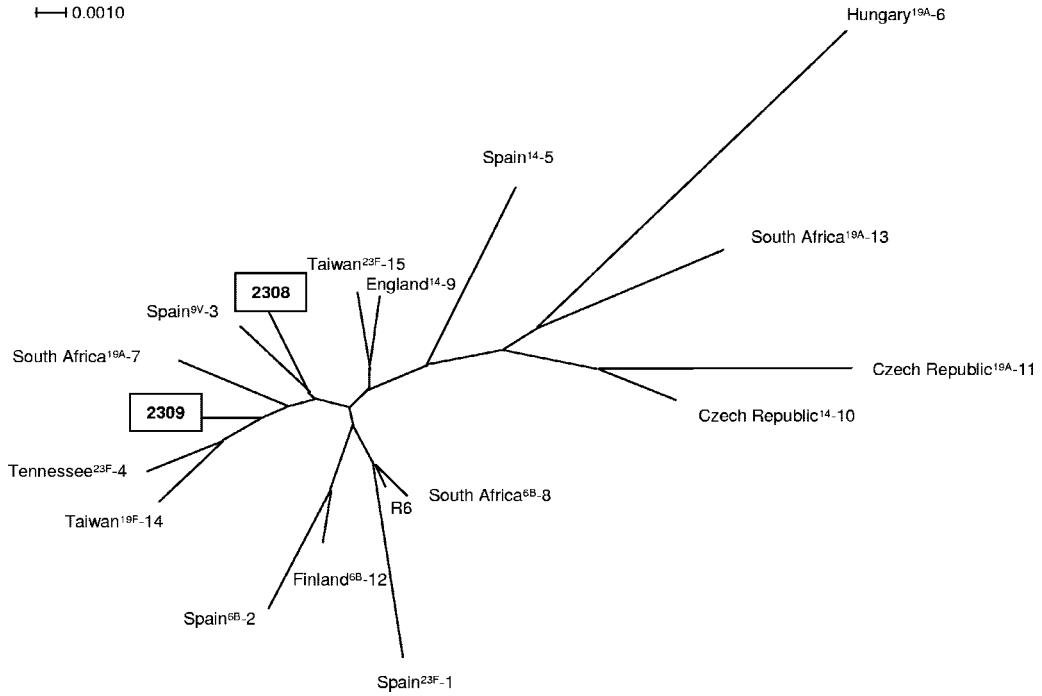

FIG. 2. Genetic relatedness of the chimpanzee *S. pneumoniae* clones to major clones of *S. pneumoniae* as described recently (21). Clones are identified by the country of isolation, followed by the serotype and the clone identification number. A SplitsTree representation is shown based on concatenated sequences of the seven housekeeping genes used for MLST analysis (10). The STs 2308 and 2309 were assigned to the chimpanzee *S. pneumoniae* clones.

panzee samples and a selection of major clones of human *S. pneumoniae* isolates representing major recognized clones worldwide (21) is shown in Fig. 2.

In order to investigate whether the putative *S. pneumoniae* clones also occur in humans working in proximity to the chimpanzees, a total of 39 samples from 28 African and European workers of the Taï chimpanzee project taken at different time points between 2004 and 2006 were screened for *S. pneumoniae*. All samples were preserved in liquid nitrogen. Samples from 21 workers were positive, but none contained the new *gdh* or *spi* allele identified in the chimpanzees. A search in the MLST database (http://spneumoniae.mlst.net) showed that the most closely related human isolates, all of which were isolated in Europe, differed by three alleles from the North/East clone and by at least five alleles from the South clone (Table 2). These data strongly suggest that the pneumococci identified in the chimpanzees were not transferred from humans to the animals. Although we cannot rule out that transfer occurred prior to the time when the humans were tested, it seems unlikely given the fact that close contact between humans and the wild animals is carefully being avoided.

The areas inhabited by the ape communities are adjacent to each other. Many contacts between the chimpanzees of neighboring groups have been observed, but not between the North and East groups, since they have no adjacent frontiers. In other words, the *S. pneumoniae* clone identified in the South community was distinct from that identified in the East community although contact between these groups occurred, whereas the same *S. pneumoniae* clone was associated with the North and East groups, where contacts have not been observed. This suggests that *S. pneumoniae* might also be associated with other animals in these areas. Potential candidates for this scenario are monkeys that are part of the ape diet, or perhaps small rodents, and further investigations are required to understand the occurrence of *S. pneumoniae* in animals from wild habitats.

The cause of death of the animals is likely to be multifactorial, although *S. pneumoniae* infections could play a role in the severity of the disease. The pathological and histopathologic changes were consistent with the picture of a severe purulent multifocal bronchopneumonia, lung edema, and upper respiratory tract infection. In most samples, DNA from other pathogens could also be amplified, including rRNA from *Pasteurella* spp., human metapneumovirus, and respiratory syncytial virus; details will be described elsewhere (Leendertz et al., submitted). Recent findings at a primate rehabilitation unit demonstrated that viral upper respiratory tract infections can predispose chimpanzees to invasive infections caused by *S. pneumoniae* (13). It would be important to isolate the infectious *S. pneumoniae* from the wild chimpanzees in order to elucidate further properties of these strains, such as the capsular type, and preferably genomic data should be generated.

In this study we have shown for the first time that the human pathogen *S. pneumoniae* is also associated with disease in wild apes. The focus of most previous studies on captive or wild-living nonhuman primates was on the transmission of retroviruses, such as simian immunodeficiency virus, simian T-cell leukemia virus, and foamy virus or highly acute diseases such as Ebola (11, 17, 19, 23, 26, 27), documenting that pathogens found in primates can easily spread to humans with fatal con-

sequences. Our data show that other microbial agents pathogenic for humans can be found in great apes, emphasizing the importance of monitoring of mortality rates of wild primates combined with broad pathogen-screening programs. Understanding the routes of transfer between the chimpanzees and the existence of other potential natural hosts for this human pathogen are major challenges for future research.

We thank the Ivorian authorities for long-term support, especially the Ministry of the Environment and Forests, as well as the Ministry of Research, the directorship of the Taï National Park, and the Swiss Research Center in Abidjan. We also thank the assistants of the Taï Chimpanzee Project for their help during observation of the chimpanzees. We thank Pierre Formenty for performing the necropsy of two chimpanzees of the North group considered in this paper. The skillful technical support of N. Eckhardt for necropsies and of Sonja Schröck of the Nano+Bio Center at Kaiserslautern and Ute Buwitt and Julia Tesch at the Robert Koch Institute for DNA sequencing is gratefully acknowledged.

This work was supported by the BMBF (PTJ-BIO/0313134), the EU (LSHM-CT-2003-503413), the Stiftung Rheinland Pfalz für Innovation (0580), and the Max Planck Society.

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