New *Streptococcus pneumoniae* Clones in Deceased Wild Chimpanzees

Fang Chi,1§ Michaela Leider,2,3,4§ Fabian Leendertz,2,4* Carina Bergmann,1† Christophe Boesch,4 Svenja Schenk,2,3,5 Georg Pauli,2 Heinz Ellerbrok,2 and Regine Hakenbeck1*

Department of Microbiology, University of Kaiserslautern, Paul Ehrlich Str. 23, D-67663 Kaiserslautern, Germany; Zentrum für Biologische Sicherheit, Robert Koch Institute, Nordufer 20, D-13353 Berlin, Germany; Charité-Universitätsmedizin Berlin, Hindenburgdamm 30, 12203 Berlin, Germany; Max Planck Institute for Evolutionary Anthropology, Deutscher Platz 6, D-04103 Leipzig, Germany; and Institute for Parasitology and International Animal Health, Free University of Berlin, Königsweg 67, D-14163 Berlin, Germany

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 phy, 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 Tai 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 follow-ups 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: *phy*, 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>
<thead>
<tr>
<th>Chimpanzee name</th>
<th>Date of finding dead chimpanzee</th>
<th>Age (yr/mo)</th>
<th>Community</th>
<th>ST*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loukoum</td>
<td>05/10/1999</td>
<td>27*</td>
<td>North</td>
<td>2308</td>
</tr>
<tr>
<td>Lekkas</td>
<td>05/14/1999</td>
<td>7/7</td>
<td>North</td>
<td>2308</td>
</tr>
<tr>
<td>Candy</td>
<td>02/16/2006</td>
<td>Adult*</td>
<td>East</td>
<td>2308</td>
</tr>
<tr>
<td>Vasco</td>
<td>02/09/2006</td>
<td>Adult*</td>
<td>East</td>
<td>2308</td>
</tr>
<tr>
<td>Orest</td>
<td>03/10/2004</td>
<td>5/10</td>
<td>South</td>
<td>2309</td>
</tr>
<tr>
<td>Virunga</td>
<td>03/19/2004</td>
<td>39*</td>
<td>South</td>
<td>2309</td>
</tr>
<tr>
<td>Ophelia</td>
<td>03/10/2004</td>
<td>1/4</td>
<td>South</td>
<td>2309</td>
</tr>
<tr>
<td>Ishas Baby</td>
<td>02/09/2006</td>
<td>0/2</td>
<td>South</td>
<td>2309</td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address for Regine Hakenbeck: Department of Microbiology, University of Kaiserslautern, Paul Ehrlich Str. 23, D-67663 Kaiserslautern, Germany. Phone: 49-631 205 2353. Fax: 49-631 205 3799. E-mail: hakenb@rhrk.uni-kl.de. Mailing address for Fabian Leendertz (chimpanzee questions): Zentrum für Biologische Sicherheit, Robert Koch Institute, Nordufer 20, D-13353 Berlin, Germany. Phone: 49-30-4547 2258. Fax: 49-30-4547 2605. E-mail: LeendertzF@rki.de.

† Present address: Novo Nordisk Pharma GmbH, Brucknerstr. 1, D-55127 Mainz, Germany.

§ These two authors contributed equally to this work.

* Published ahead of print on 22 June 2007.
The four sequences were identical in all cases but differed from the R6 sequences by 6 bp (South) or 7 bp (North) in all samples except for 1 nucleotide change in the North group. The amino acid sequence is indicated below (aa). Only nucleotides and amino acids that differ from those of the R6 sequence are shown.

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

<table>
<thead>
<tr>
<th>S. pneumoniae isolate</th>
<th>Allele no. for gene*</th>
<th>ST</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>North/East clone</strong></td>
<td>8 134 4 4 132* 142 74 2308</td>
<td></td>
</tr>
<tr>
<td>Closest human isolates</td>
<td>2061 (19A/Spain) 8 13 4 4 6 4 14 2109</td>
<td></td>
</tr>
<tr>
<td>6A-12 (6A/United States) 8 13 4 4 17 4 14 2154</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3852 (4/Poland) 8 70 4 4 6 116 6 2190</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>“South clone”</strong></td>
<td>8 138* 74 10 6 4 161 2309</td>
<td></td>
</tr>
<tr>
<td>Closest human isolates</td>
<td>M16 (23A/United Kingdom) 8 8 9 6 4 6 190</td>
<td></td>
</tr>
<tr>
<td>263/99 (23F/Norway) 2 10 4 10 6 4 65 322</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2828-02 (13/Kenya) 1 11 74 10 6 14 18 701</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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. 1.** Sequence of the lytA gene obtained from the chimpanzees *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.

Several oligonucleotides were used for PCR analysis of *S. pneumoniae* genes (3′→5′): *ply*, GAGGGTAAATCAGCTACCC and GACCAAAAAGGACGCTCTGC; lytA, GCCATGTGTTCGGAGCCG and CACCGGTCGTTCGTACGCC; and *pbp2x*, GAAAGAAATTGGGTGCGTACTTGTGGG, and *lytA*, GCACATTGTTGGGAACGG and CCAATTCACACGG and GGATAAAGGCAGGCAGC. 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 sequenced using bacterium-specific primers. The following oligonucleotides were used for PCR analysis of *S. pneumoniae* genes (3′→5′): *ply*, GAGGGTAAATCAGCTACCC and GACCAAAAAGGACGCTCTGC; lytA, GCCATGTGTTCGGAGCCG and CACCGGTCGTTCGTACGCC; and *pbp2x*, GAAAGAAATTGGGTGCGTACTTGTGGG, and *lytA*, GCACATTGTTGGGAACGG and CCAATTCACACGG and GGATAAAGGCAGGCAGC. 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* genes 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, Lel74 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 pneumococcal Ply, a cytolytic 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 GTCATGAAGTGGGCACC), *gki* (CTTGGATTTGGCGAGCC and GATGTCGTAATGTGGG), and *spi* (AAGCCTTAAAGAAGTTTAGG and GTTTCCTAAAAAGTTCCGATAC). 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-
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 Tai 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-

![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.](http://jb.asm.org/DownloadedFrom/10.1128/IAI.00160-07)
sequences. Our data show that other microbial agents patho-
genic 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. Under-
standing 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 Tai National Park, and the Swiss
Research Center in Abidjan. We also thank the assistants of the Tai
Chimpanzee Project for their help during observation of the chimpan-
zees. 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 Schroc-
ker 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 fur Innovation
(OS80), and the Max Planck Society.

REFERENCES
Forest: behavioural ecology and evolution. Oxford University Press, Oxford,
United Kingdom.
Romero. 2001. A novel solenoid fold in the cell wall anchoring domain of
Walker, and B. Le Guennou. 1999. Ebola virus outbreak among wild chim-
7. Hakenbeck, R., K. Kaminski, A. König, M. van der Linden, J. Paik, P.
Reichmann, and D. Zähner. 1999. Penicillin-binding proteins in β-lactam-
DeHoff, S. T. Estrem, L. Fritz, D.-J. Fu, W. Fuller, C. Geringer, R. Gilmour,
J. S. Glass, H. J. Khoo, A. R. Kraft, R. E. Lagace, D. J. LeBlanc, L. N. Lee,
E. J. Leftomaitis, J. Lu, M. Matsuhashi, S. M. McEachern, M. McNeeley, K.
McLeaster, C. Q. Mundy, T. L. Nicas, F. H. Norris, J. O’Gara, R. B. Peery,
G. T. Robertson, P. Rockey, P.-M. Sun, J. E. Winkler, Y. Yang, M. Young,
Bellido, G. Zhao, C. A. Zook, R. H. Baltz, S. R. Jaskunas, P. R. J. Rostek,
Bioinformatics 14:686–73.