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REVIEW

Approved and novel strategies in diagnostics of rat bite fever and other *Streptobacillus* infections in humans and animals

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ABSTRACT

Rat bite fever (RBF), a worldwide occurring and most likely under-diagnosed zoonosis caused by *Streptobacillus moniliformis*, represents the most prominent disease of *Streptobacillus* infections. Recently, novel members have been described, from which a reservoir in rats and other animal species and a zoonotic potential can be assumed. Despite regularly published case reports, diagnostics of RBF continues to represent a 'diagnostic dilemma', because the mostly applied 16S rRNA sequence analysis may be uncertain for proper pathogen identification. Virtually nothing is known regarding prevalence in humans and animal reservoirs. For a realistic assessment of the pathogen's spread, epidemiology and virulence traits, future studies should focus on the genomic background of *Streptobacillus*. Full genome sequence analyses of a representative collection of strains might facilitate to unequivocally identify and type isolates. Prevalence studies using selective enrichment mechanisms may also enable the isolation of novel strains and candidate species of this neglected group of microorganisms.

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Introduction

For almost a century, *Streptobacillus moniliformis* represented a monotypic species within the genus *Streptobacillus*[1] (*Streptobacillus, Fusobacteriales*) causing streptobacillary rat bite fever (RBF) and Haverhill fever (HF).[2] RBF was first noted by Wagabhatt some 2,300 y ago in India[3] and describes 2 similar yet distinct syndromes, from which the other – albeit less often – is caused by *Spirillum minus* (due to lack of a type strain not listed in the Approved List of Bacterial Names).[2,4] *Spirillum minus* infection, also known as sudoku, has been reported in Asia and is not further discussed here. The acute disease symptoms of the bacterial zoonosis streptobacillary RBF or food-borne HF include fever, malaise, muscle pain, arthritis and abscess formation, endocarditis, bacteremia, and maculopapular, petechial or pustular rash as well as vomiting and pharyngitis.[4] Most likely under-reported worldwide, streptobacillary RBF is predominantly transmitted through rat bites and scratches,[4] whereas HF is transmitted directly or indirectly by contact with rat urine.[5,6] In untreated cases RBF mortality ranges from 7 to 13%.[7-9] Approximately 50–100% of wild rats usually asymptomatically carry *S. moniliformis* in their oro- or nasopharynx and shed the organism with saliva and urine,[2,10] but abscess formation has also been described in rats and mice.[11,12] Other rodents as well as companion and exotic animal species and livestock are principally reported to be susceptible to clinical infection besides rats and mice, but mice may strain-dependently develop disease.[4,11,13-22]

Detection of streptobacillosis due to *S. moniliformis* is referred to as a "diagnostic dilemma"[23] because reasons for under-diagnosing in susceptible host species are missing notice of a rodent bite or contact, non-specific clinical symptoms,[6] fastidious growth of the widely unknown microorganism and a lack of reliable diagnostics, non-notifiable disease and broad chemotherapeutic susceptibility. With respect to known diagnostic difficulties with this microorganism this review summarizes diagnostic approaches to detect streptobacillary infection in humans and animals.

Properties of the agent

Host spectrum

S. moniliformis has been isolated from various animal species. It is frequently found in wild rats (*Rattus norvegicus*),[10] but also in rats housed as pets,[12,24] and has also been found

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in laboratory rats.[25-27] Isolates exist also from laboratory mice (*Mus musculus*)[11,28-30] and from turkeys (*Meleagris gallopavo*).[13,16,18,22] Isolates from rats, mice, turkeys and humans were shown to belong to the same species. [31] Reports on possible infections in other livestock species date decades back into the last century.[17,20] However, as these strains are physically not available, reports are not fully in congruence with the genotypic and phenotypic properties of *S. moniliformis*. Moreover, as recent studies have even found large quantities also in genital tracts from cows and ewes,[32] there is some doubt if streptobacilli in livestock are identical with the RBF organism. Anecdotally, streptobacilli have been described from exotic host species like gerbils and squirrels that were occasionally named "Streptothrix paraxeri cepapi" after Smith's bush squirrel (*Paraxerus cepapi*),[33-35] partially with involvement of human infection resembling RBF,[33,36] but these isolates have also not been stored. Based on metagenomics data from cotton rats[37] it may be possible that streptobacilli in other rodent species might in fact represent separate species (Fig. 1). Further proof of *S. moniliformis* in exotic species was recorded from a koala and macaques.[19,21,38] Carnivores like dogs, cats, weasels and ferrets were occasionally found to be colonized or even suffer from *Streptobacillus* infection.[14,15,39] It remains unclear whether such findings really represent *S. moniliformis*, although identified after mouthing wild rats, or if *Streptobacillus* species other than *S. moniliformis* may be involved that belong to the mouth microbiota and occasionally cause also disease in dogs and cats. *S. moniliformis* is an important zoonotic agent and is usually transmitted to

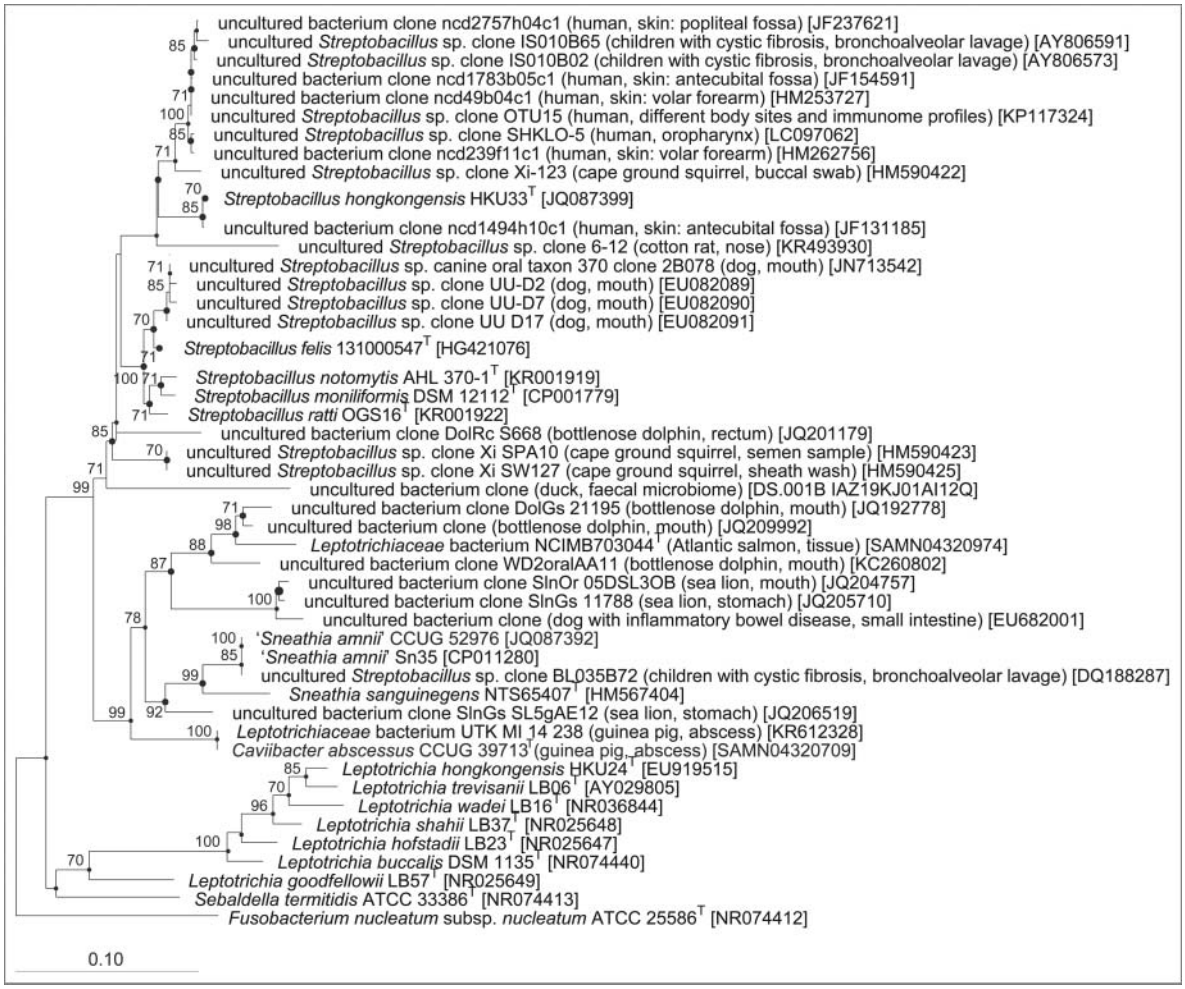

Figure 1. Maximum parsimony (MP) tree showing the phylogenetic relationship of cultured *Leptotrichiaceae* species and those only represented by environmental 16S rRNA gene sequences. The tree was calculated in ARB using DNAPARS and based on 16S rRNA gene sequences spanning at least gene termini 97 to 1356.[138] Shorter sequences were added after tree construction without changing the overall tree topologies. Large circles represent nodes that were at least also present with high bootstrap support in the Maximum likelihood (ML) tree. Small circles mark nodes that were also present in the MP and neighbor joining tree, but in the ML tree only supported by bootstrap values <70%. GenBank/EMBL/DDBJ accession numbers of the sequences are given in parentheses. Numbers at branch nodes refer to bootstrap values >70% (100 replicates). *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586^T was used as outgroup. Bar: 0.1 nucleotide substitutions per nucleotide position. ^{T} marks type strain sequences.

humans by direct contact with rats (rat bite fever), but infection of humans is also possible through contaminated food (HF).[40-42] Contact with carnivorous animals is, however, only rarely believed to lead to human RBF.[43-48]

After the genus *Streptobacillus* was held monotypic for almost a century, a second species, *S. hongkongensis*, has been isolated from 2 humans with peritonsillar abscess and septic arthritis.[49] Recently, a third species was isolated from the lungs of a cat with pneumonia[50] which has been described as *S. felis*.[51] Some other isolates formerly assigned to *S. moniliformis* exist, from which *S. notomytis* from a spinifex hopping mouse (*Notomys alexis*) and from black rats (*Rattus rattus*) and *S. ratti* from an asymptomatically colonized black rat were recently described.[52,53] Contrarily to Nolan et al.,[54] various potentially novel *Streptobacillus* species and phylotypes consistent with operational taxonomic units have been identified in the last few years from Atlantic salmon[55] and microbiomes of digestive tracts in dolphins and sea lions,[56-58] upper respiratory tracts in cotton rats,[37] digestive tracts in dogs,[39,59,60] intestinal tract of a ducks,[61] genital tracts in livestock,[32] and skin and gut microbiomes in humans (Fig. 1).[62,63] This fuels the assumption that *Streptobacillus* species are far more distributed in the environment aside from their natural hosts than previously thought. Contrarily, former *Streptobacillus*-like organisms from fish[55] and guinea pigs[64-67] are even more distantly related to classical *Streptobacillus* species and indeed represent novel genera, that have been recently described.[68,69]

Virulence factors

Despite recent advances in decoding the complete genome of *S. moniliformis* and further *Streptobacillus* species no designated virulence associated genes have been described.[54] Concerning pathogenicity one might refer to possible virulence properties in an α-hemolytic strain.[27] Indeed, hemolytic strains of *S. moniliformis, S. hongkongensis*, and *S. felis* were involved in clinical disease in a rat,[70] a dog,[15] a cat,[50] and a human,[51] but other clinical isolates, especially those causing severe or even fatal disease turned out to be non-hemolytic so that other virulence factors apparently play a more important role. These might include the extraordinary high amount of DNase in all strains, which is released independently from bacterial growth.[71] Further reflections on virulence regard the lipopolysaccharide[27] and the agglutination of erythrocytes. However, as depicted in the chapter on hemagglutination the experiments unequivocally suggest the presence of adhesins, a mechanism involved in bacterial pathogenicity which is a prerequisite for the "successful" infection of a host. Indeed, there appear to remain other factors besides adhesins as can be concluded from the fact that hemagglutination could principally also be observed in non-host species for *S. moniliformis*. These might include not yet identified genetic factors at the host side which can be concluded from differences in susceptibility to infection like for instance the genetically diverse, highly susceptible C57BL/6 J mice compared to BALB/c mice.[11,30] C57BL/6 J mice are known to show an exacerbated release of IL-12 compared to BALB/c mice, if Toll-like receptor (TLR) 2 agonists on the surface of *Listeria monocytogenes* are stimulated.[72] This could also explain a more severe pro-inflammatory response in C57BL/6 J mice by TLR-mediated recognition of *S. moniliformis*.[73] However, although neutrophils seem to represent the predominating leukocyte cell fraction in RBF patients, mouse macrophages are known to be killed earlier in the presence of engulfed *S. moniliformis*.[74]

Diagnostics

Direct techniques for detection of infection

Phenotypic identification

Bacterial cultivation from clinical samples. *Streptobacillus* infection is mostly diagnosed by isolation of the organism from blood, synovia, pus or other fluids, nevertheless the organism is difficult to grow in culture and requires specific media and incubation conditions. An anticoagulant in blood cultures, sodium polyanethol sulfonate (SPS; trade name "Liquoid"), used to grow bacteria from blood samples from patients suspected of bacteremia inhibits growth of the organism in concentrations as low as 0.0125%.[75] Therefore, other additives are necessary for isolation of *S. moniliformis*.[76] Good growth of all species can be achieved on Columbia agar supplemented with 5% sheep blood after 2–5 d of incubation at 37°C in the presence of 5–10% CO_2, but initial culture of the organism from clinical specimens can be difficult due to overgrowth by faster growing and less fastidious bacteria. We had the best culturing results using tryptone soy agar or broth (containing 30 g tryptone soy (Oxoid), 5 g yeast extract (Merck, Darmstadt, Germany), 800 ml Aqua dest., optionally 12 g agar and the addition of 200 ml decomplemented horse serum (Oxoid) after autoclaving). Growth of streptobacilli can further be improved by a 5–20% supplementation of common media with serum or ascitic fluid. [70] Some authors have used media supplemented with colistin, sulfamethoxazole-trimethoprim and nalidixic acid for primary

isolation from colonized mucosal sites by suppressing Gram-negative contaminating flora.[10,11,24] However, work in our lab revealed that *S. hongkongensis* DSM 26322^T is the only member of the genus that is trimethoprim/sulfamethoxazol-sensitive as well as nalidixic acid-sensitive *Streptobacillus* strains occurred.[31,51] In liquid media (e.g. tryptone soy broth) with addition of serum, streptobacillary growth can be detected after 2–7 d as typical "puff-ball" or "bread crumb-like" appearance.[2,4]

Suboptimal growth at least for some strains of *S. moniliformis* and *S. hongkongensis* can still be detected in an aerobic and anaerobic atmosphere.[26,49,70] Isolates from guinea pigs were historically associated with *S. moniliformis* and reported to grow under strictly anaerobic conditions.[64] Investigations in our lab have now shown that these strains from guinea pigs are indeed obligate anaerobes and now form a novel taxon, *Caviibacter abscessus*, within the family *Leptotrichiaceae*.[69]

Growth characteristics

Colonies of *Streptobacillus* are tiny, drop-like, shiny, slightly convex, 0.1–0.4 mm in diameter after 48–72 h of incubation in a capnophilic atmosphere. Some of the colonies show a "fried-egg" appearance indicating the presence of L-forms.[2] Whereas L-form variants arise spontaneously on agar media, the formation of cell wall deficient bacteria is thought to be a consequence of specific immunity *in vivo* and thus are responsible for clinical relapses and resistance to antimicrobial agents that interfere with cell wall synthesis.[77] The L-forms, which are sometimes regarded as non-pathogenic variants *in vivo* [78] and which can be induced *in vitro* on sheep blood agar by addition of 2 IU/ml penicillin,[79] spontaneously revert into bacillary forms after subculturing.

The vast majority of strains are non-hemolytic, but strains with slightly α-hemolytic colonies are known to occur [15,27] and the type strains of *S. felis* 131000547^T, *S. ratti* OGS16^T and *S. hongkongensis* DSM 26322^T all typically demonstrate α-hemolysis on sheep blood agar. [31,50,51,53]

Morphologic features

The microscopic features are consistent with Gram-negative, pleomorphic, fusiform to filamentous, non-spore forming, non-encapsulated, non-acid-fast rods which are arranged in chains and clumps. Sometimes, especially in aged cultures, irregular, lateral bulbar swellings can be seen, that resemble a "string of beads" or a necklace, which is the translation of the Latin word *moniliformis*. The 0.1–0.7 × 1–5 μm sized bacteria tend to pleomorphism and might form up to 150 μm unbranched filaments in stains from cultures compared to stains from infection sites (Fig. 2).[4]

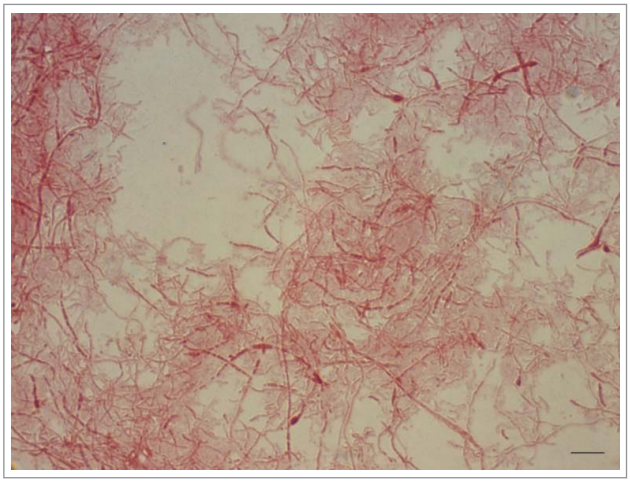

Figure 2. Gram-negative, pleomorphic cells of a 6-day-old culture of *Streptobacillus moniliformis* DSM 12112^T are arranged in chains and clumps and display irregular, lateral bulbar swellings, that resemble a 'string of beads' or a necklace – the translation of the Latin word *moniliformis*. The 0.1–0.7 × 1–5 μm sized bacteria tend to pleomorphism and might form up to 150 μm unbranched filaments. Oil immersion, ×1000 magnification. Bar, 5 μm.

Electron microscopy was carried out with one strain of a '*S. moniliformis*-like organism' isolated from a calve suffering from interstitial pneumonia.[17] The "bread crumb-like" floccules from liquid media appeared as "densely staining filaments and swollen bodies" which could also be appreciated by light microscopy. This isolate was not subjected to molecular analysis or sequencing and other key characteristics were missing, e.g., being dependent on a capnophilic atmosphere or pathogenicity for mice.

Own transmission electron micrographs (JEM-1011; JEOL, Freising, Germany) of cells of *S. moniliformis* DSM 12112^T after growth on sheep blood agar at 37°C for 7 d show oval to elongated cells in a diameter range of 0.3–0.7 μm and lengths from 0.9 to 5.2 μm, without any flagella, but with a recognizable cell envelope, and partly an arrangement in chains (Fig. 3 a). The comparison of electron micrographs from the recently described species *S. hongkongensis* DSM 26322^T, *S. felis* 131000547^T, *S. notomytis* AHL307–1^T, and *S. ratti* OGS16^T grown under the same conditions and incubation times do not show discernible differences (Fig. 3 b-e).

Biochemical properties

For a review of biochemical tests that need to contain the addition of serum to the respective media see refs.[2,4,31] In our opinion the best conventional biochemical results

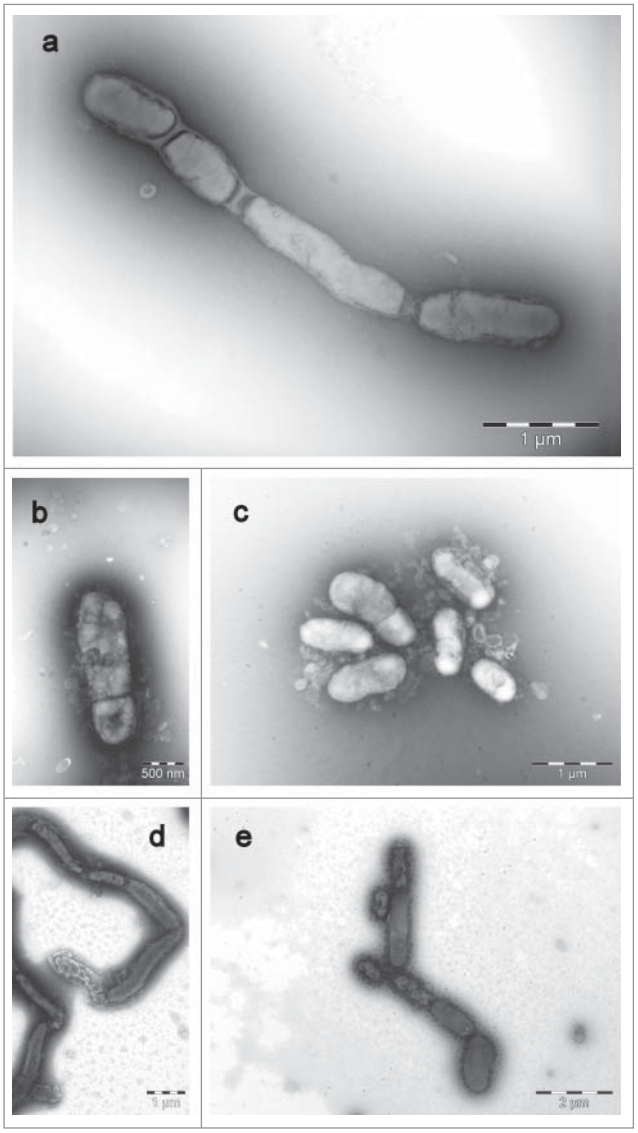

Figure 3. Transmission electron micrographs (JEM-1011; JEOL, Freising, Germany) of cells of a: *Streptobacillus moniliformis* DSM 12112^T, b: *Streptobacillus felis* 131000547^T, c: *Streptobacillus ratti* OGS16^T, d: *Streptobacillus notomytis* AHL 370–1^T and e: *Streptobacillus hongkongensis* DSM 26322^T. All strains were grown on sheep blood agar at 37°C for 7 d. Images were taken with negative contrast (PTA method) at ×3000 to ×10,000 magnifications. Bars, 500 nm, 1000 and 2000 nm, respectively.

were obtained with phenol red broth base (Difco, distributed by Becton Dickinson [Heidelberg, Germany]) supplemented with carbohydrates to be tested. The original recipe from Difco gives better results than the later modification by Becton Dickinson. All tests should be read after prolonged incubation at 37°C for up to 7 d. Some authors suggested to obtain biochemical profiles with commercially available systems, e.g. API-E (bioMeriéux, Nürtingen, Germany),[10] but use of those commercial biochemical platforms remains controversial.[70] However, a set of *S. moniliformis* field and reference strains in our laboratory was mostly in accordance with known patterns.[2,4,10,11,26,27,70,80] In contrast to biochemical assays, enzymatic pattern testing does not require proliferating bacteria. Most studies assessing enzymatic profiles of streptobacilli were using the API-ZYM system (bioMeriéux).[24,27,80] Positive reactions were recorded for alkaline phosphatase, butyrate esterase, caprylate esterase, myristate lipase, leucine arylamidase, chymotrypsin, acid phosphatase, and glucuronidase activities.[24] We have recently employed another commercially available biochemical system (Merlin Micronaut, Bornheim, Germany) that was specifically adapted to the growth of *Streptobacillus*.[31] A choice of suitable biochemical tests is given in Table 1. In summary, *Streptobacillus* species can be indicated by a combination of growth and micromorphological characteristics together with some important, congruently negative resulting key reactions like cytochrome oxidase, catalase, urease, and nitrate reduction, Voges-Proskauer-reaction and indole production. Further biochemistry might be variable between strains of the same species and is not adequate to differentiate different *Streptobacillus* species.

Table 1. Physiological characteristics of *Streptobacillus* species known to date obtained by VITEK2-compact with the NHI card†, API-Zym‡ (both bioMeriéux) and classical reactions§ (modified after[53]); Taxa: 1, *Streptobacillus moniliformis* DSM 12112^T; 2, results from 6 *Streptobacillus moniliformis* reference strains (ATCC 27747, ATCC 49567, ATCC 49940, NCTC 11194, CIP 55–48 and CIP 81–99); 3, *Streptobacillus hongkongensis* DSM 26322^T; 4, *Streptobacillus felis* 131000547^T; 5, *Streptobacillus notomytis* AHL 370–1^T; 6, *Streptobacillus ratti* OGS16^T; +, positive; -, negative; +/− variable.

Compound	1	2	3	4	5	6
Hemolysis on SBA§	−	+/−	+	+	−	+
Phosphatase (unspecified)†	−	−	+	−	−	−
Phenylalanine arylamidase†	+	+	−	−	+	+
Ala-Phe-Pro arylamidase†	+	+	−	−	+	+
Alkaline phosphatase‡	w	+/−	+	+	−	−
Esterase (C4)‡	w	+/−	w	+	+	−
Esterase lipase (C8)‡	+	w/+	w	+	+	+
Leucine arylamidase‡	−	+/−	−	−	w	−
α-Chymotrypsin‡	+	w/+	−	−	+	+
Acid phosphatase‡	w	−/w	+	+	−	−
Naphthol-AS-BI-phosphohydrolase‡	−	−	w	−	−	−
α-Glucosidase‡	−	−	−	−	−	−

#score values 0–5 indicate strength of enzymatic intensities (0–2: negative [−], 3: weak [w], 4–5: positive [+]).

Chemotaxonomic pattern

Fatty acid profiles obtained by gas-liquid chromatography, together with characteristic growth, have been used for rapid identification of *S. moniliformis*.[80-86] The major cellular fatty acid peaks are tetradecanoic acid (14:0), palmitic acid (16:0), octadecanoic acid with linoleic acid (18:2) and oleic acid

Table 2. Cellular fatty acid pattern of *Streptobacillus* species known to date (modified after [53]); Taxa: 1, *Streptobacillus moniliformis* DSM 12112^T; 2, *Streptobacillus hongkongensis* DSM 26322^T; 3, *Streptobacillus felis* 131000547^T; 4, *Streptobacillus notomytis* AHL 370–1^T; 5, *Streptobacillus ratti* OGS16^T. Biomass for fatty acid analysis was harvested after 3 d of growth in capnophilic environment with 10% CO_2 on Columbia sheep blood agar at 36°C.

Fatty acid	1	2	3	4	5
$C_{14:0}$	1.5	–	1.5	1.6	1.5
$C_{15:0}$*iso*	3.9	3.0	2.1	–	–
$C_{16:0}$	27.8	26.5	28.2	29.4	28.7
$C_{17:0}$	1.5	–	1.5	–	1.5
summed feature 5 $C_{18:0}$ ANTE/$C_{18:2}\omega6,9c$	13.3	5.6	12.1	13.0	8.5
$C_{18:1}$ $\omega6c$	2.2	–	2.0	–	5.9
$C_{18:1}$ $\omega9c$	25.1	30.2	24.1	26.6	23.6
$C_{18:0}$	23.5	34.7	21.6	29.4	26.3
$C_{20:4}\omega6,9,12,15c$	1.2	–	1.1	–	–

For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. *cis* isomers are indicated by the suffix *c*.

(18:1), and stearic acid (18:0).[84,85] Fatty acid profiles obtained with use of gas chromatography coupled with mass spectrometry showed major peaks for C16:0, C18:2, C18:1, and C18:0 fatty acids, a profile characteristic of *S. moniliformis*.[82] In light of additional species of *Streptobacillus* this must be scrutinized, because these species cannot be differentiated by their fatty acid patterns alone. A comprehensive comparison of fatty acid profiles from all *Streptobacillus* species known to date is presented in Table 2.[53]

Polar lipids are poorly understood in *Streptobacillus* species. A lack of quinones and a specific polyamine pattern different from those of the α-, β- and γ-subclasses of the proteobacteria was proposed by Hofmann and Wullenweber et al.[70,71] The protein profiling from whole cell preparations displayed a species specific pattern of 40–50 proteins ranging from 18 to 100 kDa. Four major protein bands in the region 60–67 kDa were formed that accounted for 20–30% of the total protein.[87] Earlier assumptions of protein-based strain differences between human and murine isolates as well as between Haverhill and rat bite fever strains[87] were not confirmed by other authors.[80,88] On the other hand, especially differences of HF- versus RBF-strains are unlikely because rats represent the source of infection in both cases and disparities could better be explained by different gene expression following oral or parenteral infection[4] or simply by too few HF-strains under study. Additionally, the time between infection and strain isolation from the host following rat exposure is usually too short to facilitate adaptation of strains and expression of a different phenotype.

Experimental infection

Historically, the classical foot pad test was performed for confirmation of *S. moniliformis* infection by injection into mice, which led to septic arthritis within few days.[70] This painful procedure is now obsolete as better *in vitro* diagnostics are available. A number of studies have, nevertheless, proven that *S. moniliformis* strains isolated from susceptible host species were able to cause infection in rodents, thereby partially fulfilling Koch's postulates.[11,22]

Hemagglutination

Screening for adhesive properties was performed for 14 *S. moniliformis* strains and for *S. notomytis* AHL370-1^T by hemagglutination experiments using erythrocytes from 11 different host donor species, i.e. red blood cells from humans, BALB/c and C57B1/6J mice, rats, turkeys, guinea-pigs, hamsters, chicken, sheep, horses, pigs and cattle were included.[31,71] Adhesive properties were detected in all *S. moniliformis* strains tested. Erythrocytes of the different vertebrate species were agglutinated with varying intensity. The strongest reactions could be observed in erythrocytes from turkeys, humans, guinea-pigs and pigs, followed by rats and chickens. C57BL/6J mice, known to represent a highly susceptible mouse strain toward streptobacillosis,[11] were less strongly agglutinated compared to erythrocytes from the more resistant BALB/c mice. By adding mannose, a known agonist of a common adhesin receptor, no significant differences could be observed indicating mannose-resistant agglutination in all cases. No differences were observed between agglutination of erythrocytes from 'original' host species (from which respective strains were originally isolated) and other host red blood cells, but susceptibility was generally highest in species of potential hosts compared to non-host species.

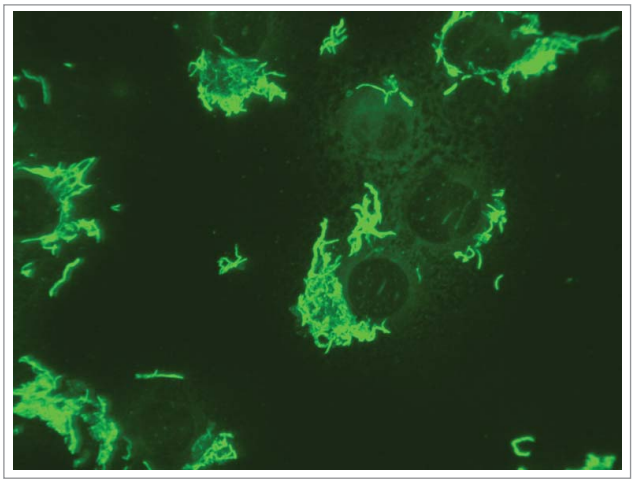

Figure 4. Positive immunofluorescence reaction with *Streptobacillus moniliformis* ATCC 14647^T. Suspended L929 cells are infected, pipetted to glass slides and incubated for 4 hours until complete adherence.

There were no differences in the hemagglutinating behavior between RBF and HF strains of *S. moniliformis*.[71]

Serum agglutination

For direct identification of *S. moniliformis* agglutination reactions with specific serum have been used in the past.[89] Direct immunofluorescence was also employed to achieve identification by staining *S. moniliformis* bacteria with a polyclonal antiserum (Fig. 4).[7] None of these tests is commercially available and specificity of such assays remains to be reviewed.

Mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was found to be a fast and reliable tool for species identification of *S. moniliformis*.[50] In the meantime, this technique has proven to contain enough discriminatory information to differentiate all currently known species of *Streptobacillus*.[31,53] Likewise, commercial databases do not contain spectra of all members, but respective spectra can be obtained via MALDI-UP, a user-to-user dedicated database platform.[90] A representative cluster analysis of MALDI-TOF spectra is depicted in Figure 5. In a repeatedly culture-negative clinical case of RBF, employment of PCR and electrospray ionization followed by mass spectrometry (PCR/ESI-MS) proved to be a useful tool in detection of *S. moniliformis* from a synovial fluid but not from the patient's serum. The authors highlight that this technique was culture-independent and even successful in specimens obtained following initiation of antimicrobial therapy.[91]

Fourier transform-infrared spectroscopy (FT-IR)

As a vibration-spectroscopic technique, FT-IR is using the mid-infrared region of the electromagnetic spectrum to analyze the total composition of dried films of microorganism cells. In comparison with the protein-fingerprints obtained by MALDI-TOF MS, FT-infrared-spectra mirror information about the sum of biomolecules, like carbohydrates, lipids, proteins and other cell components.[92] This method had already been

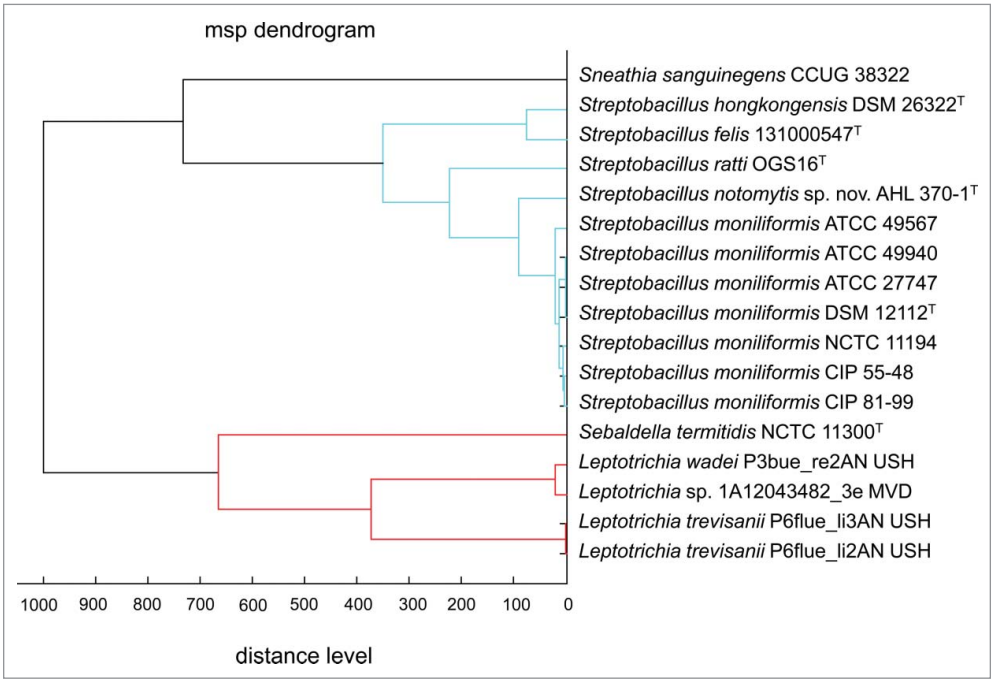

Figure 5. Dendrogram including all main spectra peak lists (MSP) of the family *Leptotrichiaceae* available in the Bruker Taxonomy Database; spectra of *Streptobacillus ratti* OGS16^T, *Streptobacillus notomytis* AHL 370–1^T, *Streptobacillus hongkongensis* DSM 26322^T, *Streptobacillus felis* 131000547^T, *Streptobacillus moniliformis* and *Sebaldella termitidis* NCTC 11300^T reference strains were recorded using the direct transfer protocol. The dendrogram was generated using the BioTyper MSP Dendrogram Creation Standard Method (v1.4) of the MALDI BioTyper OC Software (v3.1, build 66). The database used (DB 5627, BrukerDaltonics) comprised solely 24 spectra from *Streptobacillus moniliformis* DSM 12112^T; ^{T} type strain, ATCC: American Type Culture Collection, Rockville, USA, DSM: Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany, CIP: Collection of Institute Pasteur, Paris, France, NCTC: National Collection of Type Cultures, London, UK.

used as a fingerprint-based tool for rapid and reliable classification of a large number of clinically relevant pathogens[93-95] including recently described *Streptobacillus* species (Fig. 6).[31,51] However, at present, the lack of enough strains from the novel species makes it difficult to validate and test the performance of the species identifying methods.

Antimicrobial properties

Despite its good response to various antimicrobial treatments one should attempt susceptibility testing for every isolate. Most studies employed disk diffusion testing where diameters of zones with growth inhibition are recorded according to a norm (e.g., according to the German standard DIN 58940).[11,85,96] Some authors have used minimum inhibitory concentration (MIC) testing with the agar incorporation test[80] or a breakpoint method,[70,71] that gave mostly congruent results compared with the disk diffusion method.[70] With respect to MIC testing with automatic systems the slow growth of *Streptobacillus* hinders a reliable end point measurement and can only be read by visual interpretation during off-label use. One has to take into account, that no official breakpoints specific for *Streptobacillus* have been published to date. Therapeutics of choice are generally penicillin, streptomycin and tetracycline.[70] Therapy in mice was successfully initiated with 1 g ampicillin/L drinking water given for 2 weeks, followed by chlortetracycline for 1 week.[11] Expectably, MIC testing results are too vague and – like biochemistry – not appropriate to differentiate species.

Storage of bacteria

To keep *Streptobacillus* in strain collections, freezing of fresh bacterial biomass and supplementation of respective medium with 20% serum or in pure cattle serum with 6% glucose at −70°C works well for re-cultivation even after several years. Deep freezing in brain heart infusion (BHI) broth supplemented with 10–20% (w/v) of glycerol can also be advised (own observations).[88] Lyophilization in fetal calf serum with 6% glucose is also a good option for long-term storage in our laboratory. Resuscitation of sub-lethally damaged strains was successfully achieved by centrifuging the previously frozen organism onto a human endothelial cell culture where regular growth could be initiated.[97]

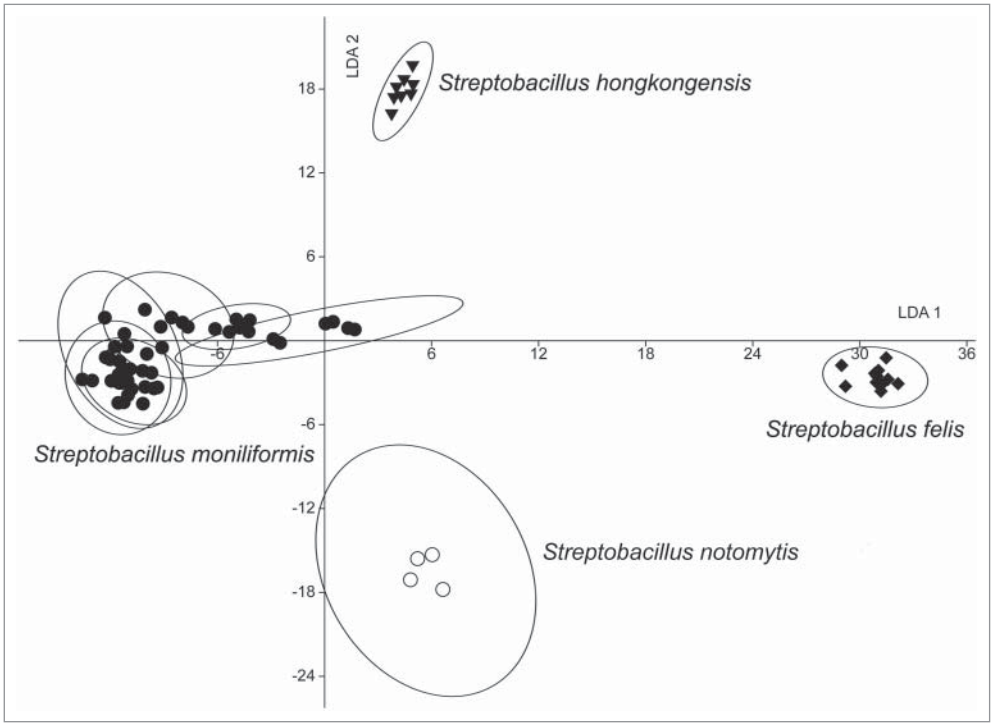

Figure 6. Linear discriminant analysis (LDA) analysis of 69 infrared spectra of 10 *Streptobacillus* isolates obtained by Fourier-transform infrared-spectroscopy (FT-IR) using OPUS Software (vers. 4.2, BrukerOptics, Ettlingen). The wave numbers 550–1800 and 2800–3200 cm^{-1} of second derivative spectra were selected and vector normalized. After a principal component analysis, the first 30 components were used for the LDA. In this LDA every isolate was defined as one group. Spectra of *Streptobacillus notomytis* AHL 370–1^T are represented by circles, *Streptobacillus moniliformis* by dots, *Streptobacillus hongkongensis* DSM 26322^T by triangles and *Streptobacillus felis* 131000547^T by diamonds.

Molecular identification

Molecular properties

Determinations of guanine/cytosine (G/C) contents of 23 *S. moniliformis* as well as the novel species revealed a nearly identical G/C content of 25.7–28.9 mol% in all investigated *Streptobacillus* strains.[31]

A high level of DNA-DNA-homology (DDH) between 14 strains of *S. moniliformis* could be shown by Hofmann.[71] As concluded from an earlier definition all of the investigated *S. moniliformis* had DDH levels above 70% thus indicating them as members of a single species.[98,99] According to this definition *S. notomytis* AHL370-1^T revealed 68% homology to the *S. moniliformis* type strain, thereby at best justifying a separate subspecies.[98] We therefore believe that DDH of *Streptobacillus* species gives weak results.[31] Instead, average nucleotide identity (ANI) was carried out according to the method described by Goris et al.,[100] with which analogous results could be confirmed. They could demonstrate a close relationship between DDH values and ANI in that the recommended cut-off point of 70% for species discrimination corresponded to 95% ANI. The 95–96% species boundary is also supported by Richter & Rosselló-Móra,[101] who have developed an alignment free interface to calculate ANI also used in this study. In contrast to the highly homologous group of *S. moniliformis* strains all novel members could be unequivocally discriminated.

Species specific PCR for S. moniliformis

PCR protocols published to date for the detection of *S. moniliformis* in clinical samples and for identification focus on respective partial gene sequences of the 16S rRNA gene.[10,12,97,102-104] The method by Boot et al.[102] amplified a 296 bp fragment and showed considerable sensitivity but also some flaws in specificity due to amplicon sequence similarities with *Leptotrichia* sp., *Fusobacterium necrogenes* and *Sebaldella termitidis*.[102,103] Although these non-specificities could be solved by macro restriction with the endonuclease *Bfa*I, Kimura et al.[10] advanced this PCR with respect to specificity by improving oligonucleotide primers according to Table 3. Thus, a 269 bp fragment was amplified and cross reactivity with the above mentioned bacterial species was no longer detected. We have further modified the method by Kimura et al.[10] slightly by changing the annealing temperature to 53°C for 1 min. We have calculated a diagnostic sensitivity of 2 $\times$ 10^2 bacteria with this PCR by detecting as few as 10 pg of serially diluted purified DNA lysate of *S. moniliformis* DSM 12112^T endowed in homogenized rat lung tissue. Details on our PCR have been previously described.[50] A different PCR protocol employing primer pair SbmF/SbmR (Table 3) yielded a significantly longer amplicon (1,222 bp),[12] thereby further improving specificity. Both PCR protocols were suitable to detect all *S. moniliformis* strains from humans, rats, mice and turkeys and also *S. felis, S. notomytis* and *S. ratti*, but *S. hongkongensis* was not detected in the PCR by Rohde et al.[12,51-53] Summarizing, all the mentioned PCR systems must presently be regarded rather genus than species specific. In an era of easy access to sequencing techniques it is always desirable to sequence amplicons. *S. moniliformis* and *Leptotrichia* sp. turned out to non-specifically cross-react in a fluorescence *in situ* hybridization assay (FISH) for rapid identification of *Fusobacterium* spp.[105] which in turn suggests its use also for the direct detection of *S. moniliformis*.

Marker gene sequencing

16S rRNA gene

A number of studies have used full length or partial 16S rRNA gene sequencing as a diagnostic tool for species determination of *S. moniliformis* in clinical samples[10,39,97,106-109] as well as for laboratory confirmation of suspicious isolates.[24] Because of the extraordinary role of 16S rRNA gene sequencing in bacterial taxonomy this gene enables to compare isolates and phylotypes obtained in microbiome studies. On the other hand, 16S rRNA gene sequencing can be insufficient for definite species resolution.[110] For unequivocal identification of *Streptobacillus* species in particular, this gene should always – especially in the highly homologous species *S. moniliformis, S. felis, S. notomytis* and *S. ratti* – be confirmed by another gene locus or method such as those described below. Contrarily, in *S. moniliformis* with a sufficient choice of strains, intraspecies heterogeneity was too low to distinguish strains from different origins and hosts (Fig. 1).[31]

Other housekeeping genes

Species specific *gyrB* primers were designed to amplify a 514 bp fragment of the gene for gyrase B in order to identify *S. moniliformis*.[24] Within the phylum Fusobacteria sequencing of 16S rRNA, 16S-23S rRNA internal transcribed spacer, *gyrB, groEL, recA, rpoB*, conserved indels and genes for group-specific proteins, 43 kDa outer membrane protein and zinc protease have been proposed for species identification or phylogenetic analysis[111-120] and more than 31 whole genome sequences have been released in GenBank. On the basis of next generation sequencing a number of functional genes was tested for their phylogenetic potential.[31] To overcome the above mentioned uncertainties, we have used *groEL, recA* and *gyrB* in addition to 16S rRNA, which

Table 3. Oligonucleotide primer sequences and PCR conditions of the target genes used for the detection of *Streptobacillus* species.

Target gene	Oligo-nucleotide primer	Sequence	PCR program[†:]	Expected size of PCR product (bp)	Reference
16S rRNA	LPW8385	5′-GAACGCTGACAGAATGCTTA-3′	1	1425	[111]
	LPW8387	5′-CCAATCACTATCCACACCTTA-3′	1		
chaperonin (*groEL*)	LPW8389	5′-GTTGTGGAAGGNATGCARTTYGA-3′	1	555	[111]
	LPW8441	5′-CAGCTCCAACTTTTATTACAGCT-3′	1		
gyrase subunit B(*gyrB*)	LPW10271	5′-GGAAMWGAYRTAAGAGAAGG-3′	1	796	[111]
	LPW8399	5′-TTCATTTCTCCTAGNCCYTTRTA-3′	1		
recombinase subunit A (*recA*)	LPW8402	5′-GGTGCCGTTATGAAAYTNGGNGA-3′	1	813	[111]
	LPW10124	5′-GAACCAGGCTCCAGCTTT-3′	1		
DNA-directed RNA polymerase subunit B (*rpoB*)	LPW8697	5′-AAATGGCACTTGAGCTGT-3′	1	768	[111]
	LPW8698	5′-CAATTCCAACAGTAATTCCA-3′	1		
16S rRNA	LPW57	5′-AGTTTGATCCTGGCTCAG-3′	2	*	[49]
	LPW205	5′-CTTGTTACGACTTCACCC-3′	2		
16S rRNA	LPW26378	5′-AGGACAATGRAAAKAGAAG-3′	2	*	[49]
	LPW26129	5′-TATCTCAGTCCCCTTGTG-3′	2		
16S rRNA	LPW26128	5′-AAGTTGGGGACTCTAATG-3′	2	*	[49]
	LPW26379	5′-CTATTCATTTCYCATTGTCC-3′	2		
recombinase subunit A (*recA*)	LPW18647	5′-GGWKCYRTHATGAARYTYGGWGA-3′	2	*	[49]
	LPW18648	5′-ARCTRAACCAYGMWCCRCT-3′	2		
16S rRNA	S5	5′-CATACTCGGAATAAGATGG-3′	3	269	[10]
	AS2	5′-GCTTAGCTCCTCTTTGTAC-3′	3		
16S rRNA	SbmF	5′-GAGAGAGCTTTGCATCCT-3′	4	1222	[12]
	SbmR	5′-GTAACTTCAGGTGCAACT-3′	4		
gyrase subunit B (*gyrB*)	MZK-F	5′-AAGATAGGGTAATGCTTACAGAAGGAG-3′	5	1316	[24]
	MZK-R	5′-AATCTACCTTGTTTTGCAGATCCAC-3′	5		
16S rRNA		5′-AGAGTTTGATGGCTCAG-3′	6	1400	[139]
		5′-GGAACGTATTCACCGTAGCA-3′	6		

[†:]PCR program:
1:x40 (94°C, 60 secs, 55°C, 60 secs, 72°C, 120 secs), x1 (72°C, 600 secs);
2:*
3:x1 (95°C, 180 secs), x35 (95°C, 20 secs, 53°C, 60 secs, 72°C, 60 secs), x1 (72°C, 420 secs) (annealing of this PCR was modified to 53°C (60 secs));
4:x1 (94°C, 240 secs), x35 (94°C, 60 secs, 50°C, 60 secs, 72°C, 60 secs), x1 (72°C, 420 secs);
5:*
6:*
*not provided in publication

unequivocally could discriminate *Streptobacillus* strains to species level.

Indirect techniques for detection of infection

Although the authors are unaware of any seroprevalence surveillance studies in humans to assess the number of atypical or subclinical cases with the potentially lethal RBF microorganism, various serologic approaches exist to detect antibodies against *S. moniliformis* in serum. Among these are direct slide agglutination and complement fixation techniques with human or naturally or experimentally infected animal sera.[15,40,74,121-128] Compared to modern methods, these tests show a flaw in sensitivity and specificity. Nevertheless, agglutinating antibodies were also used early in taxonomic studies to type *S. moniliformis* strains.[125] A complement fixation test[74,129,130] and an indirect immunofluorescence assay (IFA)[11,27,75] have been employed in intravenously infected mice. The latter was prepared by using air-dried and heat fixed bacterial suspensions with an OD of 0.2 on IFA slides as antigen. Alternatively, antigen for serology can be prepared by adding a bacterial suspension to cells (e.g., L929, HeLa cells) grown on IFA slides (Fig. 4). This has the advantage that bacteria adhere to the cells so that unspecific reactions can easily be identified.

A protocol for an enzyme-linked immunosorbent assay (ELISA) was evaluated[26,88] and propagated for testing mouse and rat colonies.[88] The authors used washed and merthiolate-inactivated bacteria cultured in broth. This test was found superior in detecting significantly more positive animals compared to culture.[26] Different immunoglobulin subclasses could be detected with the usual shift from IgM to IgG with duration of infection by using different secondary antibodies.[26] Unfortunately, this test failed to unequivocally detect true infection due to lack in specificity and therefore immune blotting (IB) with whole cell antigens was advised to confirm positive or doubtful reactions from ELISA.[25] Interestingly, antibodies in sera of guinea pigs were also detected by ELISA, but guinea pigs were resistant to oral or nasal infection with a rat strain of *S. moniliformis*[131] and strains infecting guinea pigs formerly assigned to *S. moniliformis* were found to represent a novel species,[69]

thereby suggesting the possibility of cross reactivity of this ELISA with other species.[88]

Use of membrane proteins of *S. moniliformis* instead of whole bacterial cells reduces the background reactions in ELISA and increases its specificity (Nicklas, unpublished data). Proteins can easily be prepared as described by Livingston et al. [132] for *Helicobacter hepaticus*. These proteins can also be coupled to polystyrene microspheres (Luminex Corporation distributed by Diamex, Heidelberg, Germany) and applied in bead-based multiplex serology. We use this test as primary test for health surveillance of rodent colonies and IFA with cells grown on IFA slides after infection with *S. moniliformis* as a confirmatory test (Fig. 4).

Employing SDS-PAGE of *S. moniliformis* whole cell preparations, a species specific pattern of 40–50 proteins was derived.[87] By IB, however, a number of approximately 10 different antibodies to respective immunogenic antigens of the 18–87 kDa range were demonstrated.[25] Rodent sera were considered positive if an antibody activity against at least 2 antigens of the 32–55 kDa range could be detected. With this assumption IB yielded a diagnostic sensitivity of 78% and a diagnostic specificity of 85%.[25] Interestingly, though *S. notomytis* AHL370-1^T displayed a unique profile in electrophoretic protein patterns[87] no antigenic differences could be observed for this species compared to *S. moniliformis*.[88]

In contrast to viral serology, there are few publications which emphasize serology for bacterial infections, especially to confirm the microbiological status of laboratory rodent colonies. Serology has the advantage that no animals have to be sacrificed and that serum samples can easily be shipped to external laboratories. On the other hand, the risk of false-positive and false-negative reactions must be considered. None of these serological tests is currently commercially available.

However, antibodies can routinely be found in infected rats after 2 to 4 weeks post infection.[26] Conversely, mice strain-dependently suffered from natural infection and even died before antibodies could be detected.[11] Especially susceptible were C57BL/6J mice usually displaying cervical lymphadenitis in contrast to BALB/cJ, C3H/He, CB6F1, B6D2F1 and DBA/2J mice. Seroconversion following oral infection was only observed in C57BL/6J, C57BL/10, AKR/N, B6D2F1 and DBA/2J and in all of the tested mouse strains after intravenous injection, despite severe clinical symptoms were only observed in all C57BL/6J and some DBA/2J mice.[11,70] After intranasal application, that represents the natural way of infection, antibodies were not reliably detected in mice and rats,[11,133] but experiments were terminated already 4–6 weeks after infection. We experimentally infected BALB/c intranasally and detected antibodies by ELISA and IFA usually after 8 weeks or later (Nicklas, unpublished data). Other authors came to the conclusion that genetic factors as well as individual resistance were responsible for different strain susceptibility in mice.[11,70] Intravenous and subcutaneous infection of non-specified mice led to a weak neutrophilia and maximum antibody titers not exceeding 1:640. Despite an effect that homologous antibodies existing prior to infection prolonged the incubation period, the authors concluded that the organism was in some fashion resistant to phagocytosis *in vivo*.[74]

With respect to specificity (serological) cross reactions to most other rodent bacteria could be excluded for ELISA and IFA, except for some members of the order Mycoplasmatales.[70,88] An age-dependent effect was reported for the ELISA so that routine monitoring of rats should be done up to an age of 16 weeks to prevent false positive reactivity.[70]

Discussion

RBF is occurring worldwide and is believed to be under-recognized and under-diagnosed in humans.[4] The risk of infection with any organism following a rat bite is 1–10%,[8] but the risk of developing streptobacillary RBF and the infectious dose are unknown. According to another survey 40,000 rat bites are noticed every year[134] and approximately 2% of rat bites are followed by an infection.[135] Untreated RBF is associated with a case fatality rate of up to 13%.[70] The reasons for under-diagnosing streptobacillosis in man and animals include organism as well as host specific factors like unsuitable diagnostic tools, the fastidious growth and broad chemotherapeutic susceptibility of this microorganism, a non-notifiable disease as well as missing notice of a rodent bite, non-specific clinical symptoms, especially in chronic infections and a broad spectrum of differential diagnoses. Additionally, only very severe clinical causes will be diagnostically worked up and few laboratories and physicians are experienced with RBF or are even aware of the disease. In addition to the natural reservoir of rats, mice and other rodents, streptobacillary infections have also been reported to occur in livestock as well as zoo animals like calves, a pig, turkeys, non-human primates, and a koala.[4,13,16-22] Recently, various publications suggest that *Streptobacillus* species might be far more common and distributed in the environment or as commensal microbiota than previously thought.[32,37,59-63] It could recently be shown that the natural reservoir for the very rare cases of human *S. hongkongensis* infection known to date is indeed the human oropharynx* and presumably not an unidentified animal or environmental reservoir.[49] Despite a certain amount of annually published case reports, most of which have solely used 16S rRNA

sequencing alone for definite diagnosis, there has not been much progress in the diagnosis of acute clinical cases of RBF in the last decades. Moreover, as pointed out earlier, 16S rRNA sequencing alone may be insufficient for unequivocally determining the involved pathogen to species level. In conclusion, the better knowledge of the global spread of *Streptobacillus* and its variability demands both a higher awareness as well as better diagnostic approaches in human as well as in animal diagnostics. Hence, the present review focused on a large spatiotemporal collection of *S. moniliformis* isolates from different host species and also included all currently known novel members of the genus. We aimed to analyze whether well-established diagnostic tools still fit the demands of an increasing diversity of *Streptobacillus* members and host species and also report our experiences with modern as well as little-known diagnostics.

Primary isolation by culture methods remains difficult as colonies are small and shiny and appear only after incubation for several days in an atmosphere enriched with CO_2. Isolation from clinical samples on blood agar is unlikely in mixed cultures (e.g., oral cavity, nasopharynx, intestinal tract) due to overgrowth by less fastidious organisms but is easily possible from otherwise sterile environments (e.g., blood, joint fluid, abscesses). Colonies may differ in size as L-form variants arise spontaneously. They are usually non-hemolytic but strains with a weak α-hemolysis do occur. In liquid media containing serum or ascitis fluid bacteria show typical "puff-ball" or "bread-crumb-like" appearance as a sediment and a clear supernatant. The yield can be improved by adding antimicrobial agents like for instance colistin, sulfamethoxazole-trimethoprim and nalidixic acid to the culture medium, but even then the isolation rate can be low. Although Kimura et al.[10] could detect a prevalence of up to 92% by PCR they succeeded to culture only 7 isolates from more than 1000 suspicious colonies. We have earlier shown that physiological parameters are problematic for typing *Streptobacillus*. In general, carbohydrate fermentation tests and other tests requiring proliferating bacteria may be difficult to read as some strains grow poorly resulting in very weak reactions. Biochemistry is furthermore largely dependent on the test system itself, possible batch-to-batch variation and the person reading the tests and even with commercial test systems it was not unequivocally possible to get identical results for the same species or even the same strains in repeated experiments.[31] Nevertheless, it is possible to use information from biochemistry together with growth characteristics, when working with suspicious isolates, but the characters are generally too weak to differentiate species. If standardized test systems are employed, one should use tests determining end-point measurements (e.g. API ZYM®, VITEK2-compact® (NHI profile), Merlin Micronaut *Streptobacillus* profile) that are thus independent from bacterial growth compared to tests requiring viable bacteria. Important key reactions for all members of the genus *Streptobacillus* are cytochrome oxidase, catalase, urease, nitrate reduction, Voges-Proskauer and indole production (all negative). Chemotaxonomic analysis was slightly deviant from other studies for *S. moniliformis* that have found homologous major fatty acid profiles of tetradecanoic acid (14:0), palmitic acid (16:0), octadecanoic acid with linoleic acid (18:2) and oleic acid (18:1), and stearic acid (18:0).[82,84,85] In contrast, we have detected relatively uniform fatty acid patterns of $C_{16:0}$, $C_{18:0}$, $C_{18:1}\omega 9c$, summed feature 5 $C_{18:0}$ ANTE/ $C_{18:2}\omega 6,9c$ and $C_{18:1}\omega 6c$ for all *Streptobacillus* species.[53] Antimicrobial resistance profiles – albeit also not suitable for species or genus discrimination – have revealed that *S. moniliformis* – since no β-lactamase activity could be demonstrated so far[54] – is susceptible to all β-lactam antibiotics.[31,80] Penicillin G was repeatedly reported being the most efficient antimicrobial substance *in vitro* and *in vivo*, which further supports its use as the drug of choice in the treatment of RBF and HF, followed by tetracycline.[2] The successful use of the combination of clindamycin with rifampin (for enhanced tissue concentration) has been described in a case of abscess formation.[136] Because of generally low MIC values the strains from our study confirmed a generally good therapeutic basis, but, nevertheless, some isolates were *in vitro* resistant or intermediate resistant to ciprofloxacin, erythromycin, gentamicin, nalidixic acid and streptomycin.[31]

Hence, these very similar, non-discriminatory physiological results suggest that other traits should be propagated for the identification of *Streptobacillus* species. Based on spectral differences our group could recently show that 23 *S. moniliformis* strains from at least 5 different host species isolated over the past 90 y from almost all subcontinents as well as the type strains of *S. hongkongensis* and *S. felis* were unequivocally differentiated by MALDI-TOF MS and also by FT-IR, where the spectral information mirrors information from a broad variety of main component biomolecules.[31,137] This was also true for the 2 recently described novel species *S. notomytis* and *S. ratti*.[52,53] Together with the ease, expense and availability of these methods in the microbiological laboratory nowadays, at least MALDI-TOF MS should presently be regarded as the new gold standard in species discrimination, but – on the other hand – usually requires previous

cultivation of the organism. Nevertheless, by the worldwide spread of the MALDI-TOF MS technology in clinical microbiological laboratories, we expect a significantly higher number of reliable diagnoses for *Streptobacillus* sp. This is particularly true, since relevant entries for a specific database extension are available.[90] The published PCR assays are genus rather than species specific. A diagnostic sensitivity of less than 2×10^2 cfu was calculated for homogenized rat lung tissue. Diagnostic flaws concerning specificity toward other genera were successfully corrected in the meantime. Based on the novel members, a real species specific real-time PCR remains to be developed, thereby further improving also sensitivity. Phylogenetic results largely confirmed the findings with spectroscopy. Molecular data derived from 16S rRNA gene sequencing as well as multiple protein-coding phylogenies of *groEL, gyrB* and *recA* resulted – independent of the treeing method – in almost identical phylogenetic trees for respective nucleotide and amino acid alignments (data not shown). Intraspecies homology is high as can be concluded from G/C contents and average nucleotide identities. With respect to epidemiology and virulence factor analysis there is still a great demand for a broader insight into multiple *Streptobacillus* genomes. Studies using selective enrichment steps like for instance antibody enhanced isolations may facilitate the acquisition of novel strains from different host species in order to fill these gaps.

Conclusion

Rat bite fever represents a significant public health threat that is under-diagnosed in humans and animal species. Novel species of the genus made it necessary to critically review diagnostic tools with respect to species specificity. We have provided an update in diagnostics to improve detection and isolation of these neglected microorganisms. All members of the recently extended genus can be reliably differentiated from a pure culture by MALDI-TOF MS, FT-IR spectroscopy and also by sequence analysis of selected functional genes. 16S rRNA sequencing alone is adequate to allocate the pathogen to the correct genus, but may be insufficient for definite species diagnosis since also other *Streptobacillus* species except *S. moniliformis* are adapted to the rat oropharynx. Contrarily, growth characteristics and classical phenotypic methods and also standardized biochemistry are laborious and also only suitable for genus determination, but do not possess enough discriminatory power to sufficiently differentiate *Streptobacillus* on the species level. Based on additional full genome sequences, the detection of further housekeeping genes will enable the development of new tools like multilocus sequence typing (MLST) or multilocus sequence analysis (MLSA) for both, molecular epidemiology as well as in-depth infra-species resolution and the determination of clonality.[110] Further genetic studies on *Streptobacillus* should also include investigations on possible virulence determinants and differences in pathogenic mechanisms between strains.

Abbreviations

ANI	average nucleotide identity
API	analytical profile index
ATCC	American Type Culture Collection, Rockville, USA
BHI	brain heart infusion
bp	base pair
CIP	Collection of Institute Pasteur, Paris, France
DDH	DNA-DNA homology
DIN	Deutsche Industrienorm (German standard)
DSM	Deutsche Sammlung für Mikroorganismen, Braunschweig, Germany
ELISA	enzyme-linked immunosorbent assay
FISH	fluorescence *in situ* hybridization
FT-IR	Fourier transform-infrared spectroscopy
G/C	guanine/cytosine (contents)
HF	Haverhill fever
IB	immunoblotting
IFA	immunofluorescence assay
IU	international units
kDa	kilo Dalton
L-form	cell wall deficient variant, derived from Lister (L) institute
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MLSA	multilocus sequence analysis
MLST	multilocus sequence typing
MIC	minimum inhibitory concentration
NCTC	National Collection of Type Cultures, London, UK
OD	optical density
PCR	polymerase chain reaction
RBF	Rat bite fever
S.	Streptobacillus
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPS	sodium polyanethol sulfonate
T	type strain
TLR	Toll-like receptor
w/v	mass/volume

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Ethical statements

The authors state that we complied with all of the legal requirements pertaining to the German Cancer Research Center (Deutsches Krebsforschungszentrum) in which animal experiments were done. The procedures were approved by the Ethics Committee of Animal Experimentation in Germany (notification no. A10-02 [Regierungspräsidium Karlsruhe]).

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