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Molecular Approaches in the Detection and Characterization of *Leptospira*

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Abstract

Conventional diagnosis of leptospirosis and characterization of *Leptospira* spp. relies mainly on serology. A major drawback of serology in diagnosis is that it needs sufficient levels of anti-*Leptospira* antibodies, thus jeopardizing confirmation in the early acute phase of disease. The cross agglutinin absorption test (CAAT) that determines *Leptospira* serovars is a technically demanding and laborious method and therefore is only performed at a few laboratories. Novel molecular diagnostic tests mainly rely on the polymerase chain reaction (PCR). PCR perfectly complements serological testing, since it is especially sensitive in the first 5 days after onset of symptoms. Real time PCR is rapid and has been validated for their high clinical accuracy. The introduction of molecular techniques for defining *Leptospira* species revolutionized the categorization of strains in this genus, as species and serogroups appeared to show little correlation. The reference test in molecular speciation is based on determining DNA homology. This approach is tedious and user-unfriendly and therefore is increasingly replaced by other techniques. To date, a wealth of molecular typing methods is available. Most attractive are those techniques that provide direct digital and electronically portable data. Such techniques comprise fluorescent amplified fragment length polymorphism (FAFLP), array typing, multilocus variable number of tandem repeats analysis (MLVA) and sequence-based phylogeny, and to some extent pulsed field gel electrophoresis (PFGE). Multilocus sequence typing (MLST) is the most robust method for determining *Leptospira* strain diversity and in the future will probably only be surpassed by phylogeny on whole genome sequences.

Keywords: *Leptospira*; Leptospirosis; Diagnosis; Characterization; Serovar; Molecular typing; Serological typing; Species

Background

Leptospirosis is a zoonotic disease of global distribution with a whole range of animals acting as carriers or intermediate hosts. It is considered an emerging global public health problem both in local populations and in returning travellers [1]. The disease ranges from mild to lethal courses in its clinical spectrum and probably has a high proportion of sub-clinical and asymptomatic infections. Recently, WHO has established a Leptospirosis Burden Epidemiology Reference Group (LERG) [2] which estimated the mean global burden of endemic human leptospirosis on an incidence of 5 per 100,000 populations, recognizing this as an underestimation because it is based on severe and scarcely notified cases only and does not include epidemic leptospirosis [3]. Leptospirosis was first described by Adolf Weil in 1886 [4] but may already have been reported in 1745 [5]. A typical case of the disease is characterized by sudden onset of malaise, often intense muscular pains, and high fever for several days, followed by jaundice and renal failure and haemorrhages [6]. Weil's name is still attached to a severe form of leptospirosis but at present, it is preferable to refer to all leptospiral infections as leptospirosis regardless of clinical symptoms and signs [7]. Leptospires were first observed in kidney tissue of a patient suspected of yellow fever by silver staining [8]. Later on the organism was found in a filtrate from stagnant water and designated as *Spirocheta biflexa* [9]. At the end of 1914 Inada and co-workers detected the microorganism in the liver of a guinea pig injected with the blood of a patient suffering from Weil's disease and isolated and identified '*Spirochaeta icterohcemorrhagiae*' as the causative agent of the Japanese form of Weil's disease [10]. Noguchi [6] succeeded to isolate the organism from American wild rats, with the pathogen supposed to be identical to Japanese one.

Serological diagnosis and classification

Conventional diagnosis comprises culturing, observation of leptospires by dark field microscopy and several serological tests. Leptospires are fastidious organisms that take a long time to grow. Therefore, culturing is not frequently used and does not contribute to early diagnosis. Dark field microscopy on blood samples from febrile patients is notoriously unreliable and is not recommended as the sole diagnostic test [7]. Several methods detect anti-*Leptospira* antibodies as a sign of infection. The most commonly used tests are the microscopic agglutination test (MAT) and the IgM ELISA [7]. The MAT still has unsurpassed serovar specificity and represents the gold standard in leptospirosis diagnosis. A drawback of serological approaches is that anti-*Leptospira* antibodies only become detectable in the late acute phase of the disease. Hence serological confirmation comes too late for effective antibiotic treatment that should be started as early in the disease as possible. In contrast, most molecular detection methods can be applied in the early acute phase of the disease.

Conventionally leptospires are separated into serovars based on differences of serological features. The serovar is the basic systematic

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unit in serological classification, which is represented by a reference strain [11]. The serological characterization is of diagnostic and epidemiological significance. The MAT performs optimal when executed with a panel of strains reflecting locally circulating serovars, while protection, either by natural infection or through vaccination, is thought to be serovar specific and requires knowledge on local serovars. In addition, serovars might be associated with chronic carrier species. Hence, information on serovars represents an important contribution to the development of prevention and control measures. The cross agglutinin absorption test (CAAT) is the standard assay for establishing the serovar status [11]. Serologically related serovars are placed into serogroups, which do not have a formal taxonomic status. To date, more than 250 pathogenic serovars placed in 25 serogroups of the genus *Leptospira* have been identified [12,13]. In literature, serovars are sometimes referred to as serotypes but the term serovar will be used throughout this paper following the current recommended serological nomenclature [14].

Molecular detection and characterization of leptospires

In the last decades, typing and detection based on molecular techniques have been introduced and applied widely to the field of leptospires. Moreover, the molecular methods were exploited as alternative or supplementary approach to the currently existing serological methods. Molecular characterization separates *Leptospira* into species. Currently 20 species have been recognized consisting of nine pathogenic, five intermediate, five saprophytic and one species, *L. meyeri*, consisting of a mixture of putative pathogenic and saprophytic serovars (Table 1). Previous reviews described molecular methods as part of a general overview or within a broad framework of serotyping, genotyping and, post-genomic typing methods [12,15-18] but a literature review focussed on molecular detection and typing methods has not been presented. In this paper, we review molecular detection and typing methods as well as combined approaches that have been utilized for diagnosis and the identification and characterization of leptospires.

Molecular detection: diagnosis

Dot and in situ hybridization techniques: Molecular detection of leptospires comprises a number of methods including dot and in situ hybridization using labelled whole genome DNA [19–22] or specific DNA segments as probes [23–25]. The main drawback of DNA hybridization is the use of radio-active isotopes as a label. This requires special safety facilities and well-equipped laboratories. Enzymatic staining assays using biotin-labelled DNA hybridization probes form a

No	Pathogenic	Reference	Designation method
1	*L. interrogans*	[91]	DNA relatedness using hydroxyapatite method
2	*L. santarosai*	[91]	DNA relatedness using hydroxyapatite method
3	*L. weilii*	[91]	DNA relatedness using hydroxyapatite method
4	*L. borgpetersenii*	[91]	DNA relatedness using hydroxyapatite method
5	*L. noguchii.*	[91]	DNA relatedness using hydroxyapatite method
6	*L. kirschneri*	[90]	DNA relatedness using quantitative slot blot hybridization method
7	*L. alexanderi*	[93]	DNA relatedness using hydroxyapatite method
8	*L. alstonii** (*L. genomospecies1*)	[93]	DNA relatedness using hydroxyapatite method
9	*L. kmetyi*	[127]	Phylogenetic analysis of the 16S rRNA confirmed by DNA-DNA hybridization
	Non pathogenic		
10	*L. biflexa*	[91]	DNA relatedness using hydroxyapatite method
11	*L. wolbachii*	[91]	DNA relatedness using hydroxyapatite method
12	*L. vanthielii** (*L. genomospecies3)*	[93]	DNA relatedness using hydroxyapatite method
13	*L. terpstrae** (*L. genomospecies4*)	[93]	DNA relatedness using hydroxyapatite method
14	*L. yanagawae** (*L. genomospecies5*)	[93]	DNA relatedness using hydroxyapatite method
	Mixture of putative pathogenic and saprophytic serovars		
15	*L. meyeri*	[91] [170] [128]	DNA relatedness using hydroxyapatite method Multi-lucus sequence based genotyping 16S rRNA gene sequencing and PFGE
	Opportunistic/intermediate		
16	*L. inadai*	[91]	DNA relatedness using hydroxyapatite method
17	*L. fainei*	[121]	Mapped restriction site polymorphisms in the rrs (165 rRNA) gene, Arbitrarily primed PCR, PFGE , PCR specific for the rrs gene of pathogenic leptospires, DNA-DNA and Phylogenetic analysis of 16S rRNA sequences
18	*L. broomii*	[183]	DNA–DNA relatedness, 16S rRNA gene sequence data and PFGE
19	*L. wolffii*	[126]	Phylogenetic analysis of the 16S rRNA
20	*L. licerasiae*	[184]	16S ribosomal RNA gene sequencing, pulsed-field gel electrophoresis, and DNA-DNA hybridization analysis.

* *Leptospira genomospecies* 1, 3, 4 and 5 as renamed according to International Committee on Systematics of Prokaryotes; Subcommittee on the taxonomy of *Leptospiraceae* Minutes of the closed meeting, 18 September 2007, Quito, Ecuador.

Table 1: *Leptospira* species and method of determination.

more practical tool for routine diagnosis [26]. In situ hybridization on tissue samples is a useful approach for determining infection in carriers or confirming leptospirosis in fatal cases but otherwise is not very helpful in diagnosis. Millar et al. [19] successfully applied radioactive labelled whole genomic DNA from serovar Pomona to detect the heterologous serovars Hardjo and Tarassovi in body fluids. Terpstra and co-workers [20,22] modified the in situ DNA hybridization technique of Langer et al. [27] into a method detecting pathogenic *Leptospira* with biotin-labelled genomic DNA as a probe in liver smears, plasma sediment and urine dotted on a nitrocellulose membrane filter. This study was followed up by the use of specific recombinant probes in dot-blot and in situ hybridization assays [28]. Zuerner and Bolin [23,24] cloned a repetitive sequence element from serovar Hardjo type Bovis for use as a diagnostic probe for bovine leptospirosis in North America. These studies together concluded that specific recombinant DNA probes might provide proper tools for routine diagnosis and classification in cases of infections with distinct serovars but have wide applicability for the genus *Leptospira* in general [23–25,28–33].

Nucleic acid amplification techniques: Most molecular diagnosis tests rely on amplification of *Leptospira*-specific nucleic acids from clinical samples that contain leptospires at an early acute phase of the disease. Such methods include isothermal amplification methods the Nucleic Acid Sequence-Based Amplification (NASBA) in the early 1990's [34]. and the recent loop-mediated isothermal amplification (LAMP) [35]. NASBA amplifies multicopy RNA which provides the potential of a high diagnostic sensitivity. The isothermal approach evading the use of expensive and complicated thermal cyclers and the possibility of reading results by eye was used to propagate the method as a simple and affordable diagnostic tool. However, in practice the method appeared inferior to the polymerase chain reaction (PCR) because of the more general use of PCR, its equipment and enzymes as well as knowledge on effective primers and probes were much more advanced. Simplicity and affordability are also used to propagate LAMP as an affordable alternative to PCR [35]. However, like NASBA, LAMP cannot as yet compete with the diagnostic sensitivity and specificity of PCR [36], probably for the same reason; PCR is more widely used and therefore better developed. Speed as well as technical and financial aspects may play a minor role as continuous improvement on PCR equipment and reagents has led to the availability of technically and diagnostically well performing rapid formats at affordable costs. Of note, current DNA extraction methods present the main limitation to the clinical performance of enzymatic amplification approaches. Therefore, more attention should be directed to efficient *Leptospira* DNA extraction methods from clinical material that effectively remove reaction inhibitors and increase the DNA recovery ratio.

The polymerase chain reaction: Conventional PCR: The PCR is a revolutionary technique developed in 1983 by Mullis, which amplifies specific targets of DNA to more than a billion-fold [37,38]. Conventional PCR was introduced as a promising molecular detection method of leptospires in biological materials as early as 1989 [39]. Primarily this method employed a primer pair deduced from genomic libraries by differential hybridization to specifically detect serovar Hardjo type Bovis [39]. The study suggested usefulness of the PCR both for diagnosis and large-scale epidemiological studies. Several other primer pairs have been deduced from genomic libraries [40–42]. Additionally, primer pairs have been deduced from a variety of defined genes, i.e. *rrs* [43,44], *rrl* [45,46], *flab* ([47], *gyrB* [48] and *ompL1* [49]. The introduction of genomics enabled the identification of genes confined to pathogenic *Leptospira* species [50] and hence the design of pathogenic-specific primers. Targets comprised the genes *lig* [51], *lipL32/hap1* [52], *lipL21* and *lipL32* [53,54] and *lipL41* [55]. Other approaches comprised the increase of the sensitivity of the PCR by using primer pairs targeting repetitive elements [56–60], or of both sensitivity and specificity by applying nested PCR primers [61,62] or using a subsequent southern hybridization step with a specific internal [32,39,42,44]. Whereas a multitude of conventional PCRs have been described, only few have been subjected to clinical evaluation at a limited scale. The PCR with combined primer sets G1/G2 and B64-I/B64-11 [42] was compared to culturing on blood and urine samples from 71 cases with acute leptospiral infection [63]. PCR was more sensitive (62%) than culture (48%). Additionally a *rrs*-based PCR [64] was evaluated in two separated studies with MAT as the reference test using clinical samples from 200 and 90 patients with suspected leptospirosis, respectively [65,66]. The study on 90 cases found 14 patients positive PCR, supported by a seroconversion in the follow up samples [66]. However, it was not clear whether additional PCR positive scores, not supported by a positive serology, were due to a more sensitive PCR or should be attributed to (cross) contaminations. In the other study, PCR was positive in only 44% of MAT confirmed cases and again the significance of PCR positive scores in serological negative samples is unclear [65]. Both the limited evaluations itself and the sub-optimal performance of the PCR as revealed by these evaluations have left the value of conventional PCR for the laboratory diagnosis unclear. A major drawback of the conventional PCR is that it is particularly prone to contamination, which easily leads to false positive outcomes [62,67].

Real-time PCR: Real-time (rt) PCR refers to PCR-based amplification of DNA that is monitored during the amplification process. It uses several types of dyes and probes for the detection of amplification. The most available formats are TaqMan probes, Molecular Beacons, Scorpions, Light Upon eXtension technology (LUX) and SYBR Green 1 dye. In all of these chemistries, PCR products are detected by generation of a fluorescent signal. TaqMan probes, Molecular Beacons and Scorpions rely on Förster Resonance Energy Transfer (FRET) in which energy transmitted by a "donor fluorophore" (the reporter) is absorbed by a nearby "acceptor" (the quencher) present on the probe but the mechanisms of releasing the fluorescent signals are different. In case of TaqMan probes the quencher becomes separated from the reporter when the 5' nuclease activity of the Taq polymerase enzyme degrades the annealed probe during amplification. Molecular beacons are hairpin shaped oligonucleotides bound to a fluorescent reporter and a quencher at the opposite ends. When the hairpin is intact, the reporter and quencher dyes are proximal to one another, preventing FRET. During annealing and amplification, the hairpin opens thus separating reporter and quencher. LUX is much similar to molecular beacons but uses one self-quenching fluorogenic primer labelled with a single fluorophore close to a fluorescence quenching hairpin structure proximal to the 3' end. Upon extension of the primer during annealing, the hairpin loop is stretched, resulting in fluorescence emission [68]. SYBR Green is an intercalating agent, which emits a strong fluorescent signal upon binding to double-stranded DNA. Accumulation of double stranded DNA thus results in an increasing signal.

SYBR Green and LUX-based rtPCRs merely depend on specific primer annealing for generating amplicon-specific fluorescence signals, whereas the probe-based chemistries gain specificity by additional specific probe annealing. Dye and LUX-based rtPCR therefore need subsequent melting curve analysis to determine the specific melting temperature Tm of the product. This has the advantage that this Tm

to some extend enables speciation [69]. For probe-mediated rtPCRs, several distinct fluorophores are available as reporter allowing the execution of multiplex rtPCRs targeting several loci in one reaction. rtPCRs have been introduced into the field of leptospires as a rapid and sensitive alternative to conventional PCR methods. These rtPCRs target a variety of genes, including rRNA genes, housekeeping genes, specific leptospires sequences and genes confined to pathogenic *Leptospira* [45,48,51,67,69-72,74-77] (Table 2). A few of these have been validated, Slack et al. [70] modified and evaluated a previous *rrs*-based Taqman rtPCR [71] against culture and *Leptospira*-specific IgM enzyme-linked immunosorbent assay (ELISA) on patient sera. This rtPCR was later on compared with a *lipL32*-deduced Taqman rtPCR [73,74]. In addition, a *secY*-based SYBR Green PCR was evaluated against culture and serology [67] following the OIE protocol [78]. All validations indicated a high diagnostic accuracy of rtPCRs on blood samples at the early acute phase of the disease, which is most relevant for clinical decision-making.

Molecular characterization of *Leptospira*

DNA-DNA relatedness technique: Initially, the genus *Leptospira* [79] was defined by morphological and cultural characteristics (Box 1). The genus was conventionally separated into *L. interrogans* and *L. biflexa* for pathogenic and saprophytic species, respectively [7], based on animal tests and phenotypic features [80]. To date, *Leptospira* spp. are assigned to DNA homology categories determined via DNA-DNA hybridization as the gold standard. The hybridization method has been applied for a variety of micro-organisms [81] and was introduced into prokaryote systematics from the 1960s onwards by various groups [82–87]. Haapala and co-workers [88] were the first to explore the use of DNA-DNA hybridization techniques as a speciation method for the genus *Leptospira*. They employed the DNA thermal elution technique

Method	Target sequence	Reference
Fluorescence resonance energy transfer	*rrl* gene	[45]
SYBR Green 1 dye	*gyrB* gene	[48]
SYBR Green 1 dye	*LipL32* gene	[72]
SYBR Green 1 dye	locus LA0322 of serovar Lai	[69]
SYBR Green 1 dye	*rrs* gene	[76]
SYBR Green 1 dye	*secY* gene	[67]
TaqMan Probe	*23S* rRNA gene	[75]
TaqMan Probe	*ligA* and *ligB* genes	[51]
TaqMan Probe	*LipL32* gene	[73]
TaqMan Probe	*rrs* gene	[71]
TaqMan Probe	*rrs* gene	[70,71]
Light Upon eXtension technology (LUX)	*lipL32* gene	[77]

Table 2: Real-time PCRs for leptospires detection.

Box 1. Morphology and cultural characteristics of genus *Leptospira:*

Flexible, helicoidal organisms usually 6–20µm long, 0.1µm diameter; one or both ends hooked or straight; not visualised by direct light, but by darkfield microscopy; not easily stained. There are two subterminal periplasmic flagella, one at each end. G+C% ratios 35.3–43.4mol%. Characteristic motility. Aerobic and microaerophilic; utilise ammonium salts as a nitrogen source, unsaturated fatty acids as a carbon source, and purines. Growth optimum pH 7.2–7.6, at 28–30°C. Optimum growth in 6–14 days, but variable, in liquid media; subsurface slow colonial growth. Strains may be parasitic or free living [80].

Box 2. Criteria of genomic speciation of *Leptospira*

Five parameters can be used to genetically define a species: (i) relatedness at conditions optimal for DNA re-association, (ii) relatedness at conditions less than optimal for DNA re-association (at which only highly complementary sequences can re-associate), (iii) divergence in related nucleotide sequences, (iv) genome size, and (v) G+C content of DNA. The first three parameters are exclusive; e.g., if strains of a given species are 90% inter-related, they cannot be equally related to any other species, or if their related sequences show 2% divergence, they must exhibit a greater level of divergence to all other species. The last two parameters are not exclusive [92].

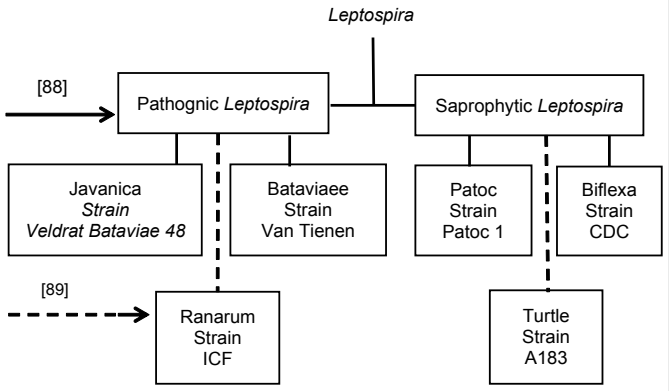

Figure 1: Early speciation by DNA-DNA hybridization. The first applications of DNA-DNA hybridization techniques as speciation method for genus *Leptospira* resulted in four genetically distinguished groups comprising pathogenic and saprophytic *Leptospira* [88], subsequently supplemented with other two groups [89].

to measure the DNA homology on the basis of duplexes trapped in agar. This was followed up by Brendle et al. [89] and Ramadass et al. [90], utilizing hybridizations on membrane blots to determine differences in hybridization potential between strains and species. Yasuda et al. [91] and Brenner et al. [92] employed the hydroxyapatite (HA) column approach and applied the three exclusive parameters to genetically define a species as proposed previously (Box 2). The introduction of hybridization techniques into *Leptospiraceae* had a strong impact on its taxonomy because the genomic characterization showed little correlation with the existing serological classification of serovars and serogroups. As an initial consequence the genus *Leptospira* was separated into four genetically distinguished groups [88], supplemented by two additional species [89] (Figure 1). However, the major contribution to molecular speciation came from the groups presented by Brenner et al. [93]. Yasuda et al. [91] and Ramadass et al. [90] dividing *Leptospira* into 15 species. Additional species have been identified in separate studies applying *rrs* sequencing and phylogeny either or not combined with DNA hybridization or other molecular approaches (Table 1). To date, *Leptospira* are separated into 20 species (Table 1) grouped into pathogenic species, with *L. interrogans* as a major species, intermediate species with unclear pathogenic status, and saprophytic species, including *L. biflexa* consisting of only 3 serovars [94]. DNA hybridization is a complicated method that requires the use of considerable amounts of isotope-labelled DNA of high quality. Therefore, its application is currently limited to a single laboratory. As a consequence, other techniques, notably single or multilocus sequence analysis are gaining importance as molecular tools for the speciation of *Leptospira*.

Bacterial restriction-endonuclease DNA analysis: Bacterial restriction-endonuclease DNA analysis (BRENDA) is a technique that

generates a characteristic agarose gel pattern of DNA fragments after electrophoresis of DNA cleaved into distinct fragments by a restriction endonuclease. Marshall et al. [95] were the first to demonstrate the usefulness of this method for leptospires characterization in 1981 rapidly followed by others to test several serologically related and cross-agglutinating serovars. The most appealing example is probably the demonstration of marked genetic differences via distinct BRENDA patterns of serovar Hardjo, strain Hardjoprajitno, isolated from man compared to isolates of serovar Hardjo from cattle in New Zealand [96]. Separation of serovar Hardjo into the two species *L. interrogans* (Hardjo type Prajitno) and *L. borgpetersenii* (Hardjo type Bovis) has been confirmed by several other studies and is a well-accepted fact to date. In another study BRENDA was used to challenge and refute the hypothesis of Yanagawa and Takashima [97] stating genetic rearrangements can change serovar Hebdomadis into Kremastos under pressure of the immune system [98]. Exposure of serovar Hebdomadis to immune serum did not result in marked changes of DNA features towards those of Kremastos [98]. BRENDA applied on serovar Pomona isolated from animals in USA, Canada and New Zealand revealed homology with Pomona strain Kennewicki but was distinct from the reference strain Pomona [99], implying that Kennewicki presents a subtype of Pomona. In addition, it was shown that BRENDA patterns of isolates from North America did not concord with their serovar nominations in the serogroups Mini and Sejroe [100]. Ellis et al. [101] used 20 restriction enzymes for BRENDA on pig isolates belonging to the Australis serogroup and were able to show differences at the subserovar level for both serovars Bratislava and Muenchen. They concluded that BRENDA results are more consistent with epidemiological observations than features based on serology. In spite of the application of BRENDA contributed much to the understanding of the molecular epidemiology of leptospirosis, its difficult interpretation and standardization of profiles, hampering exchange of results between laboratories and the need of large quantities of high quality genomic DNA, has hampered a wide application.

Southern blot hybridization: Complex BRENDA profiles can be simplified by applying subsequent Southern blotting. The degree of simplification depends on the specificity of the hybridization probe. Various probes have been used for genotyping of leptospires, comprising amongst others radioactive labelled *Eco*R I digested genomic DNAs [102], pathogen specific recombinant probes selected by differential hybridization [32] and repetitive elements [30,57,103,104]. Classification of *Leptospira* strains and isolates by Southern blotting largely corresponded to those established by the standard DNA homology determination methods [88,89,93,105] and, like BRENDA, confirmed the divergence between molecular and serological typing [32,98]. Thus observation of strongly limited numbers of restriction enzyme generated DNA fragments in fingerprints by southern blotting appeared an attractive approach for the typing of *Leptospira*.

Ribotyping: Basically, ribotyping is a southern blotting method as described above, albeit that the method uses a general probe that has been deduced from a homologous rRNA coding sequence. Ribotyping is based on the concept that the conserved nature of rRNA genes allows the use of a single probe for typing bacteria of any phylogenetic position [102,106–110]. Both *Escherichia coli rrs* and *rrl* deduced biotin-labelled probe and digoxigen-labeled *rrs* and *rrl* probes from *L. interrogans* serovar Icterohaemorrhagiae have been used for ribotyping on *Leptospira* [106,111,12]. Bioinformatics software was employed to determine the sizes of the fragments. The restriction patterns clustered the leptospires serovar into separated species that correlated with DNA homology classes [91] but, as to be expected, did not concord the serogroup concept with an exception for serogroup Ballum [112]. However, the method showed limitations since it did not distinguish several serovars [112,113]. The relatively simple banding profiles, good reproducibility and possibility for use of an automatic system are advantages of this method [113].

Sequencing and phylogeny on rRNA encoding genes: The introduction of the sequence-based determination techniques has made a tremendous contribution to the effort of elucidating the evolution of micro-organisms and, specifically, contributed to new insights into the molecular epidemiology of leptospires and the taxonomy of *Leptospira*. In a general study a comparative analysis of 16S rRNA sequences was used to explore prokaryote phylogeny [114], ^{32}P-labelled 16S rRNA from several genera and species was digested with ribonuclease T1 and the resulting oligonucleotides were resolved by two-dimensional paper electrophoresis and then sequenced. Phylogeny revealed that spirochetes represented one of the most ancient branches of eubacteria with *Leptospira* forming one of the deepest evolutionary clades within the spirochete branch [115,116]. Remarkably, there were only one or two copies of the rRNA encoding genes in *Leptospira* that were not organized in an operon [117-119]. The unique feature of rRNA genes within *Leptospira* may indicate an important feature in the evolution and molecular classification of leptospires. Using *rrs* sequences it was shown that within the family of Leptospiraceae *Leptospira parva incertae sedis* (now *Turneriella parva H*) and *Leptonema illini* form distinct genera from *Leptospira*, while *Serpulina hyodysenteriae* and *S. innocens* (now genus *Branchyspira*) have been placed in the family Spirochaetaceae together with *Treponema* and *Borrelia* [120]. The genus *Leptospira* was presented in three major clades comprising pathogenic, intermediate and saprophytic species [120–122]. Although currently several, more variable genes are targeted for *Leptospira* phylogeny, the *rrs* gene is the most commonly used one to date [120–128].

Pulsed-field gel electrophoresis: Pulsed-field gel electrophoresis (PFGE) has been described as an agarose gel electrophoresis system utilizing perpendicularly oriented, non-uniform, alternately pulsed electrical fields [129]. This technique is suitable for large DNA fragments, separating them with greater resolution than conventional electrophoresis. PFGE has been used to study the size of the *Leptospira* genome consisting of a large and a small replicon, with sizes varying from 3.1 to 5 Mb and 0.35 Mb, respectively depending on study or species [130-134]. By physical mapping, it was shown that both the large and small replicons contain essential genes, suggesting that these are chromosomes. Considerable re-arrangements and consequent intraspecies differences in *L.interrogans* became evident [134]. These early observations have now been confirmed by available genome sequences of *L. interrogans*, *L. borgpetersenii* and *L. biflexa* [50]. In the early 1990's, PFGE was explored as a tool for *Leptospira* [135,136]. The technique, when applied on a panel of serovars from most serogroups, appeared to generate profiles that coincided with the distinct serovars, with few exceptions. Additionally, PFGE successfully confirmed the serovar status of several isolates from humans and animals. Almost two decades later, the validation and application of a modified PFGE assay in *Leptospira* reference strains and clinical isolates was reported [137,138]. The modifications included the use computer software for fragment pattern analysis and the creation of a database. It was suggested that application of a standardized procedure followed by submission of digital images to central site for normalization and evaluation would enable analysis without shipping strains between laboratories. PFGE has been advocated as a suitable

adjunct or even substitution to serotyping [136,138]. However, a word of caution should be at place. The serovar is mainly determined by the composition and structure of LPS located at the outer face of the cell wall. A general genomic approach like PFGE using the high plasticity of the *Leptospira* genome might generate information that generally coincides with the serovar status but cannot replace serological serovar identification. Moreover, not all serovars can be distinguished by the method [135,136] while others are separated into distinct (genome-based) species [17]. Reliance on PFGE alone might thus cause incorrect serovar identification. Only a molecular tool using polymorphisms in LPS coding DNA would justify its claim as substitute to the tedious serological serovar determination. However, attempts to achieve this have only been partially successful to date [139,140].

Application of PCR-based techniques for typing

PCR-based techniques were utilized by many researchers to categorise leptospires or to analyse genome compositions. The technique is either coupled to subsequent sequencing of the amplicon or accompanied with other molecular techniques including Restriction Fragment Length Polymorphisms (RFLP), Low Stringency Single Primer PCR (LSSP-PCR), Single Strand Conformation Polymorphism analysis (SSCP) and hybridisation with specific labelled probes. PCR-based typing methods can be divided into two groups, i.e. (i) PCR for characterization and (ii) PCR combining diagnosis and characterization.

(i) PCR for characterization: For characterization of *Leptospira* a variety of approaches have been described, including the use of repetitive elements [60,141–143], insertion elements [58,144–146] and restriction enzyme fragmented DNA as amplification targets [147–149] as well as arbitrarily primed PCR (AP-PCR) and randomly amplified polymorphic DNA (RAPD) of whole genomic DNA [29,150–155].

Amplification of repetitive elements for example differentiated serovar Hardjo type Bovis from Hardjo type Prajitno [60] and it was suggested that the variations in pathogenicity, host range, serology and biochemistry between these two types of serovar Hardjo might be related to the mutational effects of the repetitive element but proof was not provided. The *Leptospira* genome contains multiple copies of distinct insertion elements with highly variable distribution [156]. Application of PCR with outwards directed primers on insertion elements may develop fingerprints that characterize serovars [58,144]. On one hand, this method has the advantage of enabling typing of *Leptospira* serovars in clinical material without the need of isolation bacteria from pure culture. On the other hand, too few strains and isolates have been investigated to judge on its general applicability within *Leptospira*.

Amplified Fragment Length Polymorphisms (AFLP) either or not combined with fluorescent-labelled primers (FAFLP) cleaves genomic DNA with distinct restriction enzymes. Subsequently, restriction fragments are bound to adapters and amplified with primers matching the adapters. The products are then electrophoretically separated on polyacrylamide gels and analysed by eye or by appropriate software. The technique can be applied on any organism because it does not require prior knowledge of the DNA composition. The technique has been employed in the epidemiological typing of many Gram-positive and Gram-negative bacteria [157] and has been applied in three studies on *Leptospira* [147–149]. The method appeared less suitable for studying pathogen evolution of the genus [147] but appeared well applicable for studying clonality in regionally confined outbreaks [147,149]. Single nucleotide polymorphisms (SNPs) are highly attractive targets for *Leptospira* characterization. SNPs were used in a simple one tube typing array system, making use of a multiplex ligase-depended probe amplification (MLPA) in which the distinct products are detected via hybridization to specific probes bound to a solid support [158]. The assay could discriminate between saprophytic, intermediate, and pathogenic species. Additionally, it enabled unambiguous detection of strains of seven pathogenic species and revealed discrepancies in previous speciation and culture collections. The method is promising as it is affordable and generates electronically portable digital data and can further developed when more *Leptospira* sequences become available for identifying additional SNPs.

Numerous papers mention the use of AP-PCR on *Leptospira* [154,155,159]. AP-PCR may include the use of labelled primers for easy analysis [29]. The method is useful, but claims on its applicability for identification of serovars and subtypes [155] need to be seen with caution. The approach suffers from a main drawback in that it requires high quality DNA, that it is difficult to standardize and that it does not directly generate digital data that can be exchanged via internet [152].

(ii) Combined detection and characterization: PCR can amplify target DNA at several levels of specificity, mainly depending on the choice of primers or probes and thus enables combining the detection of leptospires with their identification. Murgia et al. [43] and Woo et al. [45] applied PCRs based on *rrs* and *rrl* sequences, respectively, to discriminate between pathogenic and saprophytic *Leptospira*. Both studies used two primers sets; one set specifically annealing to DNA from pathogenic leptospires and the other set specifically binding to saprophytic DNA. In addition, various *rrs*-based PCRs have been presented that could detect leptospires at the genus or species level [43,44,64,160]. Reitstetter et al. [49] suggested seven species-specific primer sets, deduced from *ompL1* to detect and differentiate leptospires by conventional PCR. Combined detection and typing can also be achieved with a single primer such as iRepI [143]. iRepI was deduced from a repetitive element of serovar Copenhageni and provisionally seemed to enable differentiation of serogroups.

In most of the other approaches, PCR products detecting leptospires in clinical samples are used for subsequent characterization of the infectious agent. Often this is done by sequencing and subsequent phylogeny but other methods include the analysis of mapped restriction site polymorphisms (MRSP, alternatively referred to as PCR-RFLP) in PCR-amplified loci genes [121,153,161], PCR-SSCP [151,162,164], differential electrophoretic mobility of amplicons on non-denaturing polyacrylamide gels [164] and LSSP-PCR [165]. LSSP-PCR applies low-stringency PCR on the diagnostic amplicon using a single diagnostic primer, thus generating strain-characteristic fingerprints on polyacrylamide gels. Both PCR-SSCP and LSSP-PCR produced reliable results, consistent with standard speciation and serovar determination, but in contrast to PCR-RFLP, are too complicated and laborious for a wide application.

Multiple-locus variable number of tandem repeats analysis

Multiple-locus variable number of tandem repeats analysis (MLVA) is a distinct PCR format using repeats enabling a simple and rapid method for categorization of strains and isolates. However, it requires knowledge of variable number of tandem repeats (VNTR) present on the genomes of the microorganisms under investigation. Currently, the genomes of only four pathogenic strains of *L. interrogans* and *L. borgpetersenii* have been sequenced (see section Whole genome

sequencing), largely limiting the applicability of the method to few closely related *Leptospira* species [149,166]. Majed et al. [167] and Salaün et al. [168] developed MLVA for analysing amplified fragments of strains from *L. interrogans*, *L. kirschneri* and *L. borgpetersenii* by agarose gel electrophoresis. The validation of the method on clinical isolates suggested that MLVA is useful for epidemiologic investigations of leptospirosis in regions of high endemicity. Another independently developed MLVA utilizing a combination of primers bound to distinct multi-coloured dyes and pooled capillary electrophoresis [148,169] demonstrated the usefulness of this method in the molecular epidemiological study of *Leptospira interrogans* serovar Australis in Queensland, Australia. An automated MLVA with labelled primers was applied on *L. interrogans* to study genetic affinities and ancestral origins among *Leptospira* strains [147]. It discriminated all serovars and grouped them well according to the serogroups represented in this species and might thus be useful for serovar prediction. Zuerner et al. [166] identified a unique VNTR marker for analysis of isolates from California sea lion presumably identified as serovar Pomona. All isolates shared a common VNTR profile despite temporal and spatial separation and were distinct from other isolates of serovar Pomona included in the study, suggesting that Californian sea lions are a maintenance host of *L. interrogans* serovar Pomona. This shows that MLVA is a powerful tool for epidemiological studies. However, it has drawbacks such as the subjective judgement by eye of fragment sizes when using agarose gel electrophoresis [167], limitation to a few *Leptospira* spp., or otherwise, the need for large panels of amplification primers. Similar to FAFLP, MLVA is not suitable for exploring global epidemiological investigations but might be well-applicable to study regional epidemiology and clonality in outbreak situations.

Multilocus sequence analysis

Multilocus sequence genotyping or multilocus sequence typing (MLST) is a highly robust and efficient method in identifying ancestral relationships and segregating outbreak associated strains according to their genome species status. The use of multiple loci avoids misinterpretations because of horizontal DNA transfer [50,156,170,171] that might be frequent in *Leptospira*, to date; five such schemes are available for *Leptospira*. The first one published employed six loci on three housekeeping genes, two genes encoding outer membrane proteins and *rrs* [172]. This scheme comprises all pathogenic *Leptospira* spp. and beyond and has made a database with sequences of nearly 300 strains available for off-line phylogenetic analysis. This scheme was applied in a large study on genetic affinities within a large global collection of *Leptospira* strains representing global dispersal and corresponding to a diverse array of hosts and compared MLST with FAFLP and MLVA. MLST was found to be a superior tool for gaining insights into the evolution and phylogeographic affinities of leptospires. One other scheme covering all pathogenic species addressed 4 loci within the *spc-S10-α* locus, encoding ribosomal proteins [170]. This scheme was not intended for epidemiological purposes but merely served the confirmation of the conserved nature of the operon within the genus *Leptospira*. The study revealed a high discriminative power of *secY*, one of the loci included in its scheme, and enabled the reclassification of two reference strains, i.e. strain H6 of the intermediate species *L. inadai* was reclassified as *L. interrogans*, while strain ICF of the saprophytic species *L. meyeri* showed unambiguous pathogenic features [170]. Three other schemes are limited to *L. interrogans* and the closely related *L. kirschneri*. One scheme using a super locus composed of 4 loci, including two that coded for surface proteins, had only been tested *in silico* [173]. It demonstrated correct speciation for 34 of 38 pathogenic *Leptospira* strains. Leon and coworkers [174] based their genotyping method on 7 housekeeping genes. The method was only used in a small study in which the species identity of 50/51 isolates was confirmed and lacks a website, thus hampering a wide application.

In contrast, the third MLST scheme is placed on a publicly accessible website [175]. It uses 7 loci from housekeeping genes that are distributed around the genome and were not under positive selection. This 7 loci scheme was successfully applied to demonstrate the clonality of a leptospirosis outbreak in Thailand [175]. A recent joint study compared the two major MLST schemes on a set of strains from *L. interrogans* and *L. kirschneri* [176]. The comparison revealed a similar good performance of both schemes. Of note, the stringent use of housekeeping genes is advocated in the philosophy of a 'true' MLST as neutral loci provide the best estimates of the true relationships between strains. Remarkably, however, none of the 6 loci in the genus-wide scheme, including *lipL41* and *lipL32* genes encoding surface proteins were under positive selection suggesting their suitability as neutral loci in MLST.

Whole genome sequencing

During the last decade, several complete genome sequences of *Leptospira* species were published and some of their genetic features were studied in detail providing valuable information of *Leptospira* species genetic composition, and facilitating several sequence based molecular typing methods. These studies comprise the genomes of *L. interrogans* serovar Lai [177], *L. interrogans* serovar Copenhageni [178,179], the genome sequencing of two serovar Hardjo type Bovis strains L550 and JB197 of *L. borgpetersenii* [156], and the genome sequence of *Leptospira biflexa* [50]. *L. interrogans* genome data varied widely from pathogenic spirochetes, *Treponema pallidum* and *Borrelia burgdorferi*, although they show considerable similarities in the genes that are responsible for their unique morphological features. Comparative genomics revealed both overall genetic similarities and significant structural differences, including a large chromosomal inversion and extensive variation in the number and distribution of insertion sequence elements, thus confirming genome plasticity as suggested by several previous studies [161,170,171,180,181]. The genomes of *L. borgpetersenii* showed approximately 700 kb reduction compared to *L. interrogans* and revealed substantial differences in genetic content and organization. It also suggested that *L. borgpetersenii* is undergoing a process of insertion sequence (IS)-mediated genome reduction and might be evolving towards dependence on a strict host-to-host transmission cycle.

Obviously, the availability of whole genome sequences enables unsurpassed detailed studies on pathogen evolution and distribution. Surely the latest generation sequencing platforms is expected to increase the accessibility to whole genome sequencing and, hence, whole genome typing.

The future of *Leptospira* genotyping

The MLST technique probably is the most robust and useful tool to assess *Leptospira* strain diversity as well as the taxonomic organization and accuracy of the reference collections. The technique will significantly contribute to gaining insights into the evolution and phylogeographic affinities of leptospires. However, current multiplicity of available schemes leads to confusion in the field of leptospirosis researchers. For that reason, efforts have been joined to come to a single generally supported website based MLST scheme.

Currently, MLST presents the most important tool in *Leptospira* genotyping and may only be bypassed by whole genome sequence analysis.

Conclusion

Conventional diagnosis of leptospirosis and characterization of *Leptospira* strains relied mainly on serology, with several drawbacks, such as too late confirmation of the disease and complicated and laborious typing procedures. Molecular approaches have greatly contributed to a revolution in both *Leptospira* detection and characterization. Currently well-validated rtPCR assays are available that can confirm leptospirosis in the early acute stage of illness. Molecular characterization complements the conventional serological one but has a large variety of techniques that are often easy to apply in a broad field. Current speciation is based on DNA homology studies. However, the technique is too complicated and demanding and therefore is increasingly substituted by others, often in a combined approach [121,126–128,182-184]. Multilocus sequence analysis is the most robust method for determining *Leptospira* strain diversity that might only be surpassed in the future by phylogeny on whole genome sequences.

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