Reclassification of *Leptospira meyeri* serovar Perameles to *Leptospira interrogans* serovar Perameles through serological and molecular analysis: evidence of a need for changes to current procedures in *Leptospira* taxonomy

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It has been recognized that there is heterogeneity among *Leptospira* isolates in culture collections worldwide, causing confounding results for researchers utilizing these organisms; one such culture is *Leptospira meyeri* serovar Perameles. The serovar reference strain Bandicoot 343 was previously identified to the species level by DNA–DNA hybridization; however, subsequent published studies demonstrated results that contradicted the initial speciation. In this study, initial serological testing was performed with isolates from the culture collections of the Centers for Disease Control (CDC), Atlanta, USA (strain Lepto0214), and the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia (strain Bandicoot 343), and the original serovar Perameles hyperimmune antiserum produced in 1964. The results indicated that strain Lepto0214 was not serologically reactive to the antiserum. However, further investigations revealed an alternative serovar Perameles strain held in the CDC collection (Lepto0213) that yielded titres against the antiserum. 16S rRNA gene sequencing of the three strains revealed that Lepto0214 had significant sequence similarity with previously sequenced *L. meyeri* strains; however, strains Lepto0213 and Bandicoot 343 had significant similarity with *Leptospira interrogans* strains. 16S rRNA gene sequencing results were confirmed by pulsed-field gel electrophoresis; Lepto0214 had a pattern similar to that of *L. meyeri* serovar Hardjo strain Went 5, and the pattern differed significantly from those of Lepto0213 and Bandicoot 343. This research provides evidence for the reclassification of serovar Perameles from *L. meyeri* to *L. interrogans*. This reclassification highlights a need for changes to how reference *Leptospira* serovars are identified, disseminated and stored, with the aim of reducing heterogeneity of reference strains between culture collections.

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Abbreviations: CAAT, cross-agglutination absorption test; MAT, microscopic agglutination test; PFGE, pulsed-field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of *L. interrogans* serovar Perameles strain Bandicoot 343 and the 16S rRNA gene sequences of *L. interrogans* strain Lepto0213 and *L. meyeri* strain Lepto0214 are AB368964–AB368967, respectively.

A 16S rRNA gene sequence alignment and a neighbour-joining tree based on the alignment are available as supplementary material with the online version of this paper.

INTRODUCTION

Leptospirosis is the zoonotic disease caused by members of the genus *Leptospira*. It is a widespread disease with a global distribution, with large outbreaks occurring in both developed (Morgan *et al.*, 2002; Slack *et al.*, 2006a) and undeveloped (LaRocque *et al.*, 2005; WHO, 1999) countries. The disease is transmitted by direct contact with infected animal body fluids or by indirect contact with contaminated water or soil (Levett, 2001).

Members of the genus *Leptospira* are helical spirochaetes, of which there are 20 recognized species as delineated by

DNA–DNA hybridization (Brenner *et al.*, 1999; Levett *et al.*, 2006; Matthias *et al.*, 2008; Perolat *et al.*, 1998; Slack *et al.*, 2008, 2009; Yasuda *et al.*, 1987). 16S rRNA gene phylogenetic analysis of *Leptospira* species has resulted in the broad classification of the species into pathogenic, saprophytic and intermediate species (Perolat *et al.*, 1998). *Leptospiras* are also divided serologically through the relatedness of cell antigens using the cross-agglutination absorption test (CAAT); over 200 serovars have been described for the genus.

Leptospira serovar Perameles strain Bandicoot 343 was isolated in 1958 from the bandicoot species *Perameles nasuta* in far-northern Queensland, Australia. It was later described by Wolff & Bohlander (1964) and subsequently characterized by factor analysis, which confirmed its unique serovar status (Kmetz, 1977; Wolff & Bohlander, 1964). Serovar Perameles was assigned to the species *Leptospira meyeri* by DNA–DNA hybridization using isolates taken from the Centers for Disease Control (CDC) *Leptospira* culture collection (Brenner *et al.*, 1999). 16S rRNA gene sequencing found significant nucleotide differences in three strains tested from different culture collections (the CDC, the Institut Pasteur, France, and the University of Trieste, Italy), suggesting heterogeneous distribution of this serovar (Postic *et al.*, 2000). More recently, Smythe *et al.* (2002) described the use of a specific TaqMan probe to detect pathogenic *Leptospira* species and found that the *Leptospira* serovar Perameles strain from the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Australia, produced an amplification product, whereas other *L. meyeri* strains did not, leading to some confusion as to the taxonomy of this serovar (Smythe *et al.*, 2002). In this paper, we report the recharacterization of the serovar into its appropriate taxonomic position using standard serological techniques with confirmation using 16S rRNA gene sequencing and pulsed-field gel electrophoresis (PFGE).

METHODS

Serovar Perameles strain Bandicoot 343 isolates were sourced from the culture collections of the CDC (strains Lepto0213 and Lepto0214)

and the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis (WHO Reference Centre) (strain Bandicoot 343). For further strain histories, refer to Table 1. All isolates were cultured aerobically in Ellinghausen McCullough Johnson Harris (EMJH) medium (Difco) at 30 °C. Hyperimmune antiserum against serovar Perameles was prepared in rabbits using a method described previously (Graves & Faine, 1970).

Cultures were prepared for DNA extraction using a method described previously (Slack *et al.*, 2006b) and DNA was extracted using the ChargeSwitch gDNA bacteria mini kit (Invitrogen) according to the manufacturer's instructions. PCR amplification and sequencing of the 16S rRNA gene was performed using a method described previously (Slack *et al.*, 2006b, 2007). Confirmation of the 16S rRNA gene sequence-based speciation was performed using a PCR methodology targeting the DNA gyrase subunit B gene (*gyrB*), which has been shown to be specific for pathogenic *Leptospira* strains (Slack *et al.*, 2006b). Cycle-sequencing products were purified using the sodium acetate/alcohol precipitation method according to the manufacturer's instructions (Applied Biosystems). Purified products were forwarded to the Griffith University DNA Sequencing Facility (Brisbane, Australia) for capillary electrophoresis using an ABI 3130xl instrument. The sequences were assembled and trimmed to a minimum of two contiguous sequences using the Vector NTI software (Invitrogen). The sequences from the three strains and 20 representative sequences from the genus *Leptospira* were aligned with CLUSTAL W (Thompson *et al.*, 1994). The DNA sequence alignment is available as Supplementary Fig. S1 in IJSEM Online. Estimates of evolutionary divergence between sequences were made using the Jukes–Cantor method in MEGA4 (Jukes & Cantor, 1969; Tamura *et al.*, 2007). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 1278 positions used in the 16S rRNA gene sequence analysis. A phylogenetic tree was constructed from the aligned 16S rRNA gene sequences using the neighbour-joining method (Saitou & Nei, 1987), bootstrapped 1000 times, and was rooted using *Leptonema illini* serovar Habaki strain Habaki^T as an outgroup (Supplementary Fig. S2).

PFGE analysis was performed as described previously (Galloway & Levett, 2008). Briefly, late-exponential-phase cultures were centrifuged to concentrate the bacteria used to prepare the agarose plugs. Agarose plugs were digested with 30 U *NotI* restriction enzyme at 37 °C for 2 h. Plug slices containing digested DNA were placed in the wells of a 1 % agarose gel and electrophoresed in a Bio-Rad CHEF Mapper XA or CHEF-DRIII apparatus for 18 h at 14 °C with recirculating TBE buffer under the following conditions: 2.16 s initial switch time, 35.07 s final switch time, 120° angle, 6.0 V cm⁻¹ gradient, linear ramping factor. *Salmonella enterica* serovar Braenderup strain H9812 was digested with 50 U *XbaI* for use as a

Table 1. Strain histories of the isolates used in this study

Sources: CDC, Bacterial Zoonoses Branch, Coordinating Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA; WHO, WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Western Pacific Region, Brisbane, Australia.

Strain	Source	History
Bandicoot 343	WHO	Reference strain from the original isolation maintained as part of the reference centre culture collection. All WHO hyperimmune antisera were raised against this strain and confirmed using the original hyperimmune antiserum from 1964.
Lepto0213	CDC	Serovar Perameles strain not in use at CDC but stored at –70 °C since 1976. Origin of isolate unknown.
Lepto0214	CDC	Operating as the current reference strain for serovar Perameles at the CDC and used for species determination by DNA–DNA hybridization (Brenner <i>et al.</i> , 1999). All CDC hyperimmune antisera were raised against this isolate. Origin of isolate unknown.

DNA size standard (Hunter *et al.*, 2005). The gel was stained with ethidium bromide following PFGE and photographed under UV transillumination (Fig. 1). Gel analysis and determination of Dice band-based coefficients were performed using the BioNumerics software (Applied Maths).

RESULTS AND DISCUSSION

Initially, microscopic agglutination test (MAT) analysis was performed using hyperimmune antiserum against serovar Perameles prepared in 1964, which is considered to be the original antiserum for this serovar, given that it was used by the WHO Reference Centre to type the original isolate, and also hyperimmune antiserum made against strain Bandicoot 343 by the CDC in 1978. Both antisera were run against strains Bandicoot 343 and Lepto0214; strain Bandicoot 343 produced significantly higher titres against the 1964 WHO hyperimmune antiserum and no titre against the CDC antiserum made in 1978. Strain Lepto0214 from the CDC collection produced the opposite result, recording a higher titre to the antiserum made by CDC but no reaction against the antiserum made by the WHO reference lab (Table 2). CAAT was not performed as there was no MAT relatedness between the two strains. Additionally, strain Lepto0214 failed to yield a titre when reacted against antiserum to *Leptospira interrogans* serovar Szwajizak strain Szwajizak, a member of the Mini Serogroup, whereas the WHO isolate did yield a detectable titre (not shown). Once it was established that the two serovar Perameles isolates were not identical, further isolates from the CDC culture collection were cultured and examined by MAT (Table 2). One of the isolates examined, CDC isolate Lepto0213, produced significant titres to the original hyperimmune serum (Table 2).

Evolutionary divergences were determined and phylogenetic analysis was performed using nearly complete 16S rRNA gene sequences of the three strains Bandicoot 343, Lepto0213 and Lepto0214 and 19 other strains representative of the genus *Leptospira* (Table 3). Strains Bandicoot 343 and Lepto0213 were found to group with *L. interrogans* and other pathogenic *Leptospira* strains, whereas strain Lepto0214 was found to group with *L. meyeri* and the other non-pathogenic *Leptospira* strains. The 16S rRNA gene sequences from this study were compared with those

reported previously for other Bandicoot 343 strains (Postic *et al.*, 2000). Strains Bandicoot 343 and Lepto0213 showed 100 % similarity with strains from the Institut Pasteur, France (GenBank accession no. AF157082), and the University of Trieste, Italy (AF157081), identified by Postic *et al.* (2000) as *L. interrogans* and Lepto0214 showed 100 % similarity with the CDC isolate (AF157085) identified as *L. meyeri*. Additionally, a real-time PCR targeting the *gyrB* gene specific for pathogenic *Leptospira* strains (Slack *et al.*, 2006b) failed to amplify a product from strain Lepto0214 but yielded a product from strain Bandicoot 343 which was later identified by sequencing as being derived from *L. interrogans*. The 16S rRNA and *gyrB* sequence analysis was confirmed by PFGE, which showed that Lepto0214 produced a similar electrophoretic pattern to that of *L. meyeri* serovar Hardjo strain Went 5. Strains Lepto0213 and Bandicoot 343 produced significantly different patterns from that of Lepto0214, with more bands and a more complex pattern (Fig. 1). It is interesting to note the two-band difference between Lepto0213 and Bandicoot 343; under the PFGE interpretation scheme described by Tenover *et al.* (1995), this two-band difference would designate the two isolates as closely related.

Heterogeneity of isolates between *Leptospira* culture collections is not a new problem and indeed has been recognized previously as a confounding problem associated with studies of *Leptospira* (Postic *et al.*, 2000). This problem has been recognized officially by the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Leptospiraceae* (TSCL) (Levett & Smythe, 2006, 2008). In the case of serovar Perameles strain Bandicoot 343, we have been able to resolve that it belongs to the species *Leptospira interrogans* through the use of 16S rRNA gene sequencing combined with serological comparison of the two conflicting cultures using the original hyperimmune antiserum made in 1964. Without the use of the original antiserum, the species identification could be challenged with the opposite argument that the isolate from the WHO Reference Centre is not the correct serovar Perameles strain and that the CDC collection strain is the correct strain.

It is accepted practice for the publication of novel *Leptospira* serovars that the reference culture is deposited

Fig. 1. PFGE gel and derived dendrogram for the three serovar Perameles isolates Bandicoot 343, Lepto0213 and Lepto0214 and typical *L. meyeri* reference strains.

Table 2. Results of MAT reactions for isolates Lepto0213, Lepto0214 and Bandicoot 343 against serovar Perameles hyperimmune antisera

Antiserum	Bandicoot 343	Lepto0213	Lepto0214
WHO, 1964	51 200	12 800	<50
CDC, 1978	<50	<50	102 400

into two recognized culture collections. This study highlights the need for the subsequent distribution of the hyperimmune antiserum (created from the reference culture) along with the culture into two TSCL-endorsed *Leptospira* culture collections. This original hyperimmune antiserum could then be used in the future for confident verification, at least serologically, of the identity of an isolate. In addition, the PFGE pattern of the initial isolate could be saved and compared to any later isolates that come into question. The following recommendations could be made for storage and dissemination of isolates. Firstly, multiple vials of the original culture should be stored in liquid nitrogen, with one culture remaining unopened and the others used as 'back-up' cultures, maintaining the integrity of the original isolate and adding further redundancy to the collection system. Secondly, an effort must be made by all laboratories to trace and document the

history of their isolates, particularly the original source of the isolate. Thirdly, the sourcing of isolates from third-party laboratories not recognized by the TSCL needs to be addressed to prevent the dissemination of incorrect cultures to other laboratories.

Though not directly highlighted in this study, there is a need for approved reference DNA sequences for the commonly used 16S rRNA gene from each of the 20 recognized *Leptospira* species. These sequences could be used to perform comparisons of unknown sequences and species determinations in preference to the multitude of correct, incorrect or outdated sequences available from public-domain databases such as GenBank/EMBL/DDBJ (Becker *et al.*, 2004; Janda & Abbott, 2002). This could be performed via a public information database dedicated to *Leptospira* reference serovars. This database could contain information on which collections hold the reference strains, antisera, sequence data and links to publications associated with reference *Leptospira* serovars.

In conclusion, this research provides evidence for the reclassification of *L. meyeri* serovar Perameles strain Bandicoot 343 as *L. interrogans* serovar Perameles strain Bandicoot 343. The original classification by DNA–DNA hybridization was probably performed with an incorrect culture. Whilst this research provides a definitive answer to

Table 3. Estimates of evolutionary divergence between 16S rRNA gene sequences of strains Bandicoot 343, Lepto0213 and Lepto0214 and *Leptospira* strains

Percentages of sequence similarity are shown, based on pairwise analysis of 23 sequences.

Strain	Accession no.	Bandicoot 343	Lepto0213	Lepto0214
<i>L. interrogans</i> RGA ^{T*}	AY631894	100.0	100.0	89.2
<i>L. alexanderi</i> A23 [†]	AY996803	99.1	99.1	88.8
<i>Leptospira</i> genomospecies 1 79601 [‡]	AY631881	99.2	99.2	88.8
<i>L. weilii</i> Celledoni ^T	AY631877	99.5	99.5	89.0
<i>L. kmetyi</i> Bejo-Iso9 ^T	AB279549	99.2	99.2	88.7
<i>L. kirschneri</i> 3522C ^T	AY631895	99.9	99.9	89.2
<i>L. noguchii</i> CZ 214 ^T	AY631886	99.6	99.6	89.1
<i>L. santarosai</i> LT 821 ^T	AY631883	99.1	99.1	89.1
<i>L. borgpetersenii</i> Veldrat Batavia 46 ^T	AY887899	99.5	99.5	89.1
<i>L. licerasiae</i> VAR 010 ^T	EF612284	95.9	95.9	87.8
<i>L. wolffii</i> Khorat-H2 ^T	EF025496	95.7	95.7	87.6
<i>L. fainei</i> BUT 6 ^T	AY631885	95.7	95.7	87.9
<i>L. inadai</i> 10 ^T	AY631896	95.6	95.6	88.2
<i>L. broomii</i> 5399 ^T	AY796065	95.4	95.4	88.0
<i>L. biflexa</i> Patoc I ^T	AY631876	89.3	89.3	99.8
<i>L. meyeri</i> Iowa City Frog ^T	AY631878	89.2	89.2	100.0
<i>Leptospira</i> genomospecies 3 WazHolland [‡]	AY631897	89.3	89.3	99.8
<i>Leptospira</i> genomospecies 4 LT 11-33 [‡]	AY631888	89.2	89.2	99.8
<i>Leptospira</i> genomospecies 5 Sao Paulo [‡]	AY631882	89.2	89.2	100.0
<i>L. wolbachii</i> CDC ^T	AY631879	89.3	89.3	99.8

*Status as type strain currently subject to a Request for an Opinion (Landon & Tang, 2004).

[†]Type strain unavailable.

[‡]Genomospecies reference strain.

the identity of this particular isolate, it also highlights the need for changes in the dissemination, storage and identification of *Leptospira* isolates.

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