PCR-based 'serotyping' of *Legionella pneumophila*

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Currently, several PCR assays based on 16S rRNA and virulence-associated genes are available for detection of *Legionella pneumophila*. So far, no genotyping method has been published that can discriminate between serogroups and monoclonal subgroups of the most common *L. pneumophila* serogroup 1. Our first approach was to analyse LPS-associated genes of seven *L. pneumophila* serogroup 1 strains, and we developed two PCR-based methods specific for serogroup 1. Specific DNA fragments could be amplified from all the serogroup 1 strains ($n=43$) including the strains from the American Type Culture Collection. In contrast, none of the strains from serogroups 2–15 ($n=41$) contained these specific gene regions. In a second approach, primers specific for the *lag-1* gene, encoding an O-acetyltransferase, which is responsible for the presence of the LPS epitope recognized by mAb 3/1, were designed and tested for their ability to differentiate between mAb 3/1-positive and -negative strains. All mAb 3/1-positive strains ($n=30$) contained the *lag-1* gene, but in turn 4 of 13 tested mAb 3/1-negative strains were also positive in the PCR. Thus, the discrimination between mAb 3/1-positive and mAb 3/1-negative subgroups could not be achieved for all strains. In a third approach, two intergenic regions expected to be specific for monoclonal subgroup Knoxville and closely related subgroups Benidorm/Bellingham were identified and used for selective genotyping. These intergenic regions could not only be amplified in every tested strain belonging to the subgroups Knoxville, Benidorm and Bellingham, but also in some strains of other unrelated subgroups. The two PCR approaches with primers specific for serogroup 1 genes definitely represent a valuable tool in outbreak investigations and for risk assessment. They also might be used for culture-independent diagnosis of legionellosis caused by *L. pneumophila* serogroup 1.

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INTRODUCTION

Legionnaires' disease, the pneumonic form of legionellosis, is acquired by inhalation or aspiration of legionellae from contaminated environmental sources. Potable water and cooling towers are important sources of both nosocomial and community-acquired *Legionella* infections (Fields *et al.*, 2002). The majority of infections are caused by strains belonging to *Legionella pneumophila* serogroup 1 (Helbig *et al.*, 2002; Yu *et al.*, 2002).

Due to the ubiquitous prevalence of legionellae in water supply systems, strains isolated from patients and environmental sources have to be compared by molecular typing

Abbreviations: ATCC, American Type Culture Collection; SBT, sequence-based typing.

The GenBank/EMBL/DDBJ accession numbers for the LPS synthesis gene sequences of *L. pneumophila* strains Uppsala 3 and Görlitz 6543 are AM932053 and AM778127, respectively.

A table of the sequences of the primers used for analysis of the LPS synthesis gene sequences in Uppsala 3 and Görlitz 6543 strains is available as supplementary data with the online version of this paper.

techniques to confirm or exclude a suspected environmental reservoir as the source of the infection. These techniques include mAb typing, analysis of various DNA restriction patterns (amplified fragment length polymorphism, or PFGE) (Helbig *et al.*, 1997; Fry *et al.*, 1999) and more recently sequence-based typing (SBT) (Gaia *et al.*, 2005; Ratzow *et al.*, 2007). Although SBT has the potential to be the 'gold standard' for establishing an epidemiological link to environmental sources, mAbs are still necessary for serotyping and monoclonal subtyping. Genetic fingerprinting performed directly from clinical samples seems to be very promising in cases of culture-independent detection of *L. pneumophila* DNA (Fry *et al.*, 2006; Lück *et al.*, 2007). Since the current SBT scheme cannot define the serogroups and monoclonal subgroups, we tried to develop PCR-based assays for differentiating between the serogroups and monoclonal subgroups of *L. pneumophila*.

METHODS

Bacterial strains and serotyping. Altogether 84 *L. pneumophila* strains were taken from the strain collection of the German *Legionella*

Reference Laboratory or the European Strain Collection (EUL strains), including 43 serogroup 1 strains and 41 strains of other serogroups (Table 1). Serological typing of these strains was performed using the Dresden panel of mAbs as described previously (Helbig *et al.*, 1997, 2002). Monoclonal subgroups of serogroup 1 strains were named according to the international subgrouping scheme (Joly *et al.*, 1986; Helbig *et al.*, 2002). In addition to serogroup 1, two monoclonal subgroups each were determined for serogroups 4, 5 and 6 (Helbig *et al.*, 2002). They were named after the American Type Culture Collection (ATCC) strain Los Angeles 1 or Portland 1 for serogroup 4, the ATCC type strain Dallas 1 E or the National Collection of Type Cultures type strain Cambridge 1 for serogroup 5, and the ATCC type strain Chicago-2 or our strain Dresden-1 (Helbig *et al.*, 1997) for serogroup 6. A total of 30 of the 43 serogroup 1 strains were mAb 3/1-positive, 13 of them mAb 3/1-negative. The strain collection consisted of 56 clinical isolates and 28 environmental isolates.

Sequence analysis of the LPS synthesis region in different serogroup 1 strains. Based on the published genomes of serogroup 1 strains Philadelphia-1 (mAb subgroup Philadelphia), Lens (mAb subgroup Benidorm), Paris (mAb subgroup Philadelphia) and Corby (mAb subgroup Knoxville), as well as for the LPS synthesis cluster of strain RC1 (mAb subgroup OLDA) (Chien *et al.*, 2004; Cazalet *et al.*, 2004; Glöckner *et al.*, 2008; Lüneberg *et al.*, 2000), we designed primers. The sequences of the primers used in this study are shown in Supplementary Table S1. They were used in different combinations to amplify and sequence overlapping DNA fragments of the LPS gene locus comprising 30 ORFs of two additional *L. pneumophila* strains Uppsala-3 (mAb subgroup Knoxville) (Bernander *et al.*, 2003) and Görlitz 6543 (mAb subgroup Bellingham). These strains were selected because at the beginning of our study no sequence information was available for strains belonging to these mAb subgroups. Primers were obtained from MWG. DNA sequences were determined using a cycle-sequencing procedure on an automatic DNA sequencing apparatus (Applied Biosystems 310). The alignments of the single sequences and the comparative analysis were carried out with the computer programs Lasergene 7.1 (DNASTar) and BioNumerics 5.0 (Applied Maths).

Serogroup 1-specific PCRs. According to Southern blotting data (Lüneberg *et al.*, 2000), the published genome sequences, and the sequence analysis of strains Uppsala 3 and Görlitz 6543, it was assumed that the region ORF 8–12 represents a genomic region specific for *L. pneumophila* serogroup 1. Since ORF 9, ORF 10 and ORF 11 were present in all the seven analysed strains, Uppsala 3, Görlitz 6543, Philadelphia-1, Lens, Paris, Corby and RC1 (Fig. 1), primers for ORF 9 and 11 were designed (Table 2) and tested for all strains using PCR.

lag-1 gene sequences in serogroup 1 strains. The *lag-1* gene from ten strains was amplified, sequenced and analysed (Fig. 2). The design of the primers used for PCR and DNA sequence analysis has been published elsewhere (Zou *et al.*, 1999; Lück *et al.*, 2001; Bernander *et al.*, 2003). Based on the sequences obtained for each of the similarity groups presented in Fig. 2, primer pairs with presumed specificity for each group were designed and named 'lag-1 Philadelphia', 'lag-1 Knoxville' and 'lag-1 Allentown' (Table 2). The primer pairs 'lag-1 all' were used as 'consensus' primers for all strains.

Putative mAb subgroup-specific PCRs. Depending on the variation in the presence or absence of two intergenic regions in the LPS synthesis gene clusters, putative subgroup-specific primers were designed and tested for all strains (Fig. 1, Table 2).

RESULTS AND DISCUSSION

Sequence analysis of LPS synthesis genes in *L. pneumophila* serogroup 1 strains

The complete ORF 1 to ORF 30 region (corresponding to lpg 0779 – lpg 0746 in the Philadelphia genome) was sequenced and assigned for strain Uppsala 3 (Fig. 1). For strain Görlitz 6543 the regions ORF 1–6, ORF 8–11 and ORF 14–30 were sequenced. In spite of the two 'gaps' within this region (Fig. 1) the obtained sequences could be used for further analysis. The sequences are deposited in the EMBL database under the accession numbers AM932053 (strain Uppsala 3) and AM778127 (ORF 1–6 of strain Görlitz 6543).

The LPS synthesis region of strains Philadelphia-1, Paris, Lens, Corby and RC1 has been published elsewhere (Cazalet *et al.*, 2004; Chien *et al.*, 2004; Glöckner *et al.*, 2008; Lüneberg *et al.*, 2000). All seven regions exhibited remarkable similarities. In particular, the gene locus from ORF 13 to ORF 30 showed homologies higher than 91 % between all seven analysed strains without any deletions or insertions. These findings are in concordance with the results of microarray analysis that were published recently (Cazalet *et al.*, 2008). The *neuA* gene currently used in the European SBT system is part of this region and could be detected by PCR in all *L. pneumophila* serogroup 1 strains (Gaia *et al.* 2005; Ratzow *et al.*, 2007). Obviously the region is highly conserved in strains belonging to this serogroup. In contrast, this gene could not be amplified from several strains belonging to serogroups 2 to 15. This might be due to either a low level of homology in the DNA sequences or the absence of the gene as suggested by the microarray data (Cazalet *et al.*, 2008). Further studies are needed to clarify this.

In contrast, the region of ORF 1–ORF 12 reveals several heterogeneous or absent loci among the compared strains (Fig. 1). We assumed that these differences might be a suitable basis for PCR-based subgrouping reflecting the serological diversity among serogroup 1 strains. The entire genomic region comprising ORF 8 to ORF 12 (Lüneberg *et al.*, 2000) was only present in strains RC1, Philadelphia-1 and Paris, all belonging to the mAb subtype Philadelphia or OLDA. In contrast, ORF 8 was replaced by a different region in strains Corby and Uppsala 3 (both belonging to mAb subtype Knoxville). As another example of genome variability the region ORF 7 was found to be different in strains Lens and Görlitz 6543 belonging to mAb subtypes Benidorm and Bellingham, respectively.

PCR-based differentiation of serogroup 1 versus non-serogroup 1 strains

It can be assumed that our selection of 84 tested *L. pneumophila* strains reflects the approximate spectrum and distribution of clinical and environmental strains. From all 43 serogroup 1 strains (mAb 3/1-positive and -negative)

Table 1. *L. pneumophila* isolates ($n=84$) and the presence of different LPS regions detected by direct amplification related to the reactivity with mAbs

Strain	Serogroup, mAb subgroup*	PCR system							
		ORF 9	ORF 11	<i>lag-1</i>				Intergenic region ORF 7–9 (Knoxville)	Intergenic region ORF 6–8 (Benidorm/Bellingham)
				All	Philadelphia	Knoxville	Allentown		
RC1†	1, OLDA	+	+	–	–	–	–	–	–
OLDA†	1, OLDA	+	+	–	–	–	–	–	–
Charite 16297†	1, OLDA	+	+	–	–	–	–	–	–
Erl. 361/1†	1, OLDA	+	+	–	–	–	–	–	–
Uppsala 3†	1, Knoxville	+	+	+	+	–	+	+	–
Corby†	1, Knoxville	+	+	+	+	+	–	+	–
L02-456†	1, Knoxville	+	+	+	+	+	–	+	–
L01-389†	1, Knoxville	+	+	+	+	–	+	+	–
L01-472†	1, Knoxville	+	+	+	+	+	+	+	–
Berlin 7248-99†	1, Knoxville	+	+	+	+	–	+	+	–
Knoxville-1†	1, Knoxville	+	+	+	+	–	+	+	–
EUL 128†	1, Knoxville	+	+	+	+	–	+	+	–
Oxford 4032‡	1, Oxford	+	+	–	–	–	–	–	–
Berlin P12†	1, Oxford	+	+	–	–	–	–	–	–
Philadelphia-1†	1, Philadelphia	+	+	+	+	–	+	–	–
EUL 073†	1, Philadelphia	+	+	+	+	–	+	–	–
Barcelona†	1, Philadelphia	+	+	+	+	–	+	–	–
EUL 042†	1, Philadelphia	+	+	+	+	–	+	–	–
Hamburg P5†	1, Philadelphia	+	+	+	+	+	–	–	–
Paris†	1, Philadelphia	+	+	+	+	–	+	–	–
EUL 028†	1, Allentown	+	+	+	+	–	+	–	–
Allentown 1†	1, Allentown	+	+	+	+	+	+	–	–
EUL 004†	1, Allentown	+	+	+	+	–	+	–	–
EUL 129†	1, Allentown	+	+	+	+	–	+	–	–
EUL 130†	1, Allentown	+	+	+	+	–	+	–	–
Bellingham†	1, Bellingham	+	+	–	–	–	–	–	+
EUL 092†	1, Bellingham	+	+	+	+	–	–	–	+
Görlitz 6543†	1, Bellingham	+	+	+	+	+	–	–	+
EUL 018†	1, Bellingham	+	+	+	+	–	–	–	+
Lens†	1, Benidorm	+	+	+	+	+	–	–	+
L01-354†	1, Benidorm	+	+	+	+	+	–	–	+
EUL 120†	1, Benidorm	+	+	+	+	+	–	–	+
Benidorm†	1, Benidorm	+	+	+	+	+	–	–	+
EUL 117†	1, Benidorm	+	+	+	+	+	–	–	+
EUL 124†	1, Benidorm	+	+	+	–	–	+	–	+
Münster 6850†	1, Benidorm	+	+	+	+	–	+	–	+
W07-508‡	1, Benidorm	+	+	+	–	+	–	–	+
EUL 030†	1, France	+	+	+	–	+	–	–	+
France 5811†	1, France	+	+	+	–	+	–	–	+
L01-520†	1, France	+	+	+	+	–	+	–	–
Camperdown-1†	1, Camperdown	+	+	+	–	–	–	+	–
Denver†	1, Denver	+	+	–	–	–	–	+	–
Heysham-1†	1, Heysham	+	+	–	–	–	–	+	–
Togus 1†	2	–	–	–	–	–	–	–	–
W08-231‡	2	–	–	–	–	–	–	–	–
W08-259-2‡	2	–	–	–	–	–	–	–	–
Bloomington‡	3	–	–	–	–	–	–	–	–
L05-299†	3	–	–	–	–	–	–	–	–
W03 705-2‡	3	–	–	–	–	–	–	–	–

Table 1. cont.

Strain	Serogroup, mAb subgroup*	PCR system							
		ORF 9	ORF 11	<i>lag-1</i>				Intergenic region ORF 7–9 (Knoxville)	Intergenic region ORF 6–8 (Benidorm/Bellingham)
				All	Philadelphia	Knoxville	Allentown		
Los Angeles†	4, Los Angeles	—	—	—	—	—	—	—	—
Heidelberg P3†	4, Portland	—	—	—	—	—	—	—	—
W03-496-1‡	4, Portland	—	—	—	—	—	—	—	—
Cambridge†	5, Cambridge	—	—	—	—	—	—	—	—
W03-737‡	5, Cambridge	—	—	—	—	—	—	—	—
W582-4‡	5, Cambridge	—	—	—	—	—	—	—	—
Dallas 1E‡	5, Dallas	—	—	—	—	—	—	—	—
U7W‡	5, subsp. Pascullei	—	—	—	—	—	—	—	—
Chicago 2†	6, Chicago	—	—	—	—	—	—	—	—
W07-587‡	6, Chicago	—	—	—	—	—	—	—	—
W 1239‡	6, Dresden	—	—	—	—	—	—	—	—
Heidelberg P1†	6, Dresden	—	—	—	—	—	—	—	—
W 981‡	6, Dresden	—	—	—	—	—	—	—	—
Münster K133/2‡	6, Chicago	—	—	—	—	—	—	—	—
ATCC‡	7	—	—	—	—	—	—	—	—
W06-494-3‡	7	—	—	—	—	—	—	—	—
Concord-3†	8	—	—	—	—	—	—	—	—
W06-092-1‡	8	—	—	—	—	—	—	—	—
W07-432-2‡	8	—	—	—	—	—	—	—	—
W08-774-4‡	8	—	—	—	—	—	—	—	—
ATCC†	9	—	—	—	—	—	—	—	—
Frankfurt 9‡	9	—	—	—	—	—	—	—	—
Leiden†	10	—	—	—	—	—	—	—	—
W05-177-1‡	10	—	—	—	—	—	—	—	—
W05-191‡	10	—	—	—	—	—	—	—	—
ATCC†	11	—	—	—	—	—	—	—	—
W704‡	11	—	—	—	—	—	—	—	—
ATCC†	12	—	—	—	—	—	—	—	—
W08-285-2‡	12	—	—	—	—	—	—	—	—
ATCC†	13	—	—	—	—	—	—	—	—
W04/560‡	13	—	—	—	—	—	—	—	—
ATCC†	14	—	—	—	—	—	—	—	—
Lpz LUA 36‡	14	—	—	—	—	—	—	—	—
Lansing ATCC†	15	—	—	—	—	—	—	—	—
W04-065‡	15	—	—	—	—	—	—	—	—

*Strains of the monoclonal subgroups Allentown, Benidorm, France, Knoxville and Philadelphia are mAb 3/1-positive; strains of the monoclonal subgroups Bellingham, Camperdown, Denver, Heysham, OLDA and Oxford are mAb 3/1-negative.

†Clinical isolates.

‡Environmental isolates.

ORF 9 and ORF 11 regions could be amplified using the designed primers (Table 1). Our data were recently confirmed by microarray analysis that showed that 150 serogroup 1 strains harboured ORF 11 and ORF 9 (Cazalet *et al.*, 2008). In a Danish study ORF 2 was used as the target gene for detecting serogroup 1 strains. They

reported a positive PCR in 51/51 *L. pneumophila* serogroup 1 isolates, and in the reference strains for serogroup 7 and 11, but not in any of 49 non-serogroup 1 isolates (Kuchana *et al.*, 2007). In agreement with these results, none of the strains belonging to other serogroups ($n=41$), including the type strains of serogroup 7 and 11,

Fig. 1. Schematic representation of regions of the LPS synthesis cluster in published *L. pneumophila* serogroup 1 strains and localization of the serogroup 1-specific (indicated by arrowheads and circled numbers 2 and 3) and subgroup-specific primer pairs (indicated by arrowheads and circled numbers 1, 4 and 5), see also Table 2. The regions are: (1) *lag-1* all, *lag-1* Philadelphia, *lag-1* Knoxville, *lag-1* Allentown, (2) ORF 9, (3) ORF 11, (4) Benidorm/Bellingham ORF 6–8, (5) Knoxville ORF 7–9.

were positive in our serogroup 1-specific PCRs (Table 1). Thus, our PCR assays seem to be more specific for serogroup 1. Again, these data are in agreement with the microarray analysis published recently (Cazalet *et al.*, 2008).

Differentiation of serogroup 1 mAb 3/1-positive versus serogroup 1 mAb 3/1-negative strains

In concordance with the mAb subtyping of all the seven compared strains, the *lag-1* gene encoding an *O*-acetyltransferase of the LPS *O*-chain of *L. pneumophila* serogroup

Table 2. Serogroup 1- and subgroup-specific primers

Region (region no.)*	Forward primer/sequence (5'→3')	Reverse primer/sequence (5'→3')	Fragment size (bp)
<i>lag-1</i> all (1)	Lag all up/ ATGTAYAATAAACTCACAAC	Lag all do/ ATAAGCTAACTTRTTTGATG	1065
<i>lag-1</i> Philadelphia (1)	Lag Phil up/ AGTGAAAGCGGATTGGCA	Lag Phil do/ TTAGCCACTCGCGAACTACG	725
<i>lag-1</i> Knoxville (1)	Lag Corby up/ AGTATGGGTGGATTGGTG	Lag Corby do/ TCATCCACTCACGAACCAGT	725
<i>lag-1</i> Allentown (1)	Lag Allentown up/ AGTGGAGGCAGAGTTGGCA	Lag Phil do/ TTAGCCACTCGCGAACTACG	725
ORF 9 (2)	LPS Orf 9 up2/ CAGGATTACCGCTCATTATTG	LPS Orf 9 do/ GTAATTCCCAGCCATTTACCAGATC	561
ORF 11 (3)	LPS Orf 11 up2/ GCATAGATCATCACGCTGCAG	LPS Orf 11 do/ GGATGGGGGCGATAAAGAATAAC	280
Intergenic region between ORF 6 and ORF 8 (subgroup Benidorm/Bellingham) (4)	Lens 6-8 do/ GATACTAACAGCCAAGGTG	Lens 7 up1/ TCCAACCCAAGGAATTCCTG	979
Intergenic region between ORF 7 and ORF 9 (subgroup Knoxville) (5)	Corby 7-9 up/ CTAGATCACATTCGTATCGTC	Corby 7-9 do/ GTTGACGAGATTGTATCTC	1010

*Regions are as shown in Fig. 1.

Fig. 2. UPGMA dendrogram of sequence similarities found among *lag-1* genes (1074 bp) in different *L. pneumophila* serogroup 1, mAb 3/1-positive strains. Alle, Allentown; Beni, Benidorm; Knox, Knoxville; Phil, Philadelphia.

1 was not present in strain RC1 belonging to subgroup OLDA (Lüneberg *et al.*, 2000) and was truncated in strain Görlitz 6543 (subgroup Bellingham). Vice versa, this gene was present in all five mAb 3/1-positive strains belonging to the mAb subgroups Knoxville, Benidorm and Philadelphia (Table 1). In strains Philadelphia, Paris and Uppsala 3 the *lag-1* gene is bordered by a direct repeat (Fig. 1). This specific structure is the basis for the deletion of this region in epidemiologically related strains (Bernander *et al.*, 2003) or in a spontaneous mutant of strain Philadelphia (Zou *et al.*, 1999).

For all mAb 3/1-positive strains ($n=30$) *lag-1* gene fragments could be detected by the 'lag-1 all' PCR (Table 1). However, 4 of 13 mAb 3/1-negative strains were also positive in this *lag-1* specific PCR. Three of these strains belong to monoclonal subgroup Bellingham, and one to subgroup Camperdown. Thus, these strains contained at least partial sequences of the *lag-1* gene. Most remarkably, strain Görlitz 6543 carried almost the complete *lag-1* gene with a change of only two bases in the start codon ATG. Due to this mutation that isolate probably does not express the *O*-acetyltransferase responsible for the mAb 3/1 epitope and therefore does not react with mAb 3/1. Based on previous data we assume that a switch from monoclonal subgroup Benidorm to Bellingham might have occurred (Wagner *et al.*, 2007). A mutation within the *lag-1* gene that leads to an inactive *O*-acetyltransferase has been described previously for strain Corby, and might be another reason for the inability to bind mAb 3/1 (Lück *et al.*, 2001).

Similar results were recently reported from the Centers for Disease Control and Prevention, Atlanta, GA, USA (Kozak *et al.*, 2007). These authors also found a significant number of environmental strains positive in a *lag-1* PCR but not reacting with mAb 2, which shows the same reactivity as mAb 3/1. In contrast, the Danish investigation showed a clear correlation between the presence or absence of the *lag-1* gene and the reactivity with mAb 3/1. In their study, *lag-1* PCR was positive for 33/33 mAb 3/1-positive strains

and negative in 18 mAb 3/1-negative strains (Kuchana *et al.*, 2007). In conclusion, the *lag-1* PCR in combination with serogroup-1 specific PCR can be used to detect mAb 3/1-negative strains (100 % negative predictive value). Further experiments should elucidate the DNA sequence of these regions in the mAb 3/1-negative but *lag-1* PCR positive strains. As expected, all non-serogroup 1 strains were negative in this PCR (Table 1).

***lag-1* gene sequences/PCRs in serogroup 1 strains do not correlate with the mAb subtype**

Based on the published genomes, we assumed a correlation between the DNA sequences of the *lag-1* gene and the mAb subtypes in different strains. Therefore, the differences of these gene sequences in ten additional strains were analysed and putative subgroup-specific primers were designed (Fig. 1, Table 2). By comparison, all *lag-1* sequences of selected mAb 3/1-positive strains showed high homologies with at least 89.4 % identity (Fig. 2). Primer sequences for subgroups Knoxville, Allentown and Philadelphia were very similar to each other, with only a few mismatches. Subtyping by *lag-1* PCR assays ('*lag-1* Philadelphia', '*lag-1* Knoxville' and '*lag-1* Allentown') could not be achieved for the three subgroups mentioned above (Tables 1 and 2), maybe due to this fact, but maybe also as a result of spontaneous mutations and partly because of a different genetic relatedness of *lag-1* in identical mAb subgroups (Table 1, Fig. 2). For instance, the divergent *lag-1* gene sequence of the strain Uppsala 3 belonging to monoclonal subgroup Knoxville was almost identical to the *lag-1* gene of strains Philadelphia and Paris (Fig. 2). Interestingly, strains Philadelphia, Paris and Uppsala 3 containing the direct repeats before and after the *lag-1* gene (Fig. 1) clustered in one group, whereas strains Corby and Lens not containing this particular structure clustered in another group. This might explain why the putative *lag-1* subgroup Knoxville-specific PCR yielded negative results in several strains of this mAb subgroup (Table 1). In summary, no correlation between the DNA sequence/PCRs of the *lag-1*

gene and the monoclonal subgroup of the strains could be detected (Fig. 2). However, the genetic variations of *lag-1* among identical monoclonal subgroups detected are probably strain-specific differences and might be useful during subtyping studies in epidemiological investigations.

Intergenic region PCR-based genotyping

The identical 1.6 kb regions between ORF 7 and ORF 9 instead of ORF 8 in sequenced strains Uppsala 3 and Corby suggest that there may be a genetic element specific for subgroup Knoxville. In fact, in all eight Knoxville isolates of our strain collection this fragment could be amplified with appropriate primers. However, this DNA element was also present in the ATCC strains Camperdown, Denver and Heysham (Table 1). Another unique gene region was found in strains Görlitz 6543 (mAb subgroup Bellingham) and Lens (mAb subgroup Benidorm) between ORF 6 and ORF 8 instead of ORF 7 in strains RC1, Philadelphia and Paris. The length of this region seems to be considerably larger in strain Görlitz 6543 than in strain Lens (2.1 kb). So far, this large genetic element could not be amplified completely in strain Görlitz 6543. The designed PCR (intergenic region 6–8) was positive for all isolates of mAb subgroups Benidorm ($n=8$) and Bellingham ($n=4$). Again, as an exception to these rules two strains of mAb subgroup France were also positive in this PCR (Table 1). The latter results might be explained by the fact that monoclonal subgroup France is closely related to subgroup Benidorm, as a single transposon insertion in strain Wadsworth 130b (mAb subtype Benidorm) in either ORF 8 or ORF 11 of the LPS synthesis gene locus leads to a switch from mAb subtype Benidorm to mAb subtype France (Wagner *et al.*, 2007). As expected none of the tested non-serogroup 1 strains yielded any PCR products in Knoxville and Benidorm/Bellingham subtyping assays.

Conclusions

Subtyping within outbreak investigations of human infections caused by *L. pneumophila* is currently performed by mAb typing and SBT (Gaia *et al.*, 2005; Ratzow *et al.*, 2007). Currently both methods are mostly applied to cultured strains, isolated from both clinical and environmental sources. Both mAb typing and SBT might be used in clinical samples (Fry *et al.*, 2006; Lück *et al.*, 2007). However, the sensitivity is a limiting factor for this application. Therefore, the purpose of the present study was to establish simple and rapid genotyping methods for culture-independent discrimination between serogroups of *L. pneumophila* and monoclonal subgroups of serogroup 1, the most common serogroup. As indicated above, our study identified a serogroup 1-specific genomic region. Thereupon, we developed two independent and suitable PCR assays for detecting serogroup 1 strains of *L. pneumophila*. Both DNA loci (ORF 9 and ORF 11) could be amplified successfully in all samples of our strain

collection belonging to serogroup 1, irrespective of the monoclonal subgroup type, but not in any of the serogroup 2–15 strains. In conclusion, this simple and specific PCR-based approach described here can be applied directly to clinical samples to confirm or exclude *L. pneumophila* serogroup 1 as the causative agent. Furthermore, it can be used in epidemiological investigations as a reliable method for rapid identification of suspected environmental reservoirs harbouring *L. pneumophila* serogroup 1 strains as the source of infection. Since this PCR approach can be performed within 1 day, it may help to prevent further infections.

In contrast, a reliable PCR method for discrimination between mAb 3/1-positive and mAb 3/1-negative isolates could not be achieved for all strains. Therefore, currently the use of the phenotypical marker (reactivity with mAb 2 or 3/1) is needed to subtype serogroup 1 strains.

In addition, we demonstrated in this study that selective genotyping by means of *lag-1* differences in several mAb 3/1-positive subgroups could not be accomplished due to the high rate of genetic variations and varying degree of *lag-1* relatedness among monoclonal subgroups. Finally, we used two genomic regions in different monoclonal subgroups for the implementation of PCR-based determination of subgroup clusters. However, none of these tested selective *lag-1* and intergenic PCR assays could define each individual monoclonal subgroup of serogroup 1 strains. Our results showed a high degree of diversity in different strains. Thus, our approach might be useful for genetic fingerprinting of individual strains rather than in serotyping.

Currently, we do not know all the genes that are involved in the biosynthesis of the LPS and the resulting antigen diversity. It might be speculated that not all genes are located in the investigated 30 kb DNA locus.

Our results serve as the first steps for the development of a PCR-based 'serotyping' system for *L. pneumophila*. Similar PCR-based methods have been developed for capsule polysaccharide-encoding genes in *Streptococcus pneumoniae* (Brito *et al.*, 2003; Yu *et al.*, 2008), LPS synthesis genes in *Actinobacillus actinomycetemcomitans* (renamed *Aggregatibacter actinomycetemcomitans* in 2006) (Kaplan *et al.*, 2001), and capsule-encoding genes in *Haemophilus influenzae* (Satola *et al.*, 2007). The aim of future studies will be to identify further genetic loci for DNA-based differentiation of *L. pneumophila* serogroups and subgroups related to the substrates for mAbs, and to apply the developed methods in epidemiological studies.

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