Variable genetic element typing: a quick method for epidemiological subtyping of

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Abstract: A total of 57 isolates of *L. pneumophila* were randomly selected from the German National *Legionella* strain collection and typed by monoclonal antibody subgrouping, seven gene locus sequence-based typing (SBT) scheme and a newly developed variable element typing (VET) system based on the presence or absence of ten variable genetic elements. These elements were detected while screening of a genomic library of strain Corby as well as taken from published data for PAI-1 (pathogenicity island) from strain Philadelphia. Specific primers were designed and used in gel based PCRs. PCR amplification of the *mip* gene that served as a control. The endpoint was the presence / absence of a PCR product on an ethidiumbromide stained gel. In the present study the index of discrimination was somewhat lower than that of the SBT (0.87 versus 0.97). Nevertheless, the results obtained showed as a 'proof of principle' that this simple and quick typing assay might be useful for the epidemiological characterization of *Legionella pneumophila* strains.

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1 Concise Article

2 **Variable Genetic Element Typing: A quick method for**

3 **epidemiological subtyping of *Legionella pneumophila***

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1 **Abstract:**

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4 3 *Legionella* strain collection and typed by monoclonal antibody subgrouping, seven gene locus
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7 4 sequence-based typing (SBT) scheme and a newly developed variable element typing (VET)
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9 5 system based on the presence or absence of variable genetic elements. These elements were
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11 6 detected while screening of a genomic library of strain Corby as well as from PAI-1
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13 7 (pathogenicity island) from strain Philadelphia. Specific primers were designed and used in
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15 8 gel based PCRs. The results obtained proved the usefulness of this simple and quick typing
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17 9 assay for the epidemiological characterization of *Legionella pneumophila* strains.
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1 Introduction

2 Members of the genus *Legionella* are Gram-negative bacteria and normally occupy natural
3 aquatic environments where they survive as intracellular parasites of protozoa. Currently the
4 genus *Legionella* contains more than 50 species. Among them the species *L. pneumophila* is
5 the most frequent [1;2]. *L. pneumophila* serogroup (sg) 1 is the most common cause of
6 pneumonia occurring as sporadic or endemic disease that may be acquired from different
7 environmental sources [3]. For public health authorities it is essential to detect the source of
8 infection promptly by comparing clinical and environmental strains of *L. pneumophila* to
9 conduct decontamination measures and to prevent further cases. For this numerous
10 phenotypic and genotypic typing methods have been applied to the epidemiological typing of
11 *L. pneumophila* in the recent years [4]. This include monoclonal antibody (MAb)
12 subgrouping as a rapid screening method [3]and genotyping methods such as amplified
13 fragment length polymorphism (AFLP) [4], pulsed-field gel electrophoresis (PFGE) [4;5] and
14 sequence based typing [6-8]. In contrast to band based typing methods that are difficult to
15 standardize the developed sequence based typing (SBT) has the potential of excellent
16 typeability, interlaboratory reproducibility and epidemiologic concordance[9]. However the
17 SBT is rather expensive and might therefore not applicable to all strains and in all
18 laboratories. The specific gene content of each strain sequenced so far is constituted of 7-11%
19 of each genome [10-12]. In addition, variable elements/ pathogenicity island are present in
20 some strains [13].

21 The purpose of this study was to investigate the usefulness of a binary typing system (VET)
22 based on the presence/ absence of defined genetic elements by using gel based PCR. These
23 data were compared to the European Working Group on Legionella Infections (EWGLI) SBT
24 system.

Materials and Methods

***Legionella pneumophila* strains.** A total of 57 isolates of *Legionella pneumophila* were randomly selected from the Dresden strain collection including the strains from which the complete genomes are published. From strain Corby we used a panel of variants (mutants) (EUL 135 to 139) that differ in their ability to multiply in amoebae and macrophages showed different reactivity patterns with MAbs due to a mutation in a lipopolysaccharide synthesis gene or that were resistant to rifampicin [7]. 40 strains were by definition unrelated to any other isolates in the study, i.e. they were isolated from sporadic cases or if they were part of an outbreak or cluster of legionellosis only one isolate was included in the analysis. 32 isolates were obtained during epidemiological studies (Table 1) and comprise twelve sets of related strains (A to M). Some of them were indistinguishable by MAb subgrouping and SBT thus proving a transmission from the environmental source to the patient. Sets C and H differed in both markers, whereas sets K, L, and M were indistinguishable by genetic typing but expressed a different monoclonal subtype which is related to the deletion of the *lag -1* gene [5] (Table 1).

Serotyping and sequence based typing (SBT)

Serological typing of *L. pneumophila* strains was performed by using the Dresden panel of monoclonal antibodies and the results were published previously [3;5;8]. Genotyping was performed by using the seven genes SBT [7;8]. Briefly, seven gene targets comprising *flaA*, *pilE*, *asd*, *mip*, *momp*, *proA* and *neuA* were amplified by PCR and sequenced. The assignment of the sequence type (ST) was carried out by using SBT database or the online sequence type checker (http://www.hpa-bioinfotools.org.uk/legionella/php/sbt_query1.php).

Variable element typing (VET). Target sequences for variable element typing were selected from a genomic library of strain Corby according to Heuner et al. [14] with minor modifications. Briefly, chromosomal DNA of *L. pneumophila* Corby (sg 1) was partially

digested with *Sau3A*. Fragments ranging from 1.0 to 4.0 kb were ligated into the *Bam*HI
 restriction site of vector pUC19 and transformed into *E. coli* DH5 α . The DNA sequence of
 the inserted Corby-DNA was determined by using standard m13 primers and primer walking.
 Fragments that could not be aligned to the Philadelphia genome available at that time were
 selected. Primer specific for these genetic elements were designed and used for typing (Table
 2). For *magA* and *traD* sequences from the pathogenicity island [13] were used.
 DNAs from *L. pneumophila* strains was prepared as for sequenced based typing [7]. The
 VET- PCR were performed with 10pmol of each primer, 10 μ l of DNA-puffer, 5U of Gold-
 Taq-Polymerase, 2 μ l DNA of each strain ,and 200pmol of each dNTP at a final volumes of
 50 μ l. The annealing temperature was 58°C. Gel electrophoresis was performed on 1%
 agarose gels. Gels were stained with ethidium bromide and analyzed under UV-light. PCR
 products of the expected size were considered positive. At the beginning of these experiments
 the specificity of the PCR products was confirmed by DNA sequencing [7]. In each run the
mip gene PCR of the SBT scheme were used as a positive control [7]. All VET PCR assays
 were run at least twice.
 A VET-index was applied as the summary of the binary results (presence/absence of a PCR
 product). Hereby, these results of each three PCR assays were put together. If a band was
 identified on the gel, the first PCR received the number 1; the second get the number 2 and
 the third the number 4. If no PCR product was obtained the number 0 was assigned.
 As a result each set of the three PCR assays could be summarized as one digit between 0 and
 7. All ten PCR assays could therefore presented as a 4 digit number. The advantage of this
 VET-index is a reduced size, a more clearly arrangement and the possibility to exchange these
 data between laboratories.

Results and Discussion

The primary aim of this study was to evaluate the utility of VET as a genotyping method for *L. pneumophila*. The primers developed and used in this study were aligned to the four available complete genomes of *L. pneumophila* [10;11;12] by using the Basic Local Alignment Search Tool (BLAST) available online (www.ncbi.nlm.nih.gov/BLAST) [15]. The results of this search demonstrated that the binding sites of some primers were present some of the four genomes. The investigation of the strains Philadelphia-1, Lens, Paris and Corby by using the new VET-PCRs confirmed the expected results, i. e. all PCR product predicted by the BLAST search could readily detected by using the ten VET-PCR assays. The variable elements used in this typing assay are relatively randomly distributed in the genome of the strains from which complete sequenced genomes are available (Fig.1).

In addition, all five variants of the Corby strains that differ in the cultivability in amoebal hosts, point mutations in the *rpoB* and the *lag-1* genes showed the same VET results (data not shown) thus arguing that the markers detected in the VET are stable as were the SBT results [7].

For 57 of the strains investigated by using variable element typing (VET) results were obtained for 51. In six strains none of the elements could be detected by the current assays (Tab. 1, Fig.2). Consequently the VET index of these strains was 0000. Since the positive control PCR using the *mip* primers of the SBT scheme gave positive results we are sure that the genetic elements or at least one of the primer binding sites were readily absent (or to less identical). From a total of 57 strains, 32 related strains were merged into 12 sets (A to M) (Table 1, Fig 2). From these sets of related strains the results of seven sets (A, B, D, E, F, G, and J) showed that related strains were indistinguishable by using for MAb typing, SBT and VET. For further two sets (C and H), the serotype/MAb subtype matched. However, SBT and VET of the environmental isolates were different from that of the patient's isolates. Vice versa three sets (K, L, and M) showing the same genetic fingerprints by using SBT and VET

were distinguishable by MAb typing. It has been shown previously that changes in genes involved in the LPS synthesis might change the reactivity with MAbs. Thus the loss of the *lag-1* gene coding for an O-acetyltransferase is responsible for the switch from MAbtype Philadelphia/ Knoxville to the MAb type OLDA/Denver [5;16;17].

Taking into account the moderate number of unrelated strains (n=40) in our study an moderate index of discrimination (IOD) of 0.87 was calculated. This is lower than that published previously for SBT, but higher than that for MAb typing and AFLP [4;7].

Interestingly, in some cases strains belonging to different serotypes were indistinguishable by VET. For example, the most frequently occurring VET-Index 0250 was found in several strains belonging to different serogroups/ MAb-types and SBT variants. Thus strains Uppsala 21, Uppsala 59, L04-567 or L04-564 showed different serotypes/monoclonal subtypes like OLDA, Philadelphia or serogroup 10. In addition, strains with the VET-Index 0251 (e.g., L02-298, L05-129) or VET-Index 4411 (e.g., Heidelberg P1, P9) belonged to different serogroups (Table 1).

Due to the limited number of strains a general conclusion concerning the correlation between these two genotyping methods is difficult. It is not surprising that a complete correlation does not exist between both genotyping methods, since SBT detects variations in seven genes belonging to the core genome whereas VET detects the absence or presence of variable genetic elements like PAIs. Therefore, it is not surprising that some strains of sequence type (ST) 1 investigated here, had a different VET-Index.

SBT is a powerful tool for subtyping *L. pneumophila* strains and is currently the gold standard within the European Working Group on Legionella Infections (EWGLI). Data obtained in different countries can easily be compared and the European data base currently containing hundreds of types can be used both in epidemiological investigations and to assess the risk associated with environmental reservoirs that are contaminated with illness-associated strains. However SBT is relatively expensive and therefore might not be applicable to all laboratories.

The aim of the present study was to evaluate an alternative rapid and cheap PCR approach based on the absence or presence of defined genetic elements in different strains. A recent study from Australia suggested that in all strains a similar repertoire of virulence genes exist [18]. In accordance with this study our results demonstrate that the presence of the *lvh* locus, the *trb-1* locus and the pathogenicity island I is variable in different strains. So far no association of the presence of these genetic elements with the putative virulence i. e. the origin of the strains could be demonstrated.

The usefulness of the VET approach as a technique for subtyping of strains belonging to different serogroups of *L. pneumophila* was clearly demonstrated. The possibility to express the data as binary codes allows the standardization and portability of these results as shown for *Staphylococcus aureus* and *Escherichia coli* [19;20]. It remains an open question and needs to be experimentally proven with a larger number of strains to whether the use of VET is indeed suitable for the epidemiological subtyping of *L. pneumophila*. Finally, it must be pointed out that these genetic elements might serve as candidates for a DNA chip based typing system similar to that for *St. aureus* [21]. In summary, our current results are encouraging. The VET scheme showed good typeability and discriminatory power for *L. pneumophila*. However, further variable elements must be identified to increase both, the typeability and the discriminatory potential.

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Table 1. *Legionella pneumophila* strains

Strain designation	Serogroup, Monoclonal Subtype	Sequence Type (ST)	Allels profile of <i>flaA</i> , <i>pilE</i> , <i>asd</i> , <i>mip</i> , <i>ompS</i> , <i>proA</i> and <i>neuA</i>	VET- Index	Origin of strains
Lens	1 Benidorm	15	12,9,26,5,26,17,15	0210	Clinical isolate community acquired pneumonia, unrelated
Augsburg-1	1 Benidorm	42	4,7,11,3,11,12,9	7331	Clinical isolate community acquired pneumonia, unrelated
Berlin 11	1 Benidorm	425	2,10,3,15,9,4,11	0240	Clinical isolate nosocomial pneumonia, unrelated
L02-287	1 Benidorm	181	3,7,1,12,14,9,9	3011	Clinical isolate community acquired pneumonia, unrelated
L02-298	1 Benidorm	42	4,22,11,3,11,12,9	0251	Clinical isolate community acquired pneumonia, unrelated
L04-412	1 Knoxville	9	3,10,1,3,14,9,11	0041	Clinical isolate travel-associate pneumonia, unrelated
Corby	1 Knoxville	51	6,10,15,28,9,14,6	7341	Clinical isolate community acquired pneumonia, unrelated
L01-443	1 Knoxville	9	3,10,1,3,14,9,11	6141	Clinical isolate nosocomial pneumonia, unrelated
Berlin 4	1 OLDA	1	1,4,3,1,1,1,1	2740	Clinical isolate nosocomial pneumonia, unrelated
Charite 16297/2000	1 OLDA	1	1,4,3,1,1,1,1	6650	Clinical isolate community acquired pneumonia, unrelated
Goettingen 12/98	1 OLDA	1	1,4,3,1,1,1,1	0650	Clinical isolate community acquired pneumonia, unrelated
L03-610	1 OLDA	7	1,4,3,1,1,1,6	0240	Clinical isolate community acquired pneumonia, unrelated
L04-567	1 OLDA	1	1,4,3,1,1,1,1	0250	Clinical isolate nosocomial pneumonia, unrelated
Paris	1 Philadelphia	1	1,4,3,1,1,1,1	0050	Clinical isolate community acquired pneumonia, unrelated
Philadelphia	1 Philadelphia	36	3,4,1,1,14,9,1	0430	Clinical isolate community acquired pneumonia, unrelated
L04-280	1 Philadelphia	23	2,3,9,10,2,1,6	7340	Clinical isolate travel-associate pneumonia, unrelated
L02-034	10	440	3,10,1,28,14,9,1	0000	Clinical isolate nosocomial pneumonia, unrelated
L02-303-1	15	337	10,22,7,28,16,18,6	2011	Clinical isolate nosocomial pneumonia, unrelated
Heidelberg P9	4 Portland	67	1,10,3,28,9,4,1	4411	Clinical isolate nosocomial pneumonia, unrelated
W 01/1993	5 Cambridge	245	1,10,3,13,9,4,1	7310	Environmental isolate, unrelated

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6	Chicago 2	6 Chicago	30	3,10,1,3,14,9,9	0660	Clinical isolate nosocomial pneumonia, unrelated
7	Finnl. 10	6 Chicago	68	3,13,1,28,14,9,3	3651	Clinical isolate community acquired pneumonia, unrelated
8	Heidelberg P1	6 Dresden	330	7,10,17,3,4,11,3	4411	Clinical isolate nosocomial pneumonia, unrelated
9						
10	L04-507	1 Philadelphia	332	7,10,17,6,14,11,3	0420	Clinical isolate community acquired pneumonia, unrelated
11						
12	L04-506	1 Philadelphia	332	7,10,17,6,14,11,3	0420	Clinical isolate community acquired pneumonia, unrelated
13						
14						
15	W05-007	6 Chicago	424	7,10,17,3,13,14,9	0050	Set A: Hospital water isolate
16	L04-597	6 Chicago	424	7,10,17,3,13,14,9	0050	Set A: Patients isolate nosocomial pneumonia
17						
18	L05-008	6 Chicago	424	7,10,17,3,13,14,9	0050	Set A: Patients isolate nosocomial pneumonia
19						
20						
21	W04-952	1 OLDA	1	1,4,3,1,1,1,1	0250	Set B: Hospital water isolate
22	W04-954	1 OLDA	1	1,4,3,1,1,1,1	0250	Set B: Hospital water isolate
23						
24	W04-956	1 OLDA	1	1,4,3,1,1,1,1	0250	Set B: Hospital water isolate
25	Erl. 361/1	1 OLDA	1	1,4,3,1,1,1,1	0250	Set B: Patients isolate nosocomial pneumonia
26						
27	L04-541	1 OLDA	1	1,4,3,1,1,1,1	0250	Set B: Patients isolate nosocomial pneumonia
28						
29						
30	W05-67-1	1 Benidorm	426	2,6,17,14,12,11,11	0010	Set C: Environmental isolate working place
31	W05-67-4	1 Benidorm	426	2,6,17,14,12,11,11	0010	Set C: Environmental isolate working place
32						
33	L04-497	1 Benidorm	407	6,10,15,3,21,14,9	7000	Set C: Patients isolate community-acquired pneumonia
34						
35						
36	L97-229/1	1 Philadelphia	387	2,6,17,14,13,11,11	0060	Set D: Patients isolate community-acquired pneumonia
37	L97-229/2	1 Philadelphia	387	2,6,17,14,13,11,11	0060	Set D: Patients isolate community-acquired pneumonia
38						
39						
40	W04-989	10	245	2,10,3,13,9,4,1	0010	Set E: Environmental isolate
41						
42	W04-990	10	245	2,10,3,13,9,4,1	0010	Set E: Environmental isolate
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6	L04-564	10	245	2,10,3,13,9,4,1	0010	Set E: Patients isolate community-acquired pneumonia
7	L04-565	10	245	2,10,3,13,9,4,1	0010	Set E: Patients isolate community-acquired pneumonia
8						
9						
10	W05-192	10	246	2,10,3,28,9,4,11	0010	Set F: Hospital water isolate
11	W05-191	10	246	2,10,3,28,9,4,11	0010	Set F: Hospital water isolate
12						
13						
14	Koper 1	1 Knoxville	146	1,10,18,10,2,1,6	4251	Set G: Environmental isolate from a hotel
15	L02-435	1 Knoxville	146	1,10,18,10,2,1,6	4251	Set G: Patients isolate travel-associated pneumonia
16						
17						
18						
19	W05-174-1	10	248	3,13,1,1,14,9,11	0000	Set H: Environmental isolate
20	W05-177-1	10	248	3,13,1,1,14,9,11	0000	Set H: Environmental isolate
21						
22	L05-129	10	247	1,4,3,5,1,1,11	0251	Set H: Patients isolate community-acquired pneumonia
23						
24						
25	L01-127	1 OLDA	1	1,4,3,1,1,1,1	0250	Set J: Clinical isolate nosocomial pneumonia
26	W01/1967	1 OLDA	1	1,4,3,1,1,1,1	0250	Set J: Environmental isolate
27						
28						
29						
30	Uppsala 3	1 Knoxville	9	3,10,1,3,14,9,11	0000	Set K: Clinical isolate nosocomial pneumonia
31	Uppsala 10	1 OLDA	9	3,10,1,3,14,9,11	0000	Set K: Clinical isolate nosocomial pneumonia
32						
33						
34	Uppsala 59	1 Philadelphia	1	1,4,3,1,1,1,1	0250	Set L: Clinical isolate nosocomial pneumonia
35	Uppsala 60	1 OLDA	1	1,4,3,1,1,1,1	0250	Set L: Clinical isolate nosocomial pneumonia
36						
37						
38						
39	Uppsala 21	1 Philadelphia	1	1,4,3,1,1,1,1	0250	Set M: Clinical isolate nosocomial pneumonia
40	Uppsala 22	1 OLDA	1	1,4,3,1,1,1,1	0250	Set M: Clinical isolate nosocomial pneumonia
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Table 2. Primers used for variable element typing (VET)

NR	Primer designation	Sequence (5'- 3')	Size of the PCR Product/ gene	Reference
1	TrbII-F	TAA GGA GTG AGA CTG ATG AAT C	1247/ <i>trbII</i>	12
	TrbII-R	CAT TGC ACT CCT CCT GTA TCA AT		
2	TraG1-F	TGG AGA CGG TCA ATG AGC TTG A	1279/ <i>traG1</i>	12
	TraG1-R	CAT ATC GCG AGC CGA TGC GCT G		
3	MRR-F	AAT GAG AAG TCT TGG CTA TAC TTA	835/ <i>lpc0219</i>	This study
	MRR-R	GAT ATT CTC TAG CTT CGT TCG		
4	R33-F	CGA CTA GGC GCT TAA TTC TTG	830/ <i>lpc2786</i>	This study
	R33-R	ACG GAA CCA TTA AGA CAG GTT		
5	F2-F	ACT GGC ATG AGC AAT CAG TAG	1017/ <i>pacL</i> (<i>lpc2098</i>)	This study
	F2-R	ATG ATC ACC TGT CAG GAC AGA		
6	TraD-F	GCT TAT CAT CAC TTG CCC TTT	633/ <i>traD</i>	13
	TraD-R	GCA GAG ATA CAC CAC CAA TCC GA		
7	LvhB10-F	GCA ATC GGA CTC AGG TTG CTA	710/ <i>lvhB10</i>	12
	LvhB10-R	CTG CCA AAG CGC TCG AAG AAA		
8	MagA-F	CTC TAT CGC TAA CGC ACA AGG	469/ <i>magA</i>	13
	MagA-R	CGT TGA AGT AGT TAG TGA AAG		
9	127-2-F	AAG CGA TTA GCA TAT TAC GGT TTC	127/ <i>pacL</i>	This study
	127-2-R	CAT TGA TGA GAA CAA CTG CTA TTA TAA		
10	298-F	GAT CTT TTA TGG CTG TTG TTT GAG C	298/ <i>lpc1159-60</i>	This study
	298-2-R	GCC GCC TAT TTT TCG CAC TTT A

Fig. 1. Scheme of the location of the variable genetic elements in the four published genomes of *L. pneumophila* strains Philadelphia, Paris, Lens, and Corby

Fig. 2. UMPGA dendrogram generated from the VET pattern (Dice similarity coefficient) computed with the BioNumerics software for 57 strains. ■ - Variable genetic element present. ST, sequence type; Set A to M epidemiologically related strains (Table 1)

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1 **Abstract:**

2 A total of 57 isolates of *L. pneumophila* were randomly selected from the German National
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4 *Legionella* strain collection and typed by monoclonal antibody subgrouping, seven gene locus
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6
7 sequence-based typing (SBT) scheme and a newly developed variable element typing (VET)
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10 system based on the presence or absence of ten variable genetic elements. These elements
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12 were detected while screening of a genomic library of strain Corby as well as taken from
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14 published data for PAI-1 (pathogenicity island) from strain Philadelphia. Specific primers
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17 were designed and used in gel based PCRs. PCR amplification of the mip gene that served as
18
19 a control. The endpoint was the presence / absence of a PCR product on an ethidiumbromide
20
21
22 strained gel. In the present study the index of discrimination was somewhat lower than that of
23
24 the SBT (0.87 versus 0.97). Nevertheless, the results obtained showed as a 'proof of
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27 principle' that this simple and quick typing assay might be useful for the epidemiological
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29 characterization of *Legionella pneumophila* strains.
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1 Introduction

2 Members of the genus *Legionella* are Gram-negative bacteria and normally occupy natural
3 aquatic environments where they survive as intracellular parasites of protozoa. Currently the
4 genus *Legionella* contains more than 50 species. Among them the species *L. pneumophila* is
5 the most frequent [1;2]. *L. pneumophila* serogroup (sg) 1 is the most common cause of
6 pneumonia occurring as sporadic or endemic disease that may be acquired from different
7 environmental sources [3]. For public health authorities it is essential to detect the source of
8 infection promptly by comparing clinical and environmental strains of *L. pneumophila* to
9 conduct decontamination measures and to prevent further cases. For this numerous
10 phenotypic and genotypic typing methods have been applied to the epidemiological typing of
11 *L. pneumophila* in the recent years [4]. This include monoclonal antibody (MAb)
12 subgrouping as a rapid screening method [3] and genotyping methods such as amplified
13 fragment length polymorphism (AFLP) [4], pulsed-field gel electrophoresis (PFGE) [4;5] and
14 sequence based typing [6-8]. In contrast to band based typing methods that are difficult to
15 standardize the developed sequence based typing (SBT) has the potential of excellent
16 typeability, interlaboratory reproducibility and epidemiologic concordance [9]. However the
17 SBT is rather expensive and might therefore not be applicable to all strains and in all
18 laboratories. The specific gene content of each strain sequenced so far is constituted of 7-11%
19 of gene specific to each genome [10-12]. In addition, variable elements/ pathogenicity island
20 are present in some strains [13].

21 The purpose of this study was to investigate the usefulness of a binary typing system (VET)
22 based on the presence/ absence of defined genetic elements by using gel based PCR. These
23 data were compared to the European Working Group on Legionella Infections (EWGLI) SBT
24 system.

Materials and Methods

***Legionella pneumophila* strains.** A total of 57 isolates of *Legionella pneumophila* were randomly selected from the Dresden strain collection including the strains from which the complete genomes are published. From strain Corby we used a panel of variants (mutants) (EUL 135 to 139) that differ in their ability to multiply in amoebae and macrophages showed different reactivity patterns with MAbs due to a mutation in a lipopolysaccharide synthesis gene or that were resistant to rifampicin [7]. 40 strains were by definition unrelated to any other isolates in the study, i.e. they were isolated from sporadic cases or if they were part of an outbreak or cluster of legionellosis only one isolate was included in the analysis. 32 isolates were obtained during epidemiological studies (Table 1) and comprise twelve sets of related strains (A to M). Some of them were indistinguishable by MAb subgrouping and SBT thus proving a transmission from the environmental source to the patient. Sets C and H differed in both markers, whereas sets K, L, and M were indistinguishable by genetic typing but expressed a different monoclonal subtype which is related to the deletion of the *lag -1* gene [5] (Table 1).

Serotyping and sequence based typing (SBT)

Serological typing of *L. pneumophila* strains was performed by using the Dresden panel of monoclonal antibodies and the results were published previously [3;5;8]. Genotyping was performed by using the seven genes SBT [7;8]. Briefly, seven gene targets comprising *flaA*, *pilE*, *asd*, *mip*, *momp*, *proA* and *neuA* were amplified by PCR and sequenced. The assignment of the sequence type (ST) was carried out by using SBT database or the online sequence type checker (http://www.hpa-bioinfotools.org.uk/legionella/php/sbt_query1.php).

Variable element typing (VET). Target sequences for variable element typing were selected from a genomic library of strain Corby according to Heuner et al. [14] with minor

modifications. Briefly, chromosomal DNA of *L. pneumophila* Corby (sg 1) was partially digested with *Sau3A*. Fragments ranging from 1.0 to 4.0 kb were ligated into the *Bam*HI restriction site of vector pUC19 and transformed into *E. coli* DH5 α . The DNA sequence of the inserted Corby-DNA was determined by using standard m13 primers and primer walking. Fragments that could not be aligned to the Philadelphia genome available at that time were selected. Primer specific for these genetic elements were designed and used for typing (Table 2). For *magA* and *traD* sequences from the pathogenicity island [13] were used. DNAs from *L. pneumophila* strains was prepared as for sequenced based typing [7]. The VET- PCR were performed with 10pmol of each primer, 10 μ l of DNA-puffer, 5U of Gold-Taq-Polymerase, 2 μ l DNA of each strain ,and 200pmol of each dNTP at a final volumes of 50 μ l. The annealing temperature was 58°C. Gel electrophoresis was performed on 1% agarose gels. Gels were stained with ethidium bromide and analyzed under UV-light. PCR products of the expected size were considered positive. At the beginning of these experiments the specificity of the PCR products was confirmed by DNA sequencing [7]. In each run the *mip* gene PCR of the SBT scheme were used as a positive control [7]. All VET PCR assays were run at least twice.

The binary results (presence/absence of a PCR product) were recorded as a VET type and compared to both standard methods (monoclonal antibody typing and SBT) (Table 1). These binary data is offer the possibility to exchange these data between laboratories.

Results and Discussion

The primary aim of this study was to prove the principle of VET as a genotyping method for *L. pneumophila*. The primers developed and used in this study were aligned to the four available complete genomes of *L. pneumophila* [10;11;12] by using the Basic Local Alignment Search Tool (BLAST) available online (www.ncbi.nlm.nih.gov/BLAST) [15]. The results of this search demonstrated that the binding sites of some primers were present some of the four genomes. The investigation of the strains Philadelphia-1, Lens, Paris and Corby by using the new VET-PCRs confirmed the expected results, i. e. all PCR product predicted by the BLAST search could readily detected by using the ten VET-PCR assays. The variable elements used in this typing assay are relatively randomly distributed in the genome of the strains from which complete sequenced genomes are available (Fig.1).

In addition, all five variants of the Corby strains that differ in the cultivability in amoebal hosts, point mutations in the *rpoB* and the *lag-1* genes showed the same VET results (data not shown) thus arguing that the markers detected in the VET are stable as were the SBT results [7].

For 57 of the strains investigated by using variable element typing (VET) results were obtained for 51. In six strains none of the elements could be detected by the current assays (Table 1). Since the positive control PCR using the *mip* primers of the SBT scheme gave positive results we are sure that the genetic elements or at least one of the primer binding sites were readily absent (or too less identical). From a total of 57 strains, 32 related strains were merged into 12 sets (A to M) (Table 1, Fig 2). From these sets of related strains the results of seven sets (A, B, D, E, F, G, and J) showed that related strains were indistinguishable by using for MAb typing, SBT and VET. For further two sets (C and H), the serotype/MAb subtype matched. However, SBT and VET of the environmental isolates were different from that of the patient's isolates. Vice versa three sets (K, L, and M) showing the same genetic fingerprints by using SBT and VET were distinguishable by MAb typing. It has been shown

previously that changes in genes involved in the LPS synthesis might change the reactivity with MAbs. Thus the loss of the *lag-1* gene coding for an O-acetyltransferase is responsible for the switch from MAbtype Philadelphia/ Knoxville to the MAb type OLDA/Denver [5;16;17].

Taking into account the moderate number of unrelated strains (n=40) in our preliminary study a moderate index of discrimination (IOD) of 0.87 was calculated. This is lower than that published previously for SBT, but higher than that for MAb typing and AFLP [4;7].

The most frequently occurring VET-type 0,0,0,0,1,0,1,0,1,0, was found in several strains belonging to different MAb-types and but belonged all to ST 1. Interestingly, some strains belonging to different serotypes were indistinguishable by VET (e.g., L02-298, L05-129) (Table 1).

Due to the limited number of strains a general conclusion concerning the correlation between these two genotyping methods is difficult. It is not surprising that a complete correlation does not exist between both genotyping methods, since SBT detects variations in seven genes belonging to the core genome whereas VET detects the absence or presence of variable genetic elements like PAIs. Therefore, it is not surprising that some strains of sequence type (ST) 1 investigated here, had a different VET-type.

SBT is a powerful tool for subtyping *L. pneumophila* strains and is currently the gold standard within the European Working Group on Legionella Infections (EWGLI). Data obtained in different countries can easily be compared and the European data base currently containing hundreds of types can be used both in epidemiological investigations and to assess the risk associated with environmental reservoirs that are contaminated with illness-associated strains. However SBT is relatively expensive and therefore might not be applicable to all laboratories. The aim of the present study was to evaluate an alternative rapid and cheap PCR approach based on the absence or presence of defined genetic elements in different strains. A recent study from Australia suggested that in all strains a similar repertoire of virulence gene exist

[18]. In accordance with this study our results demonstrate that the presence of the *lvh* locus, the *trb-1* locus and the pathogenicity island I are variable in different strains. So far no association of the presence of these genetic elements with the putative virulence i. e. the origin of the strains could be demonstrated.

The usefulness of the VET approach as a technique for subtyping of strains belonging to different serogroups of *L. pneumophila* was clearly demonstrated. The possibility to express the data as binary codes allows the standardization and portability of these results as shows for *Staphylococcus aureus* and *Escherichia coli* [19;20]. It remains an open question and needs to be experimentally proven with a larger number of strains to whether the use of VET is indeed suitable for the epidemiological subtyping of *L. pneumophila*. Finally, it must be pointed out that these genetic elements might serve as candidates for a DNA chip based typing system similar to that for *St. aureus* [21]. In summary, our current results are encouraging. The VET scheme showed good typeability and discriminatory power for *L. pneumophila*. However, further variable elements must be identified to increase both, the typeability and the discriminatory potential.

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Table 1. *Legionella pneumophila* strains

Strain designation	Serogroup, Monoclonal Subtype	Sequence Type (ST)	Allels profile of <i>flaA</i> , <i>pilE</i> , <i>asd</i> , <i>mip</i> , <i>ompS</i> , <i>proA</i> and <i>neuA</i>	Variable element typing VET	Origin of strains
Lens	1 Benidorm	15	12,9,26,5,26,17,15	0000101000	Clinical isolate community acquired pneumonia, unrelated
Augsburg-1	1 Benidorm	42	4,7,11,3,11,12,9	1111101101	Clinical isolate community acquired pneumonia, unrelated
Berlin 11	1 Benidorm	425	2,10,3,15,9,4,11	0000100010	Clinical isolate nosocomial pneumonia, unrelated
L02-287	1 Benidorm	181	3,7,1,12,14,9,9	1100001001	Clinical isolate community acquired pneumonia, unrelated
L02-298	1 Benidorm	42	4,22,11,3,11,12,9	0000101011	Clinical isolate community acquired pneumonia, unrelated
L04-412	1 Knoxville	9	3,10,1,3,14,9,11	0000000011	Clinical isolate travel-associate pneumonia, unrelated
Corby	1 Knoxville	51	6,10,15,28,9,14,6	1111100011	Clinical isolate community acquired pneumonia, unrelated
L01-443	1 Knoxville	9	3,10,1,3,14,9,11	0111000011	Clinical isolate nosocomial pneumonia, unrelated
Berlin 4	1 OLDA	1	1,4,3,1,1,1,1	0101110010	Clinical isolate nosocomial pneumonia, unrelated
Charite 16297/2000	1 OLDA	1	1,4,3,1,1,1,1	0110111010	Clinical isolate community acquired pneumonia, unrelated
Goettingen 12/98	1 OLDA	1	1,4,3,1,1,1,1	0000111010	Clinical isolate community acquired pneumonia, unrelated
L03-610	1 OLDA	7	1,4,3,1,1,1,6	0000100010	Clinical isolate community acquired pneumonia, unrelated
L04-567	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Clinical isolate nosocomial pneumonia, unrelated
Paris	1 Philadelphia	1	1,4,3,1,1,1,1	0000001010	Clinical isolate community acquired pneumonia, unrelated
Philadelphia	1 Philadelphia	36	3,4,1,1,14,9,1	0000011100	Clinical isolate community acquired pneumonia, unrelated
L04-280	1 Philadelphia	23	2,3,9,10,2,1,6	1111100010	Clinical isolate travel-associate pneumonia, unrelated
L02-034	10	440	3,10,1,28,14,9,1	0000000000	Clinical isolate nosocomial pneumonia, unrelated
L02-303-1	15	337	10,22,7,28,16,18,6	0100001001	Clinical isolate nosocomial pneumonia, unrelated
Heidelberg P9	4 Portland	67	1,10,3,28,9,4,1	0010011001	Clinical isolate nosocomial pneumonia, unrelated
W 01/1993	5 Cambridge	245	1,10,3,13,9,4,1	1111101000	Environmental isolate, unrelated

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6	Chicago 2	6 Chicago	30	3,10,1,3,14,9,9	0000110110	Clinical isolate	nosocomial pneumonia, unrelated
7	Finnl. 10	6 Chicago	68	3,13,1,28,14,9,3	1100111011	Clinical isolate	community acquired pneumonia, unrelated
8	Heidelberg P1	6 Dresden	330	7,10,17,3,4,11,3	0001001001	Clinical isolate	nosocomial pneumonia, unrelated
9							
10	L04-507	1 Philadelphia	332	7,10,17,6,14,11,3	0000010100	Clinical isolate	community acquired pneumonia, unrelated
11							
12	L04-506	1 Philadelphia	332	7,10,17,6,14,11,3	0000010100	Clinical isolate	community acquired pneumonia, unrelated
13							
14							
15	W05-007	6 Chicago	424	7,10,17,3,13,14,9	0000001010	Set A: Hospital water isolate	
16	L04-597	6 Chicago	424	7,10,17,3,13,14,9	0000001010	Set A: Patients isolate	nosocomial pneumonia
17							
18	L05-008	6 Chicago	424	7,10,17,3,13,14,9	0000001010	Set A: Patients isolate	nosocomial pneumonia
19							
20							
21	W04-952	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Hospital water isolate	
22	W04-954	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Hospital water isolate	
23							
24	W04-956	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Hospital water isolate	
25	Erl. 361/1	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Patients isolate	nosocomial pneumonia
26							
27	L04-541	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Patients isolate	nosocomial pneumonia
28							
29							
30	W05-67-1	1 Benidorm	426	2,6,17,14,12,11,11	0000001000	Set C: Environmental isolate	working place
31	W05-67-4	1 Benidorm	426	2,6,17,14,12,11,11	0000001000	Set C: Environmental isolate	working place
32							
33	L04-497	1 Benidorm	407	6,10,15,3,21,14,9	1110000000	Set C: Patients isolate	community-acquired pneumonia
34							
35							
36	L97-229/1	1 Philadelphia	387	2,6,17,14,13,11,11	0000000110	Set D: Patients isolate	community-acquired pneumonia
37	L97-229/2	1 Philadelphia	387	2,6,17,14,13,11,11	0000000110	Set D: Patients isolate	community-acquired pneumonia
38							
39							
40	W04-989	10	245	2,10,3,13,9,4,1	0000001000	Set E: Environmental isolate	
41							
42	W04-990	10	245	2,10,3,13,9,4,1	0000001000	Set E: Environmental isolate	
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6	L04-564	10	245	2,10,3,13,9,4,1	0000001000	Set E: Patients isolate community-acquired pneumonia
7	L04-565	10	245	2,10,3,13,9,4,1	0000001000	Set E: Patients isolate community-acquired pneumonia
8						
9						
10	W05-192	10	246	2,10,3,28,9,4,11	0000001000	Set F: Hospital water isolate
11	W05-191	10	246	2,10,3,28,9,4,11	0000001000	Set F: Hospital water isolate
12						
13						
14	Koper 1	1 Knoxville	146	1,10,18,10,2,1,6	0010101011	Set G: Environmental isolate from a hotel
15	L02-435	1 Knoxville	146	1,10,18,10,2,1,6	0010101011	Set G: Patients isolate travel-associated pneumonia
16						
17						
18						
19	W05-174-1	10	248	3,13,1,1,14,9,11	0000000000	Set H: Environmental isolate
20	W05-177-1	10	248	3,13,1,1,14,9,11	0000000000	Set H: Environmental isolate
21						
22	L05-129	10	247	1,4,3,5,1,1,11	0000101011	Set H: Patients isolate community-acquired pneumonia
23						
24						
25	L01-127	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set J: Clinical isolate nosocomial pneumonia
26	W01/1967	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set J: Environmental isolate
27						
28						
29	Uppsala 3	1 Knoxville	9	3,10,1,3,14,9,11	0000000000	Set K: Clinical isolate nosocomial pneumonia
30	Uppsala 10	1 OLDA	9	3,10,1,3,14,9,11	0000000000	Set K: Clinical isolate nosocomial pneumonia
31						
32						
33						
34	Uppsala 59	1 Philadelphia	1	1,4,3,1,1,1,1	0000101010	Set L: Clinical isolate nosocomial pneumonia
35	Uppsala 60	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set L: Clinical isolate nosocomial pneumonia
36						
37						
38	Uppsala 21	1 Philadelphia	1	1,4,3,1,1,1,1	0000101010	Set M: Clinical isolate nosocomial pneumonia
39	Uppsala 22	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set M: Clinical isolate nosocomial pneumonia
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Table 2. Primers used for variable element typing (VET)

NR	Primer designation	Sequence (5'- 3')	Size of the PCR Product/ gene	Reference
1	TrbII-F	TAA GGA GTG AGA CTG ATG AAT C	1247/ <i>trbII</i>	12
	TrbII-R	CAT TGC ACT CCT CCT GTA TCA AT		
2	TraG1-F	TGG AGA CGG TCA ATG AGC TTG A	1279/ <i>traG1</i>	12
	TraG1-R	CAT ATC GCG AGC CGA TGC GCT G		
3	MRR-F	AAT GAG AAG TCT TGG CTA TAC TTA	835/ <i>lpc0219</i>	This study
	MRR-R	GAT ATT CTC TAG CTT CGT TCG		
4	R33-F	CGA CTA GGC GCT TAA TTC TTG	830/ <i>lpc2786</i>	This study
	R33-R	ACG GAA CCA TTA AGA CAG GTT		
5	F2-F	ACT GGC ATG AGC AAT CAG TAG	1017/ <i>pacL</i> (<i>lpc2098</i>)	This study
	F2-R	ATG ATC ACC TGT CAG GAC AGA		
6	TraD-F	GCT TAT CAT CAC TTG CCC TTT	633/ <i>traD</i>	13
	TraD-R	GCA GAG ATA CAC CAC CAA TCC GA		
7	LvhB10-F	GCA ATC GGA CTC AGG TTG CTA	710/ <i>lvhB10</i>	12
	LvhB10-R	CTG CCA AAG CGC TCG AAG AAA		
8	MagA-F	CTC TAT CGC TAA CGC ACA AGG	469/ <i>magA</i>	13
	MagA-R	CGT TGA AGT AGT TAG TGA AAG		
9	127-2-F	AAG CGA TTA GCA TAT TAC GGT TTC	127/ <i>pacL</i>	This study
	127-2-R	CAT TGA TGA GAA CAA CTG CTA TTA TAA		
10	298-F	GAT CTT TTA TGG CTG TTG TTT GAG C	298/ <i>lpc1159-60</i>	This study
	298-2-R	GCC GCC TAT TTT TCG CAC TTT A

Fig. 1. Scheme of the location of the variable genetic elements in the four published genomes of *L. pneumophila* strains Philadelphia, Paris, Lens, and Corby

Fig. 2. UMPGA dendrogram generated from the VET pattern (Dice similarity coefficient) computed with the BioNumerics software for 57 strains. ■ - Variable genetic element present. ST, sequence type; Set A to M epidemiologically related strains (Table 1)

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Statements to the reviewer's comments

Reviewer #1: This is a clear, and mainly well-presented, description of a binary typing method for *Legionella pneumophila* based on presence or absence of ten defined genetic elements. I have only two minor comments:

Since the number and diversity of the isolates examined is quite small and restricted in range, this study could not be described as an evaluation of the method but rather a 'proof of principle'. As such the last line of the abstract '. proved the usefulness..' does not seem to be supported by the data presented.

CL: The abstract has been changed: Now the phrase is

"The results obtained showed that this simple and quick typing assay might be useful for the epidemiological characterization of *Legionella pneumophila* strains".

It also has been changed at the first line in the results and discussion section.

Perhaps it should be moderated a little to reflect the fact that this is a very preliminary evaluation. Also as written the abstract is not very informative. Since this is usually the only bit of a paper that most readers attend to, it might be helpful to add some data (even if it is only to say how many elements are looked at and that endpoint is presence/absence of a PCR product on a gel).

CL: The abstract was rewritten to clarify the points raised.

I may have missed something, but to me the VET-index seems an overly complex way of expressing binary data. Rather than group the ten 'scored' elements into three sets of three plus a singleton, to achieve a four digit index (the last digit of which can only ever be 0 or 1), why do the authors not leave it as a binary number? If writing a ten digit number is considered to be burdensome, or prone to transcription error, then simply express it as a decimal giving a single value for each profile running from 0-1,023?

CL: I agree with the reviewer that the introduction of an index is too complicated. Keeping in mind the relatively low number of strains this seems to be not indicated. One might consider this again when a larger number of strains have been typed. The text and table 1 were changed accordingly.