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Concise Article

Variable Genetic Element Typing: A quick method for epidemiological subtyping of *Legionella pneumophila*

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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 strained 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.

Introduction

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

The purpose of this study was to investigate the usefulness of a binary typing system (VET) based on the presence/ absence of defined genetic elements by using gel based PCR. These data were compared to the European Working Group on Legionella Infections (EWGLI) SBT 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 *fla*A, *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 *Sau*3A. 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* DH5α. 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 *mag*A and *tra*D 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, 10µl of DNA-puffer, 5U of Gold-Taq-Polymerase, 2µl DNA of each strain ,and 200pmol of each dNTP at a final volumes of 50µl. 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 antibodiy 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 *rpo*B 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 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 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 cheep 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 *fla*A, *pil*E, *asd*, *mip*, *omp*S, *pro*A and *neu*A	**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

Chicago 2	6 Chicago	30	3,10,1,3,14,9,9	0000110110	Clinical isolate nosocomial pneumonia, unrelated
Finnl. 10	6 Chicago	68	3,13,1,28,14,9,3	1100111011	Clinical isolate community acquired pneumonia, unrelated
Heidelberg P1	6 Dresden	330	7,10,17,3,4,11,3	0001001001	Clinical isolate nosocomial pneumonia, unrelated
L04-507	1 Philadelphia	332	7,10,17,6,14,11,3	0000010100	Clinical isolate community acquired pneumonia, unrelated
L04-506	1 Philadelphia	332	7,10,17,6,14,11,3	0000010100	Clinical isolate community acquired pneumonia, unrelated
W05-007	6 Chicago	424	7,10,17,3,13,14,9	0000001010	Set A: Hospital water isolate
L04-597	6 Chicago	424	7,10,17,3,13,14,9	0000001010	Set A: Patients isolate nosocomial pneumonia
L05-008	6 Chicago	424	7,10,17,3,13,14,9	0000001010	Set A: Patients isolate nosocomial pneumonia
W04-952	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Hospital water isolate
W04-954	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Hospital water isolate
W04-956	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Hospital water isolate
Erl. 361/1	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Patients isolate nosocomial pneumonia
L04-541	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set B: Patients isolate nosocomial pneumonia
W05-67-1	1 Benidorm	426	2,6,17,14,12,11,11	0000001000	Set C: Environmental isolate working place
W05-67-4	1 Benidorm	426	2,6,17,14,12,11,11	0000001000	Set C: Environmental isolate working place
L04-497	1 Benidorm	407	6,10,15,3,21,14,9	1110000000	Set C: Patients isolate community-acquired pneumonia
L97-229/1	1 Philadelphia	387	2,6,17,14,13,11,11	0000000110	Set D: Patients isolate community-acquired pneumonia
L97-229/2	1 Philadelphia	387	2,6,17,14,13,11,11	0000000110	Set D: Patients isolate community-acquired pneumonia
W04-989	10	245	2,10,3,13,9,4,1	0000001000	Set E: Environmental isolate
W04-990	10	245	2,10,3,13,9,4,1	0000001000	Set E: Environmental isolate

L04-564	10	245	2,10,3,13,9,4,1	0000001000	Set E: Patients isolate community-acquired pneumonia
L04-565	10	245	2,10,3,13,9,4,1	0000001000	Set E: Patients isolate community-acquired pneumonia
W05-192	10	246	2,10,3,28,9,4,11	0000001000	Set F: Hospital water isolate
W05-191	10	246	2,10,3,28,9,4,11	0000001000	Set F: Hospital water isolate
Koper 1	1 Knoxville	146	1,10,18,10,2,1,6	0010101011	Set G: Environmental isolate from a hotel
L02-435	1 Knoxville	146	1,10,18,10,2,1,6	0010101011	Set G: Patients isolate travel-associated pneumonia
W05-174-1	10	248	3,13,1,1,14,9,11	0000000000	Set H: Environmental isolate
W05-177-1	10	248	3,13,1,1,14,9,11	0000000000	Set H: Environmental isolate
L05-129	10	247	1,4,3,5,1,1,11	0000101011	Set H: Patients isolate community-acquired pneumonia
L01-127	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set J: Clinical isolate nosocomial pneumonia
W01/1967	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set J: Environmental isolate
Uppsala 3	1 Knoxville	9	3,10,1,3,14,9,11	0000000000	Set K: Clinical isolate nosocomial pneumonia
Uppsala 10	1 OLDA	9	3,10,1,3,14,9,11	0000000000	Set K: Clinical isolate nosocomial pneumonia
Uppsala 59	1 Philadelphia	1	1,4,3,1,1,1,1	0000101010	Set L: Clinical isolate nosocomial pneumonia
Uppsala 60	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set L: Clinical isolate nosocomial pneumonia
Uppsala 21	1 Philadelphia	1	1,4,3,1,1,1,1	0000101010	Set M: Clinical isolate nosocomial pneumonia
Uppsala 22	1 OLDA	1	1,4,3,1,1,1,1	0000101010	Set M: Clinical isolate nosocomial pneumonia

Table 2. Primers used for variable element typing (VET)

NR	Primer designation	Sequence (5'- 3')	Size of the PCR Product/ gene	Reference
1	TrbI1-F	TAA GGA GTG AGA CTG ATG AAT C	1247/*trbI1*	12
	TrbI1-R	CAT TGC ACT CCT CCT GTA TCA AT		
2	TraG1-F	TGG AGA CGG TCA ATG AGC TTG A	1279/*traG1*	12
	TraG1-R	CAT ATC GCG AGC CGA TGC GCT G		
3	MRR-F	AAT GAG AAG TCT TGG CTA TAC TTA	835/*lpc0219*	This study
	MRR-R	GAT ATT CTC TAG CTT CGT TCG		
4	R33-F	CGA CTA GGC GCT TAA TTC TTG	830/*lpc2786*	This study
	R33-R	ACG GAA CCA TTA AGA CAG GTT		
5	F2-F	ACT GGC ATG AGC AAT CAG TAG	1017/*pacL (lpc2098)*	This study
	F2-R	ATG ATC ACC TGT CAG GAC AGA		
6	TraD-F	GCT TAT CAT CAC TTG CCC TTT	633/*traD*	13
	TraD-R	GCA GAG ATA CAC CAC CAA TCC GA		
7	LvhB10-F	GCA ATC GGA CTC AGG TTG CTA	710/*lvhB10*	12
	LvhB10-R	CTG CCA AAG CGC TCG AAG AAA		
8	MagA-F	CTC TAT CGC TAA CGC ACA AGG	469/*magA*	13
	MagA-R	CGT TGA AGT AGT TAG TGA AAG		
9	127-2-F	AAG CGA TTA GCA TAT TAC GGT TTC	127/*pacL*	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/*lpc1159-60*	This study
	298-2-R	GCC GCC TAT TTT TCG CAC TTT A		

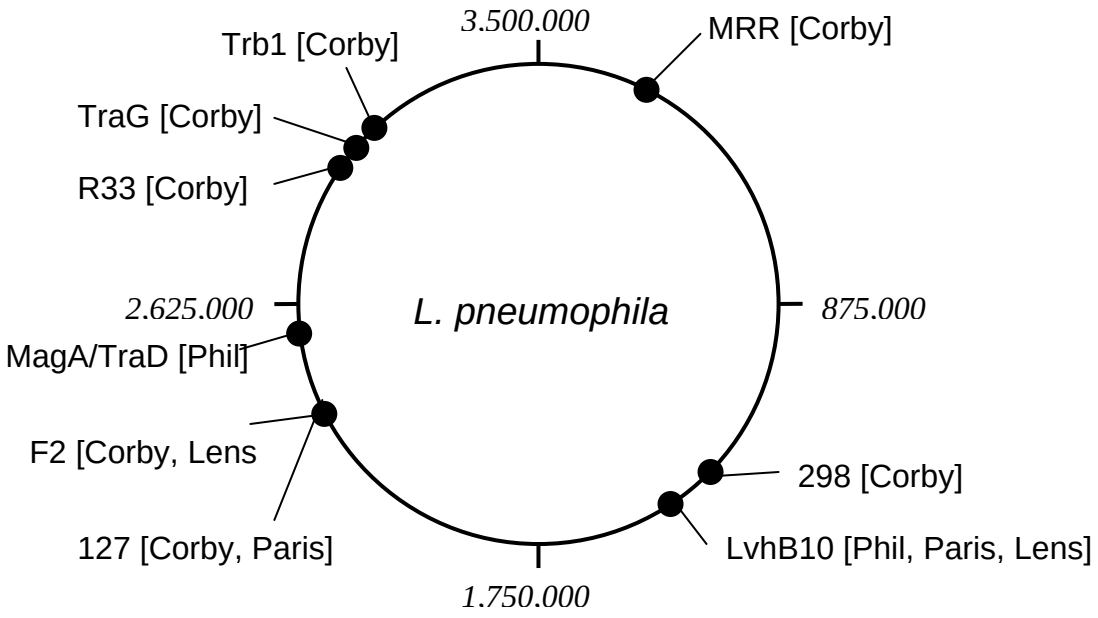

Fig. 1. Scheme of the location of the variable genetic elements in the four published genomes of *L. pneumophila* strains Philadelphia, Paris, Lens, and Corby

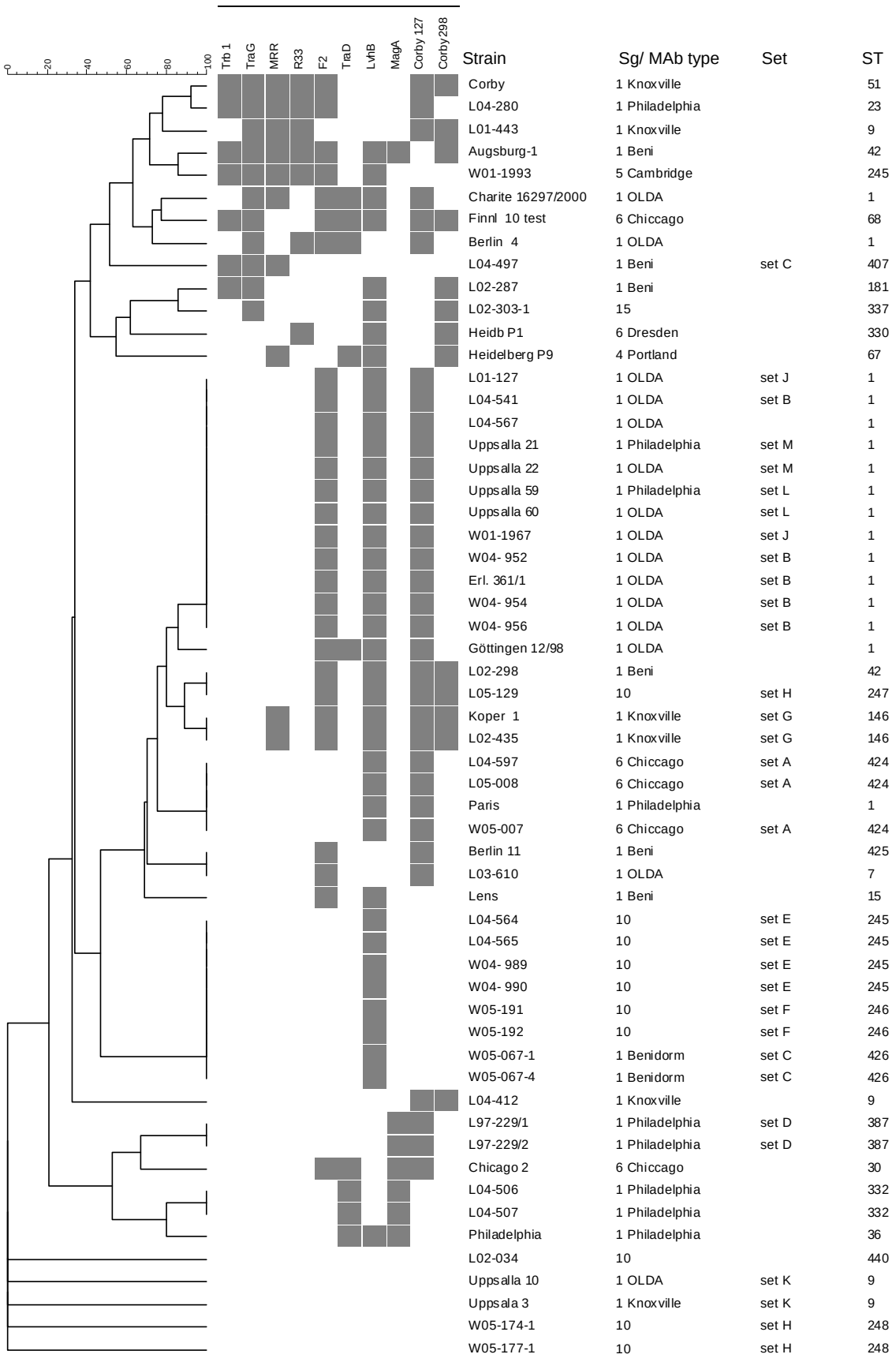

Fig. 2. UMPGA dendrogram generated from the VET pattern (Dice similarity coefficient) computed with the BioNumerics software for 57 strains. ▓ - Variable genetic element present. ST, sequence type; Set A to M epidemiologically related strains (Table 1)

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