Veterinary Microbiology 231 (2019) 11–17

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Development of multi locus sequence typing (MLST) of *Rodentibacter pneumotropicus*

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ARTICLE INFO

Keywords:
Rodentibacter pneumotropicus
Rodents
MLST
Sequence type
House-keeping enzymes

ABSTRACT

The aim of the investigation was to develop a definitive typing system for *Rodentibacter pneumotropicus.* A total of 79 strains including the type strain of *R. pneumotropicus,* all associated with rodents were used to develop a multi-locus sequence typing scheme (MLST). Primers were designed for conserved regions of seven house-keeping genes (*atpG, frdB, gdh, pgi, pmi, recA*, *zwf*) and internal fragments of 399–839 bp were sequenced for all strains. The genes were also extracted in full length from whole genomic sequences of 14 strains of which 10 were sequenced in the current study. The number of alleles at the different loci ranged from 5 to 7 and a total of 20 allelic profiles or sequence types were recognized amongst the 79 strains. Analysis of the MLST data showed that some STs have been stable over many years probably circulating in the same colonies and probably transferred between colonies. We assume that this MLST scheme may provide a high level of resolution and might be an excellent tool for studying the population structure and epidemiology of *R. pneumotropicus*. Further development of the scheme is expected by including more genes and more strains and involve whole genomic sequencing.

1. Introduction

Rodentibacter pneumotropicus, previously named [*Pasteurella*] *pneumotropica* is the type species of the new genus *Rodentibacter* (Adhikary et al., 2017a). It is a non-motile, non-haemolytic, facultative anaerobic, fermentative, Gram-negative coccobacillus which usually colonizes the mucosa of the nasopharynx, trachea, lungs, vagina, uterus, urinary bladder, intestines and other organs of laboratory animals (Needham and Cooper, 1975; Ward et al., 1978; Saito et al., 1981; Manning et al., 1991; Goelz et al., 1996). The pathogenicity of this organism in immune-competent laboratory mice and rats is known to be weak although it can occasionally produce clinical disorders in immuno-deficient animals (Needham and Cooper, 1975; Artwohl et al., 2000). High morbidity and mortality has recently been demonstrated in immune-competent mice by a virulent strain of *R. pneumotropicus* (Fornefett et al., 2018). Wild mice are carriers of this agent (Nicklas, 2007). Therefore, it is included as a routine test organism for microbiologic monitoring of laboratory rodents (Mähler et al., 2014).

Previously, *R. pneumotropicus* was classified as two biotypes, Jawetz and Heyl and various molecular typing procedures have been reported for bio-typing of these two groups. A subsequent restriction fragment length polymorphism (RFLP) analysis based on the *gyrB* gene sequence showed good resolution for molecular identification and typing (Hayashimoto et al., 2007). Recently, a method based on a combination of a real-time PCR assay amplifying a variable region of the 16S rRNA sequence with high-resolution melting curve analysis has been developed to identify and differentiate the biotypes (Miller et al., 2015). The genetic diversity of strains and isolates of [*P.*] *pneumotropica* observed by using amplified 16S rDNA restriction analysis (ARDRA) and pulse-field gel electrophoresis (PFGE) was also described (Kodjo et al., 1999; Sasaki et al., 2006). At the very recent, clonal relationship in disease outbreak of biotype Heyl has been documented by PFGE (Adhikary et al., 2017b). Moreover, Weigler and colleagues reported the usefulness of the randomly amplified polymorphic DNA polymerase chain reaction assay (RADP-PCR) for molecular epidemiologic investigation of this bacterium in laboratory rodent colonies (Weigler et al., 1996).

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https://doi.org/10.1016/j.vetmic.2019.02.025
Received 8 December 2018; Received in revised form 4 February 2019; Accepted 19 February 2019

However, many factors ($MgCl_2$, nucleotides & thermostable DNA polymerase concentrations) can affect reproducibility and variability of (randomly amplified polymorphic DNA) RAPD reactions (Meunier and Grimont, 1993). Besides, these methods are unable to share and compare information across laboratories that may necessitate repeated examinations or testing (Blackall and Miflin, 2000).

Multilocus sequence typing (MLST) represents a definitive alternative option to the methods described above, because it produces unambiguous and precise molecular typing data for characterization of bacterial isolates (Maiden et al., 1998; Enright and Spratt, 1999). Furthermore, this technique is highly portable as any laboratory can compare the sequences of the seven loci in their isolates with those in a central database and thereby obtain the allelic profile of each isolate. MLST results can give insight into the population structures of bacterial isolates. Until now, there has been no attempt to develop a MLST scheme that is only associated with rodent isolates. However, a MLST scheme of *Streptococcus pneumoniae* based on human isolates is available and includes rodent isolates, collected from infection (van der Linden et al., 2009).

In this study, we report the development of the first MLST scheme able to detect the population structure of the most important member of rodent *Pasteurellaceae*, *R. pneumotropicus*.

2. Materials and methods

2.1. Bacterial strains

A total of 79 strains were included in this study. The strains were collected from mouse (65), rat (12) and rabbit (2) and originated from the Netherlands, Denmark, Germany, Australia, Spain and USA during a period from 1948-2016. The isolates were mainly cultured from conjunctiva, the respiratory or the genital tract (Table 1). Out of 79, 65 strains were included in a previous study on the reclassification of [*Pasteurella*] *pneumotropica* as *Rodentibacter pneumotropicus* (Adhikary et al. (2017a,b). The remaining 14 strains were formerly described and analyzed as well (Benga et al., 2013; Sager et al., 2015).

2.2. Extraction of DNA for PCR

Bacteria from frozen stocks in bovine serum containing 6% glucose at - 80 °C were plated on 5% sterile calf blood agar (Blood Agar Base, CM55; Oxoid, Basingstoke, UK) and incubated under micro-aerophilic conditions in sealed plastic bags at 37 °C for 24 h. Four strains incapable to grow on blood agar were cultured on chocolate agar plates to prepare DNA because these strains were V-factor dependent. A single colony was subsequently grown in brain heart infusion broth (BHI; Difco, Heidelberg, Germany) for 24 h at 37 °C in an aerobic atmosphere. From the culture, 1.5 ml BHI culture was transferred into a tube and pelleted by centrifugation for three minutes at 15,000 × g, after which the supernatant was discarded. DNA was purified with the DNeasy Blood & Tissue Kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified DNA was frozen at −20 °C until PCR was carried out. The DNA concentration was determined with Nanodrop 1000 (Thermo Fisher Scientific, Wilmington DE, USA).

2.3. Genes for MLST

The house-keeping genes were selected based on the existing Rural Industries Research and Development Corporation (RIRDC) MLST scheme of *P. multocida* (Curated by Patrick J. Blackall, Queensland Alliance for Agriculture and Food Innovation, Australia) (Subaaharan et al., 2010). The genome of the reference strain of *R. pneumotropicus* NCTC8141^T (NCBI acc. no. ARBW01000000) was searched for annotated nucleotide sequence data. The three genes, *pmi, gdh* and *zwf* could be identified on the annotated genome of the reference strain. The selection of four additional genes *atpG, frdB, pgi and recA* was based on the MLST scheme of *Haemophilus influenzae* (Meats et al., 2003).

2.4. Gene amplification and sequencing

All primer sets used in this study were designed using the Primer3 software (http://primer3.ut.ee) using the nucleotide sequence of the respective gene in the reference strain NCTC 8141^T. The details of the primer sets are presented in Table 2. A volume of 25 µl PCR mixture contained DreamTaq DNA polymerase (ThermoFisher Scientific, Waltham, MA USA), DreamTaq Green (ThermoFisher Scientific) buffer, 0.4 mM each of dATP, dCTP, dGTP, 4 mM $MgCl_2$, 0.1 µM of each primer and 1 µl genomic DNA. For all PCRs the following reaction conditions were used: 30 s of denaturation at 94 °C, 30 s of annealing at 48 °C and 1 min of extension at 72 °C for 30 cycles. A last cycle of elongation of 5 min at 72 °C was used. The two enzymes, Exonuclease1 (Cat no.EN0581) and Fast AP (Cat no. EF0651) were used to purify the PCR product. Both enzymes were from ThermoFisher Scientific. The sequencing of the PCR reaction products was carried out by using Macrogen Europe service (Amsterdam, the Netherlands). The sequences were assembled by CLC Main Workbench (QIAGEN, Aarhus A/S) and imported as FASTA file for further analysis.

A total of 13 strains were whole genome sequenced according to Adhikary et al. (2017a). Two published genomic sequences were already available for the type strain of *R. pneumotropicus* (Table 1). The details of three of the genomes were described by Adhikary et al. (2017a). The coverage of the 10 genomes sequenced for the current study (Table 1) ranged from 27 to 67.

The genome scale data was used to develop the Bacterial Isolate Genome Sequence Database (BIGSdb) (Jolley and Maiden, 2010) (Curated by Henrik Christensen, Denmark) to facilitate analysis of epidemiology, ecology and population structure including the isolate background and phenotype. The database is hosted at https://ivsmlst.sund.ku.dk/.

2.5. MLST analysis

For each house-keeping gene analyzed, a multiple alignment of DNA sequences was constructed by ClustalX2 (Larkin et al., 2007) and BioEdit (Hall, 1999). For each locus, different sequences were assigned as distinct alleles. This resulted in a 7-digit allelic profile for each isolate. Each unique allelic profile was manually assigned as a sequence type (ST) with a random number. Isolates with the same allelic profile were assigned as the same ST.

Global optimal eBURST (goeBURST) (Francisco et al., 2009) implemented by PHYLOViZ (Francisco et al., 2012) was used to cluster STs with triple locus variant (TLV) limitations, generating a minimum spanning tree to visualize possible evolutionary relationship between STs. Two different STs sharing six of the seven loci established a single-locus variant (SLV) and a double locus variant (DLV) contained two STs differing in two loci. A clonal complex (CC) was composed of at least three strains with only SLVs or DLVs. When only two STs belonged to the same SLV they were termed a doublet. The remaining STs, which had no SLV with other STs, were termed singletons.

For linkage analysis: LIAN 3.0 was used from http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query (Haubold and Hudson, 2000). The G + C content, number of polymorphic sites and the average synonymous/ non-synonymous rate ratio (dS/dN) were calculated with DnaSP Version 5.10 (Librado and Rozas, 2009). The significance of the synonymous/ non-synonymous rate ratio (dS/dN) was evaluated by Fischer's exact test in MEGA (Tamura et al., 2011).

3. Results

For all 79 strains, PCR products of the expected size were obtained for the seven genes analyzed (*atpG, frdB, gdh, pmi pgi, recA and zwf*) using the single primer set for each respective gene shown in Table 2.

Table 1
Strains investigated in *Rodentibacter pneumotropicus* to develop the MLST scheme.

Strain	ID[a]	ST[b]	CC	Host	Organ	Origin and year of isolation	NCBI acc. no. of whole genome sequences
NCTC 8141^T (= CCUG 12398^T = DSM 21403 ^{T}= ATCC 35149 ^{T} = P421 ^{T})[c]	1 2	1	1	Mouse		Laboratory, USA, 1948	ARBW01000000 (KB904043) BBIX01000000
Ppn270 (10)[c]	16	1	1	Mouse		Wild, the Netherlands, 1995	
Ppn289[c]	17	1	1	Mouse		Wild, the Netherlands, 1995	
Ppn265[c]	18	1	1	Mouse		Wild, the Netherlands, 1995	
Ppn267[c]	19	1	1	Mouse	Trachea	Wild, the Netherlands, 1995	
Ppn283[c]	20	1	1	Mouse		Wild, the Netherlands, 1995	
Ppn261[c]	21	1	1	Mouse	Lung	Wild, the Netherlands, 1995	
40047-111[c]	22	1	1	Mouse		Denmark, 1989	
Ppn291[c]	23	1	1	Mouse	Trachea	Wild, the Netherlands, 1995	
394_12[d]	12	1	1	Laboratory mouse	Lung, Liver, Abscess	Dusseldorf, Germany, 2012	QXND01000000
2004520021[c]	54	1	1	Laboratory mouse	Caecum	Germany, 2004	
2015017061[c]	55	1	1	Laboratory mouse	Caecum	Germany, 2015	
2009167061[c]	56	1	1	Laboratory rat	Nasopharynx	Germany, 2009	
217_08[d]	24	1	1	Laboratory mouse	Nose	Dusseldorf, Germany, 2008	
567_11[d]	25	1	1	Laboratory mouse	Nose	Dusseldorf, Germany, 2011	
1009_11[d]	26	1	1	Laboratory mouse		Dusseldorf, Germany, 2011	
602_16[d]	13	1	1	Laboratory mouse	Nose	Dusseldorf, Germany, 2016	QXNE01000000
603_16[d]	27	1	1	Laboratory mouse	Trachea	Dusseldorf, Germany, 2016	
P441 (=JF3364)[c]	3	2		Mouse			MKZZ01000000
2013126011[c]	57	2		Laboratory mouse	Vagina	Germany, 2013	
3880_84[c]	28	3	3		Abscess	Denmark	
FD1202[c]	29	3	3	Rat	Nasopharynx	W. Australia, 1989	
FD989[c]	30	3	3	Mouse	Nasopharynx	W. Australia, 1987	
FD1158[c]	31	3	3	Rat	Nasopharynx	W. Australia, 1989	
FD1190[c]	32	3	3	Mouse	Nasopharynx	W. Australia, 1989	
FD1189[c]	4	3	3	Mouse	Nasopharynx	W. Australia, 1989	QXNF01000000
1994426011 (= J426011)[c]	58	3	3	Laboratory Rat	Lung	Germany, 1994	
1989323011[c]	59	3	3	Laboratory Rat	Vagina	Germany, 1989	
2010477013[c]	60	3	3	Mouse	Conjunctiva	Pet shop, Germany, 2010	
2004136011[c]	61	3	3	Laboratory mouse	Lung	Germany, 2004	
2001025091[c]	62	3	3	Laboratory mouse	Nasal cavity	Spain, 2001	
2007353011[c]	63	3	3	Laboratory mouse	Nasopharynx	Germany, 2007	
2008293051[c]	64	3	3	Laboratory mouse	Nasopharynx	Germany, 2008	
2013016012[c]	65	3	3	Laboratory mouse	Nasopharynx	Germany, 2013	
2009183011[c]	66	3	3	Laboratory mouse	Nasopharynx	Germany, 2009	
1596_07[d]	9	3	3	Laboratory mouse	Nasopharynx	Dusseldorf, Germany, 2007	QXNG01000000
607_10[d]	10	3	3	Laboratory mouse	Abscess	Dusseldorf, Germany, 2010	QXNH01000000
695_11[d]	33	3	3	Laboratory mouse		Dusseldorf, Germany, 2011	
847_11[d]	34	3	3	Laboratory mouse	Trachea	Dusseldorf, Germany, 2011	
1012_11[d]	35	3	3	Laboratory mouse		Dusseldorf, Germany, 2011	
1526_12[d]	36	3	3	Laboratory mouse	Nose	Dusseldorf, Germany, 2012	
1550_12[d]	37	3	3	Laboratory mouse	Prepuce	Dusseldorf, Germany, 2012	
530_13[d]	38	3	3	Laboratory mouse	Nose	Dusseldorf, Germany, 2013	
2009019091[c]	67	3	3	Laboratory mouse	Nasal cavity	Germany, 2009	
Ac84[c]	8	3	3	Mouse	Trachea	Wild, the Netherlands	QXNI01000000
691_11[d]	11	3	3	Laboratory mouse	Nose	Dusseldorf, Germany, 2011	QXNJ01000000
2002017091[c]	68	3	3	Laboratory rat	Vagina	Germany, 2002	
FD987[c]	5	4	1	Rat	Nasopharynx	W. Australia, 1987	QXNK01000000
FD986[c]	39	4	1	Rat	Nasopharynx	W. Australia, 1987	
Ppn271[c]	6	5	1	Mouse		Wild, the Netherlands, 1995	QXNL01000000
Ppn95[c]	7	6	15	Rabbit	Lung	Wild, the Netherlands	QXNM01000000

(*continued on next page*)

Table 1 (*continued*)

Strain	ID[a]	ST[b]	CC	Host	Organ	Origin and year of isolation	NCBI acc. no. of whole genome sequences
Ac63[c]	14	6	15	Mouse	Lung	Wild, the Netherlands, 1985	MLHR01000000
LN316_4[c]	15	10	1	Rat	Nasopharynx	W. Australia, 1986	MJMB01000000
Ac54[c]	40	11	15	Mouse	Lung	Wild, the Netherlands, 1985	
Ac59[c]	41	11	15	Mouse		Wild, the Netherlands, 1985	
Ppn259[c]	42	13	15	Mouse	Nasopharynx	Wild, the Netherlands, 1995	
Ppn258[c]	43	14	15	Mouse	Lung	Wild, the Netherlands, 1995	
Ac53[c]	44	15	15	Mouse	Ear	Wild, the Netherlands, 1985	
Ac55[c]	45	15	15	Mouse	Lung	Wild, the Netherlands, 1985	
Ac58[c]	46	15	15	Mouse	Lung	Wild, the Netherlands, 1985	
Ac60[c]	47	15	15	Mouse	Lung	Wild, the Netherlands, 1985	
Ac62[c]	48	15	15	Mouse	Nose	Wild, the Netherlands, 1985	
Ac50[c]	49	15	15	Mouse	Lung	Wild, the Netherlands, 1985	
Ppn100[c]	50	15	15	Rabbit	Nasopharynx	Wild, the Netherlands	
1995272012[c]	69	15	15	Mouse	Cecum	Wild, Germany,1995	
LN316_6[c]	51	16	1	Rat	Nasopharynx	W. Australia, 1986	
LN316_5[c]	52	16	1	Rat	Nasopharynx	W. Australia, 1986	
Ppn120[c]	53	17	15	Mouse	Trachea	Wild, the Netherlands, 1990	
1994416042[c]	70	19	15	Mouse	Lung	Wild, Germany, 1994	
1993231021[c]	71	20		Laboratory mouse	Lung	Germany, 1993	
2014383011[c]	72	21		Laboratory rat	Nasopharynx	Germany, 2014	
2014835011[c]	73	21		Laboratory mouse	Trachea	Germany, 2014	
1990443021[c]	74	22		Laboratory mouse	Lung	Germany, 1990	
1993116011[c]	75	23		Laboratory mouse	Trachea	Germany, 1993	
H2010154011[c]	76	24	24	Mouse	Nasopharynx	Pet shop, Germany, 2010	
H2010438021[c]	77	24	24	Mouse	Nasopharynx	Pet shop, Germany, 2010	
H2010385021[c]	78	24	24	Mouse	Nasopharynx	Pet shop, Germany, 2010	
2010385021[c]	79	24	24	Mouse	Caecum	Pet shop, Germany, 2010	
H2010385011[c]	80	25	24	Mouse	Caecum	Pet shop, Germany, 2010	

[a] ID in MLST database (https://ivsmlst.sund.ku.dk).
[b] STs 7–9, 12 and 18 are currently not used in the database.
[c] Adhikary et al. (2017a,b).
[d] Benga et al. (2013).

Table 2
Details of loci used in the MLST scheme and PCR primers used for amplification and sequencing of the genes.

Locus	Protein encoded by gene (locus) with alternative name in parenthesis	Primer set	Length of PCR product (bp)	Length of sequences in BIGSdb	No. of alleles	G + C (%)	No. of polymorphic sites	dS/dN ratio
atpG	FOF1 ATP synthase subunit gamma	F:GACAGACCGTGGAATGTGTG R:GGAAGCAAGGTTATCCACCA	399	870	6	39.0	7	25.4
frdB	Fumarate reductase iron-sulfur subunit	F: GAACCTTCCCTTTCCTACCG R: CACCCCGTTTTTACCGTTTA	413	771	5	41.8	25	43.6
gdh	Glutamate-1-semialdehyde-2.1-aminomutase	F: TTTGCCCTTTTTCAGCGAGC R: GGTGGCAGTTTAATTCGCCC	839	1008	6	44.7	60	0.553
pgi	Phosphoglucose isomerase (glucose-6 phosphate isomerase)	F:GTGGTGAATATCGGGATTGG R:TTGGAAATAAGCGGCAAAAC	518	1650	7	40.9	96	18.0
pmi	Phosphomannose isomerase (mannose-6-phosphate isomerase)	F: ATTTGGGGTGGAGAGCATTA R:GCCCACGTAAATAAGCGTGT	676	1010	5	39.0	18	7.45
recA	Recombinase A	F:CACGCCTAATGTCTCAAGCA R:TTGTTCAGGATTTGCAACCA	408	851	5	40.8	9	7.74
zwf	Zwischenferment (Glucose-6 phosphate dehydrogenase)	F: ACGATTAGGCGGGAACTTCT R: ATTTTGCCGTTCACTTCACC	731	1485	5	44.2	19	65.6

The number of alleles found at each locus ranged from 5 (*frdB, pmi, recA, zwf*) over 6 (*atpG, gdh*) to 7 (*pgi*). Sequence alignment of each of the seven loci showed no insertion/deletion, and the numbers of polymorphic sites varied from 7 (*atpG*) to 96 (*pgi*). The dS/dN indicated that positive selection was not found for any of the alleles (Table 2).

A total of 20 STs were recognized within the 79 *R. pneumotropicus* strains (Table 1). ST1 and ST3 included multiple isolates, 18 and 27, respectively. ST15 was the third largest with 8 isolates followed by ST24 which was associated with four isolates and ST2, ST4, ST6, ST11, ST16 and ST21 consisting of two isolates. Half of the STs (10) were singletons (Table 3). The V-factor dependent strains were associated to ST24 and ST25 which formed at doublet.

Fig. 1 shows the relationships between the 20 ST based on goeBurst analysis of the allelic profiles of all the 79 strains. STs connected by lines share at least one common allele and the STs were assigned into three major and one minor CCs (Fig. 1). A clonal complex (CC) is defined as a group of at least 3 strains with STs sharing 5, 6 or all 7 loci. CC1 and CC3 included 5 and 1 STs, respectively and were composed of 18 and 27 strains, respectively whereas CC15 included 6 STs with 16 strains and CC24 2 STs with 5 strains. In CC1, the most predominant founder was ST1 with 75% of the total number of strains. This ST only included one rat strain and the others were from mice originating from Germany, the Netherlands, USA and Denmark obtained during the period of 1948 to 2016. CC3 exclusively included ST3 and most strains were from mouse with only five from rats which were obtained from Germany, the Netherlands, Australia, Denmark and Spain and their time of collection included 1987 to 2013. ST15 was the predominant founder in CC15 which mainly included wild mouse isolates originating from the Netherlands. Finally CC24 only included pet shop isolates from Germany collected in 2010.

Table 3
Analysis of the seven MLST loci in the 79 isolates examined in this study.

No. of isolates	ST	Allelic profile						
		atpG	*frdB*	*gdh*	*pgi*	*pmi*	*recA*	*zwf*
18	1	1	1	1	1	1	1	1
2	2	2	2	2	2	2	2	2
27	3	3	3	3	3	3	3	3
2	4	1	1	1	4	1	1	1
1	5	1	1	4	1	1	1	1
2	6	4	4	5	5	4	4	4
1	10	6	1	1	7	1	1	1
2	11	3	4	5	5	4	3	2
1	13	3	4	5	5	4	3	4
1	14	3	4	5	5	4	4	2
8	15	3	4	5	5	4	1	2
2	16	6	1	1	1	1	1	1
1	17	3	3	5	5	4	1	2
1	19	3	4	5	5	3	1	4
1	20	1	2	3	5	4	3	3
1	21	5	5	6	5	5	5	5
1	22	1	3	3	1	4	3	3
1	23	1	3	3	5	4	3	1
4	24	1	3	1	2	3	1	3
1	25	1	3	1	2	3	1	1

4. Discussion

MLST schemes have previously been published for at least five species of *Pasteurellaceae* and these schemes have successfully stimulated to further investigations of population genetics and epidemiology (Christensen et al., 2014). The current study was undertaken to develop a MLST scheme of *R. pneumotropicus*. We used the same set of primers for both amplification and sequencing, an approach that has been used in other MLST schemes such as *S. pneumoniae*, *Pseudomonas aeruginosa* and *Streptococcus suis* (Enright and Spratt, 1998; King et al., 2002; Curran et al., 2004). All seven loci were without positive selection, as the synonymous substitutions at synonymous sites compared to changes in non-synonymous at non-synonymous sites (dS/dN) were not significantly lower than 1. This MLST scheme successfully identified all related isolates and was able to compare strains and results across the continents. The creation of the MLST scheme of *R. pneumotropicus* represents a major step forward in the ability of scientists around the world to type and compare isolates of *R. pneumotropicus* from all rodents. The strain collection used to set up the MLST is probably the best representation of the global genetic diversity of *R. pneumotropicus* including representatives from six countries and three continents. Hopefully the collection can be complemented by strains from Southeast Asia in the future.

The *R. pneumotropicus* population included in this study was studied by allelic profiles analysed by a minimum spanning tree. We found the association of both mouse and rat isolates in the same clonal complexes (CC1, CC3) and ST types (ST1, ST3, ST6, ST15, ST21) (Table 1). It indicated that the associated strains of mouse and rat have identical genetic background.

Recently, phylogenetic analysis of housekeeping genes and 16S ribosomal DNA indicated that the members of *Pasteurellaceae* were characterized on the basis of their host associations (Christensen and Bisgaard, 2004; Christensen et al., 2004; Korczak et al., 2004). The epidemiology of [*P.*] *pneumotropica* by ARDRA (Sasaki et al., 2006), RAPD typing (Weigler et al., 1996) and the utilization of carbon sources (Sasaki et al., 2009) were clearly defined depending on the source of animals, suggesting host specificity. The host-specific infection of the species of *Rodentibacter* was previously reported and some strains are more host specific than others whereas other variants seem to be able to

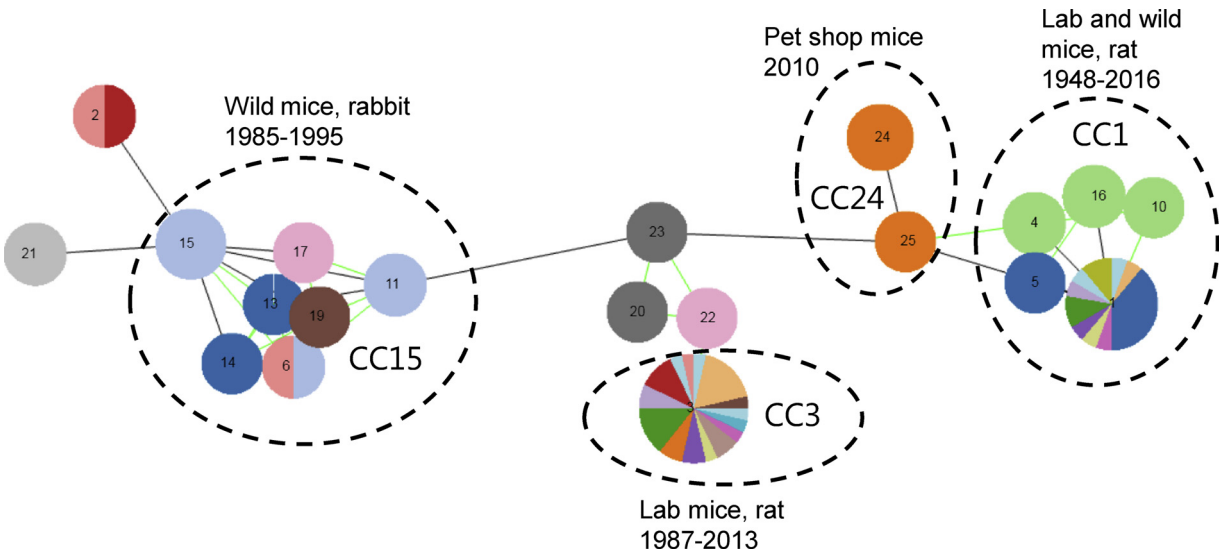

Fig. 1. Minimum spanning tree of the 20 STs inferred by PHYLOViZ on the basis of allelic profiles. Each circle indicates a ST (node), and a larger size of the circle corresponds to a larger number of strains included (circles are not fully scaled with the number of strains) (Table 1). The edges between the STs represent their relationships including SLVs and DLVs. The colors indicate years of isolation (Table 1). The dashed lines indicate major CCs.

cross the host species barrier (Nakagawa et al., 1981; Nicklas, 2007; Boot, 2010; Benga et al., 2017).

The most interesting epidemiological observation was that CC1 and CC3 included isolates from different hosts, countries and years. CC1 included seven wild mouse isolates but only one was included in CC3. Research using mice and rats was until 1960 invariably carried out on animals which have a so called conventional or "normal" microbiology. The origin of ST1 and CC1 could be of such original *R. pneumotropicus* since samples included wild mouse and the type strain from 1948. From 1960 these animals have gradually been replaced by animals from hysterectomy-derived barrier-maintained SPF-colonies. Animals from SPF- colonies have an abnormal microbiology and physiology, compared to conventional counterparts. SPF mice and rats housed within the same facility may become contaminated /infected by each others “foreign” bacteria. The foreign bacteria may later be expelled by better fitting “host specific” strains. In the wild, mice and rats will live separated and mutual infection seems very unlikely. CC3 could be a “new” clone of *R. pneumotropicus* which developed after the current way of breeding laboratory rodents was initiated. This could point to a common source of introduction from central breeding colonies. Persistence of a certain clone of *R. pneumotropicus* would be another explanation for ST1 and ST3. Such a persistence has been documented for members of *Rodentibacter heylii* (Adhikary et al., 2017b).

In conclusion, this study represents the first MLST scheme that has been established for rodent *Pasteurellaceae.* As MLST is portable and easily comparable across laboratories we suggest that MLST is accepted as the “gold standard” typing method for *R. pneumotropicus.* Further development will be needed to sequence more whole genomes to adapt the existing MLST to whole genome MLST (wgMLST) in order to include potential virulence factors into the MLST scheme and to obtain a higher resolution with respect to clonal diversity.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Acknowledgement

We would like to thank Ms Katrine Aagaard for excellent technical assistance in the laboratory.

References

Adhikary, S., Nicklas, W., Bisgaard, M., Boot, R., Kuhnert, P., Waberschek, T., Aalbæk, B., Korczak, B., Christensen, H., 2017a. *Rodentibacter* gen. nov. Including *Rodentibacter pneumotropicus* comb. nov., *Rodentibacter heylii* sp. nov., *Rodentibacter myodis* sp. nov., *Rodentibacter ratti* sp. nov., *Rodentibacter heidelbergensis* sp. nov., *Rodentibacter trehalosifermentans* sp. nov., *Rodentibacter rarus* sp. nov., *Rodentibacter mrazii* and two genomospecies. Int. J. System Evol. Microbiol. 67, 1793–1806.

Adhikary, S., Bisgaard, M., Dagnæs-Hansen, F., Christensen, H., 2017b. Clonal outbreaks of [*Pasteurella*] *pneumotropica* biovar Heyl in two mouse colonies. Lab Anim. (NY) 51, 613–621.

Artwohl, J.E., Flynn, J.C., Bunte, R.M., Angen, O., Herold, K.C., 2000. Outbreak of *Pasteurella pneumotropica* in a closed colony of STOCK-Cd28^{tm1Mak} mice. Contemp. Top. Lab. Anim. Sci. 39, 39–41.

Benga, L., Benten, W.P., Engelhardt, E., Bleich, A., Gougoula, C., Sager, M., 2013. Development of a multiplex PCR assay based on the 16S-23S rRNA internal transcribed spacer for the detection and identification of rodent Pasteurellaceae. J. Microbiol. Methods 95, 256–261.

Benga, L., Sager, M., Christensen, H., 2017. From the [*Pasteurella*] *pneumotropica* complex to *Rodentibacter* spp.: an update on [*Pasteurella*] *pneumotropica*. Vet. Microbiol. 217, 8121–8134.

Blackall, P.J., Miflin, J.K., 2000. Identification and typing of Pasteurella multocida. Avian Pathol. 29, 271–287.

Boot, R.A., 2010. *Pasteurella pneumotropica*strain of mouse origin colonizes rats but is out competed by a*P. pneumotropica* strain of rat origin. Scand. J. Lab. Anim. Sci. 37, 261–266.

Christensen, H., Bisgaard, M., 2004. Revised definition of *Actinobacillus sensu stricto* isolated from animals. A review with special emphasis on diagnosis. Vet. Microbiol. 99, 13–30.

Christensen, H., Kuhnert, P., Olsen, J.E., Bisgaard, M., 2004. Comparative phylogenies of the housekeeping genes *atpD*, *infB* and *rpoB* and the 16S rRNA gene within the Pasteurellaceae. Int. J. Syst. Evol. Microbiol. 54, 1601–1609.

Christensen, H., Kuhnert, P., Nørskov-Lauritsen, N., Planet, P.J., Bisgaard, M., 2014. Family *Pasteurellaceae*. In: 4th ed. In: Stackebrandt, E., Rosenberg, E., Delong, E., Lory, S., Fabiano Thompson, F. (Eds.), The Prokaryotes, vol. 9. Springer, New York, pp. 535–564.

Curran, B., Jonas, D., Grundmann, H., Pitt, T., Dowson, C.G., 2004. Development of a multilocus sequence typing scheme for the opportunistic pathogen Pseudomonas aeruginosa. J. Clin. Microbiol. 42, 5644–5649.

Enright, M.C., Spratt, B.G., 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 144, 3049–3060.

Enright, M.C., Spratt, B.G., 1999. Multilocus sequence typing: tracking the global spread of virulent and antibiotic-resistant strains of bacteria. Trends Microbiol. 7, 482–487.

Fornefett, J., Krause, J., Klose, K., Fingas, F., Hassert, R., Benga, L., Grunwald, T., Müller, U., Schrödl, W., Baums, C.G., 2018. Comparative analysis of humoral immune responses and pathologies of BALB/c and C57BL/6 wildtype mice experimentally

infected with a highly virulent *Rodentibacter pneumotropicus* (*Pasteurella pneumotropica*) strain. BMC Microbiol. 18, 45.
Francisco, A.P., Bugalho, M., Ramirez, M., Carriço, J.A., 2009. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. BMC Bioinformatics 10, 152.
Francisco, A.P., Vaz, C., Monteiro, P.T., Melo-Cristino, J., Ramirez, M., Carriço, J.A., 2012. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics 13, 87.
Goelz, M.F., Thigpen, J.E., Mahler, J., Rogers, W.P., Locklear, J., Weigler, B.J., Forsythe, D.B., 1996. Efficacy of various therapeutic regimens in eliminating *Pasteurella pneumotropica* from the mouse. Lab. Anim. Sci. 46, 280–285.
Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
Haubold, H., Hudson, R.R., 2000. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Bioinformatics 16, 847–848.
Hayashimoto, N., Ueno, M., Takakura, A., Itoh, T., 2007. A specific polymerase chain reaction based on the *gyrB* gene sequence and subsequent restriction fragment length polymorphism analysis of *Pasteurella pneumotropica* isolates from laboratory mice. J. Am. Assoc. Lab. Anim. Sci. 46, 54–58.
Jolley, K.A., Maiden, M.C., 2010. BIGSdb: scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11, 595.
King, S.J., Leigh, J.A., Heath, P.J., Luque, I., Tarradas, C., Dowson, C.G., Whatmore, A.M., 2002. Development of a multilocus sequence typing scheme for the pig pathogen *Streptococcus suis*: identification of virulent clones and potential capsular serotype exchange. J. Clin. Microbiol. 40, 3671–3680.
Kodjo, A., Villard, L., Veillet, F., Escande, F., Borges, E., Maurin, F., Bonnod, J., Richard, Y., 1999. Identification by 16S rDNA fragment amplification and determination of genetic diversity by random amplified polymorphic DNA analysis of *Pasteurella pneumotropica* isolated from laboratory rodents. Lab. Anim. Sci. 49, 49–53.
Korczak, B., Christensen, H., Emler, S., Frey, J., Kuhnert, P., 2004. Phylogeny of the family *Pasteurellaceae* based on *rpoB* sequences. Int. J. Syst. Evol. Microbiol. 54, 1393–1399.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and clustal X version 2.0. Bioinformatics 23, 2947–2948.
Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452.
Mähler, M., Berard, M., Feinstein, R., Gallagher, A., Illgen-Wilcke, B., Pritchett-Corning, K., Raspa, M., 2014. FELASA working group on revision of guidelines for health monitoring of rodents and rabbits: FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. Lab. Anim. 48, 178–192.
Maiden, M.C.J., Bygraves, J.A., Feil, E.J., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M., Spratt, B.G., 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proc. Natl. Acad. Sci. U. S. A. 95, 3140–3145.
Manning, P.J., DeLong, D., Gunther, R., Swanson, D., 1991. An enzyme-linked immunosorbent assay for detection of chronic subclinical Pasteurella pneumotropica. Lab. Anim. Sci. 41, 162–165.
Meats, E., Feil, E.J., Stringer, S., Cody, A.J., Goldstein, R., Kroll, J.S., Popovic, T., Spratt, B.G., 2003. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J. Clin. Microbiol. 41, 1623–1636.
Meunier, J.R., Grimont, P.A.D., 1993. Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. Res. Microbiol. 144, 373–379.
Miller, M., Zorn, J., Brielmeier, M., 2015. High-resolution melting curve analysis for identification of *Pasteurellaceae* species in experimental animal facilities. PLoS One 10, e0142560.
Nakagawa, M., Saito, M., Kohjima, K., 1981. Mutual transmission of *Pasteurella pneumotropica* between mice and rats. Jpn. J. Vet. Sci. 43, 937–940.
Needham, J.R., Cooper, J.E., 1975. An eye infection in laboratory mice associated with Pasteurella pneumotropica. Lab. Anim. 9, 197–200.
Nicklas, W., 2007. *Pasteurellaceae*. In: 2nd ed. In: Fox, J.G., Davisson, M.T., Newcomer, C., Quimby, F.W., Smith, A.L. (Eds.), The Mouse in Biomedical Research, vol. 2. Elsevier, Amsterdam, pp. 469–505.
Sager, M., Benten, W.P., Engelhardt, E., Gougoula, C., Benga, L., 2015. Characterization of biofilm formation in [*Pasteurella*] *pneumotropica* and [*Actinobacillus*] *muris* isolates of mouse origin. PLoS One 10, e0138778.
Saito, M., Kojima, K., Sano, J., Nakayama, K., Nakagawa, M., 1981. Carrier state *of Pasteurella pneumotropica* in mice and rats. Exp. Anim. 30, 313–316.
Sasaki, H., Kawamoto, E., Okiyama, E., Ueshiba, H., Mikazuki, K., Amao, H., Sawada, T., 2006. Molecular typing of *Pasteurella pneumotropica* isolated from rodents by amplified 16S ribosomal DNA restriction analysis and pulsed-field gel electrophoresis. Microbiol. Immunol. 50, 265–272.
Sasaki, H., Kawamoto, E., Tanaka, Y., Sawada, T., Kunita, S., Yagami, K., 2009. Comparative analysis of *Pasteurella pneumotropica* isolates from laboratory mice and rats. Ant. Van Leeuwenh. 95, 311–317.
Subaaharan, S., Blackall, L.L., Blackall, P.J., 2010. Development of a multi-locus sequence typing scheme for avian isolates of Pasteurella multocida. Vet. Microbiol. 14, 354–361.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
van der Linden, M., Al-Lahham, A., Nicklas, W., Reinert, R.R., 2009. Molecular characterization of pneumococcal isolates from pets and laboratory animals. PLoS One 4, e8286.
Ward, G.E., Moffat, R., Olfert, E., 1978. Abortion in mice associated with Pasteurella pneumotropica. J. Clin. Microbiol. 8, 177–180.
Weigler, B.J., Thigpen, J.E., Goelz, M.F., Babineau, C.A., Forsythe, D.B., 1996. Randomly amplified polymorphic DNA polymerase chain reaction assay for molecular epidemiologic investigation of *Pasteurella pneumotropica* in laboratory rodent colonies. Lab. Anim. Sci. 46, 386–399.