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Development of a multi-locus sequence typing scheme for avian isolates of *Pasteurella multocida*

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ABSTRACT

A total of 63 isolates of *Pasteurella multocida* from Australian poultry, all associated with fowl cholera outbreaks, and three international reference strains, representing the three subspecies within *P. multocida* were used to develop a multi-locus sequence typing scheme. Primers were designed for conserved regions of seven house-keeping enzymes – *adk*, *est*, *gdh*, *mdh*, *pgi*, *pmi* and *zwf* – and internal fragments of 570–784 bp were sequenced for all isolates and strains. The number of alleles at the different loci ranged from 11 to 20 and a total of 29 allelic profiles or sequence types were recognised amongst the 66 strains. There was a strong concordance between the MLST data and the existing multi-locus enzyme electrophoresis and ribotyping data. When used to study a sub-set of isolates with a known detailed epidemiological history, the MLST data matched the results given by restriction endonuclease analysis, pulsed-field gel electrophoresis, ribotyping and REP-PCR. The MLST scheme provides a high level of resolution and is an excellent tool for studying the population structure and epidemiology of *P. multocida*.

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1. Introduction

Fowl cholera, caused by *Pasteurella multocida*, is a common and widely distributed disease of poultry and is of major economic importance (Glisson et al., 2008). There is considerable evidence that *P. multocida* is genetically diverse e.g. on the basis of DNA:DNA hybridization and phenotypic properties three subspecies, *gallicida*, *multocida* and *septica*, have been recognised (Mutters et al., 1985), NAD-dependent isolates of *P. multocida* have been recognised (Krause et al., 1987) and 16 different biochemical types, termed biovars, have been recognised within the species (Blackall et al., 1997; Fegan et al., 1995).

The traditional method for typing *P. multocida* has been serotyping – with a range of serotyping schemes including the Heddleston somatic (Heddleston et al., 1972) and Carter capsule (Carter, 1955) typing schemes. However, the discrimination power of serotyping is very low e.g. most avian isolates belong to only two capsular types and three somatic types (Glisson et al., 2008). In addition, for the somatic serovars, there is no correlation between serovar distribution and overall population structure (Blackall et al., 1998). The use of DNA-based typing methods has been a major advance in typing. These DNA typing methods include restriction endonuclease analysis (REA), ribotyping, pulsed-field gel electrophoresis (PFGE) and PCR methods such as ERIC-PCR and RAPD-PCR and have been reviewed for their advantages and disadvantages when applied to *P. multocida* (Blackall and Miflin, 2000). While these methods have proven useful, they all share a major limitation – comparisons across laboratories and even across time are difficult to impossible to perform (Blackall and Miflin, 2000). This is a major limitation that leaves laboratories unable to share information – forcing repeated examinations or testing.

Multi-locus enzyme electrophoresis (MLEE) is a typing method that detects variations in the amino acid sequence of enzymes associated with essential house-keeping

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Table 1
Results of the MLST analysis of the isolates and strains of *P. multocida* used in this study.

Strain	ST	Allelic profile						
		adk	*est*	*pmi*	*zwf*	*mdh*	*gdh*	*pgi*
PM1	1	1	1	1	1	1	1	1
PM12		1	1	1	1	1	1	1
PM13		1	1	1	1	1	1	1
PM14		1	1	1	1	1	1	1
PM91		1	1	1	1	1	1	1
PM2	2	2	2	2	2	2	2	2
PM3		2	2	2	2	2	2	2
PM4		2	2	2	2	2	2	2
PM5		2	2	2	2	2	2	2
PM6		2	2	2	2	2	2	2
PM7		2	2	2	2	2	2	2
PM8		2	2	2	2	2	2	2
PM9		2	2	2	2	2	2	2
PM10		2	2	2	2	2	2	2
PM11		2	2	2	2	2	2	2
PM15		2	2	2	2	2	2	2
PM16		2	2	2	2	2	2	2
PM17		2	2	2	2	2	2	2
PM18	5	3	4	3	3	4	2	4
PM77		3	4	3	3	4	2	4
PM27	7	2	6	4	4	6	4	6
PM48		2	6	4	4	6	4	6
PM52		2	6	4	4	6	4	6
PM63		2	6	4	4	6	4	6
PM19	8	4	5	2	5	5	3	5
PM35		4	5	2	5	5	3	5
PM40		4	5	2	5	5	3	5
PM45		4	5	2	5	5	3	5
PM69		4	5	2	5	5	3	5
PM75		4	5	2	5	5	3	5
PM78		4	5	2	5	5	3	5
PM80		4	5	2	5	5	3	5
PM86		4	5	2	5	5	3	5
PM87		4	5	2	5	5	3	5
PM95		4	5	2	5	5	3	5
PM136		4	5	2	5	5	3	5
PM37	9	2	7	5	6	4	5	7
PM46	10	5	8	6	7	1	6	8
PM49	11	2	9	7	8	4	3	9
PM51	12	6	10	8	9	8	3	8
PM97		6	10	8	9	8	3	8
PM120		6	10	8	9	8	3	8
PM55 (NCTC 10322)	13	7	11	9	10	4	7	8
PM57 (NCTC 10204)	14	8	10	2	11	4	8	10
PM59 (CIP A125)	15	1	5	10	1	1	1	11
PM64	16	9	12	11	12	9	9	12
PM65		9	12	11	12	9	9	12
PM67	17	4	6	12	8	4	4	8
PM71	18	2	13	13	4	6	8	13
PM72	19	10	14	14	13	10	10	14
PM73	20	2	6	7	4	6	3	8
PM132		2	6	7	4	6	3	8
PM76	21	9	12	11	12	9	9	15
PM79	22	4	16	15	14	11	11	4
PM81	23	10	14	14	13	10	1	14
PM83	24	2	7	16	6	4	5	7
PM84		2	7	16	6	4	5	7
PM85	26	4	5	2	5	5	3	16
PM88	28	2	17	7	8	7	3	8
PM96	29	4	6	7	17	4	4	8
PM131	30	1	18	18	8	12	12	11
PM133	31	6	19	12	18	5	13	17
PM137		6	19	12	18	5	13	17
PM135	32	11	20	16	5	1	1	5
PM138	33	1	21	18	1	1	12	11
PM140	34	12	22	19	1	1	5	18

activities of cells (Selander et al., 1986). MLEE has allowed the recognition of a population structure within avian *P. multocida* (Blackall et al., 1998). Multi-locus sequence typing (MLST) is a sequence-based approach that takes MLEE to the level of a genotypic method (Spratt, 1999). Even more importantly, because MLST uses sequence knowledge, the typing achieved by MLST is totally portable – making comparisons across laboratories and across time easy and routine in nature (Enright and Spratt, 1998). Indeed, it has now been claimed that MLST is the "gold standard" for typing bacterial pathogens (Maiden et al., 1998).

In this study, we report on the development of an MLST scheme for *P. multocida*. We have developed this scheme using 63 Australian avian *P. multocida* and the formal reference strains for the three subspecies of *P. multocida* – *gallicida*, *multocida* and *septica*. As well, we evaluate the retrospective use of the MLST scheme to investigate a well-characterised set of *P. multocida* isolates involved in outbreaks of fowl cholera on seven Australian turkey farms (Blackall and Miflin, 2000).

2. Materials and methods

2.1. Bacterial isolates and strains

A total of 66 organisms (Table 1) previously identified as *P. multocida* were used in this study. The cultures were obtained from the culture collection of the Microbiology Research Group at the Animal Research Institute. The somatic serovar, biovar and electrophoretic type (ET) properties of these strains are detailed in Blackall et al. (1998). Three of the isolates were the formal taxonomic reference strains for *P. multocida* subspecies *multocida* (PM55 – NCTC 10322), *P. multocida* subspecies *gallicida* (PM57 – NCTC 10204) and *P. multocida* subspecies *septica* (PM59 – CIP A125). All the remaining 63 isolates were from Australian poultry.

2.2. DNA extraction

P. multocida genomic DNA was extracted using the DNeasy™ Tissue Kit (QIAGEN Cat # 69506). The protocol for animal tissues as described in the kit instructions was used for all extractions. The extracted DNA was stored at −20 °C until used. The extracted DNA was visualized for quality and quantity by electrophoresis in a 2% DNA grade agarose gel in 1× TAE (Tris Glacial Acetate EDTA) buffer, stained with ethidium bromide (10 mg/ml) and

photographed using ultra-violet light. A low DNA mass ladder (Invitrogen 10068-013) was included in the gel to estimate the quantity of the extracted DNA.

2.3. Genes for MLST

The selection of house-keeping genes was based on the genetic diversity of the relevant enzymes as shown in the prior MLEE study (Blackall et al., 1998). The *P. multocida* genome sequence database (PM 70 strain), located at National Centre for Biotechnology Information (NCBI) website [<http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=170>] was searched for annotated nucleotide sequence data. The five genes selected from the pre-existing MLEE study that could be confidently identified on the annotated genome were *est*, *mdh*, *pgi*, *pmi* and *zwf*. Existing MLST schemes for *Niesseria meningitidis* (Maiden et al., 1998) and *Streptococcus pneumoniae* (Enright and Spratt, 1998) were also consulted to aid in selection of the final two genes. The two genes selected on this basis were *adk* and *gdh*.

2.4. Gene amplification and sequencing

All primer sets used in this study were designed using the Primer 3 primer designing tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). All primers were designed against the nucleotide sequence of the respective gene in the PM 70 strain. The details of the eight primer sets used in this work are presented in Table 2. Two primer sets were used for *zwf*.

For all PCRs except the PCR for *gdh*, 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. For the *gdh* PCR, the only change was that the annealing temperature was 58 °C. For all PCR reactions, an initial denaturation of 5 min at 94 °C was performed. Two successful products of the same reaction were combined to give a 100 μl total volume. The QIAquick PCR purification kit (QIAGEN Cat. No. 28104) was used to purify the product. The procedure was carried out as in the protocol except that 30 μl of elution buffer was used to elute the DNA instead of 50 μl (resulting in a more concentrated DNA preparation).

The sequencing PCR reaction was carried out using the PRISM Ready Reaction Dyedeoxy™ Terminator Sequencing Kit (Applied Biosystems, Australia) as per the manufacturer's instructions. For each PCR amplified gene fragment two sequencing reactions were carried out separately, one with the forward primer and one with the reverse primer. The raw sequence chromatograms were visually screened with editing software (EditView, v.0.1 ABI PRISM) for the quality of sequences. The sequences were imported into Sequence Alignment software (SeqED, v 1.0.3). The forward and reverse complimented sequences were aligned and carefully cross-checked for complementarity.

2.5. MLST analysis

For each house-keeping gene analysed, a multiple alignment was manually created in SeqEd from all of the isolates sequenced. The edited sequences were saved into a text file for analysis. The sequence files were later converted into different formats such as FASTA for analysis using the START suite of programs (Sequence Type Analysis and Recombination Tests, available at the MLST website (http://pubmlst.org/software/analysis/).

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.

The START suite of programs was downloaded from the MLST website. The allelic profiles and the STs were then used in the MLST data analysis within the START suite. The instructions for START analysis were obtained from the MLST website (http:/pubmlst.org/software/analysis/). A recombination test, the Index of Association (both I_A and I_A^S), was performed as well. The construction of a dendrogram (based on the concatenated DNA sequences) by the Neighbour Joining method was also performed with the START program. Simpson's index of discrimination (*D*) was calculated for each allele and for the STs as previously described (Hunter and Gaston, 1988). The eBURST program (http://eburst.mlst.net/) was used to identify clonal complexes with a clonal complex being defined as STs that shared 6 or more loci.

Table 2
Primer sets designed for the amplification and sequencing of seven *P. multocida* house-keeping genes.

Gene and location on chromosome (1000 bp)	Primer sequence (5′–3′) (location within gene)		Fragment size (bp) Amplicon/MLST
	Forward	Reverse	
adk (323.7–324.3)	TTTTTCGTCCCGTCTAAGC (44–54)	GGGGAAAGGGACACAAGC (596–603)	570/466
est (102.9–104.9)	TCTGGCAAAAGATGTTGTCG (54–73)	CCAAATTCTTGGTTGGTTGG (675–694)	641/535
pmi (978.5–979.7)	TGCCTTGAGACAGGGTAAGC (111–130)	GCCTTAACAAGTCCCATTCG (830–849)	739/602
zwf (1751.7–1753.2)	AATCGGTCGTTTGACTGAGC (99–118)[a]	TGCTTCACCTTCAACTGTGC (887–906)[a]	808/500
	TGTTAGGTGTGGCAAGAACG (128–147)[b]	TTGCAACAAATGGTTTTGGA (722–741)[b]	614/500
mdh (0.9–3.2)	ATTTCGGGATCAGGGTTAGC (1400–1419)	GGAAAACCGGTAATGGAAGG (2000–2019)	620/521
gdh (45.6–47.0)	ATCGACTTCTTCCGCAGACC (154–173)	GCGGGTGATATTGGTGTAGG (836–855)	702/530
pgi (488.4–490.1)	ACCACGCTATTTTTGGTTGC (598–617)	ATGGCACAACCTCTTTCACC (1362–1381)	784/560

[a] This primer set is termed ZWF-F1 (forward) and ZWF-R1 (reverse).
[b] This primer set is termed ZWF-F2 (forward) and ZWF-R2 (reverse).

Table 3
Comparison of typing methods using 22 avian isolates of *P. multocida*.

Farm[a]	Outbreak[a]	Strains[a]	Date[a]	Subspecies[a]	Biovar[b]	REA type[a]	Ribotype[a]	PFGE type[c]	REP-PCR type[c]	MLEE[d]	MLST[e]
1	I	PM 137	10/92	*multocida*	3	I	i	19ii	20ii	36/A	31
	II	PM 1	2/93	*multocida*	2	VI	vi	4	4	42/B	1 (A)
		PM 12, 13, 14	3/93	*multocida*	2	VI	vi	4	4	43/B	1 (A)
2	III	PM 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	2/93	*multocida*	3	VII	vii	20	21	38/A	2
		PM 15, 16	3/93	*multocida*	3	VII	vii	20	21	38/A	2
3	IV	PM 132	10/92	*multocida*	3	II	ii	20	22	33/A	20
4	V	PM 133	10/92	*multocida*	3	I	i	19i	20i	35/A	31
6	VII	PM 135	12/92	*septica*	10	IV	iv	17	17	47/B	32 (A)
7	VIII	PM 136	12/92	*multocida*	3	V	v	16	16	25/A	8

[a] As defined previously by Blackall et al. (1995).
[b] As defined previously by Blackall et al. (1995) and subsequently modified by Blackall et al. (1997).
[c] As defined previously by Gunawardana et al. (2000).
[d] As defined previously by Blackall et al. (1998).
[e] As defined in this study.

Primer information, PCR conditions, allele sequences, sequence types and isolate information have been made available at the RIRDC *P. multocida* MLST website (http://pubmlst.org/pmultocida/) sited at the University of Oxford (Jolley et al., 2004).

2.6. Epidemiological study

The 21 isolates of *P. multocida* involved in the epidemiological evaluation have been subjected to extensive phenotypic characterisation, as well as REA and ribotyping using the enzyme HpaII (Blackall et al., 1995). While the original study (Blackall et al., 1995) involved 22 isolates, only 21 isolates were available for the current study. The 21 isolates were obtained from 15 different birds on six meat turkey farms. Based on the field information, these 21 isolates represented seven outbreaks. Multiple isolates were obtained from Farms 1 and 2 (five and 12 isolates, respectively), while only single isolates were available from Farms 3, 4, 6 and 7 (Blackall et al., 1995). The five isolates from Farm 1 were obtained from five birds and the 12 isolates from Farm 2 were from six birds. A summary of the previously recorded results (Blackall et al., 1995) for biotyping, REA and ribotype of the 21 isolates is shown in Table 3. The same 21 isolates were subsequently part of the first population structure study for *P. multocida* (Blackall et al., 1998) and the MLEE typing results of the 21 isolates are also shown in Table 3. In a further study, Gunawardana et al. (2000) typed the same 21 isolates (as part of larger study on the typing of *P. multocida*) using two further techniques – pulsed-field gel electrophoresis (PFGE) and REP-PCR. The results of the PFGE and REP-PCR typing are also shown in Table 3.

3. Results

For all 66 strains, PCR products of the expected size were obtained for six genes (*est*, *mdh*, *pgi*, *pmi*, *adk*, and *gdh*) using the single primer set for each respective gene shown in Table 2. However, for *zwf* gene, the initial primer set (ZWF-F1 and ZWF-R1) failed to give a product for 10 strains. A second set of primers (see Table 2, termed ZWF-F2 and ZWF-R2) were designed and these primers resulted in a product of the expected size for these 10 strains.

The number of strains for each allele for all seven genes is shown in Table 4. The gene with the most alleles was *est* (20 alleles) while the genes with the least alleles was *mdh* (11 alleles). Nucleotide sequence diversity within the gene loci varied from 4% (*adk*) to 12% (*est*) with the index of discrimination varying from 0.90 (*est* and *pgi*) to 0.78 (*adk*).

A total of 29 STs were recognised within the 66 *P. multocida* strains in this study (see Table 1). As the MLST website is now active and has additional strains not included in this study, the STs and alleles of the strains used in this study are not necessarily consecutive. The relationship between each ST was displayed in a dendrogram created from the concatenated sequences (Fig. 1). Most STs (19) consisted of single isolates. STs 1 and 2 had multiple isolates and are discussed in detail later. ST 8 was the second largest ST in terms of isolates (12 isolates in total). The members of ST 8 have no known epidemiological connection – being collected over a time period from 1973 to 1992 and being sourced from chickens, turkeys and a duck and being obtained in quite diverse geographical regions of Australia (Queensland and New South Wales). The index of discrimination calculated for the STs was 0.92. Four clonal complexes were identified by the eBURST program – with all having only two members (STs 8 and 26; STs 9 and 24, STs 16 and 21 and STs 19 and 23).

Linkage disequilibrium between alleles was estimated with the I_A statistic (analysing STs). The I_A was 1.0774 and the I_A^S was 0.1796 (V_O 0.9469; V_E 0.4558, $P = 0.000$), indicating that significant linkage disequilibrium was detected.

Most biovars were represented by only low numbers in the collection of isolates examined, with only biovars 1 (six isolates), 2 (8 isolates) and 3 (39 isolates) having more than 5 isolates. Biovar 3, the most common biovar (39/66 strains) was widely distributed across the dendrogram shown in Fig. 1. Within the 10 STs that consisted of more than one isolate (STs 1, 2, 5, 7, 8, 12, 16, 20, 24 and 31), all members were always allocated to a single biovar with the exception of ST 2. In ST 2, one strain (PM 17) was biovar 2 while the other 11 members of this ST were biovar 3.

Table 4
Analysis of the seven MLST loci in the 66 *P. multocida* strains examined in this study.

Allele	Number of *P. multocida* strains with the indicated allele for the following gene loci						
	adk	*est*	*pmi*	*zwf*	*mdh*	*gdh*	*pgi*
1	8	5	5	8	10	8	5
2	25	13	27	13	13	15	13
3	2	–	2	2	–	20	–
4	16	2	4	7	9	6	3
5	1	14	1	14	15	4	13
6	5	8	1	3	7	1	4
7	1	3	5	1	2	1	3
8	1	1	3	4	3	2	10
9	1	1	1	3	3	3	1
10	2	4	1	1	2	1	1
11	1	1	3	1	1	1	3
12	1	3	3	3	1	2	2
13	–	1	1	2	–	2	1
14	–	2	2	1	–	–	2
15	–	–	1	–	–	–	1
16	–	1	3	–	–	–	1
17	–	1	–	1	–	–	2
18	–	1	2	2	–	–	1
19	–	2	1	–	–	–	–
20	–	1	–	–	–	–	–
21	–	1					
22	–	1	–	–	–	–	–
Total alleles for each gene	12	20	18	16	11	13	17
No (%) of polymorphic sites	19 (4.1)	62 (11.6)	33 (5.5)	44 (8.8)	16 (3.1)	46 (7.1)	30 (5.4)
D value	0.78	0.90	0.82	0.89	0.86	0.84	0.89

In terms of isolates that could be confidently assigned to the recognised subspecies of *P. multocida*, the subspecies *gallicida* and *septica* were present only in very low numbers (two strains each) in the strain set examined. This meant that no association of particular subspecies within the dendrogram shown in Fig. 1 was possible. The three STs containing the taxonomic reference strains did not include any Australian field isolate.

Of the 13 strains in this study assigned to MLEE Cluster B by Blackall et al. (1998), 12 were assigned to the MLST Cluster marked as A in Fig. 1. The only strain assigned to MLEE Cluster B that was not assigned to MLST Cluster A was PM 17 which, by MLST analysis, was housed in ST 2. In terms of ribotypes, MLST Cluster A (marked in Fig. 1) contains all the Ribotype Cluster 1 isolates as defined by Blackall et al. (1998) except the two isolates allocated to ST 31.

The comparison of the typing results (all methods) and the available field epidemiological information is presented in Table 3. For Outbreaks II, VII and VIII, all the typing methods agree that the outbreaks are distinct and not connected. All the typing methods agree that the two outbreaks seen on Farm 1 (Outbreaks I and II) are different. Four of the methods, biovar, REA, ribotyping, and MLST, indicate that Outbreaks I and V are connected with the outbreaks being associated with strains that are indistinguishable. By PFGE and REP-PCR, the strains from Outbreaks I and V showed a slight difference (as indicated by the use of Roman numerals). These PFGE and REP-PCR differences were subtle and not sufficient to allocate the strains to different types. A similar slight difference was seen with MLEE – the two strains being allocated to different ETs that linked at a genetic distance of 0.25 (Blackall et al., 1998).

Five of the six molecular typing methods, REA, ribotyping, REP-PCR, MLEE and MLST, agree that Outbreaks III and IV are distinct and unconnected. Multiple isolates were examined within Outbreak III. By REA, ribotyping and MLEE and MLST, no variation was found within any of the 12 strains examined. PFGE and REP-PCR identified a minor variation in PM 11 that was not detected by any other method.

The following evaluation is set in two contexts – an ability to separate unrelated strains and an ability to link related isolates. In terms of an ability to link related isolates, MLST performed strongly. MLST indicated that all 12 strains from Outbreak III all belonged to the same ST (ST 2). Similarly, MLST confirmed all four isolates from Outbreak II were related – assigning all four strains to the same ST (ST 1). The identity of the four strains involved in Outbreak I was confirmed by REA, ribotyping, PFGE and REP-PCR.

MLST indicated that Outbreaks I and V were connected – with both outbreaks being associated with the same ST (ST 31). The connection between these outbreaks was confirmed by REA and ribotyping which found the strains associated with the two outbreaks to be identical. Hence, while minor differences were detected by PFGE, REP-PCR and MLEE, all typing methods essentially agree with the MLST result – Outbreaks I and V are connected and associated with the same strain.

In terms of an ability to separate unrelated strains, MLST also performed well. MLST typing indicated that Outbreaks II, III, IV, VII and VIII were all distinctly different outbreaks with no sharing of strains between the outbreaks. This was the conclusion also reached by REA, ribotyping, REP-PCR and MLEE.

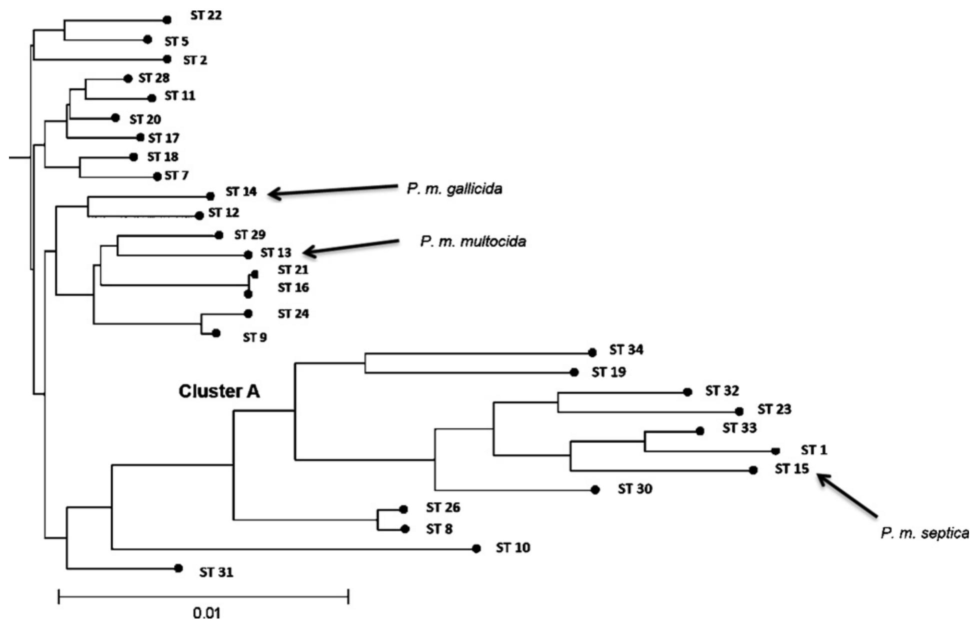

Fig. 1. Dendrogram of the 29 STs of *P. multocida* recognised in this study. The reference strains for the three subspecies of *P. multocida* are found as the sole strains allocated to ST 13 (subsp *multocida* NCTC 10322), ST 14 (subsp *gallicida* NCTC 10204) and ST 15 (subsp *septiica* CIP A125).

4. Discussion

This study was undertaken to develop an MLST scheme for *P. multocida* using 66 strains of *P. multocida*. The strains we used were deliberately selected from the prior MLEE study of Blackall et al. (1998).

We attempted to develop a simplified PCR approach for this MLST scheme and were mostly successful. We used the same set of primers for both amplification and sequencing, an approach that has been used in other MLST schemes e.g. the *S. pneumoniae* scheme (Enright and Spratt, 1998). We found that a single set of primers was sufficient to amplify six of the seven genes from all 66 strains examined. Single sets of primers for all gene loci involved in an MLST scheme is the preferred option and is a feature of MLST schemes for organisms such as *Pseudomonas aeruginosa* (Curran et al., 2004) and *S. suis* (King et al., 2002). However, the requirement to use additional primer sets, as was found necessary for the *zwf* locus of *P. multocida*, has been also necessary in other MLST schemes for organisms such as *A. baumannii* (Bartual et al., 2005) and *C. jejuni* (Manning et al., 2003). We also originally aimed at using a single set of PCR amplification conditions for all seven gene loci – a common methodology in MLST schemes. We achieved this aim for six of the loci – with a different annealing temperature being necessary for the *gdh* locus. Other MLST schemes for organisms such as *Salmonella enterica* (Sukhnanand et al., 2005), *S. suis* (King et al., 2002) and *S. uberis* (Zadoks et al., 2005) have encountered a similar need for variable PCR conditions.

The MLST study revealed a population structure that matched closely the population structure recognised by both MLEE and ribotyping as reported previously (Blackall et al., 1998). Specifically, MLST Cluster A corresponds with the previously recognised MLEE Cluster B and Ribotype Cluster 1 of Blackall et al. (1998). Hence, this MLST study provides further evidence of a differentiation within avian *P. multocida.* Strains within MLST Cluster A represent two subspecies (*multocida* and *septica*), show unusual biochemical properties and are notable for lacking strains belonging to the dominant biovar of *P. multocida* – biovar 3. Earlier studies have also identified the possibility of distinct lineages within the species. Davies (2004) recognised two lineages, A and B, within *P. multocida* based on 16S rRNA gene sequences. Kuhnert et al. (2000), also on the basis of 16S rRNA gene sequences have suggested that *P. multocida* subsp *septica* is quite distinct from the other two subspecies. Further studies, using both MLST and the sequence analysis of conserved genes such as the 16S rRNA gene sequencing of a larger collection of isolates is required to resolve the issue of lineages within *P. multocida*.

Of the 66 strains examined in both the MLST study reported here and the previous MLEE/ribotyping study of Blackall et al. (1998), only one strain showed a markedly different allocation – strain PM 17. In the MLEE study, PM 17 was assigned to ET 45 within MLEE Cluster B. In contrast, MLST analysis assigned PM 17 to ST 2. There is considerable evidence that the MLST placement is correct while the MLEE placement is erroneous. PM 17 is from the same turkey farm as isolates PM 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 15 and 16 – the other members of ST 2. All the isolates were obtained at the same time in 1993 in an outbreak of fowl cholera. Ribotyping agreed with MLST – placing PM 2–11 and PM 15–17 in ribotype 7 (Blackall et al., 1998). Hence, the known epidemiology (a confined outbreak on a single farm) and the ribotyping results all support the conclusion of the MLST study i.e. that PM 17 should be aligned with strains PM 2–11 and PM 15 and 16.

According to Ogle et al. (1987), an epidemiological marker should (1) be sufficiently sensitive to distinguish all unrelated isolates, (2) specifically identify all related isolates and (3) be stable. We have used these same criteria – sensitivity, specificity and stability (all in an epidemiological sense) – to evaluate MLST typing in the investigation of fowl cholera outbreaks. As the examination was a retrospective study involving a series of well-characterised outbreaks (in terms of field and laboratory investigations), we were able to compare MLST with almost all forms of commonly used molecular typing methods for *P. multocida*. This comparison indicated that MLST typing is sensitive (separates unrelated strains), specific (links related strains) and is stable (only one minor allele change of a single nucleotide was detected amongst twelve isolates from a single outbreak). Similar high specificity, sensitivity and stability were demonstrated by the other molecular methods (REA, ribotyping, PFGE and REP-PCR). The distinct advantage of MLST is portability and an ability to compare strains and results across the continents. The creation of the MLST scheme for *P. multocida* represents a major step forward in the ability of scientists around the world to type and compare isolates of *P. multocida* from all hosts.

This study represents the first formal proposal of an MLST scheme for avian *P. multocida*. However, there have been two previous studies that have looked at the sequences of house-keeping genes (Christensen et al., 2004; Davies et al., 2004). Davies et al. (2004) examined 36 bovine isolates using seven house-keeping enzymes. Davies et al. (2004) concluded on the basis of this analysis that the method did not achieve a high level of strain differentiation and that outer membrane typing was more discriminatory. The current study was focused on poultry isolates of *P. multocida* and shared only four house-keeping genes in common. Hence, it is difficult to compare the results of the current study with the study of Davies et al. (2004).

The study of Christensen et al. (2004) involved the analysis of the gene sequences of two house-keeping genes (*rpoB* and *infB*) for taxonomic and phylogenetic purposes. Christensen et al. (2004) reported that the analysis of these two genes showed a good correlation with each other and that the analysis provided a deeper resolution at the species level than achieved by analysis of the gene typically used for species resolution – the 16S rRNA gene.

In conclusion, this study represents the first time an MLST scheme has been established for avian *P. multocida*. The study also confirmed that MLST typing is highly specific, sensitive and stable (in an epidemiological sense) and performs as well as any other available typing method. As MLST is totally portable and easily comparable across laboratories, we suggest that MLST should now be accepted as the "gold standard" typing method for *P. multocida*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2010.01.017.

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