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Multilocus Sequence Typing for Characterization of *Staphylococcus pseudintermedius*

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***Staphylococcus pseudintermedius* is an opportunistic pathogen in dogs. Four housekeeping genes with allelic polymorphisms were identified and used to develop an expanded multilocus sequence typing (MLST) scheme. The new seven-locus technique shows *S. pseudintermedius* to have greater genetic diversity than previous methods and discriminates more isolates based upon host origin.**

Staphylococcus pseudintermedius, recently classified as a member of the *Staphylococcus intermedius* group (SIG), is the most common opportunistic pathogen in dogs. In this host it is frequently associated with pyoderma and other infections, such as those of the urinary tract, wounds, and otitis externa (1–3). It has also been isolated from infections in cats (4, 5), has zoonotic potential (6–8), and is an important nosocomial pathogen causing postsurgical infections in veterinary clinics (9, 10, 33). The incidence of methicillin-resistant *S. pseudintermedius* (MRSP) has increased significantly in the past few years (1, 5, 11–19). MRSP has emerged as an important problem worldwide because of multidrug resistance and the limited number of drug choices remaining to treat infections caused by this organism. Multilocus sequence typing (MLST) has been used extensively to define the population genetic structure of *Staphylococcus aureus* and other bacterial species. This information has been used to predict founder strains as well as track the spread of methicillin resistance and identify epidemic clones (2, 20). Likewise, the widespread application of this technique to *S. pseudintermedius* should help to identify methicillin-sensitive *S. pseudintermedius* (MSSP) progenitors of MRSP clones and provide a mechanism to track their spatial and temporal distribution (21). The identification of successful and/or virulent clonal populations of *S. pseudintermedius* may facilitate research into characteristics which provide a selective advantage and inform efforts to develop alternative methods of treatment and control, such as vaccines or phage therapy targeting the major clonal populations of *S. pseudintermedius* associated with disease.

MLST is well established as a valuable method for genotyping bacteria based on the sequence variation of housekeeping genes (9, 22). It provides accurate, portable data useful for global epidemiology studies and studies of evolution and population genetics (22–26). MLST techniques applied to diverse species of bacteria generally use at least seven loci (22, 24–27). Sequence typing based on four loci (MLST-4) has provided insight into the overall genetic structure of the SIG (1). The development of an MLST method expanded to seven loci (MLST-7) for *S. pseudintermedius* was undertaken to increase its discriminating power with the same number of loci used for other species of staphylococci (24, 32).

DNA extracts from 125 previously characterized isolates of *S. pseudintermedius* from dogs (114 isolates), cats (5 isolates), and human beings (6 isolates) from diverse geographical regions (North America, $n = 62$; Europe, $n = 57$; and Asia, $n = 6$) were used in this study (Table 1) (1, 2, 18). DNA was extracted from overnight cultures grown at 37°C on blood agar plates using either commercial DNA extraction kits (1, 18) or glass bead extraction. For the latter, bacteria derived from a single colony were suspended in 0.5 ml of 1 M Tris EDTA buffer (pH 7.5), mixed with an equal volume of glass beads, and subjected to pulsed vortexing for 5 min for cell disruption and DNA release. Supernatant of this mixture was stored at −20°C and used as the template in PCR assays.

Open reading frames were identified for 45 genes from contiguous sequences obtained using pyrosequencing technology (Vanderbilt University, Genome Technology Core [Illumina technology] and the University of Florida Interdisciplinary Center for Biotechnology Research [Roche 454 technology]), with genomic DNA isolated from *S. pseudintermedius* isolate 06-3228, a canine strain representative of the MRSP strains most frequently isolated by the bacteriology service at the University of Tennessee College of Veterinary Medicine and used previously as an in-house reference isolate (2, 28). *S. aureus* genome sequences available in GenBank (accession numbers CP000255, CP000732, CP000253, CP000730, AP009324, AP009351, CP000736, and CP000045) were aligned using the BLASTN algorithm with the National Center for Biotechnology Information (NCBI) online resource and used as a reference to predict which *S. pseudintermedius* genes were housekeeping genes and most likely to have polymorphic sites. A total of 45 candidates were identified and selected for evaluation. To amplify portions of the candidate genes, PCR primers

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TABLE 1 *S. pseudintermedius* isolates and their allelic profiles

Isolate	Host	ST based on MLST-7	ST based on MLST-4	Loci and corresponding alleles						
				tuf	*cpn60*	*pta*	*purA*	*fdh*	*sar*	*ack*
M 1332/03	Canine	1	5	1	2	1	1	2	2	1
M 543/06	Canine	2	44	1	2	1	1	3	1	1
NA 128	Canine	3	20	1	2	1	4	2	1	3
N 910/201	Human	4	20	1	2	1	4	4	2	1
3414	Feline	5	5	1	2	1	16	2	2	2
8478	Canine	6	57	1	2	2	4	2	1	2
96-032996	Canine	7	42	1	2	2	4	2	1	4
94072/594	Canine	8	37	1	2	2	4	2	3	1
4229	Canine	9	59	1	2	2	18	3	1	1
8239	Canine	10	56	1	2	7	3	2	1	1
NA 67	Canine	11	159	1	2	8	1	2	1	2
94060294	Canine	12	35	1	2	12	19	2	1	4
8639	Canine	13	62	1	3	1	7	2	2	17
96022/296	Canine	14	40	1	7	1	5	2	1	5
95072195	Canine	15	39	1	7	1	5	2	4	1
8193	Canine	16	39	1	7	1	5	4	1	6
95062/195	Canine	16	39	1	7	1	5	4	1	6
Can 10	Human	17	70	1	7	1	13	1	1	1
BH 04	Canine	18	66	1	7	4	1	2	2	4
V0709390114	Canine	19	114	1	7	4	4	1	1	1
M629/06	Canine	20	45	1	7	8	1	4	1	20
BH 47	Canine	21	45	1	7	8	14	2	1	1
08.2211	Canine	22	92	1	8	1	6	2	1	2
07.367C	Canine	23	97	1	8	1	7	2	1	2
690	Feline	24	55	1	8	5	3	1	2	20
ED 99	Canine	25	2	1	9	1	1	2	1	3
94-062394	Canine	26	36	1	9	1	7	1	4	3
95-062295	Canine	27	2	1	9	1	8	3	4	3
2431	Canine	28	60	1	9	3	3	3	4	3
07.676 C	Canine	29	68	1	10	4	1	2	1	1
08.547a	Canine	30	68	1	10	4	5	1	1	1
AV 8010	Canine	31	27	1	11	1	8	1	4	3
M407/03	Canine	32	4	1	11	1	8	2	1	1
96 030 796	Canine	33	41	1	13	1	4	2	1	4
M 741/99	Canine	34	7	1	13	1	8	2	6	2
93071493	Canine	35	34	1	13	11	14	2	2	3
96041096	Canine	36	43	1	14	1	3	5	5	6
8016	Canine	37	47	1	16	1	5	2	1	1
9318	Canine	38	61	1	20	1	3	2	1	1
08.153	Canine	39	61	1	20	1	7	1	1	4
M 1337/03	Canine	40	8	1	21	2	7	3	4	20
13	Canine	41	69	1	25	1	1	3	1	11
BH 34	Feline	42	67	1	27	2	3	1	1	3
08.1781	Canine	43	99	1	27	8	5	3	2	4
06.1705a	Canine	44	96	1	32	23	1	1	1	5
3279	Canine	45	29	2	2	1	2	2	1	3
V08801393	Canine	45	29	2	2	1	2	2	1	3
8.1791	Canine	46	103	2	9	1	8	1	2	4
96022396	Feline	47	33	2	2	1	13	1	1	12
AV 8033	Canine	48	29	2	2	1	17	2	2	1
HT20030686	Canine	49	18	2	2	4	13	2	1	3
M 721/99	Canine	50	11	2	3	1	4	2	1	2
08.1988b	Canine	51	15	2	6	1	4	1	1	16
HT20030683	Canine	52	15	2	6	1	15	1	1	1
HT20030685	Canine	53	17	2	6	4	4	4	1	1
07.1447	Canine	54	100	2	7	1	1	2	1	1
M1333/03	Canine	54	9	2	7	1	1	2	1	1
NA 31	Canine	56	100	2	7	1	4	3	1	1
NA 47	Canine	56	100	2	7	1	4	3	1	1
V0805157	Canine	58	111	2	7	1	4	4	2	5
AV 8024	Canine	59	28	2	7	1	13	4	2	1
AV 8002	Canine	60	25	2	7	2	4	3	1	6
N940276	Human	61	21	2	7	2	20	2	8	1
N940453	Human	62	22	2	7	4	7	4	2	1
HT20030684	Canine	63	16	2	7	8	14	2	1	1
NA 11	Canine	64	118	2	7	11	5	2	2	2
NA 12	Canine	64	118	2	7	11	5	2	2	2

(Continued on following page)

TABLE 1 (Continued)

Isolate	Host	ST based on MLST-7	ST based on MLST-4	Loci and corresponding alleles						
				tuf	*cpn60*	*pta*	*purA*	*fdh*	*sar*	*ack*
M 1351/03	Canine	65	3	2	7	18	1	2	1	1
N 900260	Human	66	19	2	8	1	1	2	2	7
08.1752a	Canine	67	19	2	8	1	4	1	2	8
Can 5	Canine	68	68	1	10	4	1	1	1	1
M06-13	Canine	68	68	1	10	4	1	1	1	1
NA 10	Canine	68	68	1	10	4	1	1	1	1
NA 24	Canine	68	68	1	10	4	1	1	1	1
NA 27	Canine	68	68	1	10	4	1	1	1	1
NA 37	Canine	68	68	1	10	4	1	1	1	1
NA 50	Canine	68	68	1	10	4	1	1	1	1
NA 64	Canine	68	68	1	10	4	1	1	1	1
Sp80	Canine	68	68	1	10	4	1	1	1	1
07.182a	Canine	69	101	2	8	1	4	2	1	3
8185	Canine	70	51	2	8	4	1	2	1	20
7577–06	Canine	71	71	1	9	2	1	1	2	3
BD19698-06	Canine	71	71	1	9	2	1	1	2	3
NA 126	Canine	71	71	1	9	2	1	1	2	3
NA 23	Canine	71	71	1	9	2	1	1	2	3
Gi1	Canine	71	71	1	9	2	1	1	2	3
2080806063	Canine	71	71	1	9	2	1	1	2	3
2080812013	Canine	71	71	1	9	2	1	1	2	3
2081218007	Canine	71	71	1	9	2	1	1	2	3
2090401029	Canine	71	71	1	9	2	1	1	2	3
V0703973	Canine	71	71	1	9	2	1	1	2	3
HH4	Feline	71	71	1	9	2	1	1	2	3
M575/06	Canine	72	24	2	9	1	1	4	1	4
NA 85	Canine	73	158	2	9	1	3	2	1	1
07.188a	Canine	74	103	2	9	1	4	2	1	1
06.2735a	Canine	75	103	2	9	1	8	1	1	4
M 695/99	Canine	77	6	2	9	1	8	4	1	1
AV 8001	Canine	78	24	2	9	1	13	1	5	7
129Ab	Canine	79	58	2	9	2	2	6	1	1
3708	Canine	80	58	2	9	2	4	4	5	14
07.169a	Canine	81	104	2	9	2	8	1	1	4
9162	Canine	82	54	2	9	4	1	2	1	1
7.5066	Canine	83	105	2	10	1	2	2	1	18
NA 4	Canine	84	26	2	10	1	5	2	1	2
NA 43	Canine	84	26	2	10	1	5	2	1	2
NA 45	Canine	84	26	2	10	1	5	2	1	2
NA 49	Canine	84	26	2	10	1	5	2	1	2
NA 6	Canine	84	26	2	10	1	5	2	1	2
NA 3	Canine	85	26	2	10	1	5	2	5	1
AV 8012	Canine	86	26	2	10	1	13	2	5	4
NA 20	Canine	87	NPA[a]	2	13	1	1	1	1	1
V07008754	Canine	88	106	2	13	1	4	4	1	5
2081204035	Canine	89	106	2	13	1	4	4	2	5
2090123017	Canine	89	106	2	13	1	4	4	2	5
07.281a	Canine	90	108	2	13	1	9	3	3	4
06.2381a	Canine	91	107	2	13	1	10	2	1	10
08.3174a	Canine	92	156	2	13	4	3	1	1	1
KM241	Canine	93	73	2	13	19	1	1	2	1
M657/06	Canine	94	46	2	15	4	8	2	1	1
388	Canine	95	52	2	18	4	2	1	1	20
9075	Canine	96	53	2	19	4	13	4	4	1
BH 01	Canine	97	72	2	26	1	8	1	1	4
07.257a	Canine	98	110	2	27	1	3	2	4	10
95011195	Canine	99	38	5	13	11	7	2	1	2
LY19990344	Human	100	23	9	6	1	8	4	7	1

[a] NPA, not previously assigned.

were designed using the Primer3Plus program (29) and obtained commercially (Integrated DNA Technologies). A panel of 15 *S. pseudintermedius* isolates with different genetic backgrounds, typed in our laboratory based on MLST-4 (1), was used for loci selection. The PCR consisted of 95°C for 90 s, followed by 35 cycles of 30 s at 52°C, 1 min at 72°C, 30 s at 94°C, and then 52°C for 30 s, and a final extension at 72°C for 5 min. PCR products were resolved and visualized by electrophoresis in gels containing 1.4% agarose and 0.5 μg/ml ethidium bromide. PCR amplicons of expected sizes, when produced from all isolates in the panel, were

TABLE 2 Primers for PCR amplification of the 7 loci for MLST typing of *S. pseudintermedius*

Locus	Primer	Primer sequence 5′–3′	Product size (bp)	Source or reference
tuf	*tuf* forward	CAATGCCACAAACTCG	500	1
	tuf reverse	GCTTCAGCGTAGTCTA		1
cpn60	*cpn60* forward	GCGACTGTACTTGCACAAGCA	552	1
	cpn60 reverse	AACTGCAACCGCTGTAAATG		1
pta	*pta* forward	GTGCGTATCGTATTACCAGAAGG	570	1
	pta reverse	GCAGAACCTTTTGTTGAGAAGC		1
purA	*purA* forward	GATTACTTCCAAGGTATGTTT	490	This study
	purA reverse	TCGATAGAGTTAATAGATAAGTC		This study
fdh	*fdh* forward	TGCGATAACAGGATGTGCTT	408	This study
	fdh reverse	CTTCTCATGATTCACCGGC		This study
ack	*ack* forward	CACCACTTCACAACCCAGCAAACT	680	This study
	ack reverse	AACCTTCTAATACACGCGCACGCA		This study
sar	*sar* forward	GGATTTAGTCCAGTTCAAAATTT	521	This study
	sar reverse	GAACCATTCGCCCCATGAA		This study

treated using enzymes to destroy single-stranded DNA (ExoSAP-IT; USB Corp., Cleveland, OH) and sequenced at the University of Tennessee Molecular Biology Resource Facility (Knoxville, TN) or BaseClear (Leiden, The Netherlands). Sequences were analyzed using the commercial software packages Lasergene (DNAStar, WI) and BioNumerics 6.1 (Applied Maths, Belgium).

Four of 45 candidate genes that produced PCR products from all isolates and generated the greatest number of alleles were included in MLST-7. These loci, the proteins they encode, and their position within a reference GenBank sequence are as follows: *purA*, adenylosuccinate synthetase positions 568 to 968 in JF275101; *fdh*, formate dehydrogenase positions 888 to 1236 in JF275100; *ack*, acetate kinase positions 1 to 648 in JX501660; and *sar*, sodium sulfate symporter positions 246 to 683 in JF275103. The primers used to produce the PCR products containing these loci and the sizes of the amplicons are listed in Table 2. The three housekeeping genes in MLST-4, *tuf*, *cpn60*, and *pta*, were included in MLST-7 because their usefulness for population genetic analysis has already been established and data for these loci have been obtained from numerous isolates in several studies (1, 2, 4, 8, 18).

Within the isolates used in this study, the *purA* gene sequences had 11 polymorphic sites and 20 alleles (see Table S1 in the supplemental material). Five polymorphic sites were detected in *fdh* yielding 5 alleles (see Table S2 in the supplemental material). The *ack* gene had 10 polymorphic sites and 19 alleles (see Table S3 in the supplemental material). Eight polymorphic sites were detected in the *sar* gene, resulting in 8 alleles (see Table S4 in the supplemental material). No nonsynonymous substitutions were found in *fdh*; there was one each in *ack* and *sar*, and there were two in *purA* alleles. The low numbers of nonsynonymous substitutions suggest the genes are under purifying selection. There are, however, only two sequenced *S. pseudintermedius* genomes (30, 31) and no studies to date of the ratio of nonsynonymous to synonymous substitutions within this species to serve as a reference.

Among the 125 isolates tested, MLST-7 identified 98 unique sequence types (STs) (Table 1). Sixty-one STs had a single representative based on MLST-4 and, therefore, could not be further distinguished. The isolates from 17 STs which had two or more representatives based on MLST-4 yielded 37 STs with MLST-7. Each set of two or more isolates produced additional STs with MLST-7, except for isolates with ST71 and ST118 (MLST-4 classification). With MLST-7, all feline and human isolates tested except one ST71 feline isolate have STs distinct from those obtained from other host species. MLST-4 sequence types ST9 and ST100 are no longer differentiated with MLST-7; however, ST100 is further divided into MLST-7 ST54 and ST56.

It is of interest that ST71, the most commonly identified MRSP clone spreading worldwide to date, could not be further distinguished using the new MLST-7 scheme. Although only 11 isolates were included in this study, this may be an indication of the rapid global spread of a specific MRSP ST71 clone. It is assumed that, as a greater number of samples are tested and as the clone evolves, genetic variants will be identified. However, if greater resolution is desired to further differentiate ST71, additional loci, such as those under positive selection, could be examined for this ST. ST68, the predominant MRSP isolate from the United States, is further divided into three STs. ST68 and ST71 designations were maintained to provide continuity between the MLST-4 and MLST-7 typing systems, with ST68 assigned to what appears to be the predominant form of the clonal complex (Table 1).

In this study, we report the development of a 7-locus MLST scheme for *S. pseudintermedius*. We propose that the data generated by this method can be globally shared to obtain a clearer picture of the spread of methicillin resistance among *S. pseudintermedius* and can improve our understanding of the population genetics of this species. Vincent Perreten (vincent.perreten@vetsuisse.unibe.ch) serves as curator for assignment of STs for the *S. pseudintermedius* research community and has generously agreed to continue to do so for the expanded MLST system.

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