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Development of a Rapid Multiplex PCR Assay To Genotype *Pasteurella multocida* Strains by Use of the Lipopolysaccharide Outer Core Biosynthesis Locus

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***Pasteurella multocida* is a Gram-negative bacterial pathogen that is the causative agent of a wide range of diseases in many animal species, including humans. A widely used method for differentiation of *P. multocida* strains involves the Heddleston serotyping scheme. This scheme was developed in the early 1970s and classifies *P. multocida* strains into 16 somatic or lipopolysaccharide (LPS) serovars using an agar gel diffusion precipitin test. However, this gel diffusion assay is problematic, with difficulties reported in accuracy, reproducibility, and the sourcing of quality serovar-specific antisera. Using our knowledge of the genetics of LPS biosynthesis in *P. multocida*, we have developed a multiplex PCR (mPCR) that is able to differentiate strains based on the genetic organization of the LPS outer core biosynthesis loci. The accuracy of the LPS-mPCR was compared with classical Heddleston serotyping using LPS compositional data as the "gold standard." The LPS-mPCR correctly typed 57 of 58 isolates; Heddleston serotyping was able to correctly and unambiguously type only 20 of the 58 isolates. We conclude that our LPS-mPCR is a highly accurate LPS genotyping method that should replace the Heddleston serotyping scheme for the classification of *P. multocida* strains.**

Pasteurella multocida is the primary causative agent of a wide range of economically important diseases, including hemorrhagic septicemia in ungulates, atrophic rhinitis in pigs, fowl cholera in birds, snuffles in rabbits, and enzootic pneumonia and shipping fever in cattle, sheep, and pigs (1). *P. multocida* also causes opportunistic infections in humans, often following cat or dog bites, and plays a contributory role, together with other pathogens, in a range of lower respiratory tract infections and sporadic septicemias in ungulates (1).

P. multocida strains have classically been differentiated using serological techniques. Strains can be classified into five capsular serogroups (A, B, D, E, and F) using an indirect hemagglutination test (2) and into 16 somatic or lipopolysaccharide (LPS) serovars (serotypes) using the Heddleston gel diffusion precipitin test (3). Both of these schemes have been widely used. Isolates are commonly assigned a combined designation, such as A:1 (capsular serogroup A and LPS serovar 1) or B:2 (capsular serogroup B and LPS serovar 2).

P. multocida LPS is an immunodominant antigen critical for homologous protection stimulated by bacterin (killed-cell) vaccines (4). Furthermore, in the *P. multocida* strain VP161, a full-length LPS molecule is essential for the ability to cause acute disease (5, 6). Heddleston serotyping is currently the only method used to differentiate *P. multocida* strains on the basis of LPS type. However, the accuracy of Heddleston serotyping has never been objectively tested, as the precise LPS structures produced by different strains have not been known. Indeed, there have been many informal as well as formal reports that the Heddleston system fails to type many isolates and lacks accuracy and reproducibility (7, 8). Furthermore, Heddleston serotyping is time-consuming and requires access to good-quality, serovar-specific antisera.

We have recently carried out a comprehensive analysis of the LPS structures expressed by the 16 Heddleston type strains and identified the genes required for LPS assembly in each strain (9–16). These combined analyses showed that the LPS produced by all strains consisted of a highly conserved inner core and a variable outer core and revealed that each of the 16 Heddleston type strains expresses structurally distinct LPS. Importantly, these analyses also showed that only eight unique LPS outer core biosynthesis loci are found in the 16 Heddleston type strains (Fig. 1). We have designated these genetic loci L1 through to L8. The type strains of Heddleston serovars 1, 2, 3, 5, 6, 8, 9, 12, and 16 express full-length or "parent" LPS structures, and the type strains of Heddleston serovars 4, 7, 10, 11, 13, 14, and 15 express truncated LPS—the result of mutations within the LPS outer core biosynthesis loci.

The partial differentiation of *P. multocida* strains on the basis of LPS biosynthesis genes has been reported previously (17). Using PCR-restriction fragment length polymorphism (RFLP) analysis, *P. multocida* strains were grouped into 5 PCR-RFLP types. However, only 11 of the 16 Heddleston serovars were included in

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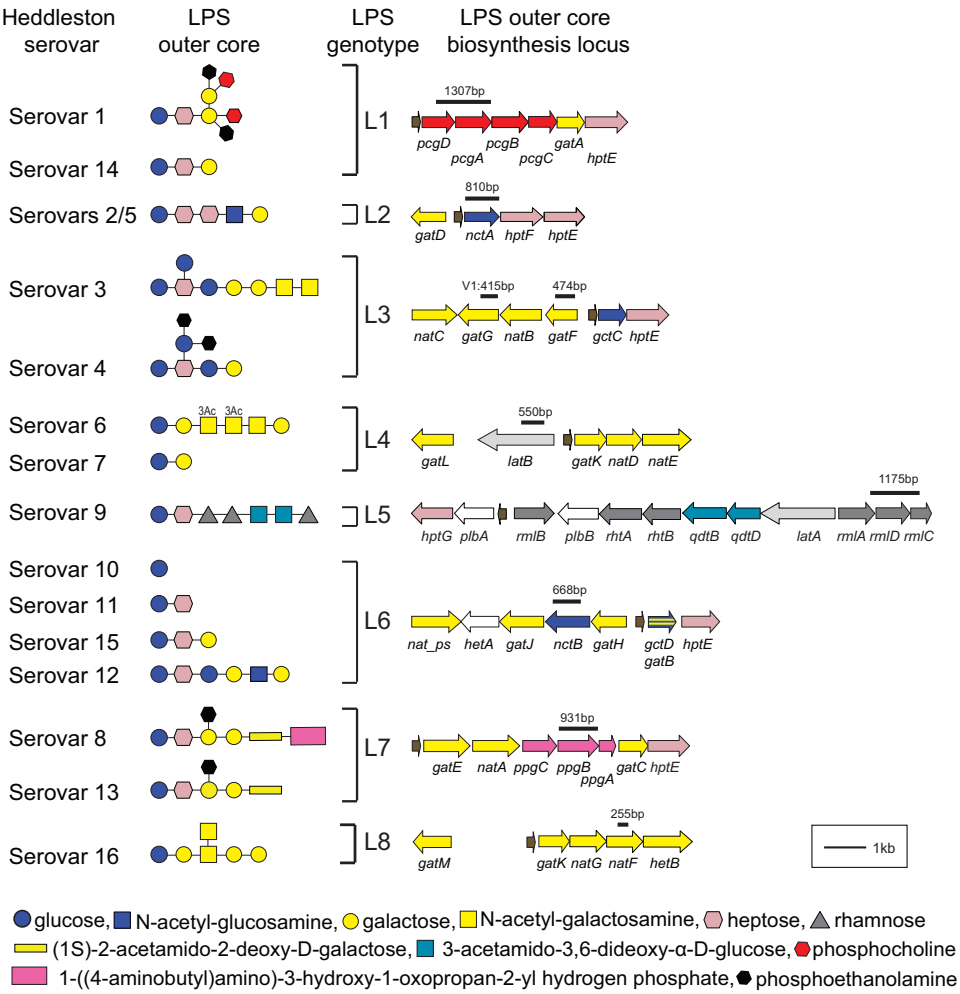

FIG 1 LPS outer core structure produced by each of the Heddleston serovar type strains and the genes responsible for LPS outer core biosynthesis in each strain. (Left) Schematic representation of the outer core LPS structures produced by each of the Heddleston serovar type strains. The last residue (glucose) of the conserved LPS inner core is shown on the far left as a reference point. Specific linkages between each of the residues are not shown. (Right) LPS genotype and genetic organization of each LPS outer core biosynthesis locus. The relative position and size of each genotype-specific PCR amplicon are shown above each LPS outer core biosynthesis locus. Each gene is color coded according to its known/predicted role in LPS biosynthesis; *gctD* and *gatB* (yellow and blue striped) in locus L6 differ by only a single nucleotide and are involved in the addition of glucose or galactose, respectively, to the outer core heptose. The *rpL31_2* gene, encoding ribosomal protein L31, is not involved in LPS biosynthesis and is colored brown.

the study. Here we report the development and testing of a multiplex PCR (mPCR) using the full set of Heddleston serovar type strains, which can accurately differentiate *P. multocida* strains into one of the eight distinct LPS genotypes. By comparing the results of Heddleston serotyping and the LPS-mPCR to the LPS structures predicted from LPS compositional analysis by mass spectrometry (MS), we have determined the accuracy of this mPCR and Heddleston serotyping for predicting LPS type. The LPS-mPCR gave a result that was indicative of LPS genotype >98% of the time, and we propose that the LPS-mPCR assay should be used to differentiate strains into their appropriate LPS genotype and, together with the *cap* mPCR, form a new molecularly based typing system for accurate strain differentiation of *P. multocida*.

MATERIALS AND METHODS

Strains used. All *P. multocida* strains were grown at 37°C in heart infusion (HI) (Oxoid, Basingstoke, United Kingdom) liquid broth with shaking or on solid HI medium containing 1.5% agar. The *P. multocida* strains used in the study are described in Table 1. All field isolates were confirmed as *P. multocida* by use of one of two *P. multocida*-specific PCR assays (18, 19).

Serotyping. Each isolate of *P. multocida* was serotyped via the Heddleston method as described previously (3).

Molecular biology techniques. Genomic DNA was purified from 1 ml of *P. multocida* overnight culture using the RBC genomic DNA purification kit (RBC, Taiwan). Each of the final LPS-mPCRs (50-μl final volume) was performed in 1× *Taq* polymerase buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl [Roche Diagnostic GmbH, Mannheim, Germany]) containing 0.4 μM each primer (Table 2), 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1.7 U *Taq* polymerase (Roche Diagnostic GmbH, Mannheim, Germany). For each colony PCR, material from 2 to 3 well-isolated *P. multocida* colonies (obtained from overnight growth of each isolate at 37°C on an HI–1.5% agar plate) was collected using a sterile tip on a 20-μl micropipette (volume set at 20 μl) inserted into the middle of each colony. The collected material was then added to a 50-μl PCR mixture and mixed thoroughly by pipetting. For PCR using genomic DNA, approximately 50 ng of column-purified DNA was added to each PCR mixture. All reaction mixtures were mixed briefly then centrifuged

TABLE 1 Characteristics of the strains used in this study[a]

Strain no.	Heddleston serovar designation(s)[b]	Isolation date	Host species
X73	H1 type strain	Prior to 1943	Chicken
M1404	H2 type strain	Prior to 1943	Bison
P1059	H3 type strain	Prior to 1943	Turkey
P1662	H4 type strain	1968	Turkey
P1702	H5 type strain	1971	Turkey
P2192	H6 type strain	1971	Chicken
P1997	H7 type strain	1971–1973	Herring gull
P1581	H8 type strain	1971–1973	Pine siskin
P2095	H9 type strain	1971–1973	Turkey
P2100	H10 type strain	1971–1973	Turkey
P903	H11 type strain	1971–1973	Pig
P1573	H12 type strain	1971–1973	Human
P1591	H13 type strain	1971–1973	Human
P2225	H14 type strain	1971–1973	Cattle
P2237	H15 type strain	1971–1973	Turkey
P2723	H16 type strain	1974	Turkey
PM1	H3 (H3, H4)	1993	Turkey
PM3	H15 (H4, H10, H15)	1993	Turkey
PM8	H10 (H10)	1993	Turkey
PM18	NT (H3)	1986	Chicken
PM19	H13 (H3)	1986	Chicken
PM36	H14 (H14)	1985	Unknown
PM37	H3 (H3)	1988	Chicken
PM45	NT (H3, H4)	1986	Chicken
PM46	H6 (H6)	1992	Chicken
PM48	H3 (H3, H4)	1983	Chicken
PM49	NT (H1, H15)	1984	Chicken
PM51	H9 (H4, H12)	1984	Chicken
PM64	NT (H3)	1979	Chicken
PM67	H3 (H3, H12)	1969	Turkey
PM72	NT (H3, H14)	1977	Chicken
PM120	H12 (H12)	1993	Chicken
PM135	H8, H13 (H13)	Unknown	Turkey
PM140	NT (H13, H14, H15)	1994	Chicken
PM147	H7 (H7)	1993	Chicken
PM878	H1, H4	2001	Chicken
PM993	H8	2002	Duck
PM995	H3	2002	Chicken
PM1075	H16	2004	Chicken
PM1098	H15	2004	Unknown
PM1099	H10	2004	Unknown
PM1103	H10	2004	Unknown
PM1113	NT	2004	Avian
PM1120	NT	2005	Chicken
PM1124	H1, H4, H12	2005	Unknown
PM1128	H10	2005	Bovine
PM1132	H1, H3, H4, H10, H14	2005	Pig
PM1153	H1, H3, H7	2005	Avian
PM1165	H1	2006	Duck
PM1193	H3	2006	Duck
PM1205	H1	2007	Emu
PM1258	NT	2010	Chicken
PM1268	NT	2010	Chicken
PM1300	H4	2009	Turkey
PM1304	H1	2009	Chicken
PM1315	H1	2009	Chicken
PM1316	H4	2009	Unknown
PM1317	H3	2009	Unknown
PM1320	H10, H13, H14	2010	Chicken
PM1369	H1	2010	Chicken
PM1396	H1, H3	2010	Unknown
PM1398	H1	2010	Chicken
PM1405	NT	2010	Chicken
PM1417	H4	2010	Chicken
PM1434	NT	2010	Chicken
PM1435	NT	2010	Chicken
PM1439	NT	2010	Chicken
PM1441	H2	2010	Turkey
PM1455	H1	2011	Chicken
PM1456	H14	2011	Chicken
PM1457	NT	2011	Chicken
PM1458	H14	2011	Chicken
PM1470	H1	2011	Turkey
PM1474	H12	2011	Duck

[a] Included are the Heddleston serovar type strains and details on the Australian *P. multocida* field isolates, including Heddleston serotyping results, isolation date, and host species.

[b] The format in which multiple numbers are separated by a comma indicates that a precipitin line was observed with more than one type of serum. The presence of results in parentheses indicates that two distinct and separate serotyping assays were performed: the result in the parentheses is the first result with this isolate. NT, nontypeable by Heddleston serotyping.

(10 s, 13,000 × *g*). All PCRs were performed in an Eppendorf Mastercycler. For colony PCR, the cycling conditions were 96°C for 10 min, followed by 30 cycles of 96°C for 30 s, 52°C for 30 s, and 72°C for 2.5 min, with a final extension at 72°C for 5 min. For PCR using genomic DNA as the template, the cycling conditions were identical to those in the colony PCR, except that the initial denaturation step at 96°C was reduced to 5 min.

The PCR products generated from the LPS-mPCR were analyzed by gel electrophoresis using 2% agarose–Tris-acetate-EDTA (TAE) gel in 1× TAE buffer for 90 min at constant voltage (70 V).

For the initial LPS-mPCR (LPS-mPCR version 1 [LPS-mPCRv1]), all primers were used at a concentration of 0.3 μM, except for the L6 primers, which were used at 0.5 μM. The cycling conditions for the LPS-mPCRv1 using bacterial colony material as the template were 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 2.5 min, with a final extension at 72°C for 2 min.

Nucleotide sequences were determined by direct sequencing from genomic DNA and/or by sequencing of amplified PCR fragments as described previously (10). Sequencing reactions were analyzed using the Applied Biosystems 3730S genetic analyzer, and sequencing chromatograms were analyzed and the LPS loci assembled using Vector NTI Advance 11 (Invitrogen). Bioinformatic analyses, including amino acid sequence alignments, were conducted using BLAST and ClustalW2.

LPS sugar compositional analyses. For compositional analysis of LPS produced by the Australian field isolates, small quantities of LPS were isolated from plate-grown cells as described previously (20). O-deacylated LPS (LPS-OH), core oligosaccharide (OS), and completely deacylated LPS were all isolated and purified from LPS as described previously (21). The sugar composition of the LPS from selected strains was determined by mass spectrometry as previously described (22). The predicted LPS structures produced by the *P. multocida* isolates were determined using MS compositional analysis and comparison with the known compositions and structures of the 16 Heddleston serovar type strains (9–16).

RESULTS

Design of a first-generation mPCR capable of differentiating *P. multocida* strains based on the genetics of LPS biosynthesis. We have shown previously that the 16 unique LPS outer core structures produced by the *P. multocida* Heddleston type strains are

TABLE 2 DNA sequence and genetic location of the primers used in the LPS-mPCR

Locus	Primer	Sequence	Location	Product size (bp)
Oligonucleotides used in final LPS-mPCR typing assay				
L1	BAP6119	ACATTCCAGATAATACACCCG	Forward primer in *pcgD*	1,307
	BAP6120	ATTGGAGCACCTAGTAACCC	Reverse primer in *pcgB*	
L2	BAP6121	CTTAAAGTAACACTCGCTATTGC	Forward primer in *nctA*	810
	BAP6122	TTTGATTTCCCTTGGGATAGC	Reverse primer in *nctA*	
L3	BAP7213	TGCAGGCGAGAGTTGATAAACCATC	Forward primer in *gatF*	474
	BAP7214	CAAAGATTGGTTCCAAATCTGAATGGA	Reverse primer in *gatF*	
L4	BAP6125	TTTCCATAGATTAGCAATGCCG	Reverse primer in *latB*	550
	BAP6126	CTTTATTTGGTCTTTATATATACC	Forward primer in *latB*	
L5	BAP6129	AGATTGCATGGCGAAATGGC	Forward primer in *rmlA*	1,175
	BAP6130	CAATCCTCGTAAGACCCCC	Reverse primer in *rmlC*	
L6	BAP7292	TCTTTATAATTATACTCTCCCAAGG	Forward primer in *nctB*	668
	BAP7293	AATGAAGGTTTAAAAGAGATAGCTGGAG	Reverse primer in *nctB*	
L7	BAP6127	CCTATATTTATATCTCCTCCCC	Forward primer in *ppgB*	931
	BAP6128	CTAATATATAAACCATCCAACGC	Reverse primer in *ppgB*	
L8	BAP6133	GAGAGTTACAAAAATGATCGGC	Forward primer in *natG*	255
	BAP6134	TCCTGGTTCATATATAGGTAGG	Reverse primer in *natG*	
Oligonucleotides used only in LPS-mPCRv1[a]				
L3	BAP6123	TCCTTATCTGACATTGAAATCG	Forward primer in *gatG*	415
	BAP6124	CTAGACATCTGGTGGTTGCG	Reverse primer in *gatG*	
L6	BAP7039	AATATCTTTATAATTATACTCTCCC	Forward primer in *nctB*	668
	BAP6132	AATGAAGGTTTAAAAGAGATAGC	Reverse primer in *nctB*	

[a] These L3 and L6 primer sets were used in the initial LPS-mPCRv1 but were replaced in the final LPS-mPCR.

generated from only eight distinct genetic loci, which we have named L1 to L8 (Fig. 1) (9–15, 20). We have therefore designated the following Heddleston serovar type strains as the LPS genotype type strains: X73 (L1), P1702 (L2), P1059 (L3), P2192 (L4), P2095 (L5), P1573 (L6), P1581 (L7), and P2723 (L8). Within each LPS genotype, strains displaying variation and/or truncation of the LPS structure can arise from random point mutations or deletions, in almost all cases, within the LPS outer core biosynthesis genes. These mutations can result in a change of function (sugar or donor specificity) or a total loss of function resulting in early termination of LPS assembly (10, 11). Given the random nature of LPS mutations, it was concluded that designing a PCR specific for each precise LPS structure identified was not possible. Thus, an mPCR assay was designed that was capable of differentiating the eight different LPS genotypes.

In all of the *P. multocida* strains so far examined, the genes required for synthesis of the LPS outer core are located between the conserved non-LPS genes *priA* and *fpg* (23). Each LPS outer core biosynthesis locus contains between 5 and 13 genes, including the highly conserved *rpL31_2* gene, encoding ribosomal protein L31, which is not involved in LPS assembly (Fig. 1). To design an mPCR specific for the LPS outer core biosynthesis loci, a bioinformatic comparison was first performed using all the predicted protein sequences from each of the eight LPS outer core biosynthesis loci. One unique (or least similar) protein sequence was selected, and the corresponding nucleotide sequence was compared with the entire nucleotide sequence of each of the eight LPS loci. Where some similarity was observed in the selected region with the nucleotide sequence from another locus, nucleotide alignments were generated and the alignments visually inspected to identify the most divergent DNA sections. This information was used to design a set of eight primer pairs specific for each of the eight LPS biosynthetic loci (Table 2). Each primer pair was also designed to generate a distinct amplicon size for optimal electrophoretic separation on 2% agarose gels.

Testing of the LPS-mPCRv1. All primer sets were initially tested in separate PCRs using either genomic DNA or colony-derived cells from each of the Heddleston type strains as the template. All PCRs amplified a product of the correct size when the appropriate type strains were used as the template (e.g., when the L1 primers were used against the L1 strains X73 and P2225). The different primer pairs were then combined and used in a single mPCR using either genomic DNA or colony-derived cells from each of the Heddleston type strains as the template. Following PCR optimization, a reproducible mPCR (LPS-mPCR version 1 [LPS-mPCRv1]) was developed that generated a single product of the expected size for all strains (Fig. 2A).

Testing of the LPS-mPCRv1 against Australian *P. multocida* field isolates. To test the reproducibility and accuracy of LPS-mPCRv1, 58 *P. multocida* field isolates were typed using both LPS-mPCRv1 and classical Heddleston serotyping. The 58 field isolates included strains obtained from a range of Australian poultry farms and other sources between 1977 and 2011. In total, 33 of the strains were recorded as being isolated from chickens, 8 from turkeys, 4 from ducks, and 1 from an emu. The host species was not recorded for 10 of the isolates. One bovine isolate and one porcine isolate were also included (Table 1).

Historical strains were serologically typed using the Heddleston serotyping system when they were first received at the Australian reference laboratory (Agri-Science Queensland), and the typing was then repeated again for this study. For some strains, the serovar determined when the Heddleston serotyping was re-

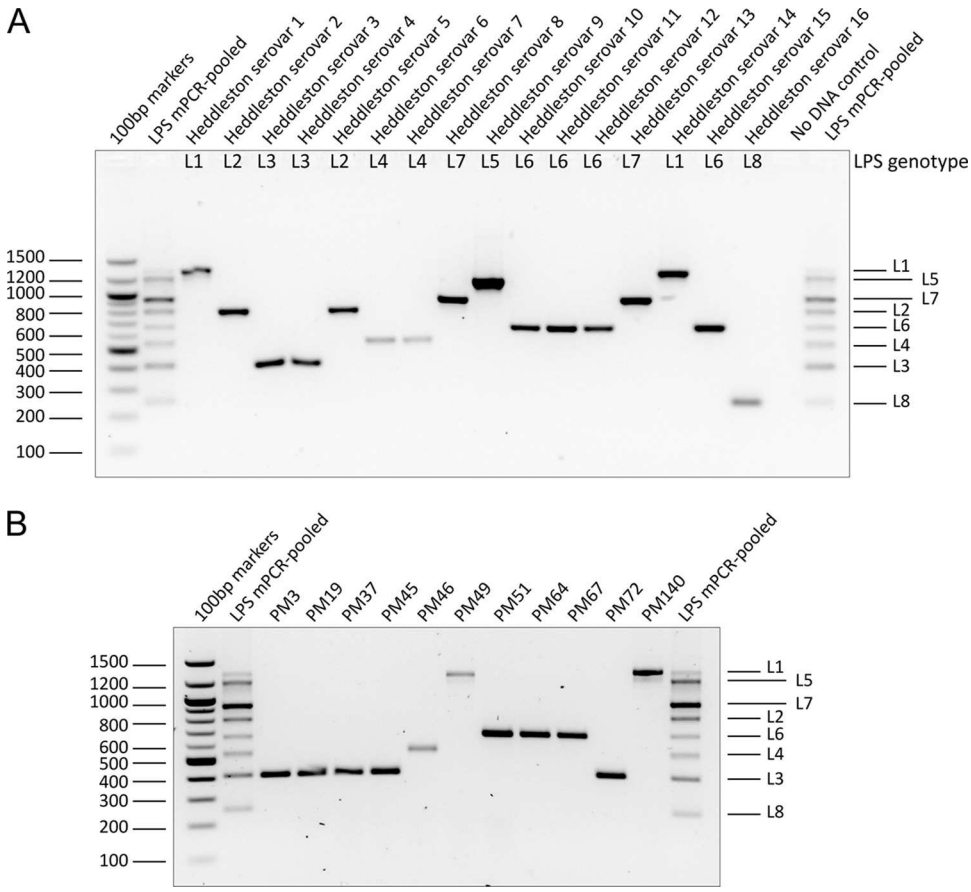

FIG 2 Gel electrophoresis separation of LPS-mPCRv1 products amplified using template of lysed colonies from each of the Heddleston type strains (H1 to H16) (A) or template derived from lysed colonies from selected *P. multocida* field isolates (B). A 100-bp ladder marker was loaded in lane 1 of each gel. Pooled amplicons, generated from separate PCRs using each of the LPS genotype type strains as the template, are shown on either side of each gel for comparison. Each LPS genotype amplicon is shown on the right, and the products are labeled L1 to L8.

peated was not in agreement with the initial serovar typing result (Table 1). Of the 58 strains, 32 gave an unambiguous Heddleston serovar result (55%) (Table 1), 17 gave an ambiguous result of two or more possible serovars, and 9 were nontypeable (no precipitin line observed). The most common serovars identified unambiguously were serovars 1 and 3.

All strains were then LPS genotyped using the LPS-mPCRv1. An example of an LPS-mPCRv1 result is shown in Fig. 2B. Of the 58 strains tested, the LPS-mPCRv1 gave an unambiguous LPS genotype for 48 of the strains (Table 3), but no PCR product could be generated for 10 strains (nontypeable). A comparison of the LPS-mPCRv1 genotype and Heddleston serovar designations of each strain (Table 3) revealed that there was complete agreement between the typing methods for only 16 of the 58 strains. Partial agreement was obtained for a further 11 strains (where serotyping gave an ambiguous result and the LPS-mPCRv1 result was in agreement with one of the serotyping results). For 15 strains, the LPS-mPCRv1 gave a locus designation that was incompatible with the serovar designation (Table 3). These data indicate that there were clear discrepancies between Heddleston serotyping and LPS genotype, as determined by the LPS-mPCRv1. Importantly, the LPS-mPCRv1 consistently assigned strains to a single genotype, whereas serotyping frequently assigned strains to multiple Heddleston serovars.

In order to determine whether Heddleston serotyping or the LPS-mPCRv1 gave a more accurate representation of the LPS produced by each strain, the LPS composition from a set of selected strains was analyzed by mass spectrometry. The strains examined included five strains from the agreement group, all strains from the nonagreement group, 9 of the 11 strains from the partial agreement group (where Heddleston serotyping gave ambiguous results), and all strains that remained nontypeable in one or both typing systems. As we have reported previously, these analyses identified a number of strains belonging to the L3 genotype, which expressed multiple LPS glycoforms (10). When interpreting the typing results, the LPS glycoform that contained the largest number of sugars/residues was deemed to be representative of the most extended LPS structure produced by the strain, and any additional glycoforms observed that contained fewer sugars (but common to the largest glycoform) were considered truncated variants.

As expected, the five strains analyzed within the agreement group (Table 3) gave LPS-mPCRv1 designations that were in agreement with both LPS composition and with serotyping. The LPS composition of the nine strains examined within the partial agreement group correlated always with the genotype designation but correlated with one of the multiple Heddleston serovar designations in only 6 of the 9 strains (Table 3). The LPS composition of two strains in the agreement group (PM120 and PM1193) and two

TABLE 3 Comparison of strain typing of 58 Australian field isolates by Heddleston serotyping and LPS-mPCR

Parameter and strain no.	Serotyping[a,b]	LPS-mPCRv1	Final LPS-mPCR (Heddleston serovars within each genotype)[c]	LPS composition[d]
LPS-mPCR and serotyping in agreement				
PM36	H14 (H14)	L1	L1 (H1, H14)	No LPS analysis
PM37	H3 (H3)	L3	L3 (H3, H4)	No LPS analysis
PM45	NT (H3, H4)	L3	L3 (H3, H4)	No LPS analysis
PM46	**H6** (**H6**)	L4	**L4** (H6, H7)	3HexNAc, 2Hex (**H6**)
PM120	H12 (H12)	L6	**L6** (H10, H11, H12, H15)	2Hex, 1Hep
PM1165	H1	L1	L1 (H1, H14)	No LPS analysis
PM1193	**H3**	L3	**L3** (H3, H4)	4Hex, Hep
PM1300	H4	L3	L3 (H3, H4)	No LPS analysis
PM1304	H1	L1	L1 (H1, H14)	No LPS analysis
PM1315	H1	L1	L1 (H1, H14)	No LPS analysis
PM1316	H4	L3	L3 (H3, H4)	No LPS analysis
PM1317	H3	L3	L3 (H3, H4)	No LPS analysis
PM1398	**H1**	L1	**L1** (H1, H14)	2PCho, 2Hex, Hep (**H1**)
PM1417	H4	L3	L3 (H3, H4)	No LPS analysis
PM1455	**H1**	L1	**L1** (H1, H14)	2PCho, 2Hex, Hep (**H1**)
PM1458	H14	L1	L1 (H1, H14)	No LPS analysis
LPS-mPCR and serotyping in partial agreement				
PM3	H15 (H4, H10, H15)	L3	**L3** (H3, H4)	1Hex, Hep/2Hex, Hep
PM19	H13 (H3)	L3	**L3** (H3, H4)	No LPS analysis
PM49	NT (**H1**, H15)	L1	**L1** (H1, H14)	2PCho, 2Hex, Hep (**H1**)
PM51	H9 (H4, H12)	L6	**L6** (H10, H11, H12, H15)	2Hex, Hep
PM67	H3 (H3, **H12**)	L6	**L6** (H10, H11, H12, H15)	1HexNAc, 3Hex, Hep (**H12**)
PM72	NT (**H3**, H14)	L3	**L3** (H3, H4)	3Hex, Hep (**H4**)/4Hex, Hep/1HexNAc, 4Hex, Hep (**H3**)/2HexNAc, 4Hex, Hep
PM140	NT (H13, H**14**, H15)	L1	**L1** (H1, H14)	1 Hex, Hep (**H14**)
PM878	H1, H4	L1	**L1** (H1, H14)	No LPS analysis
PM1124	**H1**, H4, H12	L1	**L1** (H1, H14)	2PCho, 2Hex, Hep (**H1**)
PM1132	H1, H3, H4, H10, H14	L6	**L6** (H10, H11, H12, H15)	3Hex, Hep/HexNAc, 3Hex, Hep (**H12**)
PM1396	**H1**, H3	L1	**L1** (H1, H14)	2PCho, 2Hex, Hep (**H1**)
LPS-mPCR and serotyping not in agreement				
PM8	H10	L3	**L3** (H3, H4)	2Hex, Hep/Hex, Hep/Hep
PM64	NT (H3)	L6	**L6** (H10, H11, H12, H15)	1HexNAc, 3Hex, Hep (**H12**)
PM147	H7 (H7)	L3	**L3** (H3, H4)	2Hex, Hep
PM993	H8	L6	**L6** (H10, H11, H12, H15)	No outer core (**H10**)
PM995	H3	L6	**L6** (H10, H11, H12, H15)	1HexNAc, 3Hex, Hep (**H12**)
PM1098	H15	L3	**L3** (H3, H4)	3Hex, Hep (**H4**)
PM1099	H10	L3	**L3** (H3, H4)	3Hex, Hep (**H4**)/4Hex, Hep/1HexNAc, 4Hex, Hep (**H3**)
PM1103	H10	L3	**L3** (H3, H4)	4Hex, Hep/1HexNAc, 4Hex, Hep (**H3**)
PM1128	H10	L3	**L3** (H3, H4)	1HexNAc, 4Hex, Hep (**H3**)
PM1205	H1	L3	**L3** (H3, H4)	3Hex, Hep (**H4**)
PM1320	H10, H13, H14	L3	**L3** (H3, H4)	Hex, Hep/2Hex, Hep (**H4**)
PM1441	H2	L3	**L3** (H3, H4)	3Hex, Hep (**H4**)/4Hex, Hep/1HexNAc, 4Hex, Hep (**H3**)
PM1456	H14	L4	**L4** (H6, H7)	1Hex (**H7**)
PM1470	H1	L3	**L3** (H3, H4)	1HexNAc, 4Hex, Hep (**H3**)/2HexNAc, 4Hex, Hep
PM1474	H12	L3	**L3** (H3, H4)	3Hex, Hep (**H4**), 4Hex, Hep
Nontypeable using Heddleston serotyping				
PM1113	NT	L4	**L4** (H6, H7)	1Hex (**H7**)
PM1268	NT	L3	**L3** (H3, H4)	2Hex, Hep, 3Hex, Hep (**H4**)
PM1405	NT	L1	**L1** (H1, H14)	2PCho, 2Hex, Hep (**H1**)
PM1435	NT	L1	**L1** (H1, H14)	2PCho, 2Hex, Hep (**H1**)
PM1439	NT	L3	**L3** (H3, H4)	3Hex, Hep (**H4**)
PM1457	NT	L4	**L4** (H6, H7)	3HexNAc, 1Hex/3HexNAc, 2Hex (**H6**)

(Continued on following page)

TABLE 3 (Continued)

Parameter and strain no.	Serotyping[a,b]	LPS-mPCRv1	Final LPS-mPCR (Heddleston serovars within each genotype)[c]	LPS composition[d]
Nontypeable using initial LPS-mPCRv1[e]				
PM1	**H3** (**H3, H4**)	NT	**L3** (H3, H4)	3Hex, Hep (**H4**)/4Hex, Hep/1HexNAc, 4Hex, Hep (**H3**)
PM18	NT (H3)	NT	**L3** (H3, H4)	2Hex, Hep
PM48	**H3** (**H3**, H4)	NT	**L3** (H3, H4)	3Hex, Hep (**H4**)/4Hex, Hep/1HexNAc, 4Hex, Hep (**H3**)
PM135	H8, **H13** (**H13**)	NT	NT, L7 sequence[f]	1HexNAc, 2Hex, Hep (**H13**)
PM1075	H16	NT	**L3** (H3, H4)	No outer core
PM1120	NT	NT	**L3** (H3, H4)	No outer core
PM1153	H1, **H3**, H7	NT	**L3** (H3, H4)	3Hex, Hep (**H4**)/4Hex, Hep/1HexNAc, 4Hex, Hep (**H3**)/2HexNAc, 4Hex, Hep
PM1258	NT	NT	**L3** (H3, H4)	No outer core
PM1369	H1	NT	**L3** (H3, H4)	3Hex, Hep (**H4**)/4Hex, Hep
PM1434	NT	NT	**L3** (H3, H4)	1HexNAc, 4Hex, Hep (**H3**)

[a] The format in which multiple numbers are separated by a comma indicates that a precipitin line was observed with more than one type serum. Results in parentheses indicate that two distinct and separate serotyping assays were performed: the result in parentheses is the first result with this isolate. NT, not able to be typed by this method (i.e., no precipitin line was observed using serotyping, or no amplicon was produced by mPCR).
[b] A Heddleston serovar shown in boldface correlates with both LPS composition and LPS genotype.
[c] An LPS genotype shown in boldface correlates with LPS composition.
[d] Outer core LPS sugar composition as predicted by MS/MS compositional analysis. The Heddleston serovar within the designated genotype that matches the LPS outer core composition is shown in boldface and in parentheses. Those compositions without a Heddleston serovar designation shown in parentheses do not precisely match any of the Heddleston type strain LPS structures. Multiple LPS glycoforms when detected have been separated by "/." Hex, hexose (glucose or galactose); HexNAc, *N*-acetyl hexosamine [*N*-acetylglucosamine, *N*-acetyl galactosamine, or (1*S*)-2-acetamido-2-deoxy-D-galactose]; Hep, heptose; PCho, phosphocholine. Nonstoichiometric phosphoethanolamine additions to the outer core have not been determined.
[e] Sequencing of the LPS outer core biosynthesis locus revealed significant nucleotide differences where the LPS-mPCRv1 primers were located.
[f] Sequencing of the PM135 LPS outer core biosynthesis locus revealed a large deletion in the region where the L7 LPS-mPCR primers were located.

in the partial agreement group (PM3 and PM51) did not correlate precisely with the serovar-specific LPS structures within the assigned LPS genotype. However, in each case the LPS composition did correlate with a truncated version of the LPS structure specific to the assigned LPS genotype (10, 11).

For the strains where serotyping and PCR were in nonagreement, the LPS compositional analysis was always compatible with the LPS genotype assigned using the LPS-mPCRv1. In contrast, the Heddleston serotyping designation did not correlate with the predicted LPS composition for any strain in this group, clearly showing that Heddleston serotyping is unreliable for prediction of LPS composition. Importantly, the LPS-mPCRv1 gave unambiguous LPS genotyping results (producing only a single amplicon) that always correlated with LPS composition (Table 3).

Redesign of the LPS-mPCR to increase coverage. The LPS-mPCRv1 gave an unambiguous LPS genotype for 48 of 58 field strains (Table 3) but failed to amplify a product from 10 isolates. PCR and nucleotide sequence analyses of the LPS outer core biosynthesis locus in each strain revealed that nine of the untypeable strains contained an L3 LPS locus but with significant nucleotide differences within *gatG*, where the L3 primers were located. The tenth strain, PM135, contained an L7 LPS locus but with a major deletion of 2,210 nucleotides (14) that included *ppgB*, where the L7 primers for the LPS-mPCRv1 were located.

To improve the strain coverage of the LPS-mPCRv1, the nucleotide sequence of the L3 type strain (P1059) was used to design new primers in a region within *gatF* that shared 100% identity with the nine L3 strains that were nontypeable using the LPS-mPCRv1 (Fig. 1). Substitution of the *gatF* L3 primers in the multiplex PCR resulted in amplification of all locus-specific products from the appropriate templates, except for locus 6, where only weak amplification of the product was observed for some strains (data not shown). To improve the L6 amplicon yield, the L6 primers were slightly modified. This final typing PCR was designated the LPS-mPCR. The full set of the final LPS-mPCR primers and amplicon sizes is shown in Table 2.

The final LPS-mPCR was used to genotype the 16 Heddleston type strains and was able to accurately differentiate all of these strains into the eight LPS genotypes (Fig. 3). The LPS-mPCR was then used to genotype the 58 field isolates. This final LPS-mPCR gave a single reproducible amplification product for 57 of 58 strains (strain PM135 was nontypeable), including the nine L3 strains that were nontypeable using the LPS-mPCRv1 (data not shown). All positive LPS-mPCR results were compatible with the LPS compositions that were determined (Table 3).

DISCUSSION

In this study, a multiplex PCR was designed to differentiate *P. multocida* strains on the basis of the LPS genotype. The final LPS-mPCR was able to unambiguously type the 16 Heddleston type strains and 57 of the 58 field isolates. However, strain PM135 remained untypeable, due to a large deletion in the region where the L7 LPS-mPCR primers were located (14). The failure of the LPS-mPCR due to large deletions within the LPS loci where primers are located cannot be avoided. However, our previous analyses of the Heddleston type strains and field isolates containing LPS gene mutations indicate that such large deletions are rare; most mutations within the *P. multocida* LPS outer core biosynthesis loci involve single point mutations (9–11) and would be unlikely to compromise PCR amplification.

During the testing of the LPS-mPCR for differentiation of *P. multocida* isolates, mass spectrometry analysis was used as the "gold standard" to assess the composition of the LPS produced by individual strains. This method of LPS analysis identifies sugar

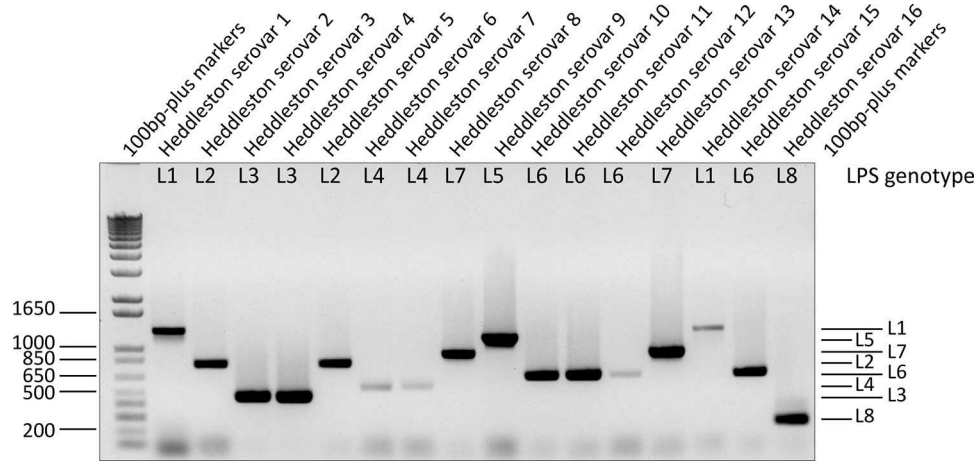

FIG 3 Gel electrophoresis separation of products generated using the final LPS-mPCR with the template derived from lysed colonies from each of the Heddleston type strains (H1 to H16). The relative size of each LPS genotype PCR amplicon remains unchanged from that of the LPS-mPCRv1, with the exception of the L4 amplicon, which is now 474 bp.

type and overall sugar content, which are then compared to the LPS compositions of the fully elucidated Heddleston LPS structures to predict the LPS glycoforms expressed by any particular strain.

Our previous analyses of the LPS genetics and structure in *P. multocida* showed that strains with the same serological designation can produce structurally distinct LPS (9–16). These analyses also showed that the LPS structures produced by many *P. multocida* strains are truncated variants of the full-length or "parent" LPS structure and that there is significantly more LPS diversity in the field than is represented by the current 16 type strains (9–12, 14). Interestingly, many *P. multocida* field isolates belonging to the L3, L4, and L6 genotypes produce a wide range of LPS glycoforms, including some which have a significantly truncated LPS outer core or no outer core at all. Many of these strains were isolated from poultry exhibiting clear signs of fowl cholera (data not shown), indicating that strains belonging to these genotypes do not require a full-length LPS molecule to cause disease. Our studies on the L1/serovar 1 strain VP161 have shown that any shortening of the LPS outer core structure in this strain results in attenuation of virulence in chickens (24), but it is possible that isolates expressing truncated LPS may be able to persist in some host niches but are not as virulent as parent strains expressing full-length LPS. Indeed, infection of chickens with a VP161 *hptE* LPS mutant (which produces a highly truncated outer core) showed that this mutant could persist at the site of muscle injection but could not be recovered from the blood, thus supporting this hypothesis (20). Importantly, many of the structures expressed by L3 and L6 genotype strains mimic host glycosphingolipids, and this may allow the bacteria to avoid recognition by the components of the innate immune system (10, 11).

Our comparison of LPS composition, Heddleston serotyping, and LPS-mPCR typing showed clearly that the Heddleston serovar designation frequently failed to correlate with the composition of the LPS produced by each strain. In contrast, the LPS-mPCR assay, specific for the identification of the LPS outer core biosynthesis loci, always correlated with LPS composition. Knowledge of the LPS genotype allows for the identification of the LPS type and possible range of LPS structures that strains can produce. However, the LPS-mPCR cannot predict the precise LPS structures produced by individual strains as random mutations within the LPS locus often lead to changes in LPS structure. We propose that the high diversity in numbers and types of LPS molecules produced by *P. multocida* strains indicates that a typing system exactly predictive of LPS structure is not feasible. If knowledge of the precise LPS structure is important for diagnosis and the control of outbreaks, then following mPCR analysis, further experiments would need to be conducted, such as carbohydrate-specific silver staining of cell lysates to assess the relative size of the LPS produced. Alternatively, for more detailed analysis, nucleotide sequencing of the LPS biosynthesis locus to identify the specific LPS gene mutations combined with carbohydrate mass spectrometry could be employed. However, these detailed analyses are beyond the scope of diagnostic laboratories.

The LPS-mPCR developed here is a highly reproducible typing system for differentiating *P. multocida* strains. We have also shown that many field isolates produce multiple LPS glycoforms simultaneously; these naturally occurring "multivalent" strains could be excellent candidates for killed-cell vaccines as they may show broader protective efficacy than strains expressing single LPS molecules. We are currently assessing this possibility.

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