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FINAL REPORT

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PROJECT LEADER: **Dr. John Boyce**

Sub-Project Title: Rapid multiplex PCR assay for differentiating *Pasteurella multocida* serovars

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Rapid multiplex PCR assay for differentiating Pasteurella multocida serovars Sub-Project No. 1.2.1

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In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

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Plain English Compendium Summary

Sub-Project Title:	**Rapid multiplex PCR assay for differentiating *Pasteurella multocida* serovars**
Sub-Project No.:	**1.2.1**
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Sub-Project Overview	We developed a new typing system to differentiate *Pasteurella multocida* strains. Our typing system accurately and unambiguously typed 57 of 59 *P. multocida* field isolates. We also showed that the protective efficacy of killed vaccines (bacterins), which are widely used to protect against fowl cholera, is exquisitely sensitive to the structure of expressed surface molecules. Conversely, live vaccines can give protection against strains expressing different surface structures.
Background	*Pasteurella multocida* is the causative agent of fowl cholera, an important disease of poultry. *P. multocida* strains have classically been differentiated serologically into 16 Heddleston serovars based on lipopolysaccharide (LPS) antigens. Heddleston typing is widely used as it is believed that protective immunity elicited by bacterin vaccines is LPS-type specific. Neither the accuracy of Heddleston typing, nor the specificity of bacterin vaccines for LPS structure, has ever been objectively assessed.
Research	Using our knowledge of LPS biosynthesis in *P. multocida* we developed a multiplex PCR (mPCR) able to differentiate strains based on genetics of LPS biosynthesis. We compared the accuracy of our mPCR with classical Heddleston serology using LPS structural data as the gold standard. The new mPCR correctly typed 57 of 59 isolates; Heddleston serology correctly typed only 20 of the 59 strains. Our mPCR is a significant improvement on Heddleston serology. We also assessed the ability of both live and killed vaccines to protect against fowl cholera caused by virulent strains expressing different surface LPS molecules. The efficacy of bacterin vaccines was exquisitely sensitive to LPS structure; these bacterin vaccines only provided protection against strains expressing identical LPS. Conversely, live vaccines gave solid protection against selected strains that expressed different LPS structures.
Implications	The mPCR typing assay we have developed should replace current Heddleston serology. As bacterin vaccines give no cross-protection against *P. multocida* strains expressing different surface LPS structures we predict that outbreaks in bacterin-vaccinated flocks likely arise following introduction of new strains with different LPS, or following mutation of resident strains resulting in LPS changes. However, live vaccines can provide good protection against strains expressing different LPS.
Publications	1. **Harper et al.,** 2014. Structural analysis of lipopolysaccharide produced by Heddleston serovars 10, 11, 12 and 15 and the identification of a new *Pasteurella multocida* LPS outer core biosynthesis locus, L6. Glycobiology **In Press**. 2. **Harper et al**., 2013. Structure and biosynthetic locus of the lipopolysaccharide produced by *Pasteurella multocida* serovars 8 and 13 and the identification of a novel phospho-glycero moiety. Glycobiology **23:**286-294.

Final Report

Introduction

Fowl cholera is an important disease of both domestic and wild birds. The causative agent is the Gram-negative bacterial pathogen *Pasteurella multocida*. *P. multocida* infections can result in acute disease, which generally causes death of the birds within 24-48 h, or chronic disease, where birds show a range of low grade signs and reduced egg production. With the expanding free range and organic broiler and layer sectors there is an increasing problem with fowl cholera, both in Australia and worldwide. In recent studies in Denmark, *P. multocida* was identified as one of the major causes of mortality in commercial free range organic layers (3, 15) and large outbreaks have also been observed in free range flocks in Australia (16). However, outbreaks are not limited to organic and free range birds as both chronic and acute outbreaks occur in caged layer hens and valuable breeder flocks. Treatment of fowl cholera relies upon the use of antibiotics such as oxytetracycline, but even with rapid treatment, very large numbers of mortalities may be recorded.

P. multocida strains have classically been differentiated into serogroups (A, B, D, E and F) based on capsule antigens (2) and further differentiated into 16 serovars (1-16) based on lipopolysaccharide (LPS) antigens using the Heddleston typing scheme (12). LPS forms the outer leaflet of the outer membrane of Gram-negative bacteria and it is an immunodominant and highly variable carbohydrate antigen that plays a clear role in the ability of strains to cause acute disease (4, 6).

At the beginning of this project there were no commercially available vaccines in Australia and the poultry industry relied on autogenous bacterins developed against local isolates. While an Australian live attenuated vaccine is now available (Vaxsafe® PM Vaccine; Bioproperties), autogenous bacterins continue to be used by many poultry producers. It is widely believed that these killed whole cell vaccines elicit strong protective immunity against strains with related LPS structures. Therefore, Heddleston serotyping has been used to predict the LPS type of both outbreak and vaccine strains in order to determine whether the currently used bacterin will provide protection against newly identified outbreak strains. Implicit in this process is the expectation that Heddleston serology gives an accurate representation of *P. multocida* LPS structure and that the protection elicited by killed vaccines is LPS type-specific. However, these points have never been objectively tested as the precise LPS structures expressed by different strains have not been known.

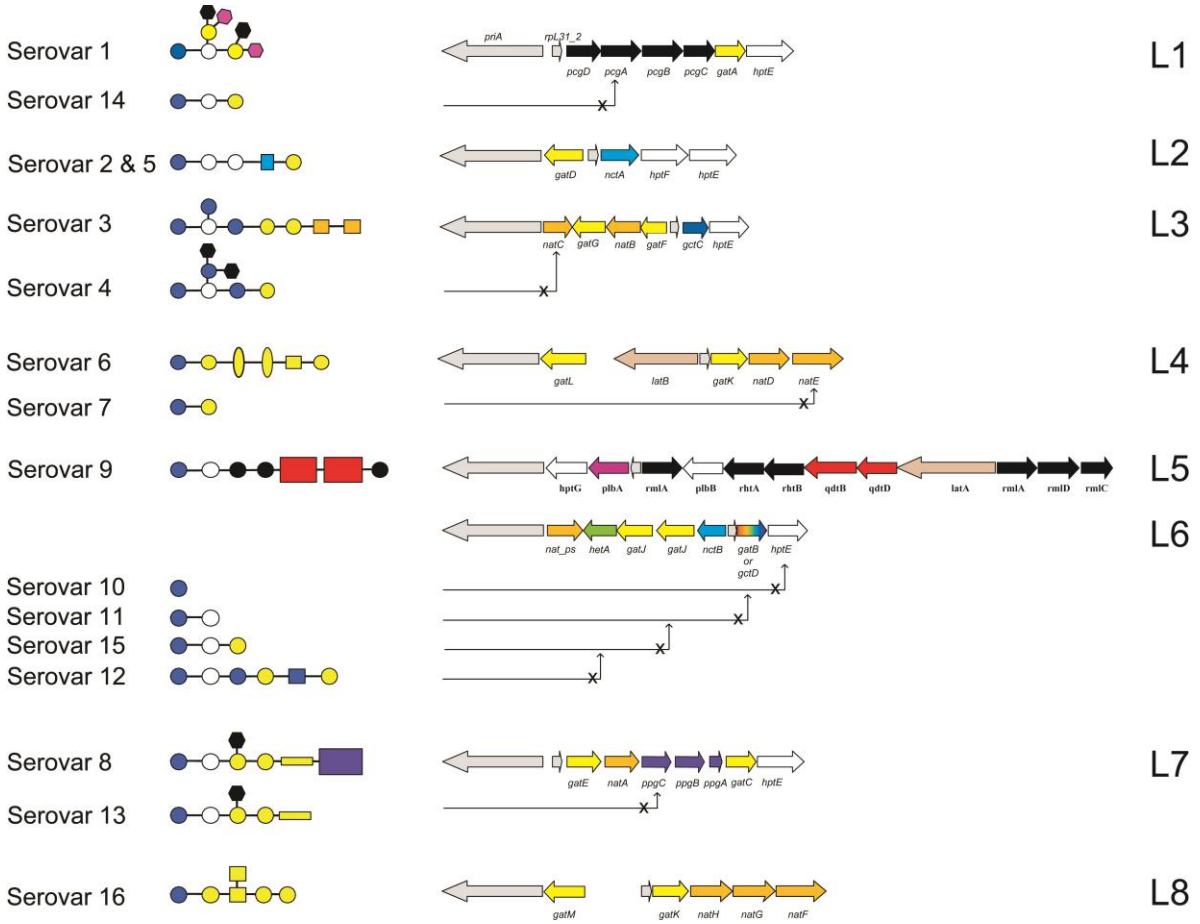
Heddleston serotyping is currently commercially performed in only one Australian laboratory (Blackall and Turni, Agri-Science Queensland). It relies on the availability of antisera “specific” for each Heddleston serovar type strain and is labour intensive. Moreover, this

typing laboratory, and others throughout the world, has lost confidence in the accuracy of Heddleston typing and believe it to be unreliable. Therefore, in this project we aimed to assess the accuracy and reproducibility of Heddleston serotyping, develop a replacement typing system based on the genetics of LPS biosynthesis and assess the ability of killed and live vaccines to elicit protection against strains with identical and related LPS structures.

Prior to the commencement of the CRC project 1.2.1, our group (together with Dr. Andrew Cox at the National Research Council, Canada) determined the LPS structures expressed by each of the 16 Heddleston type strains and identified the genes required for LPS biosynthesis in each strain (7-11). This work showed that while the 16 Heddleston type strains do indeed express structurally distinct LPS molecules, some strains expressed highly related molecules. Furthermore, we identified that only eight distinct genetic loci encode the biosynthetic genes responsible for synthesis of all 16 known LPS structures (Fig. 1). This is possible because eight Heddleston type strains express “parent” LPS structures and eight express truncated LPS structures; the result of mutations within the LPS biosynthetic loci.

Importantly, this detailed understanding of *P. multocida* LPS structure and genetics gave us a unique opportunity to assess the accuracy of Heddleston serology and identify LPS biosynthesis genes unique to each LPS type that could be targeted in an LPS-specific diagnostic multiplex PCR (mPCR) assay. Indeed, in preliminary work we had developed a working multiplex PCR (LPS-mPCRv1) that could differentiate the 16 Heddleston reference strains into the eight LPS genotypes (termed L1 through to L8). In this project we compared the accuracy and reproducibility of our LPS-mPCR with Heddleston serotyping, further refined the PCR to expand its strain coverage, and determined that this test is an accurate and reproducible diagnostic tool. This LPS-mPCR will be available, through Agri-Science Queensland, from 2014 onwards. Finally, we assessed the efficacy of live and killed vaccines for stimulating protection against strains expressing identical and related LPS structures.

Figure 1. Symbolic representation of the structure of the LPS outer core region (with conserved inner core glucose shown far left) and genetic organization of the LPS outer core loci (L1 to L8) from each of the 16 Heddleston serovar type strains. Right-angled arrows with crosses indicate key genes mutated in particular strains. Serovars 2 and 5 share the same LPS outer core loci but differ by one residue in the inner core.



○ Heptose, ● Glucose, ● Galactose, ● Rhamnose, ■ N-acetylgalactosamine ■ N-acetylglucosamine, ⬮ O-acetylated GalNAc, ▬ (1S)-2-acetamido-2-deoxy-D-galactose, ⬣ Phosphocholine, ⬣ Phosphoethanolamine, ■ 3-acetamido-3,6-dideoxy-a-D-glucose ■ 1-((4-aminobutyl)amino)-3-hydroxy-1-oxopropan-2-yl hydrogen phosphate.

Objectives

The specific aims of the project were

AIM 1. To test the robustness and validity of our prototype LPS-mPCR on *P. multocida* field isolates and develop the PCR into a diagnostic test capable of accurately and reliably typing *P. multocida* strains.

AIM 2. To determine whether the classification of strains based on LPS-specific genotypes (as generated by the LPS-mPCR) is predictive of the protective efficacy of killed and/or live attenuated vaccines.

In Aim 1 we tested the robustness and validity of our prototype LPS-mPCR on a range of *P. multocida* field isolates and compared its accuracy and reproducibility with Heddleston serotyping. Mass spectrometry LPS compositional analysis, followed by structural prediction using the known Heddleston LPS structures as reference, were used as the gold standard against which the accuracy of the LPS-mPCR and Heddleston serotyping results were assessed. These data clearly showed that the mPCR was superior to Heddleston serotyping both in reproducibility and accuracy. However, our version 1 LPS-mPCR failed to type 13% of the 58 strains analysed. We then refined the LPS-mPCR to increase its strain coverage; the final mPCR (version 5) was able to accurately type 57 of 59 strains. We then developed a full set of operating procedures for this final mPCR and transferred the assay to the current serotyping laboratory at Agri-Science Queensland under the direction of Dr. Conny Turni. This LPS-mPCR can now be used to accurately classify *P. multocida* strains based on the genetics of LPS biosynthesis and without any need for serology.

In Aim 2 we tested the ability of whole-cell killed vaccines and live vaccines to stimulate protective immunity against virulent challenge strains expressing identical or different LPS structures. For this analysis we focused on strains of direct relevance to the poultry industry, namely strains belonging to Heddleston serovars 1, 14, 3 and 4. Initially we showed that protective immunity elicited by bacterin vaccines is exquisitely sensitive to LPS structure, indicating that birds vaccinated with bacterins are not protected from infections with strains expressing even slightly different LPS. We then showed that protective immunity conferred by live strains is not dependent on exact LPS structure. These results clearly show that the use of killed bacterins is unlikely to give long-term full protection against fowl cholera.

Methodology

Strains used

All *P. multocida* strains were grown in Heart infusion liquid broth at 37°C with shaking or on solid medium containing 1.5% agar. When required the media were supplemented with the following antibiotics; streptomycin (50 µg/ml), kanamycin (25 µg/ml), spectinomycin (50 µg/ml) or tetracycline (2.5 µg/ml). The strains VP161 (A:1; LPS genotype L1) and P1059 (A:3; L3) were used for mutagenesis experiments. The field isolates used in the study are described in Chapter 1,Table 1.

Molecular biology techniques

Genomic DNA was purified from *P. multocida* strains using the RBC genomic DNA purification kit (RBC, Taiwan). Restriction enzymes were purchased from NEB (Beverly, MA) or Roche diagnostics (Mannheim, Germany) and used according to the manufacturer's instructions. Purification of plasmid and PCR-amplified DNA was carried out using Qiagen spin columns (Qiagen, GmbH, Germany). Polymerase Chain Reaction (PCR) was carried out using Taq DNA polymerase on a Eppendorf thermal cycler using cycling conditions as specified in Appendix A.

Single-crossover LPS biosynthesis mutants in strain VP161 were generated as previously described (1, 5). Directed Sigma Targetron® mutants were also constructed as previously described (14)

LPS sugar compositional analyses

The sugar composition of the LPS from selected strains was assess by mass spectrometry as previously described (5).

Vaccination trials

All animal experiments were approved by the CSIRO animal ethics committee. Groups of commercially obtained 10-14 week old Hy-Line Brown chickens were allowed to acclimatize in the animal facility for 7 days before the first vaccination. During this time birds were tagged, withdrawn from antibiotic containing feed and a sample of birds (3) from each group bled from the wing vein to give a pre-vaccination control serum sample. Birds were vaccinated with either heat-killed bacteria in aluminium hydroxide adjuvant or live attenuated strains. Birds were given a second booster vaccination between 10 and 14 days following

the first vaccination then challenged two weeks later. The doses and strains used for the challenge are as described in the text. Birds were monitored closely for signs of disease and euthanized when they showed late-stage fowl cholera signs (listlessness, depression, head hanging, ruffled feathers, increased respiratory rate, diarrhoea).

Chapter 1: Development of a multiplex PCR for the accurate differentiation of *P. multocida* strains

Comparison of the reproducibility and accuracy of the LPS mPCR (v1) with Heddleston serotyping

In prior work we determined the LPS structures expressed by each of the 16 Heddleston type strains and identified the genes required for LPS assembly in each strain. This work identified that although each of the Heddleston type strains express different LPS molecules, some strains express highly related molecules. Indeed, only eight distinct genetic loci (termed L1 through to L8) encode all 16 Heddleston LPS structures (Fig. 1). Eight Heddleston type strains express “parent” LPS structures and eight express truncated LPS structures; the result of mutations within the LPS biosynthetic loci. This detailed understanding of the genetics of LPS biosynthesis allowed us to identify unique genes that could be targeted in an LPS-specific diagnostic multiplex PCR assay. This knowledge allowed the development of a working multiplex PCR (LPS-mPCRv1) that could classify the 16 Heddleston serovars into the eight LPS genotypes

To test the reproducibility and accuracy of our LPS-mPCRv1 we initially obtained 58 *P. multocida* field isolates for typing both with our LPS-mPCRv1 and classical Heddleston serology (Table 1). The 58 field isolates were sourced from the extensive collection of Dr. Pat Blackall and Dr. Conny Turni and included strains obtained from a range of Australian poultry farms between 1979 and 2011. In total, 32 of the strains were recorded as being isolated from chickens, seven from turkeys, four from ducks, one from an Emu and the host species was not recorded for 11 of the isolates (Table 1). A single bovine and a single porcine isolate were also included.

All strains were differentiated by classical Heddleston serotyping at the Agri-Science Queensland. Of the 58 strains, 33 gave an unambiguous serovar result (57%; Table 1), 16 gave an ambiguous result of two or more possible serovars and 9 were non-typeable. The most common serovars identified unambiguously were serovar 1 (eight strains) and serovar 3 (7 strains).

All strains were also differentiated using the LPS-mPCRv1. A sample LPS-mPCRv1 result is shown in Fig. 2. Of the 58 strains tested, the LPS-mPCRv1 gave an unambiguous LPS genotype for 48 of the strains (83%; Table 2). We then compared the LPS-mPCRv1 genotypes and Heddleston serovar designations of each strain (Table 2 and Fig. 3). Interestingly, there was only complete agreement between the methods for 16 of the 57

strains (agreement group; Table 2) and partial agreement for a further 11 strains (Table 2). For 15 strains, the LPS-mPCRv1 gave a locus designation that was incompatible with the serovar designation (non-agreement; Table 2). These data indicate that there are clear discrepancies between the LPS serology and LPS genotype as determined by the LPS-mPCRv1. Importantly, the LPS-mPCRv1 consistently assigned strains to a single locus whereas serotyping frequently assigned strains to multiple serovars or to a non-typeable status.

TABLE 1. Information on the Heddleston serovar, isolation date, host species and MLST sequence type for 58 Australian *P. multocida* field isolates.

Strain Nº	Heddleston serovar (original serotyping results on historic strains)	Isolation date	Animal host	MLST Sequence Type [a]
1	3 (3,4)	1993	Turkey	1
3	15 (4, 10, 15)	1993	Turkey	2
8	8	1993	Turkey	2
18	NT [b] (3)	1986	Chicken	5
19	NT [b] (3)	1986	Chicken	8
36	(14)	1985	unknown	ND [c]
37	3 (3)	1988	Chicken	9
45	NT [b] (3,4)	1986	Chicken	8
46	6 (6)	1992	Chicken	10
48	3 (3,4)	1983	Chicken	7
49	NT [b] (1,15)	1984	Chicken	11
51	9 (4, 12)	1984	Chicken	12
64	NT [b] (3)	1979	Chicken	16
67	3 (3,12)	1969	Chicken	17
72	NT [b] (3,14)	1977	Chicken	19
120	(12)	unknown	Chicken	12
135	8, 13 (13)	unknown	Turkey	32
140	NT [b] (13,14,15)	1994	Chicken	34
146	(7)	unknown	unknown	ND
878	1,4	2001	Chicken	58
993	8	2002	Duck	12
995	3	2002	Chicken	29
1074	16	2004	Chicken	ND
1098	15	2004	unknown	ND
1099	10	2004	unknown	8
1103	10	2004	unknown	ND
1113	NT [b]	2004	Avian	ND
1120	NT [b]	2005	Chicken	ND
1124	1,4,12	2005	unknown	ND
1128	10	2005	Bovine	79

1132	1,3,4,10,14	2005	Pig	185
1153	1,3,7	2005	Avian	ND
1165	1	2006	Duck	ND
1193	3	2006	Duck	ND
1205	1	2007	Emu	9
1258	NT [b]	2010	Chicken	25
1268	NT [b]	2010	Chicken	142
1300	4	2009	Turkey	ND
1304	1	2009	Avian	ND
1315	1	2009	Chicken	ND
1316	4	2009	unknown	ND
1317	3	2009	unknown	ND
1320	10,13,14	2010	Chicken	ND
1369	1	2010	Chicken	5
1396	1,3	2010	unknown	30
1398	1	2010	Chicken	60
1405	NT [b]	2010	Chicken	30
1417	4	2010	Chicken	20
1434	NT [b]	2011	Chicken	7
1435	NT [b]	2011	Chicken	30
1439	NT [b]	2011	Chicken	20
1441	2	2011	Turkey	23
1455	1	2011	Chicken	30
1456	14	2011	Chicken	155
1457	NT [b]	2011	Chicken	ND
1458	14	2011	Chicken	ND
1470	1	2011	Turkey	ND
1474	12	2011	Duck	ND

[a] MLST sequence type as determined by Blackall and Turni (pers. comm.)

[b] NT = Non-typeable by serology, [c] ND = Not done.

In order to determine whether Heddleston serotyping or the LPS-mPCRv1 gave a more accurate representation of expressed LPS structure, we analysed the composition of the LPS from a set of selected strains by mass spectrometry. The strains examined included one strain from the agreement group, all strains from the non-agreement group, 9 of the 11 strains from the partial agreement group (where Heddleston serology gave multiple non-identical serovar predictions), and all strains that remained un-typeable in one or both typing systems. When interpreting the LPS composition in samples where more than one glycoform was present, the glycoform that contained the longest outer core structure was deemed the "parent" LPS and any additional glycoforms containing fewer sugars (but common to the parent glycoform) were considered truncated glycoforms of the parent LPS.

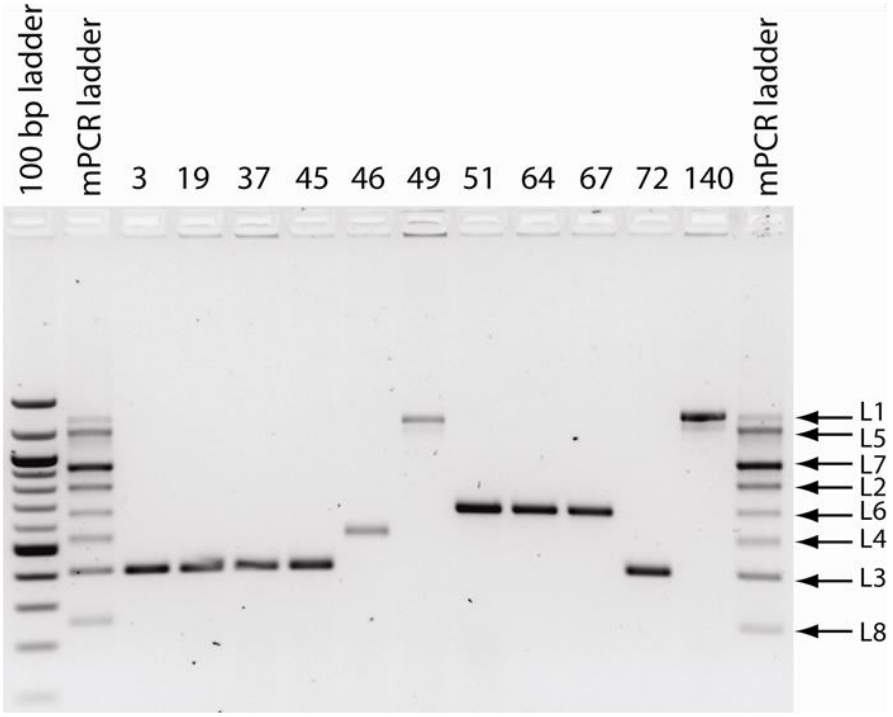


Figure 2. Gel electrophoresis separation of mPCR products derived from *P. multocida* field isolates 3, 19, 37, 45, 46, 49, 51, 64, 67, 72 and 140. A 100 bp ladder marker was loaded in lane 1 and the full mPCR ladder is shown on either side of the field isolate lanes for comparison. The different LPS-mPCRv1 loci are designated by arrows at the right and labelled L1-L8.

For the one strain from the agreement group analysed (strain 1455; Table 2), the LPS composition was in agreement with both the serology and the LPS-mPCRv1 result. For the nine strains with a partial agreement designation, the LPS composition correlated with the LPS-mPCRv1 in 8/9 strains but only correlated with one of the multiple Heddleston serovar designations in 6 of the 9 strains (Table 2). Two strains, PM3 and PM51, expressed truncated LPS glycoforms that were not compatible with any of the previously identified full-length Heddleston type strain structures. Finally, one strain in this group, PM1132, produced an LPS composition that did not correlate with the result generated in either typing system.

Interestingly, these data clearly showed that a number of strains express LPS molecules that are different from any of the LPS produced by the 16 Heddleston type strains. Indeed, many of the strains encode the LPS biosynthesis locus L3 but the LPS compositional analysis indicate that they produce only truncated LPS structures that are not identical to the LPS produced by either of the Heddleston 3 and 4 type strains. This indicates that these strains have novel mutations within the LPS biosynthetic locus. Furthermore, many of the strains that fall within the L3 group (containing serovars 3 and 4) express multiple glycoforms. Some

glycoforms are consistent with the known serovar 3 and 4 structures but other glycoforms are either shorter or longer than the type strains. This means that there is significantly more diversity in LPS structures in the field than previously believed which has important implications for vaccine formulation (see Chapter 2).

For the strains where serotyping and PCR were in non-agreement, the LPS compositional analysis was always compatible with the LPS-mPCRv1 locus designation with the exception of PM1128 where the LPS composition was not compatible with the designation assigned by either typing system. In contrast, the LPS composition was incompatible with the Heddleston serotyping designation in all cases. These data clearly show that Heddleston serotyping is an unreliable LPS typing system. In contrast, the LPS-mPCRv1 almost always gives an unambiguous result that is compatible with the LPS composition. Therefore, we can conclude that the LPS-mPCRv1 is more accurate and specific for classifying *P. multocida* on the basis of LPS phenotype than Heddleston serotyping. We also determined the DNA sequence of certain regions of the LPS biosynthetic loci of 11 of the 16 non-agreement strains and 10 of the 11 partial agreement strains (Table 2). In each case the DNA sequence obtained matched at >99% identity with the sequence predicted using the LPS-mPCRv1. Thus, these data further indicate that the LPS-mPCRv1 is reproducible and specific.

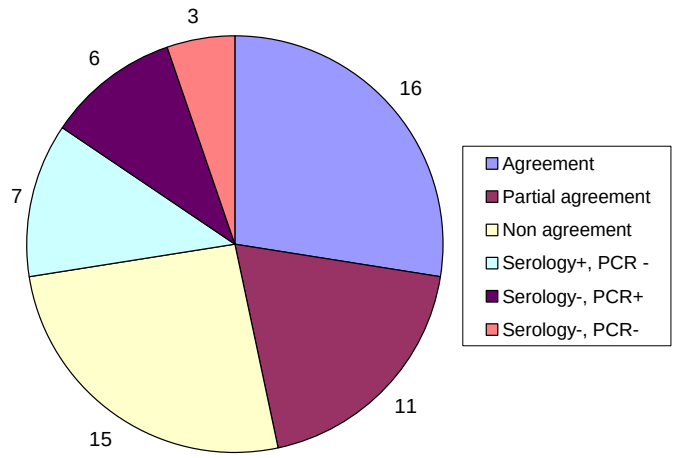


Figure 3. Pie chart showing the number of *P. multocida* strains where Heddleston serotyping and LPS-mPCRv1 agree, disagree (non-agreement) or partially agree or where one or both of the typing methods fails to give an unambiguous result (serology +, PCR -; serology –, PCR +; serology –, PCR-).

TABLE 2. Comparison of LPS typing of 58 Australian field isolates using Heddleston serotyping and the LPS-mPCRv1

Serotyping Vs LPS-mPCRv1	Strain N^{o}	Heddleston serovar [a]	LPS-mPCRv1	DNA sequence[b]	LPS outer core sugar composition [c]	LPS compatible with serovar? [d]	LPS compatible with PCR? [e]	Best typing method
Agreement	PM36	(H14)	L1 (H1,14)	ND	No LPS analysis	ND	ND	Either
Agreement	PM37	H3 (H3)	L3 (H3,4)	L3	No LPS analysis	ND	ND	Either
Agreement	PM45	NT (H3,4)	L3 (H3,4)	L3	No LPS analysis	ND	ND	Either
Agreement	PM46	H6 (H6)	L4 (H6,7)	L4	No LPS analysis	ND	ND	Either
Agreement	PM120	(H12)	L6 (H10,11,12,15)	L6	No LPS analysis	ND	ND	Either
Agreement	PM1165	H1	L1 (H1,14)	L1	No LPS analysis	ND	ND	Either
Agreement	PM1193	H3	L3 (H3,4)	L3	No LPS analysis	ND	ND	Either
Agreement	PM1300	H4	L3 (H3,4)	L3	No LPS analysis	ND	ND	Either
Agreement	PM1304	H1	L1 (H1,14)	L1	No LPS analysis	ND	ND	Either
Agreement	PM1315	H1	L1 (H1,14)	ND	No LPS analysis	ND	ND	Either
Agreement	PM1316	H4	L3 (H3,4)	ND	No LPS analysis	ND	ND	Either
Agreement	PM1317	H3	L3 (H3,4)	ND	No LPS analysis	ND	ND	Either
Agreement	PM1398	H1	L1 (H1,14)	L1	No LPS analysis	ND	ND	Either
Agreement	PM1417	H4	L3 (H3,4)	ND	No LPS analysis	ND	ND	Either
Agreement	PM1458	H14	L1 (H1,14)	L1	No LPS analysis	ND	ND	Either
Agreement	PM1455	H1	L1 (H1,14)	L1	**2PCho, 2Hex, Hep (H1)**	Y	Y	Either

Serotyping Vs LPS-mPCRv1	**Strain N^{o}**	**Heddleston serovar [a]**	**LPS-mPCRv1**	**DNA sequence[b]**	**LPS outer core sugar composition [c]**	**LPS compatible with serovar? [d]**	**LPS compatible with PCR? [e]**	**Best typing method**
Non-Agreement	PM8	H8	L3 (H3,4)	ND	2Hex, Hep[f] Hex, Hep	N	Y Y	PCR
Non-Agreement	PM64	NT (H3)	L6 (H10,11,12,15)	L6	**HexNAc 3Hex Hep (H12)**	N	Y	PCR
Non-Agreement	PM146	(H7)	L3 (H3,4)	L3	2Hex, Hep	N	Y	PCR
Non-Agreement	PM993	H8	L6 (H10,11,12,15)	ND	**no outer core (H10)**	N	Y	PCR
Non-Agreement	PM995	H3	L6 (H10,11,12,15)	L6	**HexNAc 3Hex Hep (H12)**	N	Y	PCR
Non-Agreement	PM1098	H15	L3 (H3,4)	L3	**3Hex, Hep (H4)**	N	Y	PCR
Non-Agreement	PM1099	H10	L3 (H3,4)	L3	3Hex, Hep[f] 4Hex, Hep **HexNAc, 4Hex, Hep (H3)**	N	Y Y Y	PCR
Non-Agreement	PM1103	H10	L3 (H3,4)	ND	4Hex, Hep[f] **HexNAc, 4Hex, Hep (H3)**	N	Y Y	PCR
Non-Agreement	PM1205	H1	L3 (H 3,4)	ND	**3Hex, Hep (H4)**	N	Y	PCR
Non-Agreement	PM1320	H10,13,14	L3 (H3,4)	L3	2Hex, Hep[f] **3Hex, Hep (H4)**	N	Y Y	PCR
Non-Agreement	PM1441	H2	L3 (H3,4)	L3	3Hex, Hep[f] 4Hex, Hep **HexNAc, 4Hex, Hep (H3)**	N	Y Y Y	PCR
Non-Agreement	PM1456	H14	L4 (H6,7)	L4	**1Hex (H7)**	N	Y	PCR
Non-Agreement	PM1470	H1	L3 (H3,4)	L3	**HexNAc, 4Hex, Hep[f] (H3)** 2HexNAc, 4Hex, Hep	N	Y Y	PCR
Non-Agreement	PM1474	H12	L3 (H3,4)	L3	**3Hex, Hep[f] (H4)** 4Hex, Hep	N	Y Y	PCR
Non-Agreement	PM1128	H10	L3 (H3,4)	ND	HexNAc, 2Hex, Hep[f] **HexNAc, 3Hex, Hep (H12)** (weak spectra)	N	N N	Neither

Serotyping Vs LPS-mPCRv1	Strain N^{o}	Heddleston serovar [a]	LPS-mPCRv1	DNA sequence[b]	LPS outer core sugar composition [c]	LPS compatible with serovar? [d]	LPS compatible with PCR? [e]	Best typing method
Partial agreement	PM3	H15 (H4,10,15)	L3 (H3,4)	L3	1Hex, Hep[f] 2Hex, Hep	N	Y Y	PCR
Partial agreement	PM49	NT (H1,15)	L1 (H1,14)	L1	**2PCho, 2Hex, Hep (H1)**	Equivocal	Y	PCR
Partial agreement	PM51	H9 (H4,12)	L6 (H10,11,12,15)	L6	2Hex, Hep	N	Y	PCR
Partial agreement	PM67	H3 (H3,12)	L6 (H10,11,12,15)	L6	**HexNAc, 3Hex, Hep (H12)**	Equivocal	Y	PCR
Partial agreement	PM72	NT (H3,14)	L3 (H3,4)	L3	3Hex, Hep[f] 4Hex, Hep **HexNAc, 4Hex, Hep (H3)** 2 HexNAc, 4Hex, Hep	Equivocal	Y Y Y Y	PCR
Partial agreement	PM140	NT (H13,14,15)	L1 (H1,14)	L1	**1 Hex, Hep (H14)**	Equivocal	Y	PCR
Partial agreement	PM1124	H1,4,12	L1 (H1,14)	L1	**2PCho, 2Hex, Hep (H1)**	Equivocal	Y	PCR
Partial agreement	PM1396	H1,3	L1 (H1,14)	ND	**2PCho, 2Hex, Hep (H1)**	Equivocal	Y	PCR
Partial agreement	PM19	NT (H3)	L3 (H3,4)	L3	No LPS analysis	ND	ND	ND
Partial agreement	PM878	H1, 4	L1 (H1,14)	L1	No LPS analysis	ND	NT	ND
Partial agreement	PM1132	H1,3,4,10,14	L6 (H10,11,12,15)	L6	3 Hex, Hep[f] HexNAc, 3Hex, Hep	N	N Y	Neither
Serotyping -, PCR +	PM1113	NT	L4 (H6,7)	ND	**1Hex (H7)**	NT	Y	PCR
Serotyping -, PCR +	PM1268	NT	L3 (H3,4)	L3	2Hex, Hep[f] **3Hex, Hep (H4)**	NT	Y Y	PCR
Serotyping -, PCR +	PM1405	NT	L1 (H1,14)	ND	**2PCho, 2Hex, Hep (H1)**	NT	Y	PCR
Serotyping -, PCR +	PM1435	NT	L1 (H1,14)	L1	**2PCho, 2Hex, Hep (H1)**	NT	Y	PCR
Serotyping -, PCR +	PM1439	NT	L3 (H3,4)	L3	**3Hex, Hep (H4)**	NT	Y	PCR
Serotyping -, PCR +	PM1457	NT	L4 (H6,7)	L4	3HexNAc, 1Hex, Hep[f] **3HexNAc, 2Hex, Hep (H6)**	NT	Y Y	PCR

Serotyping Vs LPS-mPCRv1	Strain N^{o}	Heddleston serovar [a]	LPS-mPCRv1	DNA sequence[b]	LPS outer core sugar composition [c]	LPS compatible with serovar? [d]	LPS compatible with PCR? [e]	Best typing method
Serotyping +, PCR -	PM1	H3 (H3,4)	NT [e]	L3	3Hex, Hep[f] 4Hex, Hep **HexNAc, 4Hex, Hep (H3)**	Y	compatible with sequence	Serotyping
Serotyping +, PCR -	PM48	H3 (H3,4)	NT	L3	3Hex, Hep[f] 4Hex, Hep **HexNAc, 4Hex, Hep (H3)**	Y (H3)	compatible with sequence	Serotyping
Serotyping +, PCR -	PM18	NT (H3)	NT	L3	2Hex, Hep	Equivocal	compatible with sequence	ND
Serotyping +, PCR -	PM135	H8,13 (H13)	NT	L7 (deletion within locus)	HexNAc, 2Hex, Hep	Y (H13)	NT	ND
Serotyping +, PCR -	PM1074	H16	NT	L3	no outer core	ND	NT	ND
Serotyping +, PCR -	PM1153	H1,3,7	NT	L3	3Hex, Hep[f] 4Hex, Hep **HexNAc, 4Hex, Hep (H3)** 2HexNAc, 4Hex, Hep	Equivocal	NT	Neither
Serotyping +, PCR -	PM1369	H1	NT	L3	3Hex, Hep[f] 4Hex, Hep	N	compatible with sequence	Neither
Serotyping -, PCR -	PM1120	NT	NT	L3	no outer core	NT	NT	ND
Serotyping -, PCR -	PM1258	NT	NT	L3	no outer core	NT	NT	ND
Serotyping -, PCR -	PM1434	NT	NT	L3	**HexNAc, 4Hex, Hep (H3)**	NT	compatible with sequence	Neither

[a] Multiple numbers separated by a comma indicates a precipitin line was observed with more than one antisera. Numbers in brackets are the original serotyping results on historic strains.

[b] We have determined the DNA sequence of a region of the LPS biosynthetic loci from each of these strains. The LPS locus (L) and Heddleston serovars (H) which matched the DNA sequence (>99%) is shown.

[c] Outer core LPS sugar composition as predicted by MS/MS analysis. Glycoforms shown in bold are compatible with a known Heddleston LPS structure (shown in brackets).

[d] LPS composition correlates exactly the LPS structure of the serovar stated.

[e] LPS composition correlates with known and possible LPS structures expressed by this locus group.

[f] Multiple LPS glycoforms detected. Hex= glucose or galactose, HexNAc=N-acetylglucosamine or N-acetylgalactosamine, Hep= heptose, PCho= phosphocholine

NT = Not able to be typed by this method,

ND= Not determined

Refining the LPS-mPCRv1 to improve strain coverage

The LPS-mPCRv1 gave an unambiguous LPS genotype for 48 of 58 field strains (Table 2). To identify why the LPS-mPCRv1 failed to amplify a product from 10 of the strains we used both direct genomic sequencing and PCR to analyse the LPS biosynthetic locus from these strains. Nucleotide sequence analysis of nine of the strains indicated that they contained genes corresponding to locus 3 (Table 2), and importantly, comparison of the DNA sequence with the known locus 3 sequence indicated that there were nucleotide differences in the region used for the design of the L3 primers. We were unable to determine any nucleotide sequence for one of the strains (pm135).

To improve the coverage of the LPS-mPCRv1 we designed new primers in a region that was common to all locus 3 strains, including those 9 strains for which the LPS-mPCRv1 had failed. In order to use a highly conserved region for locus 3-specific amplification, we changed the position of the locus 3 primers from within the *gatG* gene to within *gatF* (Fig. 1). This in-turn necessitated a change to the primers used for locus 8 such that each of the amplicons could still be readily differentiated by size using standard gel electrophoresis. This modified typing PCR was designated LPS-mPCRv3 (Table 3). However, this PCR gave only weak amplification of DNA from some of the locus 3 strains (data not shown) so we designed new locus 3 primers within *gatF.* This again changed the amplicon size which required us to revert to using the original locus 8 primers; this multiplex PCR was designated LPS-mPCRv4. The LPS-mPCRv4 PCR showed only weak amplification from some locus 6 strains (data not shown) so we extended the length of the locus 6 primers to increase the specificity of this primer pair (Table 3). This final typing PCR was designated LPS-mPCRv5. The full set of primer sequences and amplicon sizes is shown in Table 4.

The LPS-mPCRv5 was tested on the 16 Heddleston type strains and was able to accurately differentiate these strains into the eight genetic locus types (Fig. 4A). We then tested the LPS-mPCRv5 on the 10 strains which had given no amplification products with the LPS-mPCRv1 assay. Strong amplification of a locus 3-specific product was observed for 9 of these 10 strains (Fig. 4B). We then used the LPS-mPCRv5 assay to type the remaining field isolates. The LPS-mPCRv5 assay gave a single reproducible amplification product for 57 of 59 strains (97%) tested (Table 5 and Fig. 4C). This final mPCR was given the general name "LPS-mPCR" and transferred to the diagnostic service at Agri-Science, Qld.

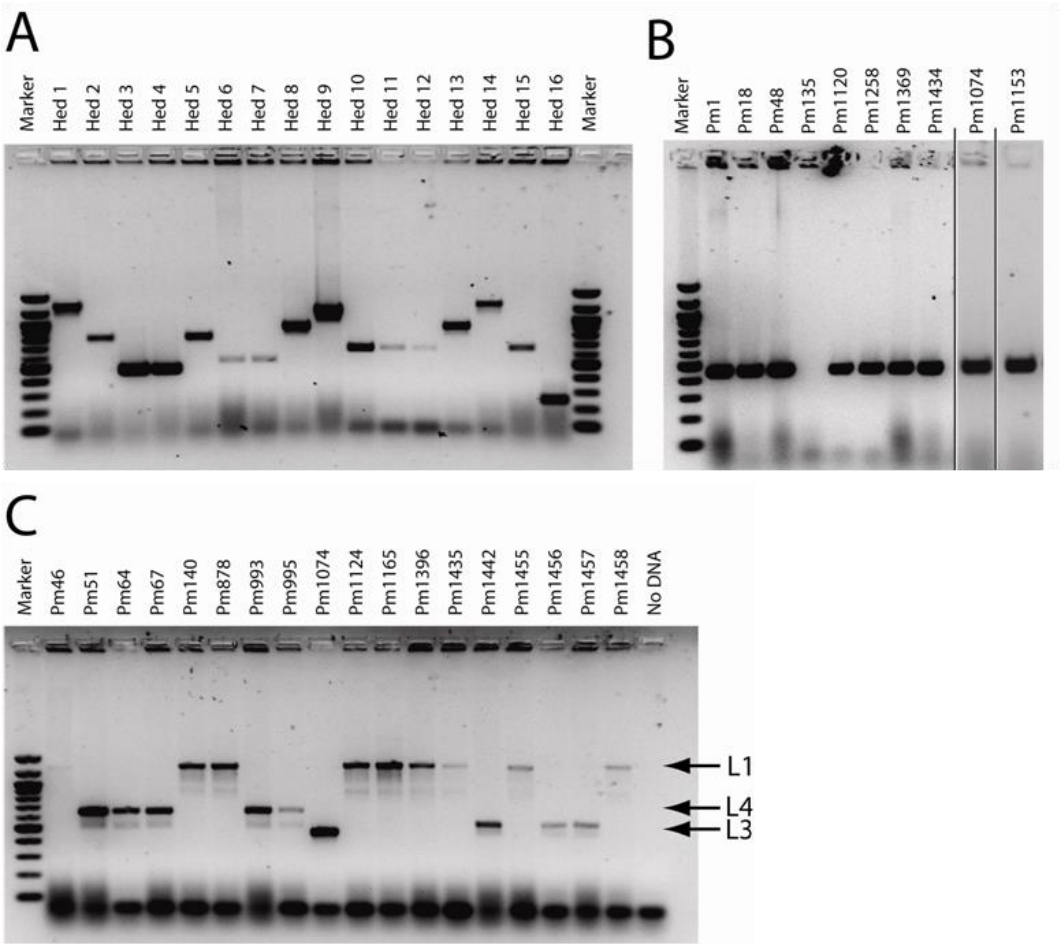


Figure 4. Gel electrophoresis separation of products derived from LPS-mPCRv5 using the following strains as template; A) Each of the 16 *P. multocida* Heddleston type strains, B) Each of the strains which was unable to be typed using LPS-mPCRv1, and C) a sample of the 59 *P. multocida* field isolates. The different LPS-mPCRv5 L1, L3 and L4-specific amplification products are designated by arrows at the right. The gel shown in panel C shows some shadowing below each DNA band; this is an artefact of the electrophoresis and is not observed in other amplifications.

Integration of the LPS mPCR into the *P. multocida* diagnostic service at Agri-Science, Qld.

The LPS-mPCR was transferred to Agri-Science Queensland and has been used to type several *P. multocida* outbreak strains when Heddleston serotyping has given ambiguous or negative results (at this stage with no fee to end user). Standard operating procedures have been developed and are attached as Appendix A.

TABLE 3. Changes to the primer sets used in the multiplex PCR

Locus	Version 1	Version 2	Version 3	Version 4	Version 5
L1	BAP6119 BAP6120				BAP6119 BAP6120
L2	BAP6121 BAP6122				BAP6121 BAP6122
L3	BAP6123 BAP6124		BAP7206[b] BAP7207[b]	BAP7213[c] BAP7214[c]	BAP7213 BAP7214
L4	BAP6125 BAP6126				BAP6125 BAP6126
L5	BAP6129 BAP6130				BAP6129 BAP6130
L6	BAP6131 BAP6132	BAP6131 BAP7039[a]			BAP7292[d] BAP7293[d]
L7	BAP6127 BAP6128				BAP6127 BAP6128
L8	BAP6133 BAP6134		BAP6133[b] BAP7205[b]	BAP6133[c] BAP6134[c]	BAP6133 BAP6134

[a] Apr., 2011 BAP7039: Extension of BAP6131 to increase T_m

[b] Nov., 2011 BAP7206 and BAP7207: Re-positioning of amplicon within locus 3 from *gatG* (*pm1139*) to *gatF* (*pm1141*), generates smaller product.
BAP7205: Re-positioning of one primer in locus 8 to generate larger product, 450 bps in size

[c] Jan., 2012 BAP7213 and BAP7214: Moved position of amplicon to within *gatF* (*pm1141*). These primers generate a 474 bp product. Reverted to using primer BAP6134 for Locus 8.

[d] Mar., 2012 BAP7292 and BAP7293: Extension of Locus 6 primers BAP6131 and BAP6132 to increase T_m.

TABLE 4. DNA sequence and genetic location of the primers used in the final multiplex PCR; LPS-mPCRv5

Locus	Primer	Sequence	Location/Description	Product Size bps
L1	BAP 6119	ACATTCCAGATAATACACCCG	Forward primer in *pcgD* of Locus 1	1307
	BAP 6120	ATTGGAGCACCTAGTAACCC	Reverse primer in *pcgB* of Locus 1	
L2	BAP 6121	CTTAAAGTAACACTCGCTATTGC	Forward primer in *nctA* of Locus 2	810
	BAP 6122	TTTGATTTCCCTTGGGATAGC	Reverse primer in *nctA* of Locus 2	
L3	BAP 7213	TGCAGGCGAGAGTTGATAAACCATC	Forward primer in *gatF* of Locus 3	474
	BAP 7214	CAAAGATTGGTTCCAAATCTGAATGGA	Reverse primer in *gatF* of Locus 3	
L4	BAP 6125	TTTCCATAGATTAGCAATGCCG	Reverse primer in *latB* of Locus 4	550
	BAP 6126	CTTTATTTGGTCTTTATATATACC	Forward primer in *latB* of Locus 4	
L5	BAP 6129	AGATTGCATGGCGAAATGGC	Forward primer in *rmlA* of Locus 5	1175
	BAP 6130	CAATCCTCGTAAGACCCCC	Reverse primer in *rmlC* of Locus 5	
L6	BAP 7292	TCTTTATAATTATACTCTCCCAAGG	Forward primer in *nctB* of Locus 6	668
	BAP 7293	AATGAAGGTTTAAAAGAGATAGCTGGAG	Reverse primer in *nctB* of Locus 6	
L7	BAP 6127	CCTATATTTATATCTCCTCCCC	Forward primer in *ppgB* of Locus 7	931
	BAP 6128	CTAATATATAAACCATCCAACGC	Reverse primer in *ppgB* of Locus 7	
L8	BAP 6133	GAGAGTTACAAAAATGATCGGC	Forward primer in *natG* of Locus 8	255
	BAP 6134	TCCTGGTTCATATATAGGTAGG	Reverse primer in *natG* of Locus 8	

TABLE 5. Differentiation of *P. multocida* field isolates by Heddleston serotyping, and the multiplex PCR typing assays LPS-mPCRv1 and LPS-mPCRv5

Strain	Heddleston typing	LPS-mPCRv1 typing (associated Heddleston types)	LPS-mPCRv5 typing
36	H14	L1 (H1,H14)	L1
49	NT (H1,H15)	L1 (H1,H14)	L1
140	NT (H13,14,15)	L1 (H1,H14)	L1
878	H1,H4	L1 (H1,H14)	L1
1124	H1,H4,H12	L1 (H1,H14)	L1
1165	H1	L1 (H1,H14)	L1
1304	H1	L1 (H1,H14)	L1
1315	H1	L1 (H1,H14)	L1
1396	H1,H3	L1 (H1,H14)	L1
1398	H1	L1 (H1,H14)	L1
1405	NT	L1 (H1,H14)	L1
1435	NT	L1 (H1,H14)	L1
1458	H14	L1 (H1,H14)	L1
1455	H1	L1(H1,H14)	L1
3	H15 (H4,10,15)	L3 (H3,H4)	L3
1	H3 (H3,4)	No Reaction	L3
18	NT (H3)	No Reaction	L3
48	H3 (H3,4)	No Reaction	L3
1074	H16	No Reaction	L3
1120	NT	No Reaction	L3
1153	H1,H3,H7	No Reaction	L3
1258	NT	No Reaction	L3
1369	H1	No Reaction	L3
1434	NT	No Reaction	L3
1442	Not done	Not done	L3
8	H10	L3 (H3,H4)	L3
19	NT (3)	L3 (H3,H4)	L3
37	H3 (H3)	L3 (H3,H4)	L3
45	NT (H3,H4)	L3 (H3,H4)	L3
72	NT (H3,14)	L3 (H3,H4)	L3
146	H7 (H7)	L3 (H3,H4)	L3
1098	H15	L3 (H3,H4)	L3
1099	H10	L3 (H3,H4)	L3
1103	H10	L3 (H3,H4)	L3
1128	H10	L3 (H3,H4)	L3
1268	NT	L3 (H3,H4)	L3
1300	H4	L3 (H3,H4)	L3
1316	H4	L3 (H3,H4)	L3
1317	H3	L3 (H3,H4)	L3
1320	H10,H13,H14	L3 (H3,H4)	L3

1417	H4	L3 (H3,H4)	L3
1439	NT	L3 (H3,H4)	L3
1441	H2	L3 (H3,H4)	L3
1470	H1	L3 (H3,H4)	L3
1474	H12	L3 (H3,H4)	L3
1193	H3	L3 (H3,H5)	L3
46	H6	L4 (H6,H7)	L4; L1[a]
1113	NT	L4 (H6,H7)	L4
1456	H14	L4 (H6,H7)	L4
1457	NT	L4 (H6,H7)	L4
1205	H1	L4 (H6,H7); L3(H3,H4)	L3
51	H9 (H4,12)	L6 (H10,H11,12,H15)	L6
64	NT (H3)	L6 (H10,H11,12,H15)	L6
67	H3 (H3,12)	L6 (H10,H11,12,H15)	L6
120	H12 (H12)	L6 (H10,H11,12,H15)	L6
993	H8	L6 (H10,H11,12,H15)	L6
995	H3	L6 (H10,H11,12,H15)	L6
1132	H1,H3,H4,H10,H14	L6 (H10,H11,12,H15)	L6
135	H13,8 (H13)	No Reaction	No Reaction [b]

[a] This strain gave two different typing results using the LPS-mPCRv5.

[b] Subsequent sequencing of this strain identified that it contained an L6 locus with a deletion in the PCR target region.

Chapter 2: Analysis of the protective efficacy of killed whole cell and live vaccines against virulent strains expressing either identical or different surface LPS molecules

Overview

It is widely believed that killed whole cell vaccines elicit protective immunity against strains with related LPS structures. However, this has never been objectively tested for *P. multocida* as the precise LPS structures expressed by different strains have not been known. Our structural analyses (Chapter 1) defined the exact LPS structures produced by the Heddleston type strains and many field isolates (Chapter 1). Furthermore, in other work we developed methods for making directed gene knockouts in *P. multocida* so that we could precisely manipulate the LPS structure produced by a particular strain (7, 14). Thus, for the first time we could determine precisely how LPS structure effects vaccine efficacy. For these analyses we focused on strains of direct relevance to the poultry industry, namely strains belonging to the genotypes L1 and L3; our mPCR analyses (chapter 1) indicated that these strains made up 26% and 56% respectively of the Australian field isolates tested. We first made sets of mutants expressing truncated LPS molecules in either an L1 (strain VP161) or an L3 (strain P1059) background. We then vaccinated chickens with either killed or live versions of these mutant strains (live strains also had the *aroA* gene inactivated) and then challenged the vaccinated chickens with virulent *P. multocida* expressing full length LPS. Both homologous (L1 vs L1 and L3 vs L3) and heterologous (L1 vs L3 and L3 vs L1) protective efficacy was assessed.

Bacterin (killed) vaccine trial using *P. multocida* strain VP161 and VP161 LPS mutants.

A set of mutants expressing modified LPS structures (Table 6) were constructed in the L1 (serovar 1) strain VP161 using single cross-over mutagenesis. Mutants constructed using this method are stable *in vitro* and suitable for use in killed-cell bacterins. However, they cannot be used in live vaccine experiments as they show a low level of reversion to the wild type phenotype (1).

The parent strain (VP161) as well as each of the single cross-over LPS mutants, were grown to mid-exponential growth phase ($O.D_{600}$=0.5) then heat-killed, diluted, and mixed with the vaccine adjuvant aluminium hydroxide (Alhydrogel). Groups of 10 chickens were vaccinated with approximately $1x10^7$ killed cells of each strain (Table 6) and an identical booster vaccination was given 2 weeks later. Birds were then challenged at 4 weeks post initial

vaccination with 250 CFU of the virulent parent strain VP161 which expresses full length LPS (L1, serovar 1) and monitored for 60 hours for signs of disease.

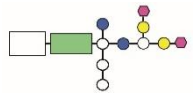
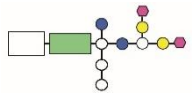
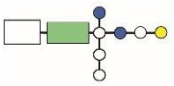
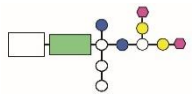
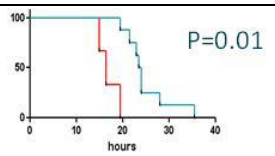
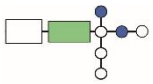
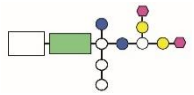
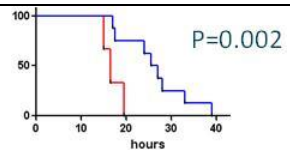
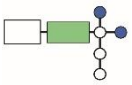
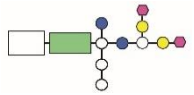
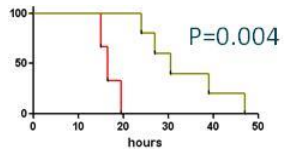
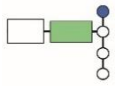
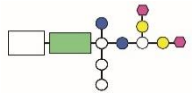
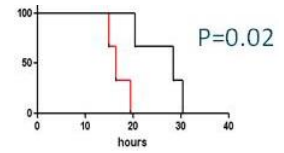
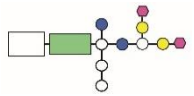
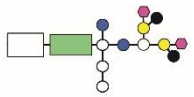
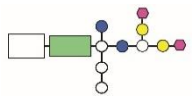
Seventy percent of birds that received the bacterin vaccine containing the killed parent strain, VP161, were protected from disease (*P* = 0.003; fisher's exact test) while there were no surviving birds in any of the groups vaccinated with killed strains expressing truncated LPS. In addition, vaccination with a bacterin containing the L1 (serovar 1) strain, X73, only afforded 30% protection. Strain X73 is a fowl cholera isolate which expresses a full-length L1/serovar 1 LPS structure that is additionally decorated with two phosphoethanolamine residues on the terminal galactose residues (Table 6) (13). Analysis of the survival times for birds in each group indicated that although vaccination with killed strains expressing truncated LPS failed to protect birds against acute fowl cholera, there was a significant extension in time to death (Table 2). These results clearly show that killed vaccines can only elicit significant protective immunity against strains expressing an identical LPS structure and will fail to protect against L1 strains that express a truncated or modified LPS. Moreover, these results suggest that single-strain bacterins are unlikely to elicit significant levels of protection against heterologous strains (strains belonging to different serovars that produce different LPS structures).

Construction of live-attenuated L1 (VP161) LPS biosynthesis mutants.

In order to construct safe live vaccines expressing truncated LPS, the single cross-over LPS mutants used in the bacterin trial (above) were further genetically modified by inactivation of the *aroA* gene. *P. multocida aroA* mutants have been proven safe for *in vivo* use by our group and others. In addition, a live vaccine (Vaxsafe PM, Bioproperties) containing a *P. multocida aroA* mutant originally constructed by our group is now commercially available in Australia. However, the effect of LPS structural changes on the protective efficacy of the live vaccine has never been tested.

To inactivate the *aroA* gene in strain VP161, and in each of the VP161 LPS mutants, we used the Sigma Targetron® system, which we have recently adapted for use in *P. multocida* (14). The Targetron® system utilizes an intron that can be genetically modified such that it can be directed specifically to any gene of interest. In each strain (VP161 parent and each of the VP161 LPS mutants) we inactivated the *aroA* gene with a specific Targetron® insertion. Integration of the Targetron® intron into the *aroA* gene in each strain was confirmed by Southern blotting and the truncated LPS profiles of each double mutant (*aroA*/LPS gene) confirmed by PAGE and carbohydrate silver stain of cell lysates (data not shown).

TABLE 6 Protective efficacy of *P. multocida* L1 killed bacterin vaccines against homologous challenge.

Bacterin strain	LPS structure on vaccine strain	LPS structure on challenge strain	Protection (n =10)	Kaplan–Meier Survival curves
VP161			70%*	
VP161 *pcgC*			0%	
VP161 *gatA*			0%	
VP161 *hptE*			0%	
VP161 *gctB*			0%	
Adjuvant/ media only			0%	
X-73			30%	

* P=0.003. Challenged dose: 250 CFU of *P. multocida* strain VP161 (ID_{50}=1-10 CFU)

Residues are: □ Lipid A, ▭ 3-deoxy-D-mannooctulosonate, ○ Heptose, ● Glucose ○ Galactose, ⬡ Phosphocholine, ⬢ Phosphoethanolamine.

Vaccine trial with live-attenuated L1 (VP161) LPS biosynthesis mutants; homologous challenge.

The VP161 *aroA* mutant and the four VP161 *aroA*/LPS double mutants were used to vaccinate groups of 15 chickens (Table 7). Briefly, each strain was grown to mid-exponential phase, diluted in Heart infusion broth and $1x10^7$ CFU injected intramuscularly into the breast muscle of each bird. Chickens were given an identical booster vaccination 10 days later with the same live-attenuated mutant, followed by the final challenge 4 weeks post initial vaccination with 110 CFU of the parent strain VP161.

In direct contrast to the results obtained in the L1 bacterin vaccine trial, all groups vaccinated with live strains expressing truncated LPS showed significant levels of protection (Table 7). Groups vaccinated with *pcgC/aroA* or *hptE/aroA* mutants showed 100% survival against challenge with the parent strain VP161 (Table 7), and 73% survival was observed in the groups vaccinated with the *gatA/aroA* or *gctB/aroA* mutants (Table 7). These results indicate that vaccination with live *aroA P. multocida* LPS mutants can give significant protection regardless of the degree of LPS truncation present on the surface of the live vaccine strains.

Bacterin (killed) vaccine trial using the L3 *P. multocida* strain, P1059, and P1059 LPS mutants.

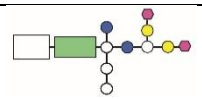
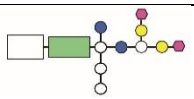
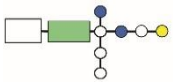
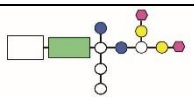
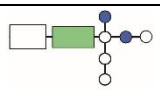
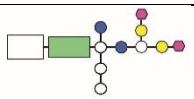
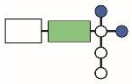
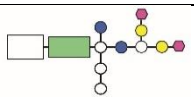
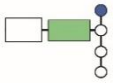
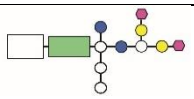
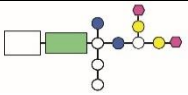
To inactivate specific LPS genes in the L3/ Heddleston serovar 3 type strain, P1059, we used the Sigma Targetron® system, which we used previously for mutagenesis of the *aroA* gene in *P. multocida* strain VP161 (see above). We independently inactivated the LPS biosynthesis genes *natB*, *gatG* and *gatF* with a specific Targetron®insertion. Integration of the Targetron®intron into the appropriate LPS gene in each strain was confirmed by PCR and direct sequencing, using a Targetron®-specific primer and genomic DNA as template (data not shown). The truncated LPS molecules expressed by each LPS mutant were confirmed by mass spectrometry of purified LPS isolated from each mutant (data not shown).

For the bacterin vaccine trial using the L3 strains, the parent strain (P1059) as well as each of the Targetron® LPS mutants, were grown to mid-exponential growth phase ($O.D_{600}$=0.5) then heat-killed, diluted, and mixed with aluminium hydroxide (final concentration 20%). Groups of 12 chickens were vaccinated with approximately $1x10^7$ killed cells of each strain (Table 8) and an identical booster vaccination was given 2 weeks later. Birds were then challenged at 4 weeks post initial vaccination with 1.5 $x10^7$ CFU of the virulent Australian field isolate, Pm1422, belonging to the same LPS genotype L3 but expressing a truncated

LPS similar to that observed in the Heddleston serovar 4 type strain P1662 (Fig. 1). Birds were then monitored for 60 hours for signs of disease.

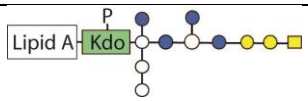
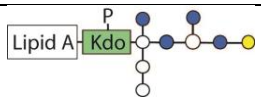
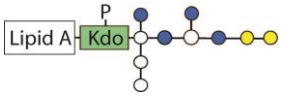
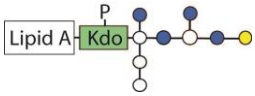
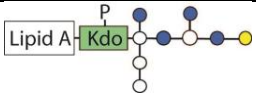
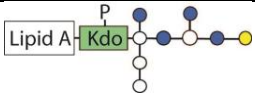
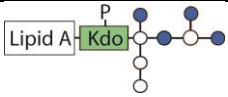
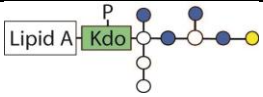
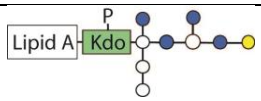
The highest level of protection was observed in the group of birds that received the bacterin vaccine containing the P1059 *gatG* mutant where 83% of birds were protected from disease (Table 2). The P1059 *gatG* mutant expresses an outer core LPS that is the same as the challenge strain, Pm1422. Thus, vaccination with a killed bacterin can give solid protection against a challenge strain with identical LPS structure. The group that received the bacterin containing an LPS structure with one additional sugar (P1059 *natB*) was also significantly protected (58% survival) but those that received bacterins with LPS truncated by one sugar (P1059 *gatF*) were not protected. Furthermore, there were no surviving birds in the group that received the P1059 bacterin. This strain expresses LPS that is two sugars longer than the Pm1422 challenge strain (Table 2). These results clearly show that L3 bacterin vaccines elicit significant protective immunity only against strains expressing identical or nearly identical LPS. This confirms our previous results with *P. multocida* L1 strains where we showed that protection was only elicited against strains expressing identical LPS. These results indicate that single-strain bacterins will not protect against heterologous strains and therefore that such bacterins will never be able to protect against the diversity of L3 strains that we now know are present in the field.

TABLE 7 Protective efficacy of live-attenuated *P. multocida* genotype L1 vaccines against homologous challenge.

L1 live vaccine strain	LPS structure on L1 live vaccine strain	LPS structure on L1 challenge strain VP161	Protection (n =15)
VP161 *aroA*			100%*
VP161 *aroA/pcgC*			100%*
VP161 *aroA/gatA*			73%*
VP161 *aroA/hptE*			100%*
VP161 *aroA/gctB*			73%*
Media only			0%

* $P < 0.01$. Challenged dose: 110 CFU of *P. multocida* strain VP161 (ID_{50}=1-10 CFU)

TABLE 8: Protective efficacy of *P. multocida* L3 killed bacterin vaccines against homologous challenge.

L3 bacterin vaccine strain	LPS structure on L3 bacterin vaccine strain	LPS structure on L3 challenge strain Pm1422	Protection (n=12) (significance)[a,b]
P1059			0%
P1059 *natB*			58% (*P* = 0.027)
P1059 *gatG*			83% (*P* = 0.006)
P1059 *gatF*			25% (NS)
Adjuvant/ media only			9% (NS)

[a] Challenge: 1.5 x 10^7 CFU *P. multocida* strain Pm1422. [b] Significance of differences in group survival were determined using Fisher's exact test; NS = not significant

Residues are: ☐ Lipid A, ▭ 3-deoxy-D-mannooctulosonate, ○ Heptose, ● Glucose ● Galactose, ■ N-acetylgalactosamine.

Vaccine trial with live-attenuated L3 (P1059) LPS biosynthesis mutants; homologous challenge.

In order to construct safe live vaccines expressing truncated LPS, Targetron® LPS mutations were generated in a previously constructed P1059 *aroA* mutant. The P1059 *aroA* mutant and the four P1059 *aroA*/LPS double mutants were used to vaccinate groups of 12 chickens (Table 9). Briefly, each strain was grown to mid-exponential phase, diluted in Heart infusion broth and approximately $1x10^7$ CFU injected intramuscularly into the breast muscle of each bird. No signs of infection were observed following the vaccinations, confirming that all strains were highly attenuated. Chickens were then given a second vaccination 14 days later with the appropriate live-attenuated mutant, followed by the final challenge 4 weeks post initial vaccination with 1.5 $x10^7$ CFU of the virulent L3 strain, Pm1422. Birds were then monitored for 60 hours for signs of disease.

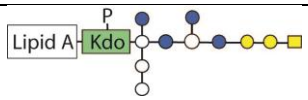
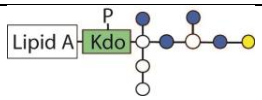
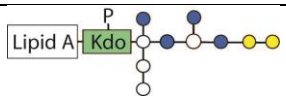
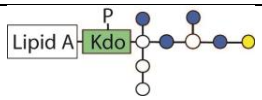
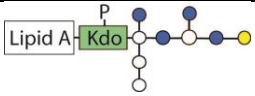
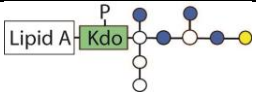
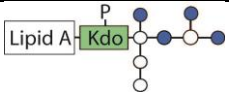
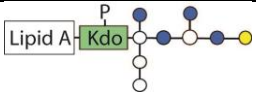
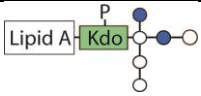
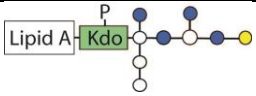
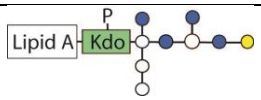
In direct contrast to the results obtained in the bacterin vaccine trial, all groups vaccinated with live strains expressing truncated LPS showed significant levels of protection (Table 9). Groups vaccinated with *aroA*/*natB*, *aroA/gatG* or *aroA/gtcC* mutants were 100% protected against challenge with the L3 strain Pm1422 (Table 9), and 83% of the birds in the group vaccinated with the *aroA/gatF* mutant were also protected (Table 9). These results show that vaccination with live *P. multocida aroA*/LPS mutants derived from strain P1059 can give significant protection against strains belonging to the same LPS genotype regardless of the LPS structure present on the surface of the live vaccine strains. Thus, protection elicited by live strains is LPS structure-independent.

Vaccine trial with live-attenuated L1, L3 LPS mutants; heterologous challenge.

For the heterologous challenge experiments using the L1 strain VP161 as the challenge strain, groups of 10 chickens were vaccinated with the P1059 *aroA* mutant or one of four P1059 *aroA*/LPS double mutants. For the heterologous challenge experiments using the L3 strain Pm1422 as the challenge strain, groups of 12 chickens were vaccinated with either the VP161 *aroA* mutant or one of the four VP161 *aroA*/LPS double mutants (Table 10). Groups of 12 birds were used for this challenge as Pm1422 is less virulent than VP161. For the vaccinations, each strain was grown to mid-exponential phase, diluted in Heart infusion broth and approximately $1x10^7$ CFU injected into the breast muscle of each bird. Chickens were then given a second vaccination 14 days later with the appropriate strain and challenged at 4 weeks post initial vaccination with either 1.5 $x10^7$ CFU of the L3 challenge strain, Pm1422 or 1.9 $x10^2$ CFU of the L1 challenge strain, VP161. Birds were then monitored for 60 hours for signs of disease.

All groups vaccinated with the live L1 VP161 *aroA*/LPS mutant strains expressing truncated LPS were fully protected against heterologous challenge with Pm1422 (Table 10). However, groups vaccinated with the L3 P1059 *aroA*/LPS mutant strains showed varying levels of protection. Groups vaccinated with P1059 *aroA* or P1059 *aroA/gatG* showed significant protection against the heterologous challenge (70 and 80 % respectively). However, there was no significant protection observed when birds were vaccinated with P1059 *aroA*/*natB*, *aroA*/*gatF* or *aroA/gtcC* mutants. These results indicate that vaccination with any live *P. multocida aroA*/LPS mutant derived from strain VP161 can give full protection against strains belonging to a different LPS genotype regardless of the degree of LPS truncation present on the surface of the live vaccine strains. Furthermore, strong protective immunity can also be elicited by some P1059 strains, including strains expressing full length and truncated LPS.

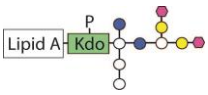
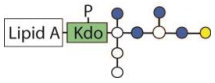
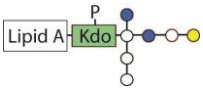
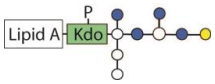
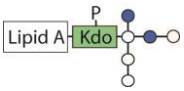
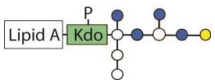
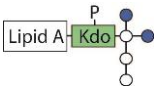
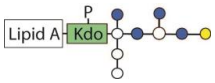
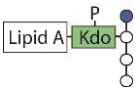
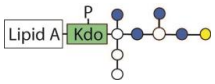
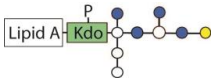
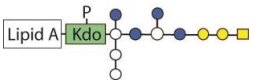
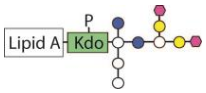
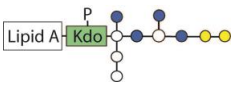
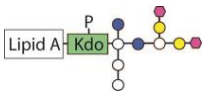
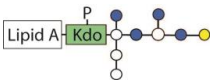
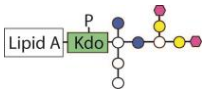
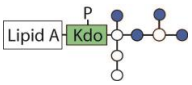
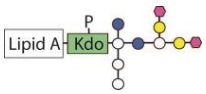
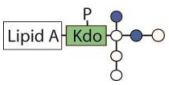
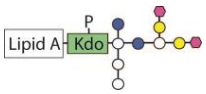
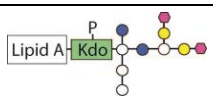
TABLE 9: Protective efficacy of live-attenuated *P. multocida* genotype L3 vaccines against homologous challenge.

L3 live vaccine strain	**LPS structure on L3 live vaccine strain**	**LPS structure on L3 challenge strain Pm1422**	**Protection (significance)** [a,b,]
P1059 *aroA*			100% (*P* = 0.0003)
P1059 *aroA*/*natB*			100% (*P* = 0.0003)
P1059 *aroA*/ *gatG*			100% (*P* = 0.0003)
P1059 *aroA*/*gatF*			83% (*P* = 0.0123)
P1059 *aroA/gctC*			100% (*P* = 0.0003)
media only			25%

[a] Challenge: 1.5 x 10^7 CFU *P. multocida* strain Pm1422. [b] Significance of differences in group survival were determined using Fisher's exact test; NS = not significant

Residues are: Lipid A, 3-deoxy-D-mannooctulosonate, Heptose, Glucose Galactose, N-acetylgalactosamine.

TABLE 10: Protective efficacy of live-attenuated L1 and L3 *P. multocida* vaccines against heterologous challenge.

L1 vaccine strain	LPS structure on L1 vaccine strain	LPS structure on L3 challenge strain Pm1422	Protection (significance) [a,b,c]
VP161 *aroA*			100% (*P* = 0.0003)
VP161 *aroA/pcgC*			100% (*P* = 0.0003)
VP161 *aroA/gatA*			100% (*P* = 0.0003)
VP161 *aroA/hptE*			100% (*P* = 0.0003)
VP161 *aroA/gctB*			100% (*P* = 0.0003)
media only			25%
L3 vaccine strain	**LPS structure on L3 vaccine strain**	**LPS structure on L1 challenge strain VP161**	**Protection (significance) [a,b,c]**
P1059 *aroA*			70% (*P* = 0.0031)
P1059 *aroA*/*natB*			40% (NS)
P1059 *aroA*/*gatG*			80% (*P* = 0.0007)
P1059 *aroA*/*gatF*			0% (NS)
P1059 *aroA/gctC*			40% (NS)
media only			0%

[a] Challenge: 1.5 x 10^7 CFU *P. multocida* strain Pm1422 for L3 challenge or 1.9 x 10^3 CFU VP161 for L1 challenge. [b] Significance of differences in group survival were determined using Fisher's exact test; NS = not significant. [c] Groups of 12 birds were used for the Pm1422 challenge and groups of 10 birds for the VP161 challenge

Residues are: Lipid A, 3-deoxy-D-mannooctulosonate, Heptose, Glucose Galactose, N-acetylgalactosamine, Phosphocholine.

Discussion of Results

Development of a mPCR typing assay for *P. multocida* strains.

We initially tested a first generation LPS-mPCR for typing *P. multocida* strains on the basis of LPS genotype. This assay was highly reproducible and specific but was unable to type 17% of the field isolates tested. We used DNA sequencing to determine the reason for PCR failure in these strains and identified that in 10 of 11 strains PCR failure was due to mutations within the L3 biosynthesis locus. Therefore, we redesigned the mPCR oligonucleotides and produced a final LPS-mPCR (version 5) that was able to unambiguously type 57 of 59 (97%) strains. This multiplex PCR is ready for use as a diagnostic tool for differentiation of Australian *P. multocida* field isolates. The multiplex PCR primers have been transferred to Conny Turni and Pat Blackall for incorporation into their commercial typing regimen.

During the testing of the mPCR for differentiation of *P. multocida* isolates, we used mass spectrometry as the gold standard to assess LPS surface structures present on individual strains. These analyses indicated that a number of field isolates expressed LPS structures different from any of the structures present on the 16 Heddleston type strains. Indeed at least four additional LPS outer core structures were identified in these field isolates which resulted from mutations within other glycosyltransferase genes. Furthermore, a number of isolates produced more than one glycoform simultaneously. Given the importance of LPS structure to bacterin vaccine efficacy (see below) this has significant importance for vaccine strain selection. These data suggest that strains that produce multiple glycoforms may be better candidates for killed vaccines as they may show broader protective efficacy.

Comparison of efficacy and LPS-specificity of killed and live fowl cholera vaccines.

The vaccine trials presented here show that the protective efficacy of bacterins derived either from L1 or L3 strains is highly LPS-structure specific, with changes of one or more sugar residues abolishing protective efficacy. These data strongly suggest that birds vaccinated with bacterins are not protected from infections with strains expressing even slightly different LPS. Furthermore, this suggests that acute outbreaks in bacterin-vaccinated flocks likely arise following introduction of new strains that express different LPS, or following mutation of resident strains resulting in LPS changes. In both scenarios, our data indicate that LPS-specific antibodies raised against the bacterin strain will not be protective against the new strain. Furthermore, as protection afforded by *P. multocida* bacterins is both LPS-mediated and LPS structure-specific protective efficacy of currently held vaccine strains

against novel outbreak strains cannot be accurately predicted by LPS genotyping alone or by conventional serology. The "gold standard" we would recommend to accurately classify strains on the basis of LPS would be LPS-mPCR followed by LPS MS structural analysis. However, a more practical approach we propose would be LPS-mPCR followed by additional typing using monoclonal antibodies directed against each of the specific LPS structures. However, appropriate monoclonal antibodies are not currently available.

In direct contrast to bacterin vaccines, we have shown that protective immunity conferred by live vaccine strains is not dependent on exact LPS structure. Indeed, unlike the bacterin experiments, all groups vaccinated with live strains expressing truncated LPS were protected against wild-type homologous challenge. Therefore, vaccination with any of the live attenuated LPS mutants elicits solid protection against the parent strain expressing full length LPS. Furthermore, vaccination with any of the live L1 *P. multocida aroA*/LPS mutants elicited full protection against strains belonging to the L3 genotype regardless of the degree of LPS truncation present on the surface of the live vaccine strains. Additionally, strong protective immunity was also be elicited by some L3 strains against L1 challenge. This clearly demonstrates that protective immunity generated against live attenuated *P. multocida* vaccine strains is mediated by antibodies predominantly raised against other, conserved protein/carbohydrate antigens and not those raised against the variable portion of the LPS structure. We predict that the live vaccine strains (preferably lacking the variable outer core region of the LPS) will also be cross-protective against other *P. multocida* strains commonly found in poultry, e.g. those belonging to LPS genotype L6 (Heddleston serovars 10,11,12 and 15).

Implications

Development of a novel typing assay for the Australian Poultry industry.

We have developed a novel *P. multocida* typing assay based on the genetics of LPS biosynthesis. This assay is now available for use in any diagnostic laboratory but will be initially used in the current *P. multocida* serotyping laboratory at Agri-Science Queensland under the direction of Dr. Conny Turni. This novel typing assay will benefit the Australian poultry industry by allowing for more accurate and rapid strain diagnosis and differentiation. In the longer term we believe that this LPS-specific multiplex PCR assay will be accepted by poultry industries around the world as the most efficient means of effective strain differentiation.

Comparison of efficacy and LPS-specificity of killed and live fowl cholera vaccines.

This project has identified that bacterins derived from either VP161 (L1) or P1059 (L3) elicit solid protective immunity against challenge with the identical strain but that bacterins derived from strains expressing truncated LPS do not give protection against the parent strain expressing full length LPS. Therefore, protective immunity elicited by bacterins is exquisitely sensitive to LPS structure, indicating that birds vaccinated with bacterins are not protected from infections with strains expressing even slightly different LPS. These data strongly suggest that acute outbreaks in bacterin-vaccinated flocks likely arise following introduction of new strains that express different LPS, or following mutation of resident strains resulting in LPS changes. In both scenarios, LPS-specific antibodies raised against the bacterin strain will not be protective against the new strain.

Conversely, we have shown that protective immunity conferred by live strains is not dependent on exact LPS structure. Indeed, unlike the bacterin experiments, all groups vaccinated with live strains expressing truncated LPS were protected against wild-type challenge. Therefore, vaccination with any of the live attenuated LPS mutants elicits solid protection against the parent strain expressing full length LPS. This shows that vaccination with live strains will likely give protection against infection with strains belonging to the same LPS genotype as well as some degree of protection against unrelated strains.

Recommendations

The Australian poultry industry still uses the Heddleston serotyping scheme to guide vaccination programs. However, we have shown here that Heddleston serotyping is unreliable as it fails to predict the correct LPS glycoform 66% of the time. Furthermore, we have also shown that there are many more LPS glycoforms than represented by the original 16 Heddleston serovars (8), many strains express more than one glycoform at a time and that there is no cross-protection elicted by killed vaccine strains against strains with different LPS. Therefore, reliance on Heddleston serology to guide bacterin vaccination programs is flawed. In this project we have developed a novel typing system based on an LPS-specific multiplex PCR (LPS mPCR) that groups *P. multocida* into 8 LPS genotypes, L1-L8. This mPCR is more reproducible, efficient and accurate than the Heddleston serotyping scheme. However, it also cannot detect LPS structural differences within strains belonging to the same LPS genotype. As an example, LPS genotype L3 contains strains that express at least 6 different LPS structures, all produced from a single LPS locus. Some LPS variation is due to random mutations in LPS genes, but the phenomena of multiple LPS structures within a single strain may also arise due to post-translational mechanisms (8). Therefore, on its own LPS mPCR can also not be used to guide strain selection for bacterin vaccination programs.

The original Heddleston serovar 3 and 4 type strains belong to the L3 genotype but express different length LPS structures. Our data indicate that a bacterin raised the Heddleston 3 type strain will not cross-protect against the Heddleston serovar 4 type strain. Therefore, we recommend for accurate LPS structure determination (essential if the poultry industry continues to use killed bacterin vaccines) the LPS-mPCR should be employed for initial strain differentiation followed by a secondary test to determine which of the specific LPS structures is being expressed. Currently mass spectrometry is the gold standard for accurate assessment of LPS structure however this method is too complex and costly for routine analysis.

With further funding it would be possible to develop a rapid and cheap test to identify LPS structure expressed by *P. multocida* strains. The generation of monoclonal antibodies specific for each of the outer core LPS structures commonly present on Australian isolates would allow a diagnostic test to be developed that combined LPS mPCR with LPS mAb serotyping. Recent closure of government laboratories and the overload makes it vital to shift, diagnostic tests to a platform that is effective and time efficient, such as Luminex technology (bead based technology), available in many diagnostic laboratories. Using the LPS mPCR together with an LPS mAb test would allow precise definition of the LPS

structure expressed by any strain and identify the appropriate strains for bacterin vaccine formulation.

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Appendix A

LPS multiplex PCR (LPS mPCR) - Standard Operating

1. Principle:

The lipopolysaccharide (LPS) multiplex PCR (LPS mPCR) is a polymerase chain reaction performed in standard 0.2 ml PCR tubes with 8 pairs of primers that amplify DNA from the LPS outer core biosynthesis locus on the *Pasteurella multocida* genome. Each primer pair in the reaction mix is specific to one of the eight unique LPS outer core biosynthesis loci, named L1 through to L8. Each amplified product generates a unique-sized fragment that can be detected and differentiated following electrophoresis of the PCR products on a 0.8% Agarose Tis-acetate-EDTA (TAE) gel stained with ethidium bromide or SYBR® Green (Life Technologies).

The LPS mPCR procedure includes:

- amplification of the LPS-specific DNA using primers specific to the LPS outer core loci
- detection of amplified products using agarose gel electrophoresis.
- Comparison of amplified products with commercial DNA ladders and standard LPS-amplicons representing all 8 LPS loci.

2. Reagents

***P. multocida* strain preparation:** Streak each of the test isolates and the Heddleston serovar type strains onto Heart Infusion media (or other suitable media) solidified with 1.5% agar. Incubate overnight at 37°C.

***P. multocida* genomic DNA preparation:** For routine testing, genomic DNA is the preferred template for the reference PCR reactions. Isolate genomic DNA from Heddleston serovar type strains representing each LPS genotype (Table 1) using a standard genomic DNA isolation kit. Store in aliquots at -20°C.

Table 1. Heddleston type strains to be used as controls for each LPS genotype

LPS Genotype	Heddleston serovars	Heddleston type strains
L1	1 and 14	X73 and P2225
L2	2 and 5	M1404 and P1702
L3	3 and 4	P1059 and P1662
L4	6 and 7	P2192 and P1997
L5	9	P2095
L6	10, 11, 12 and 15	P2100, P903, P1573 and P2237
L7	8 and 13	P1581 and P1591
L8	16	P2723

Primer Stocks (100 μM): Prepare a 100 μM stock of each primer (Table 2) using 10 mM Tris-HCl pH 8.0 as diluent. For long term storage of diluted primer, make 50 μl aliquots and store at -20°C.

Primer Set Working Mix (Contains 0.625 μM of each primer): Add 5 μl of each stock primer (16 different primers) to 720 μl 10mM Tri-HCl pH=8.0. A volume of 32 μL of working primer mix is used per 50 μl reaction.

Store primer aliquots at -20°C until use.

Table 2. LPS mPCR primer details

LPS Genotype	Primer	Primer sequence 5'-3'	PCR product size (bp)
L1	BAP 6119 BAP 6120	ACATTCCAGATAATACACCCG ATTGGAGCACCTAGTAACCC	1307
L2	BAP 6121 BAP 6122	CTTAAAGTAACACTCGCTATTGC TTTGATTTCCCTTGGGATAGC	810
L3	BAP 7213 BAP 7214	TGCAGGCGAGAGTTGATAAACCATC CAAAGATTGGTTCCAAATCTGAATGGA	474
L4	BAP 6125 BAP 6126	TTTCCATAGATTAGCAATGCCG CTTTATTTGGTCTTTATATATACC	550
L5	BAP 6129 BAP 6130	AGATTGCATGGCGAAATGGC CAATCCTCGTAAGACCCCC	1175
L6	BAP 7292 BAP 7293	TCTTTATAATTATACTCTCCCAAGG AATGAAGGTTTAAAAGAGATAGCTGGAG	668
L7	BAP 6127 BAP 6128	CCTATATTTATATCTCCTCCCC CTAATATATAAACCATCCAACGC	931
L8	BAP 6133 BAP 6134	GAGAGTTACAAAAATGATCGGC TCCTGGTTCATATATAGGTAGG	255

Deoxynucleotide (dNTPS)

Dilute commercially supplied dNTPs, either individually supplied or pre-mixed, in molecular biology grade H_2O to give a working concentration of 10 mM for each dNTP.

Taq DNA Polymerase - 5 units/μl. **Taq DNA Polymerase Buffer (commercially supplied):** 10 x Taq DNA polymerase buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl2) pH 8.3 @ 25°C

3. Multiplex PCR setup

Control PCR reactions: Use Table 1 to select the appropriate Heddleston serovar type strains that represent each of the 8 genotypes for amplification of DNA for control PCR reactions. Genomic DNA isolated from these strains can be used as template DNA instead of cell lysates. Sufficient control PCR reactions should be performed so that a product is generated that represents each LPS outer core biosynthesis loci (L1-L8).

Preparation of PCR master mix (1 sample):

Reagent	Volume (μl)		Final concentration/units
	Method A	Method B	
H_2O	11.7	10.7	
10 x Taq DNA Pol. Buffer	5	5	
dNTP mix (10 mM each)	1	1	0.2 mM
Primer mix (0.625 μM each)	32	32	0.4 μM of each primer
Taq DNA Pol. (5U/μl)	0.3	0.3	1.7 U

PCR Template preparation:

Note: Multiplex PCR can be performed with either colony material as template (method A) or cell lysate/purified genomic DNA as template (method B).

Method A: Colony PCR

- Choose 2 or 3, well isolated, *P. multocida* colonies from a fresh plate-culture. Insert a sterile 20 μl micropipette tip attached to pipettor (set to a 20 μl volume) into the middle of the colony. Place pipette tip containing cells into the 50 μl pre-prepared PCR mix and pipette up and down 3 times to ensure mixing of cells into the reaction.

- Vortex each reaction tube then spin briefly to ensure all liquid is at the bottom of the tube.

Method B: PCR using cell lysate/genomic DNA

- Using a sterile 1μl loop pick up bacterial material from 1 colony and resuspend in 200 μl molecular biology grade H_2O.
- Vortex briefly, heat at 98-100°C for 5 min.
- Spin at 13,000 x *g* for 5 min.
- Add 1 μl cell lysate (alternatively use 1 μl purified genomic DNA, approx. 50 ng) to each PCR reaction.
- Vortex each reaction tube then spin briefly to ensure all liquid is at the bottom of the tube.

PCR reaction conditions:

Method A: Colony PCR

1 cycle:	96°C for 10 minutes.	
30 cycles:	96°C for 30 sec, 52°C for 30 sec, 72°C for 2 min and 30 sec	
1 cycle:	72°C for 5 min	Holding cycle: 4°C

Method B: Standard PCR

1 cycle:	96°C for 5 minutes.	
30 cycles:	96°C for 30 sec, 52°C for 30 sec, 72°C for 2 min and 30 sec	
1 cycle:	72°C for 5 min	Holding cycle: 4°C

4. Agarose gel electrophoresis

- Prepare 1.5% Agarose gel in 1 x TAE buffer (40mM Tris, 20mM acetic acid, and 1 mM EDTA. pH 8.0).
- To 50μl of each PCR reaction add 10 μl of 6 x Gel Loading Dye.
- Load 12.5-20μl of each control and test sample.
- Load 5 μl of 1kb DNA ladder (or similar) as molecular weight markers.
- Electrophorese in 1x TAE buffer at 80-90 volts for at least 1 hour or until bands are well separated.