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: Dr Conny Turni
QAAFI, The University of Queensland

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Development of Matrix-Assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF) for serotyping *Pasteurella multocida*

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Researcher Contact Details

Conny Turni,
Queensland Alliance for Agriculture and Food Innovation,
The University of Queensland, Ecosciences Precinct,
41 Boggo Rd, Dutton Park, QLD 4102
Phone 07 3255 4296
Email: c.turni1@uq.edu.au

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

Poultry CRC Ltd Contact Details

PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

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Executive Summary

Respiratory disease caused by bacterial pathogens cause significant losses in the livestock industries including the poultry industries. Autogenous vaccines are typically used to control these pathogens but provide protection only for the serovar in the vaccine. Hence, to produce effective vaccines, the serovar of the causative bacterium needs to be known. Until recently serotyping was still done the old fashioned way by raising antisera in rabbits to the different reference strains of the relevant species.

Knowledge gained through studies on *Pasteurella multocida* from fowl has led to an extensive development of advanced molecular biology-based typing methods, such as high resolution melt analysis, Multi Locus Sequencing Typing (MLST) and Repetitive Element Palindromic PCR (rep-PCR). A recent collaborative project involving our group has confirmed that none of these molecular methods can replace conventional serotyping. In particular, the latest assay, a multiplex PCR that targets the biosynthetic locus of the lipopolysaccharide (LPS) can only separate the 16 serovars into 8 LPS types. Vaccination studies have highlighted that subtle changes in the LPS structure can lead to a lack of protection. This understanding has underpinned the search for an alternative – with the alternative explored in this work being matrix-assisted laser desorption/ionizations time-of-flight mass spectrometry (MALDI-TOF) – a method that directly examines the structures that are responsible for the serovar and immunovar specificity seen in this organism. The advances in mass spectrometry in recent years have brought this technique into the diagnostic world with MALDI-TOF now being used by many front line diagnostic laboratories to identify bacteria at the species level. As well, MALDI-TOF has the potential to be used in place of serotyping by differentiating the lipopolysaccharides (LPS) of the different serovars of a bacteria species. Publications have already proven that the structure of the LPS can be elucidated via MALDI TOF for *Vibrio fischeri*.

This project was designed to develop a method to differentiate the LPS structure of *P. multocida* and then validate the method on an extensive culture collection of field isolates with known serovars and known genetic profiles (as determined by MLST). The first step in the development of this diagnostic method was optimizing the extraction of the LPS from *P. multocida*. Initial work involved the use of a commercial extraction kit and then moved to several published methods for LPS extraction for *P. multocida*. Optimization of all extraction methods used was undertaken. Analysis by the MALDI-TOF method to differentiate the LPS structures was then attempted on the optimized extraction method. This analysis was started with a variety of solvent buffers and extended to cation exchange until optimization was achieved. The final developed method was then used on reference strains for *P. multocida*. While differentiation of the reference serovars was

achieved, the difference in the MALDI-TOF spectra peaks was not very distinct with serovars often only differing slightly in peak heights. Only a single strain per serovar (the reference strain) was used and hence no variation within the serovars was included. Given that the optimal method could only detect minor differences across these single reference strains, the diversity known to occur in the structure of the LPS of the filed isolates would overwhelm the differentiation detected in the reference strains. Hence, it was concluded that this method is not suitable for routine diagnostic analysis. Further MALDI-TOF analyses of a collection of Australian field isolates was to be undertaken both from chicken and pigs, to represent the diversity of Australian strains. This final step was not taken.

However, the isolates were serotyped and genotyped by three methods – A) LPS multiplex genotyping - which separates the 16 serovars into 8 LPS genotypes of 16 serovars; B) rep-PCR, which determines the number of strains in the collection and C) multilocus sequence typing (MLST), which makes comparison to the rest of the world possible - to look at the variability of the isolates. The serotyping and genotyping gave us the knowledge of the variability of *P. multocida* in the Australian poultry in comparison to *P. multocida* in pigs and to the rest of the world and other animal species that harbour *P. multocida* (multilocus sequencing results are on a public database). This has provided an overview of the population structure of *P. multocida* in Australia.

In summary, there was a large diversity in the chicken isolates of *P. multocida* and some of these isolates were shared with pig isolates indicating that both species are reservoirs for each other. The MALDI-TOF method could not be optimized to yield the required differentiation of the serovars necessary to separate field isolates.

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Introduction

Pasteurella multocida, the causative agent of fowl cholera, belongs to the family *Pasteurellaceae,* a family whose members are amongst some of the main causes of highly infectious diseases that negatively impact on livestock industries. As well, *P. multocida* is part of the flora of a wide range of animals and can infect humans (Wilson & Ho, 2012). *P. multocida* colonizes the nasopharynx, respiratory and gastrointestinal tracts of many animals and induces a disease known as pasteurellosis. In poultry *P. multocida* causes fowl cholera, which is a serious disease with clinical signs that include depression, ruffled feathers, fever, anorexia, mucous discharge from the mouth, diarrhea, an increased respiratory rate, septicaemia and death (Harper et al., 2006). In pigs, *P. multocida* is regarded as an opportunistic pathogen and forms part of the pig respiratory disease complex. This coinfection makes it hard to attribute symptoms due to *P. multocida*, as the clinical signs and lesions are normally superimposed on that of the primary agent. The symptoms usually involve chronic occasional cough, laboured breathing and failure to grow (Brockmeier et al., 2002). However, some signs have been solely attributed to pasteurellosis, which are the shortening and twisting of the snout, dark tear staining and pneumonia (Harper et al, 2006)

In the Australian poultry industry, killed (autogenous and commercial) vaccines are commonly used. Killed vaccines only give homologous protection against the serovar or serovars that are in the vaccine. Hence establishing the serovar of the disease causing isolate is of importance. The conventional serovar scheme used to serotype *P. multocida* is the Heddleston serotyping scheme, a traditional gel diffusion immune-precipitation technique, which requires raising antisera (traditionally in chickens) to the different serovar reference strains. Recent advances in molecular typing method have led to a shift to PCR based serotyping. A multiplex PCR targeting the lipopolysaccharide (LPS) structure of *P. multocida* has been developed. However, this PCR cannot differentiate all 16 serovars and only recognises eight different LPS types (L1 to L8) (Harper et al., 2014).

The Heddleston serotyping scheme, when performed by the classic methodology, has come under scrutiny for the following reasons – A) not being repeatable and B) some isolates cannot be typed. While the LPS PCR overcomes these issues, it has the disadvantage that it cannot differentiate between the most common serovars in poultry, serovar 3 and 4. Hence, the focus has been on using different genotyping techniques to differentiate *P. multocida* to help in specific selection of isolates that need to be included in flock specific vaccines. The main genotyping method in our laboratory is the DNA fingerprinting method known as the repetitive element palindromic polymerase chain reaction (rep-PCR). This method allows the characterisation of the genetic variability of the strains

within a population, and allows recognition of clusters or groups of strains associated with high mortality in animals (Townsend et al., 1997). The representative isolates from each group can then be selected to be included in the autogenous vaccines, and genotyping is later used to monitor the emergence of any new strain in the flock. Another typing method routinely used by laboratories for epidemiological studies is known as Multi-locus sequence typing (MLST), a sequence based methodology which makes it possible to compare typing results from laboratory to laboratory using a centralized web-based databank (Subaaharan et al., 2010). More importantly, the MLST database contains vital information on the geographical distribution of strains of *P. multocida*, and it is therefore possible to track the global spread of these strains online via the link (http://pubmlst.org/pmultocida_rirdc/).

Recent advances in mass spectrometry such as MALDI-TOF present a promising alternative to traditional serotyping. This technology is a rapid and inexpensive method, which is widely used in clinical or microbiological laboratories for bacterial identification to species level (Kuhnert et al., 2012; Seng et al., 2009). Known for its soft ionization method, MALDI-TOF is based on either the detection of proteins and peptides or the detection of nucleic acids resulting in a specific profile/fingerprint that is compared against a database for identification (Sauer & Kliem, 2010; Seng et al., 2009). Moreover, this technology has also been applied in various studies to characterise the different LPS and the lipo-oligosaccharides (LOS) of gram negative bacteria such as *Vibrio* species (Pupo et al., 2004).

The present study has focused on developing a method using MALDI-TOF technology to separate the different serovars of *P. multocida*. The aim was to develop the most suitable extraction method and then use this method to produce LPS for the analysis by MALDI-TOF. The method of extraction and analysis was then to be validated on all reference strains plus field strains from chicken and pigs to allow for the diversity of *P. multocida*. Pig isolates were also targeted for use to help understand the possible diversity of strains in Australian livestock.

Objectives

The objective was to develop a MALDI-TOF method for the differentiation of the serovars of *P. multocida*. This involved the optimization of an extraction method for MALDI-TOF and then optimizing the developed MALDI-TOF methodology and validating it on reference strains and field strains. For this genetically different field isolates were collected from chicken and pigs after analyses of their variation.

Methods

Bacteria

This study utilised the 16 serovar reference strains plus 41 field isolates from poultry and 43 field isolates from pigs (Table 1). The field isolates were collected from diseased animals and were submissions to DAF/QAAFI reference diagnostic services. The field isolates were selected for their diversity, being from different farms and different serovars.

Table 1. Reference strains and field isolates of *Pasteurella multocida* used in this project.

Reference strains			
Reference strains	**Reference strain no**		**Heddleston serovar**
PM 492	X 73		1
PM 498	M 1404		2
PM 486	P 1059		3
PM 499	P 1662		4
PM 494	P 1702		5
PM 500	P 2192		6
PM 487	P 1997		7
PM 495	P 1581		8
PM 497	P 2095		9
PM 493	P 2100		10
PM 488	P 908		11
PM 501	P 1573		12
PM 485	P 1591		13
PM 491	P 2225		14
PM 496	P 2237		15
PM 489	P 2723		16
Chicken Isolates		**Pig Isolates**	
NO	**Heddleston serovar**	**NO**	**Heddleston serovar**
1	3	1	NT
2	15	2	14, 15
3	3	3	12, 10
4	6	4	1, 8, 9
5	3	5	NT
6	NT	6	3
7	9	7	10
8	NT	8	10
9	3	9	NT
10	1,4	10	NT
11	NT	11	4
12	NT	12	4
13	1	13	13, 15, 16
14	NT	14	4, 14
15	NT	15	NT
16	NT	16	NT
17	NT	17	NT
18	13,8	18	NT
19	1	19	14
20	NT	20	NT
21	1, 4	21	NT
22	3	22	NT
23	1	23	14
24	NT	24	NT
25	10, 13, 14	25	NT
26	1	26	NT
27	1	27	NT
28	NT	28	10
29	NT	29	1
30	4	30	1, 4
31	NT	31	3, 4
32	2	32	NT
33	NT	33	3
34	14	34	1, 3
35	NT	35	NT
36	14	36	NT
37	14	37	NT
38	3	38	NT
39	1	39	NT
40	3	40	NT
41	12	41	NT
		42	NT
		43	NT

Identification and serotyping of *P. multocida*

The *P. multocida* isolates were identified by a species-specific PCR (Townsend et al., 1998) and serotyped with the Heddleston serotyping scheme as described previously (Heddleston et al., 1972). The LPS multiplex PCR, which targets the LPS outer core biosynthesis locus, developed by Harper et al. (2015) was used to assign isolates to one of the eight LPS types (termed L1 to L8). The genomic DNA for this PCR was prepared using the PrepMan Ultra Sample Preparation Reagent according to manufacturer's protocol (Life Technology – Applied Biosystems, Warrington, UK).

Genotyping by rep-PCR

A rep-PCR was performed according to Gunawardana et al. (2000) using DNA extracted by the PrepMan Ultra kit. The Bionumercis software (Bionumeric version 4.50, Applied Maths Inc, Saint-Martens-Latem, Belgium) was used to analyze the gel. If two isolates had the same genomic fingerprint, i.e. an identical band pattern including size and intensity, they were assumed to be the same strain. Within each rep-PCR genotype, a representative isolate was subjected to MLST.

Multi-locus sequence typing (MLST)

MLST was performed on each representative isolate according to Subaaharan et al. (2010), which is based on sequencing 450 to 500 base pair (bp) internal fragments of seven housekeeping genes. Each different sequence is assigned a distinct allele (a number) and the combinations of the alleles define the sequence type (ST). The RIRDC *P. multocida* MLST website (http://pubmlst.org/pmultocida/) was developed by Jolley et al. (2004) and is sited at the University of Oxford. The development of this site has been funded by the Wellcome Trust.

LPS extraction

Several methods for extraction of LPS were explored with the commercial kit (Intron) being Method 1. A significant impediment to most older extraction methods was the use of phenol. Due to the health risk associated with phenol, the use of phenol is discouraged in most laboratories. Hence, alternatives to this method were used. The Intron kit (Method 1) still contained phenol, but in very small quantities. Method 2 was a very crude extraction using only proteinase K. Method 3 was a combination of old laboratory methods and published methods and Method 4 was adapted from Pupo et al. (2004).

For each method the culturing and harvesting of bacterial cells was optimized for LPS yield and quality except that the kit was used according to manufacturer's instructions.

Culturing of bacteria to obtain 10^9 bacterial cells for Method 1 and 2

Bacteria were revived from storage onto 5% sheep blood agar (BA) plate and incubated for 24 hours at 37°C. After incubation, the bacteria were harvested from the BA and suspended into 25 ml of saline. This step was optimized, as the bacterium was at the beginning suspended into 50 ml of saline. From this saline suspension (approximately McFarland 0.5), 5 ml was transferred into 100 ml of nutrient broth and incubated for 24 hours at 37°C. After incubation 50 ml of the broth was centrifuged at 5,000 g for 10 mins and 45 ml of the supernatant removed. This step had to be optimised as the original method recommended to take only 1 ml, spin that down and remove 900 µl supernatant. After taking off the 45 ml supernatant the pellet was resuspended in the 5 ml left in the tube. Of this suspension 1 ml was used for OD reading, which should be between 0.8 – 1.2.

Method 1: Extraction of LPS using the Intron Kit

From the final culturing step, 3 ml of the bacterial cells was re-centrifuged at 13,000 g for 5 mins. The supernatant was removed and the pelleted cells loosened by tapping the tube. The LPS extraction kit (Intron Biotechnology, Korea) was used for extraction of LPS. To the loosened pellet, 1 ml of lysis buffer was added and then vigorously vortexed until the cell pellet was suspended. Next, 200 µl of chloroform was added which was again vigorously vortexed for 10 – 20 sec and incubated at room temp for 5 mins. After incubation, the solution was centrifuged for 10 mins at 4°C and 400 µl of supernatant were transferred to a new 1.5 ml tube and 800 µl of purification buffer added. After mixing well, the suspension was incubated for 10 mins at -20°C. After incubation the solution was centrifuged for 13,000 g for 15 mins at 4°C and the upper layer removed. To this upper layer, 1 ml of 70% HPLC grade ethanol was added and the pellet washed by inverting the tubes 2 – 3 times. This suspension was then centrifuged for 3 mins at 13,000 g at 4°C. The upper layer was discarded and the remaining pellet dried for 2 hours in a biosafety cabinet. The pellet was stored at -20°C until use.

Method 2: Extraction of crude LPS with proteinase K

From the final culture step, 100 µl of bacterial cells were spun for 2 mins at 13,000 g and the pellet resuspended in 36 µl of ultrapure water. Next, 4 µl of proteinase K (50 µg/ml) was added and this was incubated for one hour at 60°C. Next, 40 µl ultrapure water was added and the solution stored at -20°C until use.

Method 2 modified: Extraction of crude LPS with proteinase K

From the final culture step, 1 ml of bacterial cells were spun for 2 mins at 13,000 g. The pellet was then washed twice in 1 ml of ultrapure water. The pellet was resuspended in 36 µl of ultrapure water. Next, 4 µl of proteinase K (50 µg/ml) was added and this was incubated for one hour at 60°C. The solution was boiled for 5 mins and then spun at 13,000 g for 5 mins and the supernatant was added to a new tube and stored at -20°C until use.

Method 3: Extract crude LPS with formic acid, acetronitrile and proteinase K

The *P. multocida* strain was plated onto BA and incubated overnight at 37°C. A 1 ul loop of bacterial colonies was harvested into 200 µl of 80% ethanol and mixed. After 2 mins centrifugation at 13,000 g, the supernatant was discarded and pellet dried for 20 mins at room temperature. The weight of the pellet was recorded and 30 µl of 70% formic acid was added and the tube vigorously mixed. To this 30 µl of 100% acetonitrile was added and again vigorously mixed. The solution was then centrifuged for 2 mins at 13,000 g and the supernatant discarded. The pellet was incubated with proteinase K (50 µg /ml) at 60°C for 60 mins with a ration of 20 ug of enzyme per 10 mg of the bacterial mass. After incubation, the suspension was centrifuged at 13,000 g for 10 mins and the supernatant discarded. The precipitate was rinsed with 200 µl of ultrapure water twice and the supernatant discarded and the pellet dried in the hood at room temperature. Samples were stored at -4°C until used.

Method 4: LPS extraction according to Pupo et al. (2004)

The *P. multocida* strain was revived onto BA and incubated at 37°C overnight. About half the plate was harvested with a swab into 2 ml of PBS to an optical density of 0.9 at 650 nm. The suspension was centrifuged at 13,000 x g for 5 mins . The pellet was washed twice with 1 ml of PBS. Then the pellet was resuspended in 200 ul of lysing buffer (0.06 M Tris-base, 10 mM EDTA, 2.0% SDS, pH 6.8) and incubated in boiling water for 10 mins. After allowing cooling, 30 µl of proteinase K solution (2.5 mg/ml diluted in lysis buffer, Sigma) was added to 150 µl of boiled sample. This solution was incubated at 37°C for 16 – 24 hours. After this incubation, 1/10 volume of 3 M sodium acetate and two volumes of 100% ethanol were added and held at -80°C for 60 mins. The samples were then centrifuged at 15,000 x g for 5 mins and then washed twice with 70% ethanol and brought to a final volume of 180 µl in ultrapure water. Samples were stored at -20°C until use. Samples were stored at -20°C until used.

SDS-PAGE and Silver staining

The LPS pellet was dissolved in 40 ul of sample buffer (2X) according to the instruction of the Pierce Color Silver Stain Kit (ThermoScientific, Rockford, USA). Pellets were dissolved by boiling the samples. For non-pellet methods 3 and 4, 40 µl of sample were taken and processed. Mini gels were used for the SDS-PAGE gel run (Precise protein gel 4-20%, ThermoScientific, Rockford, USA) and 20 µl of sample was added in each well. A pre-stained protein molecular weight marker (Thermofisher, Luthuenia) was run with the samples. The gel was run at 100 V for 60 mins and then silver stained according to manufacturer's instructions, which was 30 mins in silver WS, 20 sec water rinse, 5 min in reducer WS, 5 sec rinse and 40 mins in stabilizer.

Preparation for MALDI TOF-MS

Several solvents were used to disolve the pellet, either in EDTA solution, in the sample buffer provided with the silver stain kit or in ultra pure water. The final decision was to dilute in ultra pure water (200 µl).

A desalting approach was also applied using DOWEX 50WX8-200 cation exchange resin (Sigma Aldrich). A ziptip was modified to contain 5 µl DOWEX beads above the frit. Prior to analysis the Dowex ziptip was flushed with 20 ul of 50 mM ammonium hydroxide, then 10 µl sample was applied to the top, the first 2-3 ul was discarded and then approximately 0.5 µl was applied to the prepared 0.5 M 2,5 dihydroxybenzoic acid (DHB) in methanol matrix solution spot as the droplet emerged from the ziptip.

A variety of matrixes were used including α-cyano-4-hydroxycinnamic acid (CHCA), a mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid (SDHB), sinapinic acid (THAP) and 2,5 dihydroxybenzoic acid (2,5 DHB). These matrices were applied in a number of different ways, including co-spotting (mixing sample plus matrix solution) and spotting sample onto the dried matrix, the latter was typically better for these samples in terms of getting a response. We also tried thin layer preparations, but with less success as the thin layer was generally ruined during sample application. We investigated metal chelating agents, which were variable in their effect. The MALDI-TOF analysis was done both in negative and positive mode.

Final Method including MALDI-TOF conditions

A saturated solution of DHB in acetone was diluted 1 in 10 with a solution of acetone:acetonitrile:water (6:3:1), and 1 µl of this dilution was spotted onto a polished steel target. After drying, 0.5 µl of sample was spotted over the top of the DHB crystals. Once the samples were

dry, the samples were analysed in linear positive mode MALDI TOF using a Bruker Ultraflex III over a mass range of 700 – 5000 collecting 2000 shots.

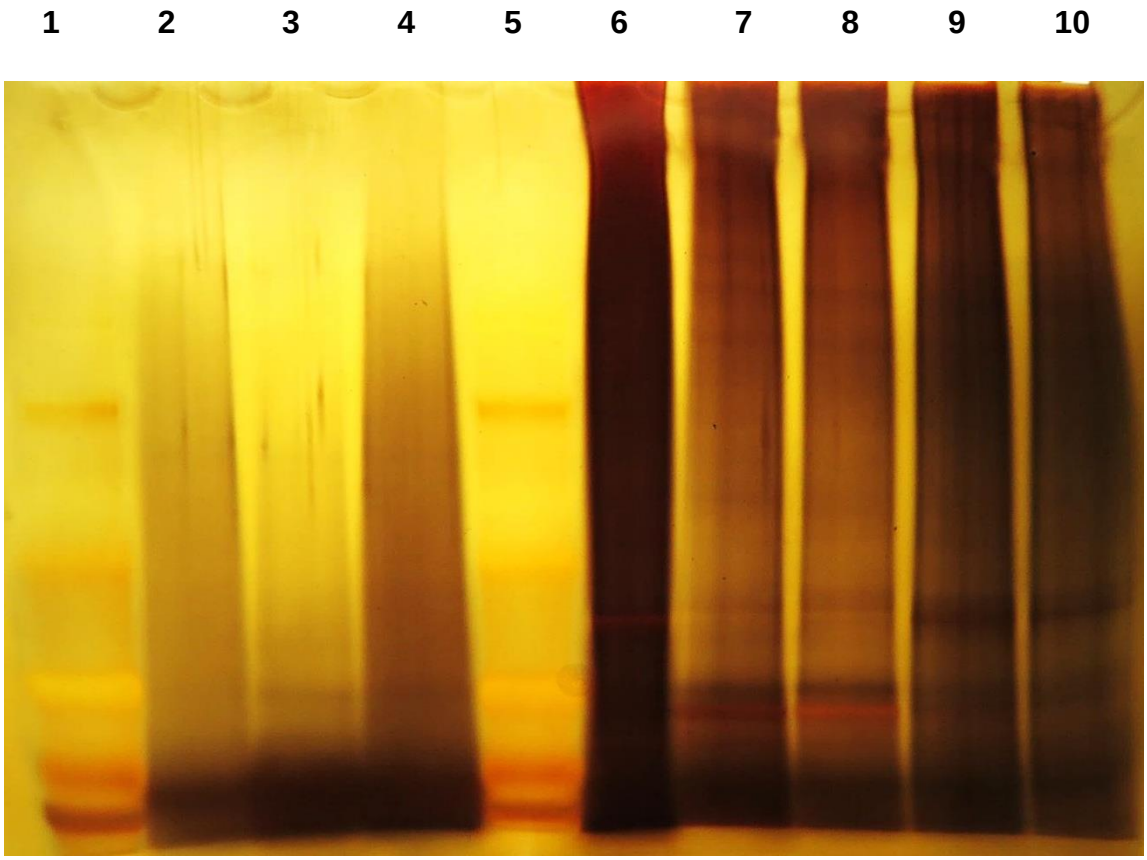
Results

MALDI-TOF

The extraction with the intron kit (Method 1) seemed to give a very weak yield compared to the other methods and despite attempts to increase the yield it was deemed largely unsuccessful (Figure 1). This poor yield also gave no peaks in the MALDI- TOF profile, despite every attempt at optimization.

Method 2 was a very crude extraction and had background in the larger band sizes, which might still be related to proteins (Figure 1).

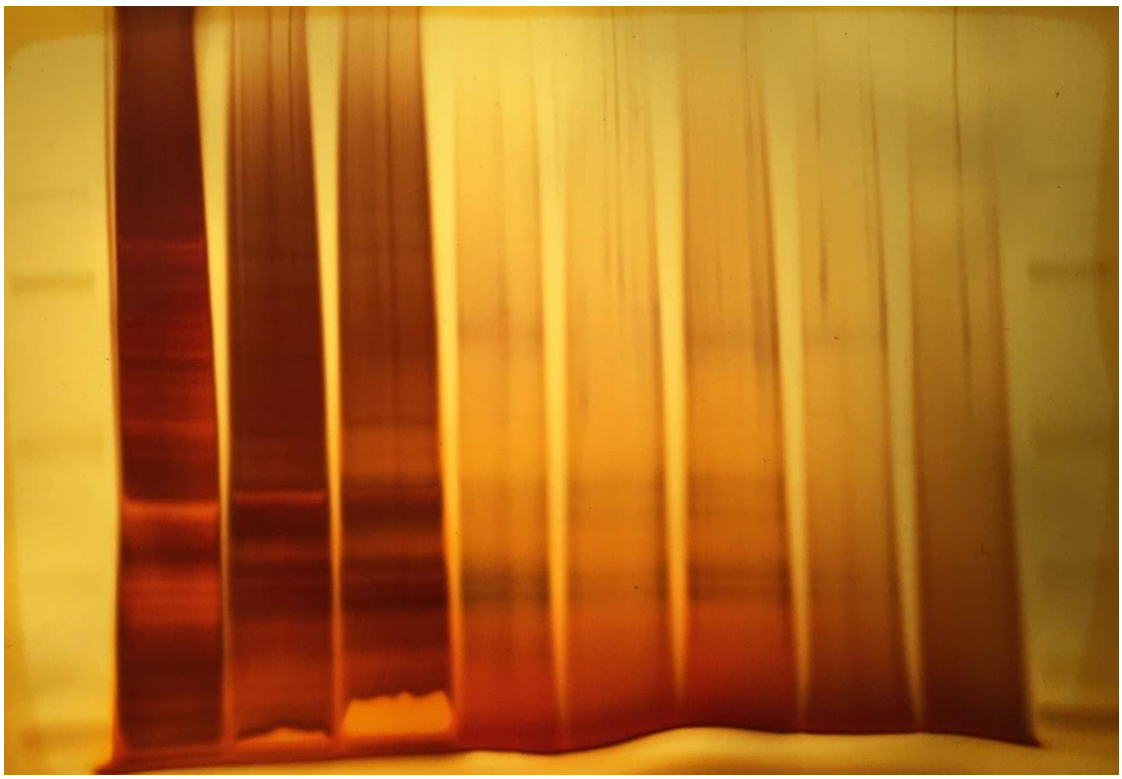
Figure 1. Comparison of the LPS extraction via the Intron kit (Method 1) and via Method 2 displayed in a silver stained gel. Lane 1 - Ladder; 2 - PM 1447 extracted by Intron kit (Method 1); 3 - PM 1628 extracted by Intron kit (Method 1); 4 - PM 1869 extracted by Intron kit (Method 1); 5 - Ladder; 6 - PM 1447 (Method 2); 7 - PM 1628 (Method 2); 8 - PM 1869 Method 2; 9 - PM 1447 1 in 5 dilution; 10 - PM 1447 (Method 2) 1 in 2 dilution.



Method 3 yielded LPS but still had a lot of protein bands despite the Proteinase K treatment (Figure 2).

Figure 2. Gel picture of silver stained gel displaying three isolates of *P. multocida* extracted by Method 3. The lanes contain the following: Lane1 - Ladder; 2 - PM 1447; 3 - PM 1628; 4 - PM 1869; 5 - PM 1447 1 in 10 dilution; 6 - PM 1628 1 in 10 dilution; 7 - PM 1869 1 in 10 dilution; 8 - PM 1447 1 in 5 dilution; 9 - Pm 1628 1 in 5 dilution; 10 - Ladder.

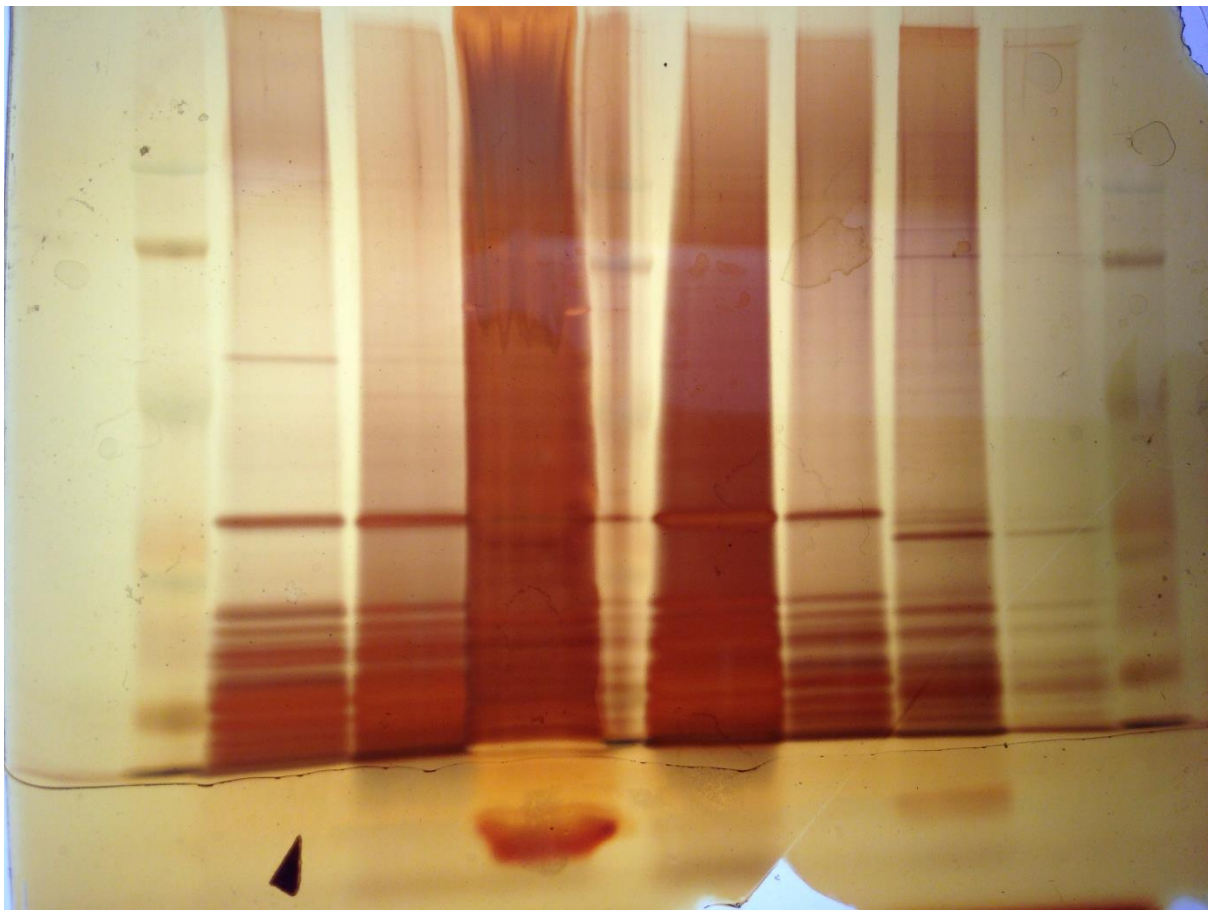
1 2 3 4 5 6 7 8 9 10



In the comparison of both versions of Method 2 - the original which started off with 100 µl of culture and the modified version which started off with 1 ml of culture – both supernatant and pellet were examined. This examination revealed that the pellet had some bands in the LPS region but only a small amount compared to the supernatant (Figure 3).

Figure 3. Comparison of Method 2 and the modified Method 2. Lane 1 – Ladder; 2 - PM 1447 (Method 2, original with additional proteinase K (i.e. 40 µl)); 3 - PM 1628 (Method 2, original with additional proteinase K (i.e. 40 µl)); 4 – PM 1447 pellet (Method 2 modified); 6 - PM 1447 (modified Method 2); 7 - PM1447 (original Method 2); 8 - PM 1628 (modified Method 2); 9 - PM 1628 (original Method 2); 10 – Ladder.

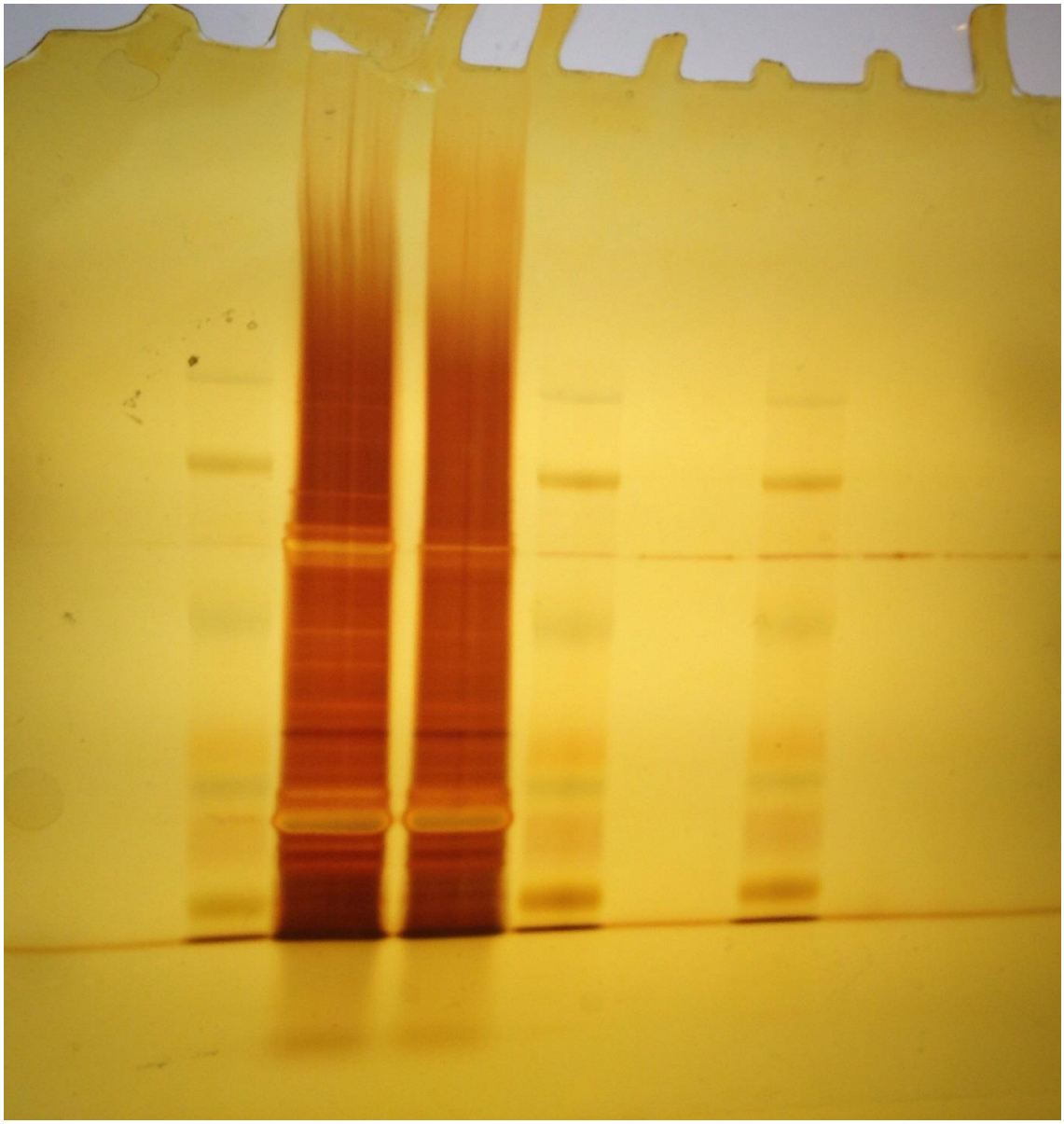
1 2 3 4 5 6 7 8 9 10



When comparing heating and not heating after resuspending the pellet in Method 4, it was found that the heating did not make a significant difference (Figure 4).

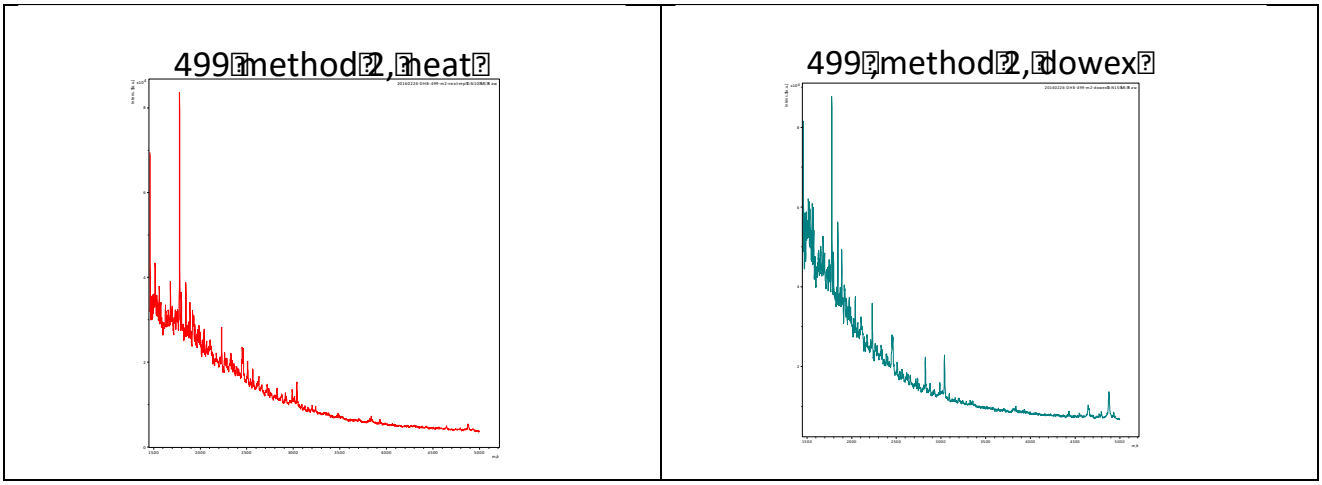
Figure 4. Comparison of heating and not heating after suspension of pellet for Method 4. Lane 1 - ladder, 2 - PM 1447 (Method 4 non- heated); 3 - PM 1447 (Method 4 heated); 4 – ladder; 5 - PM 1447 (Method 3); 6 – ladder.

1 2 3 4 5 6



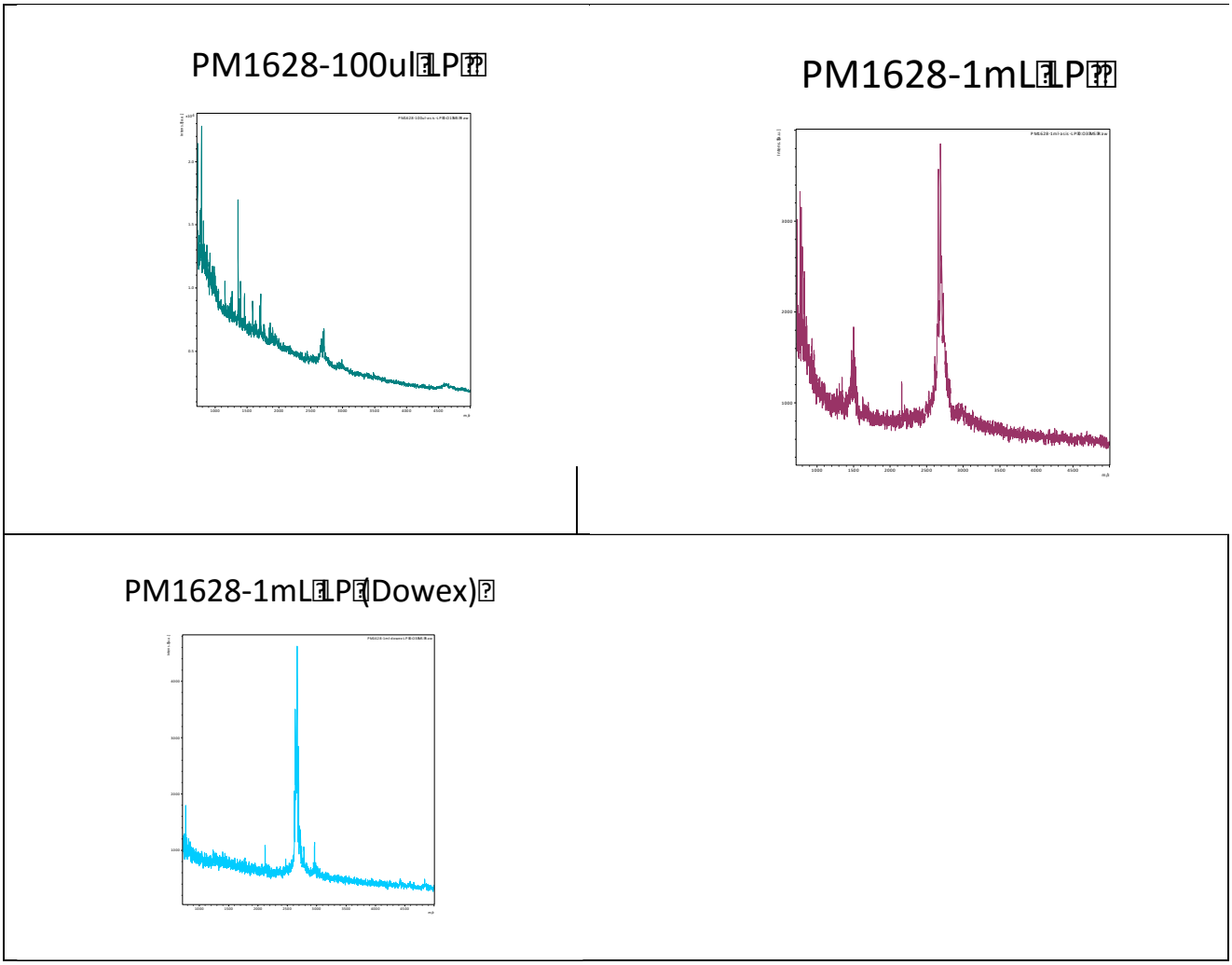
Method 2 was the first method to give MALDI-TOF results, even though the signal was not very strong. Figure 5 shows MALDI-TOF spectra of the isolates compared with Dowex (desalting), which did not improve the strength of the signal.

Figure 5. MALDI-TOF spectra comparing extraction of LPS with Method 2 using the DOWEX desalting before loading it onto the DHB matrix for *P. multocida* strain PM 499. The extract has been used either neat or with Dowex (desalting) as indicated. See complete figure in Appendix with more examples.



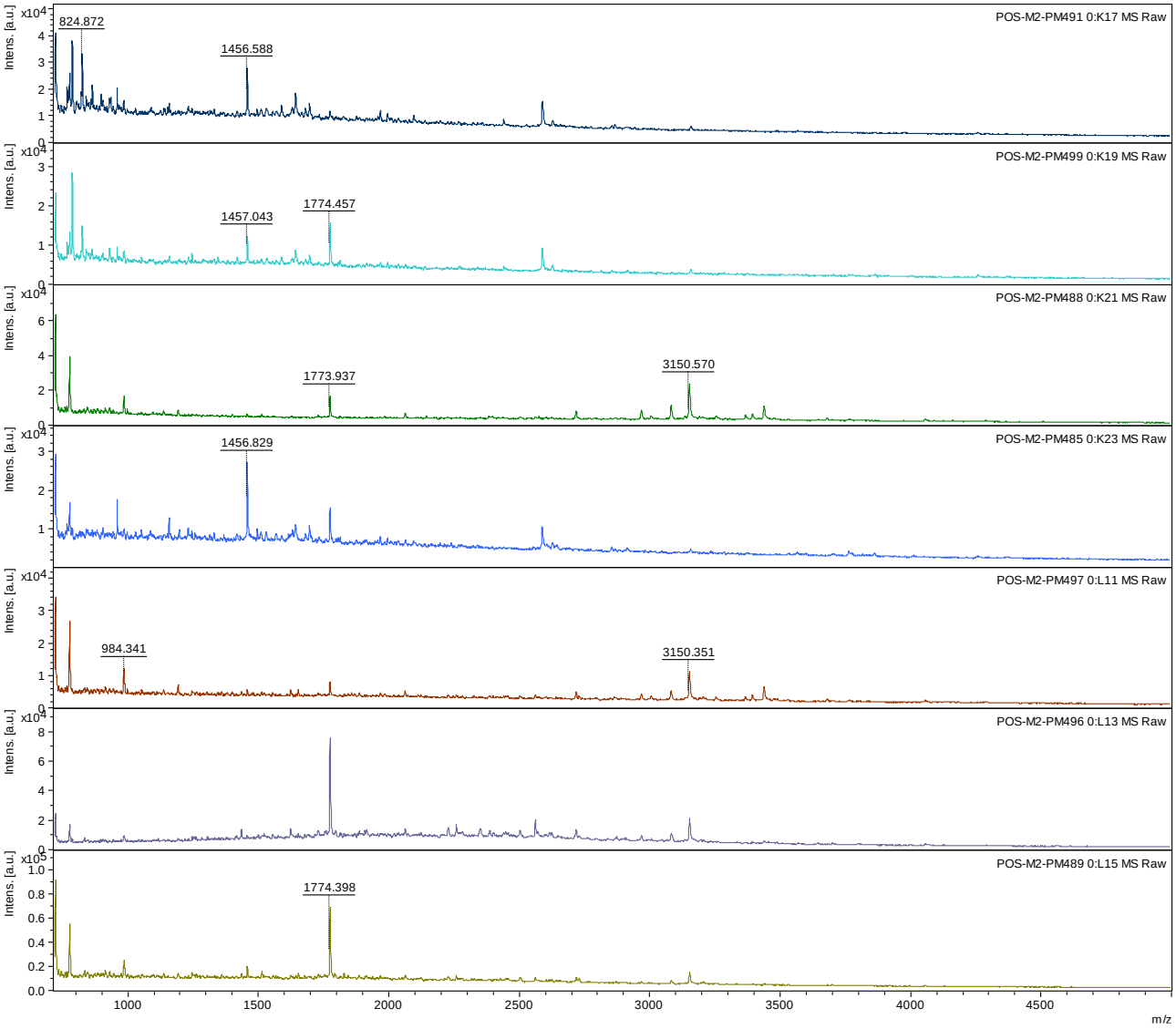
When the concentration of bacteria was adjusted at the start of the extraction of the LPS, differences in the MALDI-TOF profile were seen. The samples were acquired in linear positive mode. The matrix used was DHB with the matrix spotted and allowed to dry before the sample was spotted on top. Use of the 100 µl at the start of the method for extraction (Method 2) was not compatible with the Dowex (Figure 6).

Figure 6. Comparison of impact of adjusting the concentration of bacteria at the start of the extraction method (100 µl verses 1 ml) (Method 2 and Method 2 modified, respectively). The *P. multocida* strains used are shown in each chart. Samples were analysed in Linear Positive (LP) mode. Some samples were run through a cation exchange media (Dowex) before being analysed. See appendix for whole picture and further examples.



Seven reference strains were then used to compare the modified Method 2. Peaks were observed that could potentially differentiate the serovars (Figure 7). All samples were run as Linear Positive MALDI-TOF with DHB as matrix. (No Dowex used and 1 µl DHB solution spotted and allowed to dry, then 0.5 µl sample solution applied over the top). However, Method 2 was more variable and it was an effort to find spots for ion separation to obtain a reading.

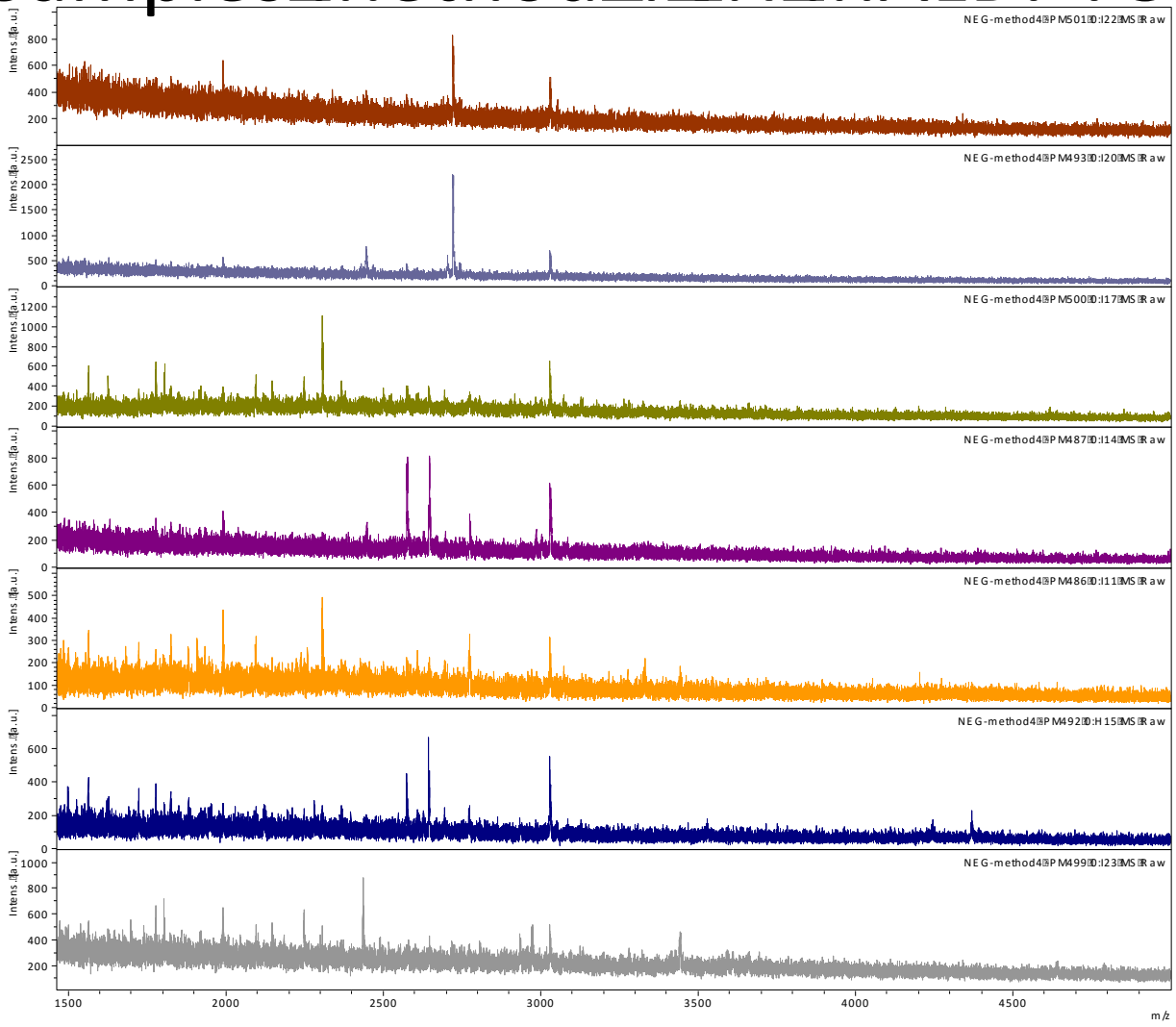
Figure 7 Comparison of the MALDI-TOF profile of the seven serovar reference strains using the modified Method 2 in positive mode. See appendix for more details.



Running the seven reference strain in negative mode (MALDI-TOF) using LPS extraction Method 4 gave the best results and some reference strains could be differentiated (Figure 8).

Figure 8 Negative mode MALDI-TOF spectra comparison of seven reference strains using LPS extraction Method 4.

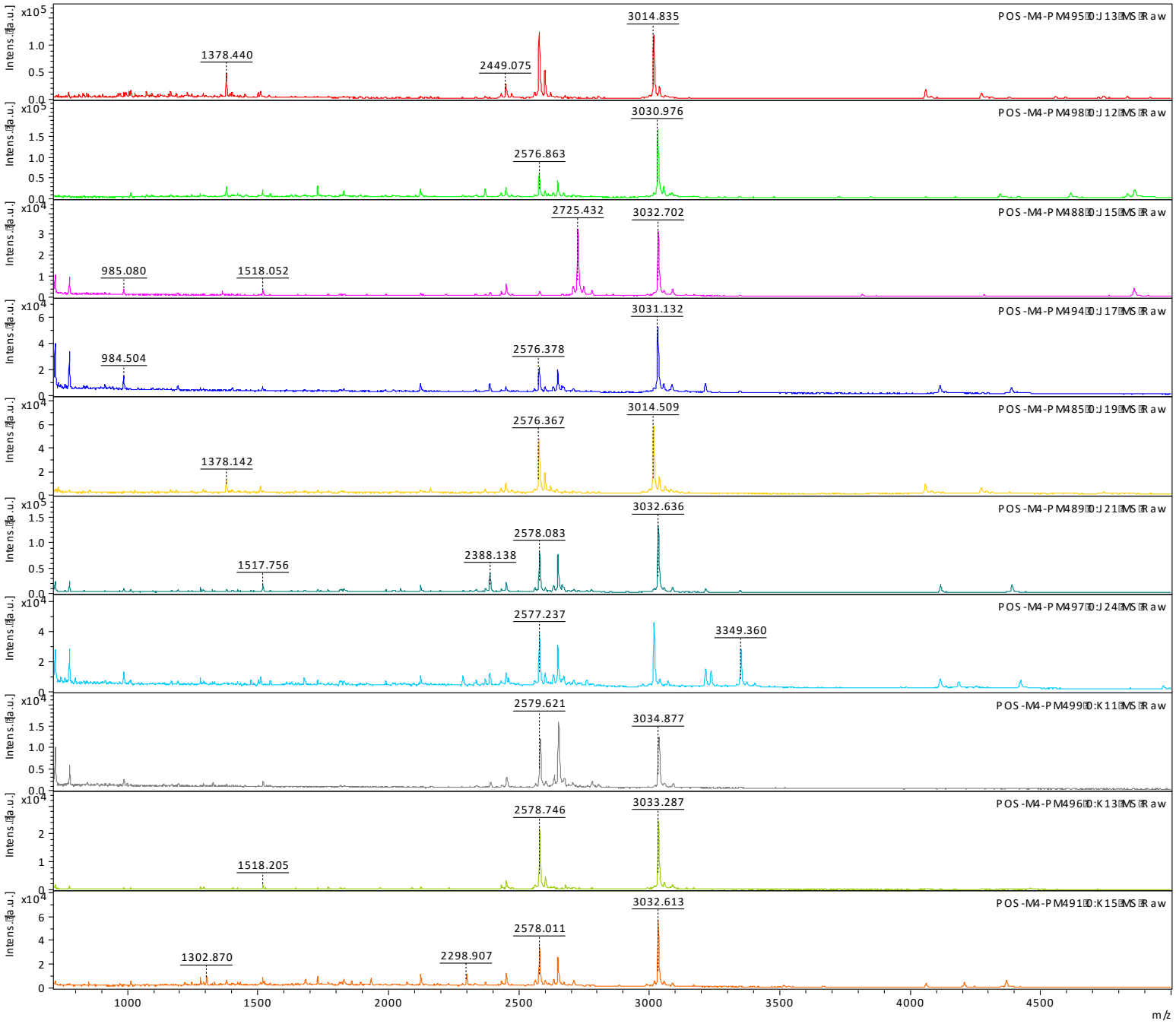
Samples Method 4 LN MALDI-TOF DHB



LPS extraction via Method 4 was the best for separating the reference serovars (Figure 8). While both linear negative and linear positive collection during MALDI-TOF work, the spectra in the positive mode revealed more definite peaks and was better for separation.

Figure 8. Analysis of LPS extracted by Method 4 from10 serovars in positive (a) and negative (b) MALDI-TOF mode.

A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x10^4
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x10^4
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x10^4
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x10^5
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x10^4
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3034.877
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x10^4
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2578.011
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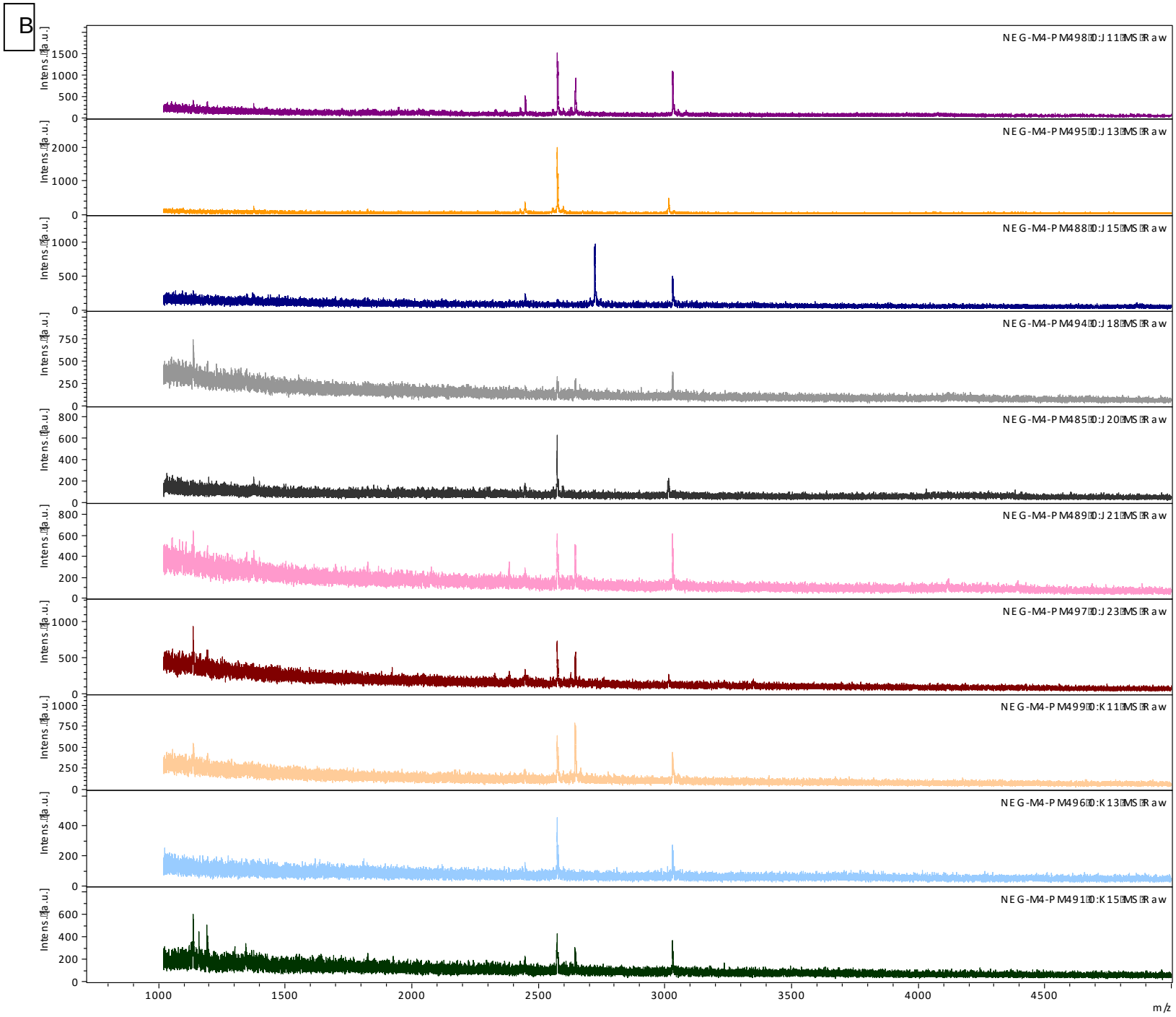
1500
2000
2500
3000
3500
4000
4500
m/z


B
Intens.[a.u.]
m/z
1000
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2000
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4000
4500

Serovar 4 and 16 are very similar except for some small differences in the size of the peaks of 1517.756. Regarding the other pattern there seems to be at least one MALDI-TOF mass spectra peak for each pattern that distinguishes it from the other serovars.

Rep-PCR

The rep-PCR revealed 20 different profiles for the pig isolates (Table 3), which would suggest a diversity of 47%.

LPS PCR

Of the eight LPS genotypes only four were detected in the chicken and pig isolates with those being L1, L3, L4 and L6 (Table 2). Remarkably, there were two chicken isolates that could not be typed by the LPS method. One of these two isolates had been typed by traditional Heddleston serotyping as cross-reacting with serovars 13 and 8. The other isolate could not be typed by either traditional Heddleston serotyping or LPS genotyping.

MLST

The results of the MLST work are presented in Table 3. There are STs shared between the poultry and the pig isolates with both host species having ST 7, 11, 20, 24 and 58 in common (Table 4). ST 20 was presented in both species as L3 and Heddleston serovar 4. Both ST 24 from the chicken and pig isolates were represented as L3 and were not typable by the Heddleston scheme. ST 58 was an L1 and 1/4 cross-reactive in the Heddleston scheme for both host species. ST 7 was present as L3 and Heddleston serovar 3, however, one isolated could not be typed in the Heddlestone serotyping. The ST 11 was presented in the chicken as none typable (NT) in the Heddleston scheme and as L1 in the LPS PCR, while in the pigs it was serovar 4 and L1 (Table 3).

Table 3. Results of the Heddleston serotyping, MLST, and LPS genotyping for all isolates for both species. If the pig isolates had the same rep-PCR profile, they were assumed to be the same isolate and that is why they were not subjected to MLST.

Poultry				
NO	Heddleston serovar	LPS genotype	ST	CC
1	3	L3	1	
2	15	L3	2	
3	3	L3	9	ST9
4	6	L4	10	
5	3	L3	7	
6	NT	L1	11	
7	9	L6	12	
8	NT	L6	16	
9	3	L6	17	
10	1,4	L1	18	
11	NT	L3	19	ST23
12	NT	L6	21	
13	1	L1	22	
14	NT	L3	24	ST9
15	NT	L3	26	ST8
16	NT	L3	28	
17	NT	NT	31	
18	13,8	NT	32	ST171
19	1	L1	33	
20	NT	L1	34	ST37
21	1, 4	L1	58	ST58
22	3	L6	29	
23	1	L3	9	ST9
24	NT	L3	25	ST78
25	10, 13, 14	L3	166	ST142
26	1	L3	5	
27	1	L1	60	ST61
28	NT	L1	30	ST30
29	NT	L3	142	ST142
30	4	L3	20	
31	NT	L3	7	
32	2	L3	23	ST23
33	NT	L3	169	ST142
34	14	L4	170	ST78
35	NT	L4	155	ST78
36	14	L1	171	ST171
37	14	L3	8	ST8
38	3	L3	179	
39	1	L1	66	ST171
40	3	L3	9	ST9
41	12	L3	8	ST8

Pigs					
NO	Heddleston serovar	LPS genotype	rep-PCR profile	ST	CC
1	NT	L3	2	124	
2	14/15	L3	8	326	ST20
3	12/10	L6	14	327	ST74
4	1/8/9	L6	15	50*	ST50
5	NT	L3	2	124*	
6	3	L6	17	167	ST50
7	10	L6	18	167	ST50
8	10	L3	1	124	
9	NT	L6	15	50	ST50
10	NT	L6	19	74	ST74
11	4	L1	9	11	
12	4	L3	4	20	
13	13/15/16	L3	4	20*	
14	4/14	L3	4	20*	
15	NT	L6	12	185	
16	NT	L3	1	124*	
17	NT	L6	12	185*	
18	NT	L3	11	328	ST13
19	14	L6	12	185*	
20	NT	L6	12	185*	
21	NT	L6	12	185*	
22	NT	L6	12	185*	
23	14	L6	12	185	
24	NT	L6	15	50*	ST50
25	NT	L3	6	24	ST9
26	NT	L3	6	24*	ST9
27	NT	L6	16	50	ST50
28	10	L6	15	50*	ST50
29	1	L6	13	27	ST74
30	1/4	L1	20	58	ST58
31	3/4	L3	4	20*	
32	NT	L3	4	20*	
33	3	L3	7	7	
34	1/3	L3	3	124	
35	NT	L3	3	124*	
36	NT	L3	6	24*	ST9
37	NT	L6	15	50	ST50
38	NT	L3	10	13*	ST13
39	NT	L6	15	50*	ST50
40	NT	L6	15	50*	ST50
41	NT	L3	5	329*	ST20
42	NT	L3	10	13	ST30
43	NT	L3	5	329	ST20
* presumed MLST ST due to same rep-PCR profile					

Table 4. Isolates of chicken and pigs that shared the same ST

Species	Isolate no	Heddleston serovar	LPS genotype	ST	CC
Poultry	5	3	L3	7	
Poultry	31	NT	L3	7	
Pig	33	3	L3	7	
Poultry	6	Nt	L1	11	
Pig	11	4	L1	11	
Poultry	30	4	L3	20	
Pig	12	4	L3	20	
Pig	13	13, 15, 16	L3	20	
Pig	14	4, 14	L3	20	
Pig	31	3, 4	L3	20	
Pig	32	NT	L3	20	
Poultry	14	NT	L3	24	ST9
Pig	25	NT	L3	24	ST9
Pig	26	NT	L3	24	ST9
Pig	36	NT	L3	24	ST9
Poultry	21	1, 4	L1	58	ST58
Pig	30	1,4	L1	58	ST58

Discussion of Results

Heddleston serotyping is the most commonly used method for sub-typing of *P. multocida* with the QAAFI/DAF laboratory offering a user pays serotyping service. The service is used actively to help develop vaccination programs as the accepted believe is that killed *P. multocida* vaccines provide protection only against those serovars in the vaccine (Glisson et al., 2008). The problem is that the Heddleston serotyping did not result in a definitive answer for a number of isolates. A total of 17 out of 41 isolates from chicken isolates were either non-typable (no reaction with any of the 16 recognised serovars) or gave a non-specific reaction (reaction with more than one serovar). Looking at the data it can also be seen that despite the LPS PCR classing them as a certain genotype, the Heddleston serovar scheme does not always agree with the LPS PCR. This lack of correlation has also been observed when the LPS PCR was developed (Harper et al., 2014) and in previous work by Singh et al. (2013). In the Harper et al. (2014) study, the gold standard method of a full chemical and structure analysis showed that LPS PCR was consistently correct while the traditional serotyping methodology was in error when these two tests were in dis-agreement.

Unfortunately, while more accurate, the multiplex LPS PCR has the disadvantage that it can only differentiate eight types. Most importantly, the LPS PCR cannot differentiate between serovars 3 and 4, which are the most common serovars in Australian poultry (Turni & Blackall, 2011). Hence, it is not possible to use the LPS PCR to provide guidance on the suitability of vaccination programs or recommend which and how many isolates need to be included in a vaccine.

The problems with the serotyping scheme make it necessary to develop an alternative system – with genotyping being the most relevant alternative. The genotyping methods so far available (all developed in previous research at the QAAFI/DAF laboratory funded by the poultry industry) are the multi-locus sequencing (MLST) and high resolution melt (HRM) analysis. Unfortunately these available established methods target housekeeping genes, which are highly conserved and are not associated with serovars. Hence, the results of MLST and HRM typing do not definitively determine whether a field isolate is of the same serovar as the vaccine strain. Similarly, MLST and HRM can confirm a current field isolate is different from one obtained in the past but cannot predict if the two strains are cross-protective.

In recent years we have undertaken extensive studies on *Pasteurella multocida* from fowl cholera outbreaks. This work has confirmed that none of these molecular methods can predict the serovar or provide predictions about cross-protection. This understanding has underpinned our search for an alternative – with that alternative being MALDI-TOF, a method that directly examines the structures that are responsible for the serovar and immunovar specificity seen in this organism. We have been involved in research using mutants that differ in just one or two sugars in the LPS that has shown these minor differences can result in total loss of protection (Harper et al., 2016).

The convenience of a kit for the extraction of the LPS was our main reason for including this method into our work. It was argued that front line diagnostic laboratories would adopt a kit easily. However, the extraction method did not yield enough LPS for the analysis via MALDI-TOF and hence proved unsuitable. The method that yielded the most LPS was the Method 4. The bands were thicker in the gel profile suggesting a higher concentration of LPS. The yield of LPS was dependent on the starting concentration of the bacteria and increasing the concentration by 10 fold improved the yield of the LPS (100 µl to 1 ml).

Other researchers have found that ion exchange resulted in better outcomes of the analysis by MALDI-TOF. However, in the case of *Pasteurella multocida* that was not the case. On contrary, at the 100 µl starting volume of bacteria, the Dowex ion exchange influenced the results negatively.

Structural analysis of serovar 2 and 5 by negative ion capillary electrophoresis electrospray MS found peaks at m/z 966.6^{3-} and 967.1^{3-} corresponded to glycoform composition of HexNAC, 3Hex, 5Hep, Kdo-P and lipid A-OH, the O-deacylated LPS (St Michael et al., 2009). Ions were also observed at m/z 1007.8^{3-} and 1008.3^{3-} corresponded to the same composition with an additional single phosphoethanolamine (PEtn) residue for the O-deacylated LPS (LPS-OH) (St Michael et al., 2009). The only peak that was observed in that range for the serovar 5 (PM 494) was at m/z 984.5. The peak in the serovar 2 is slightly higher which could possible mean the addition of a single phosphoethanolamine (PEtn) residue.

The core oligosaccharide analysis revealed smaller bands from 853.4 upwards, which represented the HexNAc, 2Hex, 5Hep, aKdo (St Michael et al., 2009). In the current study smaller bands were only seen at 776.9 and 776.6.

Other bands, like the one at m/z 1518.1, were also observed by researchers for the LPS-OH at m/z 1512.7 for serovar 2 and 5 (St Michael et al., 2009 and Harper et al., 2011). However this peak was extremely weak for the serovar 5 reference strain in this study. The lower ions correspond to the core oligosaccharides and the lipid A species (Sturiale et al., 2011). The lipooligosaccharides (LOS) are seen in the range of m/z 2600 to 3200 (Sturiale et al., 2011). Pupo et al. (2004) also found peaks in the range from 951.1 to 3766.2 for *Vibrio fischeri*. Hence the peaks observed at m/z 22298 to 3349 are LOS ion associated peaks.

It was found that Method 4 was the most suitable method for extracting the LPS in sufficient concentration and purity to give consistent results in the MALDI-TOF analysis. It seemed that most of the serovar reference strains could be separated, albeit in some cases only by one peak. Two very closely related serovars, serovars 2 and 5, could be held apart by a peak at 777.6 m/z. It is questionable if these types of minor differences can be maintained if different field isolates are investigated. In our previous collaborative studies, we have found a marked diversity in the LPS structure, particularly in fowl cholera isolates of LPS PCR type 3 (Harper et al., 2013). With this level of known field isolate diversity, it is highly unlikely that the minor differences we have detected in the 16 reference strains will be of any value. Indeed, it is our conclusion that the minor differences that exist within the reference strains will be overwhelmed by the diversity of minor

changes that are known to exist in the field isolates. Hence, at this stage, MALDI-TOF does not offer a realistic or relevant alternative when seeking to identify either serovars or immunovars.

Poultry isolates had more variation in regards to the observed STs than the pig isolates. Isolates from both species were chosen from different farms and different states to present the greatest potential diversity of isolates. For the 41 poultry isolates, 37 different STs were observed with only ST 7, 8 and 9 being represented by multiple isolates. In contrast to this, the 43 isolates from pigs were allocated to only 16 different STs, with STs 13, 20, 24, 50, 124, 167, 185 and 329 being represented by multiple isolates. Despite this diversity in STs only four of the eight LPS types were observed - L1, L3, L4 and L6. However, two of the chicken isolates could not be typed with the LPS PCR suggesting some more variation in the LPS structure.

The rep-PCR for the pig isolates also suggested that many of the isolates were the same strain and that the pool of different isolates was smaller than for the chicken isolates. Some of these isolates were shared.
Looking at the STs shared across the two host species, STs 7, 11, 20, 24 and 58, most of these STs are only represented by isolates from Australia at the RIRDC MLST website. The database for the *Pasteurella multocida* RIRDC MLST records STs 7, 11 and 20 as only occurring in Australia, with isolates from chickens and turkeys, as well as pigs. Specifically, ST7 was found in isolates from five chickens and one pig, ST11 from one turkey and one pig and ST 20 from nine chickens, seven turkeys, one pig and one cat. ST 24 was reported from two chickens in Australia and one rabbit from Italy, while ST 58 was reported from one chicken and one pig from Australia, but also from a sheep in New Zealand and one partridge from Belgium.

Of the 37 STs recognised in the *P. multocida* poultry isolates of this study, 24 STs have only been observed in poultry isolates from Australia according to the RIRDC *P. multocida* MLST database. while three STs have only been observed from poultry isolates from other countries (Table 5). Ten of the STs had several host species. The five STs that were found in this study to be shared by both species were among the 10 STs listed with different hosts. Four out of the five STs that were shared in this study had pig listed as another host species, while ST 24 has not been recorded for the pig host in the data bank.

Table 5 Isolates listed in the RIRDC *P. multocida* MLST databank for the STs found in the poultry isolates. With the highlights showing the poultry isolates from Australia.

STs of *P. multocida* from Chicken			
ST	**Host**	**No isolates**	**Country**
1	Turkey/ Chicken	5	Australia
2	Turkey	13	Australia
5	Chicken	3	Australia
5	Cattle	1	?
5	Mouse	1	?
7	Chicken	5	Australia
7	Pig	1	Australia
8	Turkey/ Chicken/ Duck	28	Australia
8	Chicken	2	Poland
8	Cattle	1	Indonesia
9	Turkey/ Chicken	4	Australia
9	Turkey/ Chicken	3	UK + USA + Denmark
9	Duck	1	UK
9	Rabbit	8	Italy + Czech Rebulic
9	Pig	1	Australia
9	Cattle	2	Scottland + India
9	Goat	1	India
9	Human	1	?
9	Lion	1	Denmark
10	Chicken	4	Australia
11	Turkey	1	Australia
11	Pig	1	Australia
12	Turkey/ Chicken	3	Australia
16	Chicken	2	Australia
17	Turkey	1	Australia
18	Chicken	1	Australia
19	Chicken	1	Australia
20	Turkey/ Chicken	16	Australia
20	Pig	1	Australia
20	Cat	1	Australia
21	Chicken	1	Australia
22	Chicken	1	Australia
23	Chicken	1	Australia
24	Chicken	2	Australia
24	Rabbit	1	Italy
25	Chicken	1	Netherland
26	Turkey	1	Australia
28	Chicken	1	Australia
29	Chicken	1	Australia
30	Chicken	1	Australia
30	Duck	1	UK
30	Cat	1	Australia
31	Turkey	2	Australia
32	Turkey	1	Australia
33	Chicken	1	Australia
34	Chicken	1	Australia
58	Chicken	1	Australia
58	Pig	1	Australia
58	Ovine	1	New Zealand
58	Partridge	1	Belgium
60	Chicken	2	?
66	?	1	Australia
66	Chicken	1	USA
142	Chicken	2	Australia
155	Chicken	2	Norway
166	Chicken	2	Australia
169	Chicken	2	Australia
170	Chicken	2	Australia
171	Chicken	2	Australia
179	Chicken	2	Australia

In summary result of this evaluation study is that analysis by MALDI-TOF for LPS of *P. multocida* does not yield the distinct differences it has shown for other species of bacteria and the difference is typically only one MALDI-TOF spectra peak difference and for two serovars the difference was in the size of the peak. This lack of major differences between the serovar reference strains, which represent one variant of each of the serovars means that there is little chance that field isolates of the various serovars can be reliably assigned to a serovar by MALDI-TOF examination.

The fact that *P. multocida* from chicken and pigs shared the same ST and had the same LPS genotype and, in most cases the same Heddleston serovar, would indicate that the pig is a potential reservoir for *P. multocida* for chickens. This has implications for biosecurity considerations, such as the need to control wild pigs around chicken farms and the need to restrict visitors and staff that have been in contact with pigs prior to entering the chicken shed.

Implications

Even though the serovar reference strains could be separated by the developed MALDI-TOF method, the fact that only minor difference existed in the MALDI-TOF spectra does indicate that this method is not an appropriate diagnostic solution for the differentiation of serovars. The hope was that this method would solve the problem of separation of the serovars and could be used to guide vaccine programs, which due to the shortcomings of the current used test is currently a problem.

There certainly seems an overlap of chicken and pig *P. multocida* strains, pointing to the potential of the pig being a reservoir for chicken *P. multocida* strain and vice versa. This brings with it biosecurity precautions when visiting pig farms and chicken farms in a short time frame of each other or visiting places where pigs are present before visiting a chicken farm. It also means that biosecurity precautions have to include considerations of wild pigs around chicken farms.

Recommendations

The rapid and confident recognition of either the serovars or the immunovars of *P. multocida* is not possible by MALDI-TOF. Nevertheless, this need for rapid, accurate serovar and immunovar recognition remains a pressing issue.

It is possible that the use of whole genome sequencing may provide the basis of knowledge to use molecular methods to address this need. The chicken meat and egg industries are currently funding such a project.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Development of Matrix-Assisted Laser Desorption Ionization Time -of - flight Mass Spectrometry (MALDI-TOF) for serotyping *Pasteurella multocida*
Poultry CRC Sub-Project No.:	1.2.7
Researcher:	Dr Conny Turni
Organisation:	QAAFI, The University of Queensland
Phone:	07 3255 4296
Fax:	-
Email:	c.turni1@uq.edu.au
Sub-Project Overview	This research attempted to validate the use of matrix-assisted laser desorption/ionizations time-of-flight mass (MALDI-TOF) spectrometry to predict the serovars and immunovars of field isolates of *P. multocida*. The work sought to detect the differences in the lipopolysaccharide (LPS) that underpin both serovar and immunovar specificity. While serovar reference strains could be differentiated with the exception of two, the differences were minor. Our recent research has shown that Australian field isolates show considerable sugar variation in the LPS. As well, we have recently shown that these minor sugar changes can result in a loss of cross-protection. On the basis of this knowledge, our finding that only subtle, minor differences could be detected between the serovar reference strains by MALDI-TOF meant that the technique has no capacity to detect the known diversity in the field isolates. Hence, MALDI-TOF does not offer a realistic or relevant approach for predicting the serovar or immunovar of field isolates of *P. multocida.*
Background	CRC research on *Pasteurella multocida* has provided PCR-based lipopolysaccharide (LPS) typing but the method cannot separate all serovars – there are 8 PCR LPS types but 16 serovars. As killed vaccines do not provide cross-protective to serovars not included in the vaccine, this severely limits the guidance provided to industry vaccination programs. CRC research has also shown that isolates of the same serovar can differ in LPS structure and can fail to be cross-protective. This research seeks to evaluate MALDI-TOF technology to assign isolates from a PCR LPS type to the correct serovar and to detect LPS structure variation within a serovar.
Research	This project was designed to develop a method to differentiate the LPS structure of *P. multocida* and then validate the method on an extensive culture collection of field isolates with known serovars and known genetic profiles (as determined by MLST). The first step in the development of this diagnostic method was optimizing the extraction of the LPS from *P. multocida*. Initial work involved the use of a commercial extraction kit and then moved to several published methods for LPS extraction for *P. multocida*. Optimization of all extraction methods used was undertaken. Analysis by the MALDI-TOF method to differentiate the LPS structures was then attempted on the optimized extraction method.

	This analysis was started with a variety of solvent buffers adding cation exchange until optimization was achieved. Several optimisations were investigated to develop the MALDI-TOF method to analyse the different LPS structures. The developed method was validated on reference strains for *P. multocida*.
Sub-Project Progress	While differentiation of the reference serovars was achieved, the difference in the MALDI-TOF spectra peaks was not very distinct with two serovars often only differing slightly in peak heights. As these were only the reference serovars and hence no variation within the serovars was included it was concluded that this method is not suitable for routine diagnostic analysis. As further analyses of a collection of Australian field isolates was to be undertaken both from chicken and pigs, to represent the diversity of Australian strains. This final step has not yet been achieved, as the method could not be optimized to give enough differences for a clear differentiation. However, the isolates were serotyped and genotyped (three methods – LPS multiplex genotyping, which can only separate 8 LPS genotypes of 16 serovars; rep-PCR, which determines the number of strains in the collection and multilocus typing, which makes comparison to the rest of the world possible) to look at the variability of the isolates. The serotyping and genotyping gave us the knowledge of the variability of *P. multocida* in the Australian poultry in comparison to *P. multocida* in pigs and to the rest of the world and other animal species that harbour *P. multocida* (multilocus sequencing results are on a public database). This has provided an overview of the epidemiology of *P. multocida* in Australia.
Implications	The key LPS structures cannot be separated by MALDI-TOF. It is possible that whole sequencing approaches could differentiate strains according to the sequence of the genes associated with the LPS structure.
Publications	None