

This is an Open Access document downloaded from ORCA, Cardiff University's institutional repository:<https://orca.cardiff.ac.uk/id/eprint/95440/>

This is the author's version of a work that was submitted to / accepted for publication.

Citation for final published version:

Aboklaish, Ali, Ahmed, S., McAllister, D., Cassell, G., Zheng, X. and Spiller, Owen Bradley 2016. Differential recognition of the Multiple Banded Antigen isoforms across *Ureaplasma parvum* and *Ureaplasma urealyticum* species by monoclonal antibodies. *Journal of Microbiological Methods* 127 , pp. 13-19.
10.1016/j.mimet.2016.05.015

Publishers page: <https://dx.doi.org/10.1016/j.mimet.2016.05.015>

Please note:

Changes made as a result of publishing processes such as copy-editing, formatting and page numbers may not be reflected in this version. For the definitive version of this publication, please refer to the published source. You are advised to consult the publisher's version if you wish to cite this paper.

This version is being made available in accordance with publisher policies. See <http://orca.cf.ac.uk/policies.html> for usage policies. Copyright and moral rights for publications made available in ORCA are retained by the copyright holders.



1 Title:

2 **Differential recognition of the Multiple Banded Antigen isoforms across *Ureaplasma***
3 ***parvum* and *Ureaplasma urealyticum* species by a panel of monoclonal antibodies.**

4

5 **Ali F. Aboklaish,^{1,2} Shatha Ahmed,^{1,3} Douglas McAllister,⁴ Gail Cassell,^{5,6} and**
6 **Owen B. Spiller^{1#}**

7

8 Institutions:

9 **1. Cardiff University, School of Medicine, Institute of Molecular and Experimental Medicine,**
10 **5th floor University Hospital of Wales, Cardiff, CF14 4XN, UK.**

11 **2. Sebha University, Faculty of Engineering and Technology, Medical Laboratory Sciences**
12 **Department, P.O. Box 68, Brak, Libya.**

13 **3. Department of Pathology, Nineveh College of Medicine, University of Mosul, Mosul, Iraq.**

14 **4. Virostat Inc., P.O. Box 8522, Portland, ME 04104 USA**

15 **5. Harvard Medical School, Department of Global Health and Social Medicine, Boston, MA**
16 **02115**

17 **6. Infectious Disease Research Institute, Seattle WA 98102**

18

19 Short title: **mAb against *Ureaplasma* species MBA**

20 **# Corresponding Author: Dr. Brad Spiller, Institute of Molecular and Experimental**
21 **Medicine, School of Medicine, Cardiff University, 5th floor University Hospital, Heath**
22 **Park, Cardiff, CF14 4XN, United Kingdom.**

23 **Phone: +44 (0)2920 742394 Fax: +44 (0)2920 744283 e-mail: SpillerB@cardiff.ac.uk**

24

25 **KEY WORDS: *Ureaplasma parvum*, *Ureaplasma urealyticum*, multiple banded antigen,**
26 **recombinant protein.**

27

28 **Abstract.**

29 Two separate species of *Ureaplasma* have been identified that infect humans: *Ureaplasma*
30 *parvum* and *Ureaplasma urealyticum*. Most notably, these bacteria lack a cell wall and are
31 the leading infectious organism associated with infection-related induction of preterm birth.
32 Fourteen separate representative prototype bacterial strains, called serovars, are largely
33 differentiated by the sequence of repeating units in the C-terminus of the major surface
34 protein: multiple-banded antigen (MBA). Monoclonal antibodies that recognise single or
35 small groups of serovars have been previously reported, but these reagents remain sequestered
36 in individual research laboratories. Here we characterise a panel of commercially available
37 monoclonal antibodies raised against the MBA and describe the first monoclonal antibody
38 that cross-reacts by immunoblot with all serovars of *U. parvum* and *U. urealyticum* species.
39 We also describe a recombinant MBA expressed by *E. coli* which facilitated further
40 characterisation by immunoblot and demonstrate immunohistochemistry of paraffin-
41 embedded antigens. Immunoblot reactivity was validated against well characterised
42 previously published monoclonal antibodies and individual commercial antibodies were found
43 to recognise all *U. parvum* strains, only serovars 3 and 14 or only serovars 1 and 6, or all
44 strains belonging to *U. parvum* and *U. urealyticum*. MBA mass was highly variable between
45 strains, consistent with variation in the number of C-terminal repeats between strains.
46 Antibody characterisation will enable future investigations to correlate severity of
47 pathogenicity to MBA isoform number or mass, in addition to development of antibody-based
48 diagnostics that will detect infection by all *Ureaplasma* species or alternately be able to
49 differentiate between *U. parvum*, *U. urealyticum* or mixed infections.

50

51 1. INTRODUCTION

52 *Ureaplasma* species are one of the smallest, free living mucosal bacteria that can be isolated
53 from the human urogenital tract. These organisms are the most common bacteria isolated
54 from infected amniotic fluid and placentas, and they contribute to adverse pregnancy
55 outcomes including preterm birth and neonatal morbidities. In a recent review, the rate of
56 *Ureaplasma* infection was reported to be almost half of the preterm infants of less than 32
57 weeks gestation in one or more compartment (respiratory, blood and/or cerebrospinal fluid),
58 indicating that these organisms were the most common pathogens affecting this population
59 [1]. Furthermore, intrauterine or perinatal infection with *Ureaplasma* species is emerging as a
60 leading risk factor for adverse pregnancy outcomes and complications of extreme preterm
61 birth such as bronchopulmonary dysplasia BPD and intraventricular haemorrhage [2] Recent
62 meta-analysis of 39 studies examining the role of *Ureaplasma* and development of BPD
63 supported a significant association between pulmonary colonization with *Ureaplasma* and
64 development of BPD in preterm infants [3].

65 However, *Ureaplasma* were initially described in isolates from male patients suffering from
66 urethritis. The initial 1954 report [4] differentiated *Ureaplasma* from *Mycoplasma* based on
67 agar plate colony morphology (initially designated T-mycoplasma for “tiny” colony, which
68 were visually distinct from the larger characteristic *Mycoplasma* “fried egg” morphology).
69 By 1982, Robertson and Stemke [5] had separated *Ureaplasma* into 14 “serovars” using a
70 panel of polyclonal rabbit anti-sera and a combination of modified metabolic inhibition test
71 and colony indirect epifluorescence methods. In the years that followed, it became clear that
72 these 14 serovars could be grouped into two separate sub-types that were initially called
73 “biovar” 1 and 2. However, in 2002 Robertson *et al.*, utilised conserved differences in DNA-
74 DNA hybridisation, distinctive RFLP patterns, and other genomic differentiators to divide the
75 14 serovars into two distinct species: *U. parvum* (serovars 1, 3, 6 and 14) and *U. urealyticum*

76 (serovars 2, 4, 5, and 7-13) [6]. A conserved PCR amplicon size difference using primers
77 recognising the promoter and coding region of the major surface protein (multiple banded
78 antigen; MBA) was found capable of separating clinical *U. parvum* (403 bp) from *U.*
79 *urealyticum* (448 bp) strains [7]. These authors also found that different sized amplicons for
80 related primer sets in this region could also separate *U. urealyticum* serovars 2, 5, 7, 8, 9 and
81 11 from *U. urealyticum* strains 4, 10, 12 and 13, as well as uniquely identifying *U. parvum*
82 serovar 6 from all other isolates [7]. The MBA is a lipid-anchored protein that is expressed
83 on the surface of *Ureaplasma* and is composed of a signal peptide, a lipid anchor addition
84 signal sequence and a relatively well conserved non-repeating region of approximately 100
85 residues at the N-terminus. However, the C-terminus region is composed of repeats that vary
86 in sequence between serovars and in repeat number amongst strains of the same serovar.
87 Kong *et al.*, [8] found that the predicted amino acid sequence for the repeat region of each *U.*
88 *parvum* serovar (1, 3, 6 and 14) was slightly different and that *U. urealyticum* serovars could
89 be separated into a unique serovar 10 repeat (genotype B, repeat TQPGSGST) and two groups
90 (with identical MBA N-terminal repeats) encompassing serovars 2, 5 and 8 (genotype A;
91 repeat TKPGSGET) and serovars 4, 12 and 13 (genotype C; repeat TSPEKPGNGT), but that
92 serovars 7, 11 (genotype E) and 9 (genotype D) could not be differentiated by consensus
93 MBA repeat sequence in their study. These defined consensus external repeats make ideal
94 targets for differentiation by antibodies and development of monoclonal antibodies against the
95 MBA have also been reported [9-13]. Some monoclonal antibodies recognise single serovars,
96 while others recognise groups of *U. parvum* or *U. urealyticum* sub-groups. However, all of
97 these antibodies belong to independent research groups and are not readily available. Here we
98 provide the first characterisation of commercially available monoclonal antibodies by
99 immunoblot against the initial prototype serovar strains and validate our results against a
100 panel of research monoclonal antibodies that have previously been published.

101

102 **2. MATERIALS AND METHODS**

103 ***2.1 Antibodies***

104 A panel of mouse monoclonal antibodies previously characterised and published [9,
105 10] (provided by Dr. Gail Cassell) were used for comparison. These antibodies included
106 clones 8A1.2 (specific for serovar 10), 10C6.6 (specific for serovar 3), 5B1.1 (specific for
107 serovars 3 and 14 only) and 8B5.2 (specific for serovars 1, 3, 6 and 14; all *U. parvum* strains).
108 Commercial monoclonal antibodies were provided by ViroStat Inc. (Portland, ME) and
109 included catalogue numbers 6522, 6523, 6525, 6527 as well as clones 4H2 and 2G9. Isotype
110 control (IgG1) monoclonal antibody was purchased from Caltag MedSystems ltd
111 (Buckingham, UK). Peroxidase-conjugated donkey anti-mouse immunoglobulin secondary
112 antibodies were purchased from Jackson ImmunoResearch Europe ltd. (Newmarket, Suffolk,
113 UK).

114 ***2.2 Bacterial strains***

115 Prototype strains representing serovars 1-14 were obtained from the American type
116 culture collection (strains 27813 (SV1); 28715 (SV3); 27818 (SV6); 33967 (SV14); 27814
117 (SV2); 27816 (SV4); 27817 (SV5); 27819 (SV7); 27618 (SV8); 33175 (SV9); 33699 (SV10);
118 33695 (SV11); 33696 (SV12) and 33698 (SV13)). *Ureaplasma* strains were cultured in
119 *Ureaplasma* selective medium (Mycoplasma Experience ltd; Reigate, Surrey, UK) as
120 previously published. Clinical isolates of *U. urealyticum* originated from a previously
121 published study examining antibiotic susceptibility for clinical isolates in England and Wales
122 between 2003-2009 [14], as were *U. parvum* strains HPA2 (SV6), HPA5 (SV3) and HPA32
123 (SV14) which have been further characterised in other investigations [15, 16].

124 ***2.3 Creation of E. coli expressing recombinant serovar 3 MBA.***

125 A codon optimised gene for expressing the serovar 3 MBA protein (only encoding 2
126 PAGKEQ C-terminal repeats) was created by utilising the Life Technologies online tool to

127 generate the DNA sequence optimised for *E. coli* expression following input of the following
128 amino acid sequence (supplementary figures 1 and 2). The promoter for the *tuf* gene from
129 serovar 3 (170 bp upstream of the AUG start codon) was then added upstream of this
130 optimised open-reading frame to promote expression. This sequence was synthesized by
131 MWG Eurofins (Ebersberg, Germany) which was provided in the ampicillin resistant plasmid
132 pEX-A2. A HindIII restriction site was engineered into the sequence just prior to the
133 PAGKEQ repeats so that digestion with HindIII and re-ligation would result in expression of
134 serovar 3 MBA that ended in FETTQPGKL rather than FETTQPGKLPAGKEQPAGKEQ.
135 One shot Top10 chemically competent *E. coli* (Invitrogen; Paisley, Scotland, UK) were
136 transformed with full or HindIII truncated plasmids, as per manufacturer's instructions. This
137 bacteria has the genotype F- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1
138 araD139 Δ (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ -. Single colonies of
139 transformed bacteria were picked from LB agar plates containing 100 mg/L ampicillin and
140 grown up in LB broth containing ampicillin for further analysis. Control bacteria containing
141 empty EX-A2 plasmid were used as controls.

142

143 **2.4 Immunoblot analysis**

144 *Ureaplasma* strains to be analysed for Immunoblot analysis were grown up in 5 ml of
145 *Ureaplasma* selective medium for 48 h and pelleted at 17,000 xg for 30 min, prior to being
146 washed by resuspension in PBS and re-pelleted (repeated 3 times). Bacterial pellets were
147 solubilised in 1% SDS (25 μ L) before addition of an equal volume of LDS-sample buffer
148 (Invitrogen) and boiled at 95°C for 5 min prior to loading on a non-reducing SDS
149 polyacrylamide gel (7.5% polyacrylamide) and separated by electrophoresis. MagicMark™
150 molecular mass protein standards were run on each gel to determine relative molecular mass
151 of proteins. Proteins were transferred electrophoretically to 0.22 μ m nitrocellulose membrane

152 and blocked for 1 h in PBS containing 0.05% Tween20 (PBST) and 10% lyophilised skim
153 milk. Monoclonal antibodies were added to a final concentration of 10 µg/ml and incubated
154 on a roller overnight at 4°C. Unbound monoclonal antibodies were removed by 3 washes in
155 PBST prior to detection with secondary antibody for 1 h at room temperature. Peroxidase
156 secondary antibodies were detected by ECL Western blotting substrate (Pierce Ltd.) and
157 exposure to X-ray film (FujiFilm). Sequencing of the *mba* gene in clinical samples to
158 determine the serovar of clinical *U. urealyticum* isolates was performed by amplifying the
159 *mba* gene by PCR using the primers revMBA Uu2 (GTTTTGTAGTTTCACCACTTCC) and
160 UMS-125 [7] and sending the purified amplicon to MWG Eurofins for sequencing. ExPasy
161 translate tool (<http://web.expasy.org/translate/>) was used to determine the amino acid
162 sequence of the *mba* gene for manual identification of repeating sequences.

163 Recombinant proteins expressed in *E.coli* were performed on a 1 ml culture grown in
164 LB broth containing ampicillin and pellets were solubilised in 1% SDS prior to addition of an
165 equal volume of LDS sample buffer (Invitrogen) and analysed by immunoblot analysis as
166 above.

167 **2.5 Immunohistochemistry analysis**

168 Pellets generated from a 10 ml HPA5 (SV3) culture, centrifuged at 17,000 g , or
169 single colonies of *E.coli* (transformed with either the recombinant MBA expression cassette
170 or empty control vector) cut from the surface of LB agar plates, were embedded in paraffin
171 using an automated processor. Sections (5 µm thick) were cut and mounted on glass slides,
172 prior to rehydration and antigen retrieval for 10mM citrate containing 0.005% Tween 80 at
173 95°C for 1 hour. Sections were then stained with 10 µg/mL primary antibody (6522, 6523,
174 6525 or isotype control) diluted in 10mM PBS containing 0.6% BSA (PBS/BSA). Following
175 removal of unbound antibody by washing in PBS/BSA, sections were incubated with 1/100
176 dilution of peroxidase-conjugated donkey anti-mouse immunoglobulin secondary antibody for

177 1 hour. Following further washing steps peroxidase was developed for 3 minutes in 0.025%
178 diaminobenzamidine containing H₂O₂. Where indicated sections were also counterstained for
179 10 sec in 0.02% picromethyl blue prior to dehydration, clearing in xylene and mounting under
180 a coverslip.

181

182 **RESULTS**

183 ***Screening prototype *Ureaplasma parvum* strains with monoclonal antibodies.***

184 Equal amounts of serovars 1, 3, 6 and 14 were separated by SDS-PAGE and transferred
185 to nitrocellulose prior to probing with monoclonal antibodies (Figure 1). Monoclonal
186 antibodies 6522, 6523 and 6527 each recognised all four serotypes of *U. parvum* (Figure 1A).
187 Whereas antibody 6525 only recognised serovars 3 and 14 and antibodies 4H2 and 2G9 only
188 recognised serovars 1 and 6. These results were validated against the pattern of MBA detection
189 by monoclonal antibodies previously published by Watson et al. [9] (Figure 1B). Monoclonal
190 antibody clone 10C6.6 (previously reported only to detect serovar 3 MBA) bound to a single
191 band of 50 kDa mass from serovar 3 (Figure 1B), while clone 5B1.1 (previously reported to
192 only detect serovar 3 and 14) bound to this band and an additional single band of mass 150 kDa
193 for serovar 14 (Figure 1B). Monoclonal antibody clone 8B5.2 (previously reported to only
194 detect MBA from all *U. parvum* serovars) detected these two bands in addition to a 72 kDa
195 band for serovar 1 and a 70 kDa band for serovar 6 (Figure 1B). As expected clone 8A1.2
196 (specific for *U. urealyticum* serovar 10) did not recognise any of these strains. Of importance,
197 the visualised relative molecular mass for each prototype strain was the same for each antibody.

198 ***Screening prototype *Ureaplasma urealyticum* strains with monoclonal antibodies.***

199 Next all antibodies were tested against the full panel of *U. urealyticum* prototype strains
200 (with *U. parvum* strains included for reference). No additional reactivity beyond that shown in
201 Figure 1 was observed for monoclonal antibodies 6525, 6527, 4H2, 2G9, 10C6.6, 8B5.2, or

202 5B1.1 (data not shown). However, antibody 6523 also reacted (to a lesser intensity) with single
203 bands of 60 and 70 kDa for serovars 7 and 11 respectively (Figure 2), whereas antibody 6522
204 recognised single bands for all strains of both *U. parvum* and *U. urealyticum* (Figure 2).
205 Repeated investigation of 6522 against all the prototype *U. urealyticum* strains as well as 11
206 additional untyped clinical *U. urealyticum* strains (Figure 3) gave the same results for the
207 prototype strains. Most of the clinical strains gave a single band and the relative mass of these
208 bands varied from strain to strain. To validate these findings duplicate blots were probed with
209 previously characterised clone 8A1.2 [9] which is specific for serovar 10. This antibody
210 recognised the same band in the prototype serovar 10 strain and bands for each of the clinical
211 strains HPA24 and 31 as identified by serovar 10 specific clone 8A1.2. Sequencing the
212 variable region of the *mba* genes from HPA 24 and 31 confirmed they were only isolate in the
213 untyped clinical collection to have the repeating TQPGSGST amino acid sequence in the C-
214 terminus of the gene also found in our prototype serovar 10 strain (data not shown).

215 ***Immunoblot analysis of recombinant serovar 3 MBA expressed by E.coli.***

216 All monoclonal antibodies were then used to analyse immunoblots containing *E.coli*
217 transformed with plasmids containing a codon optimised gene serovar 3 *mba* gene encoding
218 two PAGKEQ repeats, a truncated gene with these repeats removed, or *E.coli* transformed with
219 the empty vector (Figure 4). As expected, monoclonal antibodies that only recognise serovar
220 10 (clone 8A1.2) or serovars 1 and 6 (4H2 and 2G9) failed to react with these recombinant
221 MBA proteins. Monoclonal antibodies 10C6.6 and 5B1.1 only reacted with MBA proteins that
222 contained the PAGKEQ repeat, indicating the recognition epitope is located in the C-terminal
223 repeat region. The remaining antibodies recognised the recombinant MBA with and without
224 the C-terminal repeats equally, indicating that the epitope they recognise is a conserved
225 sequence in the non-repeating N-terminal sequence.

226

227 ***Use of monoclonal antibodies to detect MBA in paraffin-embedded sections.***

228 Next we endeavoured to see if the commercial monoclonal antibodies could detect MBA
229 antigen by immunohistochemistry in paraffin sections. Initially we examined pelleted broth
230 cultures of serovar 3 (Supplementary figure 3), which showed clear specific reactivity with
231 antibodies 6522, 6523 and 6525 relative to matched isotype controls; however, morphology of
232 centrifuged pellets were amorphous. Therefore, to prove specificity and gain a better target for
233 staining, single colonies of *E.coli* expressing the recombinant form of serovar 3 MBA utilised
234 for immunohistochemistry analysis. These paraffin-embedded colonies showed clear outlines
235 of individual bacillus that were not apparent when monoclonal antibodies were used to stain
236 control *E. coli* colonies transformed with empty vector (Figure 5). Successful staining with
237 monoclonal antibodies was only seen following antigen retrieval processing (1 h treatment with
238 citrate buffer at 95°C).

239

240 **DISCUSSION**

241 *Ureaplasma* is one of the smallest self-replicating organisms with a minimal genome, ranging
242 in size from 0.75 to 0.95 Mbp [17]. These genomes are predicted to encode on average 604 (*U.*
243 *parvum*) or 664 (*U. urealyticum*) protein encoding genes, with 515 genes universally conserved
244 across all serovars. There are several distinct phylogenetic markers that separate the *U. parvum*
245 and *U. urealyticum* species [6] and these species are readily distinguished by standard PCR
246 using primers that amplify a region from 125 bp upstream of the AUG start codon and 226 bp
247 at the 5' end of the gene [7]. The MBA protein is an excellent target for separation of the
248 *Ureaplasmas* into distinct sub-groups as it is highly expressed, often being a prominent band of
249 unique mass between strains when separated by SDS-PAGE and stained with Coomassie blue.
250 It is likely to represent a significant immunological target and the predicted amino acid
251 sequence readily separates the *U. parvum* serovars: PGKEQQ (SV1), PAGKEQ (SV3), PGKE
252 (SV6), and PAGKEQQ (SV14). However, all serovars cannot be completely resolved from
253 one another based on MBA sequence (e.g. serovars 2, 5 and 8 all have the TKPGSGET repeat).
254 Molecular methods of separating the serovars based on targets external to the *mba* gene were
255 reported to successfully separate the prototype strains [18]; unfortunately application of these
256 typing schemes to clinical isolates did not maintain clear serovar differentiation [19], likely due
257 to a propensity for *Ureaplasma* species to undergo extensive horizontal gene transfer [17].
258 There have been several reports for the development of monoclonal antibodies that recognise
259 serovars in the past. The first report was by Watson *et al.* [9] where a panel of monoclonal
260 antibodies were characterised and antibodies that recognised individual serovars 3, 8 and 10
261 were identified, and two of these antibodies have served as reference for the characterisation we
262 present here. Some of these original reference antibodies had been further characterised by
263 peptide mapping to identify the key aspects of the epitopes recognised. Amino acid sequences
264 of the repeat region identified motifs that differentiated serovar 3 specific mAbs 10C6.6

265 (KEQPA) and 3B1.5 (EQP) from an antibody (5B1.1) that recognised both serovars 3 and 14
266 equally (GK) [10]. Our results confirm that the epitopes for 10C6.6 and 5B1.1 are definitively
267 found in the repeat sequence (Figure 4); however 5B1.1 did not recognise our truncated
268 recombinant MBA which ends in PGKL, suggesting that the GK alone cannot bind the
269 antibody and significant influence is played by the preceding A or adjacent E in the conserved
270 repeat sequence unique for serovars 3 and 14. Other mAbs that have been characterised bind
271 specifically to serovar 4 [11], serovar 9 [13], and serovar 1, 3, or 6 [12]. However, the one
272 commonality of these previously characterised reagents is their sequestration in individual
273 research laboratories. Here we have characterised a panel of commercially available
274 monoclonal antibodies and found the first monoclonal antibody (6522) that can recognise all
275 strains of *U. parvum* and *U. urealyticum*. We also found an antibody that only recognises
276 epitopes common to *U. parvum* (6527) as well as one that recognises all *U. parvum* and weakly
277 binds to serovars 7 and 11 (which together form the unique MBA genotype E as previously
278 reported by Kong *et al.* [8]). Comparison of the N-terminus of all MBA sequences for
279 *Ureaplasma parvum* and *urealyticum* strains show several conserved homologous regions
280 containing hydrophilic and charged residues for both species or conserved only within *U.*
281 *parvum* (data not shown); however, exactly where monoclonal antibodies 6522, 6523 and 6527
282 bind would require an extensive mapping investigation using truncated recombinant genes or a
283 panel of peptides. The weak recognition of serovars 7 and 11 in addition to *U. parvum* by 6523
284 is difficult to explain as there is no obvious region that separates these two serovars from the
285 remaining *U. urealyticum* strains. With regards to previously published monoclonal antibodies,
286 comparison of the amino acid sequence of the repeat regions shows differences that justify how
287 antibodies could specifically recognise serovars 1, 3, 6, 9, and 10 uniquely, but the shared
288 MBA sequence of serovar 4, 10, 12 and 13 and 2, 5 and 8 make it difficult to accept antibodies
289 that uniquely detect the MBA for serovar 4 [11] and serovar 8 [9]. However, development of

290 monoclonal antibodies that specifically recognise proteins unique to serovars 2, 5, 7, 8 10, 11,
291 12 and 13, that are not raised against the MBA, have been reported [20] We have validated the
292 bands recognised by the commercial antibodies against previously characterised antibodies, to
293 confirm it is the MBA protein that the commercial antibodies are binding.

294 Using the MBA as a method to classify strains is not without potential disadvantages. The
295 MBA is phase variable. When grown in the presence of rabbit polyclonal anti-MBA
296 antibodies, expression has been found to be shut off [21], as have selection of non-adherent
297 sub-populations [22]. The mechanism of phase variation is speculated to involve tyrosine
298 recombinases (particularly XerC) and inversion of promoter regions driving expression of the
299 open-reading frames [23, 24]. The propensity for recombination can also result in multiple
300 copies of the *mba* gene being present in the genome and comparative genomic analysis has
301 suggested that repeats characteristic of different serovars can be found in a single genome of
302 the same strain [17]; however, no evidence of co-expression of separate serovar repeat
303 expression was provided in that report, and we have not found co-expression of different MBA
304 in our investigations. In our laboratory, for all cases where isolates were found to express more
305 than one MBA, we were able to purify these to single isoform expression by picking single
306 colonies from plates (data not shown); however, this does not imply that expression of two or
307 more MBA is not possible in clinical isolates.

308 The original serotyping methods utilised a modified metabolism inhibition assay where the
309 typing polyclonal anti-serovar sera were found to alter metabolism of matching strains through
310 an unknown mechanism. Watson *et al.* [9], confirmed that 4 of 6 monoclonal antibodies also
311 had differing capacities to inhibit metabolism of matching serovars. Whether different epitopes
312 on the MBA relate to metabolic inhibition, or whether only those monoclonal antibodies that
313 recognise epitopes available in the native protein inhibited metabolism remains unknown.

314 While it is difficult to speculate a mechanism for how antibody binding to a lipid anchored

315 bacterial protein would result in bactericidal or bacteriostatic activity, the future of anti-MBA
316 monoclonal antibodies as therapeutics to exploit this phenomenon remains open.
317 Here we provide a detailed analysis that differentiates between the reactivity of a panel of
318 monoclonal antibodies raised against the MBA. The results will enable other researchers to
319 speciate clinical isolates, or assign specific serovars in some cases, based on differential
320 detection by immunoblot or immunohistochemistry. More importantly, these reagents will
321 enable researchers to begin to examine correlations between MBA size or mixed MBA
322 isoforms present in each sample, rather than just nucleic acid determinations. This may be of
323 particular importance for future investigations as experimental investigations in pregnant sheep
324 have found increased pathology when less than 5 MBA isoforms were observed compared to
325 pregnant sheep infected with 9 or more MBA isoforms for the same strain [25].

326

327 **Acknowledgements:** The first two authors (AFA and SA) share equal contribution for first
328 authorship. SA was supported by a PhD studentship funded by the Ministry of Higher
329 Education, Iraq Embassy. The authors would like to thank Dr. Harold Watson and Dr. Xiatian
330 Zhang (Department of Microbiology, University of Alabama at Birmingham) for their work
331 on characterising the initial monoclonal antibodies.

332 **Disclosure of Conflict of Interests:** Douglas McAllister is the founder of Virostat Inc.

333 **Figure Legends**

334 **Figure 1.** Immunoblot analysis of whole bacterial proteins from prototype *U. parvum*
335 serovars. A. MBA proteins detected by commercial anti-MBA monoclonal antibodies
336 separated on by non-reducing SDS-PAGE. B. Validation of bands detected by commercial
337 antibodies by comparison to previously characterised antibodies from Watson *et al.* [9] known
338 to bind serovar 3 only (10C6.6), serovar 3 and 14 only (5B1.1) and all *U. parvum* MBA
339 (8B5.2). No signal was detected for serovar 10 specific clone 8A1.2. Representative blots

340 shown for multiple repeats. Molecular mass for each serovar was maintained for each
341 detected MBA, validating the reactivity of the monoclonal antibodies.

342 **Figure 2.** Immunoblot analysis of whole bacterial proteins from prototype *U. parvum* and *U.*
343 *urealyticum* strains separated by non-reducing SDS-PAGE and transferred to nitrocellulose
344 prior to probing with commercial antibodies 6522 and 6523. Relative molecular mass of MBA
345 bands for specific serovar 1, 3, 6, 7, 11 and 14 strains were identical when detected by either
346 antibody. Representative blot from multiple repeats of the experiment are shown.

347 **Figure 3.** Immunoblot analysis of whole bacterial proteins from prototype *U. urealyticum*
348 strains (A) and 11 clinical isolates (B) by 6522 following separation by non-reducing SDS-
349 PAGE and transfer to nitrocellulose. An identical blot was probed in parallel with the serovar
350 10 specific clone 8A1.2. Comparison of the detected MBA species shows bands of exactly the
351 same relative mass were detected for both antibodies, with the exception that 8A1.2 only
352 detected the higher band for serovar 10 (C) and HPA24 (D). Sequencing of N-terminus of
353 clinical isolates HPA24 and 31 confirmed they were the only strains with the unique
354 TQPGSGST repeat found to be unique to serovar 10 MBA.

355 **Figure 4.** Immunoblot analysis of whole bacterial proteins from *E.coli* transformed with
356 plasmids containing the mba gene containing two PAGKEQ repeats (R) or truncated to
357 remove the repeats (T) as well as bacteria transformed with an empty vector (C). A. MBA
358 proteins detected by commercial anti-MBA monoclonal antibodies separated on by non-
359 reducing SDS-PAGE. B. Validation of bands detected by commercial antibodies by
360 comparison to previously characterised antibodies from Watson *et al.* [9]. Representative
361 blots shown for multiple repeats.

362 **Figure 5.** Immunohistochemistry visualisation of synthetic serovar 3 MBA expressed by
363 transformed *E.coli* by monoclonal anti-MBA 6522 (B) 6523 (C) and 6525 (D) as compared to
364 6522 staining of *E.coli* that are transformed with an empty plasmid (A). Counterstaining

365 *E.coli* bacilli with Gram-stain obscures the peroxidase staining (inset C), therefore, no counter
366 stain was used in the larger images, although outline of individual bacilli at the edge of
367 colonies are distinguishable in B-D. Images are taken with oil-emersion 100x objective lens,
368 scale bar included to indicate magnification.

369

370

371 References:

372

373

374

375

376

377

378

379

380

381

382

383

384

385

386

387

388

389

390

391

392

393

394

395

396

397

398

399

400

401

402

403

404

405

406

407

408

409

410

411

412

413

414

415

416

417

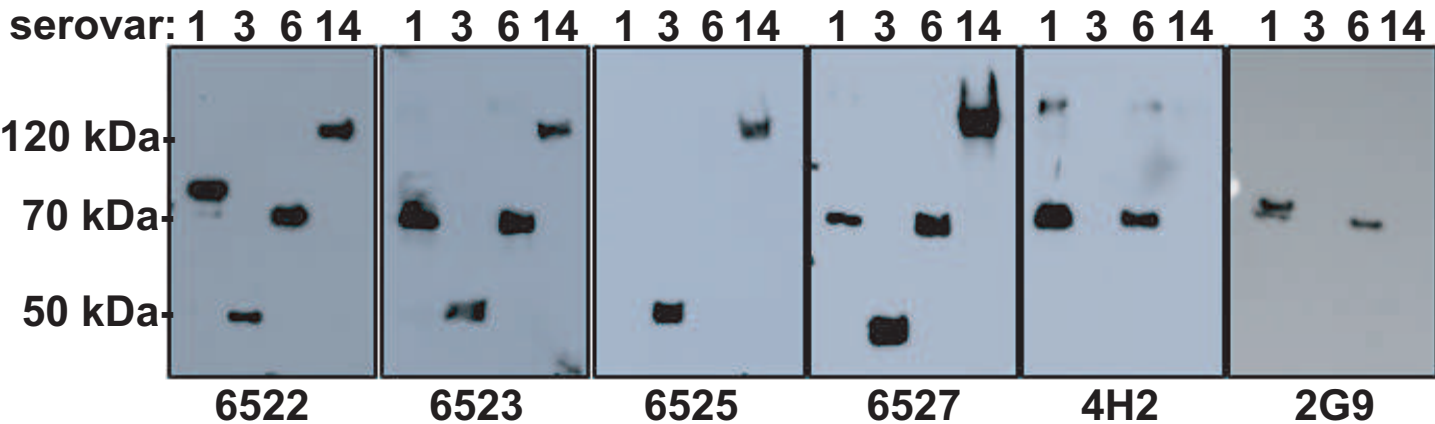
418

419

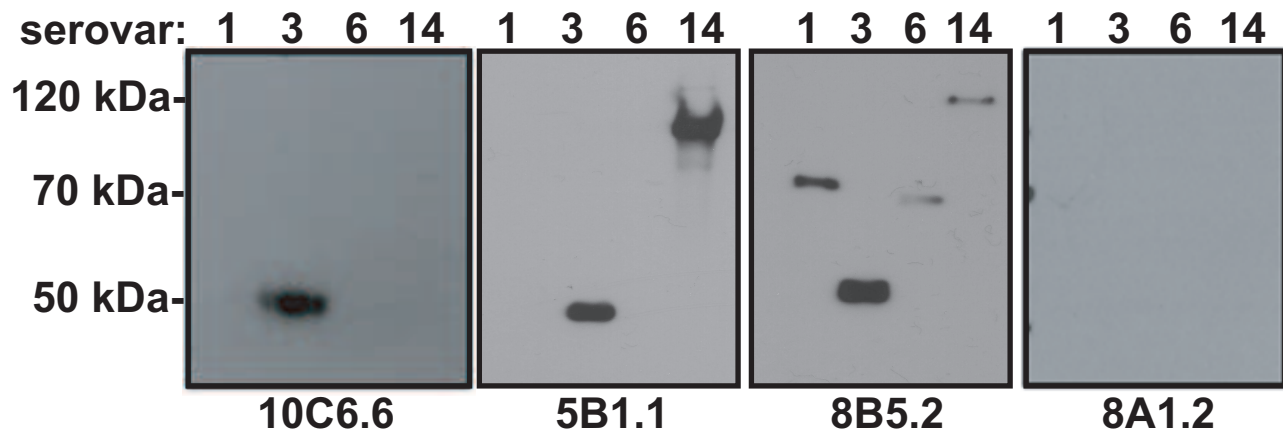
1. Viscardi RM. 2014. *Ureaplasma* species: role in neonatal morbidities and outcomes. Arch Dis Child Fetal Neonatal Ed. **99(1)**:F87-92.
2. Viscardi RM. 2010. *Ureaplasma* Species: Role in Diseases of Prematurity. Clinics in perinatology 37(2): 393-409.
3. Lowe J, Watkins WJ, Edwards MO, Spiller OB, Jacqz-Aigrain E, Kotecha SJ, Kotecha S. 2014. Association between pulmonary *Ureaplasma* colonization and bronchopulmonary dysplasia in preterm infants: updated systematic review and meta-analysis. Pediatr Infect Dis J. 33(7):697-702
4. Shepard M C. 1954. The recovery of pleuropneumonia-like organisms from Negro men with and without nongonococcal urethritis. Am J Syph Gonorrhea Vener Dis 38: 113-24.
5. Robertson JA, Stemke GW 1982 Expanded serotyping scheme for *Ureaplasma urealyticum* strains isolated from humans J Clin Microbiol. 15(5): 873–878.
6. Robertson JA, Stemke GW, Davis JW Jr, Harasawa R, Thirkell D, Kong F, Shepard MC, Ford DK. 2002. Proposal of *Ureaplasma parvum* sp. nov. and emended description of *Ureaplasma urealyticum* (Shepard et al. 1974) Robertson et al. 2001. Int J Syst Evol Microbiol. 2002 Mar;52(Pt 2):587-97.
7. Teng LJ, Zheng X, Glass JI, Watson HL, Tsai J, Cassell GH. 1994. *Ureaplasma urealyticum* biovar specificity and diversity are encoded in multiple-banded antigen gene. J Clin Microbiol. 32(6):1464-9.
8. Kong F, Ma Z, James G, Gordon S, Gilbert GL. 2000. Molecular genotyping of human *Ureaplasma* species based on multiple-banded antigen (MBA) gene sequences. Int J Syst Evol Microbiol. 2000 Sep;50 Pt 5:1921-9.
9. Watson HL, Blalock DK, Cassell GH. 1990. Variable antigens of *Ureaplasma urealyticum* containing both serovar-specific and serovar-cross-reactive epitopes. Infect Immun. 58(11):3679-88.
10. Zheng X1, Lau K, Frazier M, Cassell GH, Watson HL. 1996. Epitope mapping of the variable repetitive region with the MB antigen of *Ureaplasma urealyticum*. Clin Diagn Lab Immunol. 3(6):774-8.
11. Cheng X, A Naessens, and S Lauwers. 1993. Identification and characterization of serotype 4-specific antigens of *Ureaplasma urealyticum* by use of monoclonal antibodies. Infect Immun. 61(5): 2253–2256.
12. Cheng X, A Naessens, and S Lauwers 1994. Identification of serotype 1-, 3-, and 6-specific antigens of *Ureaplasma urealyticum* by using monoclonal antibodies. J Clin Microbiol. 32(4): 1060–1062.
13. Naessens A, Cheng X, Lauwers S, Robertson JA. 1998 Development of a Monoclonal Antibody to a *Ureaplasma urealyticum* Serotype 9 Antigen. J. Clin. Microbiol. 36(4): 1125-1127
14. Beeton ML, Chalker VJ, Maxwell NC, Kotecha S, Spiller OB. 2009. Concurrent titration and determination of antibiotic resistance in *Ureaplasma* species with identification of novel point mutations in genes associated with resistance. Antimicrob Agents Chemother. 53(5):2020-7
15. Beeton ML, Daha MR, El-Shanawany T, Jolles SR, Kotecha S, Spiller OB. 2012 Serum killing of *Ureaplasma parvum* shows serovar-determined susceptibility for normal individuals and common variable immuno-deficiency patients. Immunobiology. 217(2):187-94

- 420 16. Aboklaish AF, Dordet-Frisoni E, Citti C, Toleman MA, Glass JI, Spiller OB. 2014.
421 Random insertion and gene disruption via transposon mutagenesis of *Ureaplasma*
422 *parvum* using a mini-transposon plasmid. *Int J Med Microbiol.* 304(8):1218-25.
- 423 17. Paralanov V, Lu J, Duffy LB, Crabb DM, Shrivastava S, Methé BA, Inman J, Yooseph
424 S, Xiao L, Cassell GH, Waites KB, Glass JI. 2012. Comparative genome analysis of 19
425 *Ureaplasma urealyticum* and *Ureaplasma parvum* strains. *BMC Microbiol.* 12:88.
- 426 18. Xiao L, Glass JI, Paralanov V, Yooseph S, Cassell GH, Duffy LB, Waites KB.
427 Detection and characterization of human *Ureaplasma* species and serovars by real-time
428 PCR. 2010. *J Clin Microbiol.* 48(8):2715-23.
- 429 19. Sung TJ, Xiao L, Duffy L, Waites KB, Chesko KL, Viscardi RM. 2011. Frequency of
430 *Ureaplasma* serovars in respiratory secretions of preterm infants at risk for
431 bronchopulmonary dysplasia. *Pediatr Infect Dis J.* 30(5):379-83.
- 432 20. Echahidi F, Muyldermans G, Lauwers S, Naessens A. 2000 Development of
433 Monoclonal Antibodies against *Ureaplasma urealyticum* Serotypes and Their Use for
434 Serotyping Clinical Isolates. *Clin Diagn Lab Immunol.* 7(4): 563–567.
- 435 21. Zimmerman CU, Stiedl T, Rosengarten R, Spergser J. 2009. Alternate phase variation
436 in expression of two major surface membrane proteins (MBA and UU376) of
437 *Ureaplasma parvum* serovar 3. *FEMS Microbiol Lett.* 292(2):187-93.
- 438 22. Monecke S, Helbig JH, Jacobs E. 2003. Phase variation of the multiple banded protein
439 in *Ureaplasma urealyticum* and *Ureaplasma parvum*. *Int J Med Microbiol.* 293(2-
440 3):203-11. Zimmerman CU, Herrmann R, Rosengarten R. 2015. XerC-mediated DNA
441 inversion at the inverted repeats of the UU172-phase-variable element of *Ureaplasma*
442 *parvum* serovar 3. *Microbiol Res.* 170:263-9.
- 443 23. Zimmerman CU, Rosengarten R, Spergser J. 2013. Interaction of the putative tyrosine
444 recombinases RipX (UU145), XerC (UU222), and CodV (UU529) of *Ureaplasma*
445 *parvum* serovar 3 with specific DNA. *FEMS Microbiol Lett.* 340(1):55-64
- 446 24. Zimmerman CU, Herrmann R, Rosengarten R. 2015. XerC-mediated DNA inversion
447 at the inverted repeats of the UU172-phase-variable element of *Ureaplasma parvum*
448 serovar 3. *Microbiol Res.* 170:263-9.
- 449 25. Knox CL, Dando SJ, Nitsos I, Kallapur SG, Jobe AH, Payton D, Moss TJ, Newnham
450 JP. 2010. The severity of chorioamnionitis in pregnant sheep is associated with *in vivo*
451 variation of the surface-exposed multiple-banded antigen/gene of *Ureaplasma parvum*.
452 *Biol Reprod.* 83(3):415-26.

A. Commercially available monoclonal antibodies



B. Previously published monoclonal antibodies



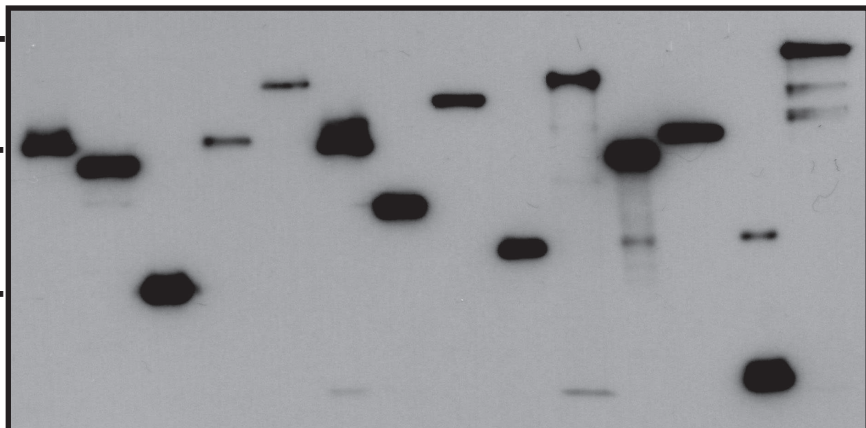
A. monoclonal antibody 6522

serovar: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

120 kDa-

70 kDa-

50 kDa-



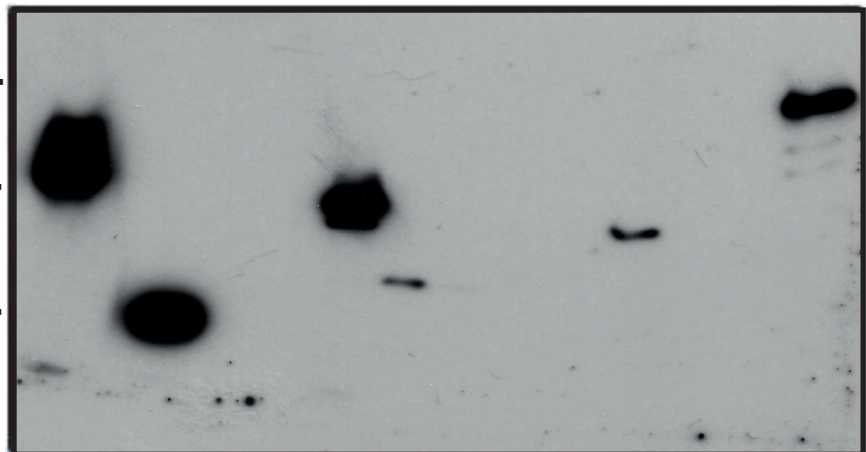
B. monoclonal antibody 6523

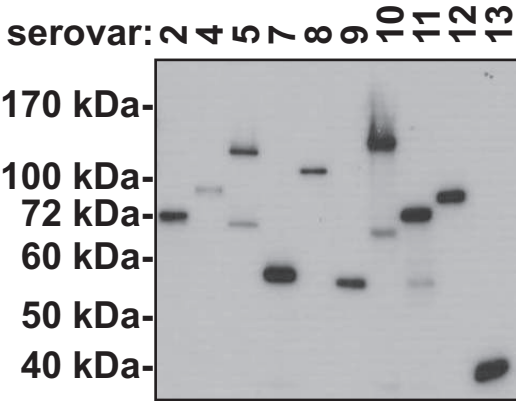
serovar: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

120 kDa-

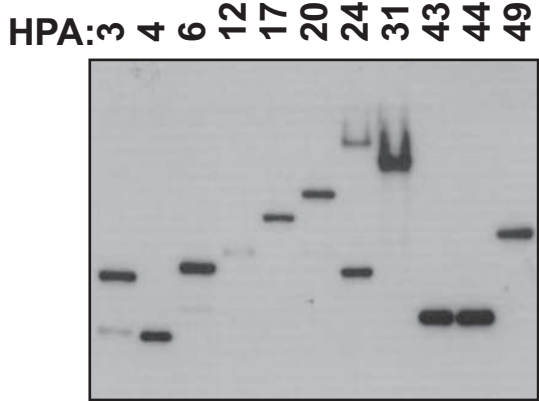
70 kDa-

50 kDa-

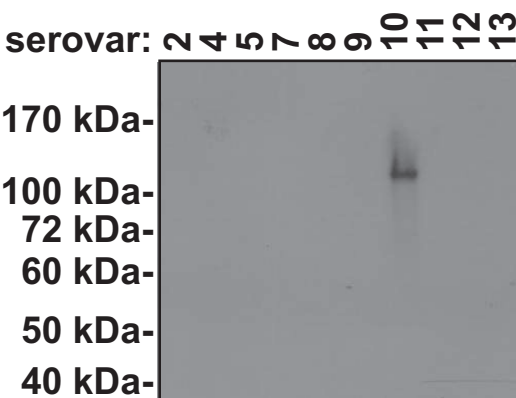




(A)



(B)



(C)



(D)

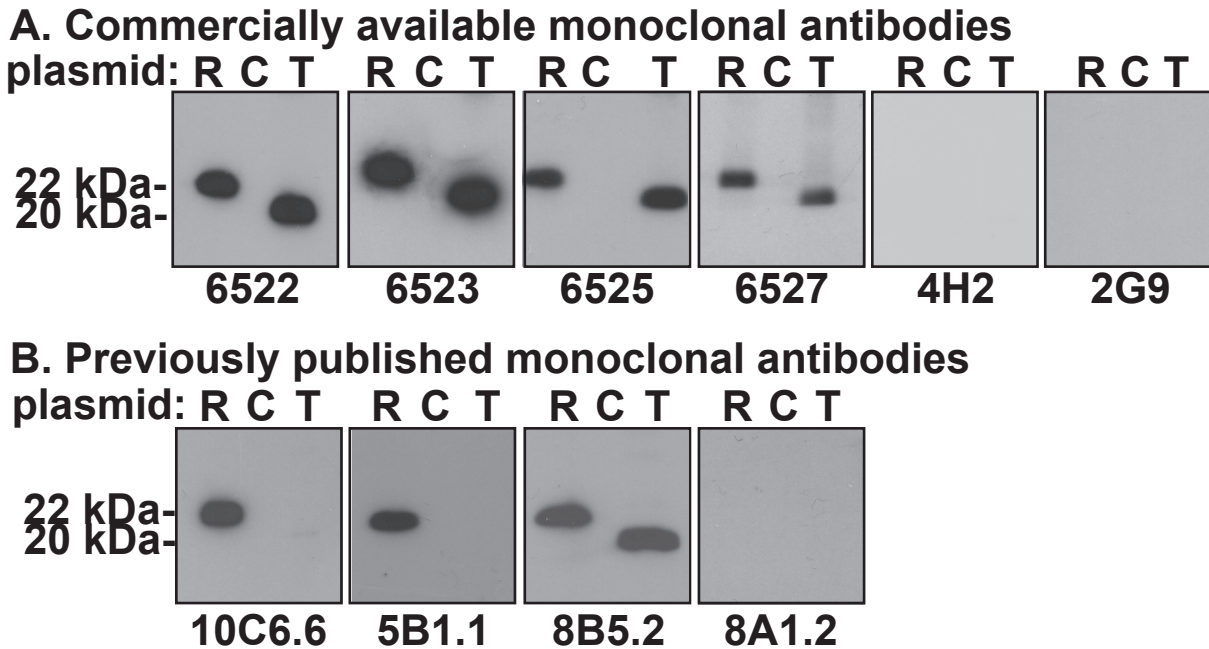


Figure 4. Immunoblot analysis of whole bacterial proteins from *E. coli* transformed with plasmids containing the *mba* gene containing two PAGKEQ repeats (R) or truncated to remove the repeats (T) as well as bacteria transformed with an empty vector (C). A. MBA proteins detected by commercial anti-MBA monoclonal antibodies separated on by non-reducing SDS-PAGE. B. Validation of bands detected by commercial antibodies by comparison to previously characterised antibodies from Watson *et al.* [9]. Representative blots shown for multiple repeats.

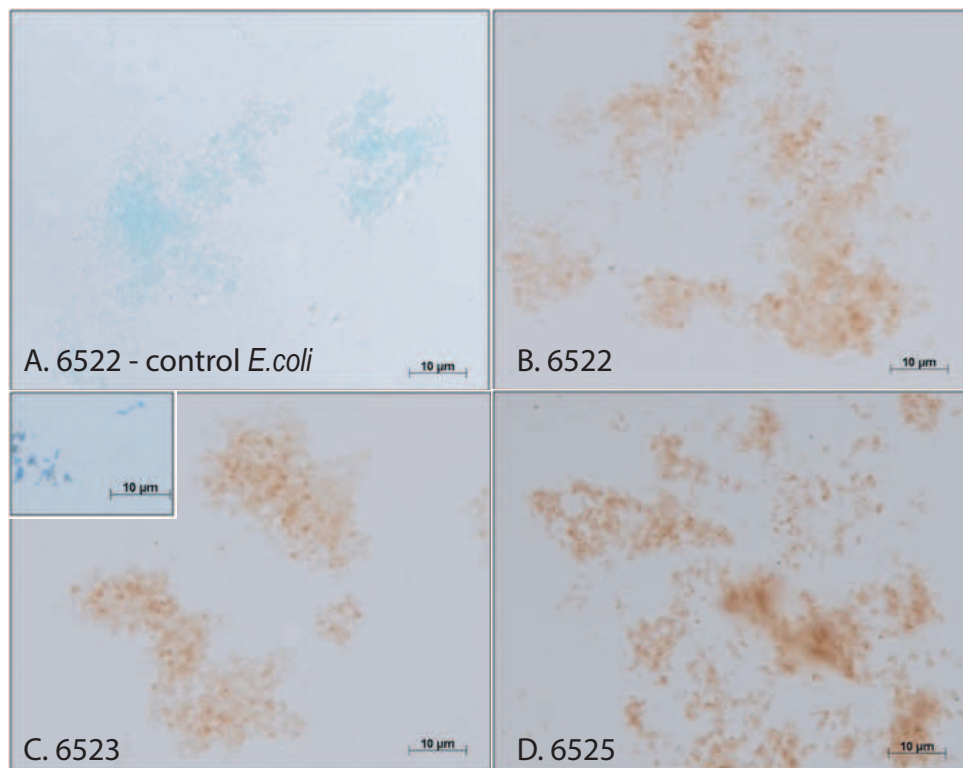


Figure 5. Immunohistochemistry visualisation of synthetic serovar 3 MBA expressed by transformed *E. coli* by monoclonal anti-MBA 6522 (B) 6523 (C) and 6525 (D) as compared to 6522 staining of *E. coli* that are transformed with an empty plasmid (A). Counterstaining *E. coli* bacilli with Gram-stain obscures the peroxidase staining (inset C), therefore, no counter stain was used in the larger images, although outline of individual bacilli at the edge of colonies are distinguishable in B-D. Images are taken with oil-emersion 100x objective lens, scale bar included to indicate magnification.