Title:

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

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

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Abstract.

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

1. INTRODUCTION

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

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

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

2. MATERIALS AND METHODS

2.1 Antibodies

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

2.2 Bacterial strains

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

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

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

generate the DNA sequence optimised for *E. coli* expression following input of the following amino acid sequence (supplementary figures 1 and 2). The promoter for the *tuf* gene from serovar 3 (170 bp upstream of the AUG start codon) was then added upstream of this optimised open-reading frame to promote expression. This sequence was synthesized by MWG Eurofins (Ebersberg, Germany) which was provided in the ampicillin resistant plasmid pEX-A2. A HinDIII restriction site was engineered into the sequence just prior to the PAGKEQ repeats so that digestion with HinDIII and re-ligation would result in expression of serovar 3 MBA that ended in FETTQPGKL rather than FETTQPGKLPAGKEQPAGKEQ. One shot Top10 chemically competent *E. coli* (Invitrogen; Paisley, Scotland, UK) were transformed with full or HinDIII truncated plasmids, as per manufacturer's instructions. This bacteria has the genotype F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-. Single colonies of transformed bacteria were picked from LB agar plates containing 100 mg/L ampicillin and grown up in LB broth containing ampicillin for further analysis. Control bacteria containing empty EX-A2 plasmid were used as controls.

2.4 Immunoblot analysis

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

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

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

2.5 Immunohistochemistry analysis

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

1 hour. Following further washing steps peroxidase was developed for 3 minutes in 0.025% diaminobenzamidine containing H_2O_2. Where indicated sections were also counterstained for 10 sec in 0.02% picromethyl blue prior to dehydration, clearing in xylene and mounting under a coverslip.

RESULTS

Screening prototype Ureaplasma parvum strains with monoclonal antibodies.

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

Screening prototype Ureaplasma urealyticum strains with monoclonal antibodies.

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

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

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

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

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

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

DISCUSSION

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

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

bacterial protein would result in bactericidal or bacteriostatic activity, the future of anti-MBA monoclonal antibodies as therapeutics to exploit this phenomenon remains open.

Here we provide a detailed analysis that differentiates between the reactivity of a panel of monoclonal antibodies raised against the MBA. The results will enable other researchers to speciate clinical isolates, or assign specific serovars in some cases, based on differential detection by immunoblot or immunohistochemistry. More importantly, these reagents will enable researchers to begin to examine correlations between MBA size or mixed MBA isoforms present in each sample, rather than just nucleic acid determinations. This may be of particular importance for future investigations as experimental investigations in pregnant sheep have found increased pathology when less than 5 MBA isoforms were observed compared to pregnant sheep infected with 9 or more MBA isoforms for the same strain [25].

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Figure Legends

Figure 1. Immunoblot analysis of whole bacterial proteins from prototype *U. parvum* serovars. 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] known to bind serovar 3 only (10C6.6), serovar 3 and 14 only (5B1.1) and all *U. parvum* MBA (8B5.2). No signal was detected for serovar 10 specific clone 8A1.2. Representative blots

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

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

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

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

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.

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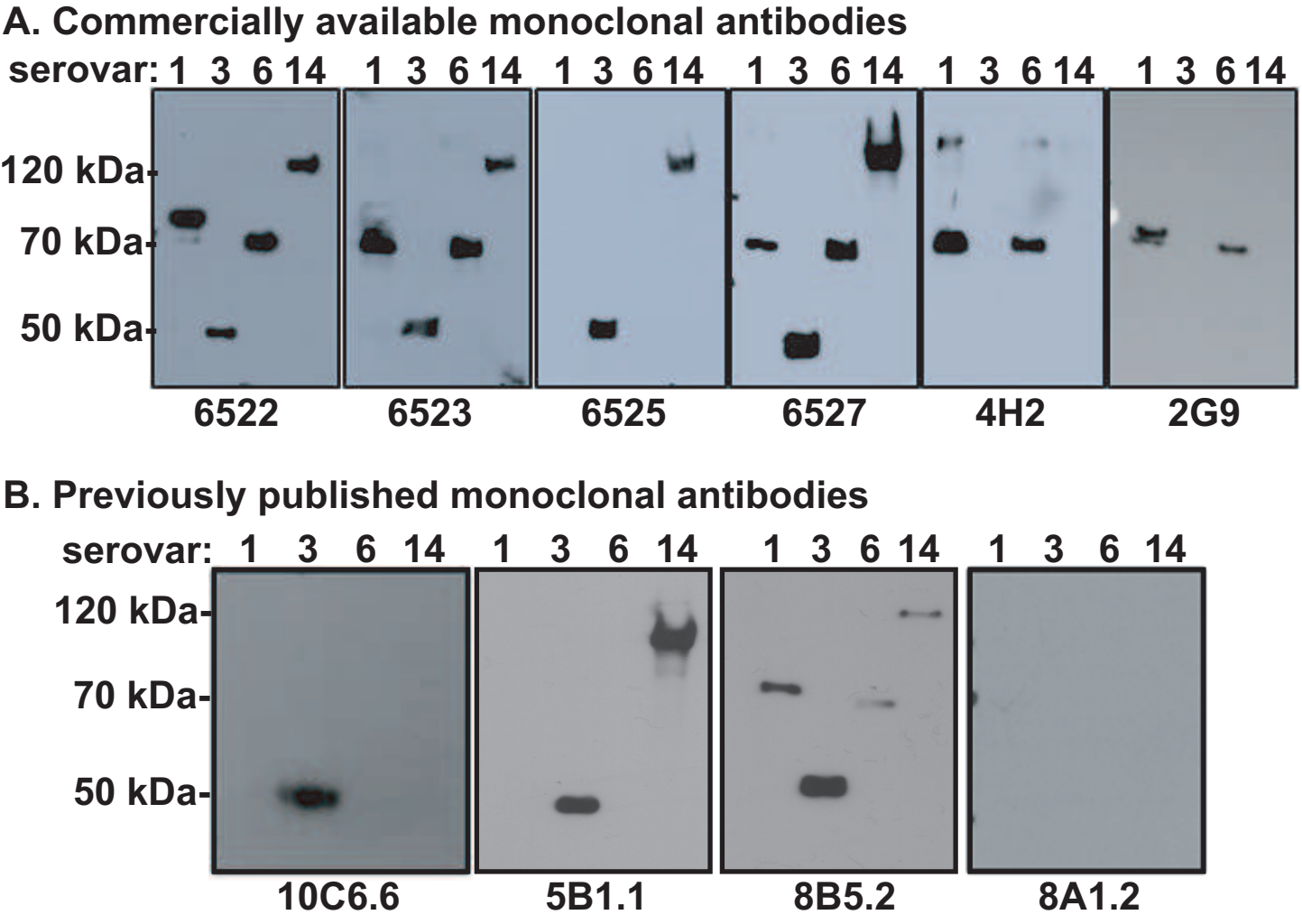
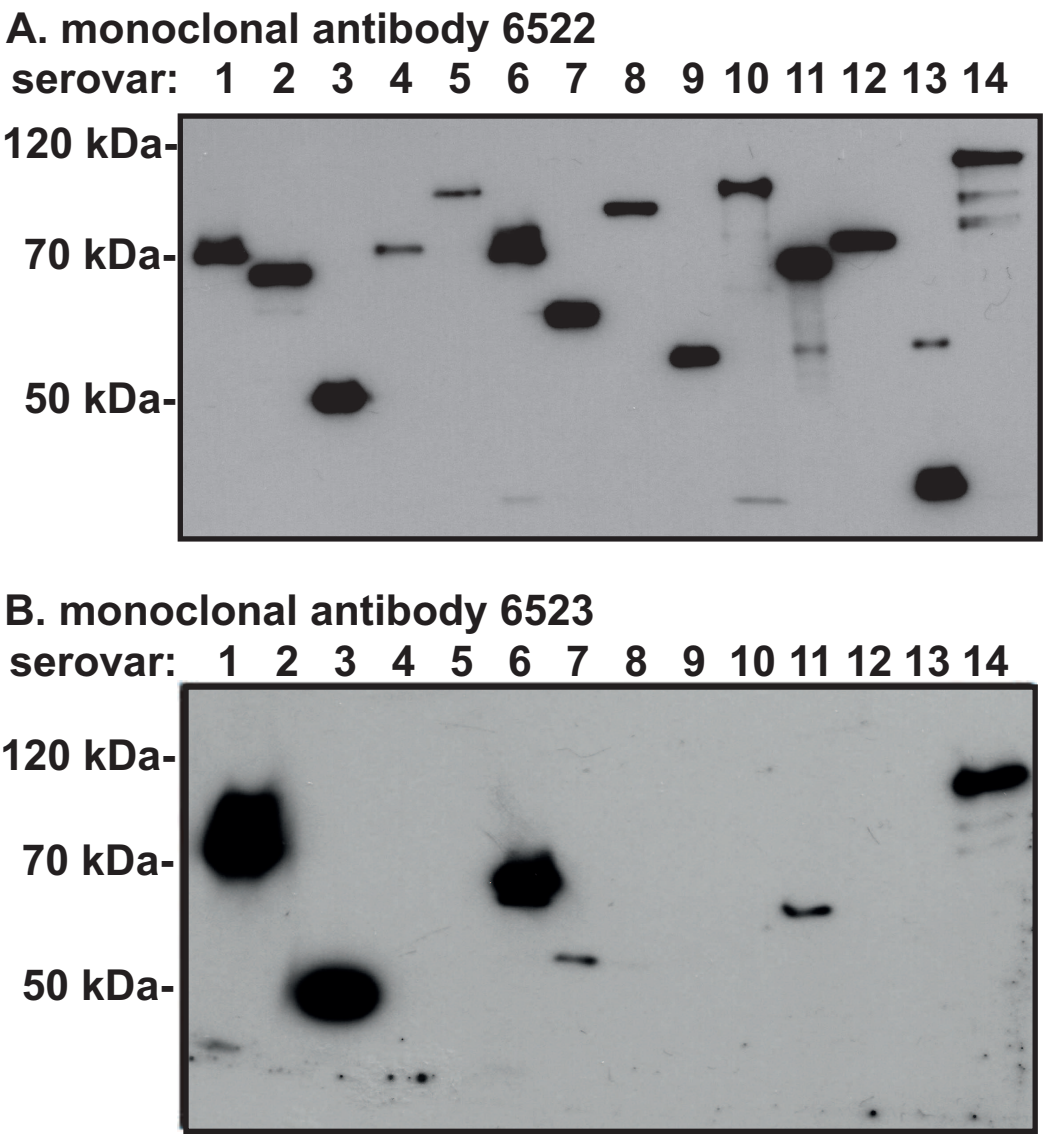

A. Commercially available monoclonal antibodies
serovar: 1 3 6 14 1 3 6 14 1 3 6 14 1 3 6 14 1 3 6 14 1 3 6 14
120 kDa
70 kDa
50 kDa
6522
6523
6525
6527
4H2
2G9
B. Previously published monoclonal antibodies
serovar: 1 3 6 14 1 3 6 14 1 3 6 14 1 3 6 14
120 kDa
70 kDa
50 kDa
10C6.6
5B1.1
8B5.2
8A1.2


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-
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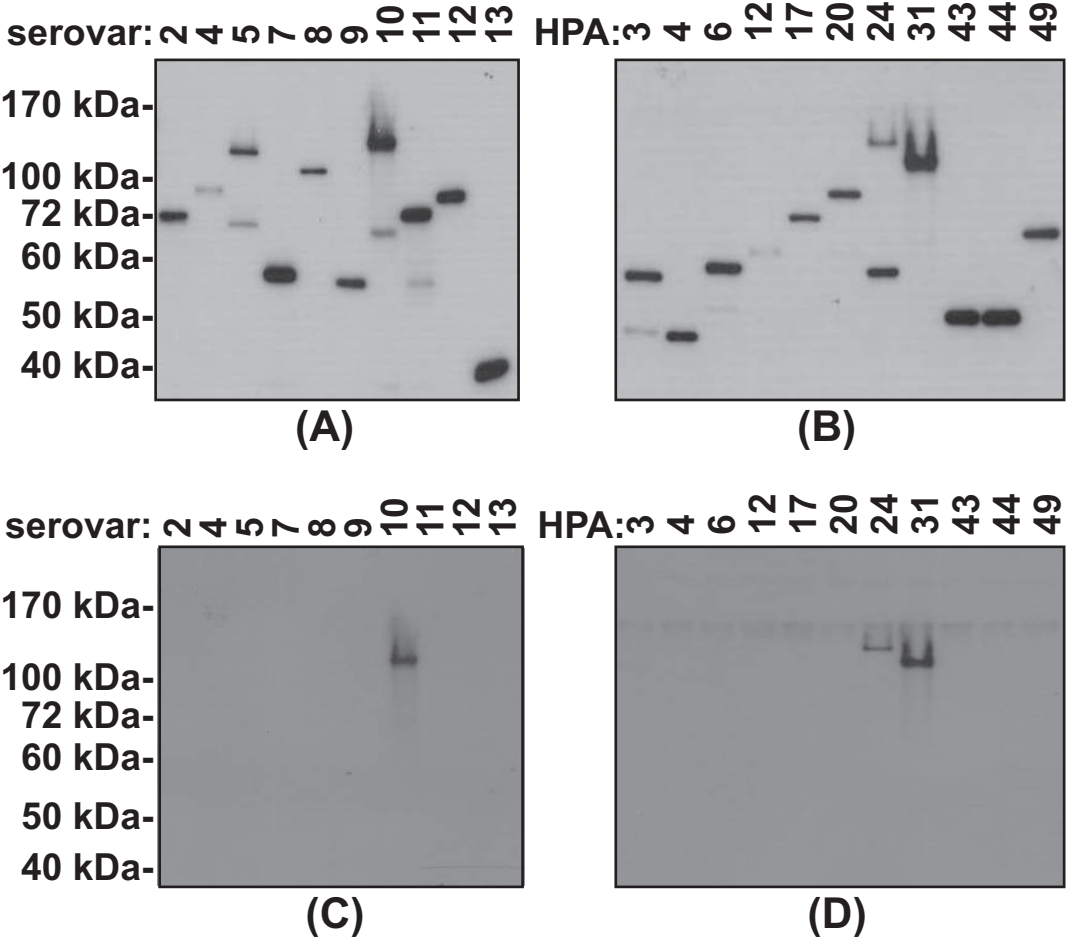
120 kDa-
70 kDa-
50 kDa-


serovar: 2 4 5 7 8 9 10 11 12 13
HPA: 3 4 6 12 17 20 24 31 43 44 49
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100 kDa-
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50 kDa-
40 kDa-
(A)
(B)
(C)
(D)

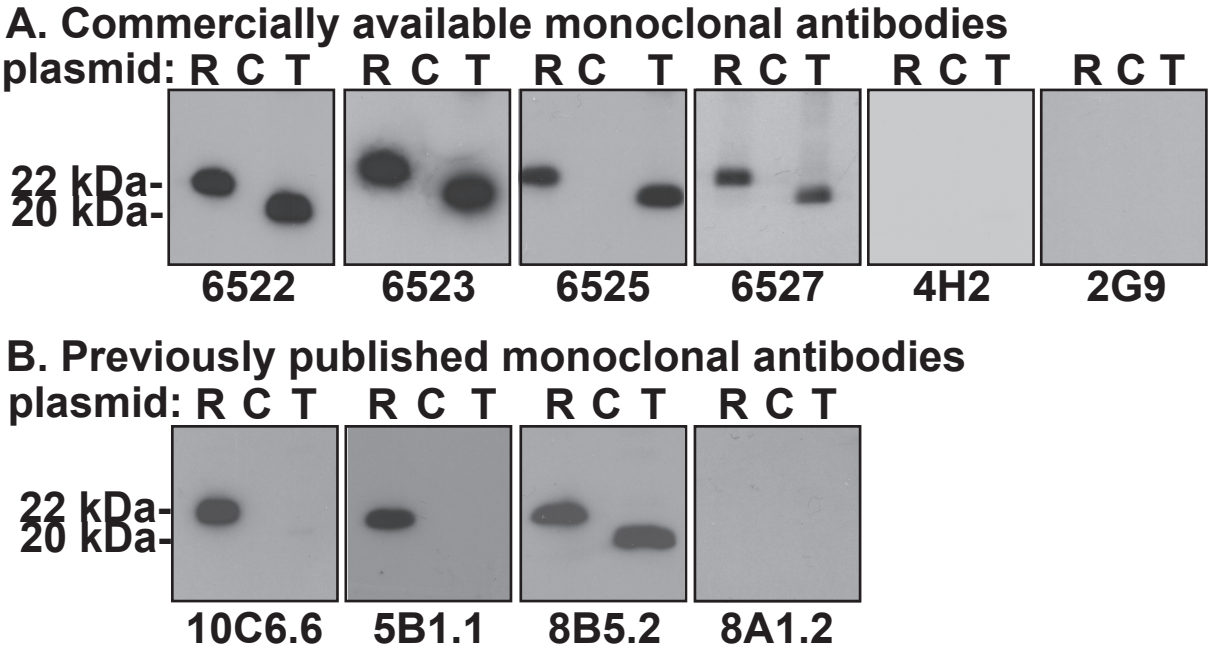

Figure 4. Immunoblot analysis of whole bacterial proteins from E.coli transformed with plasmids containing the mba gene containing two PAGKEQ repeats (R) or truncated to remove the repeats (T) as well as bacteria transformed with an empty vector (C).A. MBA proteins detected by commercial anti-MBA monoclonal 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.

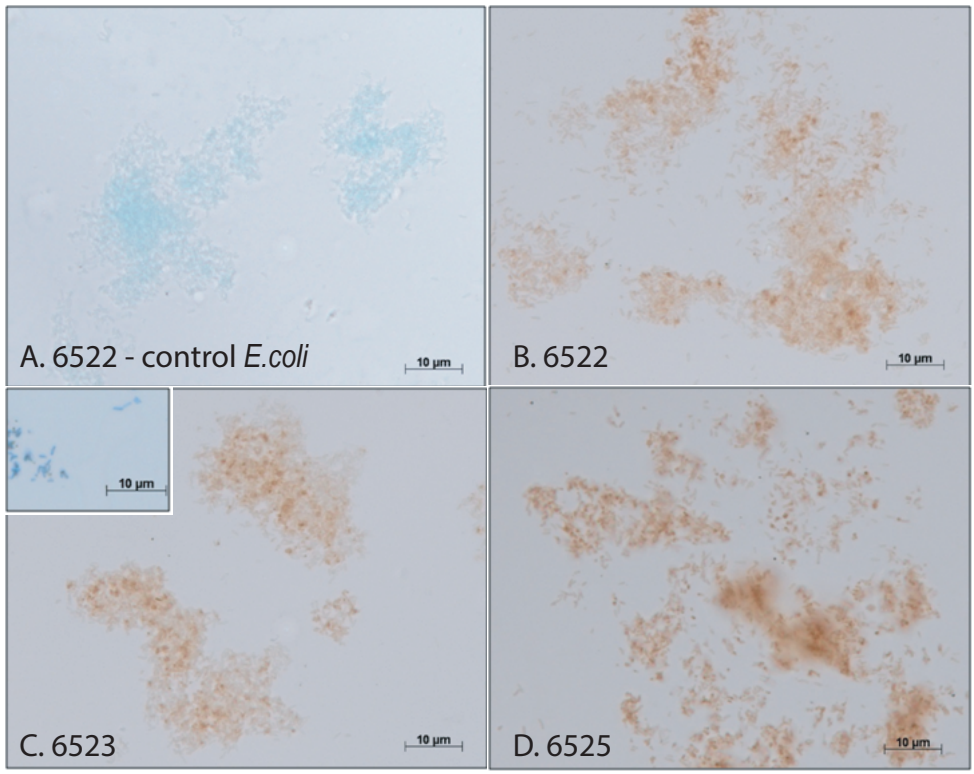

Figure 5. Immunohistochemistry visualisation of synthetic serovar 3 MBA expressed by transformed *E.coli* by monoclonal anti-MBA 6522 (B) 6523 (C) and 6525 (D) as compared to 6522 staining of *E.coli* that are transformed with an empty plasmid (A). Counterstaining *E.coli* bacilli with Gram-stain obscures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