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Differential recognition of the multiple banded antigen isoforms across *Ureaplasma parvum* and *Ureaplasma urealyticum* species by monoclonal antibodies

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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 *Escherichia 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. Viscardi (2014) reported that almost half of the preterm infants of less than 32 weeks gestation are Ureaplasma-positive in one or more compartment (respiratory, blood and/or cerebrospinal fluid), indicating that these organisms are the most common pathogens affecting this population. 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 (Viscardi, 2010). 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 (Lowe et al., 2014).

However, *Ureaplasma* were initially described in isolates from male patients suffering from urethritis. The initial 1954 report (Shepard, 1954) 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 (1982)

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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: *Ureaplasma parvum* (serovars 1, 3, 6 and 14) and *Ureaplasma urealyticum* (serovars 2, 4, 5, and 7–13) (Robertson et al., 2002). 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 (Teng et al., 1994). 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 (Teng et al., 1994). 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. (2000) found that the predicted amino acid sequence for the repeat region of each *U. parvum* serovar (Aboklaish et al., 2014; Beeton et al., 2012; Cheng et al., 1994; Robertson and Stemke, 1982) 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 (Watson et al., 1990; Zheng et al., 1996; Cheng et al., 1993, 1994; Naessens et al., 1998). Some monoclonal antibodies recognise single serovars, while others recognise groups of *U. parvum* or *U. urealyticum* sub-groups. However, no single monoclonal antibody has been reported that detects the MBA for all *Ureaplasma* species that infect humans. Further, all previously reported 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 (Watson et al., 1990; Zheng et al., 1996) (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 clone 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 (Beeton et al., 2009). Eleven clinical isolates of *U. urealyticum* originated from a previously published study examining antibiotic susceptibility for clinical isolates in England and Wales between 2003 and 2009 (Beeton et al., 2009), as were *U. parvum* strains HPA2 (SV6), HPA5 (SV3) and HPA32 (SV14) which have been further characterised in other investigations (Beeton et al., 2012; Aboklaish et al., 2014). All strains arose from infection by *U. urealyticum* only or mono-specific serovars as determined by molecular methods. *M. hominis* isolate NCTC10111 was obtained from National Collection of Type Cultures (NCTC; Held by Public Health England) as was the *M. pneumoniae* type strain NCTC10119. These were grown in Mycoplasma selective media (Mycoplasma Experience Ltd.) and used as negative controls for immunoblot analysis.

2.3. Creation of Escherichia 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 Fig. S1). 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 *Hin*DIII restriction site was engineered into the sequence just prior to the PAGKEQ repeats so that digestion with *Hin*DIII and re-ligation would result in expression of serovar 3 MBA that ended in FETTQPGKL rather than FETTQPGKLPAGKEQPAGKEQ. One shot Top 10 chemically competent *E. coli* (Invitrogen; Paisley, Scotland, UK) were transformed with full or *Hin*DIII truncated plasmids, as per the 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 ×*g* 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), quantified by the bicinchoninic acid protein assay kit (Sigma-Aldrich, UK) before addition of an equal volume of LDS-sample buffer (Invitrogen) and boiled at 95 °C for 5 min prior to loading on a non-reducing SDS polyacrylamide gel (7.5% polyacrylamide) and separated by electrophoresis (loading 15 μg per lane). SuperSignal™ Molecular Weight Protein Ladder was 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% Tween 20 (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 °C. Unbound monoclonal antibodies were removed by 3 washes in PBST prior to detection with 1:2000 dilution of secondary antibody in blocking buffer for 1 h at room temperature. Peroxidase secondary antibodies were detected by