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Shaken or stirred?: Comparison of methods for dispersion of *Mycoplasma pneumoniae* aggregates for persistence *in vivo*

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Background: Mycoplasma pneumoniae (Mpn), one of the smallest self-replicating prokaryotes, is known to readily adhere to host cells and to form aggregates in suspension. Having only one cell membrane and no cell wall, mycoplasmas present questions as to optimal aggregate disruption method while minimizing cell death *in vitro*. We compared conventional vortex mixing with other methods for disruption of bacterial aggregates and for its effect on cell viability.

Methods: Strain UAB PO1, a clinical Mpn isolate, was dispersed using a conventional vortex mixer with or without nonionic detergent (0.1% and 0.01% Tween-20), a probe-type ultrasonicator, or repeated passage through a 27-gauge needle. The resulting suspensions were assayed for recoverable colony-forming units (CFU). Flow cytometric assays were carried out to examine particle size and membrane integrity with the transmembrane potential dye $DiBAC_4$. Wet Scanning Transmission Electron Microscopy (Wet-STEM) was performed for high resolution imaging of the resultant cell suspensions. Additional Mpn strains and other human mollicute species were assayed in a similar manner. Mice were infected with either vortexed or sonicated UAB PO1 and bacterial persistence was examined *via* Mpn-specific 16S qPCR.

Results: Comparison between dispersion methods showed a 10-fold enrichment of recoverable Mpn CFU with sonication compared to other methods. Time-course analysis showed significantly lower bacterial CFU with vortexing compared to sonication at all time points. Flow cytometric analysis showed increased cellular membrane damage *via* $DiBAC_4$ staining in sonicated suspensions, but a decreased particle size. Wet-STEM imaging showed markedly improved dispersion with sonication compared to conventional vortex treatment, and surprisingly vortexing for 30 s produced up to a 100-fold drop in CFU. Results similar to UAB PO1 were obtained with three additional Mpn strains and other *Mollicutes* species, although they exhibited differential susceptibilities to disaggregation by sonication. Finally, increased persistence of the organism in a mouse model of infection was observed using sonicated suspensions for initial infection.

Conclusions: Sonication is superior to vortexing with or without nonionic detergent or repeated 27-gauge needle passage for dispersion of Mpn aggregates while preserving cell viability. Preparation of Mpn suspensions for *in vivo* experiments is best accomplished using brief sonication due to the dramatic increase in CFU produced by sonication. Dispersion methods may affect the final experimental results and should be an important consideration for future research involving mycoplasma species.

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Abbreviations: ARP, Animal Resources Program; BSL, biosafety level; CFU, colony-forming unit; d, day; dpi, days post infection; $DiBAC_4(3)$, (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol); FSC, forward scatter; hr, hour; IACUC, Institutional Animal Care and Use Committee; kHz, kilohertz; i.n., intranasal; kV, kilovolts; Mg, *Mycoplasma genitalium*; Mh, *Mycoplasma hominis*; Mpn, *Mycoplasma pneumoniae*; s, second; spp., species; rRNA, ribosomal RNA; SEM, scanning electron microscope; TEM, transmission electron microscopy; Up3, *Ureaplasma parvum* serovar 3; Uu10, *Ureaplasma urealyticum* serovar 10; Wet-STEM, wet scanning transmission electron microscopy.

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1. Introduction

Mycoplasma pneumoniae (Mpn) is a genome-reduced atypical human pathogen and a leading cause of community acquired pneumonia in hospitalized pediatric patients (Jain et al., 2015). The microbe's adhesins and accessory proteins (P1-adhesin, P30, HMW1, HMW2) are important for attachment to ciliated epithelial cells and are important virulence determinants, as the organism becomes avirulent *in vivo* upon their deletion (Balish and Krause, 2006). Due to the importance of cytadhesion for mediating virulence and persistence of the organism on mucosal surfaces, much work has focused on understanding the attachment and motility of Mpn (Drasbek et al., 2007; Kahane et al., 1982; Krause and Baseman, 1982).

In vitro models have suggested that during co-incubation of differentiated epithelial cells and Mpn, cytadhesion to the ciliated epithelial cells occurs within 30 mins of exposure (Prince et al., 2014). Adhesion occurs upon interaction of the microbe with sialic acid residues present on the epithelial cell surface, which act as receptors for host-pathogen interactions (Drasbek et al., 2007; Kahane et al., 1982). Unlike other bacteria, mycoplasmas lack a cell wall and require cholesterol for proper function of their single membrane (Balish and Krause, 2006). Combined with the importance of cytadhesion to host cells, it is of particular interest that Mpn also possesses the ability to bind to, and form biofilms on plastic and glass surfaces (Simmons et al., 2013).

Use of conventional laboratory methods for disruption of Mpn aggregates has typically been reliant upon bench-top vortex mixers or repeated passage through a 27G needle. Sonication has been used to increase the accuracy of bacterial counts within cultures by disrupting aggregates of other bacteria, *e.g. Staphylococcus aureus*(Haaber et al., 2016) and some reports in the past have documented efficient dispersion of mycoplasma suspensions using ultrasonic treatment (Furness, 1975; Furness et al., 1968; Kim et al., 1966; Uchida et al., 1981). In the current study, we sought to examine systematically the optimal means to minimize aggregation *in vitro* while preserving viability. Ideal disruption parameters on multiple Mpn isolates and additional mollicute species were assessed. We also evaluated bacterial persistence in a mouse model after inoculation of animals with Mpn suspensions prepared using each method (Wubbel et al., 1998).

2. Methods

2.1. Ethical considerations

Animal experiments were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC) and Animal Resources Program (ARP) guidelines at the University of Alabama at Birmingham (UAB), as well as the National Institutes of Health Guide for the Care and Use of Laboratory Animals under UAB animal protocol number 140,510,112.

2.2. Bacterial culture

Mpn strain UAB PO1 was utilized for all *in vitro* analysis of dispersion, infections and *in vivo* study (Simmons et al., 2013; Xiao et al., 2015). Mpn strains M129 (ATCC 29342), FH (ATCC 15531) and 54524 (UAB clinical isolate) were utilized for *in vitro* analysis of dispersion methods (Xiao et al., 2015). *Ureaplasma urealyticum* serovar 10 (Uu10, ATCC 33699), *Ureaplasma parvum* serovar 3 (Up3, ATCC 700970), *Mycoplasma hominis* (Mh, PG21, ATCC 23114), and *Mycoplasma genitalium* (Mg, G37, ATCC 33530) were used for comparison of dispersion methods between human *Mollicutes* species (Hilliard et al., 2005; Tully et al., 1986; Xiao et al., 2014). Stocks were grown in cell culture flasks in 100 mL SP4 broth for 5–7 days (mycoplasmas)(Hoek et al., 2002) or 10B broth for overnight (ureaplasmas) until color change designated approximate log phase growth (Xiao et al., 2014). Adherent bacteria were then harvested with cell scrapers (Corning Life Sciences, Corning, NY) and passaged through a 27G syringe 4–5 times to disrupt aggregates. Bacteria were then resuspended in 50 mL fresh SP4 broth. Stocks were stored at −80 °C and vials were thawed before each experiment. A8-agar and 10B broth were used for *Ureaplasma* spp., while SP4-agar and broth were used for Mh and Mg (Xiao et al., 2014).

CFU of frozen stock cultures was determined by thawing a single vial, performing serial dilutions (0.1 ml bacterial suspension in 0.9 ml SP4 or 10B) and plating 0.02 ml of each dilution on SP4 or A8 agar. Agar plates were incubated at 37 °C and 5% CO_2 between 3 and 21 days (2-3d for *Ureaplasma* spp. and Mh, 5-7d for Mpn isolates, and 14-21d for Mg). Plates were visualized using a stereomicroscope and colonies were counted on the dilutions yielding 30–300 discrete colonies.

2.3. Bacterial disruption and flow cytometric analysis

All handling of bacterial cultures was carried out in a biosafety class II cabinet with Biosafety Level II (BSL II) recommended personal protective equipment (double gloves, lab coat, mask and face shield) for aerosol containment. All hoods were disinfected with 10% bleach solution, followed by a 70% ethanol solution, and conventional sterile technique was followed to prevent spread of contaminants from the disaggregation process. For analysis of the effects of various disruption methods on bacterial CFU, stocks of bacteria were thawed and aliquoted for initial homogenous culture conditions. Aliquots were then subjected to disruption *via* vortexing (Fisher Vortex Genie 2, Cat 12-812, Fisher Scientific, Hampton, NH) (conventional disruption, two 1 s pulses at full power), vortexing with anionic detergents (0.1 and 0.01% Tween-20 solutions), passage through a 27G needle, and sonication at 20% output power at a frequency of 22.5 kHz (kHz) with a probe sonicator (Microson Ultrasonic Cell Disruptor XL2005, Heat Systems) for 1 and 5 s (power setting 2) while the tube was immersed in an ice bath. Time-course analysis of samples, either vortexed or sonicated, was carried out at 1 s, 5 s, 10 s, 20 s and 30 s from an initial homogenous stock and CFU was determined, as described above.

Prior to disruption for flow cytometric analysis, initial stocks of bacteria were diluted in fresh SP4 or 10B broth and incubated for 1 h at 37 °C to ensure optimal membrane integrity post-thaw. Flow cytometric analysis was carried out after vortexing or sonication for 10 s to test the membrane integrity. $DiBAC_4(3)$ (Bis-(1,3-Dibutylbarbituric Acid)Trimethine Oxonol) (Fisher Scientific, Hampton, NH) was utilized to detect changes in membrane potential for bacteria with analysis under channel FL1 as previously described (Xiao et al., 2014). Cell suspensions treated with 10% buffered formalin were utilized as control “damaged” cells. Flow cytometric measurements were carried out on a C6 Acuri instrument (BD Biosciences, San Jose, *CA*). FlowJo v. 10 software was utilized for analysis of bacterial flow cytometry data (FlowJo, LLC, Ashland, OR).

2.4. Wet-STEM microscopy

Wet-STEM analysis was carried out on Mpn UAB PO1 aggregates *in vitro* after disruption *via* sonication or vortexing (10 s disruption per each condition). Images were acquired with a Quanta FEG 650 Scanning Electron Microscope (SEM) using the wet Scanning Transmission Electron Microscopy (wetSTEM) detector. The samples were prepared for imaging by dropping a small aliquot of medium containing fixed bacteria on a carbon coated copper Transmission Electron Microscopy (TEM) grid. The grid was held in a Peltier cooling stage at 2 °C and pressure maintained at 4.5–5.5 Torr to help prevent evaporation during imaging using an accelerating voltage of 30 kV.

2.5. Intranasal (i.n.) infection of mice with Mycoplasma pneumoniae

Age-matched (4-week old) female BALB/cJ mice were acquired from JAX laboratories (Bar Harbor, ME) and maintained in specific-pathogen-free environments during the course of experimentation. Mice were

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