CHAPTER-9
MATERIALS AND METHODS

9.1. STUDY PERIOD

December 2011 to December 2012

9.2. BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains used in the present study are listed in Table 1. Unless otherwise stated, the wild type \textit{A.baumannii} strains and the ATCC 17978 \textit{ata} mutant strain were grown to an optical density at 650 nm (OD$_{650}$) of 0.025 in lysogeny broth (LB) broth to maximize Ata production. The complemented strain, ATCC 17978 \textit{ata-c}, was grown to an OD$_{650}$ of 0.4 in LB broth supplemented with kanamycin at 50 g/ml, and the production of Ata was induced or repressed with 2% arabinose or 0.2% glucose, respectively, for 2 h.

\textbf{Table 9.1.} Bacterial strains used in this study

<table>
<thead>
<tr>
<th>A.baumannii stain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 17978 (wild type)</td>
<td>Reference sequenced strain, susceptible to antibiotics</td>
<td>ATCC</td>
</tr>
<tr>
<td>ATCC 17978\textit{ata} (mutant)</td>
<td>ATCC 17978 derivative with an in-frame deletion of \textit{ata}</td>
<td>131</td>
</tr>
<tr>
<td>ATCC 17978\textit{ata} c</td>
<td>ATCC 17978\textit{ata} complemented with the \textit{ata} gene in pBAD18kan-Ori</td>
<td>131</td>
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<tr>
<td>S8</td>
<td>MDR clinical isolate</td>
<td>131</td>
</tr>
<tr>
<td>S11</td>
<td>MDR clinical isolate</td>
<td>131</td>
</tr>
<tr>
<td>I38</td>
<td>Clinical isolate</td>
<td>131</td>
</tr>
<tr>
<td>I25</td>
<td>MDR clinical isolate</td>
<td>131</td>
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</table>
9.3. ANTI ADHESIVE PROPERTIES OF ANTISERA TO ATA

1. Microtiter plates were coated overnight at 4°C with 5 g of collagen types IV (from Sigma) per well in 100 mM phosphate buffer at pH 7 [131].

2. Wells were washed three times with Phosphate buffer saline Tween(PBST) and then treated with blocking solution (PBST–2% nonfat dry milk) for 2 h at 37°C

3. Wells are again washed three times with PBST.

4. The ability of rabbit antibodies to Ata, prepared as described elsewhere [131], to block the binding of *A. baumannii* strains to collagen type IV was tested with wild type *A. baumannii* ATCC 17978 and the 17978 Δ*ata* and 17978 Δ*ata-c* mutant strains.

5. The wild type *A. baumannii* strains and the ATCC 17978 *ata* mutant strain were grown to an optical density at 650 nm (OD$_{650}$) of 0.025 in lysogeny broth (LB) broth to maximize Ata production. The complemented strain, ATCC 17978 *ata-c*, was grown to an OD$_{650}$ of 0.4 in LB broth supplemented with kanamycin at 50 g/ml, and the production of Ata was induced or repressed with 2% arabinose or 0.2% glucose, respectively, for 2 h.

6. All the strains are resuspended in phosphate-buffered saline (PBS) to an OD$_{650}$ of 0.4. Then, $5 \times 10^6$ CFU of these cells were preincubated with either anti Ata antibodies or normal rabbit sera (NRS; diluted 1:1,000 in PBS) in a total volume of 100 µl for 30 min before they were added to each collagen coated well

7. It was incubated for 1 h at 37°C

8. Finally, the wells were rinsed four times with PBS and treated with trypsin EDTA to release the bound bacteria.

9. The number of adherent *A. baumannii* cells was determined by serial dilution and plating
10. To test the ability of antibodies to Ata to block the binding of *A. baumannii* to various ECM/BM components, including collagen types I, III, IV, and V and laminin (all from Sigma), wild type *A. baumannii* was preincubated with either antibody to Ata or NRS at dilutions 1:100, 1:1,000, 1:10,000, and 1:100,000, and the remainder of the experiment was carried as described above for testing the anti adhesive properties of antibody to Ata against collagen IV.

### 9.4. OPSONOPHAGOCYTIC ASSAY

The opsonophagocytic killing (OPK) assay is used as a correlate for protection to measure the functional capacities of vaccine candidate raised antibodies. This in vitro assay aids selecting promising vaccines by demonstrating whether the vaccine induced antibodies drive efficient complement deposition and subsequent opsonophagocytic killing.

![Diagram of mechanism of immunity to extracellular bacteria mediated by antibodies](image)

**Figure 9.1.** Mechanism of immunity to extracellular bacteria mediated by antibodies.

1. The opsonophagocytic killing assay (OPKA) incorporated four basic components: polymorphonuclear cells (PMNs), target bacteria, test sera and a complement source. (Figure 9.1)
Polymorphonuclear cells (PMNS):

PMNs were purified from the heparinised human blood by density gradient centrifugation on a Ficoll Hypaque medium [138].

- Human blood was drawn into green topped Sodium Heparin tubes
- 5ml polymorph prep was added into sterilized 10mL glass tubes (1 tube per 5ml of drawn blood)
- Slowly 5mL blood was added to each tube on top of polymorph prep, making sure that the two layers did not mix
- Tubes were spun at room temperature 45-60 min at 2,500 RPM
- Monocyte layer (yellow layer and base) was discarded
- PMN layer (second white base and clear layer beneath) was pipetted out into 50ml conical; RBC pellet remained behind
- Volume equal to volume of PMNs of 1/2 dH2O and 1/2 MEM 1% BSA was added into conical tube
- It was split into equal volumes between two 50ml conical tubes
- It was spun at room temperature at 2500 RPM for 10 min
- Supernatant was discarded and resuspended each pellet in 10mL 1% ammonium chloride
- It was then incubated at room temperature for 10 min
- It was spun at room temperature at 2500 RPM for 10 min
- Supernatant was discarded and resuspended in 10ml MEM 1% BSA
- It was then spun at 2500 RPM for 10 min
- After last spin, pellet was resuspended in 10 ml MEM 1% BSA,
Cells counting:
- 80μl Trypan blue was mixed 20μl cell solution in 96 well plate ELISA.
- 10μl was placed in haemocytometer
- 2 corner squares were counted in haemocytometer (omitting cells on right and bottom lines)
- The total count was divided by 2 and then multiplied by dilution factor (5) and by 10⁴/ml and by total volume (10ml). It was reported in 10⁷. It was divided by 2.5 to get volume of MEM (in ml) needed to add to pellet to get final concentration of 2.5 x 10⁷/ml
- The solution was spinned at 2500 RPM for 10 min
- The supernatant was discarded and the pellet was resuspended in volume of MEM 1% BSA derived in step 16. It was kept at room temperature.

Bacteria:
- For the strain delta complemented: The strain was inoculated overnight in 1mL LB broth at 37°C. 20μl of overnight culture was inoculated in 2mL LB with in sterilized 10ml glass tubes for to prepare O.D. 0.4. Tubes were set to spin on wheel at 37°C to grow to OD 0.4. It was then induced with 20μl glucose and 200μl arabinose for 2h. After 2 hours O.D was adjusted to 0.8.

- For the strains 17978 and delta ATA: Strains were inoculated overnight in 1ml LB broth at 37°C. 100μl of overnight culture was inoculated in 100 LB broth culture in sterilized 500ml side arm flask to prepare O.D. 0.025. It was hen spinned at 3500 RPM at 4°C x 30 m. After spin, pellet was resuspended one pellet in PBS, O.D was adjusted to 0.8.

- The bacterial cells from all growth conditions were concentrated to an OD₆₅₀ of 0.8 (5× 10⁸ CFU/ml) and a 1:100 dilution (5×10⁶ CFU/ml) was
made in minimal essential medium (MEM) plus 1% bovine serum albumin for use in assay.

**Antibody:**

- The test sera was heat inactivated at 56°C for 30 min (to inactivate endogenous complement)
- It was then cooled on ice, then diluted 1:10 and 1:20 in MEM plus 1% BSA
- According to the assay design, 1ml undiluted or 1:10 dilution of polyclonal rabbit sera was absorbed 3 times with half plate of delta ATA at 4°C and was put in the rotater for 30 min (to remove antibodies not directed to target antigen).
- Tubes were always kept on ice.
- Tubes were filtered twice with 0.22μm cellulose acetate filter (to avoid bacterial contamination).

**Complement:**

- Human blood was drawn into red topped BD Vacutainer tubes.
- Tubes were spinned at 3500 RPM at 4°C for 30 min.
- 1.5 ml of first transparent yellow layer was pipetted out into 2ml ependorf.
- According to the assay design, for example 1.5ml of undiluted or any dilution of human sera was absorbed 3 times with half plate of target *A.baumannii* 17978 at 4°C and was put in the rotater for 30 min (to remove antibodies from complement source).
- Tubes were always kept on ice.
Tubes were filtered twice with 0.22µm cellulose acetate filter (to avoid bacterial contamination).

2. The actual phagocytic killing assay was performed by mixing 100 µl of PMNs, target bacteria, dilutions of the test sera, and an undiluted normal human serum as a complement source.

3. The final concentration of complement in the assay was 25%. The reaction was incubated on a rotor rack at 37°C for 90 min. Samples were taken at 0 and 90 min and diluted and plated for bacterial enumeration.

4. The percentage of bacteria killed in the OPKA was calculated by determining the ratio of the number of CFU surviving in the tubes with bacteria, leukocytes, complement, and sera after 90 min of incubation to the mean CFU in the same tube at time zero.

5. The OPKA of antisera to Ata was defined as the difference in killing activity between immune and control NRS.

6. At the final concentration of complement used in the assay (25%), complement-dependent bactericidal killing was not observed in tubes containing bacteria, diluted complement, and antibodies but lacking PMNs. In addition, controls lacking complement or antibodies were included in every assay, and bacterial growth was assessed.

9.5. COMPLEMENT DEPENDENT BACTERICIDAL KILLING

1. The ability of the antibodies to Ata to mediate the killing of A. baumannii in the presence of human complement was measured by a bactericidal assay in a plate assay format.

2. The complement source (human sera) and test sera were prepared as described before for the OPKA.
3. Cultures of *A. baumannii* were prepared as described above for the OPKA except the fact that the final concentration of the bacterial cells was $10^6$ CFU/ml.

4. In wells of sterile flat bottom microtiter plates, 10 µl of heat inactivated, absorbed test sera (NRS or rabbit immune sera to Ata at dilutions 1:10 and 1:20) was mixed with 10 µl of the bacterial suspension ($10^4$ cells/well).

5. 80 µl of undiluted human complement absorbed as described for the OPKA. The final concentration of complement in the assay was 80%.

6. The microtiter plates were then incubated with shaking for 1 h at 37°C, after which the samples were diluted and plated for bacterial enumeration.

7. The bactericidal activity of antisera to Ata was calculated as the difference in killing activity between immune and control NRS.

8. Additional controls included (i) bacteria plus heat inactivated complement and (ii) test serum plus bacteria plus heat inactivated complement.

**9.6. PROTECTION IN A MURINE PNEUMONIA ASSAY**

1. Groups of mice ($n = 8$ to 12; C57BL/6; female, 3 to 5 weeks of age) were immunized intravenously (i.v.) with 0.2 ml of either heat inactivated (56°C for 30 min) NRS or antisera to Ata sera 16 and 4 h before an intranasal infection.

2. Intranasal infection was done with 20 µl of various *A. baumannii* strains suspended in PBS.

3. At 24 h after infection, the animals were sacrificed.

4. The lungs were harvested, weighed, and homogenized in 1 ml of LB broth with 0.05% Tween, and the numbers of CFU were determined by dilution and plating and then expressed as CFU/g of lung tissue.
For the protection studies in immune suppressed mice (n =10 to 12), animals were rendered neutropenic with a single injection of the anti granulocyte monoclonal antibody (MAb) RB6-8C5(250 g, administered intraperitoneally) given 24 h before infection and the remainder of the study was carried out as described above.

9.7. HOMOLOGY OF ATA AMONG A. BAUMANNII CLINICAL STRAINS

1. The entire Ata gene was PCR amplified from genomic DNA extracted from the strains A. baumannii strains S8, S11, I38, and S25(clinical multi drug resistant strains) with high fidelity Supermix (Invitrogen) using the primers Ata F-Out and Ata R-OUT.

2. Amplicons were then sequenced with all of the primers listed, and the protein sequences were deduced from the experimentally determined nucleotide sequences of ata compared to that of A. baumannii ATCC 17978 strain using the CLUSTAL W program, version 1.83.

9.8 STATISTICAL ANALYSIS

All statistical analyses were performed using Prism 4.0 (Graph Pad Software). An unpaired Student t test was used to compare the adhesion blocking activity of antisera to Ata and NRS, and the one way analysis of variance (ANOVA) was applied for comparisons of the OPKA and bactericidal activity of antisera to Ata against wild type A. baumannii ATCC 17978 and the A. baumannii ATCC 17978 ata and ATCC 17978 ata-c mutant strains, followed by Tukey’s post hoc test for pair wise comparisons. For protection in murine pneumonia models, the data were analyzed with the Mann-Whitney U test.