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Optimization of a 96-Well Plate Format Assay to Evaluate Concentration-Dependent Activity of a Monoclonal Antibody against the O Antigen O25b from ST131-*H*30 *Escherichia coli*

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Introduction

- The *Escherichia coli* sequence type 131 (ST131) clone has spread worldwide as a cause of bacteremia, meningitis, urinary tract infection, and other extraintestinal infections.
- Among ST131 *E. coli* isolates, the *H*30 subclone has been identified as multidrug resistant.
  - This subclone expresses a conserved lipopolysaccharide (LPS) O antigen, O25b.
- Monoclonal antibodies (mAbs) targeting the LPS have been considered an attractive target for active and passive immunization approaches.
- We optimized a 96-well plate assay to test different concentrations of a mAb against an *E. coli* ST131-O25b:H4 clone that induce complement-mediated killing *in vitro* and then tested our clinical isolates in this optimized assay.

Materials and Methods

- A total of 139 *E. coli* isolates with different sequence types (STs) and O antigens were subjected to growth curves in brain heart infusion (BHI) broth and 40% active human serum (HS) over 24 hours to assess serum tolerance.
- Assay variables evaluated for optimal mAb performance included:
  - Media type: BHI broth, cation-adjusted Mueller Hinton broth (CA-MHB), and Haemophilus test media (HTM)
  - Source and concentrations of active human serum
  - Media supplementation: Ca^{2+}, Mg^{2+}, and polysorbate 80 (P80)
  - Inoculum concentration: Four ranging from 5 x 10^2 to 5 x 10^5 colony forming units (CFUs)/mL
- Each assay variable was evaluated against 12 ST131-O25b and 5 non-ST131 strains over time (8 to 10 hours) for end point determinations with 3 reading methods:
  - Plating for viable CFUs: >1.0 $\log_{10}$ reduction from growth control
  - Fluorescence: >50% reduction from growth control
  - Visual: color change of alamarBlue, a cellular metabolism indicator
- Due to wide variations in growth kinetics amongst ST131-O25b strains, the final mAb growth inhibition assay was developed in a microtiter plate format as a two-part assay that used 50% and 25% active human serum in BHI broth and 10% alamarBlue and was read after 8 and 10 hours of incubation.
  - The optimal inoculum concentration was 5 x 10^3 CFU/mL and the mAb was tested from 10-0.02 mg/L.
- The stability and reproducibility of the final mAb growth inhibition assay was assessed with one freeze-thaw cycle of the broth microdilution panels and by testing 3 separate lots of the preferred active human serum source.

Results

- After 10 hours of incubation in BHI with 40% active HS, only 36 of 69 ST131-O25b isolates exhibited visual growth from an inoculum of 5 x 10^3 CFU/mL (Figure 1A).
  - While ST131-O25b isolates appeared more readily able to grow in the presence of serum than non-O25 strains, 29% of ST131-O25b isolates were non-viable at 24 hours.
  - After 8 hours, a wide range of viable cells were recovered for ST131-O25b strains (<4 x 10^4 – 4.8 x 10^9 CFU/mL, median 1.1 x 10^6 CFU/mL; Figure 1B).
- CA-MHB and HTM at varying strengths (0.5X to 2X) did not support growth of the *E. coli* strains in active HS (data not shown).
  - Only 1 of 3 manufacturers of active HS tested contained enough complement for mAb-specific inhibition of ST131 *E. coli* isolates.
- Growth inhibition results were +/- 1 dilution from the standard assay when the media was supplemented with 15 mg/L Ca^{2+} or 15/7.5 mg/L Ca^{2+}/Mg^{2+}. However, the mAb was less active when inhibition was read in 50% serum with an additional 7.5 mg/L of Mg^{2+} (data not shown).
- Similar median end points, but varying ranges, were observed amongst the 3 reading methods (Figure 2 and Figure 4):
  - Fluorescence: median 1.25 mg/L, range 0.02-5 mg/L
  - Visual: median 1.25 mg/L, range 0.313-5 mg/L
  - Plating for CFUs: 2.5 mg/L, range 0.625-5 mg/L
- Significant lot-to-lot variability was observed between the 3 lots of active HS using the visual reading method, likely due to amount of complement in each lot (Figure 2).
- All 12 ST131-O25b isolates tested, including 1 ST131 single locus variant, were inhibited by ≤5 mg/L α-ST131 mAb in the growth inhibition assay and there were no non-ST131 strains that displayed inhibition (Figure 3).
- When mAb inhibition in the presence of active complement was compared between freshly prepared and frozen microtiter plates, 16 of 17 (94.1%) results were within a 2-fold dilution (data not shown).

Conclusions

- As monoclonal antibodies continue to be explored for difficult-to-treat bacterial pathogens, assays aimed at assessing *in vitro* performance of these mAbs should be developed.
- We developed a complement-mediated mAb assay specific to ST131-O25b *E. coli* isolates that can be read in as little as 8 hours and does not appear to cross-react with other STs.
- Each mAb would likely require significant assay development. Challenges lie in obtaining a reliable source of active complement for longitudinal studies. However, the final assay does appear to be stable once frozen, is easy to interpret, and could be performed in a clinical setting capable of broth microdilution assays.

Acknowledgements

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Figure 1A. Utilizing alamarBlue to monitor growth of *E. coli* strains in BHI and 40% active HS over 24 hours

E. coli sequence type	Number and percentage of isolates at time to color change in hours:				
	8	10	12	22	no change
ST131-O25b (69)	18 (26.1%)	18 (26.1%)	4 (5.8%)	9 (13.0%)	20 (29.0%)
ST131-O25a (9)	0 (0.0%)	0 (0.0%)	1 (11.0%)	4 (44.5%)	4 (44.5%)
ST131 non-O25 (32)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (6.0%)	30 (94.0%)
non-ST131 (29)	7 (24.1%)	0 (0.0%)	1 (3.4%)	3 (10.3%)	18 (62.1%)

Figure 1B. Viable CFU/mL recovered from *E. coli* strains grown in 40% active HS and BHI over 8 hours

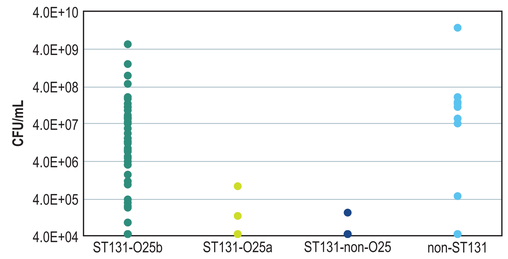


Figure 2. mAb growth inhibition assay results for ST131 strains comparing 3 reading methods and lot-to-lot variability of active HS

	ST131 strain	Active HS %	mAb growth inhibition (mg/L) with active HS lot and reading method: Lot 1 Plating	Lot 1 Fluorescence	Lot 1 Visual	Lot 2 Visual	Lot 3 Visual
1	1031823	25%	2.5	0.625	1.25	0.625	0.156
2	1044195	25%	2.5	2.5	2.5	0.313	0.313
3	1051775	50%	0.625	0.02	0.313	0.313	NI
4	1051832	25%	0.625	1.25	1.25	0.313	0.156
5	1057211	25%	5	2.5	5	0.625	0.313
6	1058957	25%	2.5	5	5	0.625	0.313
7	1061212	25%	2.5	1.25	2.5	0.625	0.313
8	1062491	50%	1.25	0.08	1.25	1.25	NI
9	1083123	25%	2.5	1.25	1.25	0.313	0.313
10	1084977	50%	1.25	0.625	2.5	2.5	NI
11	1087697	50%	2.5	1.25	5	2.5	NI
12	1046805	50%	1.25	0.313	1.25	1.25	NI

NI, not interpretable

Figure 3. Inhibition of growth assay fluorescence results for 12 ST131 and 5 non-ST131 *E. coli* isolates and the percentage of serum for the strains

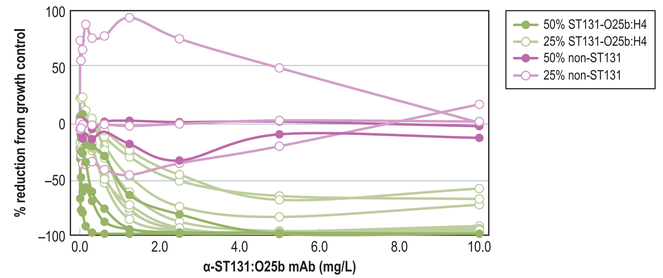
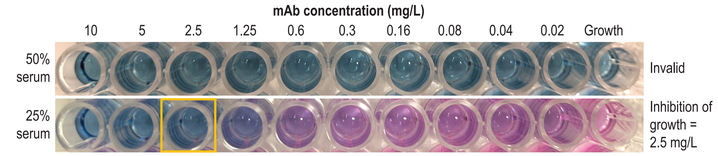


Figure 4. Example of mAb inhibition of ST131-O25b *E. coli* strain read 3 ways: visually, CFU/mL, and fluorescence



Reading method	Reduction from growth control									
Fluorescence	−95%	−98%	−89%	−63%	−32%	−23%	−20%	−16%	−21%	−25%
CFU/mL ($\log_{10}$)	−1.0	−1.3	−0.9	−0.8	−0.7	−0.3	−0.3	−0.2	−0.2	−0.4

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