Journal of Microbiological Methods 158 (2019) 52–58

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A multiplexed, indirect enzyme-linked immunoassay for the detection and differentiation of *E. coli* from other Enterobacteriaceae and *P. aeruginosa* from other glucose non-fermenters

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ARTICLE INFO

Keywords:
ELISA
Gram-negative bacteria
Bacterial detection
Latex agglutination test

ABSTRACT

Gram-negative bacteria (GNB) are important causes of community (CA) and hospital (HA)- associated infections. Here we describe the development of an indirect ELISA (I-ELISA), which can be used to detect and differentiate the Enterobacteriaceae *Escherichia coli*, and glucose non-fermenter *Pseudomonas aeruginosa* from other GNB species. The I-ELISA utilizes six antibodies for bacterial speciation, which were grouped according to their bacterial targets; Enterobacteriaceae (SL-EntA and CH1810 mAb), *Escherichia coli* (SL-EcA and 6103–46 mAb), *Pseudomonas aeruginosa* (SL-PaA and SL-PaB). The six, anti-GNB antibodies were first screened against a panel of well-characterized clinical GNB isolates to optimize assay conditions and to determine individual antibody sensitivity and specificity. When tested against a diverse, blinded panel of 94 GNB clinical isolates, the I-ELISA exhibited the following sensitivity/specificity for each target: Enterobacteriaceae (94.4%/95%), *E. coli* (82.6%/88.7%), *P. aeruginosa* (83.3%/96%). An I–ELISA to detect and differentiate the most common GNB pathogens offers advantage in terms of simplicity over diagnostic tests currently used in most clinical settings.

1. Introduction

The control and treatment of infections caused by multi-drug resistant (MDR) bacteria is a major challenge facing clinicians (Boucher et al., 2009; Vasoo et al., 2015). The challenge is particularly exemplified with the Gram-negative bacteria (GNB), which have been targeted by WHO as “Priority 1, Critical pathogens” for new antibiotics (WHO, 2017). These pathogens are becoming increasingly widespread in community settings (CA) and collectively comprise the most common causes of healthcare-associated (HA) infections (Kaye and Pogue, 2015). As a leading cause of CA-urinary tract (UTI), HA-UTI and bloodstream infection (BSI), the most frequently encountered GNB is *Escherichia coli* (Laupland and Church, 2014; Flores-Mireles et al., 2015; Buetti et al., 2017). MDR is prevalent amongst isolates of extraintestinal pathogenic *E. coli* (ExPEC), with the rising incidence of CA-UTI strains producing extended spectrum β-lactamases (ESBL) being of particular concern (Sheila Adams-Sapper et al., 2012; McDanel et al., 2017). This has led to an increased use of the carbapenems, which consequently has driven the emergence of resistance to these broad-spectrum agents amongst the Enterobacteriaceae (particularly, *Klebsiella pneumoniae*) and other clinically relevant GNB species (Gibb et al., 2002; Tzouvelekis et al., 2012; Poirel et al., 2016). Of the non-fermenting bacilli, the most common pathogen in the nosocomial setting is *Pseudomonas aeruginosa,* which accounts for around 7% of all HA-infections in the United States (Magill et al., 2014). This MDR GNB is of major clinical importance due to its ability to cause serious infections in critically ill patients, including ventilator-associated pneumonia, BSI and complicated-UTI (Juan et al., 2017).

Complications of community-acquired infections can lead to hospitalization, and many HAIs, particularly BSI, are preceded by community-onset infections (Adams-Sapper et al., 2012; Adams-Sapper, 2013). As such, the rapid and simple diagnosis of common CA-infections is critical. During diagnosis of CA-BSI and UTI, routine species level identification of causative organisms still relies predominantly on phenotypic and biochemical characterisation methods. However, these conventional characterisation methods are laborious, requiring a variety of reagents and time. For instance, differentiation of members of the family Enterobacteriaceae using the API20E biochemical testing strip can to take up to 24 h after isolation and overnight broth culture of bacteria from a patient sample (O'Hara et al., 1992; Peele et al., 1997).

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https://doi.org/10.1016/j.mimet.2019.01.014
Received 7 December 2018; Received in revised form 19 January 2019; Accepted 28 January 2019
Available online 29 January 2019

In the last decade, rapid diagnostic methods which employ PCR or mass spectrometry (MS) have been introduced, enabling the speciation of bacterial pathogens from pure cultures with high sensitivity and accuracy within a matter of hours (Buchan and Ledeboer, 2014; Angeletti, 2017). However, these novel diagnostic tools do not lend themselves to every laboratory setting, as they require both specialized equipment and training for their use. In contrast, immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) are simple, cost effective, and results can be obtained from a pure bacterial culture within one day. Other than target-specific antibodies, ELISA uses the same set of reagents to detect many varieties of microbes. Immunoassays are also adaptable to even simpler platforms, such as immunochromatographic and latex agglutination tests. If they demonstrate a high degree of specificity and sensitivity, they can become suitable diagnostic tools in most clinical settings, including outpatient clinics or private offices (Gan and Patel, 2013). Although ELISAs have been developed for the identification of various bacterial pathogens (Choi et al., 1992; Anuntagool et al., 1996; Kerr et al., 2001), none yet have been described for differentiation of GNB species commonly associated with UTI and BSI.

Here we describe the development of a highly specific and sensitive, indirect ELISA (I-ELISA) for the differentiation of major Enterobacteriaceae from glucose non-fermenters, associated with BSI and UTI. This anti-GNB indirect ELISA can be used to differentiate *E. coli* from other species of Enterobacteriaceae, such as, *Proteus mirabilis, Citrobacter freundii, Klebsiella oxytoca, Morganella morganii, Salmonella enterica* and *Klebsiella pneumoniae*. Additionally, this test can differentiate the common hospital-associated glucose non-fermenter, *P. aeruginosa*, from other clinically relevant, non-fermenting bacilli.

2. Materials and methods

2.1. Bacterial isolates and antibody collections

The GNB used in this study were BSI isolates obtained from inpatients at the San Francisco General Hospital in California (Adams-Sapper, 2013). Isolates and species included in this study can be found in Supplementary information, Tables 1 and 2. The rabbit polyclonal (SL-EntA, SL-EcA, SL-PaA, SL-PaB), and murine monoclonal (mAb) (CH1810 and 6103–46) antibodies included in this study were provided from Silver Lake Research Corporation (Azusa, CA).

2.2. Indirect ELISA

Bacterial isolates were subcultured from frozen glycerol stocks to Luria-Bertani (LB) broth and incubated overnight at 37 °C. One mL of 10^{10} CFU/mL culture was pelleted in a microfuge tube with a microcentrifuge at 13,000 rpm, and the pellet was resuspended in one mL phosphate-buffered saline (PBS, pH 7.4). This cell suspension was used as the inoculum for the indirect ELISA; after inoculation (50 μL), the cell number in the well was 5×10^8 CFU/mL. After the overnight incubation with live, whole-cell bacteria, the plate was: 1) washed three times with 200 μL of PBS with 0.05% Tween-20 (PBST); 2) blocked for one hour in 250 μL of 5% non-fat dry milk diluted in PBST (blocking buffer); 3) washed; 4) incubated for 1 h with 50 μL of primary antibody diluted in blocking buffer to a final concentration of 50 ng/mL; 5) washed three times with a 5 min incubation of 200 μL PBST; 6) incubated for 1 h with 100 μL of goat anti-mouse-IgG-HRP conjugate or goat anti-rabbit-IgG-HRP conjugate (Southern Biotech, Birmingham, AL) diluted 1:2500 in blocking buffer; 8) washed with a 5 min PBST incubation; 9) incubate for 20 min in 100 μL with substrate (SureBlue Reserve TMB Microwell Peroxidase Substrate, Kirkegaard and Perry Laboratories, Gaithersburg, MD); 10) stopped reaction with 100 μL of 1 M HCl; 11) read on a plate reader (SpectraMax M3, Molecular Devices) at OD_{450nm}.

Optimal concentrations of antibody and bacteria were determined from the receiver operating characteristic (ROC) curve analysis of data generated from differing concentrations of antibody (12.5 ng/mL, 50 ng/mL) versus differing bacteria concentrations (5×10^8 CFU/mL, 2.5×10^8 CFU/mL and 1.25×10^8 CFU/mL). The conditions that generated ROC curves with the largest area underneath the curve (AUC), were taken as the optimum conditions for the I-ELISA. Positive cut-off points at OD_{450nm} were then chosen from the ROC curves with the largest AUC to maximize sensitivity or specificity within each antibody pair. Lastly, the limit of detection (LOD) of the I-ELISA was determined by assaying serial dilutions of control strains under the optimal I-ELISA conditions. The LOD for each antibody was the lowest concentration of bacteria that would yield a positive result in the I-ELISA, at the corresponding cut-off value chosen for that antibody.

To develop the I-ELISA, we first optimized assay conditions by screening a panel of well-characterized isolates (training set) with the anti-Enterobacteriaceae, anti-*E. coli* and anti-*P. aeruginosa* antibody pairs. During the initial rounds of screening, optimal antibody and bacteria concentrations, alongside positive OD_{450} cut-off values for each antibody were determined. The I-ELISA was then validated by screening a blinded panel of 94 clinical GNB isolates, which was carried out in accordance with the optimized I-ELISA conditions.

2.3. Statistical analysis

Samples were run in triplicate during each ELISA test and raw data were used for the analysis. The corrected OD_{450nm} values were obtained by subtracting primary-secondary antibody controls and antigen-secondary antibody controls from each corresponding test well. To determine the signal-to-noise ratio (SNR), we divided the corrected OD_{450nm} value from each test well by the sum of the corrected values from the control wells. The SNRs for each antibody were used to generate ROC curves on GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com). The AUC values of the ROC curves were evaluated by the statistical operations in GraphPad Prism version 7.03 at a 95% confidence interval. The sensitivity and specificity of the blinded screen for each target organism was determined based on the positive cut-off OD_{450} values established for each antibody from the ROC curve analysis.

2.4. Latex agglutination test

Antibodies were conjugated to biotin with the Pierce Antibody Biotinylation Kit for IP (Thermo Scientific, Rockford, IL) in accordance to the kit's instructions. The biotinylated antibodies were then conjugated to Sphero™ Streptavidin Polystyrene Particles (Spherotech, Inc., Lake Forest, IL) with a mean diameter of 0.86 μm. Biotinylated antibodies and latex beads were incubated for one hour at room temperature with shaking. Immuno-latex beads were then spun down at 3000 × g for 15 min and washed twice with PBS. Bacterial isolates to be tested were streaked for single colonies onto LB agar plates and grown overnight at 37 °C. Isolates were tested by inoculating one colony into 20 μL of immuno-latex particles on a glass microscope slide. The colony-bead suspension was gently mixed by inverting the slide for 2 min, before being observed. A positive result was determined by visualising agglutination of the particles, in comparison to the negative control (immuno-conjugated beads tested against the *S. sciuri* isolate, CPP28).

3. Results

3.1. Preliminary antibody screening against characterized, clinical isolates

The ability of the presumptive anti-Enterobacteriaceae (SL-EntA and CH1810 mAb), anti-*E. coli* (SL-EcA and 6103–46 mAb), and anti-*P. aeruginosa* (SL-PaA and SL-PaB) antibodies, in detecting their respective bacterial targets was determined by I-ELISA (Section 2.2). The results of the initial antibody screens are summarized below. The identities of the

Table 1
Results obtained from screening GNB clinical isolates to determine the detection capabilities of the anti-Enterobacteriaceae antibodies, SL-EntA and CH1810 mAb.

Organism identity	*Total no. strains tested*		*Total no. strains detected*		*Total no. tests detected*		*Total no. tests undetected*	
	SL-EntA	*CH1810*	*SL-EntA*	*CH1810*	*SL-EntA*	*CH1810*	*SL-EntA*	*CH1810*
Enterobacteriaceae								
E. coli	7	7	7	7	21	21	0	0
K. pneumoniae	9	9	9	9	28	27	3	0
P. mirabilis	5	5	4	1	5	1	5	14
E. cloacae	5	5	5	5	12	15	0	0
M. morgannii	5	5	2	0	4	0	7	15
K. oxytoca	5	5	5	5	9	15	0	0
S. enterica	5	5	4	5	7	16	3	0
C. freundii	5	4	5	4	10	12	0	0
Non-Fermenters								
A. baumannii	2		1	0	2	0	3	6
P. aeruginosa	9		2	2	4	6	23	27

strains included in this section can be found in Supplementary information, Table 1.

We assayed clinical Enterobacteriaceae isolates by I-ELISA using the anti-Enterobacteriaceae antibodies, SL-EntA (46 isolates screened) and CH1810 mAb (45 isolates screened). The screening panel included the following number of target species (No. isolates screened): *E. coli* (5), *K.pneumoniae* (9), *P. mirabilis* (5), *E. cloacae* (5), *M. morganii* (5), *K. oxytoca* (5), *S. enterica* (5), and *C. freundii* (4). As a control, non-Enterobacteriaceae isolates were screened also: *A. baumannii* (2) and *P. aeruginosa* (9). Results of the screen are summarized in Table 1. Combined, SL-EntA and 1810 mAb detected all Enterobacteriaceae isolates assayed, with the exception of 3 *M. morganii* isolates. Some cross-reactivity to non-target organisms was observed with CH1810 mAb and SL-EntA. The *P. aeruginosa* isolates, SF671 and SF698 were detected with both SL-EntA and CH1810 mAb, whereas the *A. baumannii* isolate, SF476 was detected at least once with SL-EntA during multiple rounds of screening. Of the Enterobacterial isolates tested, CH1810 mAb was unable to detect *M. morganii* during repeated testing. However, two of the five *M. morganii* isolates screened were detected by SL-EntA.

The anti-*E. coli* antibodies, SL-EcA and 6103–46 mAb, were tested by I-ELISA against a panel of 31 clinical *E. coli* isolates. The non-target Enterobacteriaceae species (*K. pneumoniae, P. mirabilis, E. cloacae, M. morganii, K. oxytoca, S. enterica, C. freundii*), as well as the non-Enterobacteriaceae species, *P. aeruginosa* and *A. baumannii* were tested as a control. The results of this screen are summarized in Table 2. All *E. coli* strains were detected by both SL-EcA and 6103–46 mAb. However, some cross-reactivity to non-target Enterobacteriaceae was observed. The 6103–46 mAb antibody detected two *K. pneumoniae* isolates and one *C. freundii* isolate. SL-EcA detected six *K. pneumoniae*, two *E. cloacae*, one *S. enterica* and *C. freundii* isolate at least once during multiple rounds of screening.

The anti-*P. aeruginosa* antibodies, SL-PaA and SL-PaB were tested against a panel of 29 clinical *P. aeruginosa* isolates. Isolates from the non-target species (No. of isolates screened), *K. pneumoniae* (9), *E. coli* (7) and *A. baumannii* (2), were tested as a control. The results of this screen are summarized in Table 3. The antibody, SL-PaA detected 27 of the 29 *P. aeruginosa* isolates screened, whereas SL-PaB tested positive with all 29 isolates. Cross reactivity was observed when screening SL-PaA and SL-PaB. SL-PaA detected the following non-target organisms at least once during repeated testing: *A. baumannii*, *E. coli*, *P. mirabilis* and *C. freundii*. Likewise, SL-PaB detected isolates of *E. coli, P. mirabilis, E. cloacae, K. oxytoca, S. enterica* and *C. freundii* at least once during repeated testing.

Table 3
Results obtained from screening clinical isolates of GNB to determine the detection capabilities of the anti – *P. aeruginosa* antibodies, SL-PaA and SL-PaB.

Organism identity	*Total no. strains tested*		*Total no. strains detected*		*Total no. tests detected*		*Total no. tests undetected*	
	SL-PaA	*SL-PaB*	*SL-PaA*	*SL-PaB*	*SL-PaA*	*SL-PaB*	*SL-PaA*	*SL-PaB*
Enterobacteriaceae								
E. coli	7		4	5	4	9	17	12
K. pneumoniae	9		0	0	0	0	27	27
Other Enterobacteriaceae	7		3	5	6	9	15	12
Non-Fermenters								
A. baumannii	2		2	0	3	0	3	6
P. aeruginosa	29		27	29	75	87	16	6

3.2. Development of an I-ELISA for the differentiation of Gram-negative bacteria associated with UTI and BSI

The pairs of anti- Enterobacteriaceae, anti- *E. coli* and anti- *P. aeruginosa* antibodies were then used to further distinguish by I-ELISA, GNB commonly associated with UTI and BSI. Firstly, the optimal concentrations of both bacteria and antibody for use in an I-ELISA was determined by ROC curve analysis (as described in Section 2.1). The conditions that generated the largest area under the curve (AUC) values

Table 2
Results obtained from screening clinical isolates of GNB to determine the detection capabilities of the anti-*E. coli* antibodies, SL-EcA and 6103–46 mAb.

Organism identity	*Total no. strains tested*		*Total no. strains detected*		*Total no. tests detected*		*Total no. tests undetected*	
	SL-EcA	*6103–46*	*SL-EcA*	*6103–46*	*SL-EcA*	*6103–46*	*SL-EcA*	*6103–46*
Enterobacteriaceae								
E. coli	31		31	31	85	78	8	31
K. pneumoniae	9		6	2	10	2	18	25
Other Enterobacteriaceae	7		4	1	9	2	12	19
Non-Fermenters								
A. baumannii	2		0	0	0	0	6	6
P. aeruginosa	9		0	0	0	0	28	26

Table 4
Summary of the positive cut-off values, sensitivity and specificity and ROC curve AUC values for each anti-GNB antibody pairs.

Bacterial target	Antibody	Positive cut-off (OD_{450nm})	Sensitivity at chosen cut-off	Specificity at chosen cut-off	ROC curve AUC value[a]	95% confidence interval
Enterobacteriaceae	SL-EntA	0.123	85%	81.2%	0.916	0.872–0.961
	CH1810 mAb	0.177	78.6%	84%	0.812	0.755–0.878
E. coli	6103–46 mAb	6.797	70.6%	95%	0.944	0.916–0.972
	SL-EcA	4.320	91.4%	77.1%	0.921	0.883–0.960
P. aeruginosa	SL-PaA	1.144	81.4%	82.6%	0.934	0.899–0.970
	SL-PaB	0.401	93.5%	76%	0.937	0.902–0.971

[a] AUC values are from the ROC curve analysis of a concentration of 5×10^8 CFU/mL bacteria versus an antibody concentration of 50 ng/μL. Antibodies were screened against panels of characterized isolates by I-ELISA, to determine their specificity and sensitivity for each target organism.

for all antibodies tested, were an antibody concentration of 50 ng/mL versus a bacteria concentration of 5×10^8 CFU/mL. Under these optimized I-ELISA conditions, positive 'cut-off' OD_{450nm} value for each antibody was then determined. At the chosen cut-offs, most target species screened by I-ELISA elicited a signal which could be clearly identified as positive, while non-target species elicited a signal below this threshold. The positive, OD_{450nm} cut-off values, sensitivity and specificity and ROC curve AUC data recorded for each antibody is recorded in Table 4. The sensitivity and specificity for each antibody at the chosen cut-off values were as follows (sensitivity/specificity): Anti-Enterobacteriaceae antibodies, SL-EntA (85%/81.2%) and CH1810 mAb (78.6%/84.6%); Anti-*E. coli* antibodies, 6103–46 mAb (70.6%/95%) and SL-EcA (91.4%/77.1%); Anti-*P. aeruginosa* antibodies, SL-PaA (81.4%/82. %) and SL-PaB (93.5%/76%).

To begin validation of the antibodies to differentiate GNB by I-ELISA, we chose a panel of positive control strains. Positive control strains were chosen from the initial screening panels (Supplementary information, Table 1) due to the strong reproducible signal they elicited when tested with their respective antibody. For each antibody set, the clinical *Staphylococcus sciuri* isolate, CPP28, was included as a negative control strain (Supplementary information – Table 2). Each isolate was screened by I-ELISA using the optimal antibody/bacteria concentrations (50 ng/μL and 5×10^8 CFU/mL respectively) determined previously. Of the positive control strains, the largest variation in the SNR in relation to the mean was observed for the *K. oxytoca* isolate, SF515, when tested with SL-EntA (± 11.401). Despite the observed signal variation, the I-ELISA signal elicited by the positive control, SF515 remained above the positive OD450nm cut-off set for SL-EntA. The variation in signal may have resulted from the loss of cells during the processing of the I-ELISA, as cells are not fixed to the plate.

The LOD of each antibody was determined by I-ELISA by assaying serial dilutions of the panel of positive control strains with each of the anti-Enterobacteriaceae, anti-*E. coli* and anti-*P. aeruginosa* antibody pairs (Table 5). The LOD for each antibody is shown in Table 5. Using I-ELISA, the LOD of the anti-GNB antibodies ranged from 3×10^5 (SL-EntA) to 5×10^9 (6103–46 mAb).

3.3. Blinded screen of a panel of clinical GNB isolates to determine diagnostic accuracy of the I-ELISA in the differentiation of GNB

To validate the I-ELISA in the differentiation of GNB, we screened a blinded set of 94 clinical isolates of GNB from cases of CA-UTI and HA-BSI with the panel of anti-Enterobacteriaceae, anti-*E. coli* and anti-*P. aeruginosa* antibodies (Supplementary information, Table 3). The test was carried out in accordance with the optimized I-ELISA conditions. The results from the blinded screen were determined as negative or positive, in relation to the cut-off values (Supplementary information – Table 2) determined previously for each antibody. Controls for each antibody pair (Supplementary information – Table 2) were tested alongside the test panel of isolates during each round of blind screening. The blinded screen was analyzed as follows: Enterobacteriaceae were identified by a positive result from either of SL-EntA or CH1810 mAb. Identification of *E. coli* required a positive result from at least one of the anti-Enterobacteriaceae antibody pair, alongside a positive result from either one of the anti-*E. coli* antibodies, SL-EcA or 6103–46 mAb. Identification of *P. aeruginosa* relied on a positive result from either one of the anti-*P. aeruginosa* antibodies, SL-PaA and SL-PaB, alongside negative results from both the anti-Enterobacteriaceae and anti-*E. coli* antibody pairs. This was due to the off-target activity that the anti-*P. aeruginosa* antibodies demonstrated against some strains of Enterobacteriaceae in the initial screens (Section 3.1).

The blinded screening set included 54 Enterobacteriaceae (16 species from 8 genera) and 40 non-Enterobacteriaceae isolates (8 species from 5 genera). The Enterobacteriaceae species consisted of (No. of isolates): *E. coli* (23), *K. pneumoniae* (6), *K. oxytoca* (2), *E. cloacae* (2), *E. aerogenes* (3), *E. agglomerans* (1), *C. freundii* (2), *C. koseri* (1), *S. marcescens* (2), *S. liquefaciens* (2), *P. mirabilis* (2), *P. vulgaris* (1), *P. stuartii* (1), *P. retgerri* (2), *M. morganii* (2) and Salmonella species (2). The non-Enterobacteriaceae species included the non-fermenting bacilli, *P. aeruginosa* (18), *P. putida* (1), *A. baumannii* (11), *A. lwoffi* (5), *B. cepacia* (1), *S. maltophilia* (2), Alcaligenes (1) and one glucose-fermenting, Aeromonas isolate (1). The number of true positive/true negative isolates screened with each antibody pair in the blinded screen were as follows: anti-Enterobacteriaceae pair (54/40), anti-*E. coli* pair (23/71), anti-*P. aeruginosa* pair (18/76). Detailed results, including I-ELISA

Table 5
Limit of detection of each antibody included in the anti-GNB I-ELISA, corresponding positive control strains and I-ELISA signals at OD_{450nm}.

Antibody and corresponding target	*Positive control strain*	*LOD (CFU/mL)*	*Average I-ELISA signal (OD_{450nm}) at LOD & SD*[a]
Anti-Enterobacteriaceae			
SL-EntA	*K. oxytoca* (SF515)	3×10^5	0.197 (± 0.07)
CH1810 mAb	*K. oxytoca* (SF327)	7.8×10^7	0.521 (± 0.191)
Anti-*E. coli*			
6103–46 mAb	*E. coli* (SF499)	5×10^9	32.878 (± 26.196)
SL-EcA	*E. coli* (SF499)	3.1×10^8	8.685 (± 4.302)
Anti-*P. aeruginosa*			
SL-PaA	*P. aeruginosa* (SF827)	1.5×10^8	1.736 (± 0.492)
SL-PaB	*P. aeruginosa* (SF827)	7.8×10^7	0.979 (± 0.346)

[a] Results document intra-assay variation ($n = 3$).

Table 6
The performance of the I-ELISA in the detection of Enterobacteriaceae, *E. coli* and *P. aeruginosa*, from a blinded screen of 94 clinical isolates.

Bacterial target	Sensitivity	Specificity	Accuracy[a]	Number of target organisms detected/screened	Number of false positives/false negatives
Enterobacteriaceae	94.4%	95%	94.6%	51/54	2/3
E. coli	82.6%	88.7%	87.2%	19/23	8/4
P. aeruginosa	83.3%	96%	95.7%	15/18	1/3

The sensitivity and specificity of the I-ELISA for each target was determined by screening a blinded set of 94 clinical isolates, simultaneously with the panel of anti-GNB antibodies (anti-Enterobacteriaceae: CH1810 mAb, SL-EntA; anti-*E. coli*: 6103–46, SL-EcA; anti-*P. aeruginosa*: SL-PaA, SL-PaB). Organism identity was determined based on the positive/negative signals obtained from all 6 antibodies.

[a] Accuracy was calculated in the following manner = (total correct conditions predicted)/(total conditions) × 100 or (TP + TN)/(TP + TN + FP + FN) × 100.

OD_{450nm} values and analysis can be found in the Supplementary information, Table 3.

In the differentiation of Enterobacteriaceae species from non-Enterobacteriaceae isolates, the I-ELISA had a sensitivity of 94.4% and a specificity of 95% (Table 6). Of the blinded Enterobacteriaceae isolates screened, 3 Enterobacteriaceae isolates (SF78 - *E. aerogenes*, SF359 – *P. mirabilis*, SF582 – *M. morganii*) were not detected by either anti-Enterobacteriaceae antibody. As these isolates presented as false negative tests in the screen, it led to their mis-identification as non-Enterobacteriaceae organisms. The anti-Enterobacteriaceae antibodies elicited a false positive result when tested against SF144, an Aeromonas species and SF266, a *P. aeruginosa* isolate, during the blinded screen. This led to the mis-identification of these two isolates as Enterobacteriaceae.

In the identification of *E. coli* from other Enterobacteriaceae species, the I-ELISA had a sensitivity of 82.6%, and a specificity of 88.7%. Of the 23 *E. coli* isolates included in the blind screen, all were correctly identified as Enterobacteriaceae, with 19 being positively identified as *E. coli* by the anti-*E. coli* antibody pair. The anti-*E. coli* antibodies exhibited some off-target activity, detecting *E. cloacae* (2 isolates), *K. pneumoniae* (2 isolates), *C. freundii* (1 isolate), *S. liquefaciens* (1 isolate), *E. agglomerans* (1 isolate) and Salmonella (1 isolate) during the blinded screen. Overall, the I-ELISA had an accuracy of 87.2% in the identification of *E. coli* during the blinded screen (Table 6).

Regarding the identification of *P. aeruginosa* from non-Enterobacteriaceae organisms, the I-ELISA had a sensitivity of 83.3% and a specificity of 96% (Table 6). During the blinded screen, two isolates (SF33 and SF36) were missed by both SL-PaA and SL-PaB. The *P. putida* isolate, SF777, was mis-identified as *P. aeruginosa* during the blinded screen. One *P. aeruginosa* isolate (SF266) was mis-identified as an Enterobacteriaceae; SF266 tested positive with both SL-EntA and CH1810 mAb during the blinded screen. The *P. putida* isolate, SF777, was mis-identified as *P. aeruginosa* during the blinded screen. SL-PaA and SL-PaB exhibited off target activity against the following Enterobacteriaceae species: *P. stuartii* (1 isolate), *K. pneumoniae* (1 isolate), *E. coli* (11 isolates), *E. cloacae* (1 isolate), *M. morganii* (1 isolate), *S. liquefaciens* (1 isolate), *E. agglomerans* (1 isolate), *C. freundii* (1 isolate) and Salmonella (1 isolate). However, identification of an isolate as *P. aeruginosa* required positive signals from the anti-*P. aeruginosa* antibodies, alongside negative signals from the anti-Enterobacteriaceae and anti-*E. coli* antibody pairs. Therefore, the off-target activity of SL-PaA and SL-PaB did not result in mis-identification of the aforementioned Enterobacteriaceae during the blinded screen.

3.4. Application of the anti-GNB antibodies in a latex bead agglutination assay

We evaluated the utility of the anti-Enterobacteriaceae (SL-EntA, CH1810 mAb), anti-*E. coli* (6103–46 mAb, SL-EcA), and anti-*P. aeruginosa* (SL-PaA, SL-PaB) antibodies by the latex bead agglutination assay. Antibody-conjugated beads (immuno-beads) were tested against representative isolates of Enterobacteriaceae, *E. coli* and *P. aeruginosa*, which are described in Table 5. Results of the agglutination test are shown in Fig. 1. All strains tested were detected by their respective immuno-beads, evident by a strong agglutination reaction which was observed within 2 min of bacterial inoculation. No agglutination was observed for the negative controls, which included antibody-conjugated beads incubated with the negative control strain, *S. sciuri* CPP28.

4. Discussion

The aim of this study was to develop a simple, economical assay to differentiate common GNB species associated with CA-UTI and HAI. We developed a sensitive and specific I-ELISA which provides a simple method to separate GNB into species of Enterobacteriaceae and non-Enterobacteriaceae. From these two groups, the I-ELISA can be used to subsequently identify *E. coli* (the most common GNB associated with UTI and BSI) from other Enterobacteriaceae species, and from *P. aeruginosa,* the most common glucose non-fermenter associated with HAI. All antibodies described in this study were shown to be potentially adaptable to a latex bead agglutination assay.

Six antibodies were developed for an anti-GNB I-ELISA and were grouped according to their bacterial targets; Enterobacteriaceae (SL-EntA and CH1810 mAb), *E. coli* (SL-EcA and 6103–46 mAb), *P. aeruginosa* (SL-PaA and SL-PaB). The LOD of the anti-GNB antibodies ranged from 3×10^5 (SL-EntA) to 5×10^9 (6103–46 mAb). The optimized anti-GNB I-ELISA was validated with a diverse, blinded panel of 94 GNB isolates, consisting of 54 Enterobacteriaceae (17 species from 8 genera) and 40 non-Enterobacteriaceae (8 species from 6 genera). The anti-GNB I-ELISA had the following sensitivity/specificity for each target: Enterobacteriaceae (94.4%/95%), *E. coli* (82.6%/88.7%), *P. aeruginosa* (83.3%/96%) (Table 6). Furthermore, the anti-GNB antibodies successfully detected representative isolates of Enterobacteriaceae, *E. coli* and *P. aeruginosa* when conjugated to latex beads by the agglutination test (Fig. 1).

The anti-GNB, I-ELISA proved highly specific and sensitive in differentiating unknown isolates of GNB into Enterobacteriaceae and non-Enterobacteriaceae and from these two groups, identifying isolates of *E. coli* and *P. aeruginosa*, respectively. The ability to differentiate Enterobacteriaceae from non-fermenting organisms is important because such information can inform clinicians of appropriate class of antimicrobial drug regimen to initiate empirically. The high specificity and sensitivity of the I-ELISA in the identification of *P. aeruginosa* is also important, as this organism has been classified by the CDC as a 'serious' threat pathogen, due to the high-levels of MDR, morbidity and mortality associated with this pathogen (CDC, 2013; Thaden et al., 2017). The ability of the I-ELISA to accurately distinguish *E. coli* from other Enterobacteriaceae species is of relevance, as ExPEC is the most common cause of CA-UTI and BSI globally (Riley, 2014). Moreover, we demonstrated the functionalization of the anti-GNB antibodies in a rapid, simple, low-cost test by the agglutination assay, which would allow the identification of bacterial isolates in under 2 min.

Since biochemical tests already exist to differentiate Enterobacteriaceae organisms from non-fermenters, apart from rapid latex agglutination tests, an antibody-based test to do the same will not necessarily offer an advantage. However, antibodies provide an opportunity to adapt them into platforms not possible to do by biochemical tests. For example, antibodies can be used to capture target

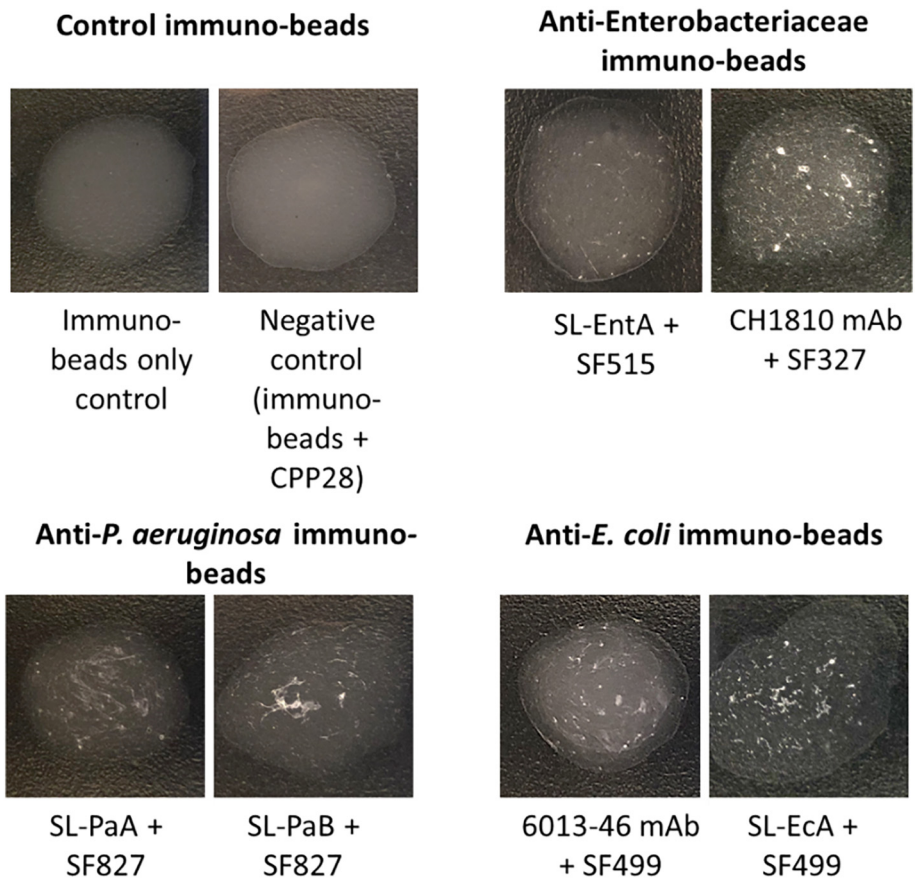

Fig. 1. Direct visualisation of the results of the agglutination assay with the anti-GNB antibodies conjugated to latex beads, compared to negative controls. Conjugated antibody and strain tested are described below each image.

bacterial cells directly from clinical specimens, which can then be further tested (e.g. for beta-lactamase production to assess resistance to beta-lactam drugs). An integrated platform using a microfluidic system to first capture bacterial cells and then assay them for enzyme production will offer a major advantage for the rapid diagnosis of UTI with urine samples that contain bacterial cells within the LOD achievable with such a test.

The limitations of this study were the absence of antibodies for the detection of other clinically important Enterobacteriaceae, such as *K. pneumoniae* and non-fermenting bacilli, such as *A. baumannii*. However, a GNB isolate identified as a non-Enterobacteriaceae organism other than *P. aeruginosa* by this I-ELISA could presumptively be identified as *A. baumannii*, another prevalent glucose non-fermenter associated with nosocomial UTI and BSI (Weiner, 2016). In this study, this presumptive identification of non-Enterobacteriaceae and non-*P. aeruginosa* GNB as *A. baumannii* was 100% sensitive.

In conclusion, we have developed an anti-GNB I-ELISA, which provides an easy means to differentiate common GNB species associated with CA-UTI and BSI. The antibody-based differentiation of GNB species could be adapted into simple, low cost platforms (such as the agglutination test) that can be used in outpatient as well as in inpatient settings to guide clinical management of cases of BSI and UTI.

Funding

This research was supported by the National Institutes of Health and the National Institute of Allergy and Infectious Diseases [grant number, RO1-AI117064].

Acknowledgements

We would like to thank Nicole J. Tarlton for taking the time to construct and subculture the panel of isolates for the blinded screen. We would also like to thank Tara R. deBoer for her general intellectual input throughout the project.

Author disclosure

Tiffany Satoorian and Mark Geisberg are both employees of Silver Lake Research Corporation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2019.01.014.

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