C-065

A Rapid Indirect Enzyme-Linked Immunosorbent Assay for Identification of *Acinetobacter* spp. From Cultured Isolates

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ASM 2008
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AMENDED ABSTRACT

Background: *Acinetobacter* spp. are important pathogens whose endemic nosocomial strains tend to express multiple drug resistance. Clinicians need an inexpensive, reliable, and rapid assay for identification of these difficult pathogens.

Methods: Polyclonal chicken IgY antibodies were developed against surface protein antigens from *Acinetobacter*. These antibodies were used to produce an indirect Enzyme-Linked Immunosorbent Assay (ELISA) capable of detecting live or chemically-fixed *Acinetobacter* spp. from colony isolates obtained after Gram staining to confirm Gram negative status. Bacteria were resuspended in PBS and added to wells in a 96-well assay plate. After binding for 30 minutes, the wells were washed and blocked with PBS + 0.1% Tween®-20 and 5 mg/mL BSA for 30 minutes. Dilute anti-*Acinetobacter* polyclonal chicken IgY antibody was added to the wells for 60 minutes. After washing, bound IgY was detected with peroxidase-linked anti-chicken IgY secondary antibody for 30 minutes. TMB substrate conversion was used to detect relative levels of enzyme activity with endpoint readout at 15 minutes.

Results: The concentrations and incubation times for bacterial immobilization, primary anti-*Acinetobacter* antibody binding, and secondary anti-chicken IgY antibody binding were optimized. After optimization, the assay could be performed in less than 4 hours, including sample preparation. Each bacterial sample was analyzed with and without primary anti-*Acinetobacter* antibody to determine the relative background contribution to the overall signal. A signal of 2-fold over background was considered positive. A collection of 35 strains of *Acinetobacter* as well as 34 clinically relevant non-*Acinetobacter* Gram negative strains were tested in the assay. The ELISA provided positive readout for 30 of the 35 *Acinetobacter* strains, and was negative on all 34 non-*Acinetobacter* Gram negative strains.

Conclusions: A rapid indirect ELISA assay for *Acinetobacter* spp. demonstrated 86% sensitivity for 35 *Acinetobacter* spp. clinical isolates and ATCC strains. Specificity of the assay was 100% on a collection of 34 common non-*Acinetobacter* Gram negative bacteria.

INTRODUCTION

Acinetobacter spp. are important pathogens whose endemic nosocomial strains tend to express multiple drug resistance. Their identification by standard culturing methods can be slow, raising the risks for critically ill patients who must receive empiric therapy until lab results become available.

The purpose of this investigation was to develop a rapid ELISA for *Acinetobacter* spp. using colony isolates.

MATERIALS & METHODS

Anti-*Acinetobacter* spp. Polyclonal Antibodies. Primary amine groups on surface proteins from *Acinetobacter* strains and isolates were labeled using the non-permeable activated biotin compound, sulfo-NHS-SS-biotin (Pierce Biotechnology). Labeled proteins were liberated from the cells by treatment with B-PER lysis reagent (Pierce). Free biotin and other small molecules were removed by gel filtration chromatography on Sephadex G-50 (Sigma-Aldrich). The biotinylated proteins were attached to Sepharose CL-6B beads (GE Life Sciences) bearing covalently linked streptavidin (Prozyme). Following binding of the biotinylated proteins, the beads were washed with PBS to remove unbound material. Chickens (Lampire Biological Products) were immunized using the *Acinetobacter* surface protein beads suspended in complete (day 0) or incomplete Freund's adjuvant (days 14, 28, 56, and 84). Eggs were collected from hens from day 43 through day 98 with yolks immediately separated and stored at -20ºC. Crude IgY was prepared from pooled egg yolks by acid precipitation to remove lipids and lipoproteins, then tangential flow filtered through 0.2 µm and 100 kDa MWCO filters. Affinity purification of specific anti-*Acinetobacter* IgY was performed using surface protein beads with specific antibodies eluted using pH 3.0 isotonic buffer. Non-specific antibodies were adsorbed on streptavidin beads, followed by adsorption on fixed bacteria.

Strain Collection. 35 *Acinetobacter* strains and isolates were obtained from ATCC®, Jones Medical Institute (JMI), and the University of Texas Health Science Center at San Antonio (UTX). The *Acinetobacter* collection contained a majority of *A. baumannii,* with at least one strain of *Acinetobacter* genomospecies 13TU (ATCC 17903). Selected isolates were verified to be of the *A. baumannii – A. calcoaceticus* complex by API 20NE (bioMérieux) but were not further differentiated to determine genomospecies. 34 non-*Acinetobacter* Gram negative strains were obtained from ATCC and JMI. The collection contained species representative of human pathogenic and normal flora commonly found in clinical specimens. Bacteria were prepared by streaking and colony isolation from Trypticase™ Soy Agar with 5% Sheep Blood (TSA II™) plates (BD), followed by log-phase growth in Trypticase Soy Broth (TSB – BD). Cultured bacteria were frozen in 15% glycerol at -80ºC.

Sample Preparation. Strains and isolates were streaked on TSA II™ blood agar plates and grown 18-24 hours at 35ºC. For each sample, several colonies were looped from the plate and suspended in 1 mL PBS to obtain a suspension containing approximately 10^8 CFU/mL (range 1x10^8 to 1x10^9 CFU/mL). Suspended cells were washed with PBS by centrifugation at 14,000xg for 2 minutes and resuspended in 1 mL PBS. Washed cells were chemically fixed in 2% formaldehyde for 15 minutes to render them non-viable. Prepared sample bacteria were stored at 4ºC until analysis.

ELISA Assay. Standard 96-well microtiter plates (Nalge-Nunc) were used for all assays. Approximately 1x10^6 CFU of fixed bacteria were added to each well in PBS for 30 minutes. Wells were washed three times with PBS containing 0.1% Tween®-20 (PBST) and blocked with BSA at 5 mg/mL in PBST for 30 minutes. Anti-*Acinetobacter* polyclonal antibodies were bound for 1 hour, followed by three PBST washes and then detected using anti-chicken IgY, peroxidase conjugated secondary antibody (KPL). SureBlue TMB substrate (KPL) was added to detect peroxidase activity and the reaction was stopped using TMB stop solution (KPL). Absorbance was read at 450 nm. All plates contained blank control wells without bacteria or primary antibody. Control wells included wells with bacteria but no primary antibody, and wells with no bacteria but with primary antibody. The controls determined the relative non-specific activity of the primary and secondary antibodies.

RESULTS

Optimization of Coated Bacteria Concentration. Various concentrations of bacteria were examined for optimal binding and detection of selected *Acinetobacter* and other Gram negative bacteria ranging from 10^4 CFU/well to 10^7 CFU/well. Maximal signals were obtained using 10^6 CFU/well.

Optimization of Primary Antibody Concentration. Various dilutions of primary antibody were examined on *Acinetobacter* strains with demonstrated low and medium reactivity. The optimal concentration for ELISA analysis was determined as the highest dilution producing positive score (greater than 2-fold over background) on the low reactivity strains.

Optimization of Binding and Development Timing. Bacterial coating duration, binding duration for primary and secondary antibodies, and development of the TMB substrate were examined with incubation times from 15 minutes to 1 hour for each step. Optimal timing for binding of bacteria to the plate was 30 minutes with a slight decrease in signal strength at 15 minutes. Primary antibody binding was optimal at 1 hour with moderate decreases in signal strength at 15 or 30 minutes. Secondary antibody binding was optimal at 30 minutes, and development of the TMB substrate with stopping at 15 minutes provided sufficient time for high and low signal determination in all tested cases. The assay could be completed through all steps in less than 4 hours, including sample preparation.

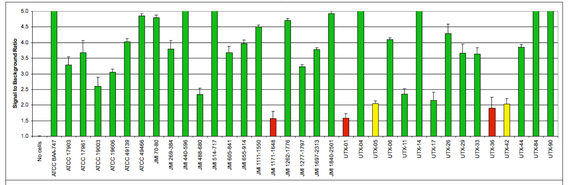

Fig. 1: ELISA Analysis of 35 Acinetobacter strains as mean signal to background ratio + standard deviation of triplicate wells.

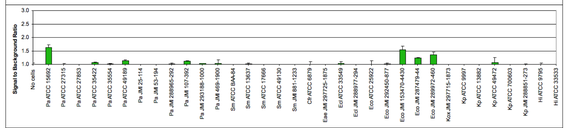

Fig. 2: ELISA Analysis of 34 non-Acinetobacter Gram negative strains as mean signal to background ratio + standard deviation of triplicate wells.

Sensitivity with 35 *Acinetobacter* Strains and Isolates. The ELISA assay was performed on the *Acinetobacter* collection in triplicate wells for each fixed bacterial sample. A positive score was defined as signal at least 2-fold over the mean background (wells that did not contain bacteria). As shown in **Fig. 1**, 30 of 35 *Acinetobacter* strains scored positive in the assay, with signal in all three wells at least 2-fold above the assay background control. Two strains had at least one well below criterion and were scored as marginal strains (yellow). Including these marginal strains as negative, along with three missed strains (red), the assay produced positive results on 86% of tested *Acinetobacter* strains.

Fig. 2 shows the results with 34 non-*Acinetobacter* strains. None of the strains demonstrated non-specific binding using the 2-fold criterion.

Lowering the criterion to 1.5-fold would increase sensitivity with some sacrifice in specificity. Studies with further improvements and larger collections will help to identify the statistically optimum cutpoint.

CONCLUSIONS

An indirect ELISA assay was developed using an affinity purified polyclonal chicken IgY antibody. The assay may be used on isolated Gram negative bacterial colonies following Gram staining to identify *Acinetobacter* spp. The assay showed 86% sensitivity on 35 *Acinetobacter* strains, and 100% specificity on 34 other Gram negative strains. The time required for assay, including preparation of sample bacteria from isolated colonies, was less than 4 hours.

Acknowledgment: We gratefully acknowledge Jim Jorgensen of the University of Texas at San Antonio for access to clinical isolates.