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Detection and quantification of Panton-Valentine leukocidin in *Staphylococcus aureus* cultures by ELISA and Western blotting: diethylpyrocarbonate inhibits binding of protein A to IgG

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Abstract

Enzyme-linked immunosorbent assay (ELISA) and Western blotting are common techniques used to detect and quantify proteins in *Staphylococcus aureus* culture supernatants, such as Panton-Valentine leukocidin (PVL). However, protein A (Spa) secreted by most *S. aureus* strains may interfere with these assays by binding to the capturing and detecting antibodies. Here, we have shown that the addition of diethylpyrocarbonate (DEPC) inhibits the binding of Spa to rabbit anti-PVL used as the capturing antibody in ELISA. In Western blotting, the presence of DEPC prevented the binding of detecting antibody to Spa. These modified ELISA and Western blot techniques should prove useful for detecting and quantifying proteins in *S. aureus* cultures supernatants.

Keywords

Staphylococcus aureus; protein A; Panton-Valentine leukocidin; Diethylpyrocarbonate; ELISA; Western Blot; Quantification; Detection

1. Introduction

A number of immunological techniques including ELISA and Western blot have been described for detecting and quantifying staphylococcal proteins. The 42 kD *Staphylococcus aureus* protein A (Spa) secreted by 99% of *S. aureus* isolates (Goding, 1978) interferes with the ELISA by binding to the Fc region of most mammalian IgGs used as the capturing antibodies in these assays. To prevent protein interference several techniques have been employed, but most have problems of their own. $F(ab')_2$ fragments have been used as capturing reagents, but Spa binds to Fab's containing V_H3 (Sasso et al., 1991; Ladhani et al., 2001). Affinity chromatography or addition and centrifugation of porcine IgG coupled to insoluble matrix has been used to remove protein A from culture supernatants (Berdal et al., 1981; Fey

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and Burkhard, 1981). Detecting rabbit antibodies have been biotinylated so that the binding site of Spa on the IgG molecule was masked (Hahn et al., 1986). Other methods have relied on the development of capturing IgG antibodies in rats (Rogemond et al., 1991) and sheep (Freed et al., 1982) that bind only weakly to Spa. In addition, chicken anti-protein A IgG has been used to sequester protein A, since chicken IgG does not bind protein A in the Fc region (Hoffman et al., 1996). These approaches developed to overcome the Spa interference require a considerable amount of time and additional expense.

Here, we describe the development of sandwich ELISA and Western blotting techniques to detect and estimate the amount of the LukS-PV component of Panton-Valentine leukocidin (PVL) secreted by community-associated methicillin resistant *S. aureus* (CA-MRSA). We show that the addition of diethylpyrocarbonate (DEPC) inhibits the interaction of protein A present in the culture supernatants containing PVL with the capturing antibody. This approach can be used to detect other proteins when interference with protein A is a problem.

2. Materials and Methods

2.1 Bacterial Preparation

Four clinical CA-MRSA isolates were used in this study: LAC (provided by F. DeLeo, NIH), SA-123, SA-109, and SA-112. LAC and SA-123 are pvl^+ strains that produce different amounts of PVL proteins; SA-109 is a pvl^- strain and SA-112 is pvl^+ but produces very low levels of Spa. Bacteria were grown in YCP (yeast, casamino acid, pyruvate) medium for 18 hours at 37° C with constant shaking from glycerol stocks stored at -30°C. Bacterial densities were determined by spectrophotometry (O.D. 600 nm) then converted to estimated colony forming units (CFUs) using a standard curve. Supernatants were collected after centrifugation (3500 rpm) for 5 minutes at 4°C.

2.2 Construction of LukS-PV recombinant protein

PVL is a bicomponent toxin encoded by two genes, *lukS-PV* and *lukF-PV* (Kaneko and Kamio, 2004). The gene encoding LukS-PV was amplified by PCR from DNA extracted (DNeasy Tissue Kit, Qiagen) from a pvl^+ *S. aureus* strain (49775, ATCC). The PCR products were ligated into pET151D/TOPO (Invitrogen) and the resultant plasmid was transformed into *E. coli* BL21 (Invitrogen) and grown in LB media with ampicillin at 37°C in the presence of 1 mM IPTG. Recombinant LukS-PV (rLukS-PV) protein was purified over a nickel-cadmium column and the His-6 tag was removed from the recombinant protein using AcTEV protease (Invitrogen).

2.3 Primary and secondary antibody preparation

Both rabbits and mice were immunized with 100 μg AcTEV-cleaved LukS-PV protein in emulsion with complete Freund's adjuvant on day 0, followed by booster immunizations in emulsion with incomplete Freund's adjuvant on days 7, 14 and 28. Antibody titers were determined by ELISA plates coated with rLukS-PV. Animals were terminally bled after satisfactory antibody titers (≥1:500,000) were reached. To rule out the possibility of cross-reactivity with other sequence related staphylococcal cytotoxins, reactivity to alpha toxin (Sigma) and native LukS-PV, LukF-PV, HlgA, HlgB and HlgC purified from culture supernatants as previously described (Prevost et al., 1995) was analyzed by SDS-PAGE and Western blotting using rabbit anti-LukS-PV as the detecting antibody (data not shown). Both rabbit and mouse antisera bound specifically to the immunizing protein only.

2.4 ELISA

Microtiter plates were coated (100 μL/well) with rabbit anti-LukS-PV diluted 1:500 in 50 mM carbonate buffer, pH 9.6 by incubating at 37°C for 2 hrs. Unbound sites were blocked with PBS containing 5% skim milk by incubating overnight at 4°C. Staphylococcal supernatants were then added to the rabbit antibody-coated plates (100 μL/well) then incubated at 37°C for 1 hour. After washing three times with PBS, the plates were incubated at 37°C with mouse anti-LukS-PV antibody diluted 1:500 in PBS containing 5% skim milk for 1 hour. After washing three times with PBS, the plates were incubated with goat anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotech) at 1:500 dilution (100 μL/well) followed by the substrate disodium p-nitrophenyl phosphate solution (Sigma) at a concentration of 1 mg/mL in 1 M diethanolamine, 0.25 mM MgCl, pH 9.8. After 1 hour of development at 37°C, the absorbance was read at OD 405 nm. To block the binding of Spa to rabbit anti-LukS-PV, rabbit antibody coated plates were incubated with DEPC (Sigma) diluted in PBS, pH 6.0 from a 5 M stock solution. To detect if Spa was binding to rabbit anti-LukS-PV coated plates, chicken anti-protein A conjugated to horseradish peroxidase (HRP, Genscript, Piscataway, NJ), was used as the detecting antibody.

2.5 Western blot for LukS-PV

Bacterial supernatants were subjected to one-dimensional SDS-PAGE on 12% polyacrylamide gels for 1 hour at 80 mV. Proteins were transferred to 0.45 μm polyvinylidene fluoride membranes (Pierce) for 30 minutes at 25 mV. Membranes were then incubated with rabbit anti-LukS-PV (1:500) diluted in PBS containing 5% skim milk, pH 6.0 in the presence of DEPC for 20 minutes at room temperature. Membranes were then washed three times with PBS, pH 7.4 prior to incubation with an anti-rabbit IgG conjugated to HRP (Pierce) at a dilution of 1:5000 in PBS with 5% skim milk, pH 7.4. Membranes were washed with PBS, pH 7.4 before the addition of the chemiluminescent substrate (Super Signal West Pico) according to the manufacturer's instructions and exposed to Hyperfilm ECL (Amersham).

3. Results and Discussion

We developed a sandwich ELISA to detect and quantify LukS-PV in culture supernatants of *S. aureus* strains. We produced rabbit and mouse antisera using rLukS-PV as the immunogen. Preliminary experiments indicated that both antisera detected purified native and rLukS-PV by ELISA when LukS-PV was directly coated on microtiter plates (data not shown). To detect LukS-PV in *S. aureus* culture supernatants, rabbit antiserum bound to a microtiter plate was used to capture LukS-PV from CA-MRSA bacterial cultures and mouse antiserum was used to detect the LukS-PV captured by the rabbit antiserum. Surprisingly, positive signals were detected with supernatants from both *pvl*$^+$ CA-MRSA isolate LAC (Figure 1; Filled squares) and *pvl*$^-$ CA-MRSA isolate SA-109 (Figure 1; Filled circles).

Spa secreted by *S. aureus* strains is known to cause interference in immunological assays (Guidry et al., 1991). To determine if Spa was responsible for the positive signal with SA-109, ELISA was repeated using chicken anti-protein A conjugated to HRP as the detecting antibody. Spa was detected in both LAC and SA-109 strains (Figure 2A; Without DEPC). These results suggest that Spa in the bacterial cultures binds to the rabbit antibodies immobilized on the ELISA plates. It is reasonable to assume that Spa in SA-109 binds to the coating rabbit antibody, and the detecting murine antibodies bind to the captured Spa yielding a positive signal.

To confirm the presence of Spa, culture supernatants from *pvl*$^+$ MRSA strains SA-123, SA-112 and LAC and *pvl*$^-$ MRSA strain CA-109 were analyzed by SDS-PAGE and Western blotting. Rabbit anti-LukS-PV detected a band of MW 32 kD corresponding to LukS-PV. In addition, an intense band of MW 42 kD, consisting of a doublet, was also detected in culture supernatants

of SA-123, SA-109 and LAC (Figure 3A). The 42 kD band was absent in SA-112, which is known to produce very little if any Spa. When the proteins were probed with chicken anti-protein A, the 42 kD band was detected in LAC, SA-109, SA-123, but not in SA-112 (Figure 3B) indicating that protein A secreted by the CA-MRSA strains is bound by rabbit anti-LukS-PV.

Haake and coworkers have shown that DEPC can inhibit Spa binding to IgG (Haake et al., 1982). When microtiter plates coated with rabbit anti-LukS-PV were incubated with 5 mM DEPC, no signal was observed with *spa*$^{+}$/*pvl*$^{-}$ CA-MRSA strain SA-109, whereas attenuated positive signal was observed with *spa*$^{+}$/*pvl*$^{+}$ LAC culture supernatants (Figure 1; dashed lines). Pre-treatment of rabbit antiserum with 5 mM DEPC for 10 min was sufficient to inhibit binding of Spa to rabbit IgG. The interference due to Spa was inhibited with increasing concentrations of DEPC with 1, 5 and 10 mM DEPC inhibiting 75%, 90% and 95%, respectively (Figure 2B).

To determine if DEPC is effective in Western blotting under denaturing conditions, culture supernatants from *spa*$^{+}$/*pvl*$^{-}$ and *spa*$^{+}$/*pvl*$^{+}$ CA-MRSA isolate culture supernatants were analyzed by SDS-PAGE and Western blotting using rabbit anti-LukS-PV as the detecting antibody in the presence of DEPC followed by goat anti-rabbit conjugated to HRP (Figure 3). The binding of anti-LukS-PV to Spa was inhibited with increasing concentrations of DEPC with 5 mM DEPC inhibiting 95% of binding (Figure 3C & 3D). The inhibition was dependent on the amount of Spa produced by the bacterial strain.

Spa binding is mediated through an exposed histidine residue at position 435 in human IgG (Deisenhofer, 1981). It has been proposed that DEPC interferes with the binding of Spa to histidine-435 in human IgG (Schroder et al., 1987). Our results indicate that DEPC prevents the direct binding of Spa to anti-LukS-PV. In addition to histidine, lysine, tyrosine, serine and threonine residues can be modified by DEPC (Hnizda et al., 2008; Mendoza and Vachet, 2008). Because these residues may be found in the antigen-binding site of antibodies, we determined if the presence of DEPC interferes with the binding of LukS-PV to anti-LukS-PV in the sandwich ELISA. To directly compare the binding characteristics, the bacterial culture supernatants and YCP media were dialyzed against PBS. The binding of anti-LukS-PV pre-incubated with 5 mM DEPC to rLukS-PV added to SA-109 (*pvl*$^{-}$) culture supernatant (Figure 4; dashed line and open diamonds) was similar to the binding of anti-LukS-PV to LukS-PV in culture media YCP (Figure 4; solid squares). However, when the binding of anti-LukS-PV pre-incubated with 5 mM DEPC to rLukS-PV added to SA-109 (pvl^{-}) culture supernatant (Figure 4; dashed line and open diamonds) was compared to the binding of anti-LukS-PV to rLukS-PV added to SA-109 (pvl^{-}) culture supernatant (Figure 4; solid line and open triangles), DEPC appeared to have a small impact on the binding of anti-LukS-PV to LukS-PV. Furthermore, when the culture supernatants were analyzed by Western blot, the band corresponding to LukS-PV in supernatant from LAC strain appeared less intense in the presence of DEPC, confirming that pretreatment of DEPC has a small impact on LukS-PV binding to its cognate antibody. It is interesting to note that the difference in binding of anti-LukS-PV to LukS-PV becomes significant only at a lower dilution of the supernatants indicating that there may be a subset of the polyclonal antibody pool affected by DEPC treatment. In this case, a higher concentration of polyclonal antiserum or a carefully chosen monoclonal antibody might be better reagents for the sandwich ELISA.

In summary, we have developed a sandwich ELISA to detect and quantify LukS-PV in culture supernatants of *S. aureus* strains. We show that DEPC can be used to inhibit binding of rabbit IgG to protein A, known to cause interference in immunological techniques. We have used this sandwich ELISA to quantify LukS-PV secreted by *S. aureus* strains in presence of different antibiotics and confirmed the quantification results by determining the relative amounts of LukS-PV secreted using Western blots (manuscript in preparation). Using DEPC should prove

useful in using antibody detection methodologies, such as ELISA and Western blotting, when protein A may cause interference.

Acknowledgments

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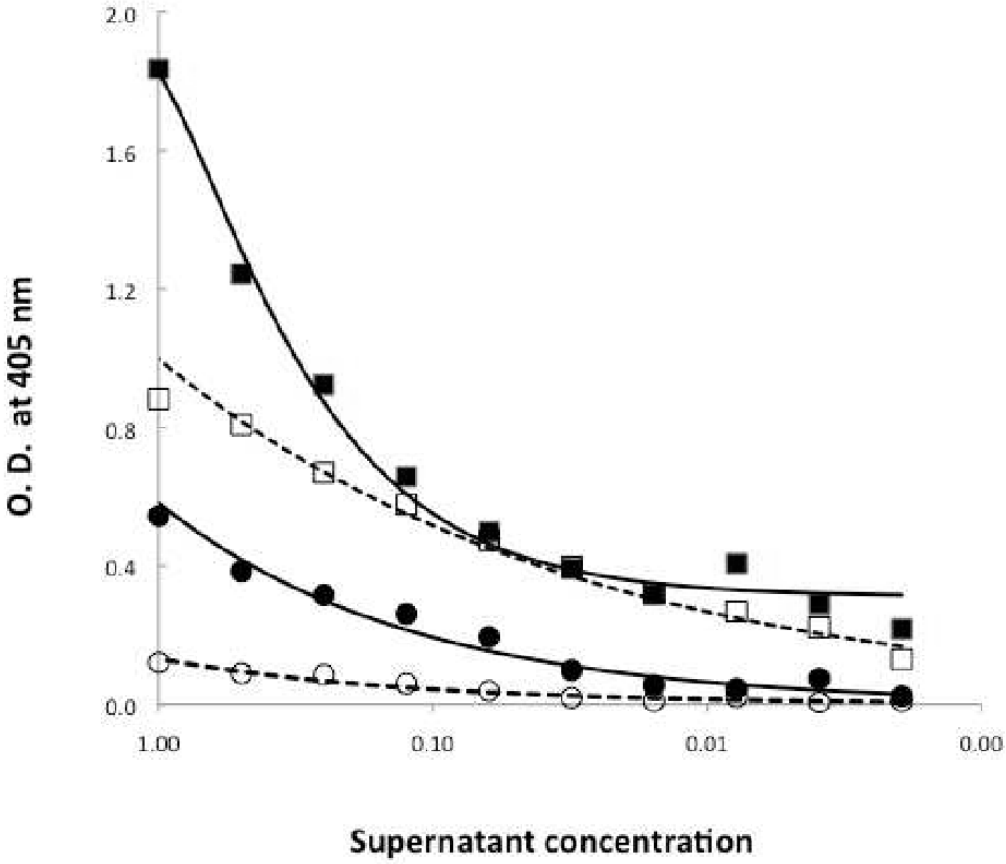

Figure 1. Sandwich ELISA to detect LukS-PV in bacterial cultures

Microtiter plates coated with rabbit anti-LukS-PV were incubated overnight with diluted culture supernatants from SA-109 (circles) and LAC (squares) CA-MRSA bacterial strains (closed symbols, solid lines), or microtiter plates coated with rabbit anti-LukS-PV were incubated for 20 min with 5 mM DEPC and incubated overnight with diluted culture supernatants (open symbols, dashed lines). The plates were incubated with mouse anti-LukS-PV followed by goat anti-mouse IgG conjugated to alkaline phosphatase and developed by adding disodium p-nitrophenyl phosphate. The optical density (O.D.) was determined at 405 nm.

Figure 2A

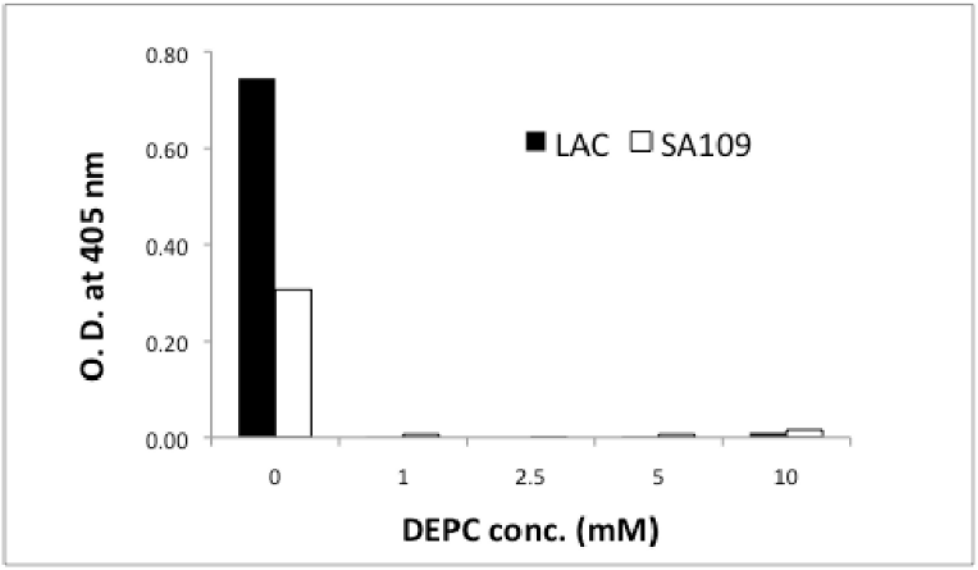

Figure 2B

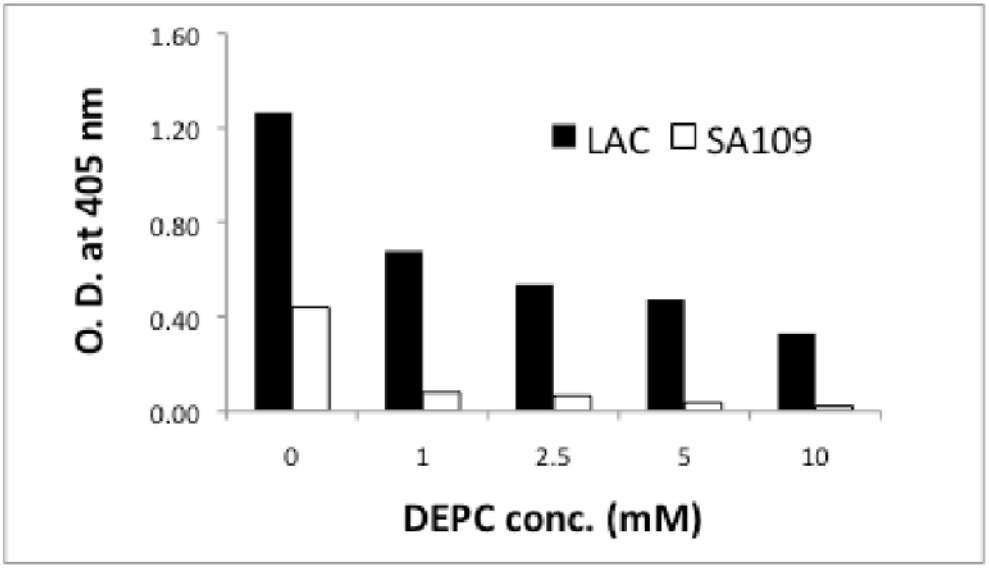

Figure 2. Sandwich ELISA to detect LukS-PV and Spa in presence of DEPC

A, Microtiter plates coated with rabbit anti-LukS-PV were incubated overnight with diluted culture supernatants. Spa bound to the capturing rabbit antibody was detected by chicken anti-protein A conjugated to HRP. **B**, LukS-PV was detected by carrying out the ELISA as described in Figure 1.

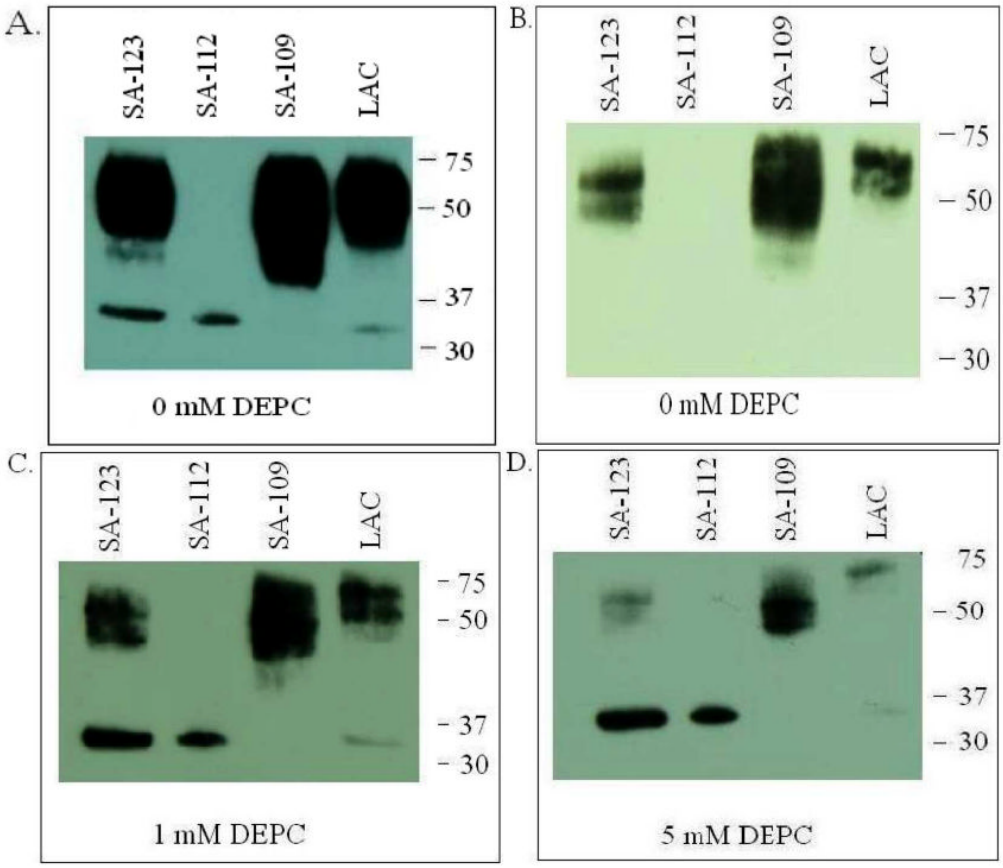

Figure 3. Western blot analysis of culture supernatants from four clinical CA-MRSA isolates

The isolates vary in the presence of genes for PVL as well as the production of protein A: pvl^+ SA-123, pvl^+ LAC producing low levels of pvl, pvl^- SA-109 and pvl^+ SA-112 producing very low levels of Spa. Proteins were separated by SDS/PAGE in 4-20% Tris-glycine gels and transferred to nitrocellulose. Nonspecific sites were blocked by incubating the blots in PBS containing 5% skim milk (PBS-milk). Blots were first incubated with (**A**) rabbit anti-LukS-PV in PBS-milk, (**C**) rabbit anti-LukS-PV mixed with 1 mM DEPC, or (**D**) rabbit anti-LukS-PV mixed with 5 mM DEPC, followed by goat anti-rabbit antibodies conjugated to HRP. (**B**) protein A was detected using chicken anti-protein A conjugated to HRP. Blots were developed by incubation with Super Signal West Pico Chemiluminescent Substrate and exposure on CL-XPOsure Film.

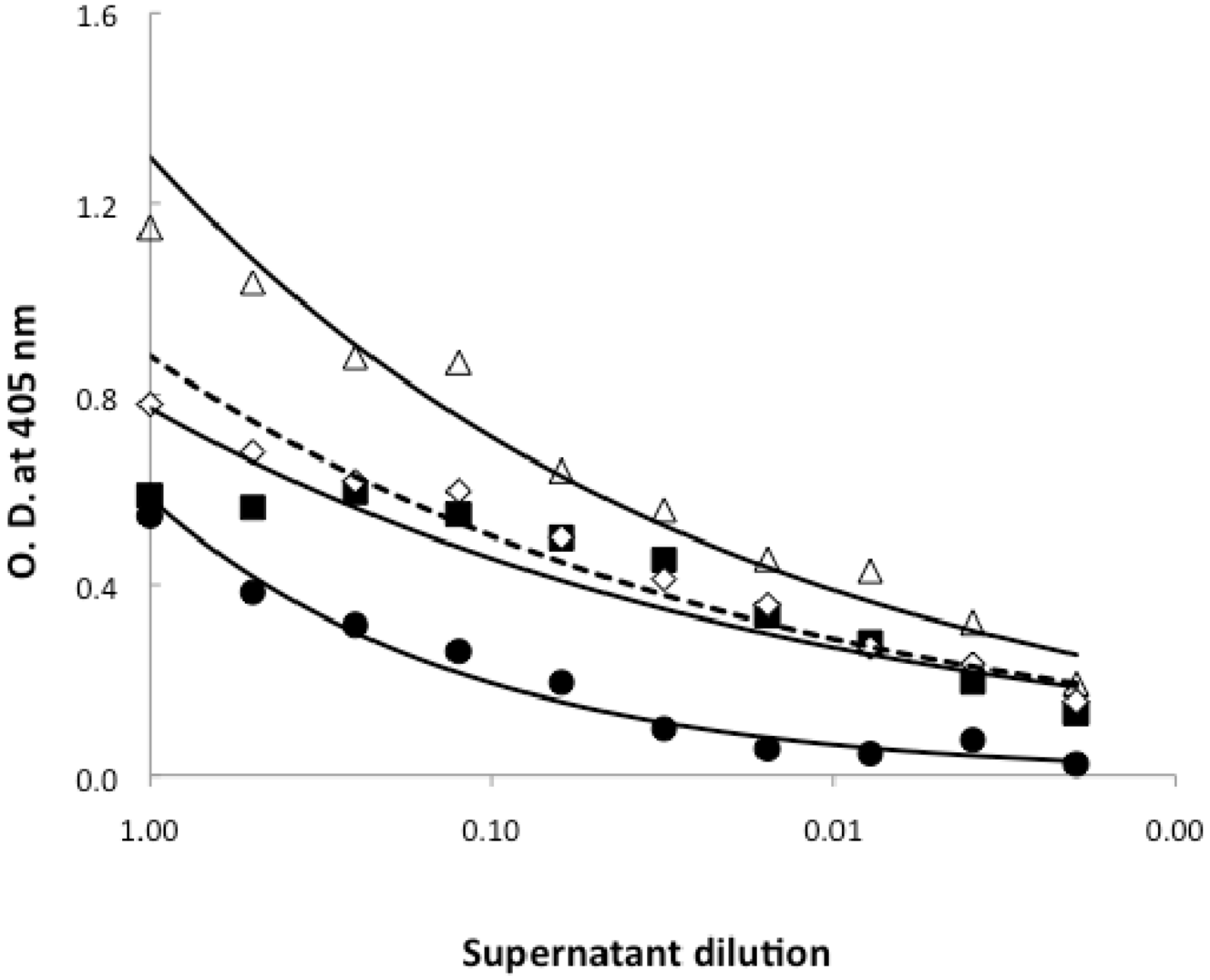

Figure 4. Binding characteristics of anti-LukS-PV pre-incubated with DEPC to rLukS-PV in YCP media and bacterial culture supernatants

ELISA was carried out as described in Figure 1. Shown is the binding of 2 μg/ml rLukS-PV in dialyzed YCP media to anti-LukS-PV (closed squares, solid lines) and the binding of 2 μg/ml rLukS-PV in dialyzed SA109 supernatant to DEPC-treated anti-LukS-PV (dashed line, open diamonds). Controls shown are dialyzed SA-109 culture supernatants binding to anti-LukS-PV (solid circles) and dialyzed SA-109 culture supernatants containing 2 μg/ml rLukS-PV binding to anti-LukS-PV (open triangles).