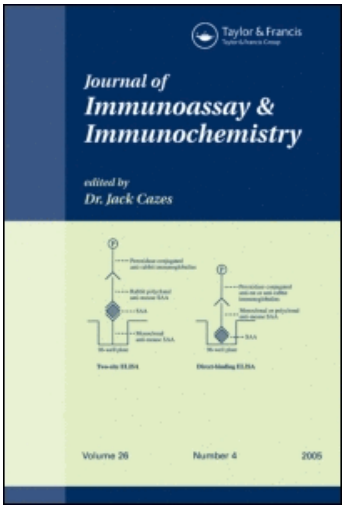

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Nielsen, Klaus(1998) 'Use of Dried Smooth Lipopolysaccharide Antigen Coated Polystyrene Plates for Diagnosis of Bovine Brucellosis by Enzyme Immunoassay', Journal of Immunoassay and Immunochemistry, 19: 1, 39 — 48
To link to this Article: DOI: 10.1080/01971529808005470
URL: http://dx.doi.org/10.1080/01971529808005470

JOURNAL OF IMMUNOASSAY, 19(1), 39-48 (1998)

USE OF DRIED SMOOTH LIPOPOLYSACCHARIDE ANTIGEN COATED POLYSTYRENE PLATES FOR DIAGNOSIS OF BOVINE BRUCELLOSIS BY ENZYME IMMUNOASSAY.

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ABSTRACT

Polystyrene plates (96 well) were sensitized with **Brucella abortus** smooth lipopolysaccharide (SLPS) antigen and then air dried at room temperature (RT) for about 1 hour to dry. Dryness was judged complete when a buffer meniscus was absent from the bottom of the well. The plates were resealed kept on the bench at RT for the duration of the study. Testing was done over 13 month by competitive and indirect ELISAs (C- and IELISA) for bovine antibody to **B. abortus** using panels of sera from **B. abortus** S19 vaccinated, unexposed and infected cattle. Testing revealed that consistent results were obtained over the test period suggesting that air drying may be a suitable alternative for storage of plates sensitized with some antigens, in particular, smooth lipopolysaccharide.

(KEY WORDS: Brucellosis, lipopolysaccharide, enzyme immunoassay, antigen preservation).

INTRODUCTION

Traditionally, most antigens immobilized on polystyrene for use in immunoassays are solubilized in a high pH, low molarity buffer and incubated with the matrix 18 hours or more (overnight) to allow maximum antigen binding. This approach works very well, however, it does have some drawbacks. Firstly, remaining concentrated antigen requires preservation to minimize bacterial growth. The antigen is usually stored frozen but repeated cycles of freezing and thawing tends to inactivate some antigens. A second disadvantage is that

assays may not be performed on the first working day. Lastly, it is inefficient to prepare small amounts of coating buffer frequently and measuring errors may occur due to the minute amounts of concentrated antigen used (the latter may not be a problem if excess antigen is used, however, for diagnostic testing, antigens are commonly titrated to save reagents and a small error in measurement may lead to a substantially different end result). One solution to these problems was to freeze plates pre-coated with antigen which allowed batch preparation of larger numbers of plates which could then be thawed as required.

This solution, however, generated another set of problems. Firstly, unless the plates were tightly sealed, evaporation occurred. This led to intraplate assay variation between replicate samples. A second problem was the amount of freezer space occupied by plates, especially in the freezing stage when the plates could not be stacked. In addition, frozen plates require shipment in dry ice, an inconvenient and expensive method for shipping larger quantities. A useful alternative would be to air dry plates precoated with antigen and then store them at room temperature. This communication reports on the long term stability of polystyrene plates, coated with **Brucella abortus** lipopolysaccharide antigen, under such conditions.

MATERIAL AND METHODS

Serum Samples: Each plate tested included four quadruplicate controls: BAC++, a positive serum reacting strongly with **B. abortus**; BAC+, a serum that gives a weak positive reaction; BAC-, a serum negative for brucellosis and Cc, a control to which no serum was added (buffer control).

For the CELISA, a panel of 5 sera each from **B.abortus** strain 19 vaccinated, negative and **B. abortus** infected animals was used. This panel included each of the 15 serum samples in quadruplicate in randomized order.

For the IELISA, a panel of sera consisting of 22 originating from S19 vaccinated animals, 6 from negative and 6 from **B. abortus** infected cattle was used. These samples were set up in duplicate on each plate.

For all assays, the serum samples were diluted in 0.01M phosphate buffer, pH 6.3, containing 0.15M sodium chloride and 7.5mM of each of EDTA and EGTA (PBS/EDTA/EGTA).

Antigen: The phenolic fraction of hot water/hot phenol extracted **B. abortus** S1119.3 cells was prepared (1). The crude lipopolysaccharide (SLPS) was treated with 5% w/v trichloroacetic acid for 15 min at RT with stirring, centrifuged at 10,000 x g for 10 min, sonicated (6 watts/1 min three times), dialized against distilled water and freeze dried. Each mg of antigen was reconstituted in 1.0 ml of 0.06M carbonate buffer, pH 9.6 prior to use and stored at 4°C or -20°C.

The biological activity of the SLPS antigen was tested against a panel of serum samples of known antibody levels and the antigen was found to give optical density values within the control limits established for each serum in the panel.

Passive Immobilization of Antigen in 96 Well Plates: NUNC 69620 polystyrene plates (2) were used. To each well, 100 ul of 1.0 ug/ml SLPS dissolved and diluted in 0.06M CO_3 buffer, pH 9.6 was added. The plate was sealed with plastic and incubated at 20+/-2°C (RT) for 18 to 20 hours. After incubation, the plates were either frozen at -20°C or the antigen preparation remaining in the wells was discarded, the plates air dried at RT and resealed with plastic. The air dried plates were stored at RT.

The air dried plates were tested at irregular intervals for the following 13 months. Frozen plates were tested at the beginning of the study and at the end to provide a 'baseline' of assay reactivity for the serum samples used. Prior to use, the frozen plates were thawed at 37°C for 30-45 min. The plates were then washed four times with 0.01M phosphate buffer, pH 7.2, containing 0.15M sodium

chloride and 0.05% Tween 20 (PBS/T). The air dried plates were not prewashed or treated before use.

Enzyme Immunoassays: Both the indirect (I) and the competitive (C) ELISAs were performed as previously described (3). In both assays, the four controls were included in quadruplicate in columns A and B of the antigen coated polystyrene plate and duplicate samples were placed in subsequent wells.

Briefly, for the IELISA, 100 ul of serum diluted 1:100 in PBS/EDTA/EGTA were added to each well. The plate was incubated at RT for 1 hour. The plate was then washed four times in PBS/T and mouse monoclonal antibody specific for bovine IgG_1 (M23), conjugated with horseradish peroxidase (4), appropriately diluted in PBS/T was added for 1 hour. After an additional four washes, 100 ul substrate/chromogen, (1mM ABTS, 4mM H_2O_2 in 0.05M citrate buffer, pH 4.5) were added for 10 min with continuous shaking. Colour development was assessed at 414 mn in a spectrophotometer.

After 6 months, the diagnostic procedure was changed slightly in that serum dilutions were changed to 1:50 and both incubation periods were adjusted to 30 min (the change is indicated in Figures 1 and 3). Data were expressed as a percentage of the reactivity of the strong positive control serum (BAC++ is 100% positive).

For the CELISA, 50 ul of serum diluted 1:10 in PBS/EDTA/EGTA were added to each well followed immediately by 50 ul of mouse monoclonal antibody to a SLPS O-polysaccharide determinant (M84), appropriately diluted in PBS/EDTA/EGTA. The plate was shaken for 3-5 min and incubated at RT for an additional 25-27 min. After four washes with PBS/T, 100 ul goat anti-mouse IgG, conjugated with HRPO (5) diluted in PBS/T were added to each well. After 30 min of incubation at RT, the plate was washed four times and colour allowed to develop as for the IELISA. Data was expressed as percent inhibition of the buffer control (BACc gives 0% inhibition).

For both assays, upper and lower control limits had been established for each control serum. These values were established by

using the dynamic mean of each replicate of control serum tested on the previous 50 plates bracketed by two standard deviations. For the data to be acceptable, the control values must fall within these limits.

Data: For each test date, the data obtained from each group of sera (negative, positive or from vaccinated cattle) were averaged for both tests. The range of each group was included. For comparison, the first and last set of data also include results obtained with frozen antigen coated plates.

RESULTS

The control sera, tested over the 13 month period, are plotted for the I- and CELISAs. Figure 1A shows that for the CELISA, the controls all fall within the upper and lower specification limits for the assay (dotted lines) and that there are only minor variations in the data over time. Similarly, Figure 1B, the results for the IELISA indicate that the controls are within the specified assay limits. Please note that the C+ serum was changed after the test had run for 5 months.

Figure 2 represents the data obtained with the CELISA. Panel A depicts the means and ranges of the sera obtained from vaccinated animals; panel B, the results obtained with sera from negative animals and panel C, those obtained with sera from **B. abortus** infected animals. In addition to the results with plates onto which antigen was dried, the initial and the last tests were also done with antigen coated, frozen plates. From the data, only minor variation between tests are in evidence and that the dried plates gave results very similar to those obtained with frozen plates. The greatest test variation was observed with sera from **B. abortus** infected cattle, however, the assay means vary very little (less than 10% inhibition over the course of the study).

Figure 3, panels A, B and C represent testing of sera from vaccinated, negative and infected cattle in the IELISA using plates with

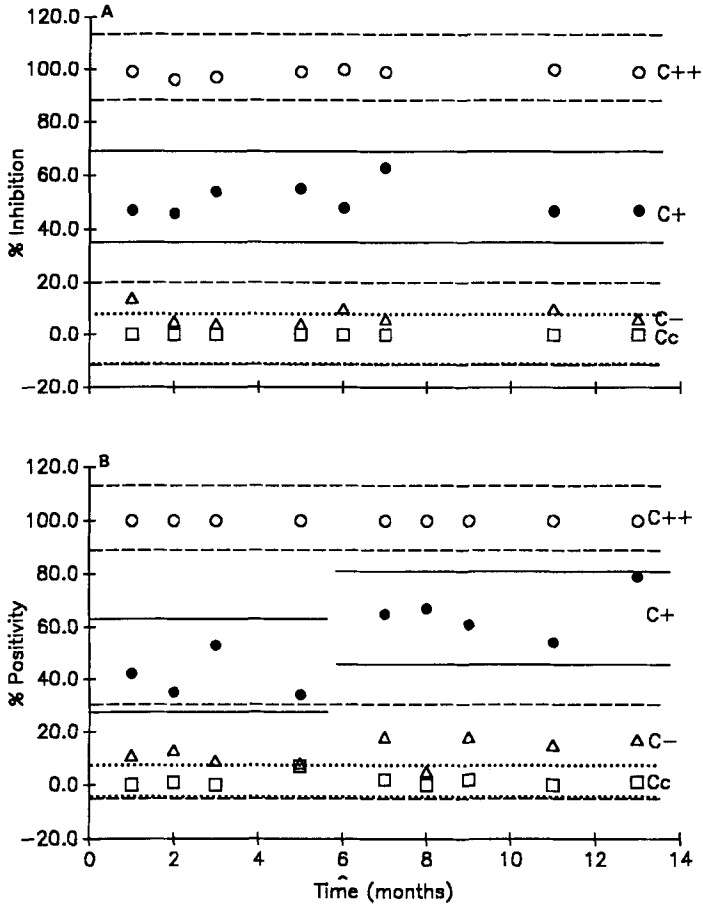

Figure 1: Results obtained with a strong positive control (C++), weak positive (C+) and negative (C-) serum controls and a no serum control (Cc) in the C- and IELISAs.
Graph A represents the % Inhibition values obtained with the control sera in the competitive ELISA, C++ (high antibody level to **Brucella abortus**, open circles), C+ (lower level of antibody to **B. abortus**, closed circles), C- (background antibody to **B. abortus**, triangles) and Cc (buffer control, no serum added, squares) over the 13 month test period. Two standard deviations, considered the upper and lower control limits, are included (dotted and dashed lines) for each control. Graph B represents the same plot for the indirect ELISA with % positivity plotted against time. The serum used as a low positive control, C+, was changed at month 5. This is reflected in the slightly higher values for this control. Please note that at month 6, the assay format was changed. This change did not cause any major changes in the controls.

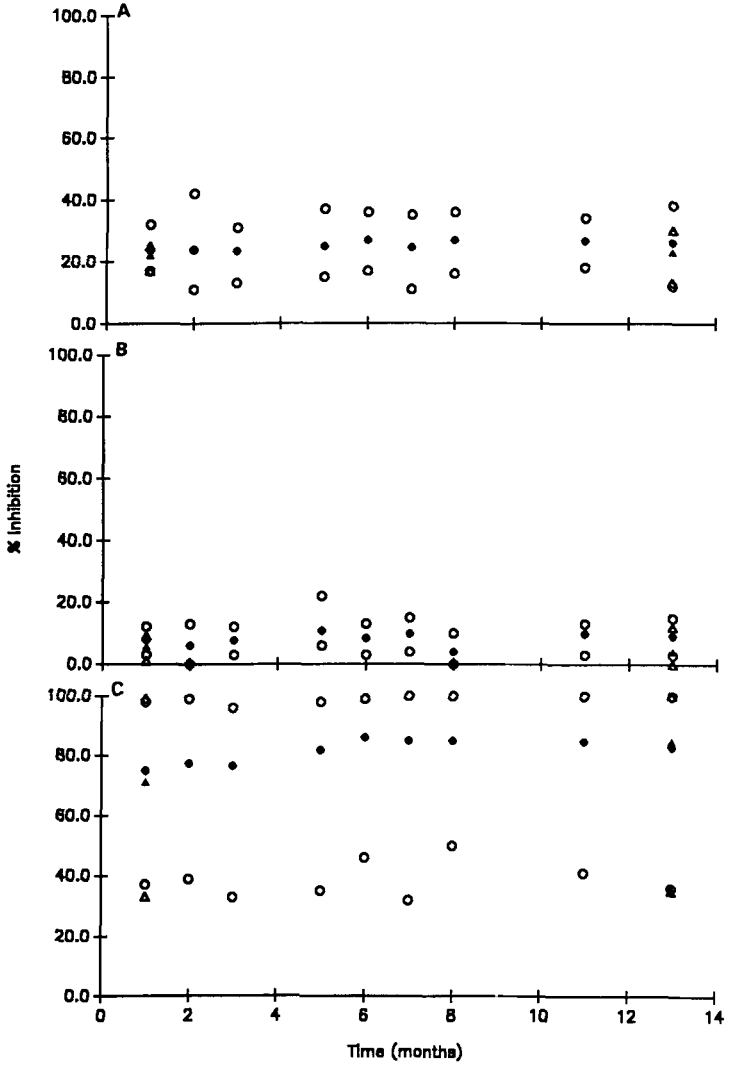

Figure 2: Competitive ELISA for detection of bovine antibody to **Brucella abortus** using antigen coated dried plates. The averaged data (closed circles) was obtained over a 13 month period using panels of sera from **B. abortus** strain 19 vaccinated animals (graph A), **B. abortus** infected cattle (graph B) and cattle not exposed to **B. abortus** (graph C). The range for each data point has been included (open circles). Month 1 and month 13 include data obtained with the same serum panels but using antigen coated frozen plates. The average of each group is represented by closed triangles and the range by open triangles.

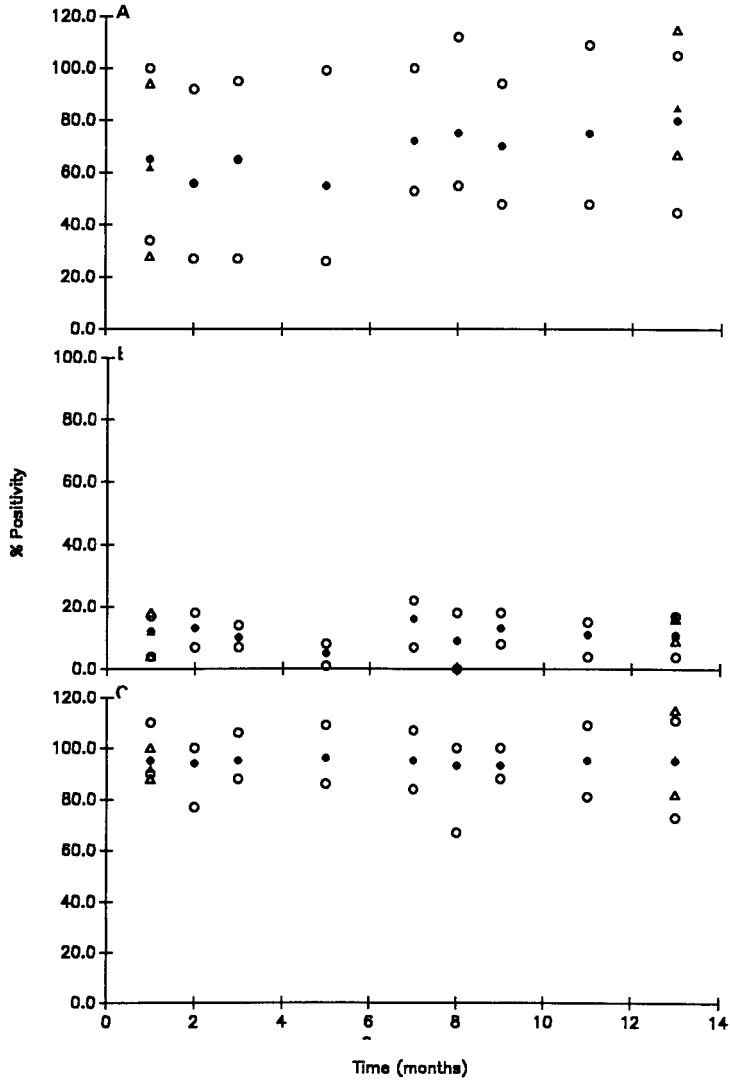

Figure 3: Indirect ELISA for antibody to **Brucella abortus** using antigen coated dried plates. Graphs A, B and C represent data from **B. abortus** strain 19 vaccinated cattle, non exposed and **B. abortus** infected cattle, respectively. The legends are the same as for Figure 2. Please note that at month 6, the assay parameters were changed. These changed did not affect the assay results.

dried antigen. Again, means and ranges of values are plotted and the first and last tests include data obtained with antigen coated, frozen plates. It is of interest to note that the change in technique at month 6 did not have an effect on the results with negative sera and those from infected cattle. However, there was an upwards trend in the means and ranges obtained with sera from vaccinated animals. The reason for this is not clear.

DISCUSSION

From the data presented, it is evident that simply air drying polystyrene plates coated with **B. abortus** lipopolysaccharide is a suit able method for storing such plates for future use.

In the competitive ELISA, using the buffer control (BACc) as 0% Inhibition, Figure 1A clearly indicates that the strong positive (BAC++) and the negative (BAC-) control sera showed very minor fluctuations over the test period. The %I for the intermediate control serum (BAC+) varied over the 13 months, however, the values were always within the established upper and lower control limits for the assay. Similarly, Figure 1B shows that the controls in the indirect ELISA fall within the established limits. It should be noted that at month 6, the assay format was changed in terms of serum dilution employed and time of incubation of both the serum and conjugated antiglobulin stages. These changes did not affect the results with the strong positive serum (BAC++) as it is set at 100% positivity. However, some variation was noted with the weak positive serum (BAC+), requiring reassessment of the upper and lower control limits for the assay. To a lesser extent, the values for the negative serum (BAC-)increased but the control limits were not affected. In all cases, the means of the control sera were within the established control limits, validating the results for the assay.

Dried antigen coated plates offer a considerable advantage over freezing as a method of storage. Thus dried plates can be stored

under the stated conditions for more than a year without taking up valuable freezer space. (At this time it is not know if other storage conditions (temperature, humidity) has an effect on the antigen activity). In addition they can be shipped without the use of coolants. This method of preservation also avoids the first wash cycle normally necessary for plates containing liquid antigen, cutting both time and expense.

ACKNOWLEDGEMENTS

The technical expertise of S. Balsevicius is gratefully acknowledged.

REFERENCES

1. Baker, P.J. and Wilson, J.B. Hypoferremia in mice and its application to the bioassay for endotoxin. J. Bacteriol. 1965; 90:903-910.

2. NUNC 69620 polystyrene plates from CIBCO/BRL, Missasauga, Ontario, Canada.

3. Nielsen, K., Kelly, L., Gall, D. et al. Comparison of enzyme immunoassays for the diagnosis of bovine brucellosis. Prev. Vet. Med. 1996; 26:17-26.

4. Henning, D. and Nielsen, K. Peroxidase labelled monoclonal antibodies for use in enzyme immunoassay. J. Immunoassay 1987; 8:297 -302.

5. Goat anti-mouse IgG (H and L chain) conjugated with horseradish peroxidase from jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA.