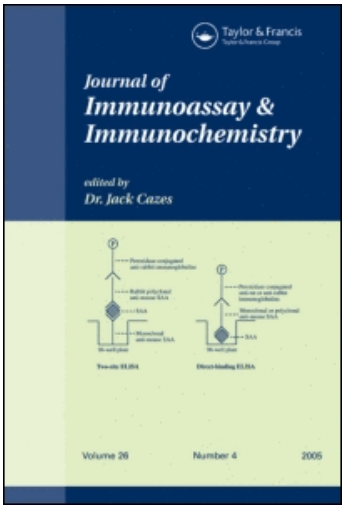

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L. Arnold Noorduyn[a]; Marc J. M. Meddens[b]; Jan Lindeman[b]; Willemien C. van Dijk[b]; Paul Herbrink[b]
[a] Department of Pathology, Free University, Amsterdam, The Netherlands [b] Department of Pathology, Medical Microbiology, and Clinical Immunology, Stichting Samenwerking Delftse Ziekenhuizen (SSDZ), Delft, The Netherlands

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FAVOURABLE EFFECT OF DETERGENT ON ANTIGEN DETECTION AND COMPARISON OF ENZYME LINKED DETECTION SYSTEMS IN AN ELISA FOR CHLAMYDIA TRACHOMATIS

L. Arnold Noorduyn[1], Marc J.M. Meddens[2], Jan Lindeman[2], Willemien C. van Dijk[2], and Paul Herbrink[2].

1:Present affiliation: Department of Pathology, Free University, Amsterdam, The Netherlands 2:Department of Pathology, Medical Microbiology, and Clinical Immunology, Stichting Samenwerking Delftse Ziekenhuizen (SSDZ), Delft, The Netherlands.

ABSTRACT

The influence of addition of detergents to the antigen on sensitivity of an ELISA for the detection of Chlamydia trachomatis was investigated. Of the detergents tested, only octyl-β-d-glucopyranoside and sodiumdesoxycholate gave respectively a two- to fourfold and an eightfold increase in sensitivity. The effect was only present within a narrow range of detergent concentrations. The optimal detergent concentration was strongly dependent on the protein concentration in the antigen preparation. For optimal detection of the bound chlamydial antigen, enzyme and biotin labeled secondary antibodies were compared. The biotin labeled antibodies were combined with enzyme labeled streptavidin-biotin complex (SBC). Color development was obtained with both types of conjugates by using either o-phenylenediamine (OPD) or an enzyme amplification system. The best results were obtained with the SBC method and OPD. (Keywords: detergent, antigen detection, Chlamydia trachomatis, ELISA, streptavidin/biotin, enzyme amplification).

INTRODUCTION

Since the introduction of the enzyme linked immunosorbent assay (ELISA) (1), many applications have been described. For the detection of microbial antigens, ELISA has been proven useful and commercial kits have been introduced. Advantages of using ELISA for the detection of microbial antigens are: 1. the absence of the need for culture; 2. no need for retaining infectivity; 3. rapid performance; 4. large numbers of specimens can be handled; and 5. it can be automated.

Chlamydia trachomatis is a micro-organism causing a broad spectrum of diseases (2,3,4,). Isolation in cell culture is difficult and time consuming. A number of alternative methods for the detection of chlamydial antigen have been described recently, including direct immunofluorescence (5,6,7,8) and ELISA (9,10,11). These rapid methods will have to attain a sensitivity and specificity which is at least comparable to culture. Because by means of these methods positivity has also been detected in culture negative specimens, the need for a combination of more than one test system has been stressed (12,13).

In this paper an ELISA for the detection of chlamydial antigen is described in which a two- to

eightfold increase in sensitivity was obtained by the addition of detergents to the antigen to be detected. In addition, to further increase sensitivity, several detection systems were compared.

MATERIAL AND METHODS

Chlamydia Antigen.

Chlamydial antigen was prepared as follows: DEAE treated Hela cells were infected with a suspension of chlamydiae, (LGV-2, strain 434B). After 3 days of incubation at 37^0C, the cells were harvested, sonicated twice for 30 seconds, and the cell debris was pelleted at low speed (10 minutes, 800 x g, 4^0C). The supernanant was centrifuged at 45,000 x g for 20 minutes at 4^0C and the pellet was suspended in a small volume of phosphate buffered saline (PBS, pH 7.3, 4.86 mM phosphate). The chlamydiae were inactivated by a 10 times freeze/thaw procedure and the protein content was determined with the SBG method (Serva, Heidelberg, FRG). As control antigen, non-infected HeLa-cells, treated as above, were used. The suspensions of chlamydial antigen and control antigen contained respectively 1.5 and 0.75 mg

protein/ml. The suspensions were aliquoted and stored at -70^{0}C.

Polyvalent Antiserum.

A polyvalent antiserum to C. trachomatis, to use as solid phase linked capture system, was raised in rabbits. The rabbits were injected intraperitoneally with a combination of 1 ml of chlamydial antigen suspension (0.4 mg/ml in PBS), prepared as described above and 1 ml of complete Freund's adjuvant. On day 14, 28 and 42, subcutaneous injections of 1 ml antigen suspension combined with 1 ml of incomplete Freund's adjuvant were given. On day 49, the rabbits were bled. The antiserum was absorbed with a HeLa/HEp2 cell mixture, bovine liver powder and guinea-pig liver powder, as described by Gardner and Mc Quillin (14). An IgG fraction was prepared by ammonium sulfate precipitation. This IgG fraction was aliquoted, lyophilized, and stored at -20^{0}C. With an immunoblot technique using chlamydial and control antigen, this IgG fraction showed a major 42 kD protein (major outer membrane protein; MOMP) reactivity.

Monoclonal Antibody

A monoclonal antibody, specific for C.trachomatis, was kindly provided by C.J.L.M. Meyer (Free University of Amsterdam, department of pathology). With an immunoblot technique using chlamydial and control antigen, this monoclonal antibody detected specifically the major 42 kD protein (MOMP).

Detergents

To study the effect of detergents on the detection of chlamydial antigen, the following detergents were added to the chlamydial antigen suspension: 1. Tween 20 (Merck, Darmstad, FRG) in concentrations between 0.015% and 1% (v/v); 2. Octylglucoside (Sigma Chemical Co., St. Louis, Missouri) in concentrations between 0.0075% and 2% (w/v); 3. Sodium dodecyl sulphate (SDS) (BDH Chemicals Ltd., Poole, U.K.) in concentrations between 0.001% and 0.12% (w/v); and 4. Sodium deoxycholate (DOC) (Merck) in concentrations between 0.0075% and 0.5% (w/v). In addition urea (Bethesda Research Laboratories, Gaithersburg, Mary land, U.S.) was tested in concentrations between 1 mol/l and 7 mol/l, and the reducing agent dithiotreitol (DTT)

(Merck) in concentrations between 0.3 mmol/l and 20 mmol/l. The detergents were dissolved in PBS, mixed with the antigen suspension, and incubated for at least 30 minutes before addition to the plates.

ELISA For Detection of Chlamydial Antigen

All incubations were performed at room temperature unless indicated otherwise. All washings were done six times, with PBS containing 0.05% Tween 20. The polyvalent anti-chlamydia IgG was diluted 1:200 in PBS and coated, 100 ul per well, in microtiterplates (Greiner, Nurtingen, FRG), overnight at 4^{0}C. After washing, the plates were incubated with two-fold serial dilutions of antigen, with or without detergent, for 1 hour with continuous shaking and washed again. The monoclonal antibody was added (ascitic fluid, diluted 1:1000 in PBS containing 0.5% gelatine) and after 30 minutes incubation, the plates were washed. For the detection of bound monoclonal antibody, four different methods were compared (Fig.1.).

1. Indirect Ortho-Phenylene-diamine (OPD) method, using peroxidase labeled anti-mouse IgG as conjugate and ortho-phenylene-diamine (OPD; Sigma) as substrate. The plates were incubated for 30 minutes

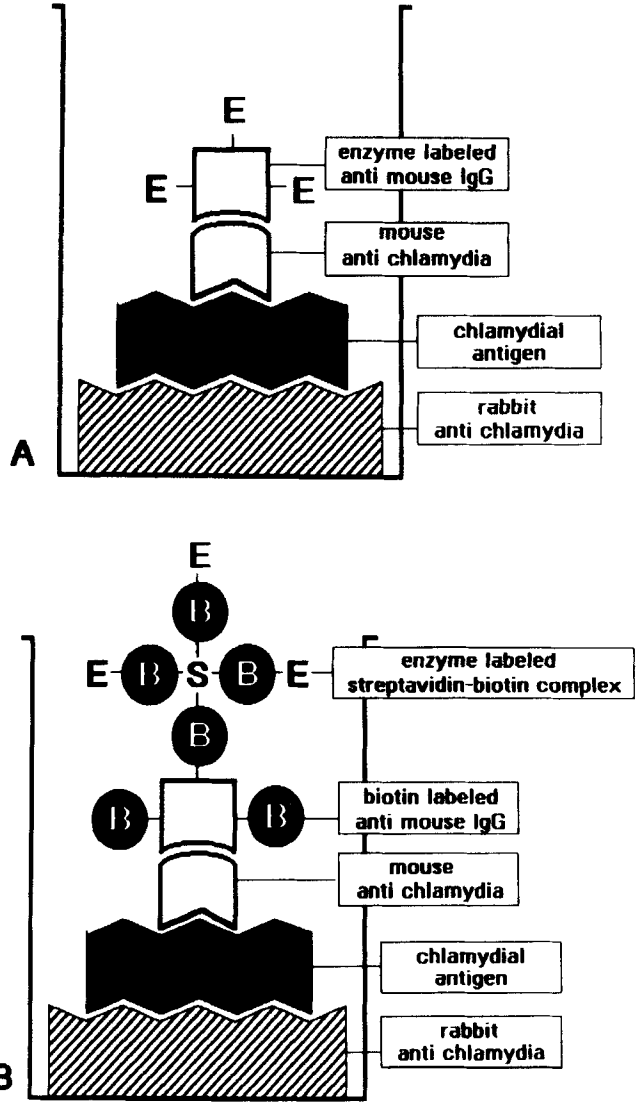

Figure 1. Characteristics of ELISA for the detection of C. trachomatis, using enzyme labeled anti-mouse conjugate (A), and of ELISA using biotin labeled antimouse antibody in combination with the enzyme conjugated SBC (B). For details see materials and methods.

with a peroxidase labeled rabbit anti-mouse IgG (Dakopatts, Glostrup, Denmark), diluted 1:200 in PBS containing 1% bovine serum albumin (BSA) and the plates were washed again. 0.1% (w/v) OPD in PBS pH 6.0, containing 0.03% H_2O_2 (0.1 ml per well) was added and after 20 minutes, the reaction was stopped with 0.1 ml 3N H_2SO_4.

2. Streptavidin-biotin-complex (SBC) OPD method, using a biotinylated anti-mouse IgG with the streptavidin-biotin complex (SBC) and OPD as substrate. The plates were preincubated with PBS containing 1% normal horse serum (NHS) for 15 minutes and emptied. The biotinylated horse anti-mouse antibody (Vector, Burlingame, California), diluted 1:1600 in PBS containing 1% BSA, 2% normal rabbit serum (NRS) and 1% NHS, was added, the plates were incubated for 1 hour and washed. A complex of streptavidin and biotinylated horseradish peroxidase (Amersham, Little Chalfont, UK) diluted 1:1000 in PBS containing 1% BSA, was added and after 30 minutes the plates were washed. OPD was added as described above.

3. Indirect enzyme amplification method, using alkaline phosphatase labeled anti-mouse IgG as conjugate, in combination with an enzyme

amplification system. The plates were incubated for 30 minutes with the alkaline phosphatase labeled rabbit anti-mouse IgG (Dakopatts), diluted 1:200 in PBS containing 1% BSA and the plates were washed again. For color development, a modification of the enzyme amplification system described by Stanley et al. (15) was used. A solution of nicotinamide adenine dinucleotide phosphate (NADP; Sigma, 0.1 ml per well, 0.1 mmol/l in 50 mmol/l diethanolamine buffer, pH 9.5), was added. After 30 minutes, 0.1 ml of amplifier solution containing 1 mg alcohol dehydrogenase (Sigma), 0.25 mg diaphorase (Biozyme, Baenaron, U.K.), 6% ethanol and 0.2 mmol/l p-iodonitrotetrazolium violet (Sigma) in 12 ml of 50 mmol/l phosphate buffer, pH 7.0, was added. Ten minutes later the reaction was stopped with 0.05 ml of 0.2 mol/l H_2SO_4.

4. Streptavidin-biotin-complex (SBC) enzyme amplification method, using a biotinylated anti-mouse IgG, in combination with a complex of streptavidin and biotinylated alkaline phosphatase and the enzyme amplification system. Incubations were done as with the peroxidase labeled SBC method except that the biotinylated anti-mouse antibody was diluted 1:3200 and the alkaline phosphatase labeled SBC (Amersham)

1:250. The enzyme amplification system was used as described above.

For all four detection methods, the absorbances were measured at 492 nm with a Titertek Multiscan (Flow Laboratories, Mc Lean, Virginia). The ELISA was considered positive if the absorbance was 0.1 optical density units above the absorbance of the control antigen ($\Delta A = A[Ag] - A[CoAg] > 0.1$ units).

RESULTS

To test the influence of the detergents and the reducing agent on antigen detection in ELISA, five antigen concentrations and one concentration of control antigen were tested with varying concentrations of all detergents. The test was performed with the SBC OPD method.

Fig.2 shows the results of the influence of the various detergents on the ΔA obtained after addition of 2 ng chlamydial antigen per well. No increase in ΔA was obtained with Tween 20 and SDS in any of the concentrations tested. The same result was obtained with urea and DTT (data not shown). However, addition of octylglucoside or DOC to the chlamydial antigen solution resulted in a strong increase in ΔA

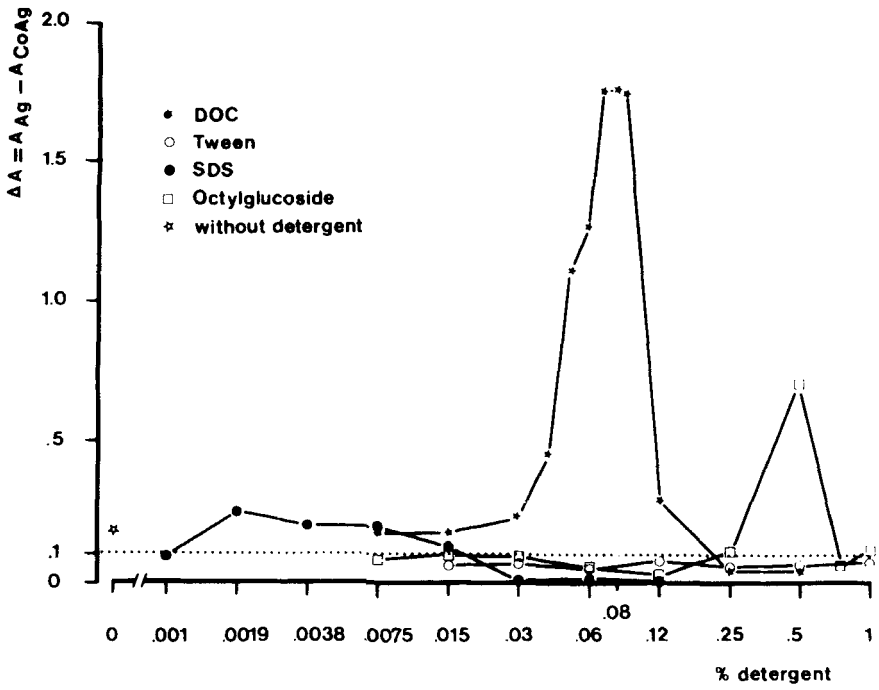

Figure 2. Effect of detergent concentration on the absorbance value in ELISA for C. trachomatis antigen. Two ng of antigen was added per well. Horizontal axis: detergent concentration. Vertical axis: ΔA = Difference between absorbance of chlamydial antigen (A[Ag]) and absorbance of control antigen (A[CoAg]). ELISA was considered positive if ΔA exceeded 0.1 optical density units. DTT and urea showed no favourable effect in any of the concentrations tested.

values. Both for octylglucoside and DOC, the effect of the detergent was restricted to a small range of concentrations. Octylglucoside showed the favorable effect in the range between 0.25% and 0.75%, DOC in the range between 0.03% and 0.12%. Optimal results were obtained at octylglucoside and DOC concentrations of 0.5% and 0.08%, respectively. The optimal concentrations were not dependent on the chlamydial antigen concentration.

The influence of DOC and octylglucoside on the sensitivity of chlamydial antigen detection is shown in Fig.3. Octylglucoside in the optimal concentration of 0.5% showed a two- to fourfold increase in sensitivity of the assay. The best results, however, were obtained with 0.08% DOC, which yielded an eightfold increase in sensitivity.

Because clinical specimens are collected routinely in transport medium, which contains protein in the form of 10% fetal calf serum, we investigated the influence of various protein concentrations and Chlamydia Transport Medium on the sensitivity improving effect of DOC. It appeared that a higher DOC concentration was needed to obtain optimal results, when higher protein concentrations were used (Fig.4).

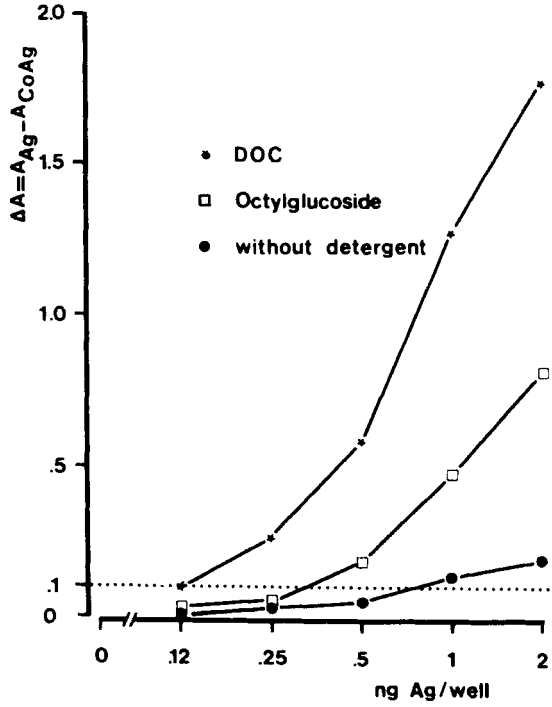

Figure 3. Effect of detergents on the sensitivity of ELISA for C. trachomatis. Optimal concentrations of octylglucoside (0.5%) and DOC (0.08%) were used. Horizontal axis: ng chlamydial antigen per well. Vertical axis: ΔA (see figure 2). ELISA was considered positive if ΔA exceeded 0.1 optical density units.

To further improve the sensitivity of chlamydial antigen detection by ELISA, four different detection sytems were compared. The results are shown in Fig.5. The indirect OPD method and the indirect

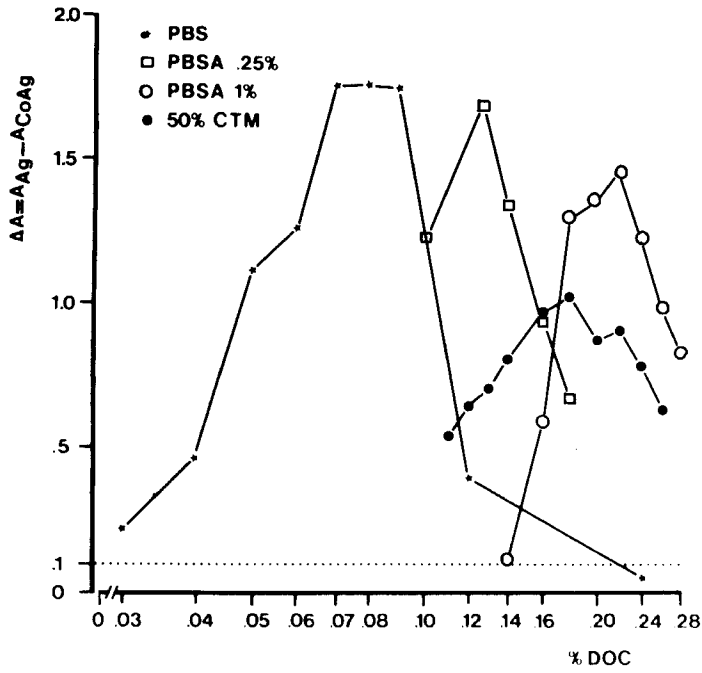

Figure 4. Effect of protein concentration and chlamydia transport medium on optimal DOC concentration. PBSA: PBS containing bovine serum albumin (percentage (w/v) indicated). CTM: Chlamydia Transport Medium. Horizontal axis: DOC concentration. Vertical axis: ΔA (see Fig. 2).

enzyme amplification method showed comparable sensitivity. However, higher ΔA values were obtained using the SBC OPD or the SBC enzyme amplification method, with the former slightly in favor. The SBC methods were approximately twice as sensitive as the indirect methods.

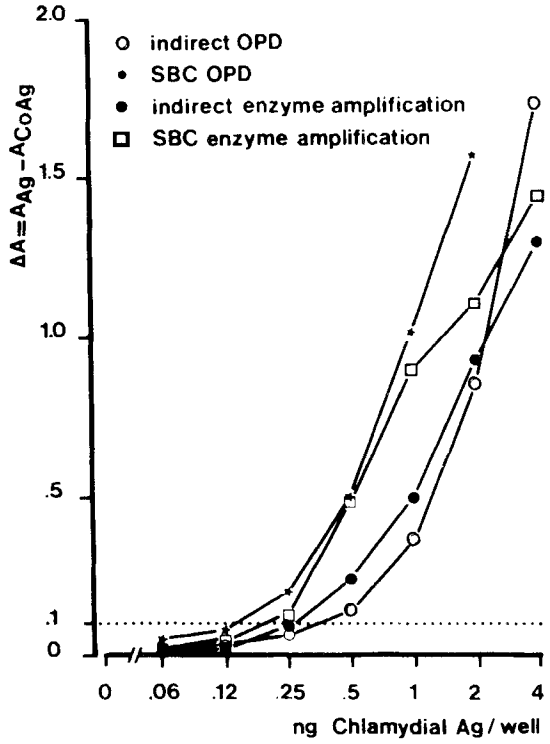

Figure 5. Effect of indirect OPD method, SBC OPD method, indirect enzyme amplification method and SBC enzyme amplification method on sensitivity of ELISA for C. trachomatis (see materials and methods for detailed explication). Antigen was dissolved in PBS containing 0.08% DOC. Horizontal axis: ng chlamydial antigen per well. Vertical axis: ΔA (see Fig. 2).

DISCUSSION

Most attempts to improve the sensitivity of ELISA's have been focused on antibodies, conjugates and substrates (16). Treatment of antigen with n-acetylcysteine or SDS to raise sensitivity has been suggested (16,17). Mahony et al (18) reported a favourable effect of addition of SDS to chlamydial antigen on the coating of microtiter plates, leading to a decrease in the amount of antigen necessary to measure antibodies against C. trachomatis. Bavoil et al (19) described the use of several detergents for solubilization of C. trachomatis outer membranes in the presence of a reducing agent. However, no application for detection of C. trachomatis antigen by ELISA was described. Our results show that a carefully chosen concentration of DOC or octylglucoside improves the sensitivity of ELISA for C. trachomatis two- to eightfold. Bavoil et al reported that the major outer membrane protein (MOMP) could be readily and fully solubilized by mild nonionic or dipolar ionic detergents in the presence of DTT. MOMP was also readily solubilized in the presence of SDS. However, DOC either in the presence or absence of DTT yielded poor solubilization of MOMP. Our results show some

discrepancies with those reported by Bavoil et al. We only observed a favourable effect of octylglucoside and DOC on C. trachomatis antigen detection by ELISA, possibly due to a better solubilization of MOMP such as shown by Bavoil et al. The optimal concentration of DOC to be used was strongly dependent on the protein concentration of the antigen preparation. This and the fact that we used an immunological detection system whereas Bavoil et al used SDS polyacrylamide gel electrophoresis, might explain the discrepancies between our results and those observed by Bavoil et al.

Of the detection systems tested, the SBC method, in combination with peroxidase and OPD or with alkaline phosphatase and enzyme amplification, showed the highest sensitivity. For reasons of ease of performance and cost effectiveness, we prefer the SBC method in combination with peroxidase and OPD.

We were unable to confirm the remarkable improvement of sensitivity by enzyme amplification as reported by Stanley et al. (15). This may be due, at least partially, to the fact that they compared the enzyme amplification system with an indirect alkaline phosphatase method in combination with p-nitrophenyl phosphate, whereas we compared the

enzyme amplification system against peroxidase and OPD, which is considered to be a more sensitive enzyme-substrate system (9,16) than the alkaline phosphatase system (20).

In conclusion, the effect of detergents on the detection of chlamydial antigen as observed in this study gives an additional approach to the optimalisation of the sensitivity of ELISA. Addition of detergents could also be useful to raise the sensitivity of ELISA's for the detection of other microorganisms.

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