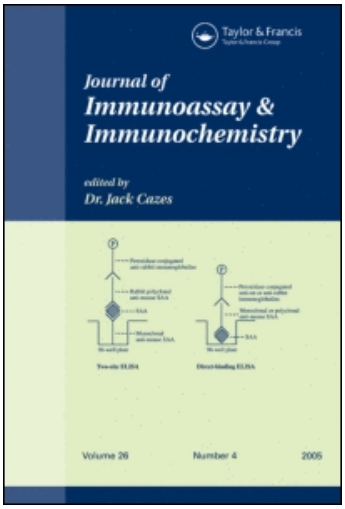


**Journal of Immunoassay and Immunochemistry**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

# Non-Instrumental Immunoassay Based on Coloured Polyacrolein Latex: Application to Group-Specific Polysaccharide of *Streptococcus Pyogenes*

Irene S. Pavlova[a]; Yuri V. Lukin[a]; Dmitri N. Avdeev[a]; Vladimir A. Kulshin[a]
[a] Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia

**To cite this Article** Pavlova, Irene S. , Lukin, Yuri V. , Avdeev, Dmitri N. and Kulshin, Vladimir A.(1995) 'Non-Instrumental Immunoassay Based on Coloured Polyacrolein Latex: Application to Group-Specific Polysaccharide of *Streptococcus Pyogenes*', Journal of Immunoassay and Immunochemistry, 16: 2, 199 — 212
**To link to this Article: DOI:** 10.1080/15321819508013558
**URL:** http://dx.doi.org/10.1080/15321819508013558

JOURNAL OF IMMUNOASSAY, 16(2), 199-212 (1995)

# NON-INSTRUMENTAL IMMUNOASSAY BASED ON COLOURED POLYACROLEIN LATEX: APPLICATION TO GROUP-SPECIFIC POLYSACCHARIDE OF *Streptococcus pyogenes*

Irene S.Pavlova, Yuri V.Lukin, Dmitri N.Avdeev and
Vladimir A. Kulshin
Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry,
Miklukho-Maklaya st. 16/10, Moscow, 117871, Russia



## ABSTRACT

Non-instrumental immunoassay methods based on immunofiltration and microtiter particle agglutination (MPA) techniques have been developed using coloured polyacrolein latex. These methods have been applied to the quantification of the group-specific polysaccharide, A-PS, of *S.pyogenes* (group A Streptococcus) and compared to the standard ELISA tests. The assay with the ability to detect the lowest concentration of antigen was MPA; as little as 0.05 ng A-PS/ml or $10^4$ cells/ml could be detected in 1.5 h. In comparison to ELISA test the sensitivity of MPA was 10 times higher and the procedure of the assay was much more simple. The sensitivity of the immunofiltration assay using both enzyme and latex markers was shown to be the same (50 ng A-PS/ml) and the duration of the assay 3-5 min. No cross-reactions of latex conjugates with non A Streptococcus cell lysates have been observed. The developed methods are easy to perform and require neither sophisticated equipment nor specially trained personal.

(KEY WORDS: microtiter particle agglutination, dot-assay, polyacrolein latex, Streptococcus A)

## INTRODUCTION

Nowadays parallel with further elaboration of traditional enzyme immunoassay rapid progress in the development of alternative non-

instrumental immunoassay techniques has been observed. While the ELISA has been extensively used for research and commercial purposes it is rather sophisticated and time-consuming method. This makes it unsuitable for application in the field or poorly equiped laboratories.

Contrary to ELISA non-instrumental tests such as agglutination and dot-assays can be simply adopted to non-laboratory settings. Enzyme labels and more recently corpuscular markers such as colloidal gold (1) or colloidal dyes (2,3) were reported for using in dot-assay. In this work we used coloured polyacrolein latex (PAL) particles of various size as a carrier in latex agglutination and a detectable label in dot-assay.



The gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus) is an important human pathogen and the causative agent of numerous suppurative infections of the throat and skin, including pharingitis, impetigo, cellulitis and necrotizing fasciitis. Of particular concern, is that the incidence of severe streptococcal diseases, including a toxic shock-like syndrome, several types of invasive infections and rheumatic fever, a nonsuppurative sequella of infection, appears to be increasing in recent years (4). The importance of *S.pyogenes* as an agent of human infection has stimulated considerable investigation into the development of rapid and sensitive methods for the diagnosis of streptococcal diseases.

We report here a new highly sensitive microtiter particle agglutination (MPA) and a rapid immunofiltration dot-assay on the basis of PAL, their application for the quantification of group-specific polysaccharide (A-PS) of *S.pyogenes*. A comparison with the sandwich ELISA has been made with respect to the sensitivity (the lowest concentration of antigen which can be detected by the test), rapidity and simplicity of the assays.

## MATERIALS AND METHODS

### Buffers

PBST was phosphate buffered saline (PBS) containing 0.05% Tween 20, PBST/BSA was PBST containing 2.0 mg/ml BSA, PBST/ovalbumin was PBST containing 1.0 mg/ml ovalbumin.

### Preparation of bacteria and antigens

All bacterial strains: *S.pyogenes* (Streptococcus A-group, M*, strain D23, No 62/59), *S.agalacticae* (Streptococcus B-group, V9 No 8/66), *S.zooepidemicus* (Streptococcus C-group, Chestle No 41/59) and *S. lenpus* (Streptococcus G-group, Valente No 22/58)) were obtained from Prague collection (Czech Republic). The groups of streptococci cells are given according to Lancefield. Streptococcus cells destroyed by *Streptomyces levoris* enzyme complex (5) were obtained from Dr.N.I. Briko (1-st Moscow Medical Institute). Cell walls were separated by centrifugation at 10000 rpm for 30 min and washed with PBS. 6 ml formamide were added per 1 g of wet cell walls, the suspension was heated at 170°C for 1 h and centrifuged. The supernatant was diluted with 10 volumes of acidic ethanol (20 g of sodium acetate per 1 l of ethanol), centrifuged and concentrated. The polysaccharides (PS) were precipitated with 5 volume of acetone, dissolved in 1% acetic acid and purified by gel-filtration on Sephadex G-50 column. The purified PS were dialyzed against water and lyophilized. The structure of the polysaccharides was confirmed by $^{13}C$-n.m.r. spectroscopy. Polysaccharide A (A-PS) was oxidated by sodium periodate and conjugated to hydrazide derivative of BSA according to reductive amination method of Gray et al. (6). The content of PS in the obtained conjugate was estimated to be 20% (7). A-PS-BSA conjugate was immobilized on Sepharose 4B by BrCN activation (8) for affinity purification of antibodies.

Preparation of antibodies

Antisera from donkeys immunized with destroyed cells of *S.pyogenes* were purchased from Moscow Institute of Epidemiology and Microbiology (Russia). The IgG fraction of the antisera was isolated by precipitation with 40% ammonium sulfate in PBS, followed by dialysis against PBS. Anti-A-PS antibodies were further affinity purified on A-PS-Sepharose 4B column followed by gel chromatography on Sephacryl S-300 column. The obtained affinity purified antibodies were used in all the experiments described below.

ELISA

HRP was conjugated to affinity purified anti-A-PS antibodies according to periodate method of Tijssen et al. (9). The wells of microtiter plate Dynatech MicroELISA (Dynatech, FRG) were coated with anti-A-PS antibodies (2.0 μg/ml) in 0.1M $NaHCO_3$ overnight at 4°C. The plates were washed five times with PBST and blocked with PBST/BSA for 30 min at 37°C. PBST/BSA solution was used for antigens (purified polysaccharides and lysates of Streptococcus cells) and antibody-HRP conjugate dilutions. Series of 2-fold dilutions of PS or 10-fold dilutions of cell lysates were prepared in the wells and incubated at 37°C for 1 h. After five washings with PBST anti-A-PS antibody-HRP conjugate was added to the wells and incubated at 37°C for 1 h . After five washings with PBST the bound peroxidase was revealed by 5-15 min incubation with 0.4 mg/ml o-phenylenediamine and 0.02% hydrogen peroxide in 0.05M citrate-phospate buffer, pH 5.0. The reaction was stopped by 1.7N sulfuric acid.

Latex conjugate preparation

Coloured polyacrolein latices (PAL) having particle diameter of 0.35 and 1.8 μm were prepared by anionic polymerization of acrolein

(10). Briefly, acrolein (4% w/v) (Fluka, FRG) was polymerized in water at pH 10.5 in the presence of 0.01-0.02% watersoluble dyes Rhodamine G or Crystal Violet (Serva, FRG) and 0-4% SDS (Serva, FRG). After 3 h stirring at 22°C the mixture was heated up to 60°C and stirred at the constant temperature for 3 h in the presence of 4% (w/v) ammonium persulfate. The obtained PAL microspheres were washed several times with water by centrifugation or microfiltration to remove unreacted compounds and finally resuspended in water at 5% concentration. The diameter of PAL microspheres was determined by submicron particle analyzer Coulter N4-MD (Coultronics, France).

PAL were coated with affinity purified anti-A-PS antibodies by two methods: covalent coupling or indirect adsorption through a protein spacer. For covalent coupling, 5 mg PAL were incubated with 5-200 μg antibody in 1 ml PBS at 22°C for 2 h under gentle stirring. After three washings with PBS/ovalbumin latex conjugates were redispersed in PBS at 0.2% (w/v). For indirect adsorption, PAL (5% w/v) was incubated with 10 mg/ml ovalbumin (Serva, FRG) in PBS at 37°C for 2 h. Modified PAL was washed 3 times with water, activated with 0.5 mg/ml tannin (Serva, FRG) in water at 37°C for 30 min, washed twice with water and incubated with antibody (1-40 μg/mg PAL) in PBS pH 6.5 at 37°C for 2 h. After three washings with PBS/ovalbumin latex conjugates were redispersed in PBS at 0.2% (w/v) and stored at 4°C.



## Microtiter particle agglutination assay

MPA assay was carried out in 96-well U-bottom microtiter plates (Nunc, Denmark) using PBS/ovalbumin for the dilution and titration of the samples. Serial two- or ten-fold dilutions of the samples were prepared in the wells of one row (25 μl per well). Then 25 μl of 0.2% PAL-antibody conjugate were added to each well. The plate

was shaken by hand and left at room temperature. After 1.5 h the agglutination picture was read by the naked eye. A positive result was observed as a diffuse coloured film of agglutinated particles evenly covering the bottom of the well. A negative result was observed as a distinct spot in the bottom of the well. A negative control was performed with pure buffer in the absence of the antigens.

Immunofiltration assay

A capture reagents were prepared by spotting 2 µl of A-PS-BSA (0.05-0.5 ng/dot) or affinity purified anti-A-PS antibody (50 µg/ml) on nitrocellulose filters AE95 (Schleicher & Schuell; pore diameter 1.2 µm). The filters were air dried and blocked with PBST/ovalbumin at room temperature for 30 min. PBST/ovalbumin solution was used for the antigens and HRP- or PAL-antibody conjugates dilutions. The blocked filters with A-PS dots were placed to the immunofiltration device (V.Tech, USA) and PAL-antibody conjugate (0.1%) was dropped on the filter and allowed to pass through the membrane. For the sandwich assay the filters with the immobilized capture antibody were placed to the immunofiltration device. Then the antigen dilutions followed by the washing buffer (PBST) and the enzyme or latex markers were dropped on the filters and allowed to pass through the membrane. After three washings with PBST bound HRP was revealed by 0.5 min incubation with 2-,4-chloro-naphtole, 0.02% hydrogen peroxide, 0.2 mg/ml diaminophenylenediamine and 0.1 mg/ml Na-bisulfite in 0.05M imidazole pH 7.5, prepared according to the method of Kobayashi et al. (11). The complete assay time was about 3 or 5 min using latex and enzyme markers, respectively. The results of the assay were read by the naked eye. A positive reaction was observed as a pink (for PAL marker) or blue (for HRP marker) spots on the filter

surface. The absence of the coloured spots on the filter was considered as a negative reaction.

## RESULTS

### Preparation of latex reagents.

The coloured PAL with particle diameters of 1.8 and 0.35 μm were prepared for use as a solid phase in MPA and immunofiltration tests respectively. Donkey anti-A-PS antibodies were immobilized on PAL by direct covalent coupling or indirect adsorption through a protein spacer. The obtained latex reagents were evaluated in specificity tests with cell lysates of *S.pyogenes* (group A), *S.zooepidemicus* (group C) and *S.lenpus* (group G). The indirectly sensitized PAL reacted with $10^6$/ml *S.pyogenes* but did not react with $10^6$/ml *S.zooepidemicus* or *S.lenpus*. The covalently coupled latex, however, not only yielded agglutination with $10^6$ *S.pyogenes*, but also with $10^6$ *S.zooepidemicus* per ml and $10^6$ *S.lenpus* per ml. Because of observed lack of specificity with the covalently coupled latex only the indirectly sensitized PAL reagent was further studied.

Experiments were performed with latex reagents to optimize conditions affecting sensitivity and specificity including optimal concentration of antibody used to sensitize the latex microspheres and microsphere size.

Preliminary investigations showed that PAL with particle diameters between 0.3-0.4 and 1.6-1.8 μm were optimal for immunofiltration and MPA assays respectively.

To determine optimal sensitization dose of antibody PAL were sensitized with donkey antibodies to A-PS at concentrations of 40, 20, 10, 5, 2,5, 1.25 and 0.6 μg protein/mg of latex. Latex reagents were then reacted in chessboard titration with doubling concentra-

tions of A-PS by immunofiltration (for small PAL) or MPA (for large PAL) assays. In case of small PAL with increasing antibody concentration up to 20 μg/mg a "plateau" (0.1 ng A-PS/dot) concentration was reached. Further increasing of antibodies concentration did not result in increasing of the sensitivity of the test. In case of large PAL with increasing antibody concentration from 0.6 to 10 μg/mg the sensitivity of MPA was increased from 1.0 to 0.05 ng/ml. However, higher antibody concentration led to partial flocculation of particles. Thus, optimal protein concentration was 20 μg/mg for small PAL and 10 μg/mg for large ones.



Detection of A-PS by immunofiltration dot-assay.

Nitrocellulose membrane with pore size 1.2 μm and red coloured PAL with particle diameter of 0.35 μm were used for dot-assay development.

Purified PS and streptococci cell lysates of various groups were tested by sandwich dot-assay using enzyme marker and latex conjugate with antibody loading 20 μg/mg PAL (table 1). The sensitivity of immunofiltration assay with both latex and enzyme markers was about the same. The minimal detecting concentration of A-PS (50 ng/ml) approximately corresponded to 0.1 ng A-PS per dot showing a good correlation with the data obtained by immunofiltration assay of captured A-PS. No positive reactions were obtained with heterologous Streptococci cell lysates that proved specificity of latex immunofiltration assay.

Detection of A-PS by MPA assay.

Blue coloured polyacrolein particles with the diameter 1.8 μm coated with donkey affinity purified antibodies to A-PS (10 μg IgG/mg PAL) were used for MPA. The sensitivity and specificity of

**TABLE 1.**
**Testing of Purified Polysaccharides and Lysates of Streptococcus Cells by Immunofiltration Using Latex (Ab-PAL) and Enzyme (Ab-HRP) Conjugates**

| Streptococcus cells group | Minimal detecting concentration | | | |
|---|---|---|---|---|
| | purified PS, ng/ml | | cell lysates, CFU/ml | |
| | Ab-PAL | Ab-HRP | Ab-PAL | Ab-HRP |
| A | 50 | 50 | $10^6$ | $10^6$ |
| B | -* | - | -** | - |
| C | - | - | - | - |
| G | - | - | - | - |

* - negative reaction at the concentration of polysaccharide < 5 000 ng/ml
** - negative reaction at the concentration of cells < $10^7$ CFU/ml



MPA were determined by testing purified PS and Streptococci cell lysates of various groups. The same antigens were tested by sandwich ELISA (table 2).

MPA allowed to detect as little as 0.05 ng/ml A-PS or $10^4$ cells/ml that was 10 times lower than could be detected by ELISA. When testing purified PS of non-A Streptococci cells no cross-reactions were observed. Cross-reactions were found with heterologous Streptococci cell lysates at concentration at least 100 times higher than those observed with homologous cells. This phenomenon could be explained by the appearance of peptidoglycan fragments in the lysate samples in the result of hydrolysis of PS on the cell surface by enzyme complex from *S.levoris*. These fragments contain

**TABLE 2.**
**Testing of Purified Polysaccharides and Lysates of Streptococcus Cells by MPA and ELISA**

| Streptococcus cells group | Minimal detecting concentration | | | |
|---|---|---|---|---|
| | purified PS, ng/ml | | cell lysates, CFU/ml | |
| | MPA | ELISA | MPA | ELISA |
| A | 0.05 | 0.50 | $10^4$ | $5.10^5$ |
| B | -* | - | $5.10^6$ | $5.10^7$ |
| C | - | - | $5.10^6$ | $5.10^7$ |
| G | - | - | $5.10^6$ | $5.10^7$ |

* - negative reaction at the concentration of polysaccharide < 100 ng/ml

N-acetylglucosamine which is immunodominant area of group-specific A-PS (5).

## DISCUSSION

In this paper some possibilities of utilizing coloured polymer latex carriers in solid phase non-instrumental methods are shown. Coloured polymer latices are of particular interest for the development of particle agglutination and dot-assay because one can easily vary the size of latex particles, insert necessary functional groups and fill them with various dyes. The surface of latex particles can be chemically modified to promote attachment of various compounds of biomedical interest.

In this paper we report development of two latex-based tests: MPA and dot-assay using coloured polyacrolein latex particles of different size. Microtiter particle agglutination is one of the most sensitive methods of non-instrumental diagnostics (10,12). In this method large polymer microspheres are used as a carrier because the duration of the analysis is limited by the sedimentation rate of the particles in the well of the microplate. In the MPA test we use polyacrolein latex with bright colour and high density of polymer (1.25 g/cm$^3$) that provides rapid sedimentation of the particles with the diameter of 1.5-2.0 μm and allows shortening of the MPA assay time down to 1.5 h. For comparison MPA with polystyrene latex requires more than 18 h (12). Besides, PAL contains reactive aldehyde groups suitable for both direct coupling of proteins or surface modification of the particles to attach an appropriate spacer arm.



In the experiments reported here antibodies covalently coupled or indirectly bound to latex particles through protein spacer, reacted equally well with A-PS antigen. The indirectly sensitized reagent appeared to be more specific in reactions with heterologous cell lysates and was therefore selected for further development of agglutination and immunofiltration tests.

The comparative characteristics of the developed immunoassay tests based on latex or enzyme markers are shown in table 3. The lowest concentration of A-PS could be detected by MPA (0.05 ng/ml). MPA is performed just in one step by mixing the sample and the latex conjugate in the wells of a plate. The results of the assay can be read in 1.5 h. MPA is the only method which is sensitive enough for primary diagnosis of streptococcus infection demanding detection of about $10^4$ CFU/ml (5).

All the other methods listed in the table 3 are less sensitive than MPA. Minimal detection concentration of A-PS reached by

**TABLE 3.**
**Comparative Characteristics of Latex and Enzyme Immunoassays**

| Method | Conjugate | Minimal detecting concentration A-PS, ng/ml | Assay duration, min |
|---|---|---|---|
| Immunofiltration dot-assay | Ab-PAL | 50.0 | 3 |
| | Ab-HRP | 50.0 | 5 |
| ELISA | Ab-HRP | 0.5 | 150 |
| MPA | Ab-PAL | 0.05 | 90 |



immunofiltration test is 50 ng/ml. Three orders of magnitude difference in the sensitivity of MPA and immunofiltration methods is compensated, however, by much shorter time of immunofitration assay (3 min). The sensitivity and the rapidity of the assay are crucial parameters when a concrete diagnostic method is selected depending on the character and conditions of the investigation.

A comparison of the sensitivity of immunofiltration assay for the detection of A-PS with enzyme and latex markers reveals approximately equal detection limits. This fact can mean that due to the short time of filtration procedure the limiting stage of the assay is antigen-antibody interaction. The enzyme based assay requires the addition of substrate solution to elicit the detectable enzyme reaction. In comparison with the enzyme marker no substrate is needed for the latex conjugate. Thus by utilizing latex marker in dot-assay instead of enzyme one of the stages is

omitted and thereby the assay procedure is significantly simplified.

Detection of A-PS by ELISA takes about 2.5 h and a microplate reader is required to evaluate the results. When reducing the incubation time of antigen and antibody-HRP down to 30 min the sensitivity of ELISA decreases from 0.5 to 5.0 ng/ml.

Several types of colloidal carriers have been used in various non-instrumental immunoassay methods (1-3). In this paper we described carriers and/or markers on the basis of coloured latex which can be successfully used in both homogeneous (MPA) and heterogeneous (dot) assay techniques. The polymer particles are chemically well-characterised and can be obtained in a wide range of size with good reproducibility that makes them an useful tool for application in various non-instrumental immunoassays.



## REFERENCES

1. Hsu,Y. Immunogold for detection of antigen on nitrocellulose paper. Anal.Biochem. 1984;142: 221-5.

2. Snowden,K. and Hommel,M. Antigen detection immunoassay using dipsticks and colloidal dyes. J. Immunol. Methods 1991;140: 57-65.

3. Beverloo,H.B., van Schadewijk,A., Zijlmans,H. and Tanke,H. Immunochemical detection of proteins and nucleic acids on filters using small luminescent inorganic crystals as markers. Anal. Biochem. 1992;203: 326-34.

4. Stevens, D.L. Invasive group A Streptococcus infections. Clin.Infect.Dis. 1992;14: 2-13.

5. Chernyakova,A.M., Shikhman,A.R., Shmakova,Z.F. and Briko,N. Enzyme immunoassay of Streptococcus pyogenes group-specific polysaccharide. Laboratornoe delo (in Russian). 1988;4: 18-22.

6. Gray, G.R. Direct coupling of oligosaccharides to proteins and derivatized gels. Arch.Biochem.Biophys. 1974;163: 426-8.

7. Axen,R., Porath,J. and Ernback,S. Coupling of peptides and proteins to polysaccharides by cyanogen halides. Nature. 1967;214: 1302-4.

8. Dubois,M., Giles,K.A., Hamilton,J.K. et al. Determination of sugars and related substances. Anal. Chem. 1956;28: 350-6.

9. Tijssen,P. and Kurstak,E. Highly efficient and simple method for the preparation of peroxidase and active peroxidase-antibody conjugates for enzyme immunoassays. Anal. Biochem. 1984;136: 451-7.

10. Lukin,Yu.V., Erokhin,E.P., Avdeev,D.N. and Zubov,V.P. Functional polymer carriers for immunodispersive diagnostics development. Reactive Polymers. 1991;15: 48.

11. Kobayashi,R. and Tashima,Y. Visualization of antigen on nitrocellulose membrane by the oxidative coupling reaction of N,N'-Dimethyl-p-phenylenediamine and 4-chloro-1-naphthol. Anal. Biochem. 1989;183: 9-12.

12. Quash,G., Roch,C., Niveleau, A. et al. The preparation of latex particles with covalently bound polyamine, IgG and measles agglutinins and the use in visual agglutination tests. J.Immunol.Meth. 1978;22: 165-74.