

Video Article

Capsular Serotyping of *Streptococcus pneumoniae* by Latex Agglutination

Barbara D. Porter¹, Belinda D. Ortika¹, Catherine Satzke^{1,2}

¹Pneumococcal Research, Murdoch Childrens Research Institute

²Department of Microbiology and Immunology, Peter Doherty Institute for Infection and Immunity, The University of Melbourne

Correspondence to: Catherine Satzke at catherine.satzke@mcri.edu.au

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Abstract

Latex agglutination reagents are widely used in microbial diagnosis, identification and serotyping. *Streptococcus pneumoniae* (the pneumococcus) is a major cause of morbidity and mortality world-wide. Current vaccines target the pneumococcal capsule, and there are over 90 capsular serotypes. Serotyping pneumococcal isolates is therefore important for assessing the impact of vaccination programs and for epidemiological purposes. The World Health Organization has recommended latex agglutination as an alternative method to the 'gold standard' Quellung test for serotyping pneumococci. Latex agglutination is a relatively simple, quick and inexpensive method; and is therefore suitable for resource-poor settings as well as laboratories with high-volume workloads. Latex agglutination reagents can be prepared in-house utilizing commercially-sourced antibodies that are passively attached to latex particles. This manuscript describes a method of production and quality control of latex agglutination reagents, and details a sequential testing approach which is time- and cost-effective.

This method of production and quality control may also be suitable for other testing purposes.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51747/>

Introduction

Streptococcus pneumoniae (the pneumococcus) is a major cause of morbidity and mortality in children under the age of five years old world-wide, particularly in resource-poor settings^{1,2}. Pneumococcal disease ranges from localized infections to life threatening conditions such as pneumonia, sepsis and meningitis^{1,2}.

More than 90 serotypes of pneumococci have been identified based on differences in their capsular polysaccharide³. Current vaccines target the capsular polysaccharide and provide protection from the major serotypes causing invasive disease⁴. Serotyping pneumococcal isolates is important for assessing the impact of vaccination on carriage and disease as well as providing broader epidemiological information^{5,6}. The current 'gold standard' method for pneumococcal serotyping is the Quellung test, but it is time consuming and requires some skill to perform⁷. Latex agglutination is an alternative serotyping method recommended by the World Health Organization⁷. Latex agglutination is quick and simple to perform, and is employed in many laboratories worldwide⁸⁻¹¹. Importantly, latex agglutination has shown comparable accuracy to the Quellung test for pneumococcal serotyping^{10,12-14}. Overall, this method is ideal for resource-poor settings as well as high throughput laboratories.

Latex agglutination reagents ('latex reagents') are created by the attachment of antibodies to latex particles¹⁵. In a positive reaction, these labeled particles agglutinate in the presence of specific antigen. Commercial latex reagents are available for a restricted range of serotypes. Latex reagents can also be prepared in-house using commercially available antisera¹⁰. Mixtures of antisera ('pools') as well as specific antisera to pneumococcal serogroups (e.g., group 19), individual serotypes (e.g., serotype 5), and to specific antigens ('factors', e.g., factor 19c recognizing serotype 19A) for further defining serotypes within groups are available¹⁶.

This manuscript outlines the major steps in the production of latex reagents using passive attachment of commercially available antisera to latex particles. The quality control (QC) aspects of this method are described, and a protocol for the use of latex reagents for pneumococcal serotyping is included.

Protocol

1. Preparation of in-house Latex Reagents

1. Preparation of glycine buffered saline (GBS)
 1. Add 1.5 g glycine, 11.7 g sodium chloride and 100 ml of distilled water to a 200 ml glass beaker.
 2. Mix to dissolve using a magnetic stirrer.

3. Transfer mixture to a 500 ml measuring cylinder and add distilled water to make a total of 200 ml.
 4. Transfer the solution back to the 200 ml beaker, check the pH and adjust to 8.2 with 5 M sodium hydroxide. NOTE: Sodium hydroxide is corrosive, wear personal protective equipment.
 5. Filter sterilize the solution using 0.22 μ M filters and several 60 ml syringes into a 250 ml preautoclaved screw capped glass bottle.
 6. Store at RT.
2. Preparation of GBS with 0.2% bovine serum albumin
 1. Add 0.2 g of bovine serum albumin (BSA) powder and 100 ml of GBS to a 200 ml glass beaker and mix to dissolve using a magnetic stirrer.
 2. Filter sterilize the solution through 0.22 μ M filters using several 60 ml syringes into a 250 ml preautoclaved screw capped glass bottle.
 3. Store at 4 °C.
3. Latex reagent preparation
 1. Label a 2 ml round bottom microfuge tube.
NOTE: round bottom tubes are used to minimize particle settling which may affect antibody attachment.
 2. Using a P1000 pipette with sterile tips add 975 μ l of GBS into the microfuge tube, then add 25 μ l of appropriate pneumococcal antiserum for the latex reagent being prepared (*i.e.*, pool, group, type or factor). Invert five times to mix.
 3. Dilute polystyrene latex particles 1:10 by adding 120 μ l of latex particles to 1,080 μ l of sterile physiological saline.
NOTE: This can be made in larger volumes, but should be made fresh each time the latex reagents are produced.
 4. Add 1,000 μ l of the 1:10 latex suspension (prepared in step 1.3.3) to the 1,000 μ l 1:40 antiserum suspension (prepared in step 1.3.2). Invert five times to mix.
 5. Incubate at 37 °C on a slowly rotating wheel (*e.g.*, 4 rpm for a wheel of 38.5 cm diameter) for 2 hr.
 6. Centrifuge for 15 min at 1,100 x g. Discard the supernatant and gently resuspend the pellet in 2 ml of sterile saline.
 7. Centrifuge for 15 min at 1,100 x g. Discard the supernatant and add 1 ml of GBS and gently resuspend the pellet.
 8. Pipette the latex suspension into a labeled 5 ml screw capped tube. Add another 1 ml of GBS.
 9. Add 2 ml of GBS containing 0.2% BSA (w/v) (pH 8.2) (prepared in step 1.2). Add 40 μ l of 10% (w/v) solution of sodium azide as a preservative.
NOTE: Sodium azide is hazardous if inhaled, and is a skin and eye irritant. It is desirable to purchase a prepared 10% solution rather than the powdered form, as this minimizes the risk of inhalation exposure. Personal protective equipment (including gloves and splash goggles) should be worn when handling this reagent. Refer to the Material safety data sheet for details.
 10. Store latex reagents at 4 °C.
 4. Preparation of negative control latex reagent
 1. Label a 2 ml round bottom microfuge tube.
NOTE: round bottom tubes are used to minimize particle settling which may affect antibody attachment.
 2. Using a P1000 pipette with sterile tips add 975 μ l of GBS into the microfuge tube, then add 25 μ l normal rabbit antiserum. Invert five times to mix.
 3. Proceed as in steps 1.3.3 to 1.3.10 above

2. Quality Control (QC) of Latex Reagents

1. Bring latex reagent to RT. Immediately before use, gently mix the latex reagent by inverting the tube several times.
2. Check reagent for autoagglutination by pipetting 15 μ l onto a glass microscope slide and proceed as in steps 4.7 and 4.8 below. There should be no agglutination of the latex particles. The reagent should appear white and smooth.
3. Test the reagent against a panel of low passage pneumococcal isolates. Use freshly grown cultures prepared as in step 3. The panel of isolates should include isolates of the 'target' serotype (the specific serotype for the reagent being tested) and 'non-target' serotypes (isolates of other serotypes that should not normally cross react with the reagent). Include at least one isolate of each target and non-target serotype for each latex reagent undergoing QC. If there is only one target or non-target serotype, test two different isolates of the particular serotype.
NOTE: The testing panel should include several isolates of each serotype, some of which are uncommon in invasive disease and/or type culture collections. It is preferable to include some carriage isolates, as they are often more demanding to serotype (data not shown), thereby further testing the capacity of the reagents and ensuring that the reagents are likely capable of serotyping both invasive and carriage isolates.
4. Proceed with testing of latex reagents with target and non-target pneumococcal isolates as per the method described in steps 3 and 4 below.
5. Quality control reagents at time of production and then at least annually thereafter.
NOTE: If a reagent does not pass QC, the batch must be discarded and a new preparation made. In the event of repeat QC failure we recommend the reagents be produced using the alternate methods described in Ortika *et al.*, 2013⁹ and in the discussion below.

3. Preparation of Pneumococcal Cultures for Serotyping

1. Using a sterile loop select a single colony of the pneumococcal isolate and streak this out onto a solid nonselective blood agar so that the primary inoculum covers approximately one third of the plate surface. Streak for single colonies.
NOTE: Plates prepared from defibrinated horse or sheep blood are suitable; but blood plates prepared with human blood or citrated animal blood are not.
2. Incubate plate O/N at 37 °C in an atmosphere of 5% CO₂.
3. Observe the growth on the plate for purity and assess that there is sufficient growth for serotyping.
NOTE: A pure culture of the pneumococcal isolate is required for serotyping. In our experience, confluent, or near confluent growth covering approximately one third of the plate surface is usually sufficient to complete serotyping. As pneumococcal cultures may be prone to autolysis,

cultures prepared for serotyping should be tested within 24 hr of subculture. Do not take the culture plate out of the incubator more than 15 to 20 min before serotyping commences.

4. Conducting Latex Agglutination Serotyping

1. Remove all latex reagents from refrigerator and allow them to warm to RT for approximately 30 min. Immediately before use, gently invert the tubes to mix the reagents.
2. Label a microscope slide.
3. Place 15 μ l of negative control latex reagent onto the microscope slide.
4. Using a sterile disposable 1 μ l loop take a sweep of the pneumococcal culture so that the loop is approximately half full.
5. Gently smear the inoculum onto the surface of the slide close to the drop of negative control latex reagent. The inoculum should be visible on the glass slide.
6. Quickly and thoroughly mix the inoculum into the negative control latex reagent using the loop. Ensure that the drop does not spread further than its initial size.
7. Pick up the slide, holding it at both ends. Rock the slide back and forth for 1 min. Take care to keep the liquid moving slowly, and ensure that the size of the drop does not increase.
NOTE: Alternatively use a plate rocker.
8. Observe for macroscopic agglutination (*i.e.*, by naked eye) and clearing of the suspension against a black background.
9. If the negative control latex reagent shows no agglutination or clearing continue testing the culture, replacing the test latex reagents for the negative control latex reagent in steps 4.1 to 4.8 above. A fresh loop must be used each time. Proceed with testing of the latex reagents starting with the pools.
10. Test each of the 'pool' latex reagents in succession until a positive test is obtained.
NOTE: The order of testing of the pools may be done in 'rounds' to reflect the local incidence of particular serotypes (*e.g.*, by testing for the three most likely pools on the first slide. If a positive reaction is not obtained, then test the next three most likely of the remaining pools on the second slide; and so on until a positive reaction is obtained). This ensures that the most common serotypes are tested first, minimizing the time and reagents needed.
11. Using the antisera manufacturer's key determine which individual antisera are represented in the positive pool.
12. Test each of the groups or types contained in the positive pool individually to determine the 'group' or 'type' as in steps 4.1 to 4.8 above.
13. Determine the result by reference to the antisera manufacturer's key. If a 'type' is determined (*e.g.*, serotype 5), then no further testing is needed and this result is recorded.
14. If a 'group' is determined (*e.g.*, group 19), then refer to the manufacturers key to determine the factors to be tested. Continue testing with the individual 'factor' latex reagents and determine the final serotype (*e.g.*, serotype 19B).

Representative Results

A positive latex reaction occurs when type-specific antibody attached to the latex particle binds to the capsule of the pneumococcus, and agglutination of the antibody labeled particles ensues¹⁵. **Figure 1A** shows a positive reaction which is characterized by visible agglutination and clearing of the background suspension. A negative reaction is characterized by the latex agglutination reagent suspension remaining smooth and white (**Figure 1B**). The reagents are optimized so that a positive reaction should be observed before the end of the one min time interval (usually detectable in approximately 20 sec). We do not recommend reading reactions after the 1 min time interval. Very occasionally, reactions display weak agglutination around the edge of the drop that is not accompanied by clearing of the background suspension, or appear 'stringy' with no background clearing (data not shown). These are most likely negative reactions. In such cases, we recommend retesting and/or further investigation using another serotyping method (for example the Quellung reaction¹⁷).

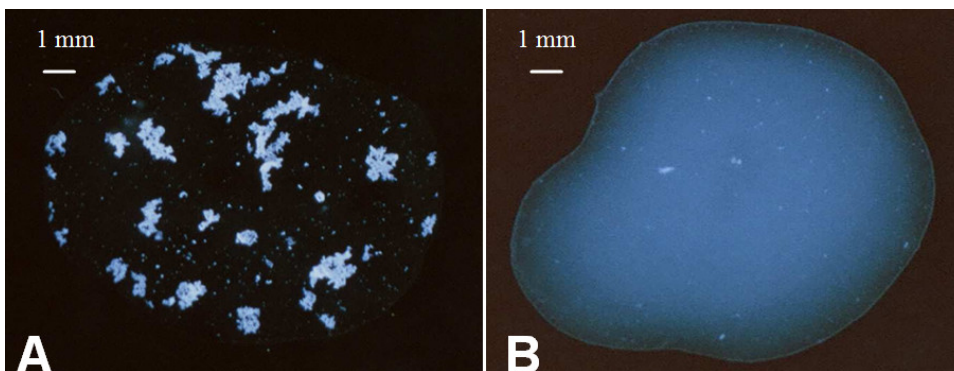


Figure 1. Positive and negative latex agglutination reactions. Preparations of a pneumococcal isolate mixed with latex agglutination reagent showing a positive reaction (**A**) or a negative reaction (**B**). Agglutination accompanied by clearing of the background is seen in the positive reaction (**A**). There is no visible agglutination with the negative control latex reagent or in a negative test reaction (**B**). Photographs⁸ used with permission. [Please click here to view a larger version of this figure.](#)

Discussion

Latex agglutination is a simple, quick and inexpensive method for pneumococcal serotyping. Commercial pneumococcal latex agglutination reagents are available, but they currently do not differentiate all known pneumococcal serotypes^{10,12}. However, latex agglutination reagents can

be easily produced in-house using purchased antisera raised against specific pneumococcal capsular antigens. In the method described here, antibodies are passively attached to latex particles to make a set of latex agglutination reagents.

A positive latex reaction occurs when specific antibodies bound to the latex particles attach to antigens on the polysaccharide capsule of the pneumococcal isolate^{18,19}. The latex particles attached to the antibodies allow the specific antibody-antigen reaction to be visualized without magnification.

Latex agglutination is a suitable method for high throughput laboratories and also for resource-poor settings. Key advantages of the latex agglutination method are that no specialized equipment is required to perform the test, reagents have a shelf life of at least 2 years when stored at 4 °C⁹, and that it is inexpensive, simple and quick to perform. Serotyping a pneumococcal isolate by latex agglutination takes approximately 10 min, and up to four latex reagents can be tested in parallel on the one slide. Furthermore, if the prevalence of serotypes is known for the relevant epidemiological setting, testing can be performed in rounds starting with the pools containing the most common serotypes similarly to the Quellung test¹⁷. This approach minimizes the number of tests needed to determine the serotype. The method is also highly reproducible between different operators (data not shown). A disadvantage of latex agglutination serotyping is that producing the reagents is time consuming, taking approximately 4 hr; although multiple reagents can easily be produced in parallel. Furthermore, some antigens are common across different pneumococcal serotypes, resulting in cross-reactions with some antisera (e.g., serotype 29, 42 and Group 35). However, these cross reactions are well characterized when using commercial antisera (which is usually preabsorbed to remove the more problematic cross-reacting antibodies) and additional reaction tables are provided by the manufacturer in order to distinguish which serotype is present. Latex reagents may also cross react if the antibodies are not aligned properly on the latex particles; these are detected as QC failures. In our experience, rigorous QC is central to the success of this method, even when commercially available antisera are used for production. QC takes approximately 15 min per reagent and relies on a set of appropriate QC isolates as described above.

The method described here results in reagents that give a positive reaction well within the test period. In our experience, false positives are rare in practice (data not shown). Occasional false negative reactions are observed; usually these relate to known issues with a particular antiserum (e.g., serogroup 6 isolates may not react with pool B antiserum), or down regulation of capsule expression by the isolate. Using the serotyping algorithm provided by the manufacturer is also important, as in most cases a false positive or negative reaction will lead to a 'blind end' result. In these cases, and when reactions are difficult to interpret, we recommend repeat-testing and/or testing by an alternative method such as the Quellung reaction.

Some troubleshooting is occasionally required to make satisfactory latex reagents. In the method described here, the antibody is attached to latex particles via passive adsorption^{15,19}, so a critical variable is the concentration of antibody used, which determines how well the surface of the latex particles is covered and the subsequent alignment of the antibody active sites^{15,20}. Consequently, if insufficient or excess antibody is attached to the latex particles, the antibody-antigen reactions will not be optimal, and unsatisfactory reagents may be produced^{15,20,21}. As antibody titers vary between different lots of antisera, a standard set of dilutions may not produce reagents that perform well⁸. A useful starting point is to use a 1:40 dilution of antiserum, and if this is not successful, dilute the antiserum further. In our experience this usually resolves the problem⁸. In a small number of cases reagents may show weak agglutination that is not accompanied by clearing of the suspension when tested with target isolates. In these cases diluting the antiserum does not improve the quality of the reagent, but satisfactory reagents can usually be produced by omitting the centrifugation and wash steps^{8,22}.

Antibody coated latex particles are used commonly in diagnostic microbiology for detection, identification or serotyping of many different microbes^{15,20}. As such, the method described here may be suitable for preparing latex reagents for other purposes, provided suitable antisera is available and adequate QC procedures are adopted.

Disclosures

The authors have nothing to disclose.

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