Capsular Serotyping of *Streptococcus pneumoniae* by Latex Agglutination

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URL: http://www.jove.com/video/51747
DOI: doi:10.3791/51747

Keywords: Immunology, Issue 91, Antisera, pneumococci, polysaccharide capsule, slide agglutination

Date Published: 9/25/2014

Citation: Porter, B.D., Ortika, B.D., Satzke, C. Capsular Serotyping of *Streptococcus pneumoniae* by Latex Agglutination. *J. Vis. Exp.* (91), e51747, doi:10.3791/51747 (2014).

**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.

**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. Preparation of Pneumococcal Cultures for Serotyping

2. Quality Control (QC) of Latex Reagents

3. Observe the growth on the plate for purity and assess that there is sufficient growth for serotyping.

2. Incubate plate O/N at 37 °C in an atmosphere of 5% CO₂.

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. Stirreat for single colonies.

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 as in steps 1.3.3 to 1.3.10 above

4. Preparation of negative control latex reagent

1. Label a 2 ml round bottom microfuge tube.

2. Using a P1000 pipette with sterile tips add 975 µl of GBS into the microfuge tube, then add 25 µl 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 µl of latex particles to 1,080 µl of sterile physiological saline.

4. Add 1,000 µl of the 1:10 latex suspension (prepared in step 1.3.3) to the 1,000 µl 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 µl 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.

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 µM filters using several 60 ml syringes into a 250 ml preautoclaved screw capped glass bottle.

3. Store at RT.

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 µl of GBS into the microfuge tube, then add 25 µl 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 µl of latex particles to 1,080 µl 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 µl of the 1:10 latex suspension (prepared in step 1.3.3) to the 1,000 µl 1:40 antiserum suspension (prepared in step 1.3.2). Invert five times to mix.

5. Centrifuge for 15 min at 1,100 x g. Discard the supernatant and gently resuspend the pellet in 2 ml of sterile saline.

6. Centrifuge for 15 min at 1,100 x g. Discard the supernatant and add 1 ml of GBS and gently resuspend the pellet.

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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. 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 µM filters and several 60 ml syringes into a 250 ml preautoclaved screw capped glass bottle.

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6. Store at RT.
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. Place 15 µl of negative control latex reagent onto the microscope slide.
3. Using a sterile disposable 1 µl loop take a sweep of the pneumococcal culture so that the loop is approximately half full.
4. 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.
5. 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.
6. 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.
7. Observe for macroscopic agglutination (i.e., by naked eye) and clearing of the suspension against a black background.
8. Test each of the ‘pool’ latex reagents in succession until a positive test is obtained. 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 other pools on the 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.
9. Using the antisera manufacturer’s key determine which individual antisera are represented in the positive pool.
10. 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. A fresh loop must be used each time. Proceed with testing of the latex reagents starting with the pools.

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 ensues\(^5\). 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 reaction\(^7\)).

![Figure 1. Positive and negative latex agglutination reactions.](image)

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. 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 °C, 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 test. 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, 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. 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. As antibody titers vary between different lots of antisera, a standard set of dilutions may not produce reagents that perform well. 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 problem. 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. Antibody coated latex particles are used commonly in diagnostic microbiology for detection, identification or serotyping of many different microbes. 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.

Acknowledgements

This work was part of the PneuCarriage project, funded by the Bill and Melinda Gates Foundation with contribution by the Victorian Government’s Operational Infrastructure Support Program.

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