

## Bacterial Agglutination Protocol

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### **Information**      **History**

When cells or other particulates become clumped together by antibodies in a solution, this is termed agglutination. In immunology, the process is differentiated from precipitation by the relative size and solubility of the objects clumped together. There are a variety of agglutination reactions in common use, including bacterial-typing reactions and blood-typing reactions.

In the 1800s, the meaning of the word agglutination was entirely different than the reaction of antibodies to clump cells together. Linguists spoke often of agglutination of words in a language, where words were glued together (5). Physicians used agglutination to describe when eyelids became glued together (15) or the edges of a wound closed.

In the history of agglutination, there are several interwoven threads. As our knowledge base grew, two different areas of study became merged into the growing field of pathogenesis and immune response. One of these areas was the research into agglutination of red blood cells, erythrocytes, and the other was the research into agglutinating bacteria and other pathogens, which led to methods for easy diagnosis of disease, evaluation of host resistance to the pathogen, and identification of specific pathogens.

Initial agglutination observations were made using the easily observable erythrocyte. In 1869, Adolf Creite described how foreign erythrocytes were attacked by serum components which were likely protein (16). His in vitro work showed that materials in serum clumped and destroyed foreign cells, but not self-cells. For example, Hughes-Jones (16) cites Creite's observation that rabbit serum did not affect rabbit erythrocytes, but that cat serum clumped rabbit erythrocytes. Hughes-Jones also identified a second early pioneer in the research into agglutination, Leonard Landois. His 1875 monograph (referenced in 16) extended the research of Creite, demonstrating in vivo and in vitro lysis of erythrocytes.

Meanwhile, a great number of researchers were traveling parallel roads leading to an understanding of the agglutination process, although they did not yet know it. In 1887 and 1889 Charrin and Roger, working with *Pseudomonas aeruginosa*, which they called *Bacillus pyocyaneus*, discovered that rabbits inoculated with heated and filtered bacterial

cultures were protected against subsequent infections (7). Williams and Cameron, in 1894, recognized the potential to develop serum-therapeutics against *P. aeruginosa*, illustrating recognition of the potential role of serum proteins in protecting against disease (33). Charrin's work also reported that the blood of immune animals was able to aggregate (clump) bacteria (6). The question at that time was: what is the mechanism of aggregation? Motile bacteria were easily aggregated and lost their motility when clumped. But when nonmotile bacteria also showed the same clumping response, the conclusion had to be that aggregation and motility were not necessarily connected (12).

In January of 1896, the term agglutination was first applied to cell clumping by Max von Gruber, in work reported by his associate Herbert Durham (11). Their research described how sera from immunized animals caused the infecting bacteria to clump. In the same year, Fernand Widal (25) communicated that he had devised a test to diagnose typhoid fever by observing the specific clumping of the typhoid bacteria by the sera of the infected person. Delepine's 1897 communication describes several of the techniques which were being used to observe bacterial agglutination, which are not very different from techniques used today (10). In his studies on natural immunity, Elie Metchnikoff (22) detected agglutination in a hanging drop preparation with a mixture of bacteria and specific immune serum.

These reports, especially Widal's, sparked a great deal of debate, refinement, and extension by many researchers. Very quickly, after Widal's work was communicated, reports of the effectiveness of his methods to test for typhoid began to be published. For example, in 1896, Johnston and McTaggart (17) noted that the respective bacteria did clump in the presence of sera from individuals who had cholera and typhoid, illustrating the specificity of the reaction.

Grunbaum (14) noted that the serum might need to be diluted in order to observe a positive test. This observation is the foundation for our agglutination tests today, a recognition that too much antibody inhibits formation of cellular clumps, what we term the prozone effect (4).

The link between diagnosis using immune sera and inducing protection by administration of immune sera was firmly made by the mid-1890s. For example, in 1897 two reports of sero-protection were published. Steele reported successful treatment of typhoid fever with antityphoid serum (26). Clubbe observed much better survival in 300 children with diphtheria who were treated with antitoxin (8).

Jules Bordet (2) introduced new terminology for agglutination in 1899. He termed the agglutinating agent agglutinine and the agglutinable substance as agglutinée.

In 1900, Karl Landsteiner (20) published a seminal paper containing a footnote reporting his initial experiments on human blood agglutination. In this work, he mixed sera and blood cells of different individuals and observed that some individuals had antibodies in their sera that were able to agglutinate other's red blood cells. It is in

Landsteiner's footnote that the two different research paths, bacterial agglutination and blood cell agglutination, merged, as it became clear that both groups of researchers were studying the same phenomenon—blood components, proteins, which were capable of causing specific cells to clump. Landsteiner extended his initial observations in a 1901 paper (21), where cells and sera from 29 individuals were examined. In this work, he described 3 blood groups, what we now call O, A, and B. A year later, von Decastello and Sturli (29) described type AB blood cells.

Since these early observations, the field of serological identification of bacteria has greatly expanded. Three cellular components, termed antigens, are commonly used in identification of bacterial cells. These are the flagellar antigen, somatic antigen, and capsular antigen.

The bacterial flagellar antigen is given the initial "H." This term was first used on *Proteus vulgaris*, coming from the German "hauch," meaning film or veil, indicating the spreading veil-like character of the bacterial growth (13). All motile bacteria have an H antigen, and most of these antigens are flagellin proteins. Both gram-positive and gram-negative bacteria can be characterized by their H antigen. Two organisms commonly characterized by their flagellar antigens are *Escherichia coli* and *Salmonella enterica*. In *E. coli*, there are at least 53 flagellar gene alleles (1 to 56; 13, 22 and 50 not used), mostly for the *fliC* gene (3, 31). *S. enterica* isolates usually have two flagellin genes (*fliC* and *fljB*), which are expressed alternately. These are described as phase 1 and phase 2 H antigens (30). McQuiston states that there are 114 recognized *S. enterica* H antigen types (23). The H antigen is destroyed by 50% alcohol and by heat (28).

Gram-negative bacteria have an outer membrane external to their thin peptidoglycan wall. A major component of this outer membrane is lipopolysaccharide (LPS). The outermost carbohydrate sequence of LPS is an extremely variable molecule, and this variability is characterized as the "O" or somatic antigen. The O came into use with discovery of nonmotile isolates of *P. vulgaris*, and comes from the German "ohne hauch," without spreading (13). O group designations for *E. coli* run from 1 (O1) to 181 (O181), including some provisional and newly characterized antigens (3). There are 46 different O serogroups in *S. enterica*. One application of a clinical agglutination test using the O antigens is the Weil-Felix reaction, which looks at the ability of patient serum to agglutinate several of the nonmotile *P. vulgaris* strains (28). High concentrations of serum from individuals with typhus fever and Rocky Mountain spotted fever caused by *Rickettsiaceae* agglutinate these bacteria.

The third most commonly used bacterial typing antigen is the heat-labile capsule. This was named "K" for "kapsule." *Salmonella enterica* typhi's and paratyphi's K antigen is termed the Vi antigen, for envelope. The capsule is the outermost layer of the microbe, and if present, it can mask the O antigen. *E. coli* has 60 different recognized K antigens (3). The K antigen is also important in characterizing *Streptococcus pneumoniae* and *Haemophilus influenzae* strains.

Variations on these typing agglutinations are used for typing other organisms. Both *S. pneumoniae* (19) and other *Streptococci* are so typed. Lancefield's *Streptococcus* groupings are based on binding of specific antisera to group-specific peptidoglycan-associated polysaccharides (32).

### **Purpose**

The agglutination reaction between surface antigens of bacteria and antigen-specific antibodies is a tool which can be used to both identify bacterial isolates and diagnose infection through the detection of bacterial-specific antibodies in samples (24).

### **Theory**

Agglutination is defined as specific clumping of particulates such as cells or cell-sized objects. Antibodies and lectins are two types of reagents which, because they bind to specific targets, can cause agglutination of particulates. Agglutination occurs when the reagent, either antibody or lectin, binds epitopes or carbohydrates on two different objects, linking them together. Epitope is the term for the region of the microbial antigen where antibodies bind.

Lectins are proteins which bind to specific carbohydrate residues. Many lectins, isolated from beans and other plants, are commercially available. Several different bacteria have been typed by their lectin agglutination patterns including *Lactobacillus* isolates (1), however, most bacterial typing tests are serologically-based and rely on antibodies that specifically recognize a given bacterial strain.

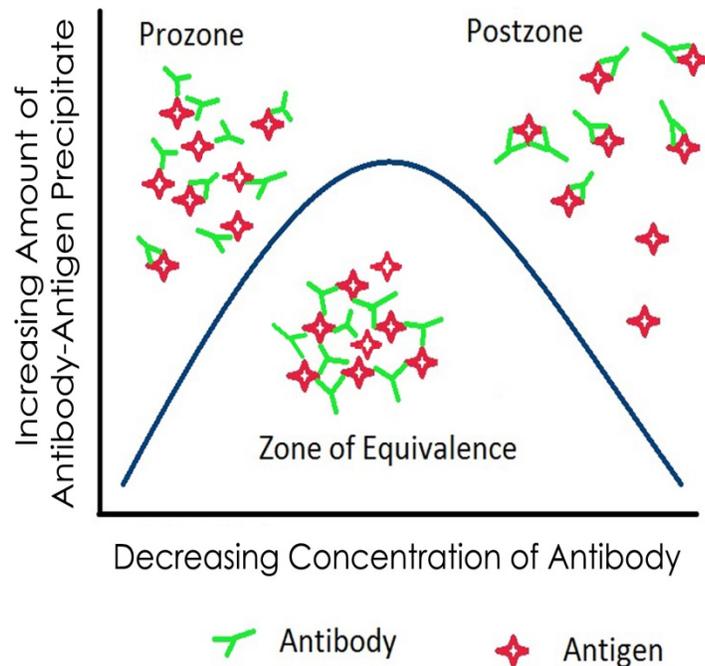
Antibodies are proteins secreted by plasma B cells. B lymphocytes are circulating white blood cells which recognize and respond to antigen epitopes. After antigen recognition, B cells differentiate into plasma B cells, secreting antibodies which recognize specific antigen epitopes. Each antibody has at least two binding sites, each of which is capable of noncovalently binding a specific antigen epitope. There are 5 classes of antibodies—IgM, IgG, IgA, IgE and IgD—and while these differ in physical structure, all have multiple epitope binding sites (9). The IgM antibody class can bind up to 10 epitopes, giving the strongest agglutination reaction. The high degree of binding specificity is exploited in clinical settings in tests to identify the causative agents of infection. In addition, agglutination is used to determine blood type based on cell surface antigens.

Agglutination reactions can be described as direct or passive assays. Direct assays involve interaction of antibody or lectin with a cellular antigen. Examples of direct assays include bacterial agglutination which is used to type strains of bacteria and diagnose infection and direct hemagglutination which would include blood typing. Examples of passive assays include passive hemagglutination or coated latex particle agglutination, measuring C reactive protein levels in blood and tissue samples (18), for example.

Serological bacterial typing or serotyping requires the preparation of

antibodies specific to the microbial antigen being characterized. This is usually achieved by immunizing an animal (mouse, rat, rabbit, goat, or even chicken) with the microbial antigen. After circulating B cells recognize the antigen, they differentiate into plasma B cells, which secrete antibodies into the bloodstream. These antibodies can then be purified from the blood, or the plasma B cells immortalized in cell culture. Antibodies can be made against proteins as well as against carbohydrates. Bacterial O and K antigens are carbohydrate antigens, while H antigens are protein antigens. Two different types of antibody preparations are in common use. Polyclonal antibodies are preparations which include antibodies with specificity to many different antigenic epitopes. Monoclonal antibody preparations are secreted by immortalized B cells in culture and consist of antibodies which all have the same antigenic specificity.

In order for agglutination between antibody and antigen to occur, the antibody and antigen epitope must be combined in the proper proportions (4), called the zone of equivalence. When this happens, the antibody molecules bind to epitopes on two or more different antigens, forming a crosslinked network. If enough antigens and antibodies are present, the mass of agglutination becomes visible to the naked eye as clumping. If the antibody molecules are in too high a concentration, they will not form the crosslinked network, even though they recognize the antigen. This is called the prozone effect. If the antigen is in excess, this will also prohibit formation of a crosslinked network, termed a postzone effect. False negative reactions can occur when antibody or antigen concentrations are in either prozone or postzone regions.



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FIG. 1. Effect of antibody and antigen concentrations on agglutination. Regions of excess antibody concentration are in the prozone region, while regions of excess antigen are in the postzone region. Antibody and antigen concentrations are optimal in the zone of equivalence.

### Materials

Physiological saline; 0.85 g of NaCl in distilled water, per 100 ml final volume

Antibacterial antibody (antiserum)

Bacterial strain to be tested. This may be an 18- to 24-hour old live slant culture or a killed preserved culture.

Microscope slide

Cover slips (optional)

Transfer loop or Pasteur pipette

Bunsen burner or equivalent (if using a nondisposable loop)

Wax pencil

Sterile 0.85% saline

Contaminated material discard

Bench disinfectant

Gloves

Goggles and lab coats when using BSL2 organisms

### Bacterial serotyping protocol (24, 27)

1. Obtain slide and other materials. Disinfect the lab bench and don the gloves.
2. Draw two circles or 1-inch squares on the slide with the wax pencil. Label one area "saline" and the other area "antiserum."
3. Place a drop of saline in the saline area and a drop of antiserum in the antiserum area.
4. Flame the transfer loop (if using a metal transfer loop) and obtain a loopful of bacteria. If the bacteria are a preserved preparation, be sure to resuspend the organisms before inserting the loop. Mix the bacteria and the saline on the slide to achieve a homogenous suspension. If using a disposable loop or pipette, place into appropriate discard. If using a metal loop, flame to sterilize.
5. Repeat the process by obtaining a loopful of bacteria and mixing the bacteria into the antiserum. Again, as above, mix the bacteria to get a homogenous suspension of bacteria without lumps. If using a disposable loop, place into appropriate discard. If using a metal loop, flame to sterilize.
6. Rock the slide in a circular motion gently for 1 minute, taking care to keep the mixtures within their sections.
7. Examine the slide and record your results. If possible, hold the slide near a bright light and view against a dark background.
8. Optional step: place a cover slip over each of the test areas on the slide. Examine under the microscope at 400X and 1,000X magnification.
9. Disinfect the bench carefully. During mixing of bacteria into saline and antiserum, small invisible droplets may be aerosolized. The antiserum will not kill the bacteria if using living organisms.
10. Discard the slide in the appropriate manner for your class, and then discard your gloves into the appropriate discard.

### Interpretation of results

Positive tests will display clumping, while negative tests will not show clumping. The clumping will be granular in appearance (Fig. 2).

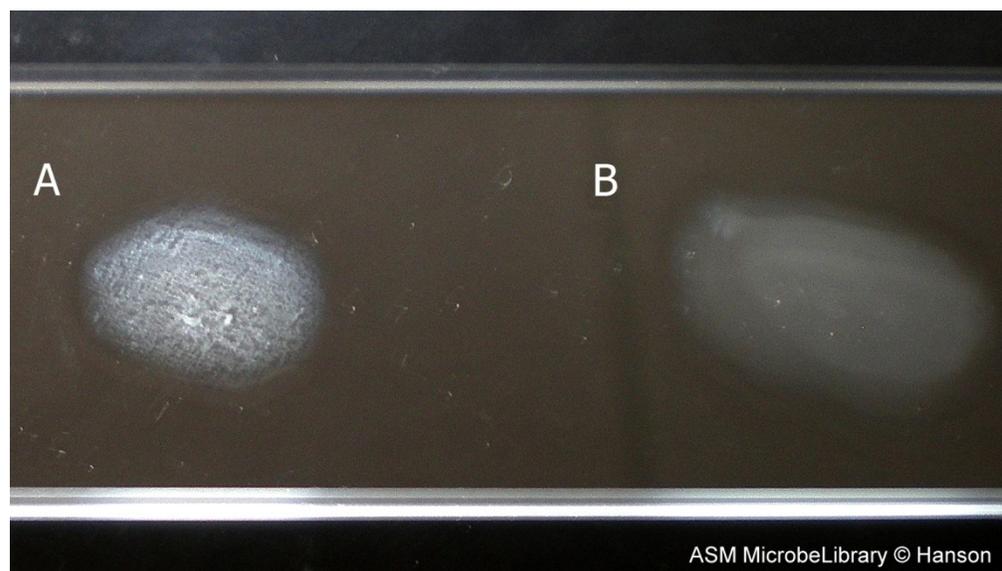


FIG. 2. Positive and negative agglutination. (A) Positive agglutination of *Salmonella* by anti-*Salmonella* antibodies is observed. B. No agglutination is observed when anti-*Salmonella* antibodies are mixed with *Escherichia coli*.

## SAFETY

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

## COMMENTS AND TIPS

1. The age of the culture to be tested should be  $\leq 24$  hours. In older cultures, cell walls will break down and diffusible components absorb into the agar.
2. Clumping in the control strain may be observed if that strain is a rough strain (no capsule) instead of a smooth strain (capsule).
3. If the sample drop dries, agglutination cannot be observed, and the test must be repeated.
4. Agglutination antiserum can be obtained from scientific supply companies and should be specific for the organism being studied.
5. The antiserum should be kept refrigerated when not in use.
6. There are kits that colorimetrically enhance visualization of the agglutination reactions. These are available from commercial sources, at varying costs. A suggested class investigation would be to determine which of these would work best in your class.

## Blood Typing

Blood typing also utilizes agglutination. Commonly available blood typing antisera include anti A, anti B, and anti Rh (D) antisera. Observation of agglutination when a drop of blood is mixed with antisera indicates that the blood cells have that specific antigen. It is recommended that blood agglutination not be performed by students who have not taken a formal microbiology course or who have no knowledge of aseptic technique, including how to properly and safely handle human blood samples. Therefore, blood typing using human blood samples should only be performed in medical microbiology courses. It is recommended that high school and introductory microbiology and immunology students learn how to perform blood typing using one of the many commercially available typing simulation kits.

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