Lancefield Whole Blood Killing Assay to Evaluate Vaccine Efficacy

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Bactericidal assays: Lancefield whole blood killing assay

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Abstract

While the Lancefield whole blood killing assay is named for the renowned streptococcal researcher Rebecca Lancefield, the protocol was first described by E.W. Todd in 1927 [1]. Initially, the assay was used to identify novel group A streptococcal (GAS) serotypes through the supplementation of non-immune human blood (often from infants) with type specific antisera prepared in rabbits [2, 3] and to demonstrate the impressive longevity of type specific immunity in patients following invasive GAS infection [4]. The modern assay is routinely used to screen defined GAS mutants [5, 6] or transposon libraries [7] for enhanced susceptibility to opsonophagocytic killing or to screen vaccine antisera [8] or other serological preparations [9] for anti-streptococcal activity.

Keywords: Lancefield assay, whole blood, bacterial killing, opsonophagocytosis, antiserum, mutant screening, multiplication factor

1 Introduction

The fundamental whole blood killing assay protocol has scarcely changed since Todd's first description [1]. Whole blood from healthy human donors is aseptically collected into tubes containing a suitable anti-coagulant and supplemented with 50-100 colony forming units (CFU) of freshly prepared GAS. The reactions are incubated at 37 °C for 3 h under constant agitation and the bacterial density at the

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beginning and at the end of the experiment are used to calculate the resulting degree of bacterial growth (denoted the multiplication factor). Multiplication factors calculated under different reaction conditions can be compared to determine, for example, the bactericidal activity of serological preparations or the role of certain genes in GAS resistance to phagocytosis.

Despite several attempts in the mid-20th century, it is clear that the human whole blood used in the assay cannot be reliably substituted with animal blood which remains only weakly bactericidal even when supplemented with human immune factors [10]. The only exception is non-human primate blood which readily kills GAS in the presence of bactericidal human antibodies [10, 3]. The specific differences between human and animal blood that are responsible for this disparity remain unclear however, Stollerman and others have provided evidence for a human specific co-opsonin that is essential for GAS killing [11-13]. Thus, the use of freshly isolated human whole blood remains an absolute requirement for a successful assay.

2 Materials

Prepare all solutions using distilled water and research grade reagents. Ethical approval covering the use of human blood from healthy donors must be obtained prior to conducting this protocol. Blood collections should be performed by a licensed physician or trained phlebotomist.

1. Frozen stocks of GAS strains of interest stored at -80°C in 20% glycerol
2. Columbia blood agar (CBA) plates (see **Note 1**): Prepare 400 ml of Columbia blood agar base in a 500 ml Duran bottle containing an autoclave safe magnetic stir bar according to the manufacturer's instructions and sterilize by autoclaving at 121 °C for 15 minutes. Cool base medium to 50°C and add 5% sterile defibrinated blood stirring continuously. Pour plates immediately and allow to set under the flame or in a laminar flow hood with the lids ajar.

3. Todd-Hewitt broth (THB): Prepare 400 ml of Todd-Hewitt broth in a 500 ml Duran bottle according to the manufacturer's instructions and sterilize by autoclaving at 121 °C for 15 minutes.
4. Phosphate Buffered Saline (PBS): Dissolve 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of dibasic sodium phosphate (Na_2HPO_4) and 0.24 g of monobasic potassium phosphate (KH_2PO_4) in 800 ml H_20 and adjust the pH to 7.4 with HCl as required. Add H_2O to 1L and sterilize by autoclaving at 121 °C for 15 minutes.
5. BD Vacutainer® Safety-Lok™ Blood Collection Sets with Pre-Attached Holder (21-gauge needle)
6. 6 ml BD Vacutainer® blood collection tubes, lithium heparin spray coated (see **Note 2**)
7. Alcohol swabs
8. Tourniquet
9. 2 ml microcentrifuge tubes (see **Note 3**)
10. An end over end rotator capable of securely holding the reaction tubes
11. A 5 % CO_2 static incubator set at 37 °C

3 Method

1. Streak GAS strain of interest onto a CBA plate and culture overnight at 37 °C in 5% CO_2
2. Inoculate a single colony into 5 ml of THB and culture overnight at 37 °C in 5% CO_2 in a static incubator
3. Inoculate 500 µl of the resulting overnight culture 1:10 into fresh, pre-warmed THB and incubate at 37 °C in 5% CO_2 in a static incubator until an A_{600} of 0.15 - 0.2 is reached (see **Note 4**)
4. Dilute culture 1:10,000 in sterile PBS. Plate 30 µl of the diluted GAS onto a CBA plate and culture overnight to determine the starting CFU/reaction

5. Aseptically collected heparinized whole blood from at least three healthy donors (see **Note 5**) and if appropriate supplement with test reagents of interest; for example, rabbit antiserum (see **Note 6**), pooled human immunoglobulin or putative bacteriostatic agents.
6. Inoculate 30 µl of the diluted GAS into 270 µl of heparinized blood and incubate for 3 h (see **Note 7**) at 37 °C in 5% CO_2 with constant end over end rotation (see **Note 8**)
7. Place reactions on ice and serially dilute 1:10, 1:100 and 1:1000 in PBS (see **Note 9**)
8. Plate 15 µl of a neat, 1:10, 1:100 and 1;1000 dilution onto CBA plates and culture overnight to determine the final CFU/reaction
9. Determine the CFU added to the reactions at 0 h and the CFU present in the reactions at 3 h (see **Note 10**) then divide the CFU/reaction (3 h) by the CFU/reaction (0 h) to give the multiplication factor.

4 Notes

1. Pre-poured Columbia blood agar plates are available for purchase from several commercial suppliers. In addition to being more convenient, purchased plates tend to be higher quality and more consistent than plates prepared in house.
2. Rothbard reported a marked reduction in bacteriostatic activity when the anticoagulant heparin was replaced with the calcium-neutralizing agents, potassium and ammonium oxalate or sodium citrate [10]. Defibrination is not recommended due to the risk of decreasing the number of leukocytes below the level required for effective phagocytosis.
3. 1.5 ml microcentrifuge tubes can also be used however it is important to ensure that the blood and streptococci mix effectively while the reactions are agitated.
4. The optical density of 0.15-0.2 is given for guidance and is based on growth of the invasive GAS isolate H305 (National Collection of Type Cultures number: NCTC8198) [14] in freshly prepared THB. The optical density that results in a starting inoculum of 50-100 CFU must be

determined empirically for each strain used. Freshly prepared cultures should be used and not frozen culture stocks.

5. The biggest hurdle that must be overcome when running a whole blood killing assays is inter-donor variation in bacterial multiplication. We and others have found that this affect can be partially ablated through prescreening of donors for ability to support GAS multiplication, or digestion of endogenous immunoglobulin prior to addition of bacteria [15, 16]. However, as discussed above, the bactericidal power possessed by the blood of some individuals cannot be explained satisfactorily by the presence of anti-GAS antibodies. If the difference in bacterial growth in whole blood is subtle between test and control conditions, there may be no alternative but to increase the number of donor blood samples until the null hypothesis can be rejected (or confirmed) with adequate statistical stringency. Three technical replicates of each donor blood sample should be set up to ensure accurate intra-donor multiplication factors are obtained. It is recommended that the assay is set up within 2 h of blood withdrawal.
6. Serum from vaccinated rabbits is most commonly used to assess the efficacy of the humoral immune response to specific antigens [17, 18]. The dilution of antiserum required to promote killing should be determined empirically and will depend upon the concentration and functionality of the test antibodies. Reactions containing pre-immune serum should be included as a control. Reactions can also be supplemented with patient serum or purified human antibodies [9]. Only scant references could be found where mouse antiserum was used in place of rabbit [19, 20].
7. While a 3 h incubation was employed in Rebecca Lancefield's seminal studies [4] and is generally accepted as the standard protocol, published incubation times range from 30 min to 24 h [21, 22, 6]. Thus, the incubation time may have to be optimized for specific applications. Fuller and colleagues noted that after 3 h the surviving streptococci multiply

rapidly to reach densities of “many millions per C.C.” within 24 h. At such densities, subtle difference between immune and non-immune reactions may be difficult to detect [22].

8. Reactions must be agitated to ensure that the leukocytes do not sediment as this will substantially reduce the apparent bactericidal activity of the blood samples. In his original publication, Todd demonstrated that the bactericidal activity of paired blood samples was reduced if incubated under static conditions, and postulated that this resulted from a reduction in contact time between the leukocytes and streptococci [1]. Sealing of tubes and/or use of a 5% CO_2 incubator is essential. If the blood is agitated in an open tube the rapid loss of CO_2 may affect the survival and growth of the GAS isolate [22].
9. Serial 10-fold dilutions in PBS can be prepared in a 96 well plate, allowing three columns of 5 µl dots to be carefully spotted onto a prewarmed blood agar plate using a 1-10 µl multichannel pipette. The dilutions will soak into the agar leaving circles which containing decreasing bacterial densities. One row of these will contain a countable number of colonies following overnight incubation at 37 °C. As undiluted blood will not soak into the agar plate, the lowest dilution that can be assayed in this manner is 1:10.
10. The starting CFU is equivalent to the number of colonies present in 30 µl of the initial GAS dilution (plated during step 4). The final CFU is equivalent to the number of colonies present in the 300 µl reaction following 3 h of incubation (an aliquot of which is plated during step 9).

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