ELISA for Alpha-hemolysin (Hla) in Methicilin-resistant *Staphylococcus aureus* (MRSA)

Varandt Y. Khodaverdian[1*] and Menachem Shoham[2]

[1]Department of Biology, Tufts University, Medford, USA; [2]Department of Biochemistry, Case Western Reserve University, Cleveland, USA
*For correspondence: varandt.khodaverdian@tufts.edu

[Abstract] Anti-virulence agents against MRSA inhibit the production of disease-causing virulence factors, such as alpha-hemolysin, but are neither bacteriostatic nor bactericidal. Here we discuss a rapid method to screen for MRSA anti-virulence agents by measuring alpha-hemolysin production through ELISA. This protocol can be used with other alpha-hemolysin producing bacteria or for other excreted toxins to which antibodies exist.

Materials and Reagents

1. *Staphylococcus aureus subsp. aureus USA300*
2. Dry ice
3. Dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific, catalog number: D128-1)
4. Polyclonal anti-Hla antibody (Abcam, catalog number: ab15948)
5. Anti-alpha hemolysin (Hla) antibody conjugated to horseradish peroxidase (Abcam, catalog number: ab15949)
6. 3,3',5,5' tetramethylbenzidine (TMB) (Sigma-Aldrich, catalog number: T4444)
7. Stop reagent (Sigma-Aldrich, catalog number: S5689)
8. Luria broth (LB) agar plates
9. Phosphate buffered saline (PBS) (pH 7.2) (Sigma-Aldrich, catalog number: P5119)
10. 10 mg/ml Bovine serum albumin (BSA) in PBS (Sigma-Aldrich, catalog number: P3688) (see Recipes)
11. 0.05% Tween 20 in PBS (Sigma-Aldrich, catalog number: P3563) (see Recipes)
12. Trypticase Soy Broth (TSB) (see Recipes)

Equipment

1. -80 °C freezer
2. 14 ml polypropylene culture tube (BD, catalog number: 352059)
3. Cotton ball or spill resistant caps
4. Microtiter 96 well EIA/RIA plates (Corning, Costar™, catalog number: 9017)

5. Microplate reader (Molecular Devices, model: SPECTRA Max M2), or any other model capable of measuring a 96 well microtiter plate at OD 650 nm
   Note: If a different model of microplate reader is used, ensure appropriate model of microtiter 96-well plate is used as the model above may not work.
6. 37 °C shaker
7. Nitrile gloves
8. Multichannel pipetman (Eppendorf)
9. 0.22 µm syringe filter (PVDF) (Thermo Fisher Scientific, Fisherbrand™, catalog number: 09-720-3)
10. 3 ml luer lock syringe (BD, catalog number: 309657)
11. 1.5 inch needle (BD, catalog number: 305187)
12. 2 ml cryotubes
13. Plastic wrap
14. Disposable inoculation loops (10 µl, PS, sterile, yellow) (LPS, catalog number: M122002)

Software

1. Microsoft Excel or other data processing program

Procedure

A. Induction of alpha - hemolysin (Hla)
   1. A frozen bacterial stock of MRSA strain USA300 is removed from the -80 °C freezer and placed directly onto dry ice.
      Note: The stock is not to thaw.
   2. Using a sterile disposable inoculator loop, inoculum is scraped off from the bacterial stock and placed into culture tube containing 1.5 ml of TSB.
      Note: Sterile technique is not required, however care should be taken to minimize possible contamination.
   3. A cotton ball is placed in the top of the culture tube and lid is put on loosely.
   4. The culture is incubated overnight, 16-18 h, at 37 °C with shaking.
      Note: The cotton ball is a precautionary measure to prevent the culture from spilling.
   5. The single overnight culture is diluted 1:100 in enough TSB to aliquot 1.960 ml diluted culture for each treatment (compounds to test and DMSO).
      Note: To determine the final volume of TSB needed for the dilution of the culture, the following calculation is used:
      (# of treatments) x 2 ml + 1 ml = ml of TSB.

6. Prepare solutions 0.05 mg/ml, 0.5 mg/ml or 2.5 mg/ml of the compounds of interested (potential MRSA anti-virulence agents) in 100% DMSO (one example of a compound of interest is Diflunisal, for more examples see Crowther, 2000).
7. 40 µl of 100% DMSO, 0.05 mg/ml, 0.5 mg/ml or 2.5 mg/ml of a compound in 100% DMSO are added to 14 ml plastic culture tubes to yield final concentrations of 2% DMSO, 1 µg/ml, 10 µg/ml, and 50 µg/ml of compound, respectively.
   Note: The concentration use varies depending on the nature of the compound of interest.
   Note: If compounds are dissolved in a different solvent, 40 µl of that solvent is used instead of DMSO.
8. 1.960 ml of the 1:100 diluted overnight culture is added to each culture tube containing compound or DMSO.
9. The remainder of the 1:100 dilution from step A5 is placed on ice.
10. As with the overnight culture, a cotton ball is placed in the top of each culture tube and the lid is put on loosely.
11. The tubes from step A8 are incubated for 6 h at 37 °C with shaking.

B. Dilution of cultures for determining colony forming units (CFU)

12. The 1:100 diluted overnight culture placed on ice in Part A is serial diluted $1:10^4$ and $1:10^5$ into TSB.
13. 100 µl of the $1:10^4$ and $1:10^5$ dilutions are spread onto LB agar plates and set aside until all of the plates are prepared. This is the zero hour time point.
14. After 6 h the treated cultures are removed from the 37 °C shaker and first processed following Part C of the protocol before conducting the following dilutions.
15. The liquid culture remaining in each treated sample is serial diluted to $1:10^4$ and $1:10^5$.
16. 100 µl of the $1:10^4$ and $1:10^5$ dilutions are spread onto LB agar plates.
17. All of the plates (including the zero hour time point plate) are incubated at 37 °C overnight.
18. The numbers of colonies in each of the $1:10^5$ dilution plates are counted.
    Note: If there are less than 30 colonies on a $1:10^5$ plate, the $1:10^4$ dilution plate of that sample is counted. If statistical power is of interest, both plates should be counted.

C. Bacterial culture sample preparation for ELISA

19. After 6 h of incubation the bacterial cultures are drawn into a luer lock syringe with a 1.5 inch needle, and the needle is removed.
    Note: The needle is removed using tweezers or hemostat, placed into bleach and disposed of in a hard plastic sharps container.
20. A 0.22 µm filter is tightly placed onto the syringe and the bacterial cultures are filtered into 2 ml cyrotubes.

Note: If there is resistance while filtering, applying gentle pressure to the plunger of the syringe will push the filtrate through. Too much pressure can result in filter failure.

21. The filtrates are immediately frozen using dry ice and stored at -80 °C for future use.
    Note: The filtrates are sterile at this point, but should still be handled with nitrile gloves throughout the protocol.

D. Alpha - hemolysin (Hla) ELISA

22. Prepare diluted 1:1,000 Hla antibody. For each 96-well plate used, 10 µl of anti – Hla antibody is added to 10 ml of cold PBS (pH 7.2) (1:1,000 dilution), and mixed gently.
23. 100 µl of 1:1,000 anti–Hla antibody in PBS is added to each microtiter 96 well, covered with plastic wrap and incubated overnight at 4 °C.
24. The supernatant is removed and briefly washed (*i.e.* rinsed) once by adding 230 µl of 0.05% Tween 20 in PBS and then removing the supernatant. Agitation is not required.
25. 230 µl of 10 mg/ml BSA in PBS block is added to each well and placed at 4 °C for 60 min.
26. The supernatant is removed and the plate is washed once by adding 230 µl of 0.05% Tween 20 in PBS and then removing the supernatant.
27. 100 µl of PBS is added to the “blank wells” in the designated row or column.
28. Test samples (filtered supernatants of bacterial cultures from step D21) are diluted 1:8 or 1:16 with PBS, and 100 µl of the diluted test samples are added to their respective wells.
29. The plate is covered with plastic wrap and foil then incubated for 1 h at room temperature with gentle rocking.
30. The supernatant is removed and 230 µl of 0.05% Tween 20 in PBS is added to each well. The supernatant is subsequently removed.
31. Step B12 is repeated two more times.
32. 100 µl of the HLA antibody conjugated to horseradish peroxidase, diluted 1:1,000 in 10 mg/ml BSA in PBS, is added to the wells, covered with plastic wrap and foil, and then incubated for 1 h with rocking at room temperature.
33. The supernatant is removed and the 230 µl of 0.05% Tween 20 in PBS is added to each well. The supernatant is subsequently removed.
34. Step D33 is repeated 2 more times.
    Note: On the final wash, using a pipette ensure the removal of all 0.05% Tween 20 in PBS.
35. 230 µl of PBS (pH 7.2) is added to each well and subsequently removed.
36. Step D35 is repeated.
37. The plate is placed upside down onto a paper towel until all of the supernatant is drained.
38. 100 µl of substrate solution 3,3’,5,5’ tetramethylbenzidine (TMB) is added to each well and incubated at room temperature for 10 min.
39. 100 µl of stop reagent is added to each well.

Note: Air bubbles may form when stop reagent is added. Prior to measuring absorbance, all air bubbles should be popped. This can be done by blowing air with a pipette, but should be done with care to avoid cross contamination between wells.

40. For each well in the plate, the OD is read at 650 nm using a microplate reader.

E. Calculations

41. The colony forming units (CFU) per ml for each treated sample is calculated by: [(# of colonies of treated sample) x (culture dilution factor)] / (# ml plated) = CFU/ml. This step can be done using Microsoft Excel or similar program.

    Notes:

    a. *0.1 ml of diluted culture is generally plated.*
    b. *The comparison CFU/ml of treated with the CFU/ml of the time zero plate is used to determine if the compounds are bactericidal, bacteriostatic, or have no effect on growth. If the CFU/ml of treated samples is statistically the same as CFU/ml of time zero, then the compound inhibits bacterial growth. If the CFU/ml of treated samples is statistically less than that of time zero, then the compound kills the bacteria.*

42. The amount of Hla production is calculated from the OD650 by: [(average OD sample) – (average OD blank)] x (sample dilution factor) / (# ml sample added) = units/ml.

    Notes:

    a. *0.1 ml of sample is generally added per well.*
    b. *This is easily done using Excel or similar program.*

43. The amount of Hla is first determined with respect to CFU by: (# units/ml) / (# CFU/ml) = units/CFU.
44. The percent Hla produced is defined as: [(units/CFU treated) / (units/CFU DMSO)] x 100 = % Hla production.

    Notes:

    a. *If % Hla production is below 100%, then the compound inhibits Hla production with respect to DMSO.*
    b. *A master Excel template (attached here) is provided with example data for a 1:8 dilution of the bacterial filtrate. The template will calculate steps E41-44 automatically once OD values and colony counts are added. Take note that the template is made for the volumes and dilutions specified in this protocol. If other volumes or dilutions are used, adjustments will need to be made in the template.*

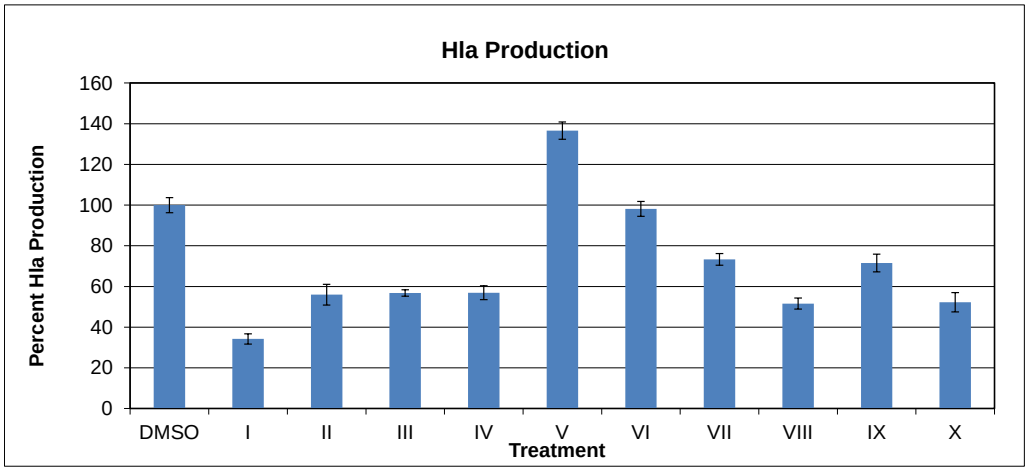

Figure 1. Example graph of percent Hla production relative to a DMSO treated MRSA bacterial culture (from example Excel template)

Recipes

1. 10 mg/ml BSA in PBS
   The solution is made as per manufacturer's directions.
2. 0.05% Tween 20 in PBS
   The solution is made as per manufacturer's directions.
3. Trypticase Soy Broth (TSB)
   17.0 g Bacto Tryptone
   3.0 g Bacto Soytone
   2.5 g Dextrose
   5.0 g sodium chloride
   2.5 g dipotassium hydrogen phosphate (pH to 7.3)

Acknowledgments

We thank Barbara Truitt and Dr. Michelle Pesho for their contributions to the development of this protocol. This protocol has been adapted from John R Crowther (2000). Funding for this work was provided by a Grant-in-Aid from the American Heart Association, Great Rivers Affiliate, and by a grant from the Steris Foundation.

References

1. Crowther, J. R. (2000). The ELISA guidebook. *Methods Mol Biol* 149: III-IV, 1-413.

2. Khodaverdian, V., Pesho, M., Truitt, B., Bollinger, L., Patel, P., Nithianantham, S., Yu, G., Delaney, E., Jankowsky, E. and Shoham, M. (2013). Discovery of antivirulence agents against methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 57(8): 3645-3652.

