Published in final edited form as:

Vet Dermatol. 2012 February ; 23(1): 57–e13. doi:10.1111/j.1365-3164.2011.01012.x.

Optimization of a *Staphylococcus aureus* adhesion assay for equine corneocytes

GC Griffeth*, N Callori*, SC Rankin†, RC Boston‡, and DO Morris*

*Department of Clinical Studies – Philadelphia, School of Veterinary Medicine, 3900 Delancey St., Philadelphia, PA 19104, USA

†Department of Pathobiology, School of Veterinary Medicine, 3800 Spruce St., Philadelphia, PA 19104, USA

‡Department of Clinical Studies – New Bolton Center, School of Veterinary Medicine, 382 West Street Rd., Kennett Square, PA 19348, USA

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes serious skin and soft-tissue infections of humans and animals. Multiple strains of MRSA have been characterized, and one in particular – designated as strain USA 500 – causes infections predominantly of horses and the people who work with them. The purpose of this study was to optimize an assay which could subsequently be used to compare the relative avidity of different *S. aureus* strains for equine corneocytes. Corneocytes were collected from the perineal skin of ten healthy horses onto adhesive discs. The discs were then incubated at 37° C with a *S. aureus* field strain at each of three concentrations [10^7 , 10^8 , and 10^9 colony forming units (CFU)/ml] and for each of three incubation periods (45, 90, and 180 min). After standardized rinsing and staining procedures, discs were examined at 1,000x magnification and areas containing confluent corneocytes were photographed. The percentage of surface area occupied by adherent bacteria was analyzed using image processing and analysis software. Significant colorspace image processing was required to distinguish bacteria from the ubiquitous melanin granules present within equine corneocytes. Objective and subjective methods were used to determine optimum conditions for specific adherence without introducing confounding factors. A bacterial concentration of 10^8 CFU/ml incubated with corneocytes for 45 min produced maximum bacterial adhesion with the least amount of inter-bacterial clumping. Future studies should utilize these conditions for optimum assay performance.

Introduction

Staphylococcus aureus is a normal bacterial inhabitant of the skin and mucous membranes of humans and some species of domestic animals. Colonization by this bacterium has been shown to increase the risk of skin and soft tissue infections (SSTI) when host defenses are compromised.¹ Resistance to multiple classes of antibiotics, as typically occurs in strains of methicillin-resistant *S. aureus* (MRSA), complicates treatment regimens and may therefore increase the morbidity associated with SSTI.^{1,2} Several clonal groups of MRSA are responsible for the majority of infections caused by this organism world-wide, and the clonal group designated by the U.S. Centers for Disease Control and Prevention as MRSA strain USA 500 causes colonization and infection predominantly of horses and the people

Corresponding author: Daniel O. Morris, DVM, MPH, School of Veterinary Medicine, University of Pennsylvania, 3900 Delancey St., Philadelphia, PA 19104, USA, domorris@vet.upenn.edu.

Conflict of interest: No conflicts of interest have been declared

who work with them.³ Because staphylococci must adhere to host tissues in order to colonize and infect, the propensity of USA 500 for horses may suggest an adaptive affinity for adhesion to equine skin and mucous membranes. This could be clinically relevant if strain-specific adhesive factors could be characterized and then subsequently targeted with anti-adhesive agents, thereby reducing reliance on antibiotic therapies.

S. aureus is known to express several protein adhesins known as MSCRAMMs (microbial surface components recognizing matrix molecules). These molecules are capable of mediating adherence of the bacteria to extracellular matrices of the host.⁴ However, bacterial adhesion is not the only virulence factor that contributes to pathogenicity. Some *S. aureus* strains have evolved specific non-adhesive mechanisms that make them more infective to ruminant animals and horses.⁵ Before investigating bacterial strain-specific adhesive mechanisms, it is important to validate an adhesion assay for equine corneocytes. Staphylococcal adhesion studies have previously validated specific incubation conditions for canine and feline corneocytes,^{6,7,8} however, it was not known if these assays could be used with equine corneocytes. The purpose of the study reported here was to optimize an *S. aureus* adhesion assay for equine corneocytes.

Materials and Methods

Corneocyte Collection

Approval for specimen collected was granted by the Institutional Animal Care and Use Committee. Ten horses were utilized as corneocyte donors, and ranged in age from four to 13 years. Five of the horses were mares (all Arabian) and five were geldings (Dutch warmblood n=1, Arabian n=2, and Arabian-quarter horse cross n=2). Corneocytes were collected from the perineal area of 10 horses using 22-mm adhesive discs (D-squame®, CuDerm Corporation, Dallas, TX, USA).⁸ The area targeted for sampling was cleansed of surface debris by the application and subsequent removal of three 5 cm adhesive tape strips applied in succession to the same sites (bilateral to the anus). Once the skin was prepared, three adhesive discs were applied side-by-side to the cleansed sites, pressed gently onto the skin, and then removed to obtain a confluent sample of corneocytes. Two additional discs were applied to and removed from the exact same sites (i.e., three disc samples collected in triplicate from each prepared skin site, bilaterally). Therefore, 18 adhesive discs were collected from each subject. Discs were stored at -70° C on D-squame® cards (provided by the manufacturer) until needed. Storage time did not exceed four weeks.

Preparation of Bacterial Suspensions

A methicillin susceptible *S. aureus* strain (ATCC 29213) was used to test *S. aureus* adhesion. The bacteria were cultured on trypticase soy agar containing 5% sheep blood (TSA, Remel, Lenexa, KS, USA) and incubated overnight at 35° C. The bacteria were harvested from the TSA plate using a sterile cotton-tipped swab, suspended in sterile phosphate-buffered saline (PBS pH 7.4), and adjusted to optical densities of 0.15, 1.0, and 1.5 (570nm). Ten-fold serial dilutions were prepared and 100ul inoculated onto trypticase soy agar plate containing 5% sheep blood in triplicate in order to estimate bacterial cell numbers at each OD. An OD of 0.15 was found to correlate with approximately 1×10^7 CFU per milliliter, OD 1.0 with 1×10^8 CFU per milliliter, and OD of 1.5 with 1×10^9 CFU per milliliter.

Optimization of the Adhesion Assay

Adhesion studies utilizing canine corneocytes have reported that an incubation time of 45 min with a bacterial suspension at OD 0.15 (570 nm) at 38° C is optimal.⁸ Therefore, the studies reported here were performed at a similar temperature (37° C). The effects of time

and bacterial concentration were tested as described previously.⁸ Briefly, one corneocyte sample from each of 10 horses was inoculated with one of three concentrations of bacterial suspension (as detailed above) and incubated at each of three time different time periods (45, 90, or 180 min) such that a total of nine discs were utilized from each of the 10 horses for the purposes of optimization. The remaining discs were maintained in storage for use in future studies that will compare the differential adhesion of different MRSA strains to equine corneocytes. For incubation, the 22-mm adhesive disc samples were placed with the corneocyte surface up in a small Petri dish. One half milliliter of each bacterial suspension was placed over the corneocyte layer. Following incubation, the samples were rinsed with PBS for 20 seconds and stained with crystal violet for ten seconds. Excess stain was removed by rinsing with running water for 10 s, and the discs were then attached to glass microscope slides using clear double-sided cellophane tape (Scotch[®] tape, 3M Stationary Products, St. Paul, MN). The slides were allowed to air dry prior to microscopic examination.

Image Acquisition and Bacterial Counting

From each adhesive disc, 15 consecutive microscopic fields that contained confluent corneocytes were photographed at 1,000x magnification.⁶ These fields were randomly selected by a standardized method,⁷ and any field in which corneocytes were either not confluent or contained other structures (i.e., hair, artifacts), or for which the entire area could not be brought into sharp focus, was rejected.⁷

Using an image processing and analysis program (ImageJ 1.44c; NIH, Bethesda, MD, USA), and a color-based image segmentation software plugin (Threshold Colour, <http://www.dentistry.bham.ac.uk/landinig/software/software.html>) which are freely available, adherent bacteria were quantified. One 1200- by 1600-pixel full-color image of each field was captured. Using ImageJ, a circle of 600-pixel diameter was selected in the center of each field, and the rest of the image was blanked. Thresholding the image was accomplished using the “Threshold Colour” plugin by filtering within the CIE L*a*b* colorspace. Briefly, an automatic filter converted the RGB image into L*a*b* colorspace, and removed all pixels which had an L* value less than 193 and/or a b* value less than 110. As the b* value lies upon a blue-yellow axis, this filtered all pixels that included any value of yellow coloration. This removed melanin granules from the image. Filtering the highest L* values removed all white and lightly stained areas. The remaining pixels were thresholded into a binary image and counted. The pixel counts for each circular area as well as the average clump size within the area were automatically entered into spreadsheet format by the software.

Analysis

In order to locate a normalizing transformation for the bacterial clump sizes we applied Tukey’s ladder test. Robustness of Tukey’s chi-square test in conjunction with the ladder of transformations to achieve normality was confirmed with the Shapiro-Wilks test. The clump size measurements were then appropriately transformed, and the possible clustering of bacterial clump sizes in terms of finite normal distributions were examined. The method followed here closely tracked the recommendations of Everitt⁹ and the specific statistical software used was the ‘fmm’ (Finite Mixture Model) procedure developed by Deb¹⁰ and implemented in Stata 11.1. A p-value of less than or equal to 0.05 was used to denote statistical significance.

Once clump sizes were shown to adhere to a definable distribution, mean disc surface areas covered by the adherent bacteria, at each of the nine combinations of incubation time and bacterial cell concentration, were calculated for each individual horse. These mean values,

for each of the ten horses, were then averaged together to produce a composite score for each of the time-concentration combinations. Results were plotted for visual inspection to determine which of the nine combinations of incubation conditions maximized bacterial adhesion to corneocytes without inter-bacterial clumping.

Results

A total of 1,350 images was analyzed to determine which combination of time and bacterial cell concentration was optimal for adhesion of *S. aureus* to equine corneocytes. Although corneocyte diameter was variable, an average of 2.3 corneocytes were present within each 600-pixel diameter image (data not shown). The ladder test showed that the appropriate transformation for clump size observations was a logarithmic transformation, and this was confirmed with the Shapiro-Wilks test. Using from two to four finite mixtures in the distributional decomposition of transformed clump sizes, it was found that two recurring clump size distributions with transformed means could explain over 90% of the total log variance of the observations. These means, expressed as square root of size units, were 2.7 (95% C.I.: 1.18, 3.67, sd. error = 0.52) and 5.0 (95% C.I.: 3.8, 5.1, sd error = 0.33), respectively. This confirmed that the technique was reproducible between specimens from different horses.

Figure 1 illustrates a typical set of processed images generated by the ImageJ 1.44c software. In Figure 2, the total aerial coverage by adherent bacteria (expressed as pixels) is compared for each combination of incubation time and bacterial cell concentration. At the lowest concentration (OD 0.15) adhesion was poor regardless of incubation time. At the highest concentration (OD 1.5), excessive clumping was subjectively observed and incubation time did not significantly alter surface area coverage. At the intermediate concentration (OD 1.0) adhesion decreased with incubation time in a linear fashion.

Discussion

The objective of this study was to optimize an assay for quantification of *S. aureus* adhesion to equine corneocytes. Optimization is a necessary preliminary step in designing experiments that aim to compare the relative avidity of different *S. aureus* strains for colonization of equine skin. As described by prior investigators, a single strain of the target bacterial species was used for optimization.^{6,8} Optimized assay conditions will subsequently be used to compare the differential adhesive potential of various *S. aureus* strains to equine corneocytes.

The corneocyte collection technique described here is simple to perform. The perineum is the only consistently hairless area of the horse that is easily accessible. This avoids the need to shave hair, and possible micro-trauma from clipper blades which could potentially damage the stratum corneum. None of the horses used in this study objected to the tape stripping or adhesive disc collection procedures. Neither chemical nor physical restraint (beyond haltering) was necessary for any horse.

It should be noted that the ubiquitous melanin granules in horse keratinocytes prevents the simple automated quantification of adherent cocci as reported in cats and dogs. This also prevents correlation of automated counts with manual (operator reported) cell counts. However, other investigators have validated similar imaging software programs for this purpose, and have reported a high coefficient of correlation between automated and manual counts.⁸ The automated color-based segmentation process used was essential to the efficient and accurate processing of the captured images. The values used to filter out non-cocci pixels were determined subjectively. Differentiating cocci from other objects in the images

was exceptionally easy, if time-consuming, when micrographs were examined by a human reviewer. Fortunately, the Threshold Colour software plugin for ImageJ is specifically designed to segment images by color, rather than by contrast and roundness, and contains multiple colorspace and filter options for this purpose. A simple methodical trial of these various colorspace and filter values was performed until values that eliminated all non-cocci pixels were determined. These values were then used to filter and segment all images before pixel quantification. This manipulation also allowed for some subjective validation of the program's efficiency in quantifying what the operator judged to be adherent cocci versus "background noise". However, the final processed images (Figure 1) take on a mosaic appearance. This again precludes quantitative comparison of automated versus manual counts, since the operator can no longer visually appreciate individual bacteria. Variations in technique or reagents could certainly change the optimal filter settings. However, it is exceptionally simple and intuitive to visually adjust the filters for use with different image capture methods, stain colors, or non-melanin confounding objects.

The data reported here suggest that *in vitro* staphylococcal adhesion studies of equine corneocytes should utilize different incubation conditions than have been validated previously for other animal species. The relatively low bacterial concentration (OD 0.15) which has been validated for studies of canine and feline corneocytes⁸ failed to produce sufficient adhesion. In addition, a relatively high bacterial concentration (OD 1.5) resulted in excessive clumping of bacteria, which has been reported by other investigators (reviewed by Lu⁸). As such, neither concentration was suitable for inter-assay comparisons. When the intermediate concentration (OD 1.0) was utilized, a decrease in proportional adhesion was noted with increasing incubation time. This was unexpected, as studies utilizing canine corneocytes for *S. intermedius* adhesion assays demonstrated a sigmoid response to increasing bacterial concentration, with a plateau in adhesion at the highest bacterial concentrations, rather than a reduction of adhesion.⁶ We hypothesize that this trend in our data represents bacterial cell-cell interference after initial equilibrium is established. Therefore, the authors recommend that an *S. aureus* suspension of OD 1.0 (10⁸ CFU/ml) be incubated with equine corneocytes for 45 min at 37° C for optimal comparison between bacterial strains.

Acknowledgments

Source of funding: National Institutes of Health/Merial Veterinary Student Summer Research Program

References

1. Diederer BMW, Kluytmans JAJAW. The emergence of infections with community-associated methicillin resistant *Staphylococcus aureus*. Journal of Infection. 2006; 52:157–8. [PubMed: 16289303]
2. Anderson ME, Lefebvre SL, Rankin SC, et al. Retrospective multicentre study of methicillin-resistant *Staphylococcus aureus* infections in 115 horses. Equine Veterinary Journal. 2009; 41:401–5. [PubMed: 19562904]
3. Weese JS, Rousseau J, Traub-Dargatz JL, et al. Community-associated methicillin-resistant *Staphylococcus aureus* in horses and humans who work with horses. Journal of the American Veterinary Medical Association. 2005; 226:580–3. [PubMed: 15742700]
4. Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. Trends in Microbiology. 1998; 6:484–8. [PubMed: 10036727]
5. Viana D, Blanco J, Tormo-Mas MA, et al. Adaptation of *Staphylococcus aureus* to ruminant and equine hosts involves SaPI-carried variants of von Willebrand factor-binding protein. Molecular Microbiology. 2010; 77:1583–94. [PubMed: 20860091]

6. Forsythe PJ, Hill PB, Thoday KL, et al. Use of computerized image analysis to quantify staphylococcal adhesion to canine corneocytes: does breed and body site have relevance to the pathogenesis of pyoderma? *Veterinary Dermatology*. 2003; 13:29–37. [PubMed: 11896968]
7. Simou C, Hill PB, Forsythe PJ, et al. Species specificity in the adherence of staphylococci to canine and human corneocytes: a preliminary study. *Veterinary Dermatology*. 2005; 16:156–61. [PubMed: 15960628]
8. Lu YF, McEwan NA. Staphylococcal and micrococcal adherence to canine and feline corneocytes: quantification using a simple adhesion assay. *Veterinary Dermatology*. 2007; 18:29–35. [PubMed: 17222237]
9. Everitt, BS. Maximum likelihood estimation: age of onset of Schizophrenia. In: Rabe-Hesketh, S.; Everitt, BS., editors. *A handbook of Statistical Analyses using Stata*. 4. New York: Chapman and Hall/CRC; 2007. p. 263-80.
10. Deb P, Trivedi PK. Demand for Medical Care by the Elderly: A Finite Mixture Approach. *Journal of Applied Econometrics*. 1997; 12:313–26.

Figure 1. Photographic representation of image processing required for quantification of *Staphylococcus aureus* adherence to equine corneocytes. Color figures represent examples of original photographs, while black-and-white figures represent images that have been processed with ImageJ software to eliminate background melanin granules and nonspecific staining. Incubation at nine combinations of time $\times$ bacterial concentration are shown. Original magnification 1,000x.

Figure 2.

Graph of the areal coverage of equine corneocytes by *Staphylococcus aureus* after incubation at 37° C for nine combinations of time × concentration. Optical density (OD) 0.15 $\approx 10^7$ colony forming units (CFU)/ml; OD 1.0 $\approx 10^8$ CFU/ml; OD 1.5 $\approx 10^9$ CFU/ml.