Journal of Microbiological Methods 132 (2017) 63–68

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Increased biofilm formation ability and accelerated transport of *Staphylococcus aureus* along a catheter during reciprocal movements

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ARTICLE INFO

Article history:
Received 11 October 2016
Received in revised form 7 November 2016
Accepted 7 November 2016
Available online 9 November 2016

Keywords:
Staphylococcus
Biofilms
Catheter
Contamination
Infection

ABSTRACT

Staphylococcus spp. is a major cause of device-related infections. However, the mechanisms of deep-tissue infection by staphylococci from the skin surface remain unclear. We performed in vitro experiments to determine how staphylococci are transferred from the surface to the deeper layers of agar along the catheter for different strains of *Staphylococcus aureus* with respect to bacterial concentrations, catheter movements, and biofilm formation. We found that when 5-mm reciprocal movements of the catheter were repeated every 8 h, all catheter samples of *S. aureus* penetrated the typical distance of 50 mm from the skin to the epidural space. The number of reciprocal catheter movements and the depth of bacterial growth were correlated. A greater regression coefficient for different strains implied faster bacterial growth. Enhanced biofilm formation by different strains implied larger regression coefficients. Increased biofilm formation ability may accelerate *S. aureus* transport along a catheter due to physical movements by patients.

1. Introduction

Catheter-, drain-, and other device-related infections are problematic in clinical settings (CDC, , 2011; Walti et al., 2013). In Europe, the incidence density of central line-associated bloodstream infections (CLABSI) is 1 to 3.1 per 1000 patient-days (Suetens et al., 2007), and approximately 80,000 CLABSI are reported among in intensive care unit patients in the United States each year (National Nosocomial Infections Surveillance System, 2004). In previous reports, the incidence of epidural abscesses formation following epidural analgesia was 0.4–0.52 per 1000 epidural catheters (Wang et al., 1999), whereas the infection rate of external ventricular drains was 7.2% (29 out of 403 patients) (Lajcak et al., 2013), and the pin site infection rate was 26.8%–57.5% (Kao et al., 2014).

Staphylococci remain a leading cause of CLABSI, epidural abscesses, and external ventricular drain and pin infections associated with serious complications (Walti et al., 2013; Wang et al., 1999; Lajcak et al., 2013; Kao et al., 2014; Fagan et al., 2013). Staphylococci appear to possess an adhesion function, as evidenced by their ability to form biofilms, which has been implicated in the frequent detection of staphylococci in device-related infections (Foster et al., 2014). A previous study suggested that the formation of biofilms enhanced the environmental survival of staphylococci by providing resistance to phagocytosis and antibiotics (Otto et al., 2014). The mechanisms underlying resistance to antibiotics include not only the ability to form biofilms but also cell wall thickening, which have enhanced the environmental survival of *Staphylococcus aureus* (Cui et al., 2003). Resistance to antibiotics in combination with biofilm formation and cell wall thickening have led to difficulties in treating device-related infections caused by *S. aureus* (Foster et al., 2014; Cassat et al., 2014; Koch et al., 2014). However, the mechanisms underlying long-distance movement by staphylococci from the skin surface to deeper tissues remain unknown because staphylococci lack flagella and are considered nonmotile.

Till date, several measures for preventing infectious complications by minimizing the number of bacteria have been reported (Mangram et al., 1999; Darouiche et al., 2010). In contrast, the repeated changing of a catheter of regional anesthesia or pin of pediatric supracondylar humeral fractures dressing for disinfection has been shown to increase the risk of device-related colonization or infection (Kao et al., 2014; Morin et al., 2005).

This discrepancy in phenomena associated with disinfection suggests that other factors besides an increase in bacterial concentration contribute to catheter-, drain-, and other device-related infections. As one of these other factors, we hypothesized that not only an increase in the concentration of bacteria but also movements of catheters, drains and other devices when changing dressing, in addition to the adhesion

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http://dx.doi.org/10.1016/j.mimet.2016.11.003
0167-7012/

function of staphylococci to the catheter-, drain-, and other device-surface are associated with the development of deeper bacterial growth. Therefore, we performed in vitro experiments to elucidate the mechanisms by which *S. aureus* are transferred from the skin surface to deeper layers of agar along epidural catheters.

2. Materials and method

2.1. Staphylococcus aureus (S. aureus)

Methicillin-sensitive *S. aureus* (MSSA) strains of the American Type Culture Collection (ATCC) 25923 and 29213, and methicillin-resistant *S. aureus* (MRSA) strains of ATCC 700698 (Mu3) and 700699 (Mu50) were provided by the Kitasato University-Laboratory of Infection Control and Research Center. The MRSA strain Oj-one (Makino et al., 2013) was provided by the Central-Laboratory for Department of Infection Control and Department of Plastic, Reconstructive and Aesthetic Surgery, Fukuoka University Faculty of Medicine. All experiments used sterile techniques.

2.2. S. aureus at catheter sites

All experiments did not use animals and human subjects and were performed in the laboratories of the Department of Anesthesiology and the Pathogenic Biology Fukuoka University Faculty of Medicine, Fukuoka University, Fukuoka, Japan. An autoclaved mannitol salt agar medium containing phenol red (Becton, Dickinson and Company, USA) was cooled to 58 °C. Fifty milliliter of the agar medium was placed in sterile, rectangular Petri dishes and allowed to solidify. One hundred millimeter-long disposable epidural polyethylene catheters (external diameter of 1.0 mm, Hakko Co., Ltd., Nagano, Japan), marked at 10 mm increments, were aseptically placed on the agar. The ends of the polyethylene catheters were loosely fixed with sterile glass slides to prevent them from moving. Autoclaved mannitol salt agar (58 °C) was poured into sterile, rectangular Petri dishes and solidified. The ends of the agar were cut to give the agar a width of 50 mm. The length of 50 mm is the typical distance from the skin to the epidural space (Mangram et al., 1999; Darouiche et al., 2010). The polyethylene catheters were not cut. Residual droplets on the insides of Petri dishes were removed using sterile cotton swabs. Minute cracks in the agar surrounding the protruding polyethylene catheter were filled with autoclaved mannitol salt agar. *S. aureus* ferments mannitol and acidifies agar, which changes the amber-red colour of phenol red in the agar to yellow, thereby facilitating the visualization and measurement of its growth in deeper layers of the agar.

MRSA: strain ATCC 700698 (Mu3) was suspended in autoclaved physiological saline at concentrations of 1×10^3, 10^4, 10^5, 10^6, 10^7, and 10^8 c.f.u. per ml. Drops of excess bacterial suspensions on the inside of Petri dishes were removed using sterile cotton swabs. Polyethylene catheter-implanted agar was positioned obliquely for 1 h; thereafter, the polyethylene catheters were moved 50 mm contralaterally to the side to which bacteria had been applied, with the polyethylene catheter tip at the opposite side of the bacterial-applied side being gripped. Bacterial growth on polyethylene catheter surfaces in the agar was measured after 72 h using the scale on a plastic ruler. Five agar specimens were prepared for each concentration, that is, the experiment was replicated five times for each concentration.

2.3. Reciprocal movements of catheter

Polyethylene catheter-implanted agar was prepared as described in the above experiment "*S. aureus* at catheter sites". The strains ATCC 700698 (Mu3) as well as ATCC 700699 (Mu50), 25923, 29213, and Oj-one were used in the above experiment. A suspension of *S. aureus* containing 1×10^8 c.f.u. per ml was applied around the sites where the polyethylene catheters protruded from the agar, as previously described.

The polyethylene catheter-implanted agar was placed obliquely and cultivated at 37 °C for 24 h, the time was defined T-0. When cultured at 37 °C for 24 h, polyethylene catheter-implanted agar was placed obliquely, and the catheters were not moved for the first 24 h. After T-0, polyethylene catheter-implanted agar was positioned obliquely to keep the sides to which bacteria had been applied facing down.

The tip of the polyethylene catheter contralateral to the side to which bacteria had been applied was initially pulled 5 mm contralaterally to the side to which bacteria had been applied, and returned to its original position. It was then pushed 5 mm ipsilaterally to the side to which bacteria had been applied and was returned to its original position. This was defined as 1 reciprocal movement with a 5-mm amplitude. Eleven reciprocal catheter movements were performed at 8 h intervals, taking 88 h in total. Polyethylene catheter-implanted agar was positioned obliquely and cultured at 37 °C after the first reciprocal catheter movement. The depth of bacterial growth along the surface of polyethylene catheters in the agar was measured every 8 h for 88 h after T-0. Five agar specimens were prepared for each *S. aureus* strain, that is, the experiment was replicated five times for each *S. aureus* strain.

2.4. Quantification of biofilm formation

A single colony grown on Tryptic Soy Agar (Becton Dickinson, Massachusetts, USA) of each strain of ATCC 700698 (Mu3), 700699 (Mu50), 25923, 29213, and Oj-one was obtained and incubated at 37 °C in tryptic soy broth (Becton Dickinson, Massachusetts, USA). Bacteria were grown to 0.57 absorbance at a wavelength of 590 nm by a spectrophotometer (GENESYS 10S VIS: Thermo SCIENTIC, Tokyo, Japan). They were diluted 1000-fold in 5 ml of broth contained in a round-bottomed polypropylene 12-ml tube (SARSTEDT, Tokyo, Japan) and cultured at 37 °C for 24 h. The supernatant (containing nonadhered cells) was removed from each tube and the biofilm on the tube surface was rinsed twice using 10 ml phosphate buffered saline (pH 7.4, 0.01 M). The biofilm was then fixed for 10 min in 10 ml of 99% ethanol, after which it was air-dried.

The biofilm was stained for 10 min with 1 ml of a 0.41% crystal violet solution (Sigma Aldrich, Tokyo, Japan). When the biofilm was stained, the tube was tilted obliquely and rotated in order to spread the crystal violet solution to the biofilm evenly. After staining, the biofilm was washed for 15 min in running tap water. Crystal violet remaining in the biofilm was extracted using 2 ml of 30% acetic acid with a vortex mixer. Its absorbance was measured using a spectrophotometer at 590 nm. Absorbance was defined as the amount of biofilm formed on the surface of the polypropylene tube.

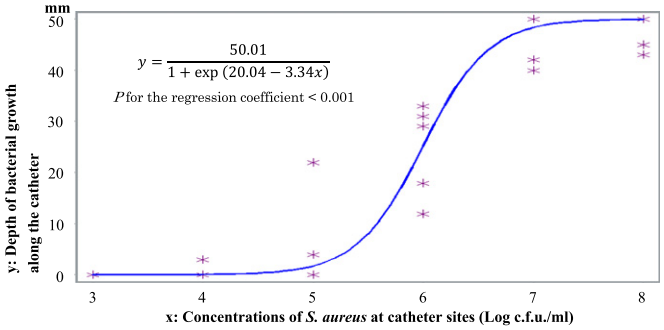

Fig. 1. *S. aureus* concentration effects at catheter sites on bacterial growth depth along a catheter moved 50 mm. The abscissa of the graph represents "x," indicating logarithmically transformed concentrations of *S. aureus* at the catheter sites. The ordinate of the graph represents "y," the depth of bacterial growth along the catheter. *: Observations. There were five specimens at each concentration (Number of samples = 5). Some overlapping data exist.