

A Novel “Inocap” Method for Homogeneous Catheter Hubs Bacterial Inoculation Using a Combination of Male Luer Caps and O-rings

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Abstract

Catheter-related bloodstream infections (CRBSIs) pose significant health risks and financial consequences for healthcare systems worldwide. In order to develop novel antiseptic technologies as alternative prophylactics for CRBSIs, a consistent and reproducible bacterial inoculation method is required for testing the technologies' antiseptic effects. This study developed and investigated a new “Inocap” model for standardized bacteria inoculation on to surfaces of catheter hubs that is exposed and highly susceptible to contamination in healthcare settings. The results showed a significantly smaller variance in the number of bacteria inoculated onto the catheter hubs by the new Inocap method than the traditional dip method ($p \approx 0.02$) while producing no significant difference in the mean value ($p > 0.25$), indicating that this new Inocap method could work as a more precise inoculation model for future disinfection studies.

Keywords: Bacteria inoculation model, disinfection, catheter related bloodstream infections, intraluminal infection, catheter hubs, contamination

Catheter-related bloodstream infections (CRBSIs) are associated with substantial morbidity and cost. In the United States alone, over 250,000 cases occur each year with a mortality rate as high as 25%, increasing both the length of hospital stay and risks for other diseases like endocarditis and osteomyelitis.¹ In addition to the direct serious health consequences, CRBSIs also come at a heavy financial price, costing the healthcare system an estimated 2.68 billion USD annually.²⁻⁵ Central line maintenance bundles—systems of interventions that often include hand-hygiene training, maximal sterile barrier precautions during central line insertion, use of chlorhexidine during skin preparation, and avoidance of the femoral placements for central lines—have generated a lot of excitement for their effectiveness at reducing rates of CRBSIs in intensive care unit (ICU) settings.⁶⁻⁹ However, compliance with the established protocols remains a key challenge with some studies reporting healthcare professionals following the recommendations only 35-45% of the time.^{10,11}

Consequently, there has been a growing imperative to investigate novel and more passive technologies, which can improve prevention rates for CRBSIs while reducing the workload placed on hospital staff. Testing these new technologies aimed at preventing CRBSIs will first require a sensitive and reproducible method of introducing appropriate contaminants into in vitro systems in order to accurately evaluate any antiseptic effects. Historically, the preferred inoculation model has been the dip method, where devices of interest such as catheter hubs or needleless connectors are immersed in a suspension of bacteria for a period of time and then allowed to dry.¹²⁻¹⁶ However, significant variability between studies in the specifics of their methods, including differences in solutions, concentrations, immersion time and drying time, make it difficult to compare their effectiveness. Moreover, many of the studies fail to quantify the bacteria inoculated, instead reporting solely the presence or absence of bacteria on inoculated surfaces after disinfection, ignoring the possibility of irregular applications.^{12,14,15,17}

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Therefore, this study aimed to test results from the conventional dip method against a novel “**Inoculation cap**” or “**Inocap**” model. The Inocap model proposed in this study was designed to have better control of the target area for inoculation around the outer surfaces of the luer hub, transfer relevant and consistent amount of bacteria that occur inside hospitals, and increase repeatability between samples by reducing human error. This model would be representative of the contamination process commonly occurring in hospital settings, leading to intraluminal infection in catheterized patients. Methicillin-resistant *Staphylococcus aureus* (MRSA) was chosen for this study due to its character as one of the most commonly found pathogens associated with nosocomial infections.¹⁸

Materials and Methods

Bacteria Stock Suspension Preparation in TSB and NSS

Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 was used as model organism because MRSA is frequently responsible for CRBSIs and represents a highly antibiotic-resistant organism. MRSA ATCC 43300 colonies were suspended and streaked onto blood agar (Biomedica, Thailand) and incubated at 37°C for 48 hours by National Healthcare Systems Co., Ltd. (Thailand). The colonies were then diluted to the desired bacteria stock concentration, 0.5 McFarland Standard or $\sim 1.5 \times 10^8$ colony-forming units (CFU)/mL, in Tryptic Soya Broth (TSB, Himedia, India) or 0.9% Normal Saline Solution (NSS) depending of the experimental groups.

Bacteria stock concentrations were measured using McFarland standards (DensiCHEK, Biomerieux, France) and further confirmed by serial dilution and spread on Tryptic Soy Agar (TSA).

Hub Inoculation

For the Inocap method, propylene vented male luer caps, no stem (Inoculation Cap Model No. 12084, Qosina, USA) were used to inoculate catheter hubs in an effort to standardize the inoculation process. To ensure firm contact with the catheter hubs, each Inocap was inserted with an O-ring made of EDTA (O-ring Store, USA). The combination of a male luer cap and an O-ring is shown as insets in Figure 1(a). One of the reasons EDTA was chosen as a material for the O-ring was for its capacity to chelate and potentiate cell walls of bacteria which would prevent biofilms from forming and better inoculate single cells.¹⁹ This was important to ensure uniform application onto each female luer.

The empty Inocaps were prepared by placing them on a tray made in-house, fully wrapped by a sheet of aluminum foil, and sterilized by autoclaving at 121°C and 15 PSI for 15 minutes. 275 μ L of bacterial stock in either TSB or NSS were then added into each Inocap and kept at 4°C for 24 hours. The refrigeration period allowed bacterial cells to adhere to the surfaces of the caps and the surface of the O-rings. The same volume of bacteria stock was then removed using a micropipette. Finally, the Inocaps were blot dried on autoclaved Kimwipe tissues to remove remaining fluid on the caps. Figure 1 illustrates the Inocap preparation procedures.

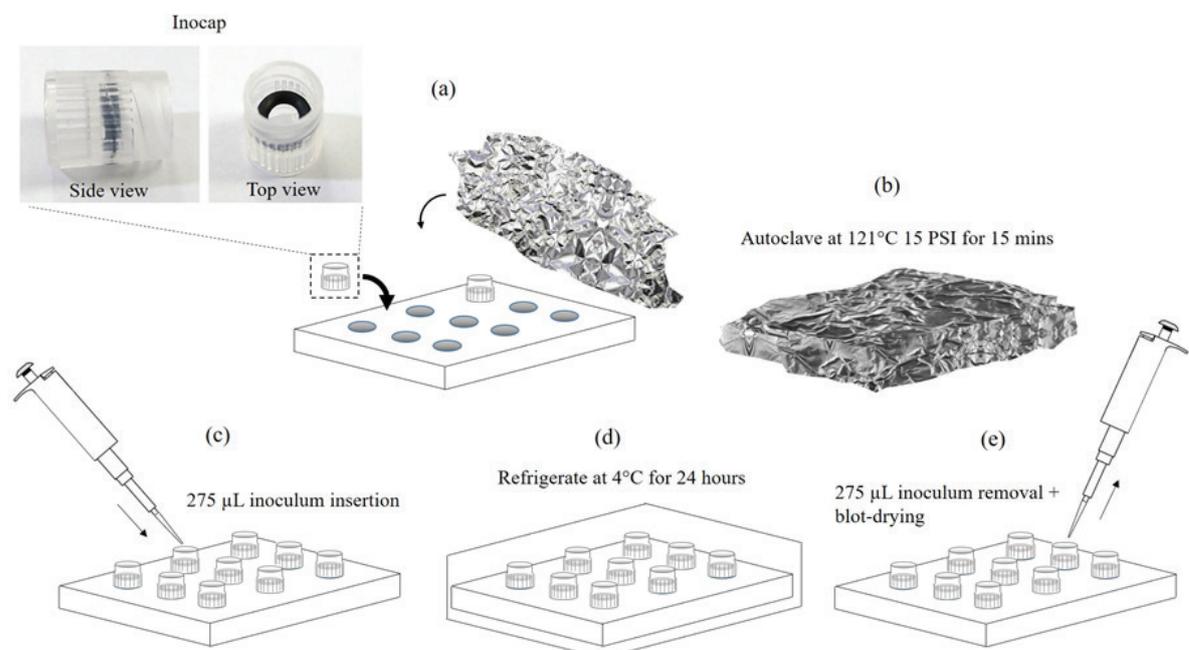


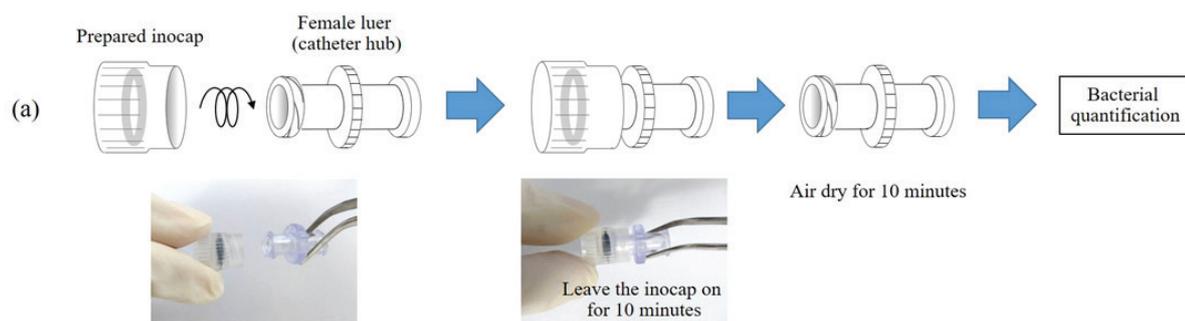
Figure 1: Inocap preparation process (a) picture of an Inocap and their placements on an autoclavable tray wrapped by aluminium foil; (b) caps sterilization; (c) addition of bacterial stock; (d) refrigeration in a sterile box; and (e) inoculum removal.

To perform the inoculation using the prepared Inocaps, the caps were screwed on to the female end of Medical-grade female luer (Nordson Medical, Fort Collins, USA), which serves as the catheter hubs in this experiment, for 10 minutes. The female luer was then allowed to air dry for 10 minutes before the bacteria quantification process was performed.

The luer inoculated using the dip method were done according to the protocol developed by Maki & Menyhay.⁸

where the female luer was inoculated by immersing the female luer threads in a suspension of MRSA containing $>1.5 \times 10^8$ colony-forming units (CFU)/mL for 10 minutes, after which the female luer was allowed to dry in a protected aseptic container for 10 minutes.¹⁴ The two inoculation methods are shown in contrast in the diagrams in Figure 2. The drying procedure was performed under standard room temperature (22 - 24°C) and humidity conditions (40 - 50%).

Inocap method



Drip method

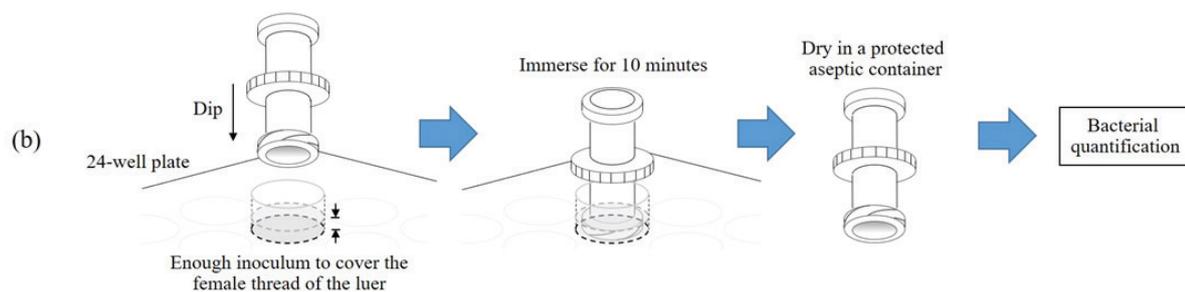


Figure 2: Inoculation with (a) the Inocap method; and (b) the traditional dip method.

Quantification of Bacteria

Each inoculated female luer (catheter hub) was transferred into 10 mL of phosphate buffered saline (PBS P4417-100TAB, Sigma-Aldrich, USA, pH 7.4) where it was vortexed for 15 seconds, sonicated for 1 minute (at 42 kHz and 110 W), and vortexed again for 15 seconds to ensure that all bacteria on the female luer is detached and suspended in PBS. The concentration of bacteria on the female luer was ascertained through serial dilutions and spread plating on TSA plates. All procedures were performed in a Class II Biological Safety Cabinet (BSC).

Statistical analysis

The effect of inoculation method and inoculum diluent were analyzed using the means and variances of the relative log reduction of the inoculated bacterial concentration from the initial bacterial stock solution concentration. The results

were compared using Equality of Variances F-Tests and then followed up with either Welch's t-tests or unpaired t-tests if equal variance was found to determine significance. Statview (v5.0) software (SAS Institute) was used to run the analysis and graphs were generated using Prism (v7.00) software (GraphPad). Alpha was set to 0.05 for all analysis and data are expressed as means \pm Standard Error of the Mean (SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Catheter Hubs Inoculated with the Inocap Method Show Greater Consistency

The inoculated bacterial count on the female luer was measured in CFU per millilitre of PBS by the procedure outlined in the bacterial quantification section. The final results were recorded in the form of log reduction which quantifies the difference between the bacterial stock concentration and

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the resulting inoculated bacterial concentration. The stock concentration of the Inocap method group and the dip method group were 1.25×10^8 and 1.64×10^8 CFU/mL, respectively.

A significant difference ($p \approx 0.02$) in the variance of log reduction between the dip method and the Inocap method was

found (Figure 3a), with those subjected to the Inocap method showing a smaller standard error (Table 1). Moreover, there was no significant difference in the means of each group ($p > 0.25$), suggesting that the Inocap method was able to produce the same desired final inoculated concentration of approximately $\sim 1.5 \times 10^5$ CFU/mL with greater reliability.

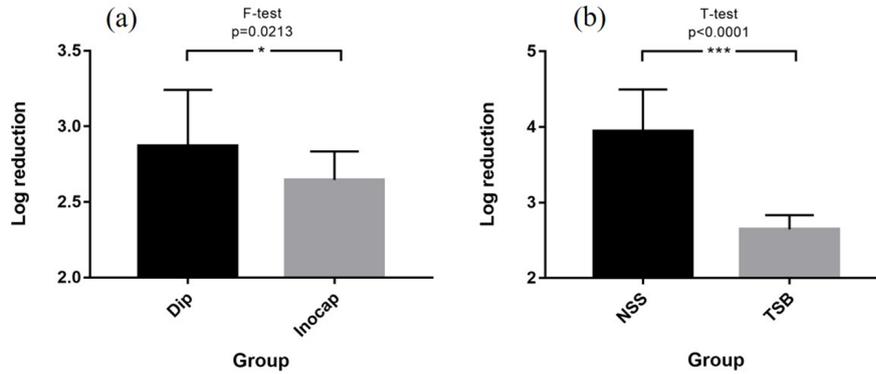


Figure 3: Log reduction showing the amount of inoculated CFU harvested from luers relative to the initial starting bacteria stock concentration. (a) Inocap and Dip methods show no significant difference in the average amount inoculated, but the dip method yields a higher variance. (b) All luers for both the TSB and NSS groups were inoculated using the Inocap method. F-test shows significant difference in variance between TSB and NSS and the TSB group showed significantly less log reduction than the NSS group.

Table 1: A summary of the standard error, average log reduction measurements, and F-test and t-test p-values for the different groups of luers organized by inoculation method and inoculum diluent

Figure	Inoculation Method / Diluent	Inoculated Bacterial Concentration (The range of CFU/mL)	Average Log Reduction	Standard Error	Number of samples	Comparison of Variance (F-Test p-value)	Comparison of Means (t-test p-value)
3 (a)	Dip method	2.22×10^5	2.869296	0.166537	5	0.0213	0.2543
	Inocap method	3.60×10^5	2.540361	0.032727	33		
3 (b)	In TSB	3.60×10^5	2.540361	0.032727	33	< 0.0001	< 0.0001
	In NSS	1.14×10^4	4.040603	0.131232	18		

Bacteria Suspensions Using TSB Inoculate Higher Concentrations for Aseptic Tests

Previous literature used various bacterial stock diluents to prepare bacteria inoculation solution.12-14 In this study, two different solutions, TSB and NSS, were used as bacterial stock diluents. The bacterial stock diluents were tested with the Inocap method for relevant and consistent of quantity of bacteria inoculated. There was a significant difference ($p < 0.0001$) in the average bacteria counts inoculated between the NSS and TSB bacterial stock diluents. The TSB solution yielded 3.60×10^5 CFU/mL, which is a 2.5-log reduction from the 1.25×10^8 CFU/ mL of stock concentration. The NSS solution inoculated lower amount of bacteria on to the female hub. It yielded 1.14×10^4 CFU/mL, which is a 4.0-log reduction from the 1.14×10^8 CFU/ mL stock concentration (Figure 3b). There was also a significant difference in the variance between the two groups ($p < 0.0001$), indicating that polymer surfaces

inoculated using TSB with the Inocap method yielded higher concentrations of bacteria and smaller variations, than those prepared with NSS.

Discussion

The results were presented as a log reduction in bacteria stock concentration to normalize for experimental variation in the starting stock concentrations between the groups. The data showed that the inocap method had no significant difference in average inoculated bacterial count as the dip method but had a lower variance (Figure 3a). This means that the inocap model can reduce uncontrollable model error in experiments testing for disinfectant efficacy studies. It is important to acknowledge that the greater variance seen with the dip method could in part be due to the smaller sample size of the dip group (5 compared to 33), but this was taken into consideration when performing statistical analysis. The

improved consistency of the inocap method can be explained by how it standardizes the inoculation procedure in two ways, first by mixing and then promptly aliquoting the stock solution to ensure equal distribution, and secondly by using an O-ring and threads along the luer connector to control the surface that comes into contact with the luer. This is in contrast with the dip method that requires scientists to manually submerge each luer sequentially in the stock solution, with no control over the concentration of bacteria currently in contact with the surface, nor the exact surface area that comes into contact with the stock solution.

The reason why bacteria stock solutions prepared in NSS inoculated much lower concentrations onto the luer connectors (Figure 3b) could be due to the viscosity of the solution or the fact that TSB is a buffered enrichment medium with proteins and nutrients, and can support bacterial activity. NSS lacks the ingredients that support bacterial activity but is still able to prevent lysis of bacterial cells. Viable bacterial cells remained suspended in NSS, and the majority is removed from the Inocaps with the removal of the inoculum fluid.

Converting from analytical solution concentration of inoculated female luer (CFU/mL) or TSB bacteria stock concentration to absolute counts (CFU), the average bacteria count on luers using NSS-Inocap method was 1.14×10^5 CFU, whereas the average bacteria count on luers using TSB-Inocap method was 3.60×10^6 CFU. The TSB-Inocap method counts were closer to the desired value of 1.0×10^6 CFU and could be fine-tuned further by adjusting the initial inoculum concentration. Bacterial count of 1.0×10^6 CFU is desired because it is sufficiently high to prove a 4-log reduction efficacy for medical device disinfection as required by the United States Food and Drug Administration (FDA) and the within the upper bound of the bacteria count commonly found in hands of medical personnel.²⁰ According to the Centers for Disease Control and Prevention (CDC) transient microflora on hands of healthcare workers from hand contact is a

common source of contamination in hospital settings.²⁰ Consequently, the use of TSB as the diluent was found to be more suitable for the Inocap method since it achieved quantities within the desired range explained above. The higher variance from NSS group indicated that bacterial stock diluent is also one of the factors that strongly affect the consistency of the inoculation concentration.

Conclusion

This study developed and investigated a new “Inocap” method for standardized bacteria inoculation on to controlled target surfaces of catheter hubs. The results showed a significantly smaller variance in the number of bacteria inoculated onto the catheter hubs by the new Inocap method than the traditional dip method ($p \approx 0.02$) while producing no significant difference in the mean value ($p > 0.25$), indicating that this new Inocap method could work as a more precise inoculation model for future disinfection studies.

TSB and NSS were also tested as the bacterial stock diluents with the Inocap method and found a significant difference in the average amount of inoculated bacteria, with TSB solutions yielding higher amount inoculated than NSS solutions. A significant reduction in the variance was also found, indicating that the use of TSB as the diluent with the Inocap method provided a better consistency during the inoculation process.

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