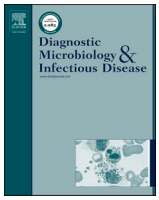
Diagnostic Microbiology & Infectious Disease 108 (2024) 116192

Contents lists available at ScienceDirect

Diagnostic Microbiology & Infectious Disease

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



Original Article

Comparative analysis of different loop types for urine culture collection: Implications for quantitative bacterial growth and culture results

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ARTICLE INFO

Keywords:
Loop
Quantitative analysis
Urine culture

ABSTRACT

We hypothesized that the loop material and size could affect the results of the culture when compared to the calibrated pipette. A total of 484 urine samples were included in the study, and each sample was plated by using different loop types and the calibrated pipette. The bacterial counts per milliliter were calculated and compared, with a focus on the important cutoff values of 10^3 and 10^4 CFU/ml for further identification. When considering the 10^3 CFU/ml as cutoff value, 1 µl and 10 µl plastic loops gave the highest sensitivity (86.8 %), whereas the 10 µl metal loop had the lowest sensitivity (64.2 %). For the 10^4 CFU/ml cutoff value, 1 µl plastic loop inoculation demonstrated the highest sensitivity (75.9 %), while the 10 µl metal loop provided the lowest sensitivity (26.5 %). These results suggest that the single use plastic loops are functional, sensitive, useful especially for critical sample.

1. Introduction

Urinary tract infections (UTIs) encompass a range of potentially life-threatening diseases, including urethritis, cystitis, and pyelonephritis. If left untreated, infections such as cystitis can lead to urogenital tract infections like prostatitis and epididymoorchitis in men. In severe cases, infections can ascend to cause pyelonephritis or urosepsis, posing a life-threatening condition [1,2]. It is crucial to determine the bacterial load and antibiotic susceptibility in a urine sample for effective clinical management.

In urine cultures, colony counting is a crucial procedure as it enables the precise identification of bacteria and facilitates the determination of antibiotic susceptibility testing testing. This method is essential for understanding infections agents and guiding effective treatment strategies.

Very early studies have considered a threshold of $\geq 10^5$ CFU/ml of pure gram-negative bacillus growth in clean catch midstream urine (CCMSU) culture to indicate acute bacterial UTI, eventhough the authors suspected that the low bacterial count in culture still might be associated with the infection [2,3]. Subsequent research in later years has demonstrated that bacterial counts as low as 10^2 CFU/ml in symptomatic women, infants, and catheterized patients may be indicative of infection. The absence of studies on urine cultures in the male population can be attributed to the limited number of patients available for such research. Different guidelines suggest varying suggested threshold for bacterial counts, ranging from $\geq 10^3$ to $\geq 10^5$ CFU/ml, based on the clinical context [4–6]. For CCMSU, microbiology guidelines recommend further identification and antibiotic susceptibility testing (AST) when the bacterial count is $\geq 10^4$ CFU/ml, while minimal identification is sufficient for counts below this threshold. In invasive urine samples (straight catheter, suprapubic needle aspiration, cystoscopy samples etc.), the limit value for growth of a single type of uropathogenic bacteria is as low as 10^3 CFU/ml [6,7]. Therefore, the accuracy of the bacterial count obtained from urine culture is crucial for determining the need for additional laboratory studies and ensuring optimal patient treatment. However, clinical microbiology guidelines acknowledge that colony counts may vary up to 100 times unless calibrated pipettes and spreaders are used [6].

In this study, we aimed to evaluate the ability of metal and plastic calibrated loops to quantify bacterial content, particularly in urine samples containing low bacterial concentrations. To investigate these potential variations, we inoculated urine samples using different-sized metal and plastic loops available on the market, as well as a calibrated pipette that is considered to accurately indicate bacterial concentration. The median values of bacterial counts detected through different

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https://doi.org/10.1016/j.diagmicrobio.2024.116192

Available online 24 January 2024

inoculation methods in culture were determined, compared with each other and assessed for further identification and antibiotic susceptibility testing using the cutoff values suggested by the guidelines. The purpose was to determine the impact of method accuracy on the laboratory practice. The present study aims to scrutinize one of the most frequently exercised but overlooked aspect of clinical microbiology practice.

2. Material and methods

The study received approval from the Istanbul Bezmialem University Non-Interventional Clinical Research Ethics Committee (Approval No: 54022451–050.01.04). The study included urine samples collected for culture between January 2018 and May 2018. All incoming urine samples were processed within half an hour and transferred to + 4 °C. Media plates showing bacterial growth of <20,000 CFU/ml with single type uniform bacteria after 24 hours were included in the study, while samples with multiple morphotypes of bacteria or growth exceeding ≥20,000 CFU/ml were excluded.

In the study, urine samples were inoculated on 5 % sheep blood agar (BD Diagnostic Systems, Sparks, MD). Plating was carried out using disposable 1 μl and 10 μl plastic ring loops (BD Diagnostic Systems, Sparks, MD) for each urine sample, while reusable 1 μl and 10 μl metal ring loops (Copan, Italy) were sterilized with fire before each plating. During sampling, the loops were held in a vertical position, and the sampling depth was limited to a maximum of 1 cm in the urine. The pipette method involved taking 10 μl of urine from each sample with a calibrated pipette (Finnpipette, Thermo Scientific, Finland) and dropping it onto the medium, followed by spreading on the surface with a single-use spreader (Interlab, Turkiye) [6]. Therefore, each urine sample was inoculated using 5 different plating methods.

2.1. Incubation and evaluation of cultures

The cultured media were incubated at 35.5 °C for 24 h, and colony counting was performed the following day. Colonies of 200 or more were considered as ≥ 200 due to the difficulty of accurate counting. Urine samples with a colony count of ≥ 200 in any culture method were excluded from data evaluation to maintain consistency. Since an exact value could not be measured for colony counts, including them would affect the central value when comparing variables across cultivation methods.

2.2. Statistical analysis of colony numbers

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the plating methods were separately calculated for two cutoff values: 10^3 and 10^4 CFU/ml, respectively.

2.3. Data analysis

The normal distribution of the data was assessed using the Kolmogorov-Smirnov test. Since the p values obtained as a result of the Kolmogorov-Smirnov test were less than 0.05, indicating that the data were not normally distributed, nonparametric tests were applied. Median differences between the pipette method and other inoculation methods were evaluated using the Wilcoxon Signed Ranks Test. Comparison of the median bacterial scores (BCM) among the inoculation methods (1 μl metal, 10 μl metal, 1 μl plastic, and 10 μl plastic) was conducted using the Friedman's test, which is suitable for non-normally distributed data. Post hoc analysis was performed using the Bonferroni test. Descriptive statistics, including median (Q1 and Q3), were presented for continuous variables. Statistical significance was set at $p < 0.05$. The IBM SPSS Statistics for Windows program, version 26 (IBM Corp., Armonk, NY, USA), was used for all statistical analyses.

3. Results

A total of 484 urine samples submitted to Microbiology Laboratory of Istanbul Bezmialem University Hospitals were included in the study. Each sample was cultured using the five different methods, resulting in a total of 2420 cultures.

Among the 484 inoculated urine samples, 50 samples with a colony count ≥200 were excluded for statistical consistency. The median and p-values of the remaining 434 samples' BCM were calculated and compared with the pipette method individually (Table 1 and Fig. 1).

According to the plating methods, the normal distribution of the BCM variable was examined with the Kolmogorov-Smirnov test, and it was observed that it did not follow a normal distribution ($p < 0.05$). Therefore, for the comparison of the BCM variable according to plating methods, the Friedman's test was employed, and pairwise comparisons of the significant results were examined using the post-hoc Bonferroni test. Additionally, the comparison of each plating method with the pipette method was conducted using the Wilcoxon Signed Ranks Test. A comparison of BCM values demostrated that median BCM of the 1 μl and 10 μl metal loops were significantly lower than the pipette ($p = 0.013$ and $p < 0.001$), BCM of the 1 μl plastic loop were significantly higher than the pipette ($p < 0.001$) while the median BCM of the 10 μl plastic loop did not significantly differ from the pipette method ($p = 0.397$).

Significant differences were found between the median BCMs among the inoculation methods (1 μl metal, 10 μl metal, 1 μl plastic, and 10 μl plastic). After the Friedman's test, a post-hoc test was applied to examine pairwise comparisons. When we compare these plating methods with each other, there was no significant difference between the BCMs calculated in the combination of 1 μl plastic and 10 μl plastic loop ($p = 0.283$), but there is a significant difference in other combinations (Table 2).

Out of the 484 urine samples included in the study, the pipette method detected growth of ≥10^3 CFU/ml in 310 samples. Sensitivity, specificity, PPV, and NPV of inoculation methods were compared to the pipette method and are presented in Table 3. The methods with the highest sensitivities were 1 μl (86.8 %) and 10 μl (86.8 %) plastic loops and the method with the lowest sensitivity was the 10 μl metal loop (64.2 %). For the same cutoff value, the method with the highest PPV was the 10 μl metal loop (86.9 %), while the 1 μl plastic loop exhibited the lowest PPV (75.8 %) (Table 3 and Fig. 2).

At Table 4, the sensitivity, specificity, PPV, and NPV of the methods are calculated with 10^4 CFU/ml as treshold colony count and the results are shown. Accordingly, the method with the highest sensitivity was the 1 μl plastic loop (75.9 %), and that with the lowest sensitivity was the 10 μl metal loop (26.5 %). Among the 83 samples with a growth of ≥10^4 CFU/ml detected by the pipette method, and in 61 samples (73.5 %), the growth was below the treshold colony count when plated with the 10 μl metal loop. Here, the method with the highest PPV was the 10 μl metal loop (100 %), while the 1 μl plastic loop had the lowest PPV (69.2 %) (Table 4 and Fig. 3).

4. Discussion

In this study, we aimed to evaluate the ability of metal and plastic calibrated loops to quantify bacterial content, particularly in urine samples containing low bacterial concentrations. Urine samples

Table 1
Median (Q1–Q3) and p values of plating methods compared to pipette (Wilcoxon Signed Ranks Test).

Plating method	CFU/ml(Q1-Q3)	Pipette CFU/ml(Q1-Q3)	p
1 μl metal loop	1000(0-3000)	1400(500-3500)	0,013
10 μl metal loop	800(300-1500)	1400(500-3500)	<0,001
1 μl plastic loop	2000(0-5000)	1400(500-3500)	<0,001
10 μl plastic loop	1500(700-3300)	1400(500-3500)	0,397

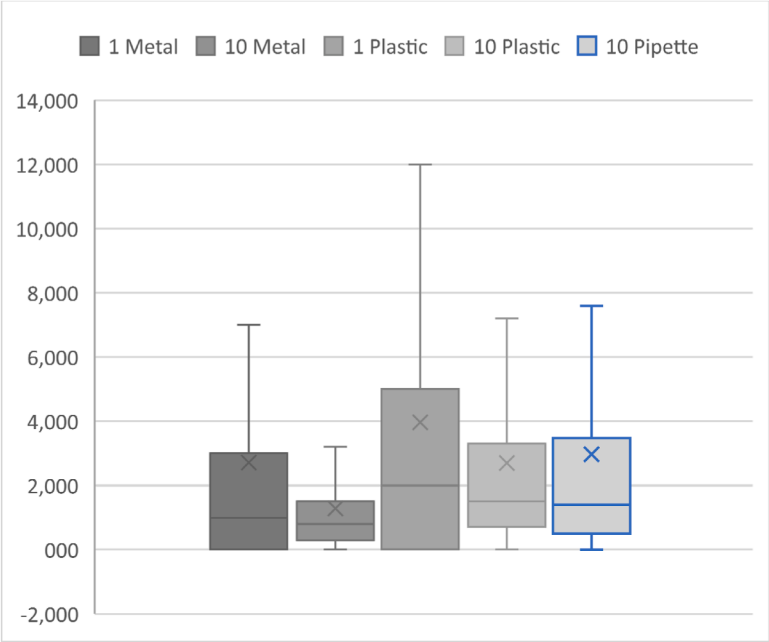

Fig. 1. Colony counts of different plating methods.

Table 2
Pairwise comparation between plating methods for BCM.

	1 µl metal-10 µl metal	1 µl metal-1 µl plastic	1 µl metal-10 µl plastic	10 µl metal-1 µl plastic	10 µl metal-10 µl plastic	1 µl plastic-10 µl plastic
p value	<0,001	<0,001	<0,001	<0,001	<0,001	0,283

Table 3
When the culture results are evaluated considering the 10^3 CFU/ml cutoff value, the sensitivity, specificity, PPV and NPV of the plating methods.

10^3 CFU/ml %95 confidence interval	1 µl metal loop (%)	10 µl metal loop (%)	1 µl plastic loop (%)	10 µl plastic loop (%)
Sensitivity	82,6	64,2	86,8	86,8
PPV	78,3	86,9	75,8	79,6
Specifitiy	59,2	82,8	50,6	60,3
NPV	65,6	56,5	68,2	71,9

Table 4
When the culture results are evaluated considering the 10^4 CFU/ml cutoff value, the sensitivity, specificity, PPV and NPV of the plating methods.

10^4 CFU/ml %95 confidence interval	1 µl metal loop (%)	10 µl metal loop (%)	1 µl plastic loop (%)	10 µl plastic loop (%)
Sensitivity	67,5	26,5	75,9	63,9
PPV	81,2	100,0	69,2	85,5
Specificity	96,8	100,0	93,0	97,8
NPV	93,5	86,8	94,9	92,9

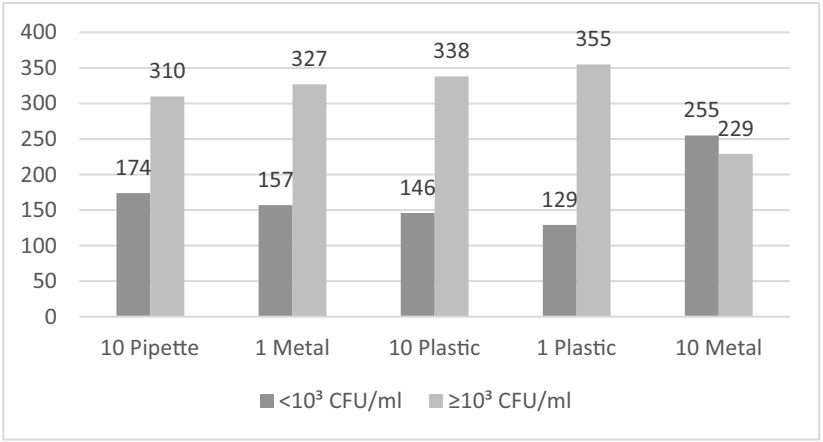

Fig. 2. Status of bacterial amounts in plating methods considering the 10^3 CFU/ml cutoff value.

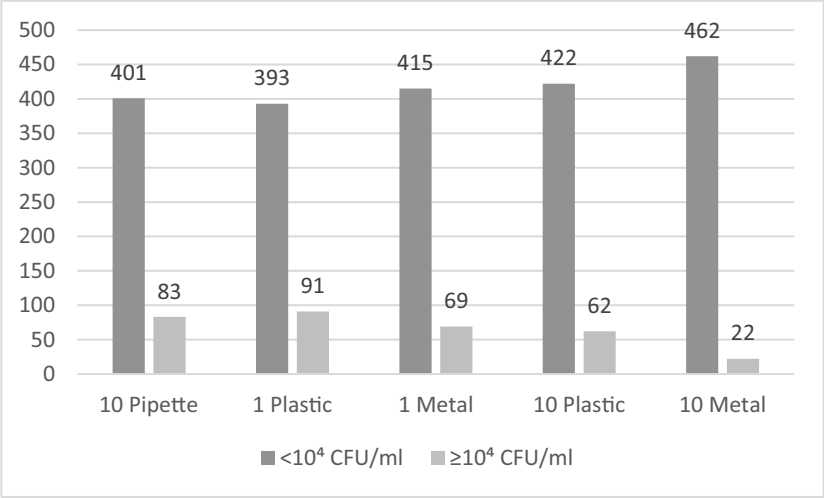

Fig. 3. Status of bacterial amounts in plating methods considering the 10^4 CFU/ml cutoff value.

received in our laboratory for culture were inoculated using various commercially available metal and plastic loops, as well as a calibrated pipette. Then, the median bacterial colony counts (BCM) observed following inoculations were calculated. Accordingly, the median BCM of the 10 µl plastic loops were not significantly different from the pipette ($p = 0.397$). Surprisingly, this median BCMs of the both 1 µl and 10 µl metal loops were significantly lower than the pipette ($p = 0.013$ and $p < 0.001$), BCM of the 1 µl plastic loops were significantly higher than the pipette ($p < 0.001$). In most high volume laboratories, plastic inoculation loops are used; however, metal loops are still used in some laboratories and therefore, our data underline the need to validate the inoculation methods at regular intervals.

The median BCM values of the culture results were compared using the breakpoints of 10^3 and 10^4 CFU/ml, which are commonly used tresholds for advancing to bacterial identification and antibiotic susceptibility testing studies in clinical microbiology laboratories. When considering 10^3 CFU/ml as the treshold value, bacterial growth in 310 out of 484 urine samples were detected in calibrated pipette group. The sensitivity, specificity, positive predictive value (PPD), and negative predictive value (NPD) of the other inoculation methods are shown in Table 3. Accordingly, the methods with the highest sensitivity were the 1 µl (86.8 %) and 10 µl (86.8 %) plastic loops, while the lowest sensitivity was observed with the 10 µl metal loop (64.2 %). Similarly, when considering 10^4 CFU/ml as the limit value, growths in 83 out of 484 urine samples plated with a pipette were observed. At Table 4, the sensitivity, specificity, PPD, and NPD of the other plating methods were evaluated based on the same limit and the results are shown. The method with the highest sensitivity was the 1 µl plastic loop (75.9 %), while the lowest sensitivity was observed in the 10 µl metal loop group (26.5 %).

In the study, angle of exit of the loop from the sample liquid, as it is known to affect the results after plating was taken into consideration. In a study conducted in 1983 by Albers and Fletcher, the accuracy of 1 µl metal loops was tested, and they reported that the amount taken by the loop can vary depending on the angle of exit [8]. Haugen et al. also found that different amounts of liquid may be taken when the loop is immersed vertically or horizontally during sample collection, and they stated that immersing it vertically yields more accurate results [9]. In our study, we made sure that the loop was exited vertically in all samplings to rule out the possible influence of this point on growth.

Similar to our study, in 2016, the performance of different devices in urine culture inoculation was quantitatively evaluated by others and the investigators used automated inoculation machines (Copan WASP [Copan, Brescia, Italy], BD InoqulA [BD Kiestra B.V., Drachten, Netherlands]) with 1 µl and 10 µl metal loops as well as calibrated pipettes. Authors reported discrepancies between methods and linked the variation to the factor that during the pick up of the sample, pipetting device had a sensor enabling a standardized amount of urine was plated. On the other hand, in the device that used the loop the amount of urine might have been influenced by the depth of sample especially in fully filled tubes. The authors concluded that depth of immersion of the loop could affect the colony counts in non-uniformly filled sample containers [10]. In our study, we considered this caveat and immersed the loops to a standardized depth of 1 cm below the liquid surface.

Earlier in another study, Frimodt-Moller and Espersen compared 1 µl and 10 µl plastic loops and reported that the 10 µl loops took approximately 1.5 times more liquid than expected, resulting in higher median BCM values. Authors concluded that the use of 1 µl loops is not appropriate for samples containing less than 10^4 CFU/ml of bacteria [11]. In our study, we made the same comparison and found that the median BCM of the 1 µl plastic loop was higher than that of the 10 µl plastic loop, but this difference was not significant ($p = 0.283$).

Clinical microbiology guidelines recommend performing antibiotic susceptibility test and full identification in case of $\geq 10^4$ CFU/ml uniform uropathogen growth in urine cultures in both gender. Also, $\geq 10^3$ CFU/ml of single-type uropathogenic bacteria growth is considered significant in women aged 14–30 years, and full identification and antibiotic susceptibility testing is advised in these samples [6]. In our study, we observed that 1 µl and 10 µl plastic loops had the highest sensitivity for $\geq 10^3$ CFU/ml, while the highest sensitivity was found with the 1 µl plastic loop for $\geq 10^4$ CFU/ml. The method with the lowest sensitivity for both limits was noticed in the 10 µl metal loop sampling. Therefore, with metal loops it would be likely that some samples might have been omitted from identification and susceptility studies. For some samples plated with metal loops, delivery of optimal patient care might adversely be affected.

Studies comparing methods in urine cultures are unfortunately rather rare in the literature. In most studies, urine samples used were prepared by spiking with various number of bacteria and the critical tresholds of breakpoints of 10^3 and 10^4 CFU/ml were usually overlooked [8,10]. In this study, we compared different inoculation methods using patients' urine samples, especially those containing low bacterial concentrations, and took into account threshold values. Therefore, our data may represent a more realistics and an actual clinical microbiology laboratory set up.

In conclusion, especially in invasive urine samples and critical patient groups such as nephrological and pediatric patients, where even low bacterial concentrations are important, plastic loops may be

preferred in order not to miss the possible infectious agent. The data presented here underlines the importance of guidelines which states that each laboratory should validate their individual plating method to be used [6]. Our study has some limitations. First of all, the Clinical Microbiology Procedures Handbook's urine culture workup guideline emphasize the importance of 10^5 CFU/ml breakpoint for 1 or 2 uropathogens in urine samples (such as voided midstream urine, indwelling catheter, suprapubic catheter, ileal conduit) in addition to the 10^3 and 10^4 CFU/ml breakpoints that we evaluated in our study [6]. The exclusion of urine samples that growth 1 or 2 uropathogens with $\geq 10^5$ CFU/ml one of the limitations of our study. Analysis according to bacterial species was not included, which may effect growth response. Future studies should address this gap by incorporating a comprehensive examination of bacterial species. As another limitation, our study was conducted in a single center with a limited number of samples. Multicenter studies with larger and more diverse samples may provide further insight and clarification to this important laboratory practice. Moreover, in our study, we used different loops and compared these with calibrated 10 μl pipettes, pipettes with higher volumes may effect the results. Further studies may give more reliable results in this subject.

Funding

The study funded by Bezmialem Vakif University Scienctific Research Unit Project NO: 12.2020/16.

CRediT authorship contribution statement

Ayse Nur Ceylan: Conceptualization, Methodology, Writing – original draft, Validation, Software, Formal analysis, Data curation, Writing – review & editing, Visualization. **Ali Toprak:** Conceptualization, Methodology, Writing – original draft, Validation, Software, Formal analysis, Data curation. **Mehmet Ziya Doymaz:** Writing – review & editing, Supervision, Project administration. **Bilge Sümbül:** Writing – review & editing, Visualization, Supervision.

Declaration of competing interest

None of the authors had benefit related to this study.

Acknowledgment

We thank Esma Koç for expert technical assistance.

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