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A Comparative Study of the Direct Gram Staining Method and The Standard Wire Loop Culture Method for Screening of Significant Bacteriuria.

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Abstract

This study attempts to compare the sensitivity of the culture method to the direct Gram staining method in the screening of significant bacteriuria. A total of 100 subjects comprised of 60 adult females, 25 adult males, 8 male children and 7 female children were screened for significant bacteriuria by the direct Gram's staining method and culture methods. In the culture method, an uncentrifuged urine sample was inoculated on Cysteine Lactose Electrolyte Deficient (CLED) agar and blood agar using a standard wire loop of 0.01mL, incubated at 37^0C for 18 to 24 hours. The direct Gram staining was carried out by plating 0.01mL of centrifuged urine sample on a clean glass slide allowing it to air dry, stained by the Gram's method and under ×100 objective lens. Of the 100 uncentrifuged urine samples processed by culture method, 21(21%) were positive for significant bacteriuria, this includes 7 adult males, 12 adult females, 1 female child and 1 male child. *Escherichia coli* and *Pseudomonas* spp were the most frequently isolated organisms with 8 of 21 (38.1%) each, followed by *Klebsiella spp* 3 of 21 (14.3%), and 2 of 21(9.5%) were *Staphylococci aureus*. In the direct Gram staining method, 18(18%) were positive for significant bacteriuria; including 7 adult males, 10 adult females 1 female child. Statistical analysis showed no significant difference in the sensitivity pattern of both methods in the diagnosis of UTIs ($p>0.05$). Though urine culture remains the best diagnostic procedure for the detection of UTIs, the direct Gram's staining method is also effective, and may be used in an emergency situation or where there are no facilities for culture to screen for significant bacteriuria.

Keywords: *Bacteriuria, Gram staining, Culture method*,

Introduction

Urinary tract infection (UTIs) is caused by the presence and growth of microorganism in the urinary tract, it is second only to respiratory tract infection as the commonest bacterial infection of mankind. It accounts for approximately 23% of all hospital acquired infection, and can occur in both male and female patients of any age with bacteria counts as low as 100 colony forming unit (CFU) per milliliters of urine. It is common in patients with symptoms of acute urethral syndrome, male with chronic prostatitis and patients with indwelling catheter. Females are however believed to be more affected; this is attributed to the shorter and wider urethra of the female gender and constant flow of menstrual blood which provides nourishment for the invading organism. The risk in female is even higher during sexual intercourse, during which the normal bacterial flora colonizing the urethral are being massaged up into the bladder. Pregnancy and child birth have been also linked to higher prevalence rate. In pregnancy, over 20% bacteriuric women develop other complications such as acute pyelonephritis. It is estimated that 2% of women develop a urinary tract infection during their lifetime, the incidence increases at puberty and remains high throughout adult life (Douglass *et al*., 2019, Zenoh *et al.*, 2018b, Robbin and Contran, 2011, Emmerson *et al.,* 1996). Enteric bacteria,

especially *Escherichia coli,* causes most of the infection in all ages, they colonize the perineum and thereafter, kidney and adjacent structure. Urinary tract infection may be either symptomatic or asymptomatic.

In previous years, two factors in particular have stimulated the research which underlies our improved understanding of urinary tract infections (UTIs), the first was the introduction of quantitative urine culture which made it possible to distinguish significant bacteriuria (SB) (Count of 10^5 CFU/mL) and urethral contaminates. This is important since urine must pass though the distal urethra, it may become contaminated by the normal flora of these regions. Isolation of more than one bacterial strain suggests such contamination, even when a single strain is isolated, quantitative culture is required to determine if it is SB. The second factor, was the discovery of a high prevalence of significant bacteriuria in apparently healthy subjects. Though lower bacterial count could be considered contaminants from urethral normal flora, studies have reported symptomatic cases in lower count ($\leqslant 10^3$ CFU/mL) among immunocompromised individuals like HIV/AIDS (Zenoh *et al*, 2019).

Lower counts may also be considered significant depending on how the sample was collected. Since above the distal urethra, the urinary tract is normally sterile, therefore any bacteria isolated from urine samples taken directly from the bladder (suprapubic aspiration), ureter or kidney must be viewed as clinically relevant (Kunin, 1993).

Diagnosis is an important factor in guiding the treatment of an individual and forms the basis of epidemiological assessment. The aim of microbiology laboratory in the management of UTIs is to provide accurate diagnosis.

The culture method has proved very effective. This procedure, is performed by spreading a standard bacteriological loopful of urine over the surface of cysteine lactose electrolyte deficient agar plate. After inoculation, the plate is left on the bench for some time, in order to allowed the urine to be absorbed into the agar medium, plate incubated at 37°C for 18-24 hours and the number of bacterial colonies counted and converted to give an estimate of the number of bacteria present per milliliter of urine expressed as colony forming unit per milliliter (CFU/mL). A count of *enterobacteriaceae* of 10^2CFU/mL or more especially for *salmonella* can be of considered significant and growth of single urinary pathogen at a concentration of $\geqslant 10^3$ CFU/mL is considered significant. The Grams staining method, named after the Danish bacteriologist, also provides an excellent way of identifying UTIs. In most laboratories, these methods are used simultaneously, in areas where facilities are poor only one method could be used. This study attempts to compare the sensitivities of both methods (Mackay, 2021, Ainsley *et al*., 2018, Nnaemeka and Iyioku, 2014, Cheesbrough, 2000, Mackay and Scollay, 1969)

Materials and methods

Study area and subjects

The study was conducted at the University of Calabar Teaching Hospital Bacteriological laboratory located in Calabar, Cross River State Nigeria, between July to August, 2020. A total of 100 patients aged ranging between 5-52 years suspected of having UTIs were examined; comprising of 60 female adults, 25 male adults and 8 male and 7 female children.

Collection of samples

Mid-stream urine samples were collected from both inpatients and outpatients attending University of Calabar Teaching Hospital using sterile leak-proof universal container. Since subjects collect their samples, they were given instructions to ensure aseptic sample collection. The need for prompt delivery of collected sample to the laboratory was thoroughly explained. The name, age and gender of the patients were properly labeled on the sterile universal container.

Diagnostic procedure

The two comparative studies; culture and Gram's staining methods, were performed at the same time on the uncentrifuged for culture, and centrifuged urine sample for gram staining. The culture method was performed first and immediately followed by the Gram's staining method so as to avoid contamination.

Culture Method

A standard wire loop of 0.01mL was used to inoculate Cysteine Lactose Electrolyte Deficient (CLED) agar and blood agar after the specimen has been thoroughly homogenized, and the agar plates were streaked, incubated for 18 to 24 hours. Following incubation, the plates were examined, colonies were counted and converted to a count per milliliter and any plates showing $\geqslant 10^5$ CFU/mL of urine were considered as significant bacteria.

Identification of isolates

Presumptive identification of bacterial isolates was based on appearance of colonies, Grams reaction, arrangement of cells. Gram positive bacteria were further subjected to catalase and coagulase test. For Gram negative, oxidase, urease and IMVIC test were performed.

Gram's staining Method.

A smear of a centrifuged urine samples was stained using Christian Gram's method and viewed with an oil immersion lens using the following steps.

i. A smear of a homogenized urine sample was made with 10 microliters (0.01ml) wire loop and allowed to air dry.
ii. The smear was then stained with 1% crystal violet for 1 minute, washed with water, then covered with Gram's Lugols iodine for 1 minute, washed with water.
iii. Decolourization was carried out with acetone (70% alcohol) and washed immediately with water. Counterstaining was carried out using 1% neutral red for 1 minute, washed with water.
iv. The back of the slide was wiped and placed on a drying tray to air dry, mounted on the microscope stage and viewed with x100 objective lens with immersion oil. A positive specimen was one presenting with one organism per oil immersion field.

Results

Of the 100-urine specimen investigated, 60 were obtained from adult females, 25 from adult males and 15 from children (7 and 8 female and male respectively). The total number positive for significant bacteriuria by the culture method was 21, including 7 males, 12 females and 2 children (a male and female). However, by the direct Gram's staining method, a total of 18 positive cases were recorded, comprised of 7 adult males, 10 adult females and 1 female child. Statistical analysis shows no significant differences in the detection rate of UTI by the two methods ($p>0.05$) as shown in table 1.

Table 1: Detection of Bacteriuria by Both Gram Staining and Culture Methods

Gender	Number examined	No Positive by Gram's staining (%)	No Positive by Culture (%)
Adults males	25	7(28)	7(28)
Adult females	60	10(16.7)	12(20)
Female child	7	1(14.3)	1(14.3)
Male child	8	0(0)	1(12.5)
Total	**100**	**18**	**21**

Distribution of Significant Bacteriuria by Ages.

A total of 15 samples from subjects aged between 5 to 14 years were processed; 2(13.3%) were positive for significant bacteriuria, comprised of 1 male and 1 female child. Subjects aged 15 to 24 years, had a total 5 samples, and 4 (80%) were culture positive; including 1 male and 3 females. Twenty subjects aged between 25 to 34 years were also investigated; 10(50%) made up of 4 males and 6 females were positive for significant bacteriuria. Twenty-two subjects aged between 35 to 44 years were investigated, and 4(18.2%), 1 male and 3 females were positive for significant bacteriuria. Among the 18 subjects aged between 45 to 54 years, only 1(5.5%) from a male subject was positive for significant bacteriuria as shown in table 2.

Table 2: Distribution of significant bacteria by ages and gender.

Age group	No. of samples processed	No. (%) positive for significant bacteriuria		
		Male	Female	Total
5-14	15	1(6.7)	1(6.7)	2
15-24	5	1(12.5)	3(23)	4
25-34	20	4(5)	6(46.2)	10
35-34	22	1(12.5)	3(23)	4
45-54	18	1(12.5)	0(0)	1
55-Above	20	0(0)	0(0)	0
Total	**100**	**8**	**13**	**21**

Significant Bacteriuria based on Gender

Table 3 shows the sensitivity level of both Gram's staining and culture methods in detecting significant bacteriuria. Of the 25 male adults and 60 adult females examined by culture method, 7(33.3%) and 12(57.1%) were positive for significant bacteriuria respectively. In the Gram staining method however, 7(28%) adults male and 10(16.7%) adult females were significantly positive for bacteriuria. Children had significantly lower cases with about 2(9.5%) of 15 subjects examined by culture method positive for significant bacteriuria comprising of 1(14.3%) of 7 female children and 1(12.5%) of 8 male children. Whereas in the Gram Staining method, only 1(14.4%) female child was significantly positive for bacteriuria.

Table 3: Distribution of UTIs according to subject investigated.

Gender	Number examined	No Positive by Gram's staining	No Positive by Culture
Adults males	25	7(28)	7(28)
Adult females	60	10(16.7)	12(20)
Female child	7	1(14.3)	1(14.3)
Male child	8	0(0)	1(12.5)
Total	**100**	**18(18)**	21(21)

Bacterial Isolates

Escherichia coli 8(38.1%) and *Pseudomonas spp* 8(38.1%) were the most isolated bacteria of the total 21 isolates followed by *Klebsiella* with 3(14.3%) of the total isolates. *Staphylococcus aureus* was the least isolated organism with a prevalence rate of 2(9.5%) (Figure 1).

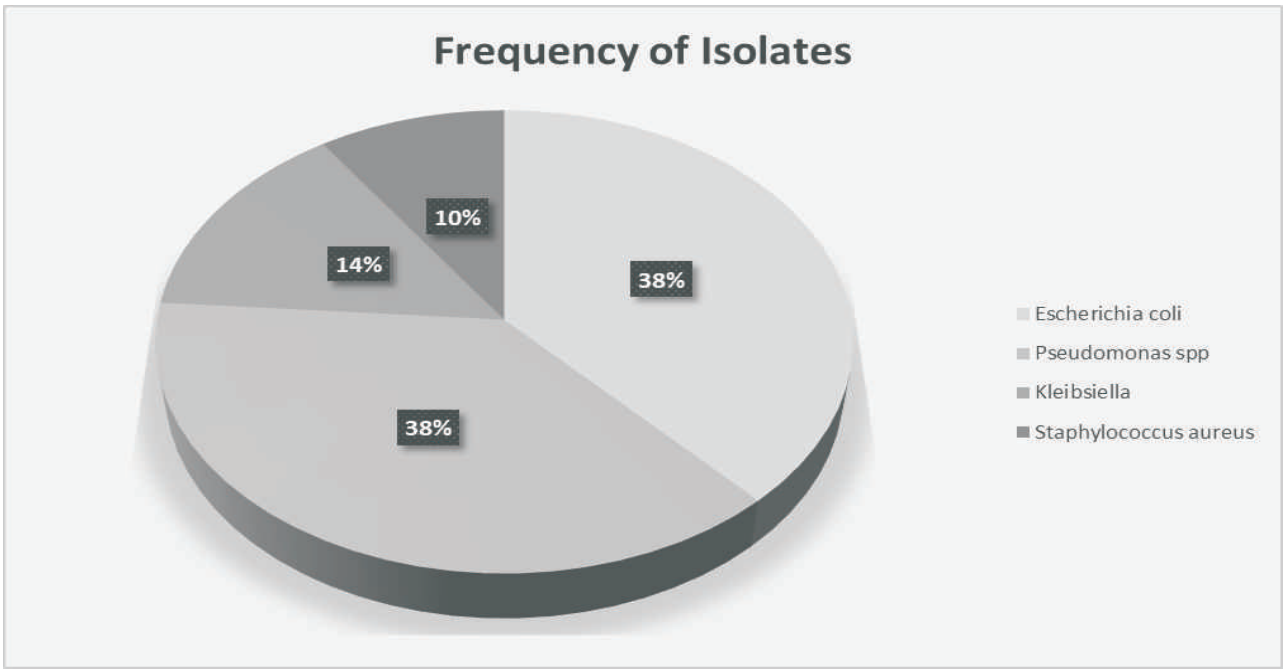

Figure 1: Frequency of Bacterial Isolates.

Discussion

This study compared the diagnostic efficacy and sensitivity of both the Gram staining and culture method in the diagnosis of urinary tract infection. Since the culture method is the most widely accepted diagnostic procedure used in detection of UTIs; this study attempts to compare the sensitivity of the direct Gram's method to the culture method in the detection of UTIs. In this study, the culture method recorded a prevalence rate of 21% across all gender and ages, while the direct Gram's method recorded 18%, with the sensitivity of 85.7% (18/21*100). Sensitivity measures the ability of a diagnostic procedure to test positive when there is actually a UTI (Michael and Loretta 2004; Zenoh *et al.*, 2018a). Michael and Loretta in their study on Laboratory Diagnosis of Urinary Tract Infections in Adult Patients reported a sensitivity of 96% for bacterial count of $\geqslant 1$ bacterial cell per high power field (hpf), and 95% for bacterial count of $\geqslant 10^5$ CFU/mL, i.e. 5 bacterial cells per hpf from a centrifuged urine sample. For an uncentrifuged urine sample, the sensitivities were 92-100%, 74% and 63% for bacterial counts of 10^5, 10^4, 10^3 CFU/mL respectively.

Zenoh *et al.* (2018a) had a relatively lower sensitivity level when they measured how urinary parameters such as the presence of pus cell and nitrite (urinalysis) associate with significant bacteriuria, they reported a sensitivity of 43% and 30% for pus cells and nitrite respectively. WHO in 2018 compared direct Gram's staining and urinalysis (Nitrite and leukocytes esterase) using the midstream urine culture (the gold standard) with a threshold of 10^5 CFU/mL as a reference standard. A Gram stain was positive if one or more bacteria were detected per oil immersed field, and a dipstick test was positive if it detected either nitrites or leucocytes. Sensitivity of Gram's method was 86%, while for urinalysis was 73%. This shows that direct Gram's staining is more reliable in the detection of urinary tracts infection than the urinalysis. Though direct Gram staining is simple, and can offer a reliable result in a very short time, studies have shown that it is less sensitive for the detection of bacteriuria in a sample with lower bacterial counts of $\leqslant 10^2$ CFU/mL, hence may not be too helpful in the detection of asymptomatic bacteriuria. Goossens *et al.* (1976) in their study of asymptomatic bacteriuria stated that neither microscopic examination nor the nitrite test can accurately detect bacteriuria, and that the quantitative urine culture by dip-slide is the easiest and most reliable way of diagnosing urinary tract infections. The direct Grams method, cannot also provide detailed identification of the organisms to species level.

Mohammed (2001) in the other hand reported a strong relationship between urine culture and direct gram staining of unspun urine sample; he reported a sensitivity of 95% compared to the culture method. Arti and Parul (2016) compared the sensitivity of diagnostic method for significant bacteriuria using 1,000 urine samples from patients in Municipal Medical College, Ahmedabad, Gujarat, India; 186 (18.6%) patients revealed urine cultures with significant bacteriuria (colony count $\geqslant 10^5$). Sensitivity and specificity of microscopic examination and strip urinalysis of bacteriuria were 96.77% and 98.52%, respectively compared to the culture method, showing close sensitivity. Barbara *et al.* (2010) identified the culture method as the gold standard method, but suggested the need to use the Sysmex UF-1000i as an alternative method for diagnosis of bacteriuria. Jenkins *et al.* (1986) recommended the use of microscopy for diagnosis of bacteriuria in their review of urine microscopy.

In this study, UTIs was more prevalent in male than female 28% vs 16% in Gram's method and 28% vs 20% in culture method. Most studies however report the opposite, as there were more female than male cases (Martin *et a*l, 2019; Zenoh *et al*, 2018b; Enrico *et al* 2012, Ekerete, 2001; Sabatth, 1980; Kunin, 1978; Stamey, 1973).

In terms of gender, younger subjects had more cases of UTIs with the exception of children aged 5-14, with prevalence rate of 13%. Sexually active subjects (aged 15-44) are at higher risk of UTIs, the prevalence rate here is over 38%. Older subjects, 45 years and above had the least prevalence rate 2.6%. This result shows that the rate of sexual intercourse increases the chances of acquiring UTIs. From the age of 45 and above, sexual desire dwindles, leading to less sexual activities, hence reduces risk of urinary tract infection.

In male, urinary tract infection occurs mostly between the ages of 25 to 34 and reduces between the ages of 45 and above. This agreed with the report of Kunin (1976) which indicated that bacteriuria is rarely seen in males before the age of 50 years in the absence of instrumentation. The two cases of UTI in children in this study were children admitted for severe malnutrition and were diarrhoeic, their history is not far from the finding of Ojuawo *et al.* (1994) that severe protein malnutrition in a child predisposes the child to urinary tract infection as a result of depressed immunity.

Escherichia coli and *Pseudomonas spp* were the most isolated organism, *Klebsiella spp* and Staphylococcus *aureus* were 3(14.4%) and 2(9.5%) respectively. This corroborates the findings of other researchers (Martin *et al.,* 2019; Zenoh *et al.*, 2018b; Enuli *et al.*, 2016; Olowe *et al*, 2013; Enrico *et al.*, 2012).

Conclusion

The culture method remains the best screening method for the diagnosis of UTIs. The direct Gram's staining method is however are also effective and can be used where there are no facilities for culture to screen for significant bacteriuria even though it has some limitations including inability to support sensitivity test.

Conflict of Interest Declaration

The authors hereby write and declare that this work was not sponsored by any individual, private or public organization. As such, there was no any form of prejudice or conflict of interest.

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