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High Frequency of Gram-negative Bacilli (GNB) Pathogens in Wounds and Other Clinical Specimens: A Grave Public Health Concern

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ABSTRACT

Background. Gram-negative bacilli (GNB) including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* are important causes of both hospital and community acquired infections in human beings. In this regard, the current study aimed to assess the frequency of GNB pathogens circulating in Hyderabad, Sindh and to obtain locally applicable data for the prevention and spread of infections caused by GNBs.

Methodology. A total of 360 clinical specimens including blood, pus, wound, urine, sputum, and body fluids from suspected indoor and outdoor patients were collected from various diagnostic centers of Hyderabad, Sindh. The isolation, identification, and characterization of GNB pathogens was performed by using standard conventional methods including morphological, cultural, and biochemical testing.

Results. A total of 143 GNBs were isolated and characterized in the current study. The data demonstrated that male patients were more affected with GNBs accounting for 55.94% ($n=80$) of infected specimens, whereas 44.06% ($n=63$) of specimens were from female patients. Moreover, specimen wise data of sample positivity revealed that 13.29% ($n=19$) of GNBs were isolated from pus specimens, 58.59% ($n=70$) from urine specimens, 34.97% ($n=50$) from blood specimens, 1.40% ($n=2$) from fluid specimens, and 1.40% ($n=2$) of GNBs were isolated from sputum specimens. Bacteriological profiling revealed that 41.26% ($n=59$) of the isolated bacteria were *E. coli*, considered as the predominant bacteria isolated from urine specimens. Whereas, *S. enterica* serover Typhi was the most frequently isolated bacteria from blood specimens accounting for 20.28% ($n=29$) of all bacteria. Other less prevalent but important pathogenic bacteria included *K. pneumoniae* accounting for 12.59% ($n=18$) of all bacteria, *P. aeruginosa* accounting for 8.39% ($n=12$) of all bacteria, *Acinetobacter* spp. accounting for 6.99% ($n=10$) of all bacteria, and *Enterobacter* spp. accounting for 2.10% ($n=3$) of all bacteria.

Conclusion. To conclude, the high frequency of GNBs isolated from clinical specimens at Hyderabad, Sindh poses an alarming situation and warrants an urgent need to monitor and control the spread of pathogenic bacteria.

Keywords: blood, Gram-negative bacilli (GNB), typhoid fever, UTI, wounds

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Highlights

1. GNBs constitute an important consortium of human pathogens.
2. *E. coli* remains a highly prevalent GNB among urine samples.
3. *S. typhi* is the predominant GNB causing bloodborne infections.

1. INTRODUCTION

Among the hospital acquired infections, a wide range of infections is caused by an important group of bacterial pathogens collectively known as Gram-negative bacilli (GNB). They have been demonstrated to cause various infectious diseases, such as UTI, wound infections, bacteremia, diarrhea, cystic fibrosis, and typhoid in human population, especially in developing world including Pakistan. The group consists of *E. coli*, *Acinetobacter* spp., *Enterobacter* spp., *Pseudomonas* spp., *Klebsiella* spp., *Salmonella* spp., and *Proteus* spp. The emergence of multidrug resistant (MDR) bacteria poses a great threat within the healthcare settings as antimicrobial therapy fails to eradicate them. Among GNB infections, the most prevalent causative agent reported in literature is *E. coli* which is associated with various infections. Whereas, *S. typhi* is another predominant GNB which acts as a causative agent of typhoid in human beings. It has been shown to remain the most frequently recovered bacteria from blood specimens in Pakistan, where typhoid fever remains highly endemic for several decades [1].

Many other GNB pathogens have been reported to cause important infections including *P. aeruginosa*, which causes a lung infection in human beings known as cystic fibrosis, as well as other infections such as wound infections including burn, skin and soft tissues, and post-surgical infections [2].

Cystic fibrosis patients tend to be susceptible to *Burkholderia cepacia* infection. *K. pneumoniae* have been reported as an opportunistic GNB causing infections, such as pneumonia. MDR *K. pneumoniae*, being resistant to carbapenems which are last resort antibiotics, appears to be a great challenge and potential threat to global health, worldwide [3]. MDR-GNB may transfer resistance to other bacterial isolates resulting in greater hindrance in antibiotic stewardship. Due to various factors including poverty, poor sanitation, unsafe water supply, and mismanagement and improper handling of food supply, infections caused by GNB bacterial pathogens are more common in Pakistan [4]. Furthermore, the misuse and abuse of antibiotics without proper diagnosis and prescription has further worsened the public health situation, which is considered as a major risk factor of GNB associated infections and dissemination of antibiotic resistance in Pakistan.

It is alarming that GNBs have built-in abilities to acquire resistance against almost all available antibiotics, such that MDR-GNB pathogens pose a serious challenge via subsequent transfer of resistance to other bacteria that makes them resistant as well to antibiotic therapy [5-7]. A wide range of MDR-GNB pathogens including extended spectrum β -lactamases producers [8-10], carbapenem-resistant *K. pneumoniae*, carbapenem resistant *A. baumannii*, and others have been

reported in previous studies [11]. The increased outbreaks of infections associated with MDR-pathogens have worsened public health worldwide. A high rate of mortality and morbidity, resulting due to nosocomial infections by GNB, has been reported in developing countries. This may possibly be due to the lack of effective vaccines as well as limited options of antibiotic treatment due to MDR isolates. The current study was, therefore, designed to evaluate the prevalence of indigenous GNB pathogens circulating in Hyderabad, Sindh, and their antibiotic susceptibility patterns to control the spread of infections and drug resistance. The outcomes of this study would help local physicians in conducting empirical therapy of GNB infections and may lead to antibiotic stewardship. Thus, this study aimed to isolate and identify GNB from a variety of clinical specimens to assess their prevalence and to recommend preventive measures for controlling the infections caused by them.

2. MATERIALS AND METHODS

2.1. Culture Media and Growth Conditions for GNB Isolation

Only Gram-negative clinical isolates were included in the current study. GNB isolates grown on solid media were incubated at 37°C for 24 hrs without shaking, while liquid cultures were incubated at 200 rpm in shaking incubator. All media used for the identification and characterization of GNB isolates including cysteine lactose electrolyte-deficient (CLED) blood agar base with 5% sheep blood, MacConkey agar, and nutrient agar/broth were purchased from Oxoid, UK.

2.2. Study Design and Location

The current cross-sectional and prospective study was carried out for one year from August 2018 to July 2019 at the Institute of Microbiology, University of Sindh, Jamshoro.

2.3. Sample Collection

All specimens of blood, urine, pus, fluids, and sputum were collected aseptically from suspected indoor (hospitalized) and outpatient door (OPD) patients attending diagnostic medical centers/laboratories of Hyderabad, Sindh. Blood culture bottles were used for collecting blood samples, while sputum and urine specimens were collected in sterile wide-mouthed bottles. Blood samples were collected through vein puncture from the suspected patients and transferred immediately to blood culture bottles. Mid-stream urine samples were collected by clean catch method. Briefly, the patients were instructed to clean the genital area thoroughly to avoid contamination which may affect the results. Wound specimens were collected either in pus form or as fluid/aspirates with the help of sterile swabs or syringes, respectively.

2.4. Processing of Clinical Specimens

All collected samples were either processed immediately at the laboratory or stored under appropriate conditions for transporting to the laboratory. Appropriate media were used to preserve the isolates during their transporting to laboratory to maintain the viable growth of suspected pathogens. Blood samples were immediately transferred to BACTEC bottles for cultivation and incubated in BACTEC 9050 series for the detection

of bacterial growth. Other body fluids and swab samples which needed enrichment or other treatment were processed accordingly.

2.5. Isolation of the Clinical GNB Isolates

All GNB isolates were cultured on diagnostic media to obtain single discrete colonies which were selected for sub-culturing on the selective medium. The pure single colonies were subjected to the standard protocol used for the identification of isolates at species level.

2.6. Phenotypic Characterization and Identification of Clinical GNB Isolates

Identification of GNB isolates was carried out based on morphological and microscopic characteristics, growth patterns on various diagnostic media, and biochemical testing, such as citrate utilization test, fermentation of sugar, urease production, patterns of hemolysis on blood agar, indole and H₂S

production, and catalase and oxidase tests. Bacteria demonstrating the phenotypic characteristics of Gram-negative bacilli or GNB were selected for further characterization.

3. RESULTS

The frequency of GNBs among different clinical samples such as pus, urine, blood, fluids, and sputum was investigated to determine the current infection rate of GNBs in Hyderabad, Sindh. The data demonstrated that 143/360 clinical samples yielded the growth of GNBs, which were selected for further characterization and assessment of antibiotic susceptibility patterns. Overall distribution of the samples revealed that the highest percentage of samples (48.95%, *n*=70) belonged to urine, followed by blood samples (34.97%, *n*=50), and pus samples (13.29%, *n*=19). Whereas, the lowest percentage belonged to sputum and fluids specimens (1.4%, *n*=2) (Figure 1).

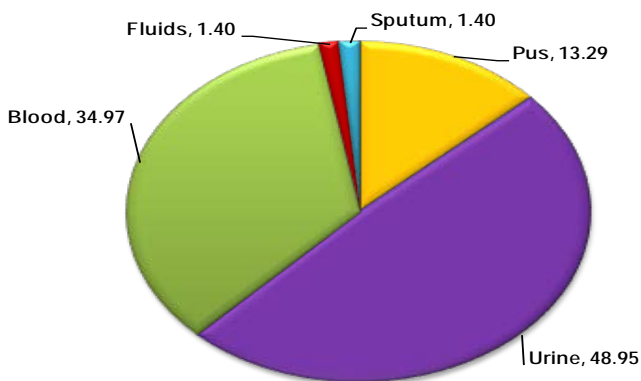


Figure 1. Overall Distribution of Clinical Specimens Yielding GNB Growth

3.1. Gender-wise Distribution of GNB Infected Patients

Gender-wise analysis of data demonstrated that female patients were

more susceptible to GNB infection, although they yielded a high frequency of GNB recovery as compared to male patients. Frequency distribution of GNB positive specimens based on gender,

age, and the type of specimen is shown in figures 2-4. Pus samples yielded the highest positivity rate in the age group of 21-40 years (Figure 2), indicating the increased prevalence of skin and soft tissue infections. The age group of 1-10 years demonstrated the lowest positivity for GNB infections (Figure 2). Similarly, the frequency of urine infection was higher in female patients

than male patients. The age group of 21-30 years demonstrated that increased urine specimens resulted in a higher rate of GNB infections (Figure 3). The rate of bloodborne GNB infections also appeared more prevalent in female patients than male patients. However, the age group of 1-10 year was found to be the most frequently infected by GNB infections (Figure 4).

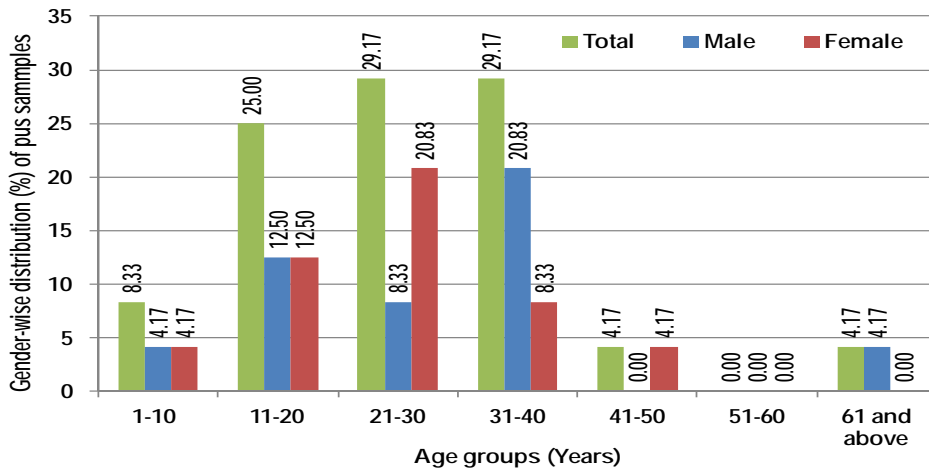


Figure 2. Age and Gender-wise Distribution of Patients Demonstrating Higher GNB Infections of Skin and Soft Tissue

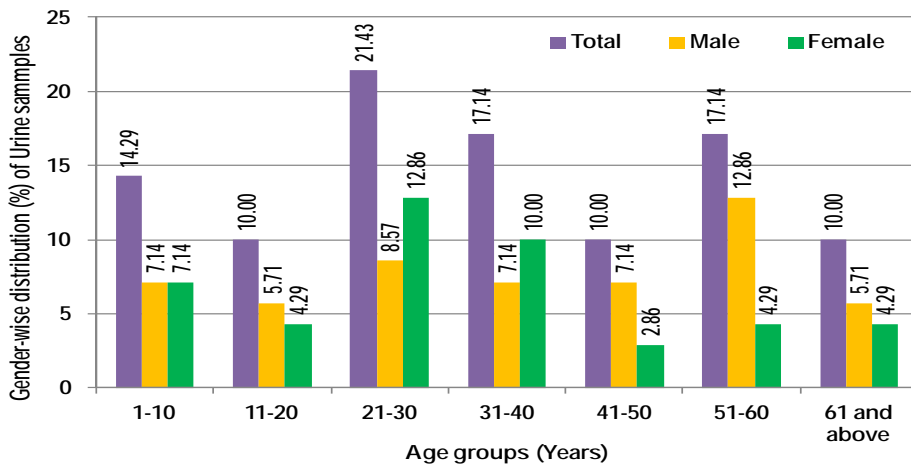


Figure 3. Age and Gender-wise Distribution of Patients with GNB Induced Urine Infection

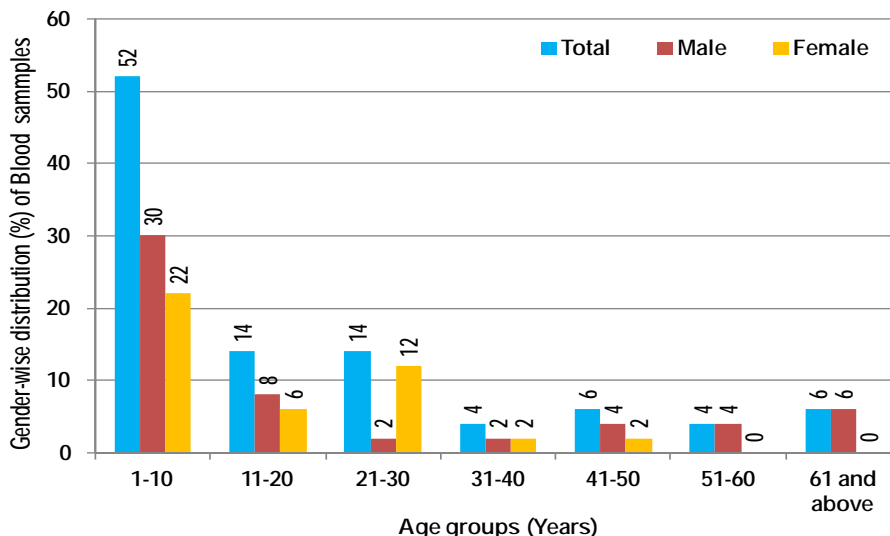


Figure 4. Age and Gender-wise Distribution of Patients with GNB Induced Blood Infection

3.2. Characterization of GNB Pathogens

A total of 143 GNB clinical isolates were selected. The specimen yielding the growth of both Gram-negative and Gram-positive bacteria were excluded, considering them as contaminated/mixed cultures. All clinical isolates were characterized using conventional techniques, such as microscopic observation of stained smear through Gram-staining. The bacterial isolates appearing as pink rods based on Gram reaction and microscopic analysis were initially identified as GNB (Figure 5). All presumptively identified clinical isolates were validated by culturing them on MacConkey agar. Moreover, GNB isolates showing flat colonies and fermented lactose in MacConkey agar were presumptively identified as *E. coli*. Whereas, bacterial cultures producing pigmented colonies and non-lactose fermenter were

identified as *Pseudomonas* spp. Likewise, GNB isolates growing as mucoid and slightly pink colonies on MacConkey agar were identified as *Enterobacter* spp. On the other hand, dark pink colonies were identified as *Klebsiella* spp. All GNB isolates were further characterized based on their biochemical characteristics.

3.3. Overall Prevalence of GNB Pathogens

Based on the data of cultural, morphological, and biochemical characterization, a total of 143 GNB bacterial isolates belonging to eight (08) different genera were identified and characterized. *E. coli* was identified as the predominant GNB (41.26%), followed by *Salmonella* Typhi (20.28%), *Klebsiella* spp. (12.59%), *Pseudomonas aeruginosa* (8.39%), *Acinetobacter* spp. (6.99%), *Enterobacter* spp. (4.90%), *Proteus mirabilis* (3.55%), and *B. cepacia*

(2.10%), respectively (Table 1). Sample-wise prevalence of isolates indicated that *E. coli* was a highly prevalent GNB in urine specimens, followed by *Klebsiella* spp. However, GNB profiling

of blood specimens demonstrated the highest prevalence of *Salmonella* Typhi, followed by *Acinetobacter* spp.

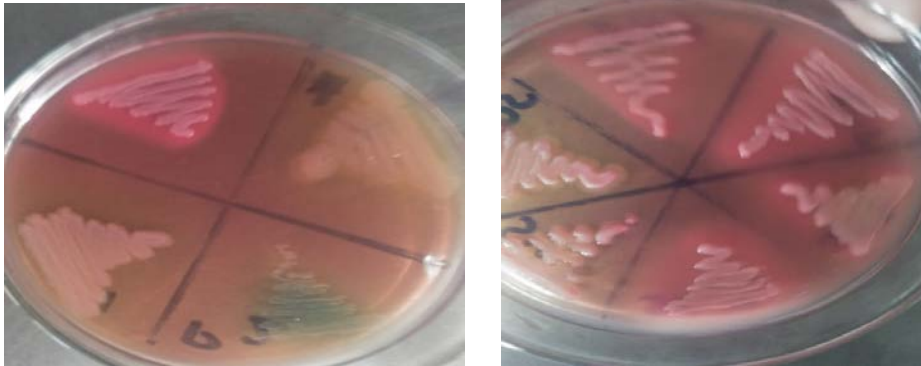


Figure 5. Pure Cultures of GNB Isolates on MacConkey Agar

Table 1. Sample-wise Distribution of Isolated GNBs

S. No.	GNB	Total (%)	Total (n)	Specimen-wise Distribution				
				Pus (n)	Urine (n)	Blood (n)	Fluids (n)	Sputum (n)
1.	<i>E. coli</i>	41.26	59	6	49	2	2	-
2.	<i>K. pneumoniae</i>	12.59	18	3	12	2	-	1
3.	<i>P. aeruginosa</i>	8.39	12	5	4	2	-	1
4.	<i>P. mirabilis</i>	3.50	5	2	2	1	-	-
5.	<i>Acinetobacter</i> spp.	6.99	10	-	1	9	-	-
6.	<i>Enterobacter</i> spp.	4.90	7	3	2	2	-	-
7.	<i>S. typhi</i>	20.28	29	-	-	29	-	-
8.	<i>B. cepia</i>	2.10	3	-	-	3	-	-
Total		100	143	19	70	50	2	2

4. DISCUSSION

The current study aimed to assess the prevalence and characterization of GNB pathogens in a wide range of hospital and community acquired infections in Hyderabad, Sindh. GNBs are frequently isolated bacteria that pose a serious threat to public health, particularly the management of certain infections in hospital settings, owing to their potential to acquire and

disseminate antibiotic resistance even against the last resort antibiotics [12]. In the current study, a total of eight (8) different genera of GNB pathogens were found circulating in Hyderabad, Sindh. A previously published study demonstrated the recovery of eleven (11) types of GNB pathogens including *Enterobacter* spp., *E. coli*, *Klebsiella* spp., *Acinetobacter* spp., and *P. aeruginosa* as frequently isolated along

with other less prevalent bacterial strains [13]. The lack of surveillance of antibiotics use, infection control measures, and an increase of carbapenemase producing Enterobacteriaceae are serious threats to the safety of patients and healthcare workers [14]. Multidrug resistance among bacteria has further aggravated public health concerns. Thus, the routine monitoring of infections caused by locally prevalent bacteria and their patterns of antibiotic susceptibility and resistance is essential. It would assist the clinicians in recommending empirical treatment based on local data for controlling the infections caused by multidrug-resistant (MDR) bacteria, particularly carbapenem resistant Enterobacter spp., *Pseudomonas aeruginosa*, and Enterobacterales [15, 16]. Among the isolated *E. coli*, the majority of isolates were recovered from urine specimens, suggesting that *E. coli* is the predominant GNB pathogen causing UTI in Hyderabad. The scenario may become even worse when these *E. coli* acquire multidrug resistance and are frequently isolated from catheterized patients, causing UTI treatment to become more complicated with an increased risk of mortality and morbidity due to hospital acquired infections [17, 18].

In the current study, gender-wise frequency of GNB infections was also recorded. It was found that the rate of infection was higher in male patients as compared to the female patients. However, previously published data demonstrated that gender-wise prevalence remains evenly distributed, without significant differences [19]. Various, age-wise distribution revealed that GNB infection was more

frequent in the elderly, causing UTI. On the other hand, in blood samples, children under ten years of age were found to be more susceptible to GNB infections. This is contrary to the previously published data which reported no association between age and GNB infections [19, 20]. High prevalence of GNB pathogens found in this study, particularly that of *S. typhi* and *E. coli*, is a matter of concern in the management and control of infections and warrants their strict surveillance and accurate diagnosis.

4.1. Conclusion

The current study demonstrated eight (08) GNB pathogens circulating in Hyderabad, Sindh. *E. coli* and *S. typhi* were found to be highly prevalent GNB pathogens among urine and blood samples, respectively. The sample-wise distribution of GNB pathogens was found to vary with their highest recovery from urine, followed by blood. The infection rate was found to be higher in male patients as compared to female patients. However, age-wise distribution showed that blood infections were highly prevalent in children under ten years of age. On the other hand, urine and pus samples yielded a higher positivity ratio among the age group 21–30 years. To conclude, *E. coli* and *S. typhi* bacterial isolates were demonstrated as leading pathogens causing urinary tract and blood stream infections, posing a serious threat to public health and limiting the treatment options even with the newest antibiotics/drugs. Thus, proper diagnosis of GNB infections is of paramount importance together with their continuous surveillance and monitoring in order to reduce their spread.

CONFLICT OF INTEREST

The author of the manuscript has no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

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