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Prevalence, Phenotypic Characterization and Antibiotic Susceptibility of Non-Fermentative Gram Negative Bacilli Isolates at a Tertiary Care Centre

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A B S T R A C T

In hospitalized patients with predisposing illnesses leads to the life threatening infections with Non-fermentative Gram negative bacteria (NFGNB) like *Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia* and *Burkholderia cepacia*. The patients are placed at high risk because the NFGNB have high intrinsic antibiotic resistance. All consecutive, non-duplicate isolates of NFGNB were collected from various clinical samples like blood, sputum, tracheal aspirate, urine, pus etc. from hospitalised patients during the study period. The phenotypic identification of various isolates was done as per standard laboratory protocol and antibiotic susceptibility interpretation was done as per CLSI guideline 2015. Out of 420 isolates the predominant sample is sputum and the predominant isolate was *Pseudomonas aeruginosa* followed by *Acinetobacter baumannii*. From the total 420 isolates, the maximum resistance was to cefepime 62 (14.8%) followed by Ceftazidime 50 (12%) and Ceftazidime-clavulanic acid 50 (12%), colistin 20 (4.8%), piperacillin-tazobactam 18 (4.3%) and gentamicin 18 (4.3%), imipenem 13 (3.1%), ciprofloxacin 12 (2.8%) and amikacin 11 (2.6%). Some of the studies from India reported the predominant isolate was *Pseudomonas* and some as *Acinetobacter*. In relation to antibiotic resistance comparatively imipenem resistance was very less than other studies that may be due to less usage of carbapenems. The main projection from our study is proper identification of NFGNBs, drug holiday and judicial usage of third and fourth generation cephalosporins and carbapenems are very important. Also implementation maintenance of strict antibiotic policy can help to fight against drug resistance by HICC.

Introduction

In hospitalized patients with predisposing illnesses leads to the life threatening infections with Non-fermentative Gram negative bacteria (NFGNB) like *Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia*

and *Burkholderia cepacia*. The patients are placed at high risk because the NFGNB have high intrinsic antibiotic resistance (Hancock, 1998). NFGNB in the last two decades showed increase in resistance to oxyimino-cephalosporins and carbapenems, because of this they belong to multi-drug resistant (MDR) organisms. There will be increased mortality, increased hospital stay and costs because of the compromised treatment of the resistance pattern produced by NFGNB (Hancock, 1998; Su *et al*., 2009; McGowan *et al*., 2006). The potent treatment of serious infections caused by these bacteria is carbapenems. The broad spectrum activity and resistance to hydrolysis by most β-lactamase producers including extended spectrum beta lactamase (ESBL's) made them to be the preferred treatment of choice. Hence the hospitals with high prevalence of ESBL's there is increase in use of carbapenems. Production of metallo-β-lactamase (MBL) which belongs to Ambler class B enzyme is increasingly being reported especially in *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* The metal chelators can inhibit the hydrolysis of carbapenems by MBL's (Ambler class B enzyme), it has broad spectrum but it can't hydrolyse Aztreonam (Franco *et al*., 2010; Govan, 2011). The MBL genes can be horizontally transferred between pathogens in hospital settings. Molecular methods for detection of MBL genes are most specific and accurate method but the resource limitations also should be considered. This study was carried out mainly for proper phenotypic characterization with basic tests available without automated systems (Table 2) and antibiotic susceptibility of NFGNB isolates and to identify the presence of carbapenemase production in different non-fermenters. So the prevention of multidrug resistant isolates in the hospital settings can be implemented properly by identification and detection of carbapenemase producing organisms.

Materials and Methods

This prospective study was carried out in the central laboratory department of microbiology of a tertiary care level hospital of Kancheepuram for a period of six months. All consecutive, non-duplicate isolates of NFGNB were collected from various clinical samples like blood, sputum, tracheal aspirate, urine, pus etc. from hospitalised patients during the study period. The phenotypic identification of various isolates was done taking in to account the colony morphology, pigment production and other biochemical tests like oxidase test, fermentation of sugars, motility, nitrate reduction, decarboxylase tests and glucose oxidative fermentative test (OF test) etc. (Fig 1,2,3,4) were done for speciation of the non-fermentative Gram negative bacilli according to standard protocol. The highlight of this study was phenotypic identification of various non-fermentative gram negative bacilli by a chart derived (Table 2) from different textbooks of diagnostic Microbiology with colony morphology and basic biochemical tests available in most of the diagnostic centres especially without automated systems for identification (CLSI, 2015; Koneman *et al*., 2006).

Antimicrobial susceptibility

The antimicrobial susceptibility of all isolates was done by Kirby-Bauer disk diffusion method according to the CLSI (M100-S25) guidelines 2015. Susceptibility other antimicrobials was done under standard conditions. Carbapenem susceptibility was seen by using imipenem (10 µg) discs (Fig 5, 6).

Results and Discussion

A total of 420 non-fermenting Gram negative bacteria (NFGNB) were collected. The species wise distribution in all samples shown that the most common isolate collected during the present study was *Pseudomonas aeruginosa* 210 followed by *Acinetobacter baumannii* 180, *Burkholderia cepacia* 18, *Stenotrophomonas maltophilia* 4, *Pseudomonas stutzeri* 4, *Alkaligenes faecalis* 2 and *Ralstonia picketti* 2 (Fig.7). The distribution of various clinical samples (Fig.8) comprised of blood, sputum, urine, pus, High vaginal swab, and other miscellaneous samples. Sputum (126) was predominant sample followed by urine (98), High vaginal swab (80), pus (72), blood (24) and other miscellaneous samples (20).

The sample distribution: Male patients were 185 (44%) and female patients were 235(56%) and hospitalized patients were 170(40%) and out patients were 250 (60%).Ward wise distribution of samples (170) show that most samples in the present study were received from surg ward 50 (≈30%) followed by ICU-surg 35 (20.5%), ICU-med 30 (17.6%), Medicine ward 25 (14.7%), TBCD ward 10 (≈5.9%), orthopaedics ward 10 (≈5.9%), OG ward 5 (2.9%) and ENT ward 5 (2.9%).

Antibiotic susceptibility testing (ABST) profile

Out of total 420 isolates, all were processed for antibiotic susceptibility testing by Kirby-Bauer disk diffusion method according to the CLSI (M100-S25) guidelines 2015. From the total 420 isolates, the maximum resistance was to cefepime 62 (14.8%) followed by Ceftazidime 50 (12%) and Ceftazidime-clavulanic acid 50 (12%), colistin 20 (4.8%), piperacillin-tazobactam 18 (4.3%) and gentamicin 18 (4.3%), imipenem 13 (3.1%), ciprofloxacin 12 (2.8%) and amikacin 11 (2.6%) (Table.1). Out of 420 isolates, 50 (12%) were ESBL producing (resistant to both Ceftazidime and Ceftazidime-clavulanic acid). The resistance pattern to nalidixic acid, nitrofurantoin and norfloxacin (urinary quinolones) were 28 (28.5%), 18 (18.35) and 5 (5.1%) out of total 98 isolates from urine. The colistin resistant (intrinsic resistance) isolates (20) were identified as *Burkholderia cepacia* (18) and *Ralstonia picketti* (02) with various biochemical analyses (Table.2). None of the *Pseudomonas species* or *Acinetobacter baumannii* isolates was resistant to colistin.

The early detection of carbapenemase producing strains and their prevention with appropriate antibiotics are very crucial steps in implementation of hospital infection control spread. For the detection of genes responsible for carbapenem resistance, various molecular techniques are available but they are not available in smaller centres. In such places, phenotypic characterisation can be done with limited resources and they are very sensitive in detection of ESBLs, carbapenemases and MBLs (Deshmukh *et al*., 2011).

A total of 420 samples were collected and analysed during the period from Jan 2016 to Aug 2016. The species wise distribution in all samples shown that the most common isolate collected during the present study was shown from increasing to decreasing order: *Pseudomonas aeruginosa* 210(50%), *Acinetobacter baumannii* 180 (42.8%), *Burkholderia cepacia* 18 (4.3%), *Stenotrophomonas maltophilia* 4 (≈1%), *Pseudomonas stutzeri* 4 (≈1%), *Alkaligenes faecalis* 2 (0.48%) and *Ralstonia picketti* 2 (0.48%).The study done at Kolkata by Rit *et al.,* and at Kolar by Malini *et al.,* showed higher prevalence of *P.aeruginosa* 50.24%and 53.8% respectively among the

non-fermenters. Whereas *A.baumannii* was the predominant isolate among non-fermenters in a study carried out by Joseph *et al.,* and Irfan *et al.,* (2008).

Considering sample wise distribution, most samples from which non-fermenters were isolated were those from sputum 126 (30%) was predominant sample followed by urine 98 (23.3%), High vaginal swab 80 (19%), pus 72 (17.1%), blood 24 (5.7%) and other miscellaneous samples 20 (4.7%). The predominant NFGNB isolates were from pus and respiratory secretions in a study carried out by Rit *et al.,* (2004) and Amudhan *et al.,* (2012) respectively.

There are various mechanisms by which Pseudomonas acquires resistance when compared to *Enterobacteriaceae* and proves their versatility. The detection of ESBL production among Pseudomonas aeruginosa and other non-fermenters are not described in Clinical Laboratory Standards Institute (CLSI) guidelines. The phenotypic confirmatory disk diffusion method using ceftazidime with and without clavulanic acid detected 14 and 16 ESBL by studies carried out by Singhal *et al.,* and various other studies respectively (Aggarwal, 2008; NitinBandekar *et al*., 2003). The epidemic outbreak among the hospitalised patients in many institutions in India, US and UK were due to 17 ESBLs. The prevalence of ESBL among non-fermenters in our study was50 (12%) and the prevalence of ESBL among*Pseudomonas aeruginosa* wereranging from 3.3% to 77.3% and among *Acinetobacter species*were ranging from 14.2% to 54.6% byvarious studies carried out in India (Jarlier *et al*., 1998).

In this study imipenem resistance among non-fermenters was seen in 13 isolates (3.1%) by Kirby-Bauer disk diffusion method. There are only few studies from India reported about imipenem resistance and they mention the prevalence of carbapenem resistance in non-fermenters varying from 36 to 90%. Out of these 13 imipenem resistant isolates, 61.5% isolates were of *A.baumannii,* 23.07% of *P.aeruginosa* and 15.38% of isolates were *Burkholderia cepacia*. A study about non-fermenters was carried out by Amudhan*et al* mentioned imipenem resistance shown by *A.baumannii* and *P.aeruginosa* as 64.08% and 34.07% respectively (Amudhan *et al*., 2012). The same finding of predominance of imipenem resistance was shown by*A.baumannii* among all non-fermenters, even though imipenem resistance was very low.

Isolate wise distribution shown out of 420, 180 isolates were *A.baumannii* during the study period were. Out of that 61.5% of *A.baumannii* isolates were imipenem resistant. The prevalence of imipenem resistance study among *Acinetobacter baumannii* by Khajuria *et al* from a centre in western Maharashtra to be at 42.11 % and Shivaprasad *et al.,* have reported 50.59% and Baran *et al.,* from a hospital in Turkey have reported 53.7%. Whereas low level of imipenem resistance among *Acinetobacter baumannii* isolates were reported by Rit *et al.,* and it was found to be 10%. Out of total 420 isolates, 210 *P.aeruginosa* were collected during the study period. Among these 210 isolates, imipenem resistant *P.aeruginosa* was 15.38%. The prevalence of imipenem resistance among the *P.aeruginosa* reported by various studies done by Bhalerao *et al.,* (67.5%), Behera *et al.,* (69%), Onguru *et al.,* (44.1%), Singh *et al.,* (21%), Carmeli *et al.,* (13%) and Lin *et al.,* (10.2%). Whereas in a study from Eastern India by Kalidas *et al* reported lower resistance of 9% to imipenem in *P.aeruginosa.*

The various antibiotic resistance profile among the ESBL producers and non-producers, imipenem resistant and sensitive isolates were showing resistance to piperacillin-tazobactam 18 (4.3%) and gentamicin 18 (4.3%), ciprofloxacin 12 (2.8%) and amikacin 11 (2.6%). ESBL and carbapenemase producing bacteria are frequently shows resistance to many other classes of antibiotics like aminoglycosides and fluoroquinolones. This is due to plasmid mediated encoding of ESBL and MBL genes for drug resistance to other antibiotics (Nathisuwan *et al*., 2001).

Table.1 Antibiotic susceptibility profile of non-fermentative gram negative bacilli

Antibiotics	Sensitive (%)	Resistant (%)
Cefepime	358 (85.2%)	62 (14.8%)
Ceftazidime	370 (88.1%)	50 (12%)
Ceftazidime-clavulanic acid	370 (88.1%)	50 (12%)
Colistin	400 (95.3%)	20 (4.8%)
Piperacillin-tazobactam	398 (94.7%)	18 (4.3%)
Gentamicin	398 (94.7%)	18 (4.3%)
Imipenem	407 (96.9%)	13 (3.1%)
Ciprofloxacin	408 (97.1%)	12 (2.8%)
Amikacin	409 (97.3%)	11 (2.6%)

Fig.1 Colony morphology of *Pseudomonas aeruginosa* in MacConkey agar showing 2-3 mm, flat, smooth, non-lactose fermenting colonies with regular margin and Alligator skin like from top view

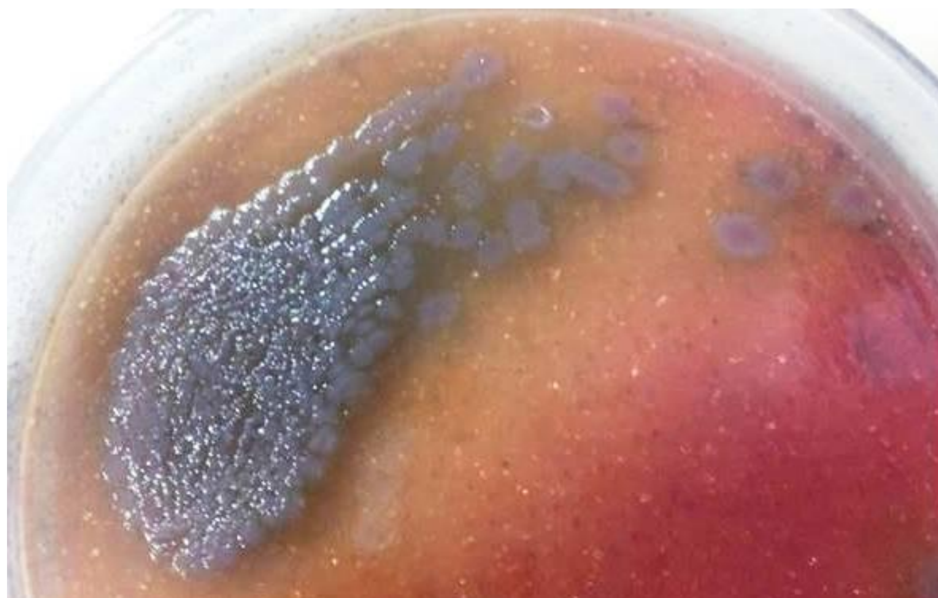


Table.2 Phenotypic identification chart for non-fermentative gram negative bacilli

Organism	Oxidase	Motility	Pigment &colony	Growth on MacConkey	Oxidize glucose	Growthat 42°C	NO_3 reduction	Indole test	Urea	Lysine decarb	Arginine decarb	Esculin	Polymyxin/ colistin
Pseudomonas aeruginosa	+	+	Bluish-green	+	+	+	+	-	+/-	-	+	-	S
Burkholderia cepacia	**W +**	+	Grey,yellow	+	+		+/-		+	+	-	+/-	**R**
Burkholderia pseudomallei	+	+	Cream to tan	+	+		+	-	+/-	-	+	+/-	**R**
Stenotrophomonas maltophilia	-	+	Lavender-purple, ammonia odour	+	+		+/-		-	+	-	+	S
Acinetobacter baumanii	-	-	No pigment	Pale pink	+	+	-	-	+/-	-	+	-	S
Alcaligenes faecalis	+	+		+	-		-	-	-			+	
Ralstonia picketti	+	+		+	+		+		+			-	**R**
Sphingomonas paucimobilis	+	+	Deep yellow	-	+			-	-			+	S
Brevundimonas diminuta	+	-		+	W +		-	-	-			-	S
Achromobacter denitrificans	+	+	No pigment	+	-		-	-	-			+	S
Shewanella putrefaciens	+	+	Orange-tan	+	W+	+	+/-		+/-			-	S
Chryseobacterium meningosepticum	+	-	Pale yellow	+/-	+		-	+	-			+	**R**
Ochromobactrumanthropi	+	+	No pigment	+	+		+ N_2		+			+/-	S
Rhizobium radiobacter	+	+	Extremely mucoid, No pigment	+ (pink)	+		+/-	-	+			+	
Roseomonas	**W +**	-	Mucoid, pink	+ (pink)	+/-		+/-		+			+	
Sphingobacterium multivorum	+	-	Pale yellow	+	+		-	-	+			+	**R**
Flavobacterium mizutaii	+	-	Yellow	-	+		**To N_2**	-	-			+	**R**
Psychrobacte rimmobilis	+	-	Rose like odor, no pigment	+/-	-		+/-	-	+				
Weeksella virosa	+	-	Butterscotch	+/-	-		-	+	-			-	S
Methylobacterium spp	+	+	Dry, coral, pink	-	+/-		+/-	-	+			-	

Gray shade- M.C encountered Non Fermenters **Oxidizes maltose, lactose-** *S.maltophilia, R.picketii, R.radiobacter* **H2S produced by** *Shewanella spp* **OF mannitol-** *B.cepacia* complex
Growth in 6.5% NaCl- *A.faecalis* **PAD positive-** *Ochrobactrum spp, R.radiobacter, Psychrobacterphenylpyruvicus* & immobilis

Fig.2 Oxidase test showing oxidase positive (left side) and oxidase negative (right side)

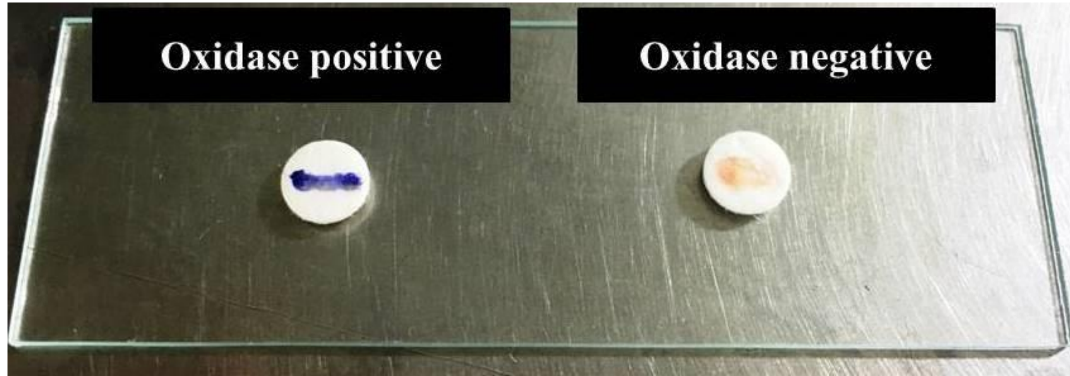


Fig.3 Various biochemical reactions for identification of non-fermentative gram negative bacilli

Fig.4 Hugh-Leifsen/ oxidative fermentative medium showing oxidative utilization of sugars – utilization of glucose e.g: *Pseudomonas* spp and *Acinetobacter baumannii* (left side) and maltose e.g: *Stenotrophomonas maltophilia* (right side)

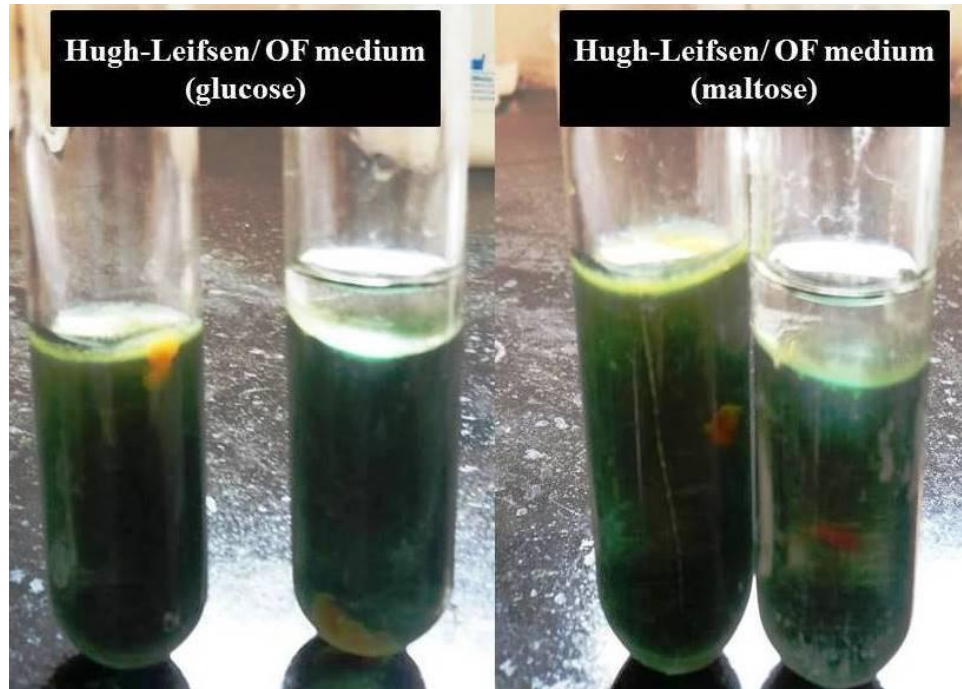


Fig.5 *Pseudomonas aeruginosa* sensitive to imipenem (IPM), piperacillin-tazobactum (PIT), gentamicin (GEN), ceftazidime (CAZ), ceftazidime-clavulanic acid (CAC), colistin (CL)

Fig.6 *Acinetobacter baumannii* resistant to imipenem (IPM), piperacillin-tazobactum (PIT), gentamicin (GEN), ceftazidime (CAZ), ceftazidime-clavulanic acid (CAC), colistin (CL), cefepime (CPM)

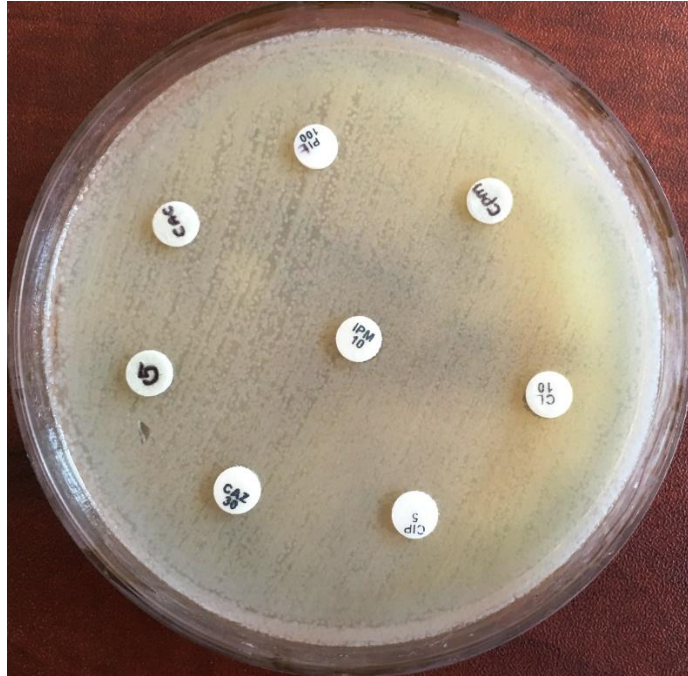


Fig.7 The species wise distribution of various isolates in all samples

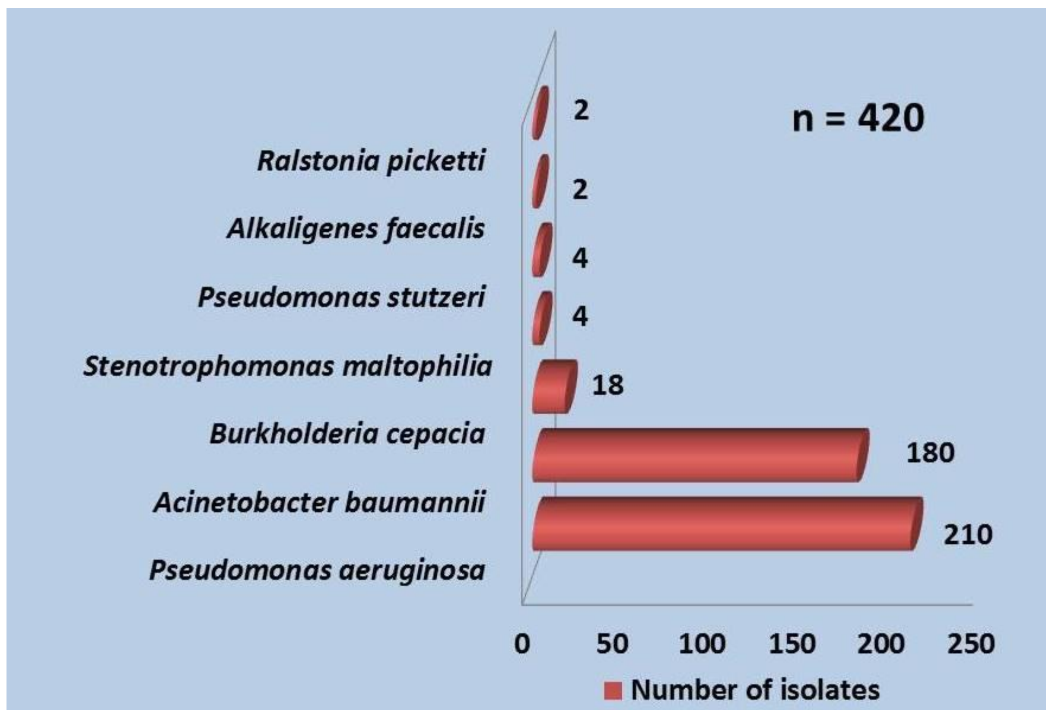
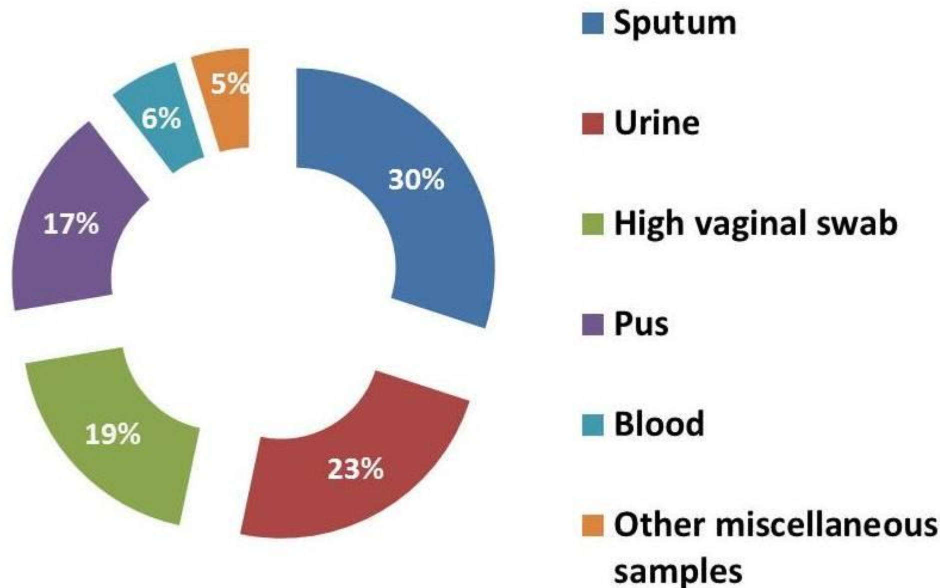


Fig.8 The distribution of various clinical samples



In conclusion, a prospective study conducted to know the prevalence, phenotypic characterization and antibiotic susceptibility of 420 non-fermentative Gram negative bacilli (NFGNBs) isolates from various clinical samples at a tertiary care centre. The majority of isolates were *Pseudomonas aeruginosa* 210 (50%) followed by *Acinetobacter baumannii* 180 (42.8%), *Burkholderia cepacia* 18 (4.3%) rest were other NFGNBs. The maximum isolates were resistant to third and fourth generation cephalosporins i.e., ESBL producers (12%) followed by piperacillin-tazobactam, ciprofloxacin, gentamicin and amikacin and imipenem resistance was 3.1%. The maximum imipenem resistance were seen in *A.baumannii* followed by *P.aeruginosa*. All the isolates were susceptible to colistin except 20 which were intrinsic resistant and were classified into *Burkholderia cepacia* and *Ralstonia picketii.*

The proper speciation of non-fermentative gram negative bacilli and antibiotic susceptibility testing as per latest CLSI guidelines for intrinsic drug resistance are very important. In this study the ESBL producers were more when compared to imipenem resistance or other antibiotics, may be because of the high usage of the third and fourth generation cephalosporins for the treatment of the patients when compared to carbapenem group of antibiotics (almost similar to drug holiday). Also regular follow up of resistance pattern, especially third and fourth generation cephalosporins and imipenem and their judicial usage in clinical practice or in hospitalized patients are important preventive measures for spread of multi drug resistant (MDR) organisms. The screening of the health care professionals and proper segregation of biomedical waste and also dedicated activity of hospital infection control committee (HICC) in a hospital with maintenance of strict antibiotic policy can help to fight against drug resistance.

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