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Phenotypic Characterization of *Burkholderia* Spp: In a Tertiary care hospital of Eastern India.

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Running title: Phenotypic and Genotypic Characterization of *Burkholderiaspp*: A Diagnostic Challenge

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ABSTRACT

BACKGROUND: The genus *Burkholderia* a gram negative bacteria, belonging to phylum proteobacteria inhabits soil and plants are pathogenic to humans. These include *B. mallei* and *B. pseudomallei* of the *B. pseudomallei* complex, which cause glanders and melioidosis, respectively. *B. cepacia* complex affecting cystic fibrosis patients causes “cepacia syndrome”.

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AIMS: To study the prevalence, risk factors, and antibiotic susceptibility pattern of the *Burkholderia* species.

METHODS: This was a hospital based prospective study conducted in the Department of Microbiology, SCB Medical College and Hospital, Cuttack, Odisha. Total 820 samples collected from hospitalized patients were processed for phenotypic characterisation and antimicrobial susceptibility testing.

RESULTS: Out of 820 samples, 505 (61.6%) were found to be culture positive with maximum culture positivity among pus samples 227 (91.5%). *Pseudomonas aeruginosa* was the predominant isolate 89 (73.5%) followed by *Acinetobacter baumannii 20* (16.6%) and 11(9.09%) *Burkholderia* spp. Prevalence of *Burkholderia* species was 11/820(1.34%). Diabetes mellitus was the commonest risk factor 8 (72.7%). All the isolates of *Burkholderia pseudomallei* were sensitive to Ceftazidime, Imipenem and Amoxi-clav while *Burkholderia cepacia complex* were sensitive to Ceftazidime, Meropenem and Levofloxacin.

CONCLUSION: *Burkholderia* spp can be identified by phenotypic methods but species level identification is more specific by molecular methods. It also guides the clinicians for instituting appropriate treatment which leads in reducing morbidity and mortality.

KEY WORDS: *Burkholderia* spp., nosocomial infection, cystic fibrosis, NFGNB, BCC

INTRODUCTION:

The genus *Burkholderia* (phylum *Proteobacteria*, class β-*Proteobacteria*, order *Burkholderiales*, family *Burkholderiaceae*) comprises more than 120 species was differentiated both on phenotypic characteristics as well as on 16S rRNA sequences, cellular lipid and fatty acid composition in 1992 [1].These organisms the majority being saprophytes inhabits soil and plants. Several *Burkholderia* species are pathogenic in humans, which includes *Burkholderia pseudomallei*, the causative agent of melioidosis; *Burkholderia mallei*, the causative agent of glanders and *Burkholderia cepacia complex* (BCC) group which causes 'cepacia syndrome'. The species in this genus differ from other Pseudomonads by exhibiting resistance to polymyxin B & colistin group of antibiotics. These are aerobic, non-sporing, non-fermenting, gram negative bacilli (NFGNB) that have emerged as opportunistic pathogens causing nosocomial infections among hospitalized and immune-compromised patients.

Melioidosis also known as 'Whitmore disease' is a life threatening infectious disease of tropics & subtropics caused by soil dwelling bacterium *B. pseudomallei*. It was first described by Alfred Whitmore & Krishnaswami in 1912 from cases of septicemia in morphine addicts in Rangoon, Burma. [2] Melioidosis emerged as an infectious disease of major public health importance in South East Asia & Northern Australia in the latter half of 20th century. [3] Cases of melioidosis have been reported from India since 1991. [4] It remains uncertain whether the occurrence of Melioidosis has increased in recent years or whether it was simply under-diagnosed & under recognized by the clinicians. Now it is an emerging infectious disease especially from the eastern & western coastal regions of India. [5] The organism has been recovered from wet soils, rice paddy fields, streams, pools and stagnant water reservoirs. The

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mode of transmission includes percutaneous inoculation, inhalation & ingestion, the commonest being direct inoculation of contaminated soil & surface water through abraded skin & inhalation of polluted water. [6] Diabetes mellitus is the most important risk factor. [7] Other high risk populations include individuals with chronic renal impairment(6-19%),tuberculosis(9-16%),immune disorders/steroid therapy (2.9-9.5%),solid tumors (0.7-10%),hematological malignancies (0.7-8%),chronic lung disease(2.8-3%),chronic heart disease(7.0%),smoking(10%),chronic alcoholism(0.7-2%),hemolytic anemia (0.7-2%) & malnutrition or anemia(8%). [8] *B .pseudomallei* infection has protean clinical manifestations and severity varies from an acute fulminant septic illness to a chronic infection that may mimic cancer or tuberculosis. The primary presenting features are pneumonia followed by genitourinary infection, skin infection, bacteremia without evident focus, septic arthritis or osteomyelitis, neurologic involvement & some may not have any evident focus of infection. [9]

Burkholderia cepacia, which was firstly described as responsible for onion soft rot by Walter Burkholder in 1942, [10] as an opportunistic pathogen for cystic fibrosis and chronic granulomatous disease patients as it causes fatal pneumonia known as "the cepacia syndrome."*Burkholderia cepacia complex* (BCC) is an important nosocomial pathogen particularly in those with prior broad-spectrum antibacterial therapy. Spectrum of infections caused by BCC includes bacteremia, urinary tract infection, septic arthritis, peritonitis & respiratory tract infection. Patients with cystic fibrosis & chronic granulomatous disease are predisposed to infection caused by BCC but it is being increasingly recognized as an important pathogen in both immune-compromised & hospitalized patients who are infected by contact with contaminated equipment during hospitalization. BCC bacteremia should be considered in febrile hospitalised patients, especially those with indwelling catheter , on ventilators, having cystic fibrosis or have immune dysfunction. [11] BCC multiplies in aqueous hospital environments including disinfectants & intravenous fluids for long periods. In India, there are no precise reports of the prevalence of BCC infection , and in most cases, ambiguously it has been reported as non fermenting gram-negative bacilli or simply *Pseudomonas* species.

Burkholderia mallei is an obligate parasite of animals primarily horses, mules & donkeys causing a respiratory tract infection known as glanders. It can be transmitted to humans through abraded skin in rare instances and may be laboratory acquired also. *B.mallei* had been used as a biowarfare agent in 2nd World War. [12] It is the only non-motile species of the genus *Burkholderia.*

Due to high intrinsic resistance and improper identification of non- fermenting gram-negative bacilli, it is difficult to treat such infections by *Burkholderia* spp, which leads to increased morbidity and mortality. Hence a prospective study has been undertaken to determine the prevalence, associated risk factors, identification and antibiogram of the *Burkholderia* species.

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Aims and Objectives:

Identification and characterization of different *Burkholderia* species, their prevalence, its risk factors and their antibiogram

MATERIALS & METHODS

Study Design & Place of Study:

The study was a hospital based prospective study conducted in the Department of Microbiology, SCB Medical College & Hospital, Cuttack, Odisha . for a period of 2 years from September 2017 to August 2019.

Study Population and Inclusion criteria:

After taking informed consent, 820 Clinical Samples i.e. (sputum, throat swab, blood, pus, urine, body fluids, ET tube tip and chest drain) from Indoor/Outdoor patients were included in the study. Detailed clinical history along with demographic data was also collected.

Collection and Processing of Various Samples:

Samples were collected following universal precautions and examined microscopically by Gram staining, inoculated on 5% Sheep blood agar , Mac Conkey agar ,CLED agar (for urine sample) & Ashdown's agar. Plates were incubated aerobically at 37^0C and observed for growth up to 5 days.

Phenotypic Identification:

Presumptive identification of *Burkholderia spp* was based on Gram staining, motility by hanging drop, colony morphologies in various media and biochemical reactions.

Key biochemical reactions to differentiate various *Burkholderia spp.* [13]

Test	*B.pseudomallei*	*B.mallei*	BCC
Oxidase	+	-	V
Motility	+	-	+
Growth at 42^0C	+	-	V
Oxidises Glucose	+	+	+
Oxidises Maltose	+	V	+
Oxidises Lactose	+	V	V
Oxidises Mannitol	+	-	+
NO3 Reduction	+	+	V
NO3 to Gas	+	-	-
Arginine Dihydrolase	+	+	-
Lysine Decarboxylase	-	-	V
Wrinkled Colonies	+	-	-

B.pseudomallei isolates were screened by a typical antibiogram as they are generally resistant to Colistin(10 µg) and Gentamycin(10 µg) but susceptible to Amoxicillin and Clavulanate (20/10 µg) [14]

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Antimicrobial Susceptibility Testing (AST)

Antibiotic susceptibility testing of the isolates were performed by using Kirby Bauer disc diffusion method as per CLSI guidelines. .Commercially available antibiotic discs manufactured by Hi-Media (Mumbai, India) were used. Additionally the antibiotic susceptibility for Levofloxacin and Chloramphenicol were determined by the MIC breakpoints (as per CLSI guidelines) by E-test [15]

Data Analysis:

The data were entered into the SPSS (24) software version. The percentage, proportion and standard deviations were observed. The results were presented in text and tables. P value (<0.05) was considered statistically significant.

Ethical issue: Present study satisfies the criteria of Institutional Ethics Committee (IEC) S.C.B Medical College Cuttack, 753007, Orissa as per the World Medical Association Declaration of Helsinki vide Institutional Ethical Committee (IEC/IRB No.—980/Dt.14-10-2019).

OBSERVATIONS:

Table No 1: Culture positivity of different samples processed

Nature of specimen	Number	Culture positive (n,%)	Culture negative (n,%)	*Burkholderia* spp isolated(n)	Prevalence (n,%)
Blood	316	165 (52.2)	151 (47.8)	6	1.9 (6/316)
Pus	248	227 (91.5)	21 (8.5)	4	1.6(4/248)
Sputum &Throat swab	124	58 (46.8)	66 (53.2)	00	00
Urine	82	34 (41.5)	48(58.5)	1	1.2(1/82)
Others	50	21(42)	29(58)	00	00
Total	820	505 (61.6)	315 (38.4)	11	1.34 (11/820)

A total of 820 samples were collected which included blood, pus, sputum, throat swab, urine and others body fluids. Out of the total 820 samples collected 505 (61.6%) were culture positive and 315 (38.4%) were culture negative. Maximum culture positivity was found among pus samples 227 (91.5%) and followed by blood 165 (52.2%). The prevalence of *Burkholderia* spp in all the samples accounted to (11/820) i.e. 1.34% percent .*Burkholderia* spp were not isolated from sputum & throat swab and from samples of other category.

TableNo 2: Non fermenting gram negative bacilli (NFGNB) isolated among the culture positive samples

Different samples analysed for culture positivity (n = 505)		
Nature of specimen	Culture positive	Non- fermenter (n, %)
Others	21	7 (33.3)
Pus	227	61(26.9)

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Urine	34	8 (23.5)
Blood	165	36 (21.8)
Sputum& Throat swab	58	9 (15.5)
Total	505	121 (24)
Different species of isolated Nonfermenters (n=121)		
NFGNB	Number	Percentage (%)
P.aeruginosa	89	73.5
A.baumanii	20	16.6
A.lwoffii	1	0.81
Burkholderia spp	11	9.09
Total	121	100

Nonfermenters were observed in 505 culture positive samples constituting 121(24%).
Most common source of Non-fermenters were samples included in others category 21(33.3%) followed by pus 61(26.9%). Only 9(15.5%) Non-fermenters were isolated from sputum.
Among the Non-fermenters isolated *Pseudomonas aeruginosa* was the most common organism 89(73.5%) followed by *Acinetobacter baumannii* 20 (16.6%) and *Burkholderia* spp accounted to 11(9.09%) only.

Table No 3: Sample wise prevalence of *Burkholderia species*

Nature of specimen	Number	*B.pseudomallei*	Prevalence (%)	BCC	Prevalence (%)
Blood	316	1	0.31 (1/316)	5	1.6 (5/316)
Urine	82	1	1.2(1/82)	0	0
Pus	248	4	1.6(4/248)	0	0
Sputum& Throat swab	124	0	0	0	0
Others	50	0	0	0	0
Total	820	6	0.73(6/820)	5	0.6 (5/820)

Maximum number of *Burkholderia pseudomallei* was obtained from pus sample (4/248) whereas BCC was only isolated from blood (5/316). The overall prevalence of *B.pseudomallei* in different clinical samples is 6/820 (0.73%) and for BCC the it was 5/820 (0.6%) respectively.
patients belong to the adults with Diabetes mellitus being the commonest risk factor associated with such infections . (Table No 3 & Fig No 1, 2,3,4)

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Figure 1 -: Growth of *Burkholderia pseudomallei* on Ashdown agar after A) 48 hrs & B) 72 hrs of incubation.

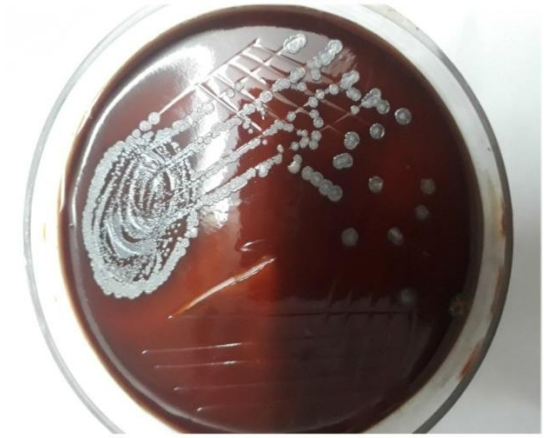

Figure 2-:Growth of *Burkholderia pseudomallei* on sheep blood agar after A) 24 hrs& B) 72 hrs of incubation.

Figure 3-: Growth of *Burkholderiacepacia complex*(BCC) A) on MacConkey agar after 24 hrs of incubation B) on Blood agar after 24 hrs of incubation.

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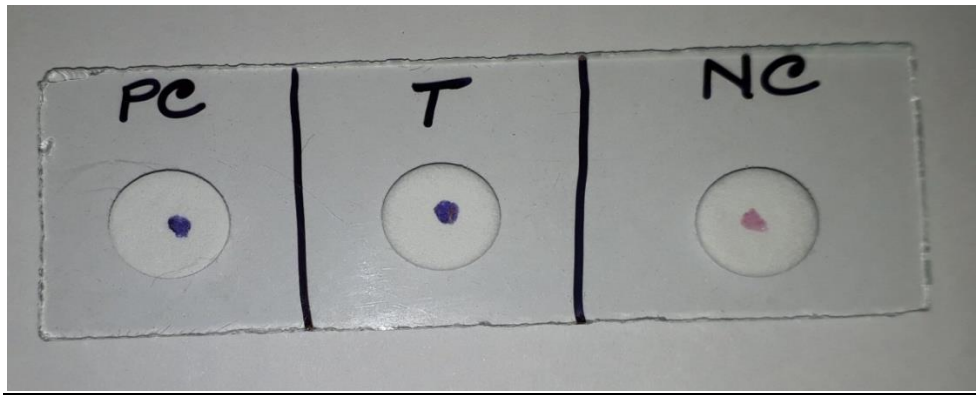

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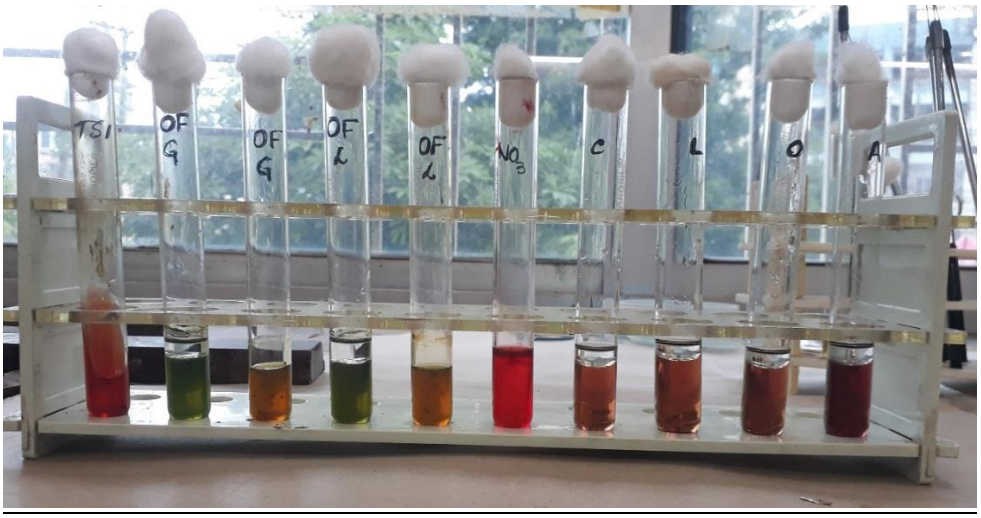

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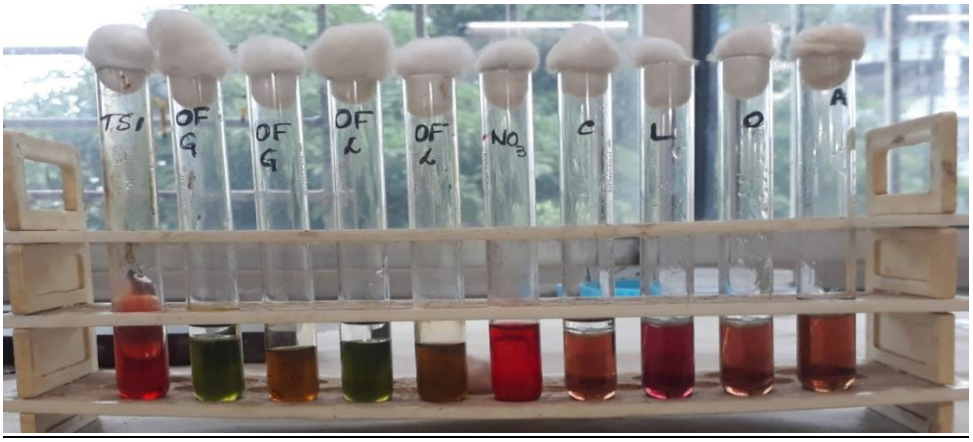

C

Figure 4 : A) Positive oxidase test B) Biochemical reactions of *Burkholderia pseudomallei* C) Biochemical reactions of *Burkholderia cepacia complex.*

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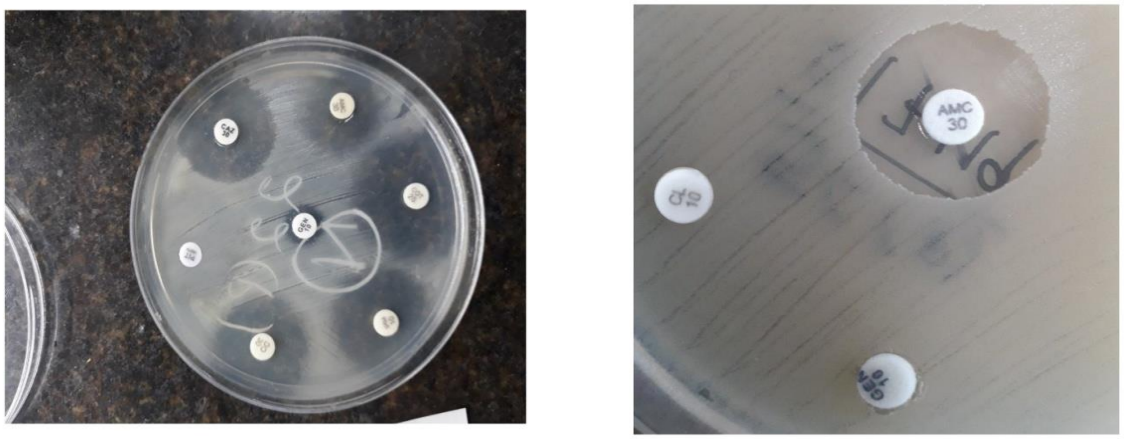
Table No 4: Antimicrobial susceptibility pattern of *Burkholderia pseudomallei and Burkholderia cepacia complex*

Antimicrobial susceptibility pattern of *Burkholderia pseudomallei*		
Antibiotic	Number sensitive	Sensitivity (%)
Ceftazidime	6	100
Imipenem	6	100
Amoxi-clav	6	100
Piperacillin-tazobactam	4	66.6
Trimethoprim-sulfomethoxazole	5	83.3
Doxycycline	4	66.6
Tetracycline	4	66.6
Gentamycin	0	0
Antimicrobial susceptibility pattern of *Burkholderia cepacia complex*		
Antibiotics	Number sensitive	Sensitivity (%)
Ceftazidime	5	100
Meropenem	5	100
Levofloxacin	5	100
Trimethoprim-sulfomethoxazole	4	80
Chloramphenicol	4	80
Minocycline	3	60



A B

Figure 5-: A)Antimicrobial susceptibility pattern of *Burkholderia pseudomallei* B) Typical antibiogram for screening of *Burkholderia pseudomallei*(colistin & gentamicin resistance and amoxi-clav sensitive)

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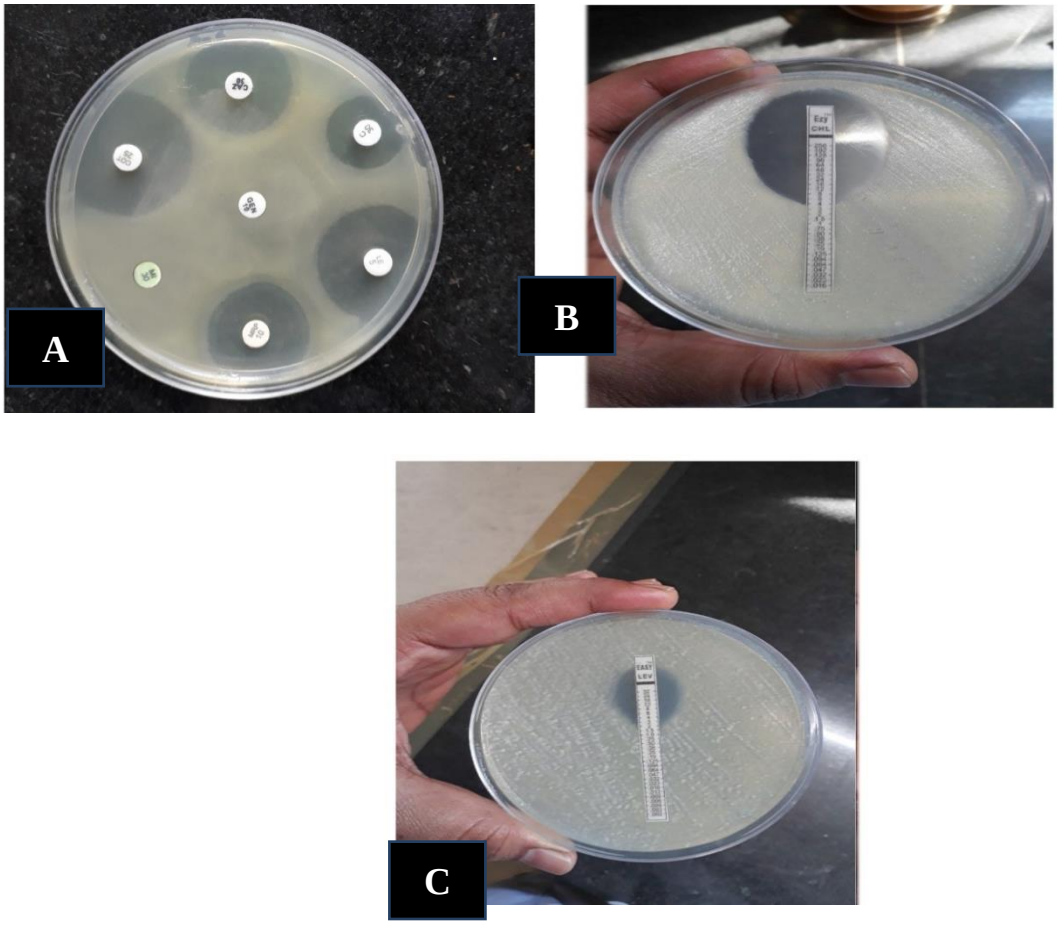

Figure 6 -:A)Antimicrobial susceptibility pattern of *Burkholderiacepacia complex* B)Determination of minimum inhibitory concentration (MIC) of Chloramphenicol by E-test. C) Determination of minimum inhibitory concentration (MIC) of Levofloxacin by E-test

All the isolates of *Burkholderia pseudomallei* were sensitive to Ceftazidime, Imipenem and Amoxi-clav whereas isolates of *Burkholderia cepacia complex* were sensitive to Ceftazidime, Meropenem and Levofloxacin. (Table No 4 & Fig 5, 6)

DISCUSSION

Burkholderia species has now emerged as significant pathogen causing serious nosocomial infection and also life-threatening infections in immune-compromised hosts. Because of the high transmissibility between hospitalized patients and their multiple drug resistance they cause serious problems in clinical settings. Amongst all, *Burkholderia pseudomallei, Burkholderia mallei, Burkholderia cepacia complex* are the pathogenic species of the genus *Burkholderia* which causes infections in humans. [16]

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In the present study, a total of 820 clinical samples like blood, pus, sputum, urine, body fluids were collected out of which 505(61.6%) samples were found to be culture positive and maximum culture positivity was found among pus samples 227 (91.5%) [Table 1], which was concordant with the findings of Sharma D *et al.* [17] In this study, 121(24%) nonfermenting gram negative bacilli (NFGNB) were isolated from 505 culture positive samples, (Table 2))which was similar with the study by Sharma D *et al.* [17], but the result was much higher than that reported by Madkey *et al.* [18] who reported an isolation rate of 5.19%.

In the current study, the commonest source of nonfermenters were samples of other category that included body fluids, ET tube tip, chest drain 7/21 (33.3%) followed by pus 61/227 (26.9%)[Table 2] which correlates with the study by Sharma D *et al.* [17]

Pseudomonas aeruginosa was the commonest nonfermenter isolated accounting for 89(73.5%) followed by *Acinetobacter baumannii* 20(16.6%). Other significant nonfermenters isolated were *Acinetobacter lwoffii* 1(0.81%) and *Burkholderia* spp 11(9.09%) [Table 2], but it differs from Madkey et *al.* [18] where the most common nonfermenter isolated was *Acinetobacter* spp accounting to 56.82% followed by *Pseudomonas* spp.*(*40.92%),*Stenotrophomonas maltophila* (1.36%) and *Burkholderia cepacia complex* (0.90%).

In the present study *Burkholderia species* accounted to 11(9.09%) among the Non-fermenting gram negative bacilli (NFGNB)[Table 1]that coincides with study by Kalidas Rit *et al.* [19] The prevalence of *Burkholderia* species in the clinical samples was 11/820(1.34%) [Table No 1 & 3]. The predominant *Burkholderia species* isolated were *Burkholderia pseudomallei* and *Burkholderia cepacia complex (BCC).* The overall prevalence of *Burkholderia pseudomallei* was 0.73% [Table No 1 & 3] . This finding is much higher as compared to study by Mary V Jesudason *et al.* [20] This difference in prevalence of isolates in different health care settings was likely and well expected as they depend on many local variables. *Burkholderia pseudomallei* was most prevalent in pus samples which accounted to 1.6% but in study by K Vidyalakshmi *et al.* [21] majority of the isolates were recovered from blood samples.

The overall prevalence of *Burkholderia cepacia complex (BCC)* in our study was 5/820(0.6%) [Table 3], which is concordant with study by Alaa Fahim Abbas. [22] BCC isolates were most prevalent in blood samples 5/316(1.6%) which is comparable to study by T.S. Shailaja *et al.* [23]

In the present study, majority of the patients belonged to the age group of 41-50 years 3/11 (28%). This is similar to the studies done by Payne GW *et al.* [16] and Sagar Chandrakar *et al.* [24] In this study *Burkholderia* species were isolated from 7/11(63.3%) male patients and 4/11(36.3%) female patients. A male preponderance was observed in our study with male to female ratio of 1.75:1 similar to the study by Alaa Fahim Abbas [22] Diabetes mellitus was found

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to be the commonest risk factor 8/11 (72.7%) followed by indwelling catheters 6/11 (54.5%), which correlates with the findings reported by Sagar Chandrakar *et al*. [24]

All the isolates of *Burkholderia pseudomallei* were uniformly sensitive 6(100%) to Ceftazidime, Imipenem and Amoxicillin-Clavulanic acid and resistant to Gentamycin. Isolates sensitive to Trimethoprim-sulfomethoxazole were 5 (83.3%) whereas 4(66.6%) of the isolates were sensitive to Piperacillin-tazobactam, Doxycycline and Tetracycline respectively. [Table 5].This finding is in accordance to study by Subarna Dutta *et al*. [25]

In this study all the isolates of *Burkholderia cepacia complex* were uniformly sensitive to Ceftazidime, Meropenem and Levofloxacin followed by Trimethoprim - Sulfomethoxazole and Chloramphenicol 4/5(80% each) where as 3/5 (60%) of them were sensitive to Tetracycline. This finding was comparable to T.S. Shailaja *et al*. [23] who reported that 97.96% of the isolates were sensitive to Ceftazidime, 71.43% to Meropenem but only 40.82% of the isolates were susceptible to Levofloxacin.

CONCLUSION

Due to constant revision of taxonomy of *Burkholderia* genus and the similarity in phenotypes of several species, correct identification is difficult and sometimes they are misdiagnosed as other nonfermenting gram negative bacilli like *Pseudomonas species* or *Acinetobacter* species from which they have to be differentiated. It has always been difficult task for a routine microbiological laboratory to identify NFGNBs and poor laboratory proficiency in identification of *Burkholderia* species prevails worldwide including India. Due to this, the microbiological reports of the disease, caused by this organism are rare in India. Although phenotypic methods could identify the genus *Burkholderia* but species level identification is possible only by molecular methods due to overlapping of biochemical characteristics between species.16S *rRNA* and *recA* gene targets significantly improves species level discrimination in Bcc.

Isolation of these non-fermenting gram negative bacilli and their antibiotic susceptibility pattern should be regarded with all seriousness in clinical practice and clinical epidemiology because by being resistant to multiple antibiotics their prevalence not only limits the treatment options but also act as a reservoir of drug resistant genes. An increased clinical vigilance with good microbial work- up would help, with improved, accurate and rapid diagnosis and also guides the clinicians in instituting appropriate treatment without delay that can significantly reduce incidences of morbidity and mortality. They are considered as emerging nosocomial pathogens. So every effort should be made for prevention and control of infections caused by them. Clinicians should adhere to the antibiotic policy and the policy should be revised regularly depending upon the antibiotic sensitivity pattern and feedback received from clinicians.

Limitations of the study:

Inadequate laboratory identification and limited treatment options are the main obstacles hindering accurate diagnosis and thus proper therapeutic outcome.

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