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RESEARCH ARTICLE

IDENTIFICATION OF NON FERMENTING GRAM NEGATIVE BACILLI FROM PATEINS IN AL-DIWANIYA CITY AND DETECTION OF THEIR VIRULENCE FACTORS.

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Abstract

Non fermenting gram negative bacilli (NFGNB) have emerged as multidrug resistant pathogens associated with life threating nosocomial infections. To assess the frequency of these pathogens, (873) different clinical specimens were collected from patients admitted to Al- Diwaniya teaching hospital during a period extended from January to December 2015. Bacterial isolates recovered from the collected sampled have identified based on biochemical tests and confirmed using Vitek2 Compact system. The predominant isolate was *Pseudomonas aeruginosa* (60%) followed by *Burkholdaria cepacia* (26%), *Acintobacter* spp.(6%). *Pseudomonas putida* , *Pseudomonas leutella*, *Sphingomonas paucimobilis*, and *Achromobacter* spp. (2%). The highest number of the NFGNB was obtained from ear swabs (36%) followed by sputum (24%), vaginal swabs and urine (12%), throat swabs (10%), and blood (6%). The NFGNB were isolated from both males and females and most of them detected in the age group 15-30 years followed by the age group 47-62 year then 31-46 year. Virulence factors, including colonization factors antigens I and III, proteases, siderophores, and capsule have identified in the isolated NFGNB. Results also showed that isolates of the predominant species, *P. aeruginosa*, were resistant to multiple antibiotics including, Ampicillin (93.98%), Ceftriaxone (82.13%), Chloramphenicol (72.09%), Piperacillin (64.31%), Ciprofloxacin (46.97%), Gentamycin (32.4%), Tetracycline (30.75%), Levofloxacin (15.17%), Amikacin (12.12%), Trimethoprim–Sulfamethoxazole (10.67%). *P. aeruginosa* isolates showed low resistance to Colistin (2.34%) and Imipenem (6.89%). The outcome of this study may be helpful to understand the epidemiology of these organisms, which is crucial for appropriate management of infections caused by NFGNB.

Introduction:-

Non fermenting gram negative bacilli (NFGNB) is a diverse group of aerobic, non spore forming, gram negative bacteria that either do not use carbohydrates as energy source or utilize them by means of metabolic pathways other than fermentation (Vijaya, Bavani *et al*. 2000, Benachinmardi, Padmavathy *et al*. 2014). This group comprises of organisms from various genera such as *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Burkholderia cepacia*, *Sphingobacterium*, *Agrobacter*, and *Weeksiella*. These organisms exist commonly in soil and water, and they are also present as harmless parasites on the mucus membranes of humans and animals (Baron, Peterson *et al*. 1994). Although the members of NFGNB are normally considered to be commensals or contaminants, the pathogenic potential of this group has been established undeniably because of their regular isolation from clinical specimens and their involvement with many diseases(Sujatha Karjigi 2013). Recent studies revealed that these organisms are now associated with serious infections, such as meningitis, ventilator associated pneumonia (VAP), surgical site infection, osteomyelitis, and wound infections (Juyal, Prakash *et al*. 2013). The NFGNB reported to form about (15%) of all bacterial isolates from clinical samples(Gokale and Metgud 2012). Infections caused by these

organisms are endogenous or exogenous origin, depending on numerous factors like abusive use of wide spectrum antimicrobial agents, the use of immunosuppressant substance, prolong surgical procedure, and inadequate instrumentation (Frota and Moreira 1998, Kaur, Gupte *et al*. 2015). Most of the NFGNB are resistant to many antibiotics because of the extensive use of the antimicrobial agents used to treat infections caused by these organisms, making them important health care associate pathogens (Gokale and Metgud 2012). They have diverse mechanisms for resistance, including: enzymes production, enzymatic deactivation of antibiotics, precise targeted enzyme which is inhibited by antimicrobial agents, modification in target locations, creation of efflux pumps, defeat of outer membrane proteins or porins, and decrease of the antimicrobial agent uptake; therefore , the therapeutic choices are strictly restricted to treat the NFGNB infections (Zahid, Akbar *et al*. 2014). This is the first study to investigate the clinical significance of NFGNB in Iraq. The objectives of this study are to: (1) Isolate and identificate NFGNB from different clinical specimens, including sputum, throat swabs, ear swabs, urine, blood, and vaginal swabs (2) Investigate the prevalence of NFGNB in Iraq, particularly in Al-Diwaniyah city (3) Identificate the various virulence factors related to infections caused by NFGNB, and (4) Test the susceptibility of the isolated and identified predominant species, *P. aeruginosa*, to the commonly used antimicrobial agents.

Methods and materials:-

Specimens collection:-

A total of 873 different clinical specimens, including sputum, throat swabs, ear swabs, urine, blood, stool, wound and vaginal swabs were collected from patients admitted to Al- Diwaniya teaching hospital during a period extended from January to December 2015. The following data were recorded for each patient: age, sex, hospitalization. The specimens were collected by standard methods as mentioned in (Forbes 2007).

Isolation and Identification:-

The specimens were cultured on MacConkey's agar and nutrient agar then incubated aerobically at 37ºC for 24-72hrs for primary isolation. Non lactose fermenting colonies on MacConkey's agar were selected and sub cultured to obtain pure culture. Bacterial isolates were identified by routine diagnostic tests including cultural, morphological and biochemical characteristics (Baron 1994, Forbes 2007). Confirmative diagnosis was done by using Gram-negative GN identification card automated Vitek2 Compact system (Biomeriux).

Virulence factors tests:-

Virulence factors were detected as described in (Cruckshank 1975, Piret 1983, Nassif 1989, Baron 1994). Haemagglutination test was carried out to detect the ability of bacterial isolates to produce colonization factors antigens. Negative staining method was performed to determine if bacterial isolates posse's polysaccharide capsule. M9 medium was used to investigate siderophore and extracellular proteases production.

Antibiotic susceptibility test:-

Antibiotic susceptibility pattern were determined by Bauer Kirby's disc diffusion method according to the Clinical and Laboratory Standard Institute (CLSI) guidelines(CLSI 2007). Mueller-Hinton plates were inoculated with a 0.5 McFarland standard suspension of bacteria, and disks were placed. Zones of growth inhibition were recorded in millimeters after overnight incubation.

Results:-

Out of 873 different clinical specimens collected during the study period, the NFGNB were isolated from 50 samples (5.72%), while other bacterial species were isolated from 563 samples to form (64.49%) of the total specimens number. No growth was detected in 260 samples with a percentage of (29.78%).Table (1).

Table(2) shows the specimens profile and frequency of NFGN bacilli isolated from sputum, throat swabs, ear swabs, urine, blood, vaginal swabs, stool, and wounds. The NFGNB were predominant in ear swabs (36%) followed by sputum (24%), vaginal swabs and urine (12%). The NFGNB were also isolated from throat swabs (10%) and blood (6%).Table (3) illustrates the specimens profile and distribution of NFGN bacilli isolates. The most common NFGNB isolated during the current study was *Pseudomonas aeruginosa* (60%) followed by *Burkholdaria cepacia* (26%), *Acintobacter* spp.(6%). *Pseudomonas putida* , *Pseudomonas leutella*, *Sphingomonas paucimobilis*, and *Achromobacter* spp.were also isolated with a percentage of (2%). Most of *Pseudomonas aeruginosa* (16 isolates) were recovered from ear swabs followed by urine (6 isolates), vaginal swabs (5 isolates), sputum (2 isolates), and throat swab (1 isolate). *Burkholdaria cepacia* was predominant in sputum samples (7 isolates) followed by throat

swabs (4 isolates) and blood (2 isolates). Two *Acintobacter* spp. isolates were recovered from sputum and one isolate from ear swab. *Pseudomonas putida* was isolated only from one blood sample, while *Pseudomonas leutella* was obtained only from a vaginal swab. A single isolate of *Sphingomonas paucimobilis* was recovered from an ear swab, and one isolate of *Achromobacter* spp. was isolated from a sputum sample.

The distribution of NFGN bacilli in relation to age and sex is demonstrated in table (4). The age range in our study was 15-63 years. The NFGNB were isolated from 26 (52%) male and 24 (48 %) female. The highest number of isolates (22 isolate) (44%) was detected in the age group 15-30 years followed by the age group 47-62 year(17 isolates)(34%) then 31-46 year (9 isolates)(18%).

Table (5) summarizes the type and percentages of virulence factors identified in the present study. Colonization factors antigenI were detectable in (50%) of *Pseudomonas aeruginosa* isolates, (33.3%) of *Acintobacter* spp., and (15.4%) of *Burkholdaria cepacia* isolates. Colonization factors antigen III were detected in (100%) of *Pseudomonas putida* , *Pseudomonas leutella*, and *Acintobacter* spp. isolates, (86.7%) of *Pseudomonas aeruginosa* isolates, and (76.9%) of *Burkholdaria cepacia* isolates. Sidrophores were identified in (83.3%) of *Pseudomonas aeruginosa* isolates and (84.6%) of *Burkholdaria cepacia* isolates. Extracellular proteases were detected in (100%) of *Pseudomonas leutella* and *Sphingomonas paucimobilis* isolates, (36.7%) of *Pseudomonas aeruginosa* isolates, and (23%) of *Burkholdaria cepacia*. Moreover, capsule was found in (100%) of *Pseudomonas putida*, *Pseudomonas leutella*, *Sphingomonas paucimobilis*, and *Achromobacter* spp. isolates, (84.6%%) of *Burkholdaria cepacia* isolates, (73.3%) of *Pseudomonas aeruginosa* isolates, and (66.6%) of *Acintobacter* spp .isolates. Our results showed that *P. aeruginosa* isolates were resistant to multiple antibiotics including, Ampicillin (93.98%), Ceftriaxone (82.13%), Chloramphenicol (72.09%), Piperacillin (64.31%), Ciprofloxacin (46.97%), Gentamycin (32.4%), Tetracycline (30.75%), Levofloxacin (15.17%), Amikacin (12.12%), Trimethoprim–Sulfamethoxazole (10.67%). The most effective antibiotics are Colistin(2.34%) and Imipenem (6.89%) (Figure1).

Table 1: Numbers and percentages of NFGN bacilli and other bacteria isolated during the current study.

Cultural result specimens	Number of specimens	%
NFGN bacilli isolates	50	5.72
Other bacteria	563	64.49
No growth	260	29.78
Total	873	100

Table 2: Specimens profile and frequency of NFGN bacilli isolates.

Types of specimens	Number of Specimens	Number of NFGN bacilli isolates	%
Sputum	117	12	24
Throat swabs	86	5	10
Ear swabs	98	18	36
Urine	381	6	12
Blood	93	3	6
Vaginal swabs	41	6	12
Stool	45	0	0
Wounds	12	0	0
Total	873	50	100

Table 3: Specimens profile and distribution of NFGN bacilli isolates.

Specimens profile / Bacterial species	Sputum	Throat swabs	Ear swabs	Urine	Blood	Vaginal swabs	Total	%
Pseudomonas aeruginosa	2	1	16	6	0	5	30	60
Pseudomonas putida	0	0	0	0	1	0	1	2
Pseudomonas leutella	0	0	0	0	0	1	1	2
Burkholdaria cepacia	7	4	0	0	2	0	13	26
Acintobacter spp.	2	0	1	0	0	0	3	6
Sphingomonas paucimobilis	0	0	1	0	0	0	1	2
Achromobacter spp.	1	0	0	0	0	0	1	2
Total	12	5	18	6	3	6	50	100

Table 4: Occurrence of NFGN bacilli in relation to age and sex.

Age-group (years)	Male		Female		Total	
	No. isolates	%	No. isolates	%	No. isolates	%
15-30	10	20	12	24	22	44
31-46	7	14	2	4	9	18
47-62	8	16	9	18	17	34
≥63	1	2	1	2	2	4
total	26	52	24	48	50	100

Table 5: Virulence factors detected in NFGN bacilli isolates

Virulence factors / Bacterial species	colonization factors antigen I	colonization factors antigen III	Sidrophores	Extracellular proteases	Capsule
Pseudomonas aeruginosa	50 %	86.7%	83.3%	36.7%	73.3%
Pseudomonas putida	0%	100%	0	0	100%
Pseudomonas leutella	0%	100%	0	100%	100%
Burkholdaria cepacia	15.4%	76.9%	84.6%	23%	84.6%
Acintobacter spp.	33.3%	100%	0	0	66.6%
Sphingomonas paucimobilis	0	0	0	100%	100%
Achromobacter spp.	0	0	0	0	100%

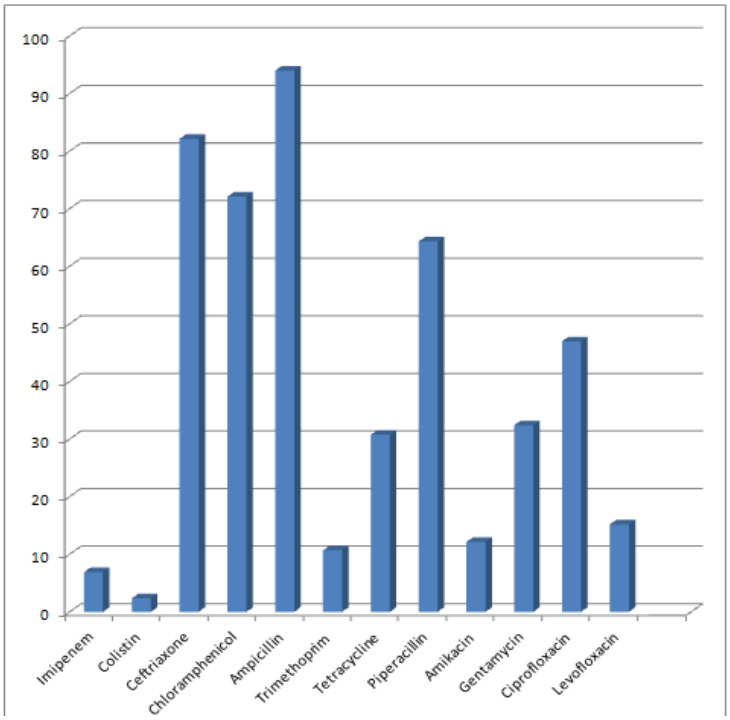

Figure 1: Antibiotic resistant profile of P. aeruginosa

Discussion:-

Non-Fermenting Gram negative bacilli have emerged as significant opportunistic pathogens that are responsible to cause many infections especially in the growing inhabitants of patients who are immunocompromised by their disease or medical treatment(Baron 1994, Enoch, Birkett *et al*. 2007). The NFGNB epidemiological complexity, tendency to cause outbreaks of infection, and antimicrobial resistance make these organisms worth mentioning and investigating (Rahbar and Hajia 2006, Taherikalani, Etemadi *et al*. 2008). Studies performed by different researchers

have illustrated that the range of isolation rate of NFGNBs vary from (9.32%) to (45.9%) (Sidhu, Arora *et al*. 2010, Juyal, Prakash *et al*. 2013). The most common non fermenting pathogens for humans are *Pseudomonas aeruginosa* and *Acinetobacterbaum-aniiare*, and infections by other bacterial species are quite uncommon *(Taneja, Maharwal et al*. 2003, Thipperudraswamy, Nadigar *et al*. 2014). In the present study, the predominant bacteria was *Pseudomonas aeruginosa* (60%) followed by *Burkholdaria cepacia* (26%). In other studies, the most frequent NFGNB isolated were *Pseudomonas aeruginosa* (70.43%) (50.24%) then *Acinetobacter calcoaciticus-baumaniicomplex* (25.44%) (24.78%) respectively (Thipperudraswamy, Nadigar *et al*. 2014, Bhargava, Kar *et al*. 2015). Among the diverse clinical specimens that we collected and processed for the isolation of pathogens capable of causing diseases, the NFGNB were predominant in ear swabs (36%) followed by sputum (24%) in comparison to other studies where the highest numbers of NFGNB were isolated from the pus exudates and body fluid samples (Bhargava, Kar *et al*. 2015) (Malini, Deepa *et al*. 2009).

Pseudomonas aeruginosa isolates were recovered from ear swabs, urine , and vaginal swabs where they can colonize and cause chronic otitis (Rowlands, Devalia *et al*. 2001) and urinary tract infection(Ferroni, Nguyen *et al*. 1998) respectively. It was also isolated from sputum and a throat swab where consider the most significant cause of morbidity and mortality of chronic airway infections, such as cystic fibrosis (Moffett 2010). We have identified two colonization factors antigens I and III, proteases, siderophores, and capsule in our *P. aeruginosa* isolates. To establish an infection, a bacterium must adhere to the epithelium and multiply; to accomplish this, *P. aeruginosa* have evolved colonization factors, such as pili or fimbria that recognize and attach the bacteria to host cells. *P. aeruginosa* produces several surface-associated adherence factors which promote attachment and contribute to the virulence of this pathogen(Hahn 1997). Proteases can cause bleeding and tissue necrosis. Siderophores are implicated in the chronic infection caused by *P. aeruginosa* by allowing the bacteria to multiply even if there is ferrous ions deficiency (Ben Haj Khalifa, Moissenet *et al*. 2011). Mucoid exopolysaccharide capsule can protect *P. aeruginosa* from host phagocytic activity and from antibiotics (Vasil 1986). *P. aeruginosa* in the present study showed high sensitivity to Colistin and Imipenem, and the results are similar to (Meghna, Bhat *et al*. 2014, Bhargava, Kar *et al*. 2015) and different from (Taneja, Maharwal *et al*. 2003) when they found high resistance to Imipenem by *P. aeruginosa*. This difference is likely because of the diversity in antibiotic susceptibility in different environmental conditions and regions. On the other hand, *P. aeruginosa* isolates were highly resistant to multiple antibiotics including, Ampicillin, Ceftriaxone, Chloramphenicol, Piperacillin; the results are consistent with(Kalidas Rit 2013).

Burkholdaria cepacia isolates were recovered from sputum, throat swabs, and blood. This bacterium has emerged as an opportunistic pathogen of pulmonary infections in patients suffering from cystic fibrosis and immunocompromised patients without cystic fibrosis (Govan, Hughes *et al*. 1996), and it is progressively more reported as a cause of healthcare associated infections, including bloodstream infections (De Smet, Veng *et al*. 2013). We also identified colonization factors antigens I and III, proteases, siderophores, and capsule in our *B. cepacia* isolates. *Acintobacter* spp. isolates were isolated from sputum and ear swab. *Acinetobacter* has appeared as a significant nosocomial bacterium causing infectious outbreaks in patients leading to high mortality and morbidity. It is associated with blood stream infections (Kaur, Gupte *et al*. 2015), acute or chronic otitis media, and otitis externa (Dadswell 1976).

Pseudomonas putida was isolated only from one blood sample, and it is reported to cause lethal case of bacteremia because of skin and soft tissue infections (Thomas, Okamoto *et al*. 2013). *Pseudomonas leutella* was obtained only from a vaginal swab; it is an uncommon opportunistic pathogen that documented as an unusual cause of infections in underlying medical disorders (Yousefi, Shoja *et al*. 2014). A single isolate of *Sphingomonas paucimobilis* was recovered from an ear swab; it is infrequently isolated from clinical specimens and it is related to a vast variety of infections. The majority *S. paucimobilis* infections reported in either hospital acquired or associated to nosocomial outbreaks (Toh, Tay *et al*. 2011). One isolate of *Achromobacter* spp. was isolated from a sputum sample. This bacterium is increasingly found in sputum of cystic fibrosis people (De Baets, Schelstraete *et al*. 2007)

Our study also revealed that the NFGNB were isolated from both males and females, and the highest number of NFGNB isolates was detected in the age group 15-30 years followed by the age group 47-62 year then 31-46 year. People at the age of 15 - 30 years are more energetic, have more public contacts, more movement from one place to another so the chances of getting infection at this age is high. The possibility of getting infections depends on number of experiences to the injurious bacteria, viruses and toxins. In contrast, at older ages, the people are limited from social contact, so the chances of developing infections is less(Akbar, Zahid *et al*. 2014).

Accurate identification and revealing of antibiotic sensitivity profile is vital for a proper management of infections caused by NFGNB (Benachinmardi, Padmavathy *et al*. 2014). It is also important to start the clinical relevance of the NFGNB members before considering as pathogens to keep people away from unnecessary use and appearance of drug resistance strains(Kalidas Rit 2013).

Conclusion:-

To the best of our knowledge, this is the first report to explore the prevalence of NFGNB in Iraq, particularly in Al-Diwaniya city. Results of this study may be useful to understand the epidemiology and the pathogenic functions of these organisms, and they are also vital for appropriate management of infections caused by NFGNB.

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