Review of Literature:

*Pseudomonas* belongs to genus of gamma proteobacteria, further belonging to the larger family of *Pseudomonads* [30]. On the basis of 16SrRNA sequence analysis, *Pseudomonas* includes strains formerly classified in the genera *Chryseomonas* and *Flavimonas*. Those strains previously classified in the genus *Pseudomonas* are now classified in the genera *Burkholderia* and *Ralstonia*. *Pseudomonas* was first isolated by a French military surgeon Sedillot in 1850 from a blue-green discharge on surgical dressing with associated infections[28]. The name *aeruginosa* stems from the green-blue coloured colonies seen among many clinical isolates[29].

**VIRULENCE FACTORS:**

*Pseudomonas aeruginosa* is a notorious organism that is difficult to control with antibiotic and detergents. It produces several metabolites that enhance the colonization and infection of the host tissue. Virulence factors includes, endotoxin[liopolysaccharides(LPS)], motility, pili, capsule and several exotoxins – proteases, haemolysins, lecithinase, elastase and DNase. The most important exotoxin is probably exotoxin A, which functions the blocking of protein synthesis.

In patients with Cystic Fibrosis, most of the *P. aeruginosa* strains produces mucoid colonies due to loosely associated layer of Alginate – a amonic polysaccharide polymer which surrounds the cells and binds them together in aggregates[28]. Despite of vast array of virulence factors, *P. aeruginosa* is still an opportunistic pathogen. The virulence factors depends upon the site and nature of infection. For example:

- Proteases play a key role in corneal ulceration.
- Exotoxin and proteases (elastase) are important in burns infections and septicemia.
- Alginate and quorum sensing molecules are associated with the formation and architecture of biofilms in chronic pulmonary colonization.
- Pyochelin and fluorescein (pyoverdin) act as important bacterial siderophores.
- Production of fluorescein in vivo allows to compete with mammalian iron-binding proteins like transferring [31].
Biofilm formation:
A significant number of Pseudomonas species produces protective alginate polysaccharides as aggregate of cells (microcolonies). This type of growth occurs on the surfaces of the devices, body tissues, that is also known as biofilm. Secreation of these polysaccharides (slime layer) helps the bacteria to protect from phagocytosis of macrophages by host cells [32]. The property of producing biofilm, helps in resistance for physical and biochemical treatment including antibiotics.

Fundaciin Lusara, et al. [33] Mexico, conducted a study to find out the relationship between biofilm forming capacities and antibiotic resistance by phenotypic methods in clinical isolates of Pseudomonas aeruginosa. He evaluated, 162 clinical isolates of P. aeruginosa for phenotypic detection of biofilm forming capabilities. Only 14% of strains produced biofilm after 8-hrs of incubation and 8% after 24hrs; the latter group showed more multidrug resistant, particularly to Piperacillin-Tazobactam and Imipenem. The frequency of biofilm formers at 8-hrs were higher among isolates which were resistant to six antibiotics.

METALLO-β-LACTAMASES
Metallo-β-lactamase belongs to class B(according to Ambler’s classification), which requires divalent cations of zinc as cofactor for enzyme activity and are therefore characterized by inhibition of metal chelators[35]. They show potent activity of hydrolyzing not only carbapenem but also against other β-lactam antibiotics with the exception to monobactams. They are not inhibited by inhibitors like as salbactum group, tazobactums, or clavulanic acid group. Carbapenems, mainly imipenem, meropenem and panipenem (available only in Japan) are potent agent for the treatment of infections due to multidrug resistant bacteria. However the prevalence of carbapenem resistant bacteria has been increasing worldwide. The IMP and VIM genes responsible for MBL production are plasmids mediated and can rapidly spread resistance to other bacteria. Thus, MBL-producing strains are considered and reported as important causes of hospital acquired infections (HAI). The level of resistance in Pseudomonas aeruginosa may be of two type, low-level to carbapenems (where MIC would be 8-32mg/L) and high- level (MIC in more than 32mg/L). Low level resistance to Imipenem is mostly due to less uptake of the drugs as a result of down-regulation of the outer membrane protein (OprD) of gram negative cell wall, which serves as
the primary route of entry for carbapenems or due to loss of the porin channel OprD 66 (i.e.) lack of ‘carbapenem specific porin’. High level resistance to carbapenems (MIC > 32 μg/ml) can be due to the presence of metallo-β-lactamase, which exhibit a broad-spectrum beta lactam resistant genes as mobile genetic cassettes with other resistance determinants inserted into class I or 3 integrons [36]. This mechanism is still uncommon towards Pseudomonas aeruginosa. Resistance to Meropenem but not Imipenem may also arise via over expression of the MexA-MexB-OprM efflux pump [37]. Although being the most recently characterized MBL in 2009 in India, has also been found in several continents, including North America and Australia.

Figure 1: The worldwide distribution of metallo-β-lactamases (Taken from Cornaglia et al., 2011).

The VIM and IMP enzymes are the most common isolated MBLs found in carbapenem-resistant bacteria. The predominance of VIM-type versus IMP-type in bacteria depends on geographical distribution, with IMP-type MBLs predominates in Asia where it was first discovered and VIM enzymes predominates in Europe though now both enzymes are now disseminated globally established. IMP and VIM possess the broadest substrate of hydrolysis range among Pseudomonas aeruginosa beta lactamases, including Penicillins, cephalosporins, cephemycin, oxacephemycins and carbapenems, but not to monobactams. With the exception of SPM-1, all the clinically related MBLs are gene cassettes encoded as part of integrons, which in are often found as part of complex transposons.
According to Zavascki AP *et al.*, from Brazil, reported that the infections by MBL producing *Pseudomonas aeruginosa* resulted higher in-hospital mortality, than those by non-MBL *Pseudomonas aeruginosa* (51.2% versus 32.1%, respectively. And their higher mortality rates 17.3 per 1000 versus 11.8 per 1000 patient-days, respectively [40].

**Types of MBLs:**

**NDM gene:**

The New Delhi metallo beta-Lactamase-1 (NDM-1) enzyme was named after New Delhi, the capital of India, it was first described by Yong et al [42] in December 2009 in a Swedish national who felt ill with an antibiotic resistant bacterial infections that he possibly acquired from India. Undoubtedly, the impact of activity of NDM-1 is threatening as today we have no potent and effective antibiotics for treating such patients.

*Enterobacteriacae* isolates having NDM-1 are now been isolated from multiple areas of India and Pakistan and the United Kingdom [44]. Such isolates have also been recently reported from three U.S. states [43]. The lancet [44] study, identified and named gram negative enterobacteriacae with resistance to carbepenems as NDM-1 isolates have been obtained from Guwahati, Mumbai, Varanasi, Bangalore, Pune, Kolkata, Hydrabad, Port Blair and New Delhi from India. Eight cities (Charsaddaa, Faizabad, Gujrat, Hafizabad, Karanchi, Lahore, Rahim Yar Khan, Sheikhupura) in Pakistan and Dhaka in Bangladesh suggesting world spread dissemination. He concluded that the cases that were found NDM-1 infected had travelled to Asian countries and they acquired infection during treatment [44]. Recently few cases have been reported from Canada who acquired NDM-1 infection but had never travelled outside Ontario state in the last decades[45].

There are similar finding without any relationship to India NDM-1 are reported from Serbia [46], from Iraq and Georgia (soldiers who were injured during the Iraq war 2007 and during the Georgian- Russian war in 2008) [47] and Italy [48].

**IMP gene:**

IMP (Imipenemase) was the first mobile MBL discovered from *P.aeruginosa* strain GN17203 in Japan during 1988, with a high MIC on Imipenem of 50μg/ml and also resistant to extended spectrum Cephalosporins with Imipenem MIC of 50μg/ml.
The resistance was found on a transferable conjugative plasmid that could be readily transferred by mutation to other Pseudomonas strains. Following that, three years later, an identical gene was found in *Serratia marcescens* from an UTI patient at Aichi hospital in Okazaki, Japan.[49].

**VIM gene:**

Second predominant group of enzyme acquiring MBL is VIM (Verona Integron encoded Metallo β-lactamases) type, which was first described in Verona, Italy from *Pseudomonas aeruginosa* isolated in 1997 those were resistant to Piperacillin, Ceftazidime, Imipenem and Aztreonam, with MIC of Imipenem >128μg/ml. The *bla*VIM-1 gene was integrated as a gene cassette into a class 1 integron. This integron also carries an integrase gene typical of class 1 integrons in addition to the *bla*VIM-1 gene cassette, it has an *aac* A4 gene cassette which encodes resistance to aminoglycosides. In *P.aeruginosa*, this integron of *bla*VIM-1 was probably located on the chromosome[50]. Similarly VIM-1 was also detected from *Pseudomonas putida* from Italy, Greece and France recently. Among several types of MBL enzymes identified, VIM type enzymes appears to be most prevalent [51]. The *bla*VIM-2 was first identified in southern France from a *P.aeruginosa* isolated from blood culture from a neutropenic patients and the isolate was found to be resistant to most of the β-lactams, including ceftazidime, Cefepime and Imipenem but remained susceptible to aztreonam. Similarly, VIM-2 producing *P.aeruginosa* were also isolated from Italy and Greece during the same period. The most common MBL identified worldwide is VIM-2 [52].

A study done by Pitout *et al.*, detection of *Pseudomonas aeruginosa* producing metallo-beta lactamase(MBL) from Canada using 241 clinical strains of imipenem sensitive *Pseudomonas aeruginosa* from the period of two years(2002 to 2004), 110/241 (46%) were MBL positive using phenotypic methods. Among 241 non-susceptible *Pseudomonas aeruginosa* strains, 110/241 (46%) were PCR positive for MBL genes, 103/241 (43%) for *bla*-VIM gene and 4/241(2%) for *bla*-IMP gene and EDTA disk screen test with meropenem showed 100% sensitivity and 97% specificity for detecting MBL in control strains and clinical strains [15].
A study done by Joseph et al. from Puducherry, India, on Ventilator Associated Pneumonia (VAP) and the role of Multidrug resistant (MDR) pathogens, showed 37 (78.7%) of the 47 VAP pathogens were multidrug resistance. Where, 20% were MBL producing Pseudomonas aeruginosa among 78.7% MDR VAP pathogens[57]. A study done by Shanthi et al. With 30 carbapenem resistant nosocomial isolates of MDR Pseudomonas aeruginosa showed, MBL production from 29 strains, death occurred in 57% of patients. Thus concluded that MBL- mediated carbapenem resistance in Pseudomonas aeruginosa is a significant threat in hospital patients [54].

Quinines-Falconi et al., showed Emergence of Pseudomonas aeruginosa strains producing metallo-beta-lactamases of VIM-2 types, from 86 carbapenem resistance P. aeruginosa isolates collected from National Institute of Respiratory Disease of Mexico city, showed presence of VIM-2 in two clonally related isolates[58]. Another study done in Hiroshima, Japan for Molecular characterization of Imipenem resistant Pseudomonas aeruginosa from six hospitals by Masaru Ohara et al., 1,058 strains of Pseudomonas aeruginosa strains showed, 100 strains were resistant to Imipenem (9.5%). Among those 100 strains, 14 (14%) were MBL positive by double disc synergy test (DDST) using Sodium mercaptoacetic acid disk and of which 6% were positive for blaVIM-2 by PCR and 32(32%) were Multidrug resistant strains.[59].

Methods of phenotypic detection of MBL:

1) Imipenem – EDTA combined disc test:
   - This combined disc Imipenem-EDTA test was first performed by Yong et al.
   - The test organism is inoculated on well dried MHA plates as recommended by the CLSI. Two 10μg Imipenem disc are placed on the plate and 10μg of EDTA solution is added to one of them to obtain the desired concentration (750μg). After overnight incubation, the inhibition zone of Imipenem and Imipenem EDTA discs were compared.[64]
   - The increase in inhibition zone with Imipenem-EDTA disc is ≥7mm than the Imipenem disc alone, is considered as MBL positive.[64]

2) Imipenem EDTA double disc synergy test (DDST):
   - Lawn culture of the test organism is made onto MHA plates as recommended by the CLSI.
A 10 μg Imipenem disc is placed 20mm center to centre from a blank disc containing 10 μg of 0.5 M EDTA (750 μg).

Plates are incubated at 37°C overnight.

Enhancement of zone of inhibition between Imipenem disc and EDTA disc in comparison with the zone of inhibition on the opposite side (another side) of the disc (drug) is interpreted as a Positive test.

Other chelating agents can be used are,

a) EDTA disc (1,900 μg)

b) Mercaptoacetiic acid (MPA) 3 μl/disc

c) Sodium Mercaptoacetic acid (SMA) 3mg/disc.

d) EDTA (750 μg) + SMA (2mg) /disc.

3) EDTA disc potentiation using Ceftazidime, Cefepime and Cefotaxime:

- DDST using ceftazidime disc has originally reported to be more sensitive than Imepenem.

- The test organism is inoculated onto MHA plates as recommended by the CLSI.

- A filter paper blank disc is placed and the following discs including Ceftazidime (30 μg), Cefepime (30 μg) and Cefotaxime (30 μg) are kept 25mm centre to centre from the blank disc. 10 μl of 0.5 M EDTA solution is added to the blank disc and the plate is incubated overnight at 37°C.

- Enhancement of the zone of inhibition between the EDTA disc alone and any one of the three Cephalosporin disc is compared with the zone of inhibition on the far side of the drug is considered as positive result.

4) Modified HODGE test:

- *Escherichia coli* (ATCC 25922) is inoculated on to the MHA plate as per as CLSI guidelines.

- Test organism is heavily streaked from centre to periphery of the plate. And keep for 15min at room temperature.

- A 10 μg Imipenem disc is placed at the centre and the plate is incubated overnight.
· The presence of distorted zone of inhibition is interpreted as positive. Performance of test is improved by the addition of zinc sulphate (140μg/disc) or to MHA to a final concentration of 70μg/ml.

5) MBL E test: (Imipenem):
· E-test MBL strip contains double sided seven gradients (dilutions) of Imipenem alone on one side (4 to 256μg/ml) and Imipenem (1 to 64 μg/ml) with a fixed concentration of EDTA on the other side is used for MBL detection.
· MIC ratio of Imipenem/ Imipenem + EDTA of >8, or reduction of Imipenem MIC by ≥ 3 log2 dilutions in the presence of EDTA or appearance of zone of inhibition indicates MBL production.

Molecular detection methods:
   a) DNA probes: Detection of Metallo β lactamase genes are performed using DNA probes that are specific for VIM, IMP, NDM and SPM enzymes.
   b) PCR: This is the easiest and most common molecular method used to detect the presence of β lactamases.
   c) Oligotyping: The first molecular method for the identification of β lactamase was the oligotyping method.
   d) PCR-RFLP: In this method amplified PCR products are digested with several restriction endonuclease enzymes and then the subsequent fragments are separated by electrophoresis.
   d) LCR: Ligase chain reaction can be used to distinguish between a number of IMP, VIM variants.
   e) Nucleotide sequencing: It remains the gold standard for determination of the specific β lactamase gene present in a strain but labour intensive and can be technically challenging.