Susceptibility to Environmental Phages With clinical Antibiotic-resistant *Acinetobacter baumannii* than Antibiotic-sensitive Strains.

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Abstract

Gram negative hospital acquired infection are a major problem since last two decades *Acinetobacter baumanii* has emerged as a highly trouble pathogen for many institutions globally. As a consequence of its immense ability to acquired antibiotic drug resistance determinant .It has justifiably been propelled to the fore front of scientific attention. Rapid spread of multidrug resistant isolate causing infection. Acinetobacter baumannii clinical strains, were selected from a new phage library for further characterization. The complete genomes of the two phages were analyzed. Both phages are characterized by broad host range and essential features of potential therapeutic phages, such as short latent period (27 and 21 min, respectively), high burst size (125 and 145, respectively), stability of activity in liquid culture and low frequency of occurrence of phage-resistant mutant bacterial cells. Genomic analysis showed that while bacteriophage with resemblance to some unclassified Pseudomonas aeruginosa phages, The newly isolated phages can serve as control A. baumannii infections.

Key words:-*Acinetobacter* isolates, Bacteriophages, Multidrug-resistant (MDR)

Introduction:-

The gene *Acinetobacter* is defined as gram negative ,cocco bacilli often difficult to destain strictly ,aerobic ,non motile ,catalase positive, and oxidase negative. some strain are micro capsulated and rarely reduce nitrate growth occur even on simple medium between 20°C to 30°C without any growth factor.Multidrug-resistant (MDR) Acinetobacter baumannii is a relatively newly emerged pathogen, notorious for its role in nosocomial wound infections [1]–[3]. The ability to form biofilms and extended survival on environmental surfaces along with broad resistance to antibiotics puts A. baumannii on the list of the medically most important pathogens [4]. The known risk factors for A. baumannii colonization or infection include prolonged hospitalization, intensive care unit (ICU) admission, recent surgical procedures, parenteral nutrition, invasive procedures, nursing home residence, and previous broad-spectrum antibiotic

use [5]–[9].In comparison to other clinically relevant bacteria, Acinetobacter spp. can develop antibiotic resistance extremely rapidly due to long-term evolutionary exposure to soil organisms that produce antibiotics [10]. Many A. baumannii strains are characterized by an impressive number of acquired mechanisms of resistance to antibiotics, including enzymatic inactivation, modification of target sites, active efflux and decreased influx of drugs [11]. In addition, A. baumannii has a naturally occurring carbapenem-hydrolyzing class D β-lactamase (CHDL) gene, intrinsic to this species, although phenotypic resistance is not associated only with the presence of this gene and normally depends on other genetic determinants as well [12].Treatment of MDR strains of A. baumannii is a challenging issue for modern medicine. Carbapenems are the antibiotics of the first choice against multidrug resistant A. baumannii infections. However, carbapenem-resistant A. baumanniii are being increasingly reported worldwide [6], [13]. Other therapeutic options include sulbactam, aminoglycosides, polymyxins and tigecycline. Recently, high resistance rates to tigecycline and polymyxins were also reported [14]–[16].Bacteriophages active against MDR strains of A. baumannii could be considered as a potential solution to meet the challenges posed by this pathogen. Indeed, a number of new phages carrying potential to combat infections caused by A. baumannii strains have been recently reported [17]–[24].In our study, we isolated and characterized two highly lytic bacteriophages, named in accordance with the proposed nomenclature for bacteriophages by Kropinski et al. [25]. The phages were characterized, according to criteria considered as essential for potential therapeutic phages and both phages proved to meet all of them.

Materials and Methods:-

Bacterial strains

In total, 34 strains of Acinetobacter spp. were used in the study.Initial identification and antibiotic susceptibility profile detection of the strains were performed . For the precise identification, partial sequencing of the rpoB gene was performed [26]. All strains were typed by rep-PCR using the kit . A cluster of closely related isolates was defined as isolates sharing ≥95% similarity [27]. All strains were screened for the presence of two genes like carbapenemases encoding genes in their genomes by Multiplex-PCR (M-PCR) [28].

Phage isolation, purification and propagation:-

K.M.hospital waste water samples were used for isolation of active phages by culture-enrichment method described in Merabishvili et al. [29], with slight modifications. Briefly 15ml of 10× concentrated LB broth was mixed with 135 ml sample water and 1 ml (108 cfu/ml) of one/different combinations of test strains in a 200 ml bottle. The mixture was incubated at 32°C

overnight. Subsequently, 3 ml of chloroform was added and the bottle was further incubated at 4°C for 2 h. The parallel streaks and agar-overlay methods [29] were used to check the lysates for the presence of phages and subsequent isolation of pure phage particles. Plaques with different morphology were touched with a sterile pipette tip, inoculated into 2 ml of sterile LB broth and incubated at 37°C for 2 h. Each phage separation procedure was repeated 10 times to ensure single phage suspensions.High titer bacteriophage stocks were prepared by the agar overlay method [29]. The A. baumannii strain .Host strain for the propagation of the bacteriophages. Further phage particles in the obtained lysates were precipitated using PEG 8000 (8%, wt/vol) + 1 M NaCl [30]. Phages for proteomic analysis were purified by centrifugation in CsCl gradients at 140,000×g for 3 h [30].

Host range analysis and determination of Efficiency of Plating (EOP):-

The parallel streaks method [29] was used to detect the susceptibility of the 34 bacterial strains for the two phages. All susceptible strains revealed by the parallel streaks method were titered against the phages to detect the EOP and the ability of the phage to propagate in the strain. EOP was calculated as the ratio of titer on the test strain to the titer on the host strain.

Phage adsorption and one-step growth parameters :-

Exponentially grown A. baumannii cells were mixed with each phage at a MOI of 0.001 and incubated at 37°C. Aliquots of 50 µl were removed after 3, 5, 8, 10, 15 and 20 min and diluted 100 times in 4.45 ml LB broth and 0.5 ml chloroform. The tubes were incubated for 30 min at room temperature and subsequently titrated to detect the number of non-adsorbed phages. The rate of adsorption was determined according to Adams [32]. The adsorption curves were constructed based on the ratio of non-adsorbed phage at different time intervals over the initial phage number.In the one-step growth experiment, A. baumannii (108 cfu/ml) was infected with each phage at a MOI of 0.001. The adsorption process was allowed to occur during 8 min at 37°C. The mixture was centrifuged (13,000×g, 1 min), and the pellet resuspended in 10 ml of fresh LB broth and incubated for 1.5 h at 37°C. The samples were taken at intervals of 3 minutes and titrated. The latent period was defined as the interval between adsorption of the phages to the bacterial cells and the release of phage progeny. The burst size of the phage was determined as the ratio of the final number of free phage particles to the number of infected bacterial cells during the latent period.

Frequency of occurrence of phage-resistant mutant bacterial cells

The frequency of occurrence of phage-resistant mutant bacterial cells was detected by the method described in Adams [32]. Briefly, 1 ml of bacterial culture at the concentration of 108

cfu/ml was mixed with the appropriate volume of phage lysate at a MOI of 100. After 10 min of incubation at 37°C, 100 µl of the mixture was spread on LB plates and phage-resistant colonies were enumerated after overnight incubation at 32°C. The emerged colonies were isolated twice to assure phage-free bacterial cultures. Further the isolated bacterial cultures were tested against phages by parallel streaks method to confirm their true resistance. The experiments were performed in triplicate and mean values calculated.

Results and Discussion :-

A. baumannii has been listed by the Infectious Diseases Society as one of the six top-priority dangerous microorganisms. The increased occurrence of A. baumannii MDR strains and in health care settings worldwide in general [9], [16] prompted us to search for alternative solutions to the problem, such as bacteriophages. A. baumannii phage collection and characterization of bacterial strains.The host range of the newly isolated phages was studied on a collection of strains including the 28 clinical A. baumannii strains and other representatives of the genus Acinetobacter,. All bacterial strains used in the study were characterized according their susceptibility profiles towards four classes of antibiotics, i.e. aminoglycosides, quinolones, carbapenems and polymyxins MDR A. Most of the studied A. baumannii strains were also characterized by resistance to aminoglycosides and quinolones and all of them revealed susceptibility to colistin A. baumannii strains (15 out of 28), each phage was able either to adsorb and/or propagate on 11 strains but 2 strains showed complete resistance to both phages. A. baumannii strains and to propagate.

Phenotypic characterization of phages :-

The frequency of appearance of phage-resistant mutants in phage-sensitive strains is one of the characteristics of major importance for therapeutic bacteriophages. the mutation frequency parameters in the host strain A. baumannii These figures indicate a low ability of the strain to obtain phage-resistance mutants.

The single-step growth experiments were performed for determination of latent periods and burst sizes of phages, as important characteristics of phage infection process. characterized with a shorter latent period of 21 min and a bigger burst size of 145 phage particles per infected host cell. The obtained physiological parameters of the newly isolated phages characterize them as phages with high activity against the susceptible strains.Stable activity of phages in broth cultures represents their ability to overcome the growth of phage-resistant mutants and gives an indication on effective titer of therapeutic preparations to be used in future.Study of phage

activity after 24 hour. Study of phage activity after 48 hours. Control: non-infected bacterial culture of A. baumannii strain .

Conclusions:-

The newly isolated A. baumannii phages Acibel004 and Acibel007 are characterized by a broad host range, a relatively low frequency of occurrence of phage-resistant mutant bacterial cells and infection parameters required by therapeutic phages in general. Close genetic relatedness of the studied phages to well-known groups of lytic phages together with the absence of the genes associated with lysogeny indicates a true virulent nature of these phages. In conclusion, Acibel004 and Acibel007, based on thorough characteristic of their physiology, genome and proteome, can be regarded as phages with a high potential to be applied for bacteriophage-based therapy against infections caused by A. baumannii strains. Further in vivo safety and efficacy of these phages produced as a therapeutic preparation need to be evaluated in clinical trials.

Reference:-

1. Antony y.Peleg ,Harald Seifert and david L.Paterson.
Acinetobacter baumanii emergence of a successful pathogen.
Vincent Trottier,Nicolas Namian,Danial G.Pust,Zeher Ronald Maning,Jr.Micheal B.Dunham,
Multi Drug Resistant (MRD)organism in intensive care unit (ICUs) are a growing concern.

2. Jones A, Morgan D, Walsh A, Turton J, Livermore D, et al. (2006) Importation of multidrug-resistant Acinetobacter spp. infections with casualties from Iraq. Lancet Infect Dis 6: 317–318.

3. Petersen K, Riddle MS, Danko JR, Blazes DL, Hayden R, et al. (2007) Trauma-related infections in battlefield casualties from Iraq. Ann Surg 245: 803–811.

4. Abbott I, Cerqueira GM, Bhuiyan S, Peleg AY (2013) Carbapenem resistance in Acinetobacter baumannii: laboratory challenges, mechanistic insights and therapeutic strategies. Expert Rev Anti Infect Ther 11: 395–409.

5. Yang HY, Lee HJ, Suh JT, Lee KM (2009) Outbreaks of imipenem resistant Acinetobacter baumannii producing OXA-23 β-lactamase in a tertiary care hospital in Korea. Yonsei Med J 50: 764–770.

6. Fishbain J, Peleg AY (2010) Treatment of Acinetobacter infections. Clin Infect Dis 51: 79–84.

7. Mihu MR, Martinez LR (2011) Novel therapies for treatment of multi-drug resistant Acinetobacter baumannii skin infections. Virulence 2: 97–102.

8. McGlone SM, Doi Y, Bailey RR, Harrison LH (2011) Economic value of Acinetobacter baumannii screening in the intensive care unit. Clin Microbiol Infect 17: 1691–1697.

9. Doi Y, Kandiah S, Hariri RS, Harrison LH (2012) Natural history of multidrug-resistant Acinetobacter baumannii carriage in intensive care units. Infect. 33: 642–643.

10. Manchanda V, Sinha Sanchaita S, Singh NP (2010) Multidrug resistant Acinetobacter.. J Glob Infect Dis 2: 291–304.

11. Peleg AY, Seifert H, Paterson DL (2008) Acinetobacter baumannii: emergence of a successful pathogen. Clin Microbiol Rev 21: 538–582.

12. Turton JF, Woodford N, Glover J, Yarde S, Kaufmann ME, et al. (2006) Identification of Acinetobacter baumannii by detection of the blaOXA-51-like carbapenemase gene intrinsic to this species. J Clin Microbiol 44: 2974–2976.

13. Pogue JM, Mann T, Barber KE, Kaye KS (2013) Carbapenem-resistant Acinetobacter baumannii: epidemiology, surveillance and management. Expert Rev Anti Infect Ther 11: 383–393.

14. Navon-Venezia S, Leavitt A, Carmeli Y (2007) High tigecycline resistance in multidrug-resistant Acinetobacter baumannii. J Antimicrob Chemother 59: 772–774.

15. Dizbay M, Altuncekic A, Sezer BE, Ozdemir K, Arman D (2008) Colistin and tigecycline susceptibility among multidrug-resistant Acinetobacter baumannii isolated from ventilator-associated pneumonia. Int J Antimicrob Agents 32: 29–32.

16. Cai Y, Chai D, Wang R, Liang B, Bai N (2012) Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. Antimicrob Chemother 67: 1607–1615.

17. Lin NT, Chiou PY, Chang KC, Chen LK, Lai MJ (2010) Isolation and characterization of phi AB2: a novel bacteriophage of Acinetobacter baumannii. Res Microbiol 161: 308–314.

18. Chang KC, Lin NT, Hu A, Lin YS, Chen LK, et al. (2011) Genomic analysis of bacteriophage φAB1, a phiKMV-like virus infecting multidrug-resistant Acinetobacter baumannii. Genomics 97: 249–255.

19. Popova AV, Zhilenkov EL, Myakinina VP, Krasilnikova VM, Volozhantsev NV (2012) Isolation and characterization of wide host range lytic bacteriophage AP22 infecting Acinetobacter baumannii. FEMS Microbiol Lett 332: 40–46.

20. Shen GH, Wang JL, Wen FS, Chang KM, Kuo CF, et al. (2012) Isolation and characterization of φkm18p, a novel lytic phage with therapeutic potential against extensively drug resistant Acinetobacter baumannii. Plos One 7: e46537 Available: .

21. Jeon J, Kim JW, Yong D, Lee K, Chong Y (2012) Complete genome sequence of the podoviral bacteriophage YMC/09/02/B1251 ABA BP, which causes the lysis of an OXA-23-producing carbapenem-resistant Acinetobacter baumannii isolate from a septic patient. J Virol 86: 12437–12438.

22. Li P, Chen B, Song Z, Song Y, Yang Y, et al. (2012) Bioinformatic analysis of the Acinetobacter baumannii phage AB1 genome. Gene 507: 125–134.

23. Jin J, Li ZJ, Wang SW, Wang SM, Huang DH, et al. (2012) Isolation and characterization of ZZ1, a novel lytic phage that infects Acinetobacter baumannii clinical isolates. BMC Microbiol 28: 156 Available:

24. Huang G, Le S, Peng Y, Zhao Y, Yin S, et al. (2013) Characterization and genome sequencing of phage Abp1, a new phiKMV-like virus infecting multidrug-resistant Acinetobacter baumannii. Curr Microbiol 66: 535–543.

25. Kropinski A, Prangishvili D, Lavigne R (2009) The creation of a rational scheme for the nomenclature of viruses of Bacteria and Archaea. Environm Microbiol 11: 2775–2777.

26. Gundi VA, Dijkshoorn L, Burignat S, Raoult D, La Scola B (2009) Validation of partial rpoB gene sequence analysis for the identification of clinically important and emerging Acinetobacter species. Microbiol 155: 2333–2341.

27. Higgins PG, Hujer AM, Hujer KM, Bonomo RA, Seifert H (2012) Interlaboratory reproducibility of DiversiLab rep-PCR typing and clustering of Acinetobacter baumannii isolates. J Med Microbiol 61: 137–141.

28. Turton J F, Ward ME, Woodford N, Kaufmann ME, Pike R, et al. (2006) The role of ISAba1in expression of OXA carbapenemase genes in Acinetobacter baumannii.. FEMS Microbiol Lett 258: 72–77.

29. Merabishvili M, Pirnay JP, Verbeken G, Chanishvili N, Tediashvili M, et al. (2009) Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. PLoS One 4: e4944

30. Sambrook J, Russell DW (2001) Chapter 2: Bacteriophage λ and its vectors. In: Molecular cloning: A laboratory manual, ed. New York: Cold Spring Harbor Laboratory Press. 2.1–2.117.

31. Imberechts H, Wild P, Charlier G, De Greve H, Lintermans P, et al. (1996) Characterization of F18 fimbrial genes fedE and fedF involved in adhesion and length of enterotoxemic Escherichia coli strain 107/86. Microb Pathog 21: 183–192.

32. Adams MH (1959) Appendix: Methods of study of bacterial viruses. In: Bacteriophages. New York: Interscience Publishers. 443–522.

33. Appelmans R (1921) Le dosage du bactériophage. Compt Rend Soc Biol 85: 1098.

34. Merabishvili M, Verhelst R, Glonti T, Chanishvili N, Krylov V, et al. (2007) Digitized fluorescent RFLP analysis (fRFLP) as a universal method for comparing genomes of culturable dsDNA viruses: application to bacteriophages. Res Microbiol 158: 572–581.

35. Besemer J, Borodovsky M (1999) Heuristic approach to deriving models for gene finding. Nucl Acids Res 27: 3911–3920.