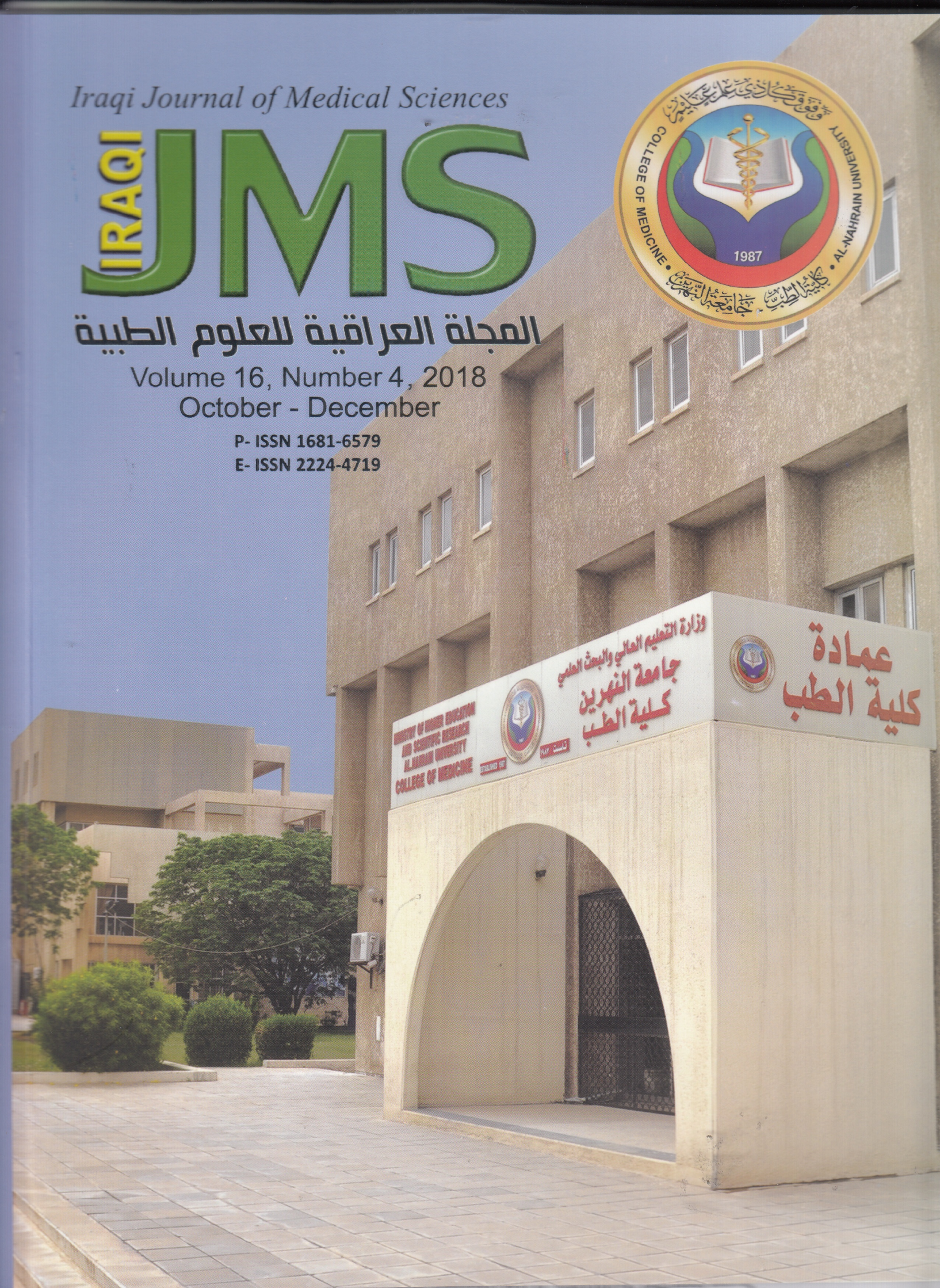
Iraqi Journal of Medical Sciences

IRAQI JMS

المجلة العراقية للعلوم الطبية

Volume 16, Number 4, 2018
October - December

P- ISSN 1681-6579
E- ISSN 2224-4719



Volume 16 (4) 2018
DOI: 10.22578/IJMS.16.4.

P-ISSN 1681-6579
E- ISSN 2224-4719

IRAQI JOURNAL OF MEDICAL SCIENCES

Editorial Board Members

Iraqi Journal of Medical Sciences

Aims and Scope

Iraqi Journal of Medical Sciences is published by College of Medicine, Al-Nahrain University. It is a quarterly multidisciplinary medical journal. High quality papers written in English, dealing with aspects of clinical, academic or investigative medicine or research will be welcomed. Emphasis is placed on matters relating to medicine in Iraq in particular and the Middle East in general, though articles are welcomed from anywhere in the world.

Iraqi Journal of Medical Sciences publishes original articles, case reports, and letters to the editor, editorials, investigative medicine, and review articles.

Mission and Vision

Mission of Iraqi JMS

To establish rapid review processes aiming to puplish scientific papers that help to augment knowledge and highlight discoveries in the field of medical sciences to be a world-wide forum in assisting the distribution of medical reasearches to career readers.

Vision of Iraqi JMS

To be pioneer national medical journal interesting in increasing the understanding of diseases and treatment.

All correspondence and subscription information requests should be addressed to:
The Editor of **Iraqi Journal of Medical Sciences**
College of Medicine
Baghdad, Iraq
Tel. + 964 7717516090
P.O. Box 70044, Kadhimiya, Baghdad, Iraq.
E-mail: iraqijms@colmed-alnahrain.edu.iq
http://www.iraqijms.net

Iraqi JMS FORMAT

INSTRUCTION TO AUTHORS

Iraqi Journal of Medical Sciences (Iraqi JMS) is a periodic, peer-reviewed journal published quarterly by College of Medicine, Al-Nahrain University. Iraqi JMS publishes manuscripts in all fields of health and medicine written in English.

Types of Contributions: Original articles, review articles, case studies, editorials, medical education, history of medicine, ethics, practical points, medical quiz, conferences, meetings and letters to the Editor.

Manuscripts:

- Submission of a manuscript implies that is not being considered for publication anywhere.
- The author should provide the following:

A. A document officially state that the current work was carried out at the site, which provides the certification. The document should be signed by the highest authorized member at that location.

B. Document stated clearly that his current work is in agreement with the medical ethics provided either from the local ethical committee in the place where he did his work or from the Ministry of Health, Department of Training and Improving skill - Research and Educational facilities, the approval has to be stated separetly in the method section.

C. Publication fees are 100,000 IDs in addition to 20,000 IDs for checking of plagiarism. Other extra fees will be taken for extra pages (6000 IDs for each additional page (more than six pages) and up to 24000 IDs only and 10,000 IDs For any Figure).

- Manuscripts submitted to Iraqi JMS are subject to editorial evaluation and revision by three referees after being checked electronically for any plagiarism.
- The format of IJMS complies with the uniform requirements for manuscripts submitted to Biomedical Journals, published by the International Committee of Medical Journals Editors (ICMJE) (Vancouver, British Colombia, 1979) and its last update in October 2001, available on the web site www.icmje.org.
- Manuscript should be typewritten font size 14, double spaced on size A4 (29.5x21 cm) paper with wide margins and line- numbered. Page should be numbered consecutively. One original and three photocopies including figures, tables, and photographs should be submitted. Begin each of following sections on separate page in the following sequence: Title page, abstract and keywords, text, acknowledgments, references, tables, and legends for illustration.
- Manuscript and figures will not be returned to the authors whether the editorial decision is to accept, revise or reject.
- Manuscripts must be accompanied by a covering paper signed by all authors that the paper has not been published in and will not be submitted to any other journal if accepted in Iraqi JMS.

- The title page should contain (a) title of the manuscript, (b) names of each author (first name, middle initial and family name) including highest academic degree, (c) official academic and/or clinical title and affiliation (d) name and address of the institution where the work was done (e) name and address (E-mail if available) of the author to whom correspondence should be sent.
- Authors can also submit the scientific publication through the official Iraqi JMS web site at (http://submit.Iraqijms.com/). Users must register when accessing the Iraqi JMS online submission system for the first time, by clicking on "Register." Three steps are involved in obtaining a personal account.

Abstract: Manuscript should include an abstract of not more than 250 words. Structured abstract typed on a separate sheet and consist of background, objective, method, results, and conclusion.

Keywords: Three to ten keywords should be provided on the same page as the abstract in English. As far as possible, be selected from the National Library of Medicine, Medical Subject Headings.

Manuscript format: It should be divided into the following parts: introduction, methods, results and discussion.

References: All references should be listed in consecutive numerical order by English numerical, in the order of citation in the text **and each reference must be followed with its DOI link.** Once a reference is cited all subsequent citations should be to the original number.

Examples

1. Standard Journal Article: use et al when the number of authors exceeds 3.
Halliwell B, Gutteridge JMC. Oxygen toxicity, Oxygen radicals, transition metals and disease. Biochem J. 1984; 219(1): 1-14.
2. Books: Mann JI, Pyorala K, Teuscher A. Diabetes in epidemiological perspective. London: Churchill Livingstone; 1983. p. 1-5.
3. Chapter in book: Phillips SJ, Whisnant JP. Hypertension and strock. In: Laragh JH, Brenner BM. editors. Hypertension: Pathophysiology, diagnosis, and management. 2nd ed. NewYork: Raven Press; 1995. p. 465-78.

- **How to find DOI for the references of your submitted article to Iraqi Journal of Medical Sciences (IJMS)**

1. First, click on this link http://www.crossref.org/guestquery/
2. Go to "search on article title"
3. Fill in the author name and the title of the reference
4. Copy and paste the found DOI (if any: as some references have no DOI) to the end of each reference in the reference list in your article to be submitted to IJMS.

That's it!!

Tables: Each table should be typed on a separate page double-spaced, including all headings, number all tables with Arabic numerals and include a short title. Vertical lines between columns are to be avoided.

Figures: All figures must be suitable for reproduction without being retouched or redrawn. Photographs must be supplied as glossy black and white prints. The top of the figures should be indicated clearly.

Legends: Captions for figures must be typed; double spaced, and must not appear on the figure.

Acknowledgments: Collate acknowledgments in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Conflict of interest: All authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. **Example** of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications\registrations, and grants or other funding. See also http://www.elsevier.com\conflictsofinterest .
Please complete and upload the conflict of interest and author declaration form with your manuscript.

Author contributions: Each author is required to declare his or her individual contribution to the article: all authors must have materially participated in the research and\or article preparation, so roles for all authors should be described. The statement that all authors have approved the final author's article should be true and included article in the disclosure.

Role of the funding source: You are requested to identify who provided financial support for the conduct of the research and\or preparation of the article and to briefly describe the role of the sponsor (s), if any, in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. If the funding source (s) had no such involvement then this should be stated.

List of abbreviation: Any abbreviations used should be listed after the abstract and defined at first use in the main body of the article. Use only widely accepted and conventional abbreviations. Avoid abbreviations in the title and abstract.

Proof Reading will be done by the secretarial office of the journal. The principal author will receive a copy of the journal. The authors are responsible for accuracy of all statements, data, and references included in the manuscript.

- After the manuscript has been accepted for publication, authors are required to supply the final version of the manuscript on CD in MS word version 6 or later.

Iraqi Journal of Medical Sciences

A Medical Journal Encompassing All Medical Specializations

Issued Quarterly

CONTENTS

Iraqi Journal of Medical Sciences

A Medical Journal Encompassing All Medical Specializations

Issued Quarterly

CONTENTS

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Liquid Biopsy- A New Prospect

Ahmed K. Mahdi *PhD Molecular Pathology*

Dept. of Pathology and Forensic Medicine, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Tissue biopsy has been the mainstay in tumor diagnosis for centuries. But due to its invasiveness and the heterogeneity of tumors there was a need for an alternative or adjuvant techniques to diagnose and assess tumors. Liquid biopsy is emerging as a new technique that will open the way for the diagnosis, tumor characterization, assess disease prognosis and individualize treatment options in cancer patients.

Keywords Biopsy, CTC, ctDNA

Citation Mahdi AK. Liquid biopsy - A new prospect. Iraqi JMS. 2018; 16(4): 353-356. doi: 10.22578/IJMS.16.4.1

List of abbreviation: CTCs = Circulating tumor cells, ctDNA = Circulating tumor DNA, RBCs = Red blood cells, WBCs = White blood cells, EpCAM = Epithelial cell adhesion molecule, CK = Cytokeratin, Her2 = Human epidermal growth factor, CD = Cluster of differentiation, FDA = American food and drug administration, CFDA = Chinese national food and drug administration, SE = Subtraction enrichment, iFISH = Immunostaining fluorescence in situ hybridization

Early detection and diagnosis of cancer represents a challenge in medical practice. Tissue biopsy is the golden stone in diagnosing tumors, but it is invasive to start with and difficult to obtain more than one biopsy along the course of the disease. Nowadays, and with the advancement in technologies it is considered as part of the routine clinical practice to detect (at the molecular level) specific drivers and mutations in tumors aiming at predicting treatment response and emergence of any drug resistance. What remains as an obstacle is the heterogeneity of tumors whether of the primary site or that in the metastases, or even at different time points during the progression of the disease. Therefore, it might be very difficult to assess tumor heterogeneity with one biopsy [1,2]. Additionally, the emergence of tumor resistance to different agents is common in clinical practice. Therefore, research teams all over the world have been working on developing techniques that help in early detection and diagnosis of cancer and assessing response to treatment and prognosis [3].

Liquid biopsy (which includes circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA)), gives the option of collecting more than one sample non-invasively along the course of the disease, which enables tumor detection and characterization, predicting and assessing the prognosis, response and resistance to treatment, and early detection of any relapse [3]. We will focus on the CTCs and its future prospect in cancer in this article.

CTCs detection and enrichment and biological properties

Thomas R. Ashworth, an Australian scientist, was the first to discover and describe CTCs in a blood sample collected from a patient with breast cancer in 1869 [4]. CTC is the term used to describe tumor cells that detach from the

primary tumor and circulate in the peripheral blood or lymphatics and grow in the blood, or settle and grow in the bone marrow, lymph nodes or in any other organ leading to secondary tumors [5]. These CTCs are exposed to very harsh conditions and a very small percentage of CTCs (< 0.01%) manage to survive these conditions leading to the development of metastases [6]. The important point regarding CTCs is that they can arise at any phase of tumor development, and hence (theoretically) they can be used as an early marker to diagnose the tumor and as a tool to monitor disease progression and any relapse [3].

The obstacle that delayed the use of CTCs for these purposes despite its discovery since the 19th century was the scarcity of the CTCs in peripheral blood. In the last few decades there have been giant leaps in technology that enabled researchers to develop techniques that helped in improving the separation and enrichment of CTCs [3]. This can be achieved through two main mechanisms:

a) Detection using the physical properties of CTCs:

This method uses physical properties of CTCs to separate them from the rest of cells in peripheral blood. These physical properties include the size of tumor cells to start with assuming that they are larger than other cells. The CTCs have a size of (~ 17-52 µm) that is larger than RBC (~ 6-8 µm) and WBCs (~ 7-15 µm) [7]. Another method that uses physical properties is gradient centrifugation. Ficoll density gradient (which is a hydrophilic polysaccharide with a high mass) separates CTCs from the rest of blood cells depending on the density difference assuming that tumor cells have higher amount of DNA and higher density [8]. Other physical parameters include malleability, migratory capacity and the electric charge of the CTCs beside the size and density. Counting on physical properties alone to separate and enrich CTCs falls short due to the huge variability among tumor cells in regard to their physical properties and this leads to the false detection of blood cells as CTCs. Therefore, this technique might have a high percentage of false-positive results [3].

b) Detection using the biological properties of CTCs:

This method exploits the biological properties of the CTCs and is based on taking advantage of surface markers of CTCs to detect them in an antibody-antigen binding pattern. A panel of markers can be used such as epithelial cell adhesion molecule (EpCAM), cytokeratin family members (CK 8, 18 and 19), human epidermal growth factor (Her2), N-cadherin and vimentin. This biological detection method is called immune capture method [9-11]. In principle, magnetic beads are covered by specific antibody to the target of interest and then these beads are mixed with the blood sample to allow the binding of the antibodies to their target antigens that are located on the targeted cells. Afterwards the blood sample is passed through a magnetic field that leads to isolation of targeted cells (or exclusion of the unwanted cells in certain cases) that are bound to the beads and pulled to the periphery of the tube under the effect of magnetic field enriching targeted cells. So we end up with the following pattern "Targeted cell-surface antigen-the specific antibody-magnetic beads". Immune capture can be achieved through positive and negative enrichment. Positive enrichment method uses metallic beads that are bound to antibodies targeting specific CTCs surface antigen [12].

Cell SearchTM System (CSS:Verdix LLC, NJ, USA) uses this positive enrichment method and it is the only approved system in the world for the detection of CTCs in malignant tumors by both the American Food and Drug Administration (FDA) and the Chinese National Food and Drug Administration (CFDA). This system uses EpCAM coated beads to isolate CTCs and thereafter further steps use other antibodies (CK 8 and 18 for epithelial cells, CD45 for WBCs and DAPI as a nuclear stain) to exclude

leukocytes and confirms the diagnosis [3,12,13]. Similar to all systems, this system has pros and cons. The pros are that it requires small amount of blood (7.5 ml only), and the results were considered reproducible, specific and sensitive. The cons are due to the reliance of the system on EpCAM for the detection of CTCs, then those cells that undergo epithelial-mesenchymal transition and lost their EpCAM will not be detected. Also EpCAM expression varies considerably in solid tumors due to tumor heterogeneity rendering some CTCs undetectable [14]. Additionally, antibody binding to CTCs leads to activation of some pathways and intracellular instability which affects further protein, genomic and molecular analyses of the CTCs. All those drawbacks lead researchers to develop negative enrichment method to overcome them. The negative enrichment system uses hypotonic lysis of RBCs in addition to removal of WBCs through anti-CD45 antibodies to isolate CTCs. Subtraction enrichment (SE) is another way that has been developed. It differs from usual negative enrichment but falls under the same umbrella and it uses non-hypotonic lysis method to remove the RBCs and relies on the use of multiple anti-WBCs antibodies (conjugated to beads) to remove the WBCs and allows non-disruptive detection of CTCs. To further improve the sensitivity of detecting CTCs with minimal disruption, the Cytelligen system was developed. It combines subtraction enrichment with immunostaining fluorescence in situ hybridization (iFISH) and has proven efficient in detecting CTCs from different tumors. Special probes detecting the centromere in chromosome 8 is one of the probes used in iFISH to help in the detection of CTCs taking in consideration that the large number of cancer cells have heteroploid chromosome 8. Side by side with other immunostaining the isolation and detection of CTCs becomes easier and much more clear [13,15].

The detection of CTCs is improving and developing and it is one of the hot topics that came under focus in the last decade. Researchers worldwide are still working on developing new and improving existing techniques for the detection and enrichment of CTCs. Cell Search system has been approved by FDA for the detection of CTCs in breast cancer, colorectal and prostate cancer [16]. Despite the large number of studies in the field of CTCs, they are still in the beginning of a long path before they become routine tests. One major obstacle is the high cost of the available techniques. Another drawback is the variability in the sensitivity of the detection capability among different techniques and studies. Analysis of CTCs detection in bladder cancer showed that the detection of CTCs in the peripheral blood in patients with lymph nodes metastases fell in the range of (29.1-91%). While it was higher in those with distant metastases (33-100%), and therefore the prognostic role of CTCs cannot be denied and opens the way for a larger role in the future [16].

References

1. Swanton C. Intratumor heterogeneity: evolution through space and time. Cancer Res. 2012; 72(19): 4875-82. doi: 10.1158/0008-5472.CAN-12-2217.
2. Gerlinger M, Rowan AJ, Horswell S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. N Engl J Med. 2012; 366(10): 883-92. doi: 10.1056/NEJMoa1113205.
3. Li J, Han X, Yu X, et al. Clinical applications of liquid biopsy as prognostic and predictive biomarkers in hepatocellular carcinoma: circulating tumor cells and circulating tumor DNA. J Exp Clin Cancer Res. 2018 Sep 3; 37(1): 213. doi: 10.1186/s13046-018-0893-1.
4. Ashworth, TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Aust Med J. 1869; 14: 1469.
5. Yin CQ, Yuan CH, Qu Z, et al. Liquid biopsy of hepatocellular carcinoma: circulating tumor-derived biomarkers. Dis Markers. 2016; 2016: 1427849. doi: 10.1155/2016/1427849.
6. Miller MC, Doyle GV, Terstappen LW. Significance of circulating tumor cells detected by the cellsearch system in patients with metastatic breast colorectal and prostate cancer. J Oncol. 2010; 2010: 617421. doi: 10.1155/2010/617421.
7. Low WS, Wan Abas WA. Benchtop technologies for circulating tumor cells separation based on biophysical properties. Biomed Res Int. 2015 2015: 239362. doi: 10.1155/2015/239362.
8. Hou HW, Warkiani ME, Khoo BL, et al. Isolation and retrieval of circulating tumor cells using centrifugal

forces. Sci Rep. 2013; 3: 1259. doi: 10.1038/srep01259.

9. Alix-Panabieres C, Pantel K. Challenges in circulating tumour cell research. Nat Rev Cancer. 2014; 14(9): 623-31. doi: 10.1038/nrc3820.

10. Pantel K, Alix-Panabieres C. Real-time liquid biopsy in cancer patients: fact or fiction? Cancer Res. 2013; 73(21): 6384-8. doi: 10.1158/0008-5472.CAN-13-2030.

11. Pantel K, Alix-Panabieres C. The clinical significance of circulating tumor cells. Nat Clin Pract Oncol. 2007; 4(2): 62-3. doi: 10.1038/ncponc0737

12. Shen Z, Wu A, Chen X. Current detection technologies for circulating tumor cells. Chem Soc Rev. 2017; 46(8): 2038-2056. doi: 10.1039/c6cs00803h.

13. Riethdorf S, Fritsche H, Muller V, et al. Detection of circulating tumor cells in peripheral blood of patients with metastatic breast cancer: a validation study of the CellSearch system. Clin Cancer Res. 2007; 13(3): 920-8. doi: 10.1158/1078-0432.CCR-06-1695.

14. Ogle LF, Orr JG, Willoughby CE, et al. Imagestream detection and characterisation of circulating tumour cells - A liquid biopsy for hepatocellular carcinoma? J Hepatol. 2016; 65(2): 305-13. doi: 10.1016/j.jhep.2016.04.014.

15. Lin PP. Integrated EpCAM-independent subtraction enrichment and iFISH strategies to detect and classify disseminated and circulating tumors cells. Clin Transl Med. 2015; 4(1): 38. doi: 10.1186/s40169-015-0081-2.

16. Khetrapal P, Lee MWL, Tan WS, et al. The role of circulating tumour cells and nucleic acids in blood for the detection of bladder cancer: A systematic review. C Cancer Treat Rev. 2018; 66: 56-63. doi: 10.1016/j.ctrv.2018.03.007.

E-mail: dr.ahmedkhairallah@gmail.com
dr.ahmedkhairallah@colmed-alnahrain.edu.iq

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Phage Cocktails Against Highly Multi-Drug Resistant *Acinetobacter baumanii*

Hayder N. Jasim *MSc*, **Ahmad S. Abdul-Ameer**[1] *PhD*

[1]Dept. of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background Phage therapy is a potential alternative treatment for infections caused by many bacterial species such as *Acinetobacter baumannii* (*A. baumannii*), a significant nosocomial pathogen, has evolved resistance to almost all conventional antimicrobial drugs in poor hygiene and conflicts areas like Iraq.

Objective Isolate and apply bacteriophages as alternative therapeutic agents against extensively drug-resistant (XDR) and pan-drug-resistant *A. baumannii* and evaluation extracted native endolysin activity.

Methods Twenty-three bacterial samples were collected in Al-Imamein Al-kadhimein Medical City hospital. Phages were isolated from different regions in Baghdad city including (soil, sewage, irrigation channels). Native endolysin was extracted from highly lytic phage that produced halo-like appearance around inhibition zone.

Results Out of 50 isolates, 23 isolates (46%) of XDR, pan-drug resistant (PDR) *A. baumanii* have been isolated from patients with various infections. 136 lytic phages specific to *A. baumanii* were isolated. Each bacterial isolate was sensitive to at least one lytic phage. Phage cocktails were formulated and were shown remarkably minimize the bacterial resistance to individual lytic phages. In addition, the endolysin native activity of lytic phages specific to *A. baumanii* evaluated in this study revealed a potent antibacterial activity (> 1 log) reduction of bacterial density in just one hour of endolysin treatment.

Conclusion Phage therapy assessed in this study was shown ability to efficiently solve the problems of "superbug" bacteria by lysing effectively most XDR, PDR bacteria in vitro. And, phage cocktails were shown to be superior over single-phage preparations in treating *A. baumanii* with much less resistance rate to therapeutic phages. Furthermore, intrinsic activity of native endolysin revealed promising results to tackling superbug pathogens.

Keywords Phage, Phage cocktails, Acinetobacter, native endolysin, extensively drug-resistant (XDR), and pan-drug resistant (PDR)

Citation Jasim HN, Abdul-Ameer AS. Phage cocktails against highly multi-drug resistant *Acinetobacter baumanii*. Iraqi JMS. 2018; 16(4): 357-371. doi: 10.22578/IJMS.16.4.2

List of abbreviations: *A. baumanii* = *Acenitobacter baumanii* CLSI = Clinical and Laboratory Standards Institute, MDR = Multiple drug resistant bacteria, PDR = Pan drug resistant bacteria XDR = Extensive drug resistant bacteria

Introduction

Antibiotic resistance is an emerging global health disaster, resulting from the constant use (and misuse) of antibiotics in healthcare [1,2]. *Acinetobacter baumannii* (*A. baumannii*) is a Gram-negative, capsulated, opportunistic pathogen that is effortlessly spread in hospital intensive care units (ICU) [3]. Most of *A. baumannii* clinical isolates are multi-drug resistant (MDR), extensively drug-resistant (XDR), and pan-drug resistant (PDR) bacteria, which greatly restricts the available treatment choices [4]. To prevent

returning to the dark "post antibiotics" era, there is an urgent need for new therapeutic agents against the MDR, XDR, PDR pathogens. To fight these bacteria, the scientists suggest a number of new therapeutics alternatives or complements to antibiotics against the "superbug" pathogens, of which *A. baumannii*. Interestingly, bacteriophage, or phage, therapy has been placed at the top of table presenting a possible alternative mean to tackle refractory bacterial infections [5].

Phage therapy refers to the utilization of phages to treat bacterial diseases [6]. Phages are very abundant in nature [7] and every bacterium is likely to have their own specific viruses that could be utilized as antibacterial agents [8-10]. The host range of a given phage is often very specific to the sub-species level, which may confer an advantage over antibiotics if infectious bacteria can be targeted without damaging commensal members of the host microbial community.

The formulation of phage cocktail could save lives of uncountable patients suffering from serious and devastating *A. baumanii* infections resistant to the conventional antibiotics. This highlights the importance of using phage cocktails especially in a country like Iraq where *A. baumanii* flourishes in poor hygiene and areas of conflicts [11].

The current study aims at testing the efficacy of phage therapy, via using a single phage and a phage cocktail, to treat infections with MDR *A. baumanii* bacteria in vitro and to extract and determine intrinsic activity of native endolysin.

Methods

Specimen collection and identification

Fifty samples of bacteria were collected in Al-Imamein Al-kadhimein Medical City Hospital in Alkademiya, Baghdad. Bacterial sampling was carried out during the period from September 2016 to November 2016. A total of twenty-three (23) different *A. baumanii* isolates (11 XDR, 12 PDR), belonging to hospitalized patients with various infections including septicemia, skin infection, severe urinary tract infection, pneumonia, and meningitis, were obtained from the Central Laboratory of the hospital. At the same day, samples were transferred to the laboratory of the Medical Microbiology Department in the College of Medicine, Al-Nahrain University to for cultivated bacteria by using nutrient, MacConkey agar and blood agar then incubate at 37 °C for 18-24 h. Next day, all bacterial isolates were subjected to a full set of diagnosis including Gram staining, culture, and bio-chemical tests including Oxidase test, Catalase test, Kligler iron agar (KIA), Indole production test, Motility test, Urease production test, Citrate utilization test, Lactose fermentation test, and growth at 44 °C test [12]. Furthermore, the results of the identification of *A. baumanii* were confirmed by API 20E system.

Antibiotic susceptibility test

Antibiotic susceptibility test was carried out on *A. baumanii* isolates using Kirby-Bauer method [13]. Seventeen types of antibiotic disks were used as following Imipenem (10 μg), Ciprofloxacin (5 μg), Colistin (10 μg), Tigacyclin (15 μg), Gentamicin (10 μg), Cefotaxime (30 µg), Ceftazidime (30 μg), Ceftriaxone (30 μg), Trimethoprim/sulphamethaxazole (10 μg), Cefepime (30 µg), Levofloxacin (10 μg), Piperacillin (100 µg), Tobramycin (10 µg), Amikacin (30 µg), Meropenem (10 µg), Aztreonam (30 µg) and Amoxicillin-clavulanic acid (20 µg). A 0.5 McFarland standards of bacteria was used and inoculated and spread by a sterile swab on Muller-Hinton agar Medium. Antibiotic discs were then placed on inoculated agar plates by forceps. The plates were left in incubator upside down at 37 °C for 18-24 h.

At Subsequent day, plates were carefully examined for any inhibition zones along with measuring their diameter in millimeters (mm) using a metric ruler. Then, classified as sensitive, intermediate, or resistant, according to the standardized table supplied by Clinical and Laboratory Standards Institute (CLSI) guidelines [14]. Furthermore, the results of antibiotics susceptibility test were confirmed by VITEK 2 System.

Bacteriophage sampling, isolation

Different crude samples for phage isolation were obtained from different regions in Baghdad city including sewage, farm soil, feces of sheep, chicken litter, and swab from surgical lounge in Al-Imamein Al-kadhimein Medical City Hospital during the period from January 2017 to April 2017. Overnight bacterial broth (100 µl) was mixed with 2-3 ml of crude samples, which were derived from sewage, cattle feces, chicken litter that might contain Acinetobacter-specific phages. Then, 2-3 ml, equal volume, of nutrient broth and 2ml of Lambda buffer were added to the mixture as well. Then, the mixture was incubated overnight at 37 °C with continuous shaking. Next day, supernatant was taken and 1:10 v/v chloroform was added with gentle shaking for 5-7 min at room temperature to lyse the remaining bacteria. Then, centrifugation at 1000 g for 3 min was carried out to produce primary phage suspension. Subsequently, one ml of overnight bacterial broth was poured onto nutrient agar plate and spread by sterile swab in order to make bacterial lawn. After 10-20 min, the lawn should have been dried. Ten (10) µl of primary phage suspension were spotted onto the surface of the bacterial lawn and were allowed to dry before incubating at 37 °C for 18-24 h in inverted state. On the next day, if zone of lysis was developed at the spot of the primary phage suspension, a lytic and specific phage for the target bacteria was identified and picked up the inhibition zone by sterile loop and put into 1 ml of Lambda buffer in 1.5 ml sterile Eppendorf tubes, then 1:10 v/v chloroform was added to the lysate with gentle shaking for 5-7 min at room temperature, then, centrifuged at 1000 g for 3 min and bacterial cell debris were pelleted, and the supernatant containing phages was transferred to 1.5 ml sterile Eppendorf tubes and stored at 4 °C for one month. The supernatant was called transient phage stock suspension [15].

Optimization and characterization of isolated phages

Plaque characteristics were determined using top layer plaque assay and according to the following parameters: a) Diameter (mm) of the plaque. b) Shape of the plaque. c) Depth of the plaque. d) Margin cut. e) Clarity or turbidity of the plaque. Accordingly, the clearest and largest plaques were selected; moreover, small or turbid plaques were subjected to optimization by conducting serial passage in top layer plaque assays; at each run, the best of the best plaques, in terms of the above-mentioned parameters, were selected in order to acquire better virulence characteristics of the isolated lytic phages. It is noteworthy to mention that not all turbid and small plaques were optimized. This depends on the potential of the phage to enhance its virulence characteristics [15]. In this approach, burst size, burst time, and infection percentage are determined according to [15]. One hundred (100) µl of 106 PFU/ml of phage were added to 100 µl of 104 CFU/ml of target bacterial broth culture at MOI equal to 100. This mixture was dispensed into a sterile 1.5 ml Eppendorf tube and then incubated at 37 °C for 5-10 min to allow the phage to enter into bacterial host (phage contact time). Then, the mixture of phage-bacteria was centrifuged at room temperature at 1000 g for 3 min. Subsequently, the pellet was re-suspended in one ml of nutrient broth (this step was repeated 3 times). The aim of this step is to remove all of the extracellular phages and to neglect the supernatant and keep only the bacterial cells infected with specific phages. Afterwards, the re-suspended tube was ten-fold serially diluted (10-1-10--2) by adding 100 µl of the suspension to 900 µl of nutrient broth. Ten µl from each dilution were spotted on target bacterial lawn at timely intervals; 20, 25, 30, 35, 40, and 45 min, then, let the plate get wet and incubated overnight at 37 °C. Next day, the plaques were counted to calculate the infection percentage, burst time, and burst size [15] as follows:

Infective percentage (IP %): refer to the percentage of specific lytic phages that invade the target bacteria. This percentage was calculated by dividing the number of plaques during the pre-burst time over the number of bacteria used in the assay. Burst time (BT): refer to the time required by the infecting

phages to burst and exist from bacterial cells. So, BT is the period before a sharp rise was detected in the number of the progeny phage particles for the certain dilution. Burst size (BS): refer to the number of the new progenies of phage per one cell of target bacteria. BS was calculated by dividing the number of plaques post- burst time over the number of plaques pre-burst time [15].

Testing of bacterial resistance rate of *A. baumanii* to infecting bacteriophages

The resistance rate of bacteria to infecting phages was measured. A piece from the same bacterial lawn of the target bacteria that is equal in diameter to phage lysis spot was cut by a sterile loop and put in 1.5 ml sterile Eppendorf tube containing one ml of normal saline. This approach is to obtain the same number of bacteria that was present in the phage spot lysis zone. Then, the tube was subjected to periodic shaking for 5 min. Then, tubes were centrifuged at 1000 g for 3 min at room temperature. Afterwards, the supernatant was removed and the precipitate was re-suspended in one ml of normal saline [15].

Ten-fold serial dilutions of the resulting bacterial suspension (10-1-10-5) were made. Then, 10 µl drop of the bacterial suspension was spotted on a nutrient agar plate inclined 45 degrees in one direction in order to spread the drop to one direction forming lines at which counting of bacterial colonies becomes much easier. The plates were incubated at 37 °C for 24 h. The bacterial resistance rate was calculated as the following [15]:

Resistance rate = Number of resistant colonies per phage lysis spot / number of bacterial colonies formed from the same size cut of bacterial lawn.

Determination of the coverage rate of bacteriophage cocktails to *A. baumanii*

In this approach, after mixed numerous phages in one suspension, randomly sampled 10 *A. baumanii* isolates were collected from patients in Al-Imamein Al-kadhimein Medical City Hospital. Ten (10) µl of bacteriophage cocktails suspension were spotted on to the surface of the overnight bacterial lawn and were allowed to dry before incubating at 37 °C for 24 h. On the next day, if a zone of lysis was developed at the spot where the phages suspension was applied, a susceptible bacterial isolate to phage cocktail was found. Then, the coverage rate of the formed bacteriophage cocktails was measured using this formula:

Coverage rate = (number of bacteria lysed by cocktails / total number of bacteria) x 100%.

The assessment of the activity of phage Endolysin on A.r baumanii bacteria

Extraction of Endolysin

About 100 ml of broth of *A. baumanii* bacteria were incubated for 18-24 h at 37 °C. Next day, 250 ml of broth medium were added to the bacterial broth and incubated for another 3 hours at titer 1×10^9 CFU/ml. Up to 10 ml of phage at titer 1×10^{11} PFU/ml (1:100 MOI) were mixed with bacteria for 20 minutes and then put them directly in ice. Centrifugation at 10,000 g for 20 minutes and take the sediment. The sediment was suspended in 10 ml of 0.05 M phosphate buffer + 5 mg deoxyribonuclease and incubated for 60min at 37 °C. And 0.005 M EDTA was added and centrifugation at 10,000 g for 1 h and then the supernatant was taken. Disodium tetrathionate (0.3 M) was added and mixed for one hour at 4ºC. Ammonium sulfate was added to 85% saturation and incubated for 18-24 h at 4 °C. Next day, centrifugation at 10,000g for 1h and resuspended in 5 ml of 0.05 M phosphate buffer saline (PH 7.5). Dialysis against 200ml of the buffer at 4 °C was conducted. The resultant solution was added to column chromatography sephadex G.100 in 0.1 M phosphate buffer saline PH 7.5, in 18×0.5 cm column. Each one ml of the resultant filtrate was collected in Eppendorf tubes. From each Eppendorf tube, 10 µl of the filtrate were dropped by automatic pipette onto *A. baumanii* bacterial lawns of the specific bacteria to see which Eppendorf tube contains the lytic and native activity of endolysin.

Measurement of the native activity of Endolysin on A. baumanii bacteria

Upon using sephadex G100 chromatography, the used phage (AB3P5) gave 10 Eppendorf tubes of one ml eluted fluid; in case endolysin was extracted, at least one of these tubes must show a lytic activity against the corresponding *A. baumanii* isolate. The endolysin activity was first checked by lysis on bacterial lawn and second by decreasing the optical density of the bacterial broth when measured by a spectrophotometer. *A. baumanii* broth was composed of bacterial cells growing at mid-log phase (OD600 = 0.6) and were centrifuged (4000 g, 30 min, 4 °C) and then re-suspended in a phosphate-buffered saline (PBS) at PH (7.5). After assigning the tube that showed lysis in the bacterial lawn assay, 30 µl of this supposed-to-be endolysin-containing elute were added to 270 µl of the prepared bacterial broth at room temperature. Then, the optical density was measured spectrophotometrically every ten minutes for 1 h at 600 nm [16].

Results

Characteristics of the isolates of *A. baumanii*

The characteristic features of the bacterial isolates used were the site of infection, patient's sex, patient's age, and the disease or lesion from which bacterial isolates were taken. As shown in table 1, the patients were infected with virulent bacteria causing serious and life-threatening diseases including urinary tract infection, septicemia, wound infection, pneumonia, and meningitis. A total of 23 *A. baumanii* isolates were collected. The specimens from which *A. baumanii* were isolated are as follows: blood 7/23 (30.4%), urine 2/23 (9%), wound swab 7/23 (30.4%), diabetic foot 3/23 (13%), sputum 2/23 (9%), and C.S.F 2/23 (9%). The diseases from which *A. baumanii* bacteria were isolated wound infection 10/23 (43.4%), urinary tract infection 2/23 (9%), septicemia 9/23 (39.1%), pneumonia 2/23 (9%), and meningitis 2/23 (9%) (Tables 1 & 2). Hence, the most prevalent disease related to *A. baumanii* was wound infection followed by septicemia. The age of patients ranged from 1 day to 70 years and male to female ratio was 2.3:1.

All bacterial isolates appeared as Gram-negative coccobacilli and occasionally arranged in diplococci. All of the isolates were tested for biochemical tests and *A. baumanii* showed negative results for oxidase, motility, indole production and urease production tests, and positive results to catalase and citrate utilization tests; Kligler iron agar test developed an alkaline slant, no change at bottom, H2S negative without gas production. Also, when *A. baumanii* isolates were cultured on MacConkey agar, they appeared as small, pale and lactose non-fermenter colonies, while on blood agar they appeared as opaque, creamy and non-hemolytic colonies. Growth at 44ºC was positive for all *A. baumanii* isolates which showed the ability to grow at this temperature degree. This test was used to distinguish *A. baumanii* (which was able to grow at this temperature) from other Acinetobacter species which are unable to grow at this temperature degree.

Antibiotic sensitivity test

The results showed that different *A. baumanii* isolates had different antibiotic sensitivity profiles; of 23 isolates included in the current study, 11 were XDR and 12 were PDR. As shown in Figure 1.

The characteristic features of the isolated and optimized phages

The characteristics of plaque assay of the isolated phages showed that plaques clarity (clear, semi-clear, turbid, semi-turbid), plaques size was varied and ranged between 0.5 mm to 6.5 mm, margin cut (regular and irregular), and plaques shape (oval and circular).

One hundred and thirty-six (136) phages specific for 23 *A. baumanii* bacteria were isolated. The specimens were obtained mainly from sewage and also from irrigation channels, then from waste water, soil, feces of sheep, chicken litter and swab from lounge. However, most of the isolated phages were highly lytic

and produced obvious inhibition zone on target *A. baumanii* bacteria where plaque size was higher than 3mm with full clarity of plaques; therefore, further optimization was not needed save for 25 phages which required further optimization in order to increase their lytic characteristics, (Table 1). The titer of the specific lytic phages isolated and optimized to the bacterial isolates were amplified and measured by using top layer plaque assay. Most phages reached high titers ranging between 108-1011 PFU/ml using top layer plaque assay. The optimized specific and lytic phages were shown to be able to completely lyse the bacterial host in whatever manner of application of phages as demonstrated in figures 2 and 3.

The characteristics of the isolated and optimized phages in terms of biokinetic assay

In the current study, 10 bacteriophages to different bacterial isolates were randomly selected to give representative values of biokinetic characteristics. The results in this study showed that the average burst time (BT) was 73.5 min ranging between 30 to 45 min. The maximum burst size (BS) of the randomly selected phages to *A. baumanii* was 245 progenies, while the minimum BS was 130 progenies and the average BS was 187.5 progeny. The average infective percentage (IP %) was 85.45% ranging between 74.4% and 94.5%, as shown in table (3).

Formation of phage cocktail to *Acinetobacter baumanii*

A phage cocktail was formed by mixing 64 phages specific for 23 *A. baumanii* isolates (AB1-AB23). One hundred, 100 µl of 10^6 PFU/ml of each phage were collected in one tube to form a bacteriophage stock containing a wide range of isolated phages. All bacterial isolate, except AB2 and AB8, were targeted by more than one phage; the most targeted isolate was AB3 where 6 different phages shared the same specificity towards this isolate.

Bacterial resistance to a single phage versus phage cocktail

Up to 18/23 (78.3%) of *A. baumanii* bacteria were completely sensitive to the applied lytic phages with zero resistant bacterial colonies. So, only 5 out of 23 isolates (21.7%) of *A. baumanii* were shown to develop some level of resistant colonies in the inhibition zone at the spot of lytic phage application. On other hand, the formed phage cocktail was shown to remarkably minimize the number of the resistant bacterial colonies appeared to individual phages. The results revealed that once *A. baumanii* isolate develops resistance to one member of phage cocktails, the bacterial isolate was still sensitive and lysed by other phage members in the cocktails as shown in figure 4.

The coverage rate of the formed phage cocktail to *A. baumanii* bacteria

Ten (10) *A. baumanii* isolates were collected from patients resided in Al-Imamein Al-kadhimein Medical City Hospital. The collected specimens were not biased towards particular disease, site of infection, or patients' age or sex. Full sets of identification were performed, then the results of identification of *A. baumanii* confirmed by API 20E system.

The formed phage cocktail was able to form a clear inhibition zone on the most tested bacterial lawns. The coverage rate of the formed phage cocktail was calculated. The phage cocktail was shown to be able to lyse 7/10 (70%) of *A. baumanii* bacteria and thus the coverage rate was 70%.

Table 1. The characteristic features of *A. baumanii* bacteria to which the lytic and specific phages were isolated

Bacterial isolate	Specimen	Age of patient	Sex of patient	Disease
AB1	Wound swab	30 years	Male	Wound infection
AB2	Urine	40 years	Female	Urinary tract infection
AB3	Blood	7 years	Female	Septicemia
AB4	Throat swab	4 days	Female	Pneumonia
AB5	Wound swab	6 years	Male	Wound infection
AB6	Wound swab	37 years	Male	Wound infection
AB7	Blood	35 years	Male	Septicemia
AB8	Blood	1 day	Male	Septicemia
AB9	Blood	5 days	Male	Septicemia
AB10	Blood	3 days	Male	Septicemia
AB11	Wound swab	2 months	Male	Wound infection
AB12	Wound swab	33 years	Male	Wound infection
AB13	Sputum	38 years	Male	Pneumonia
AB14	Diabetic foot	40 years	Female	Wound infection
AB15	Wound swab	70 years	Male	Wound infection
AB16	CSF	43 years	Female	Meningitis
AB17	Blood	2 years	Female	Septicemia
AB18	Wound swab	12 years	Male	Wound infection
AB19	Diabetic foot	41 years	Male	Wound infection
AB20	Blood	55 years	Male	Septicemia
AB21	Diabetic foot	2 years	Female	Wound infection
AB22	Urine	67 years	Male	Urinary tract infection
AB23	CSF	14 days	Male	Meningitis

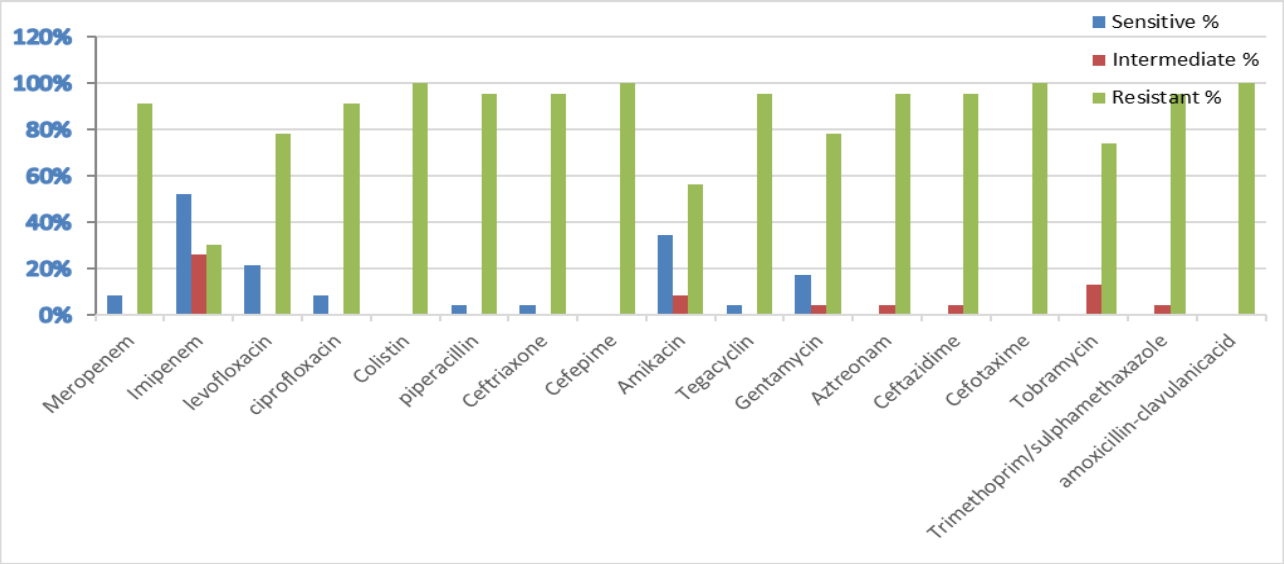


Figure 1. The rate of antibiotic sensitivity/ resistance of 23 *A. baumanii* isolates to a panel of 17 antibiotic disks commonly used in Iraq

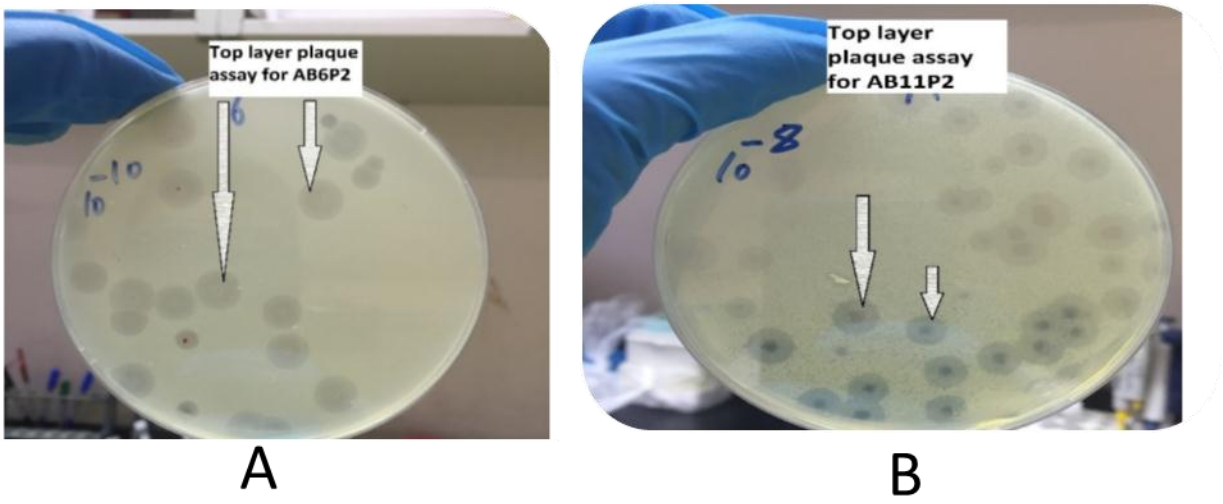


Figure 2. A) plaques produced by of AB6P2 via top-layer plaque assay B) plaques produced by AB11P2 via top-layer plaque assay

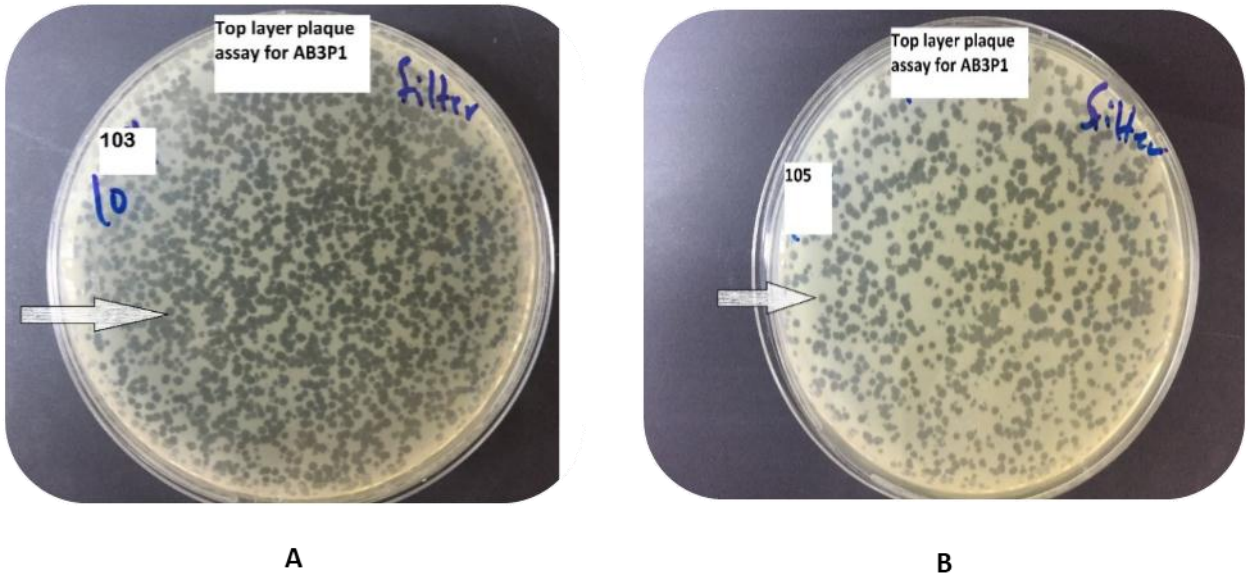


Figure 3. Top layer plaque assay for AB3P1 with different concentrations A) AB3P1 with 103 PFU/ml B) AB3P1 with 105 PFU/ml

Table 2. Morphological features of the isolated phages to *A. baumanii* bacteria before and after optimization via top layer plaque assay

Phage symbol	Plaque size (mm)		Plaque clarity		Plaque shape		Margin cut	
	Before	After	Before	After	Before	After	Before	After
AB1P1	0.5	1.5	Turbid	Semi-clear	Round	Round	Un-obvious	Regular
AB1P2	0.3	1	Semi-turbid	Clear	Round	Round	Irregular	Irregular
AB2P1	2	2.5	Semi-clear	Semi-clear	Round	Round	Regular	Regular
AB3P1	0.8	1.5	Semi-turbid	Semi-clear	Oval	Oval	Irregular	Irregular
AB3P2	0.5	0.5	Semi-clear	Clear	Oval	Oval	Regular	Regular
AB3P3	2.5	4	Semi-Clear	Clear	Round	Round	Irregular	Irregular
AB3P4	3.5	3.5	Semi-Clear	Clear	Oval	Oval	Irregular	Irregular
AB4P1	3.5	7	Clear	Clear	Round	Round	Irregular	Irregular
AB5P1	1	1.5	Turbid	Clear	Oval	Oval	Un-obvious	Irregular
AB6P1	2	2	Semi-turbid	Semi-clear	Semi-round	Round	Irregular	Regular
AB6P2	2	3.5	Semi-clear	Semi-clear	Oval	Oval	Irregular	Irregular
AB9P1	0.5	0.5	Semi-Clear	Clear	Round	Round	Regular	Regular
AB10P1	1.2	2.5	Clear	Clear	Round	Round	Regular	Regular
AB10P2	1.9	5.5	Semi-turbid	Clear	Round	Round	Regular	Regular
AB12P1	1	1	Semi-turbid	Semi-turbid	Oval	Oval	Irregular	Irregular
AB15P1	3.5	5.5	Turbid	Clear	Round	Round	Un-obvious	Regular
AB15P2	0.8	1	Semi-turbid	Clear	Round	Round	Regular	Regular
AB17P1	1.7	3.5	Semi-Clear	Clear	Oval	Oval	Irregular	Irregular
AB19P1	0.5	3	Semi-turbid	Semi-turbid	Round	Round	Un-obvious	Irregular
AB19P2	0.8	1.5	Turbid	Clear	Round	Round	Un-obvious	Regular
AB20P1	0.5	2	Semi-Clear	Clear	Oval	Oval	Regular	Regular
AB21P1	1.5	6.5	Turbid	Semi-clear	Oval	Oval	Regular	Regular
AB21P2	1	2	Semi-turbid	Semi-clear	Round	Round	Irregular	Irregular
AB22P1	0.5	1.5	Turbid	Turbid	Oval	Oval	Un-obvious	Regular
AB22P2	2.3	4.5	Semi-turbid	Semi-turbid	Oval	Oval	Irregular	Irregular

Table 3. Shown the biokinetics: Infective percentage (IP %), Burst time (BT) in minutes, and Burst size (BS) in number of progenies of the randomly selected bacteriophages to *A. baumanii* bacteria

	IP %	BT	BS
AB1P2	91.3	40	170
AB3P4	94.5	45	245
AB5P1	76.4	35	220
AB7P3	85.2	30	160
AB9P1	88	45	210
AB10P2	86.6	45	145
AB14P1	77.5	40	190
AB15P3	82	30	200
AB17P2	80	40	130
AB20P1	79	35	185

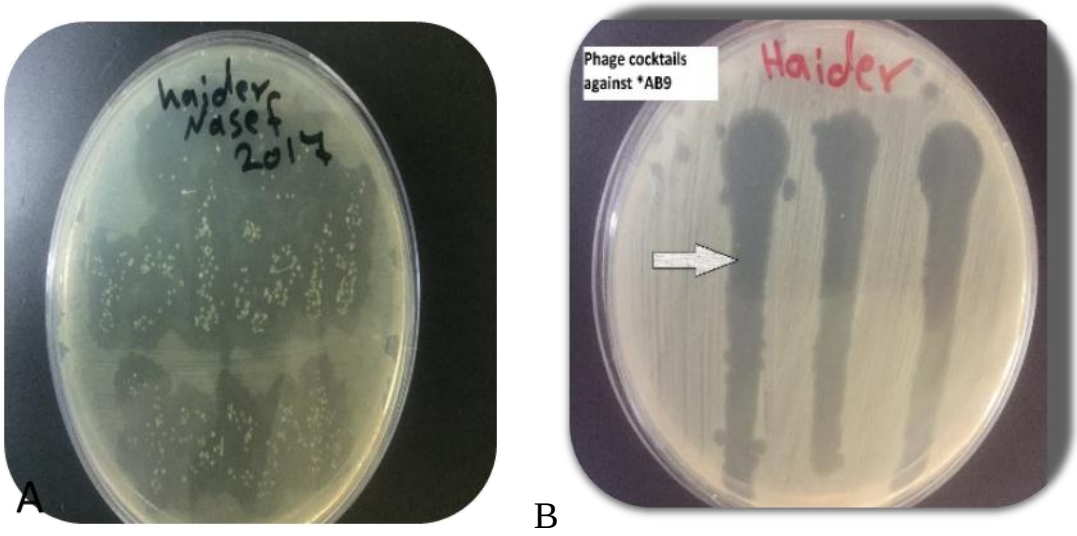


Figure 4. (A) resistant *A. baumanii* bacteria (AB3) to a single specific phage (AB3P1) (B) the phage cocktail completely lysed *A. baumanii* (AB3) bacteria without development of any resistant colonies

Determination of the native activity of phage Endolysin on *A. baumanii* bacteria

During the isolation and optimization of different phages against *A. baumanii* isolates; some phages were found to produce a halo-like appearance around the inhibition zone produced by some lytic phages as shown in figure 6. This halo-like appearance suggested a native endolysin production from phage. The findings of this study revealed that a specific phage endolysin to *A. baumanii* was extracted successfully by using sephadex G100 column chromatography. The Eppendorf tube number two showed positive results for phage

endolysin. The optical density of *A. baumanii* broth was measured initially at zero time, just before the addition of the corresponding endolysin, then it was measured every ten minutes for complete one hour and it showed obvious decline in optical density of bacterial broth with time. According to t-distribution test, there is a significant difference between the test groups, bacteria treated with endolysin and control group, bacteria alone with PBS, (P = 0.00134). Moreover, the overall enzymatic activity of extracted native endolysin was quantified by turbidometric reduction analysis, 270 µl of exponentially growing *A. baumanii* (AB3) cultures (1.4 x 108 CFU/ml) were challenged to 30 µl of extracted native endolysin at room temperature. *A. baumanii* optical density and viability counts were reduced from 0.585 to 0.031 after one hour of treatment, compared with the untreated control group that continued to grow (from 577 to 624 after one hour). It was shown to be -0.0092 ΔOD/min. By using standard curve measurements to interpolate OD values to bacterial count, it was shown that the endolysin native activity surpassed 1.4 log reduction threshold after one hour of treatment as shown in figure 5.

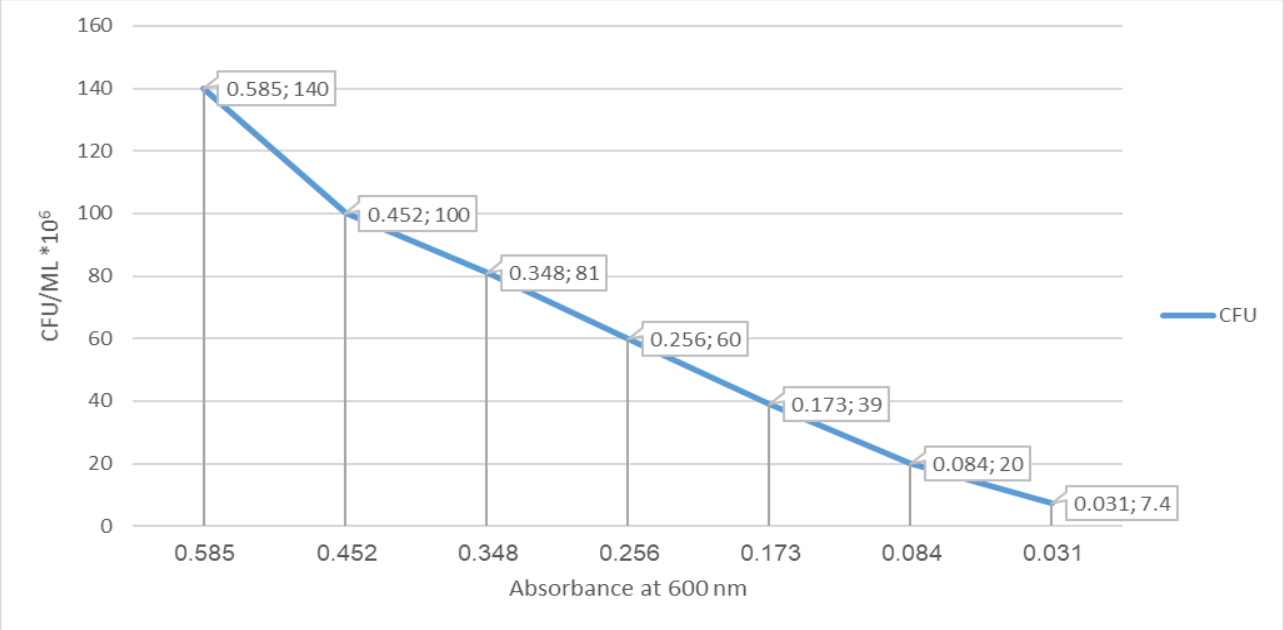


Figure 5. Interpolation of bacterial count in CFU/ml with the optical density (600nm) of *A. baumanii* broth treated with AB3 phage endolysin

Discussion

The results in the current study revealed different antibiotic resistant profiles by different *A. baumanii* isolates. All of the isolates of *A. baumanii* were shown to be fully resistant to several antibiotics tested in the current study and as follows: Cefepime, Cefotaxime, Colistin, and Amoxicillin-clavulanic acid. However, the resistance rate to other antibiotics was less than 100% and ranged from 95.65% to 30.43%. The MDR status reported in current study agrees with the findings of other recent studies carried in Iraq [17-19] but this study disagrees with a study carried out in USA which reported that approximately 50% of patients are with colistin-resistant *A. baumannii* [20]. The variation in the results may be due to sample size, sampling procedure, differences in the time of the studies, or differences in the geographic areas. This study

agrees with study in Iraq which found that, *A. baumanni* clinical isolates developed 97.3% of resistance to Aztreonam and Ceftriaxone, 89.5% to Ceftazidime, and 58.2% to Imipenem [21]. Moreover, this study agrees with another local study conducted in Iraq which found that, a high level of *A. baumanii* resistance, 88.2% to Meropenem and a lower resistance rate 52.9% to Imipenem while complete resistance was reported, 100%, to Cefepime, Azteronam, and Ceftriaxone [22]. The complete resistance of *A. baumanii* isolates collected in this study to colistin might be attributed to the major mechanism of colistin resistance in *A. baumannii*, namely modification of lipopolysaccharide (LPS) outer membrane via adding phosphor ethanol amine to the hepta - acylated lipid A structure [23,24].

The presence of β-lactamases, which are the backbone of the most principal mechanism of β-lactam resistance. These enzymes, at least in part, hydrolyze carbapenems along with other β-lactams [25]. Recently a new extended spectrum AmpC enzyme was identified in *A. baumannii* bacteria; this enzyme has been shown to be able to hydrolyze Ceftazidime, Cefepime and Aztroenam [26].

In this regard, bacteriophage or phage therapy could offer one of the best applicable solutions to overwhelm the problem of antibiotics resistance of bacteria in Iraq and in the world [27]. One of the striking merits of using bacteriophages over antibiotics in a country like Iraq is the fact that phages are self-amplifying in the site of infection so phages can be given to patients in a single dose, therefore, unnecessary to repeat doses of phages; hence, incompliance of patients will not affect the success of the course of therapy. In this study, the lytic and specific phages to *A. baumanii* were isolated from various environmental sources; the main source was sewage; this finding is in line with other studies [28,29]. Another main source of phages in this study was waste water [30]. The current study revealed that sewage was the best source to isolate highly lytic and specific phages to *A. baumanii* [31]. Moreover, phages from sewage showed good clarity and size of plaques; this might be credited to the fact that phages in sewage tolerate drastic environment which favors the induction of temperate phages, residing in high number in Acenitobacter bacteria, to lytic ones.

The current study showed successful in vitro use of both single phage and phage cocktail to lyse *A. baumanii* XDR or PDR isolates. Nevertheless, this study revealed a superiority of the phage cocktail over the single phage in lysing *A. baumanii* bacteria without development of resistant colonies to phage therapy. Consequently, such phage cocktails are powerfully supposed to prevent the emergence of phage-resistant mutants [16,32]. The results of the current study highlighted the fact that using phage cocktails provides several advantages. Firstly, phage cocktails broaden the strain-specific range of infective phages. This permits effective therapy of a broader spectrum of bacteria within the same *A. baumanii* species [33,34]. Secondly, phage cocktails solve the serious obstacle of the development of *A. baumanii* resistance to attacking phages. It was stated that using phage cocktails is the finest choice for effective phage therapy without suspicions of rapid emergence of bacterial resistance [33]. The phage cocktail used in this study ensured two important goals, covering as much as possible different strains of *A. baumanii*, and the second goal is that each bacterial isolate was recognized by multiple different phages, a necessary step to combat bacterial resistance to phage therapy. Each *A. baumanii* isolate might have more than one receptor and each receptor is recognized by a different phage to attach and invade [35]. This explains why each bacterial isolate was invaded by more than one different phage. Therefore, when a bacterial isolate develops resistance to one phage in the phage cocktail, it is still sensitive to other phages in the same phage cocktail. From the findings of the top layer plaque assay of this study, each member of the phage cocktail was different from each other, and from the findings of the bacterial resistance rate to the single phage versus the phage cocktail, the phages used in this study seem to target different receptors on the cell wall of *A. baumanii* bacteria. This provides evidence on

the preferred use of phage cocktails in the phage therapy of superbugs like *A. baumanii* XDR and PDR bacteria tested in this study.

The coverage rate of the formed bacteriophage cocktail in this study was shown to be very high, up to 70%. Such high coverage paves the road to successful and ready-to-use therapy of serious and life-threatening infections of *A. baumanii*. Nevertheless, in this study, it was proven that in few months and by a single researcher, a phage cocktail of 64 *anti-A. baumanii* specific and lytic phages was formed. The formed phage cocktail could save lives of uncountable patients suffering from serious and devastating *A. baumanii* infections resistant to the conventional antibiotics. This highlights the importance of using phage cocktails especially in a country like Iraq where *A. baumanii* flourishes in poor hygiene and areas of conflicts [36,37].

The results of the native endolysin activity in the current study are in a harmony with few studies examined the native activity of endolysin produced from bacteriophages that infect gram-negative bacteria such as *A. baumanii*, *P. aeruginosa* and *E. coli* [38-42]. The current study highlights the intrinsic antimicrobial activity of native endolysin produced from phages against G-ve bacterial pathogens. Native endolysin activity is a good candidate for the therapeutic/disinfectant endeavor to control nosocomial infections caused by multiple drug-resistant bacteria, particularly MDR *A. baumanii* bacteria [38]. The intrinsic antibacterial activity of endolysin against G-ve needs the ability of endolysin to get through the outer membrane pf these bacteria. This might explain why endolysins from phages infecting Gram-negative hosts are mostly small single-domain globular proteins (molecular mass between 15 and 20 kDa), and usually without a specific CBD module [39]. These lysins likely better fulfill the catalytic role of classical enzymes (aiding multiple catalytic reactions during cell lysis), as opposed to their Gram-positive counterparts, which are proposed to bind to one site and have a very low off-rate [43,44].

In Acinetobacter, it seems rather unusual that the lysogens would evolve a large diverse group of lysins for the sole purpose of releasing their phage progeny. Researchers guess that these lysins might in some manner be harnessed by the Acenitobacter bacteria to control their environment and fight back competing bacteria of other species [45]. Being a soil organism, *A. baumannii* shares a highly competitive niche with further bacteria, including Pseudomonas and Bacillus which have an advantage over *A. baumannii* with their capability of producing several bacteriocin molecules used to kill bacteria in proximity [46,47].

Taken together, the findings of this study indicate that *A. baumanii* bacteria in Iraq are mostly XDR and PDR bacteria; such abnormally high rate of multiple drug resistance necessities novel methods to tackle this impeding health risk on community. Therefore, the phage therapy assessed in this study was shown to be able to efficiently solve the problems of superbug resistant bacteria by lysing effectively most XDR and PDR bacteria in vitro. And, phage cocktails were shown to be superior over single-phage preparations in treating *A. baumanii* with much less rate of resistance to therapeutic phages. In addition, the endolysin native activity of lytic phages specific to *A. baumanii* evaluated in this study revealed a potent antibacterial activity (> 1 log) reduction of bacterial density in just one hour of endolysin treatment; this provided promising results to tackle Gram negative bacteria by using low molecular weight endolysins which are of high level of native antibacterial activity.

Acknowledgments

Regard and gratefulness should be presented to the staff members Department of Microbiology at the College of Medicine, Al-Nahrain University for their assistance.

Authors contribution

Dr. Abdel-Ameer: designed the research and conducted study analysis. Jasim: conducted the research.

Conflict of interest

There is no conflict of interest among authors of this manuscript.

Funding

There is no funding source for this research.

References

1. Cantas L, Shah QA, Cavaco LM, et al. A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. Front Microbiol. 2013; 4: 96. doi: 10.3389/fmicb.2013.00096.
2. Hofer U. The cost of antimicrobial resistance. Nature Rev Microbiol. 2019. 17(3): 78-89. doi: https://doi.org/10.1038/s41579-018-0125-x.
3. Murray CK, Yun HC, Griffith ME, et al. Recovery of multidrug-resistant bacteria from combat personnel evacuated from Iraq and Afghanistan at a single military treatment facility. Mil Med. 2009; 174(6): 598-604. doi: https://doi.org/10.7205/MILMED-D-03-8008.
4. Calhoun DA, Jones D, Textor S, et al. Resistant hypertension: diagnosis, evaluation, and treatment: a scientific statement from the American Heart Association Professional Education Committee of the Council for High Blood Pressure Research. Circulation. 2008; 117(25): e510-26. doi: 10.1161/CIRCULATIONAHA.108.189141.
5. O'Neill, J. Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance. 2016. Available at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf.
6. Abedon ST, Kuhl SJ, Blasdel BG, et al. Phage treatment of human infections. Bacteriophage. 2011; 1(2): 66-85. doi: 10.4161/bact.1.2.15845.
7. Hendrix RW, Smith MC, Burns RN, et al. Evolutionary relationships among diverse bacteriophages and pro-phages: all the world's a phage. Proc. Natl. Acad. Sci. U.S.A. 1999; 96: 2192-7. doi: 10.1073/pnas.96.5.2192.
8. Clokie MRJ, Millard AD, Letarov AV, et al. Phages in nature. Bacteriophage. 2011; 1(1): 31-45. doi:10.4161/bact.1.1.14942.
9. Flores CO, Meyer JR, Valverde S, et al. Statistical structure of host-phage interactions. Proc. Natl. Acad. Sci. U.S.A. 2011; 108(28): E288–E297.doi:10.1073/pnas.1101595108.
10. Ormälä AM, Jalasvuori M.. Phage therapy: should bacterial resistance to phages be a concern, even in the long run? Bacteriophage. 2013; 3(1): e24219. doi: 10.4161/bact.24219.
11. Falagas ME, Vardakas KZ, Kapaskelis A, et al. Tetracyclines for multidrug-resistant *Acinetobacter baumannii* infections. Int J Antimicrob Agents. 2015; 45(5): 455-60. doi: 10.1016/j.ijantimicag.2014.12.031.
12. Merril CR, Scholl D, Adhya SL. The prospect for bacteriophage therapy in Western medicine. Nat Rev Drug Discov. 2003; 2(6): 489-97. doi: 10.1038/nrd1111.
13. Vandepitte J, Engbaek K, Rohner p, et al. Basic laboratory procedures in clinical bacteriology. 2nd ed. Geneva, World Health Organization. 2003. p. 103-21. http://www.who.int/iris/handle/10665/42696.
14. Clinical and Laboratory Standards Institute. M100-S24 Performance standards for antimicrobial susceptibility testing: Twenty-fourth informational supplement. USA: Clinical and Laboratory Standards Institute (CLSI). 2014.
15. Knezevic P, Petrovic O. A colorimetric microtiter plate method for assessment of phage effect on Pseudomonas aeruginosa biofilm. J Microbiol Methods. 2008; 74(2-3): 114-8. doi: 10.1016/j.mimet.2008.03.005.
16. Jassim SA, Abdualamir AS, Abu baker F. Methods for bacteriophage design. In: International application published under the patent cooperation (PCT)" (W.I.P. organization, ed.), 2010. Vol.WO2010064044 A1.
17. Simmons M, Donovan DM, Siragusa GR, et al. Recombinant expression of two bacteriophage proteins that lyse clostridium perfringens and share identical sequences in the C-terminal cell wall binding domain of the molecules but are dissimilar in their N-terminal active domains. J Agric Food Chem. 2010 Oct 13;58(19):10330-7. doi: 10.1021/jf101387v.
18. ALHuraishi MAM. Molecular detection of multidrug resistance *Acinetobacter baumannii* from different clinical samples. MSc thesis. Al-Nahrain University; 2017.
19. Mashhadani EIJA. Study the activity of Bacteriocin produced from *Lactobacillus plantorum* on virulence factors of *Acinetobacter baumannii*. MSc. thesis Al-Mustasiriyah University; 2010.
20. Al-Marjani MF, Al-Ammar MHM, kadhim EQ. Occurrence of ESBL and MBL genes in Pseudomonas aeruginosa and *Acinetobacter baumannii* isolated from Baghdad, Iraq. Int J Curr Res. 2013; 5(9): 2482-6.
21. Qureshi ZA, Hittle LE, O'Hara JA, et al. Colistin-Resistant *Acinetobacter baumannii*: Beyond Carbapenem Resistance. Clin Infect Dis. 2015; 60(9): 1295-303. doi: 10.1093/cid/civ048.
22. Al-Saleem NH. Genotyping relatedness of *Acinetobacter baumannii* isolated from medical City/Baghdad. PhD thesis. College of Science. University of Baghdad; 2013.
23. Pelletier MR, Casella LG, Jones JW, et al. Unique structural modifications are present in the lipopolysaccharide from colistin-resistant strains of *Acinetobacter baumannii*. Antimicrob Agents Chemother. 2013; 57(10): 4831-40. doi: 10.1128/AAC.00865-13.
24. Arroyo LA, Herrera CM, Fernandez L, et al. The pmrCAB operon mediates polymyxin resistance in Acinetobacter baumannii ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. Antimicrob Agents Chemother. 2011; 55(8): 3743-51. doi: 10.1128/AAC.00256-11.
25. Jain R, Danziger LH. Multidrug-resistant Acinetobacter infections: an emerging challenge to

clinicians. Ann Pharmacother. 2004; 38(9): 1449-59. doi: 10.1345/aph.1D592.
26. Rodriguez-Martinez JM, Nordmann P, Ronco E, et al. Extended-spectrum cephalosporinase in Acinetobacter baumannii. Antimicrob Agents Chemother. 2010; 54(8): 3484-8. doi: 10.1128/AAC.00050-10.
27. Bragg R, van der Westhuizen W, Lee JY, et al. Bacteriophages as potential treatment option for antibiotic resistant bacteria. Adv Exp Med Biol. 2014; 807: 97-110. doi: 10.1007/978-81-322-1777-0_7.
28. Kusradze I, Karumidze N, Rigvava S, et al. Characterization and Testing the Efficiency of *Acinetobacter baumannii* Phage vB-GEC_Ab-M-G7 as an Antibacterial Agent. Front Microbiol. 2016; 7: 1590. doi: 10.3389/fmicb.2016.01590.
29. Regeimbal JM, Jacobs AC, Corey BW, et al. Personalized therapeutic cocktail of wild environmental phages rescues mice from Acinetobacter baumannii wound infections. Antimicrob Agents Chemother. 2016; 60(10): 5806-16. doi: 10.1128/AAC.02877-15.
30. Merabishvili M, Vandenheuvel D, Kropinski AM, et al. Characterization of newly isolated lytic bacteriophages active against *Acinetobacter baumannii*. PLoS One. 2014; 9(8): e104853. doi: 10.1371/journal.pone.0104853.
31. Lobocka M, Hejnowicz MS, Gagała U, et al. The first step to bacteriophage therapy–how to choose the correct phage. In: Borysowski J, Miedzybrodzki R, Górski A. (eds). Phage therapy: current research and applications. Norfolk: Caister Academic Press; 2014.
32. Jassim SA, Abdulamir AS, Abu Bakar F. Phage-based limulus amoebocyte lysate assay for rapid detection of bacteria. 2011. https://patentimages.storage.googleapis.com/4f/a1/be/5c71cee97cac20/WO2011098820A1.pdf.
33. Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. Future Microbiol. 2013; 8(6): 769-83. doi: 10.2217/fmb.13.47.
34. Kelly D, McAuliffe O, Ross RP, et al. Development of a broad-host-range phage cocktail for biocontrol. Bioeng Bugs. 2011; 2(1):31-7. doi: 10.4161/bbug.2.1.13657.
35. Chan BK, Abedon ST. Phage therapy pharmacology: phage cocktails. Adv Appl Microbiol. 2012; 78: 1-23. doi: 10.1016/B978-0-12-394805-2.00001-4.
36. Peleg AY, Seifert H, Paterson DL. Acinetobacter baumannii: emergence of a successful pathogen. Clin Microbiol Rev. 2008; 21(3): 538-82. doi: 10.1128/CMR.00058-07.
37. Lai MJ, Lin NT, Hu A, et al. Antibacterial activity of Acinetobacter baumannii phage φAB2 endolysin (LysAB2) against both gram-positive and gram-negative bacteria. Appl Microbiol Biotechnol. 2011; 90(2): 529-39. doi: 10.1007/s00253-011-3104-y.
38. Briers Y, Volckaert G, Cornelissen A, et al. Muralytic activity and modular structure of the endolysins of Pseudomonas aeruginosa bacteriophages phiKZ and EL. Mol Microbiol. 2007; 65(5): 1334-44. doi: 10.1111/j.1365-2958.2007.05870.x.
39. Miroshnikov KA, Faizullina NM, Sykilinda NN, et al. Properties of the endolytic transglycosylase encoded by gene 144 of Pseudomonas aeruginosa bacteriophage phiKZ. Biochemistry (Mosc). 2006; 71(3): 300-5.
40. Paradis-Bleau C, Cloutier I, Lemieux L, et al. Peptidoglycan lytic activity of the Pseudomonas aeruginosa phage phiKZ gp144 lytic transglycosylase. FEMS Microbiol Lett. 2007; 266(2): 201-9. DOI: 10.1111/j.1574-6968.2006.00523.x.
41. Junn HJ, Youn J, Suh KH, et al. Cloning an expression of Klebsiella phage K11 lysozyme gene. Protein Expr. Purif. 2005; 42(1): 78-84. doi: 10.1016/j.pep.2005.03.026.
42. Mikoulinskaia GV, Odinokova IV, Zimin AA, et al. Identification and characterization of the metal ion-dependent L-alanoyl-Dglutamate peptidase encoded by bacteriophage T5. FEBS J. 2009; 276(24): 7329-42. doi: 10.1111/j.1742-4658.2009.07443.x.
43. Schmelcher M, Shabarova T, Eugster MR, et al. Rapid multiplex detection and differentiation of Listeria cells by use of fluorescent phage endolysin cell wall binding domains. Appl Environ Microbiol. 2010; 76(17): 5745-56. doi: 10.1128/AEM.00801-10.
44. Loessner MJ, Kramer K, Ebel F, et al. C-terminal domains of Listeria monocytogenes bacteriophage murein hydrolases determine specific recognition and high-affinity binding to bacterial cell wall carbohydrates. Mol. Microbiol. 2002; 44(2): 335-49.
45. Lood R, Winer BY, Pelzek AJ, et al. Novel phage lysin capable of killing the multidrug-resistant gram-negative bacterium *Acinetobacter baumannii* in a mouse bacteremia model. Antimicrob Agents Chemother. 2015; 59(4): 1983-91. doi: 10.1128/AAC.04641-14.
46. Saleem F, Ahmad S, Yaqoob Z, et al. Comparative study of two bacteriocins produced by representative indigenous soil bacteria. Pak J Pharm Sci. 2009; 22(3): 252-8.
47. Hibbing ME, Fuqua C, Parsek MR, et al. Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol. 2010; 8(1): 15-25. doi: 10.1038/nrmicro2259.

Correspondence to Hayder N. Jasim
E-mail: haider.nasef@gmail.com
Received Oct. 25th 2017
Accepted Jan. 8th 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

The Comparison between the Effect of Two Hours Atropinization Versus Three Days Atropinization on the Cycloplegic Outcome in Children

Abeer A. Mohamad[1] *FICMS (Ophth.)*, **Hind A. Mahdi**[1] *FICMS (Ophth.)*, **Majid S. Younis**[2] *FRCOphth*

[1]Dept. of Surgery, College of Medicine, Al-Nahrain University, Baghdad-Iraq, [2]Dept. of Surgery, College of Medicine, University of Baghdad, Iraq

Abstract

Background Cycloplegia abolishes the accommodative power by causing paralysis of ciliary muscle by anticholinergic drugs, which will inhibit stimulation of both ciliary muscle and sphincter pupillae causing cycloplegia and mydriasis. Atropine is widely used in cycloplegic refraction despite its potential toxicity.

Objective To evaluate the possible role of two hours atropinization versus three days atropinization on the cycloplegic outcome in children.

Methods This is a clinical interventional study that included fifty children aged two to seven years' old who attended Ibn Alhaitham Teaching Hospital from October 2012 to March 2013; manual refraction was done for each child after 120 minutes of two drops atropine 1% five minutes apart and refraction was repeated after three days of twice daily atropine 1% administration by the parents. T-test was used for means comparison.

Results Fifty patients (26 males, mean age 3.89 ± 1.3) were included in the study. Spherical equivalent results obtained after three days atropinization (M = 4.2, SD = 1.85) were significantly higher than those obtained after two hours atropinization (M = 3.84, SD = 1.64) ($t(49) = -6.60$, $p < 0.05$).

Conclusion Two hour atropinization was inferior to the standard three days atropinization as it has less cycloplegic effect and so it cannot be recommended based on the current evidence.

Keywords Atropine, cycloplegia, atropinization, refraction

Citation Mohamad AA, Mahdi HA, Younis MS. The comparison between the effect of two hours atropinization versus three days atropinization on the cycloplegic outcome in children. Iraqi JMS. 2018; 16(4): 372-377. doi: 10.22578/IJMS.16.4.3

List of abbreviations: None

Introduction

Cycloplegia abolishes the accommodative power by causing paralysis of ciliary muscle by anticholinergic drugs, which will inhibit stimulation of both ciliary muscle and sphincter pupillae causing cycloplegia and mydriasis [(1)].

Cycloplegic examination will not only help us to determine the static refractive error, but also dilate the pupil allowing for full ophthalmological examination like detecting any opacity of the ocular media and for posterior segment examination [(1,2)].

Cycloplegic refraction helps determine full hyperopia in patients with accommodative esotropia and prevents overcorrection in myopic patients. It is also useful in prescribing correction in patients with limited cooperation

during subjective refraction and amblyopic patients who have chaotic accommodation [3].
Atropine can be used as drops or ointment. Maximal cycloplegia occurs at three hours and the recovery of accommodation will start after 3 days of discontinuing the drug and usually completed by 10 – 14 days [4].
The conventional recommendation of 3 drops of 1.0% atropine for either eye per day corresponds to 2-3 milligrams atropine per day (and this over three days) carries a risk of overdosing. Through the lacrimal ducts, a major proportion of the atropine passes into the nasopharyngeal cavity (where atropine is absorbed and can then act systemically). Consequently, it is not surprising that intoxications with atropine eye-drops have been repeatedly reported for decades. Also, atropine is contraindicated in patients with Down syndrome and albinism. The dosage of atropine borders on the toxic range. Additionally, parental compliance with the dosage schedule cannot be guaranteed [5-7].
Despite the fact that nowadays cyclopentolate is preferable for routine cycloplegia and has comparable results to atropine with less incidence of toxicity [2,8,9], many authors still consider atropine as the drug of choice for complete cycloplegia due to its strong cycloplegic action which may uncover additional 0.3–0.5 D of hyperopia in light, as well as deeply pigmented children with hyperopia and white children with esotropia [2,4].
The specification one drop of 0.5 or 1.0 percent atropine eye drops twice a day for three days was recommended by Duke-Elder 50 years ago [10]. There have not been many changes in the dosage recommendations for decades.
Parents who have to administer atropine at home for three days not uncommonly have difficulties in applying eye drops, and the ophthalmologist cannot always be certain whether correct application was carried out and whether full cycloplegia was really attained. Auffarth and Hunold had proposed a newer scheme for refractive measurements under atropine cycloplegia two hours after application of two drops of atropine (0.5% atropine children <2 years; 1.0% atropine children >2 years). This abbreviated scheme has the advantage of easier application by the untrained personnel, better compliance by the patients and their families, and the lesser chances of toxicity or overdose from prolonged atropine administration [11]. To our knowledge, no studies were conducted to evaluate the effectiveness of this abbreviated regimen apart of that of Auffarth and Hunold.
This study was carried to establish whether application of two eye drops of atropine and subsequent determination of refraction after two hours provided the same results as those obtained after conventional administration of atropine for three days.

Methods

This is a clinical interventional study conducted in the strabismus Unit at Ibn Alhaitham Teaching Hospital from October 2012 to March 2013. The sample was chosen by convenient randomization of the patients attending the Unit during the period of the study. All patients with strabismus and refractive error, whose age was from two to seven years, were invited to participate in this study with the exclusion of those with history of cardiac disease. A total of 50 patients, who agreed to participate, were enrolled in this study with consent from their parents.
Each patient received one drop of atropine 1% followed by another one after five minutes. Manual refraction was done after two hours and recorded. The patients and their families were instructed to apply further atropine drops that evening and to continue with twice daily atropine drops for the next two days. Refraction was recorded manually in the morning of the fourth day by the Unit refractionist.
The data were collected using a data collection form that was formulated for the purpose of the study. The data included age, sex, and presence of strabismus and/or refractive error, the refractive error recording after two drops

of atropine and the refractive error after three day atropinization.

Statistical analysis was done using IBM® SPSS® (Statistical Package for Social Sciences) Statistics version 21 on a Windows 7 Home Premium PC. Descriptive statistics were presented as (mean ± standard deviation) for the continuous variable (age, and the refractive error after atropinization) while the categorical variables (gender, presence of strabismus and/or refractive error) were presented as frequencies (numbers) and proportions (percentages). Level of significance was determined using t-test and a p value of ≤ 0.05 considered as significant difference.

Results

A total of 50 patients were enrolled in this study, 24 (48%) were males and 26 (52%) were females with almost equal male to female ratio as shown in table 1 and figure 1. The mean age of the study group was 3.86 ± 1.3 years and ranged from 2 -7 years, these findings and the age distribution are illustrated in table 1.

Table 1. Baseline characteristics of the study group

Variable		Value
Gender	Male n (%)	24 (48%)
	Female n (%)	26 (52%)
	Total	50 (100%)
Age (Years)	Mean ± Std deviation	3.89 ± 1.3
	Range	2 - 7

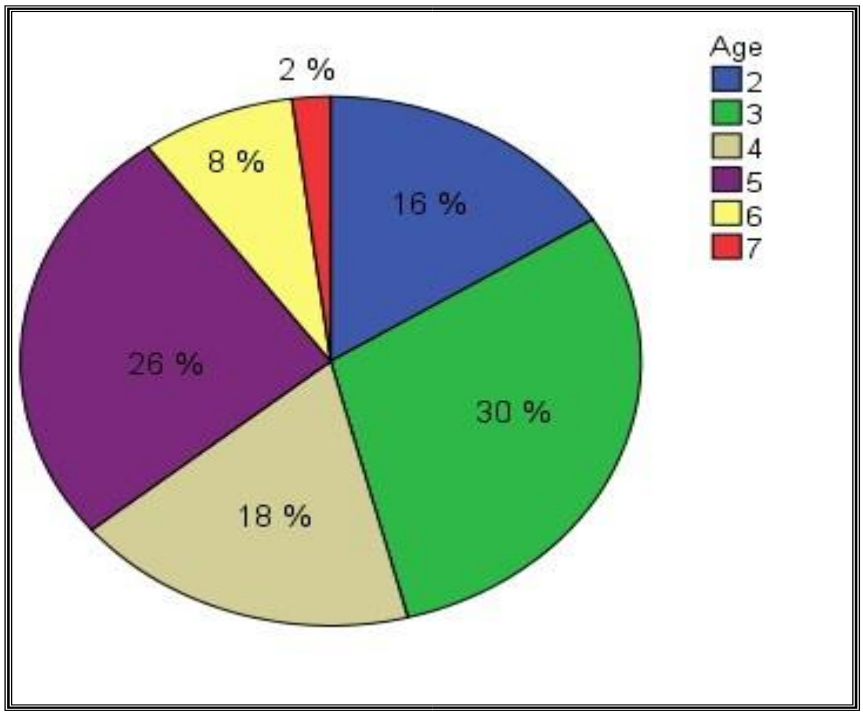


Figure 1. Age Distribution among sample

Among the study sample, 10 (20%) of the patients have strabismus and 13 (26%) have refractive error, while 27 (54%) have both strabismus and refraction as shown in table 2.

Table 2. Frequency of patients with strabismus and refractive error

Strabismus n (%)	Refractive Error n (%)	Both n (%)
10 (20%)	13 (26%)	27 (54%)

Table 3 illustrates the mean refraction obtained after two hours atropinization and that obtained after three days with 95% confidence interval.

Table 3. Mean, standard deviation and 95% confidence interval of two hours atropinization versus three day atropinization

	Mean (Diopter Sphere)	Standard deviation	95% Confidence Interval	
			Lower	Upper
Two Hours Atropinization	3.84	1.64	3.32	4.21
Three Days Atropinization	4.20	1.85	3.97	5.02

In figure 2, the frequency of the difference in the dioptric power between two hours and three days atropinization is shown. These results have demonstrated that in only 22% of patients had similar refraction with two hour atropinization as that of the three day atropinization. Nevertheless, it is quite obvious that 56% of the differences lies within 0.5 diopter and 82% within 1 diopter.

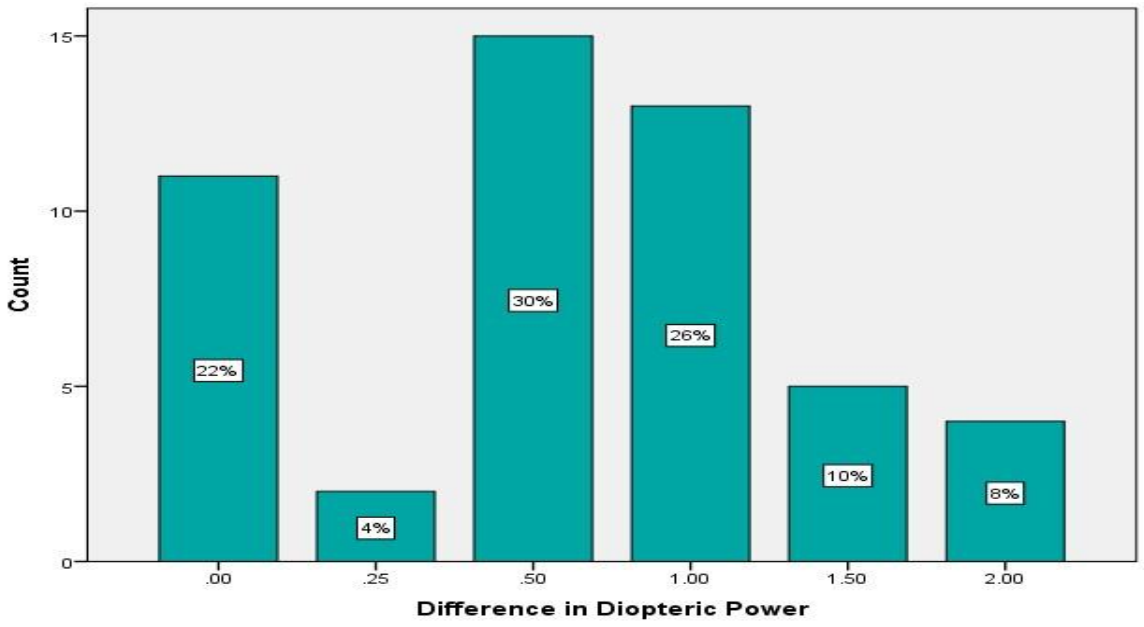


Figure 3. Difference in dioptric power between two hours and three day refraction

Spherical equivalent measured after two hours atropinization and after three days atropinization was analyzed using a dependent samples t-test. This revealed that spherical equivalent results obtained after three days atropinization (M = 4.2, SD = 1.85) were significantly higher than those obtained after two hours atropinization (M = 3.84, SD = 1.64) ($t(49) = -6.60$, $p < 0.05$).

Discussion

The results from this study showed statistically significant ($P \leq 0.05$) difference between two hour atropinization and three day atropinization. These results differ from those of Auffarth and Hunold, who had concluded that two hour atropinization has comparable results to three day atropinization without relying on statistical evidence. They assumed that with the presence of 0.5 diopter difference is enough to recommend the abbreviated scheme as this difference showed in about 80% of patient and they considered that a good correlation between the two methods [(11)].

In this study, the difference of 0.5 diopter was observed in only 56% of cases. This difference can be explained by the difference in obtaining refraction being manual in this study and automated refractometer in Auffarth and Hunold's study.

This study has its limitations. First, the small sample size may not demonstrate the true relation between the two regimens. Secondly, the use of manual refraction may have an impact on the results.

This study has concluded that two hour atropinization does not produce similar refraction as that of the standard three day atropinization.

This study recommends when atropine is used as a cycloplegic agent, the standard three day atropinization scheme will produce a more accurate refraction and is therefore it is recommended to continue using this scheme for cycloplgia with atropine till further studies confirm the non-inferiority of the abbreviated regimen.

Acknowledgments

Appreciate the help from refractionists in the squint unit at Ibn Alhaitham Eye Teaching Hospital.

Author Contribution

All authors have contributed equally to this article.

Conflict of interest

Authors have nothing to disclose.

Funding

Self-funding.

References

1. Apt L, Gaffney WL. Cycloplegic Refraction. In: William T, Edward J, (eds). Duane's Ophthalmology. Philadelphia: Lippincott Williams and Wilkins; 2008. p. 400-1.
2. McKeown CA, Davidson SL. The pediatric eye examination. In: Albert DM, Miller JW, Azar DT, et al (eds). Albert's and Jakobiec's Principles and practice of ophthalmology. 3rd ed. Philadelphia: Elsevier; 2008. p. 4141.
3. American Academy of Ophthalmology. Basic and clinical science course: pediatric ophthalmology and strabismus. San Francisco: American Academy of Ophthalmology; 2014-2015. p. 12.
4. Bowling B. Kanski's Clinical ophthalmology: a systematic approach. 8th ed. Edinburgh: Elsevier Saunders; 2016. p. 755.
5. Hopkins G, Pearson R. Ophthalmic drugs: diagnostic and therapeutic uses. 5th ed. Philadelphia: Butterworth Heinmann Elsevier.2007. p. 85-105.
6. Dollery C. Therapeutic Drugs. 2nd ed. New York: Churchill Livingstone.1999. p. 40-4.
7. O'Connor PS, Mumma JV. Atropine toxicity. Am J Ophthalmol. 1985 May; 99(5): 613-4.
8. Farhood QK. Cycloplegic refraction in children with cyclopentolate versus atropine. J Clin Exp Ophthalmol. 2012; 3 (7): 1-5. doi: 10.4172/2155-9570.1000239.
9. Zetterström C. A cross-over study of the cycloplegic effects of a single topical application of cyclopentolate-phenylephrine and routine atropinization for 3.5 days. Acta Ophthalmol (Copenh). 1985; 63(5): 525-9.
10. Duke-Elder S. The practice of refraction. Philadelphia: Blakiston Publishing Company. 1943.
11. Auffarth G, Hunold, W. Cycloplegic refraction in children: Single-dose-atropinization versus three-day-atropinization. Doc Ophthalmol. 1992; 80(4): 353-362.

Correspondence to Dr. Abeer A. Mohamad
E-mail: Aabl_81@yahoo.com
aberamir@colmed-alnahrain.edu.iq

Received 28th Dec. 2017
Accepted 6th May 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Effects of Ciprofloxacin on Male Fertility Parameters and Sperm DNA Integrity in Rats

AbdulKareem H. Abd[1] *PhD*, **Saad S. Al-Dujaily**[2] *PhD*, **Dalia A. Al-Saray**[3] *PhD*

[1]Dept. of Pharmacology, College of Medicine, [2]Biotechnology Research Center, Al-Nahrain University, Baghdad-Iraq,
[3]Dept. of Pharmacology, College of Dentistry, Babylon University, Babil, Iraq

Abstract

Background The administration of antibiotics is of great value in the therapy of infections of male genital tract, which may have an effect on fertility. Antibiotics are generally prescribed for a variety of infections. A number of patients requiring assisted conception occasionally show evidence of reproductive tract infections. While fluoroquinolones being excessively prescribed in the treatment of male genital tract infections, too little information concerning their outcome on fertility are present.

Objective To estimate ciprofloxacin effects on sperm function parameters in addition to inspect whether ciprofloxacin can affect the integrity of sperm DNA.

Methods In the present study, 48 male adult rats were enrolled. The animals were randomly allocated into six groups; four ciprofloxacin treated groups, which were treated with either (40 mg/kg/day) or (80 mg/kg/day) of ciprofloxacin and 2 control groups. For each dose, the treatment maintained for either 14 days or 28 days. At the end of each duration of treatment, certain epididymal sperm function parameters: sperm morphological normality, sperm concentration and sperm motility were analyzed together with sperm DNA integrity analysis.

Results A significant reduction in sperm concentration, motility and percentage of morphologically normal sperm (in a dose dependent manner) was observed when ciprofloxacin administered for 28 days. The level of DNA fragmentation was significantly elevated with a significant reduction in sperm chromatin quality in ciprofloxacin treated groups whereas serum testosterone level was not significantly affected.

Conclusion Ciprofloxacin can adversely influence fertility parameters in male rat.

Keywords Fertility, Ciprofloxacin, Sperm DNA

Citation Abd AH, Al-Dujaily SS, Al-Saray DA. Effects of ciprofloxacin on male fertility parameters and sperm DNA integrity in rats. Iraqi JMS. 2018; 16(4): 378-384. doi: 10.22578/IJMS.16.4.4

List of abbreviations: None

Introduction

Antibiotics are frequently prescribed in the treatment of different types of infections. Whereas some patients need assisted fertilization, in several cases, these patients display evidence of infection in male reproductive tract [(1)]. Consequently, the use of antibacterial agents is essential in the treatment of genital tract infections, which can probably influence fertility in male.

The antibiotic fluoroquinolones are frequently prescribed by fertility specialists in the therapy of numerous types of bacterial infections when high level of leukocytes is observed in the semen or before in vitro fertilization program, without taking consideration to bacterial evidence of infection [(2)].

Infertility can be defined as failure to attain pregnancy after 12 months of a usual

unprotected sexual intercourse [3]. Male infertility problem represents more than 45% of infertility problems. There are multiple factors, which can affect fertility in male; anatomical causes like varicocele or ductal obstructions represent some infertility factors in male [4]. Additionally, male infertility can be produced from abnormality in sperm; as it was predicted that defects in sperm production represent 36-75% of male infertility causes. Other factors that associated with infertility of male are reactive oxygen species, sperm antibodies, infection, cigarette smoking, radiation, heavy metals, hormonal causes, several therapeutic drugs and others [5].

Ciprofloxacin, which represent a second-generation fluoroquinolone, a broad-spectrum antibiotic used in the treatment of several gram-positive and gram-negative bacterial infections which affect the bones, joints, urinary and respiratory tracts. It generally acts by inhibition of type II topoisomerase, DNA gyrase, which is required for unwinding of replicated prokaryotic DNA. It is routinely prescribed by fertility specialists and urologists in the therapy of reproductive infections. Its side effects take place mostly in the central nervous system and gastrointestinal tract. Allergic and cardiovascular complications are other adverse effects observed during administration of ciprofloxacin [6].

In vitro and in vivo genotoxicity researches had recommended that this antibiotic is harmless for therapeutic use [7]. On the other hand, other studies have confirmed that ciprofloxacin could impair testicular structure and function [8]. Therefore, this study was intended to detect the effect of ciprofloxacin on sperm function parameters and integrity of sperm DNA.

Methods

This study involved the use of 48 male adult Albino-rats. Their weight was about (225±25) gm and the age of rats was ranged between 7-8 weeks old. The rats were housed in controlled temperature around 24 °C and 13±1 hour light-dark cycles. Rats were fed an ordinary commercial pellet. The experimental groups were equally allocated into six groups (4 ciprofloxacin treated groups and 2 control groups), ciprofloxacin was injected intraperitonially in a doses of (40 mg/kg/day) and (80 mg/kg/day) and each dose was administered in two periods, short duration of 14 days and long one (28 days) (the use of two doses was intended to detect the effect of increasing the dose on fertility parameters), in the control groups rats were injected intraperitonially with normal saline (in the same volume as ciprofloxacin): in the first control group rats were injected for 14 days and in the second control group they were injected for 28 days. At the end of each duration of treatment, rats from each group were anesthetized by diethyl ether, blood and epididymis were collected from each rat for the measurement of: sperm morphological normality, sperm concentration and sperm motility together with sperm DNA integrity analysis. The work on the animal was approved by the Institute Review Board in the College of Medicine of Al-Nahrain University.

Preparation of epididymal sperm

The caudal epididymis part of each rat was dissected and placed in (1 ml) of a previously warmed Hams F12 medium. Tearing of the tissue was made in order that the spermatozoa would swim out into the culture medium.

Microscopic examination

The microscopic observation was performed for each sample. one drop of sperm sample was put on a warm slide then covered by standard cover slip for scoring under light microscope of (40 X) objective.

Sperm function parameters analysis

Certain sperm function parameters were examined specifically; sperm motility, concentration and morphology. Motility was expressed as the fraction of progressive motility including speedy spermatozoa, grade A; slow spermatozoa, grade B; non-progressive sperm, grade C; and non-motile sperm, grade D [9].

Sperm DNA integrity (Acridine Orange Test)
Acridine orange represent a metachromatic fluorescence probe for evaluation of the degree of sperm nuclear DNA affinity for in-situ acid-induced denaturation by differentiation between native double-stranded DNA, which give green fluorescence and the red fluorescent which is produced by denatured single-stranded DNA [10]. Smears were fixed in glacial acetic acid-methanol (1:3), followed by staining with acridine orange {0.19 mg/ml, pH 2.5}.

Aniline Blue staining
Aniline blue distinctively stains histones rich in lysine and consequently detecting anomalies in sperm chromatin condensation [11]. In order to attain this, 3% buffered glutaraldehyde was used for fixation of sperm samples smears. Then stained with 5% aqueous aniline blue. Under light microscope, examination of spermatozoa was performed using a magnification of ×100 eyepiece [12].

Testosterone measurement
After centrifugation of whole blood, the serum was obtained and the concentrations of testosterone was measured by radioimmunoassay by means of a readymade kit (ichromaboditech).

Statistical analysis
The analysis of the study data was performed by SPSS software version 16. All results are expressed as mean±SE. The difference between the quantitative data was analyzed with one-way ANOVA, and followed by the Tukey test. P-value less than 0.05 were considered significant for all data in this study.

Results

Effects of ciprofloxacin on sperm concentration:
Administration of ciprofloxacin for 14 days (in a dose of 40 and 80 mg/kg/day) was not significantly affect sperm concentration, while significant decline in sperm concentration was observed when the drug was administered in a dose of (80 mg/kg/day) for 28 days as compared with the control group (Table 1).

Effects of ciprofloxacin on sperm motility
No significant alterations were seen in progressive motility or in the total motility of sperm (as compared with the control group) when the drug was administered (in 40 and 80 mg/kg/day) for 2 weeks, while a significant lessening in the total and in the progressive motility was observed when ciprofloxacin was injected for 28 days and the decline in sperm progressive motility was dose related (Table 1).

Effects of ciprofloxacin administration on sperm morphology
A significant reduction in sperm morphological normality was resulted when ciprofloxacin injected for 28 days and in high dose (80 mg/kg/day) as compared with the control group (Table 1).

Effects of ciprofloxacin on sperm DNA integrity and chromatin quality
Treatment with ciprofloxacin resulted in a significant elevation in the level of staining propensity of sperm DNA with acridine orange and this elevation was increase as the dose and /or the duration of the drug was increased, these differences as compared to the control groups (Table 2) and (Figure 1).
The fraction of sperm, which is positively stained with aniline blue was significantly elevated and in positive correlation with the dose and the duration of treatment with ciprofloxacin as compared with the control group (Table 2).

Effect of ciprofloxacin on serum testosterone level
In the present study, treatment with ciprofloxacin in two different doses and in different durations show no significant changes in the level of serum testosterone as compared with the control groups (Table 3).

Table 1. Effects of intraperitoneal injection of ciprofloxacin on sperm motility, concentration, and morphological normality in adult male rats

Treatment	Duration (days)	Progressive motility%	Total motility%	Immotile sperm%	Sperm concentration $^{\times}10^{6}$ (sperm/ml)	Morphologically normal sperm %
Control	14	49.0±1.41 (a)	80.71±0.92 (a)	19.29±0.92 (a)	40.14±1.32 (a)	91.71±0.42 (a)
	28	52.0±1.27 (a)	83.0±1.27 (a)	17.00±1.27 (a)	37.71±1.61 (a)	92.57±1.0 (a)
Ciprofloxacin (40 mg/kg/day)	14	47.86±1.92 (a)	79.43±1.84 (a)	20.57±1.84 (a)	38.0±0.8 $^{\times}10^{6}$ (a)	91.71±1.09 (a)
	28	36.57±.72 (b)	71.71±1.02 (b)	28.29±1.02 (b)	36± 0.48 $^{\times}10^{6}$ (ab)	90.71 ± 1.04 (ab)
Ciprofloxacin (80 mg/kg/day)	14	44.0±1.0 (a)	77.71±0.68 (a)	22.29±.68 (a)	37.2±0.84 $^{\times}10^{6}$ (a)	91.0±1.11 (a)
	28	29.57±0.53 (c)	68.29±1.30 (b)	31.71±1.30 (b)	32.7±0.42 $^{\times}10^{6}$ (b)	88.14± 0.63 (b)

Values are expressed as mean±standard error (n=8)
Values on the same column having the same letter (for example letter a) are not significantly different.

Table 2. Effects of intraperitoneal administration of ciprofloxacin on sperm DNA integrity and chromatin quality in adult male rats

Treatment	Duration (days)	Positive acridine orange staining %	Positive aniline blue staining %
Control	14	7.57±0.37 (a)	9.71±0.57 (a)
	28	6.29±0.42 (a)	10.14±0.94 (a)
Ciprofloxacin (40 mg /kg/day)	14	26.0±0.44 (b)	14.43±0.95 (b)
	28	42.57±0.37 (d)	20.14±0.51 (cd)
Ciprofloxacin (80mg/kg/day)	14	28.71±0.68 (c)	18.0±0.62 (c)
	28	47.0±0.53 (e)	22.14±0.8 (d)

Values are expressed as mean ± standard error (n=7)
Values on the same column having the same letter (for example letter a) are not significantly different.

Table 3. Effects of intraperitoneal administration of ciprofloxacin on serum testosterone level in adult male rats

	Serum Testosterone level (ng/ml)	
Treatment	14 days	28 days
Control	1.79±0.29 (a)	1.93±0.27 (a)
ciprofloxacin (40 mg/kg/day)	2.48±0.28 (a)	1.94±0.19 (a)
Ciprofloxacin (80 mg/kg/day)	2.25±0.25 (a)	2.06±0.28 (a)

Values are expressed as mean ± standard error (n=7)
Values on the same column having the same letter (for example letter a) are not significantly different.

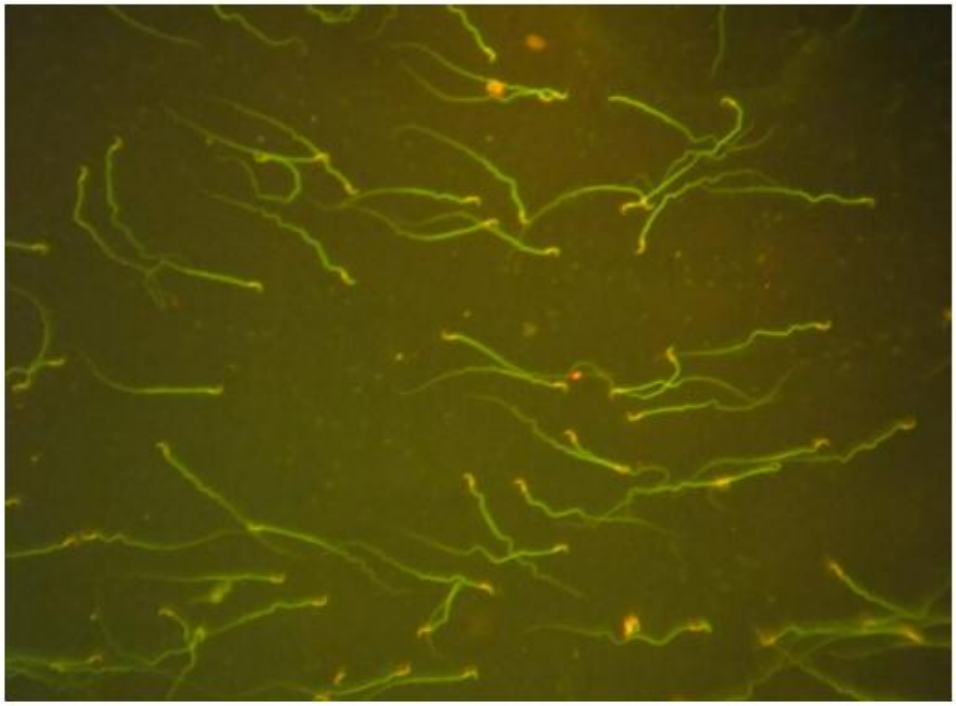


Figure 1. Fluorescence microscopic images of acridine orange stained cells, which present that spermatozoa generating green fluorescence are considered to contain normal DNA content, whereas sperms that display spectrum of yellow or orange to red fluorescence are considered to contain fragmented DNA

Discussion

In the present study, administration of ciprofloxacin resulted in significant decline in sperm concentration in a dose and time related manner, similar findings were observed by Demir et al. in 2007 as they had noticed a marked drop in sperm count after ten days of ciprofloxacin treatment [13]. Kaki et al. (2008), who recorded that treatment with ciprofloxacin for 60 days resulted in a significant reduce in the number of spermatogenic cells in seminiferous tubules [14]. There are a number of postulated mechanisms by which sperm concentration could negatively affected as direct toxicity of sperm or by suppression of cell growth or cellular production [15]. Eukaryotic cells apoptosis represents other mechanism and can be resulted from interference with the mitochondrial pathway [16], reduction in testosterone level [17], as well as, decline in chromatin quality or integrity of DNA can also have detrimental effect on sperm concentration [18]. In the current study, testosterone level was not considerably affected by ciprofloxacin, hence it cannot consider as a reason by which sperm concentration was unfavorably affected, however, when chromatin quality and sperm DNA integrity were tested, current results indicate a marked increase in the intensity of DNA fragmentation with reduction in sperm chromatin structure quality. As a result, it can be predicted that these detrimental effects on sperm genetic material can be considered as the mechanisms by which other sperm function parameters such as sperm concentration being negatively affected in the present study.

The motility of sperm depends mainly on Ca^{2+} influx and on mitochondrial oxidation process to obtain the energy necessary for hyperactivity and flagellum movement [19]. Thus, the adverse effect of ciprofloxacin on sperm motility propose that the drug may interfere with the function or the structure of Ca^{2+} channels by direct or indirect toxicity. The

other explanation is that; the drug may affect Cat Sper channels. As these channels are responsible for increasing Ca ions exhaustion which in turn stimulate sperm motility [20].

Evidences from previous reports in sperm morphology indicated that, alteration in the morphology of sperm might be resulted from modification in the compaction of chromatin [21]. These supposed mechanisms for abnormalities in sperm morphology are well-matched with the present findings as in the present study, it is founded that ciprofloxacin could adversely affect sperm chromatin structure.

The present findings showed that following ciprofloxacin administration, the level of sperms with a single stranded DNA (as indicated by acridine orange stain) and that of immature sperms (as indicated by aniline blue) were significantly elevated. Generally, the occurrence of elevated level of DNA nicks reflects cell necessity to unwind the torsional strain that produced from negative supercoiling which, on the other hand, associated with the protamines displacement as an alternative of nucleosomal histones and the modification in tertiary structure of the elongating spermatids. The presence of these nicks is not risky as they are continually ligated by topoisomerase II enzyme before spermiogenesis completion. Yet, these nicks cannot be correctly repaired if irregularity in topo II ligating activity are present or if its activity is blocked by the inhibitors of topo II enzyme [22]. Since ciprofloxacin is known as topoisomerase inhibitor [23], so it can be concluded that this drug could block the formation and ligation of DNA nicks which sequentially disturbs protamination and as a consequence stimulate internal damage in DNA by increasing its propensity to damage and prevention of its repair. This theory has been confirmed with elevation of DNA damage as indicated by the acridine orange and aniline blue staining in sperms after ciprofloxacin administration in the current study.

The current study concluded that ciprofloxacin could adversely affect the process of spermatogenesis in rats in time- and dose-dependent manner.

Acknowledgments

The authors wish to thank the staff of Biotechnology Research Center, Al-Nahrain University for their valuable and scientific support.

Author Contribution

Al-Saray: Acquisition of data with participation. All authors participated in the conception and design of the study, analysis and interpretation of data.

Conflict of interest

The authors declare no conflict of interest.

Funding

Self-funding.

References

1. Pajovic B, Radojevic N, Vukovic M, et al. Semen analysis before and after antibiotic treatment of asymptomatic chlamydia-and ureaplasma-related pyospermia. Andrologia. 2013; 45(4): 266-71. doi: 10.1111/and.12004.
2. Calogero AE, Condorelli RA, Russo GI, el al. Conservative nonhormonal options for the treatment of male infertility: antibiotics, anti-inflammatory drugs, and antioxidants. Biomed Res Int. 2017; 2017: 4650182. doi: 10.1155/2017/4650182.
3. Gurunath S, Pandian Z, Anderson RA, et al. Defining infertility- a systematic review of prevalence studies. Hum Reprod Update. 2011; 17(5): 575-88. doi: 10.1093/humupd/dmr015.
4. Olayemi, FO. A review on some causes of male infertility. Afr J Biotechnol. 2010; 9(20): 2834-42.
5. Punab M, Poolamets O, Paju P, et al. Causes of male infertility: A 9-year prospective monocentre study on 1737 patients with reduced total sperm counts. Hum Reprod. 2017; 32(1): 18-31. doi: 10.1093/humrep/dew284.
6. Sharma PC, Jain A, Jain S, et al. Ciprofloxacin: review on developments in synthetic, analytical, and medicinal aspects. J Enzyme Inhib Med Chem. 2010; 25(4): 577-89. doi: 10.3109/14756360903373350.
7. Herbold BA, Brendler-Schwaab SY, Ahr HJ. Ciprofloxacin: in vivo genotoxicity studies. Mutat Res. 2001; 498(1-2): 193-205.
8. Khaki A. Assessment on the adverse effects of Aminoglycosides and Flouroquinolone on sperm parameters and male reproductive tissue: A systematic review. Iran J Reprod Med. 2015; 13(3): 125-34.
9. Vasan SS. Semen analysis and sperm function tests: How much to test? Indian J Urol. 2011; 27(1): 41-8. doi: 10.4103/0970-1591.78424.

10. Agarwal A. Said TM. Sperm chromatin assessment. In: Gardner DK, Weissman A, Howles CM, et al (eds). Textbook of assisted reproductive techniques Laboratory perspectives. 4th ed. Vol. 1. Informa; 2012. p. 75.

11. Zidi-Jrah I, Hajlaoui A, Mougou-Zerelli S, et al. Relationship between sperm aneuploidy, sperm DNA integrity, chromatin packaging, traditional semen parameters, and recurrent pregnancy loss. Fertil Steril. 2016; 105(1): 58-64. doi: 10.1016/j.fertnstert.2015.09.041.

12. Talebi AR, Vahidi S, Aflatoonian A, et al. Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. Andrologia. 2012; 44 Suppl 1: 462-70. doi: 10.1111/j.1439-0272.2011.01206.x.

13. Demir A, Türker P, Önol FF, Sirvanci S, Findik A, and Tarcan T. Effect of experimentally induced Escherichia coli epididymo-orchitis and ciprofloxacin treatment on rat spermatogenesis. Int J Urol. 2007; 14(3): 268-72. doi: 10.1111/j.1442-2042.2007.01682.x

14. Khaki A, Heidari M, Novin MG, et al. Adverse effects of ciprofloxacin on testis apoptosis and sperm parameters in rats. Iran J Reprod Med. 2008; 6(2): 71-6.

15. Zobeiri F, Salam S, Sadrkhanlou R, et al. Role of mitochondria in ciprofloxacin-induced apoptosis in murine sperm cells. Reprod Sci. 2013; 20(9): 1090-5. doi: 10.1177/1933719113477482.

16. Abd-Allah AR, Aly HA, Moustafa AM, et al. Adverse testicular effects of some quinolone members in rats. Pharmacol Res. 2000; 41(2): 211-9. doi: 10.1006/phrs.1999.0580.

17. Elias A, Nelson B. Sub chronic evaluation of ciprofloxacin and perfloxacin on sperm parameters of male guinea pigs. Asian J Exp Biol Sci. 2012; 3: 595-601.

18. Zobeiri F, Sadrkhanlou RA, Salami S, et al. The effect of ciprofloxacin on sperm DNA damage, fertility potential and early embryonic development in NMRI mice. Vet Res Forum. 2012; 3(2): 131-5.

19. Correia J, Michelangeli F, Publicover S. Regulation and roles of Ca2+ stores in human sperm. Reproduction. 2015; 150(2): R65-76. doi: 10.1530/REP-15-0102.

20. Rahman MS, Kwon WS, and Pang MG. Calcium influx and male fertility in the context of the sperm proteome: an update. Biomed Res Int. 2014; 2014: 841615. doi: 10.1155/2014/841615.

21. Zini A, Phillips S, Courchesne A, et al. Sperm head morphology is related to high deoxyribonucleic acid stainability assessed by sperm chromatin structure assay. Fertil Steril. 2009; 91(6): 2495-500. doi: 10.1016/j.fertnstert.2008.03.032.

22. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl. 2002; 23(1): 25-43.

23. Mitscher LA. Bacterial topoisomerase inhibitors: quinolone and pyridone antibacterial agents. Chemical reviews. 2005; 105(2): 559-92.

24. Abdul Kahaleq MA, Abu-Raghif AR, Kadhim SR. Antibacterial activity of Fenugreek essential oil against Pseudomonas aeroginosa: In vitro and in vivo Studies. Iraqi J Med Sci. 2015; 13(3): 227-34.

Correspondence to Dalia A. Al-Saray
E-mail: dalyaabdulzahraa@gmail.com
Received Jan. 25th 2018
Accepted Mar. 18th 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

The Efficacy and Safety of Percutaneous Nephrolithotomy in Correlation with Different Renal Stone Burdens

Issam S. Al-Azzawi[1] *FICMS, FEBU*, **Ali W. Mohammed Zaki**[1] *FICMS*, **Hader I. Jwaid**[2] *FICMS*, **Samir L. Salih**[2] *FICMS*, **Ali A. Al-Obaidi**[2] *FICMS*

[1]Dep.t of Urology, College of Medicine, Al-Mustansiriyah University, Baghdad, Iraq, [2]Dep.t of Urology, Al-Yarmouk Teaching Hospital, Baghdad, Iraq

Abstract

Background The improvements in instruments and technique of percutaneous nephrolithotomy (PCNL) in the last 3 decades have led to expansion in its indications to include even the large and complex renal stone cases.

Objective To determine the efficacy and safety of PCNL in relation to stone complexity.

Methods In this prospective, clinical, interventional study, 51 patients with symptomatic renal stones of different sizes and locations, were admitted in our Urology Unit, fully evaluated and treated by our team with PCNL. For all the patient's data collected were: age, gender, body mass index (BMI), previous renal surgery, pre-operative stone number, stone size, stone position, stone opacity, surgical approach, operative time, stone clearance rate, postoperative residual stones and complications, hospital stay and adjuvant therapy. By using different statistical methods, correlations were made to elicit the impact of stone size, stone position, stone opacity, BMI and previous renal surgery, operative time, stone free rate, complication rate and the need for auxiliary procedures.

Results In this study, the 51 included patients were 31 males and 20 females with a mean age 40.3 year. All the stones treated were over 2 cm in their longest diameter, with 58.8% of them were ≥ 3 cm, and mean stone size was 4.2±0.99 cm. The most prevalent site of stone was the lower calyx (39.2%), followed by (37.3%) as partial staghorn, while complete staghorn calculi constituted (11.8%). The final success rate of treatment was 86.3%. (60.8%) of the cases found to be stone free at immediate postoperative period. Auxiliary procedures were used in (27.5%) patients, to improve the stone clearance. Residual stones were ultimately remained in only (7.8%). Complications occurred in 11 patients (21.6 %).

Conclusion PCNL though demanding, is an effective and reasonably safe procedure for the treatment of different renal stone burdens. Size and position of stones in the calyceal system and past renal surgery were the main determinants of operative time, stone clearance, complication rate and the need for auxiliary procedures.

Keywords Renal stone, PCNL, stone complexity, stone free rate, complications

Citation Al-Azzawi IS, Mohammed Zaki AW, Jwaid HI, Salih SL, Al-Obaidi AA. The efficacy and safety of percutaneous nephrolithotomy in correlation with different renal stone burdens. Iraqi JMS. 2018; 16(4): 385-392. doi: 10.22578/IJMS.16.4.5

List of abbreviations: BMI = Body mass index, CT = Computerized tomography, ESWL = Extracorporeal shock wave lithotripsy, IVU = Intravenous urography, PCNL = Percutaneous nephrolithotomy, UPJ = Uretero-pelvic junction, URS = Ureteroscopy

Introduction

There are many factors that should be considered in dealing with renal stones including; stone size, position, chemical composition, hardness of stone, presence of a distal obstruction, renal function indices, availability of equipment and experience of the surgeon. All these factors will dictate the type of therapy suitable for each patient. Current treatment options for renal stones are extra corporeal shock wave lithotripsy (ESWL), percutaneous nephrolithotomy (PCNL), retrograde intra renal surgery (RIRS), and to

much less extent open surgery. In addition to preventive medical treatment [1-3].

Since the first PCNL procedure described by Fernstro and Johansson in 1976 [4], till now, there was a dramatic improvement in all aspects of this procedure including the type of nephroscope used, the fluoroscopy localization technique, the intracorporeal lithotripters used (including the Holmium laser), and the camera system [5]. These improvements in instruments and technique have led to expansion in the indications of PCNL to include even the large and complex renal stone cases. Current indications for PCNL includes: Staghorn calculi, large stone burden (>2 cm), hard calculi (cystine, brushite, struvite, calcium oxalate monohydrate), impacted or large proximal ureteral calculi, calyceal diverticular calculi, ectopic renal calculi (as in horseshoe kidney, pelvic kidney, or transplanted kidney), Coexisting Uretero-pelvic junction (UPJ) obstruction and renal calculi, Lower pole renal calculi greater than 1 cm, and stones that have failed ESWL [6].

Preoperative planning is essential to identify the position and number of stones, assess the intrarenal collecting system architecture, and to evaluate the relationship of the kidney and its surrounding organs. This is achieved by using one or more imaging modalities; as plain radiograph (KUB), renal ultrasonography, intravenous urography (IVU), and computerized tomography (CT) scan [7].

Difficult Access Situations included: previous renal surgery, perinephric scarring, supracostal upper calyx, mobile kidney, calyceal diverticulum, inability to find the renal pelvis, bifid collecting system, non-dilated collecting system, horseshoe kidney and pelvic kidney [8].

The aim of the study was to determine the efficacy and safety of PCNL in relation to stone complexity (according to different patient and stone parameters).

Methods

In this prospective clinical interventional study, which was conducted during the period (November 2014 to November 2016), 51 patients with symptomatic renal stone disease, treated with PCNL in our center, were included. Inclusion criteria were patients above 18 years of age, irrespective of gender with normal renal function and stone size > 2 cm.

Exclusion criteria were untreated active urinary tract infection, uncorrected coagulopathy, severe cardiovascular disease, pregnancy, severe obesity and patients with congenital renal anomalies (such as horseshoe or ectopic kidneys).

For all the patient's data were collected: age, gender, BMI, previous renal surgery, pre-operative stone number, stone size, stone position, stone opacity, surgical approach, operative time, stone clearance rate, postoperative residual stones &complications, hospital stay and adjuvant therapy.

In addition to history, clinical examination and routine laboratory Investigations, preoperative imaging with KUB, renal ultrasonography and CT scan were used to identify the number, size, opacity and location of stones and the anatomical details of the collecting system. IVU sometimes used when CT scan not available.

Stone positions were classified into:

- Upper or middle calyx
- Pelvic or lower calyx
- Partial staghorn
- Complete staghorn

All patients were treated under general anesthesia, in prone position and a single subcostal lower pole percutaneous access was usually made by using the ''triangulation'' technique under fluoroscopic guidance. Then rigid nephroscopy was performed and stones were localized and fragmented by a pneumatic lithotriptor. Normal saline was used for continuous irrigation by using pulsatile low-pressure perfusion pump.

At the completion of fragmentation, stones were removed with grasping forceps. Stone clearance and the integrity of the collecting system were confirmed intraoperatively by nephroscope and fluoroscopy.

Finally, a 5 Fr Double-J stent was introduced antegradely into the ureter and a 16 Fr

nephrostomy tube (Foley catheter) was inserted into the renal pelvis or the involved calyx at the conclusion of the procedure. Nephrostomy tube usually removed after 24 hours if there is no urine leakage, pain, fever or residual stone and in the absence of any complications, the patient usually discharged on the second postoperative day.

When residual calculi larger than 8 mm were present, ESWL or a second PCNL was considered after 2 weeks. All complications (intraoperative and postoperative) were stated. Stone free state is defined as the absence of any visible stone fragments on the nephroscopy at the end of the procedure and on the early postoperative imaging studies.

The final success rate is defined as the absence of significant stone fragments on the postoperative imaging studies after 6 weeks.

Statistical analysis was conducted by using the Statistical Package for Social Sciences (SPSS) version 20. The continuous data were represented by mean, standard deviation and range, while the categorical data presented as frequency and percentage tables. P-Value < 0.05 was used as the alpha level of significance.

This study was approved by the ethical committee of our hospital and all the patients signed an informed consent.

Results

In this prospective clinical interventional study, out of the 51 patients included; 31 (60.8%) were males and 20 (39.2%) were females, their mean age was 40.3 years and ranged from (18-62) years.

The mean body mass index of the patients was 22.9±2.7 kg/m^2 and ranged from 18 to 28, about one-fourth (13/51) of them were overweight (≥25 kg/m^2). The study also showed that 15 out of 51 included patients (29.4%) have previous renal surgeries.

Regarding the stone characteristics in the included patients, as shown in table 1, 39/51 (76.5%) were radio-opaque, and 30/51 (58.8%) of the managed renal stones were (≥3 cm) with mean stone size 4.2±0.99 cm.

The most prevalent site of stone was the lower calyx; 20/51 (39.2%), followed by 19/51 (37.3%) as partial stag-horns occupying the pelvis & one of the calyces. Hydronephrosis was found to be mild in 22 (43.1%), moderate in 18 (35.3%) and only 3 (5.9%) had a severe grade (Table 1).

In this study, the final success rate of treatment was 86.3% (in 44 patients) and that 31/51 (60.8%) of the cases found to be stone free at immediate postoperative period. Auxiliary procedures were used in 14 (27.5%) patients, to improve the stone clearance, including 6 patients underwent ESWL, 4 patients treated by URS and staged PCNL was done in 4 patients. Residual stones were ultimately remained in only 4/51 (7.8%).

Conversion to open surgery was only needed in three (5.9%) patients. Complications occurred in 11 patients (21.6%), with no fatal or life-threatening complications (Table 2).

The operative time of PCNL procedure was found to be different and correlated with certain patients & stone characteristics. Factors that significantly affect the operative time were: stone size ($p<0.0001$), the position of the stone ($p<0.0001$) and previous renal surgery ($p=0.014$) (Table 3).

Stone free rate was also correlated & significantly higher among patients with small size stones (95.2%), in lower calyx stones (100%) and in patients with no previous renal surgery (72.2%), as shown in table 4.

The postoperative complications were found to be significantly associated with only the stone size as (36.7%) of patients with large size stones (≥ 3cm) showed complications while no one of the patients with small size stones had any complication ($p=0.005$).

Other parameters like patients' BMI, past renal surgical history, stone position and opacity did not show significant associations with postoperative complications in our series.

The need for the use of auxiliary procedures (to accomplish stone clearance) was also significantly associated with: the size of the stones ($p= 0.007$) (being used in 43.3% of

patients with large stones in comparison to 4.8% among those with stones (< 3 cm) , with the complexity of stone position (p<0.0001) (the auxiliary procedures were needed in 33.3%, 36.8% and 83.3% in upper calyx, partial and complete staghorn stones respectively) and were much more needed in patients with previous renal surgery (60%) with a P value 0.002 .

The conversion to open surgery was done in only 3 (5.9%) cases, without any significant correlation with any of the studied parameters.

Table 1. Renal stone Characteristics in the included patients

Variables	Number	Percent
Stone location		
Right	29	56.9%
Left	22	43.1%
Right: Left ratio	1.32:1	
Opacity		
Lucent	12	23.5%
Opaque	39	76.5%
Opaque: Lucent ratio	3.25:1	
Size		
< 3 cm	21	41.2%
≥ 3 cm	30	58.8%
Position		
Lower calyx	20	39.2%
Partial stag-horn	19	37.3%
Upper calyx	6	11.8%
Complete stag-horn	6	11.8%
Grade of Hydronephrosis		
No	8	15.7%
Mild	22	43.1%
Moderate	18	35.3%
Sever	3	5.9%

Table 2. Number and percentage of complications in PCNL patients

Variables	Number	Percent
Fever >38 °C	9	17.6%
Urine leakage (pcs extravasation)	7	13.8%
Bleeding requiring transfusion	6	11.8%
Visceral injury (pneumothorax)	1	2.0%

Table 3. Correlation of mean operative time with renal stone's and patient characteristics

Variables	Operation duration (Minute) Mean ± Standard deviation	p-value
Stone size		
< 3 cm	83 ± 12	<0.0001*
≥ 3 cm	102 ± 16	
Position[a]		
Upper calyx	92 ± 15	
Partial staghorn	98 ± 12	<0.0001*
Complete staghorn	123 ± 14	
Lower calyx	83 ± 11	
Opacity		
Lucent	90 ± 13	0.275
Opaque	95 ± 18	
BMI		
<25 kg/m2	93 ± 18	0.585
≥25 kg/m2	96 ± 15	
Previous renal surgery		
Yes	102 ± 13	0.014*
No	91 ± 18	

Independent t-test, [a] ANOVA test, * Significant at 0.05 level

Table 4. Correlation of the stone-free rate with renal stone's and patient characteristics

Variables	Stone free rate Yes No. (%)	Stone free rate No No. (%)	p-value
Stone size [Y]			
< 3 cm	20 (95.2)	1 (4.8)	<0.0001*
≥ 3 cm	11 (36.7)	19 (63.3)	
Position [E]			
Upper calyx	3 (50)	3 (50)	
Partial stag-horn	7 (36.8)	12 (63.2)	<0.0001*
Complete stag-horn	1 (16.7)	5 (83.3)	
Lower calyx	20 (100)	0 (0)	
Opacity [Y]			
Lucent	9 (75)	3 (25)	0.415
Opaque	22 (56.4)	17 (43.6)	
BMI [χ]			
<25 kg/m^2	26 (68.4)	12 (31.6)	0.056
≥25 kg/m^2	5 (38.5)	8 (61.5)	
Previous renal surgery [χ]			
Yes	5 (33.3)	10 (66.7)	0.01*
No	26 (72.2)	10 (27.8)	

Y Yates continuity correction of chi, E Exact test, [χ]Chi-square tests, * Significant at 0.05 level.

Discussion

The goal of surgical therapy for renal stones is to achieve maximum stone clearance with the least morbidity to the patient. Percutaneous access to the renal collecting system with improvement of endourologic instruments and intra-corporeal lithotripters has yielded greater success rates and lower complication rates and is therefore recommended as the treatment of choice for renal stones measuring >2 cm in diameter [9].

Nevertheless, PCNL considered a demanding procedure and it is only safe and effective in experienced hands [10].

Currently, complex staghorn calculi have been mostly managed with PCNL. The morbidity of PCNL in this aspect is less than open surgery with better stone-free rates [11]. In fact, staghorn calculi were the most difficult group of stones treated in this series with the least immediate stone-free rate (16.7%). It is known that PCNL for the treatment of staghorn stones is a challenging procedure. It requires considerable experience in gaining percutaneous tracts, performing delicate and judicious intrarenal manipulations, mastering all techniques of intracorporeal stone disintegration, and weighing the benefits of complete stone clearance against the risks of complications [12].

The mean operative time in our study was 102 minutes for stone size (≥ 3cm), which is longer than in other studies (Shalaby et al, 80 minutes) [13]. This could be attributed to the difference in experience, facilities & completeness of stone clearance.

A significant positive correlation was seen between stone burden and operative time, because larger stone need more time for fragmentation, as well as the position of the stone significantly affects the operation duration, being the longest in complete staghorn stones (occupying the whole pelvicalyceal system) and shortest in lower calyx stones. Access to the other calyces can be difficult through the subcostal route by rigid nephroscope. In our experience, access through a lower calyx into the upper and middle calyx was difficult and necessitates longer operative and fluoroscopy time.

The overall stone-free rate in our study at the time of hospital discharge was 60.8% and increased to 86.3% by using auxiliary procedures postoperatively. This rate is lower than that reported by Rahman's study (83% - 90.8%) [14] and higher than that reported by El-Nahas et al study (56.6%-72.7%) [12].

PCNL should aim to achieve maximum stone clearance. The reasons for residual fragments are migration of a stone or stone fragments to an inaccessible calyx, termination of the procedure because of bleeding, complex anatomy increasing the technical difficulty, and inability to visualize the stone on fluoroscopy.

The judicious use of auxiliary procedures (repeated PCNL, URS, ESWL) in the early postoperative period for removal of clinically significant stone fragments will improve the final stone clearance rate. Moreover, the combined use of rigid and flexible nephroscope (when available) facilitate stones retrieval through calyces that could not be negotiated by rigid nephroscope alone and the use of the flexible Holmium YAG laser fibers through flexible nephroscope will help in-situ disintegration of calyceal stones and improved the overall success rate [12,15].

Stone free cases in our series were significantly higher among patients with small size stones and those located in one calyx (especially the lower calyx) because in such size & position there is less possibility of having residual fragments. While in staghorn stones with multiple large branches, percutaneous access to all the calyces was somehow difficult through one tract, that's why the rate of residual stones was high.

In this study, the overall complication rate was 21.6%, which is lower than in Mousavi's study (30.3%) [16]. Fever was recorded in 9 (17.6%) patients, and all were treated conservatively with good antibiotics cover and delaying the withdrawal of the nephrostomy tube till the fever subsided. Transient post- operative fever occurs in up to 30% of patients after PCNL, which is usually related to duration of surgery and the amount of irrigation fluid used, but the rate of sepsis is much lower, ranging from 0% to 3% in patients covered with appropriate perioperative antibiotics [17].

Bleeding requiring blood transfusion has been reported to be 1% to 15% [6]. It occurred in 6 (11.8%) of our patients, with an average peri-operative blood loss of 950 ml (estimated by calculating the blood content in the sucker bottle & the drainage bag), which is higher than in Rahman's study (4.8%) [14]. The risk of hemorrhagic complications requiring blood transfusion in our study was associated with a larger tract size (for large stones), renal pelvic perforation, and total blood loss. In most of the cases the bleeding was controlled by placement of the nephrostomy tube and/or clamping the nephrostomy tube to tamponade the bleeding, but in 2 cases the procedure was converted to open surgery.

In PCNL, the overall pleural injury rate with supra-costal access was estimated to be 16%, compared to 4.5% with an infra-costal approach, and as many as 64% of patients with pleural injury require chest tube drainage [17].

The only visceral injury in this series was a Pneumothorax which occurred in one (2%) patient, due to very highly placed kidney under the rib cage, which is comparable to the rate of pleural injury in Ullah's study (1.9%) [9]. This injury was successfully treated by a thoracostomy drain but the procedure converted to open one.

The postoperative complications were found to be significantly associated with stone size as there was a tendency to higher grade complications in complex stones, which was similar to the findings in Abdelhafez's study [10], because dealing with large stones need prolonged operative time, larger volumes of irrigation fluid and difficult manipulation with a higher possibility of perforation and bleeding [18,20].

PCNL in a patient with previous renal surgery may take longer duration and lead to a higher percentage of auxiliary procedures [8,19], as in the current study, probably because of the scar tissue and anatomic changes in the kidney that lead to difficulties in tract dilation of perinephric space, in addition, intrarenal manipulation of stone will be more difficult.

This study concluded that PCNL, though demanding, is an effective and reasonably safe procedure for the treatment of different renal stone burdens.

In this study, size and position of stones in the calyceal system and past renal surgery were the main determinants of operative time, stone clearance, complication rate and the need for auxiliary procedures.

Acknowledgments

The authors express their gratitude for all the staff of the urology department of Al-Yarmouk teaching hospital for their substantial help.

Author Contribution

All authors have made substantial contributions to this study: Dr. Al-Azzawi: study concept and design, general supervision on the work, critical revision and final approval of the manuscript. Dr. Mohammed Zaki and Dr Salih: acquisition of data, analysis and interpretation of data, and drafting the manuscript. All 5 authors contributed in patient selection, admission, performing the surgical procedures, follow up and data collection for the patients included in this study.

Conflict of interest

Authors declare no conflict of interest.

Funding

There was no funding source for this research.

References

1. Srisubat A, Potisat S, Lojanapiwat B, et al. Extracorporeal shock wave lithotripsy (ESWL) versus percutaneous nephrolithotomy (PNL) or retrograde intrarenal surgery (RIRS) for kidney stones. Cochrane Database Syst Rev. 2014; issue 11. Art. No.: CD007044. doi: 10.1002/14651858.CD007044.pub3.
2. Alkan E, Ozkanli O, Avci E, et al. Effectiveness of flexible ureterorenoscopy and laser lithotripsy for multiple unilateral intrarenal stones smaller than 2 cm. Adv Urol. 2014; article ID 314954, 5 pages. doi: http://dx.doi.org/10.1155/2014/314954.
3. Kenney PA, Wotkowicz C, Libertino JA. Contemporary open surgery of the kidney. In: Kavoussi LR, Novick AC, Partin AW, et al (eds). Campell-Walsh urology. 10th ed. Vol 2. Philadelphia: Elsevier Saunders; 2012. p. 1554-1627.
4. Spann A, Poteet J, Hyatt D, et al. Safe and effective obtainment of access for percutaneous nephrolithotomy by urologists: the Louisiana State

University Experience. J Endourol. 2011; 25(9): 1421-5. doi: 10.1089/end.2010.0643.
5. Patel SR, Nakada SY. The modern history and evolution of percutaneous nephrolithotomy. J Endourol. 2015; 29(2): 153-7. doi: 10.1089/end.2014.0287.
6. Miller N, Lingeman JE. Percutaneous renal access. In: Smith JA, Howards SS, McGuire EJ, et al (eds). Hinman's atlas of urologic surgery. 3rd ed. Philadelphia: Elsevier Saunders; 2012. p. 845-53.
7. Yuhico MP, Ko R. The current status of percutaneous nephrolithotomy in the management of kidney stones. Minerva Urologica Nefrologica. 2008; 60: 159-75.
8. Marcovich R, Smith AD. Percutaneous renal access: tips and tricks. BJU Int. 2005; 95: 78-84. doi: 10.1111/j.1464-410X.2005.05205.x.
9. Ullah A, Khan MK, Rahman AU, et al. Percutaneous nephrolithotomy: A minimal invasive surgical option for the treatment of staghorn renal calculi. Khyber Med Univ J. 2012; 4(4): 156-60.
10. Abdelhafez MF, Bedke J, Amend B, et al. Minimally invasive percutaneous nephrolithLapaXY (PCNL) as an effective and safe procedure for large renal stones. BJU Int. 2012; 10(11 Pt C): E1022-6. doi: 10.1111/j.1464-410X.2012.11191.x.
11. Desai M, Jain P, Ganpule A, et al. Developments in technique and technology: the effect on the results of percutaneous nephrolithotomy for staghorn calculi. BJU Int. 2009 Aug;104(4):542-8; discussion 548. doi: 10.1111/j.1464-410X.2009.08472.x.
12. El-Nahas AR, Eraky I, Shokeir AA, et al. Percutaneous nephrolithotomy for treating staghorn stones: 10 years of experience of a tertiary-care centre. Arab J Urol. 2012; 10(3): 324-9. doi: 10.1016/j.aju.2012.03.002.
13. Shalaby MM, Abdalla MA, Aboul-Ella HA, et al. Single puncture percutaneous nephrolithotomy for management of complex renal stones. BMC Res Notes. 2009; 2: 62. doi: 10.1186/1756-0500-2-62.
14. Rahman AU, Ullah A, Khan S, et al. Percutaneous nephrolithotomy for the treatment of renal stones larger than 2.5 cm. JPMI: J Postgrad Med Instit. 2011; 25: 362-7.
15. Stoller ML. Urinary Stone Disease. In: McAninch JW, Lue TF. Smith & Tanagho's General urology. 18th ed. New York: McGraw-Hill; 2013. p. 249-79.
16. Mousavi-Bahar SH, Mehrabi S, Moslemi MK. Percutaneous nephrolithotomy complication in 671 consecutive patients: a single-center experience. Urol J. 2011; 8(4): 271-6.
17. Taylor E, Miller J, Chi T, et al. Complications associated with percutaneous nephrolithotomy. Transl Androl Urol. 2012; 1(4): 223-8. doi: 10.3978/j.issn.2223-4683.2012.12.01.
18. Akman T, Sari E, Binbay M, et al. Comparison of outcomes after percutaneous nephrolithotomy of staghorn calculi in those with single and multiple accesses. J Endourol. 2010; 24(6):955-60. doi: 10.1089/end.2009.0456.
19. Khorrami MH, Hadi M, Sichani MM, et al. Percutaneous nephrolithotomy success rate and complications in patients with previous open stone surgery. Urol J. 2014; 11(3): 1557-62.
20. De S, Autorino R, Kim FJ, et al. Percutanous nephrolithotomy versus Retrograde intrarenal surgery: a systematic review and meta-analysis. Eur Urol. 2015; 67(1): 125-37. doi: 10.1016/j.eururo.2014.07.003.

Correspondence to Dr. Issam S. Al-Azzawi
E-mail: issam_alazzawi@yahoo.com
Received Nov. 1st 2017
Accepted Apr. 1st 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Effects of N-Acetyl-Cysteine (NAC) Administration on Glucose Homeostasis Parameters in Prediabetic Patients

Ahmed R. Abu Raghif[1] *PhD*, **Yaseen A. Yaseen**[2] *FICMS*, **Muntadher H. Dawood**[3] *MSc*

[1]Dep.t of Pharmacology and Therapeutics, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [2]Dept. Dean of College of Medicine, Misan University, Iraq, [3]Dept. of Pharmacology and Toxicology, College of Pharmacy, Misan University, Iraq

Abstract

Background Prediabetes is determined on the bases of glycemic parameters, which are above normal but below diabetic thresholds. Prediabetes is associated with the presence of insulin resistance and β-cells dysfunction. N-acetyl cysteine (NAC), as a safe and inexpensive medication, is commercially accessible since long-time ago. This drug is not found in natural sources, although cysteine is present in some meals like chicken and turkey meats, garlic, yogurt, and eggs. NAC prevents apoptosis and oxygen related genotoxicity in endothelial cells by increasing intracellular levels of glutathione and decreasing mitochondrial membrane depolarization reaction.

Objective To evaluate the effects of NAC administration on glucose homeostasis parameters in prediabetic patients.

Methods This study included, 25 patients treated with dietary control and life style modifications for 12 weeks, 25 patients treated with NAC (600 mg) oral tablets twice daily plus dietary control and life style modifications for 12 weeks. Other 20 in addition to 50 patients to have an idea about the normal values of study parameters and in order to assess how much the drug used in the study were able to normalize the abnormal parameters.

Results NAC demonstrated a significant decrease in the fasting blood sugar, HbA1c, fasting Insulin and insulin resistance at the end of 12 weeks ($P<0.05$) compared with baseline measurements.

Conclusion The results of the study showed that N-acetyl cysteine has an effective effect on glycemic control.

Keywords Prediabetes , N-acetyl cysteine , glycemic control

Citation Abu Raghif AR, Yaseen YA, Dawood MH. Effects of N-Acetyl-Cysteine (NAC) administration on glucose homeostasis parameters in prediabetic patients. Iraqi JMS. 2018; 16(4): 393-399. doi: 10.22578/IJMS.16.4.6

List of abbreviations: AGEs = Advanced glycation end products, FPG = Fasting plasma glucose, GSH = Glutathione, HOMA = Homeostatic model assessment, IFG = Impaired fasting glucose, IGT = Impaired glucose tolerance, NAC = N-acetylcysteine, ROS = Reactive oxygen species

Introduction

Prediabetes is determined on the bases of glycemic parameters which are above normal but below diabetes thresholds. It is a high risk state for diabetes with an estimated annual conversion rate of 5-10%; a similar proportion is converting back to normoglycemia [(1)]. Prediabetes is associated with the presence of insulin resistance and β-cell dysfunction. These abnormalities start before glucose changes are detectable [(2)].

The high risk for developing diabetes is related to two states; impaired fasting glucose (IFG) (defined as fasting plasma glucose (FPG) of 5.7-6.9 mmol/L in the absence of impaired glucose tolerance (IGT), and to IGT (defined as post-load plasma glucose of 7.8-11.0 mmol/L based on 2-hour oral glucose tolerance test (OGTT) or

a combination of both [3]. A lower cut-off value for IFG (FPG 5.6-6.9 mmol/L) is employed by the American Diabetes Association. In addition, it has introduced hemoglobin A1c levels of 5.7-6.4% as a parameter of high diabetes risk [4]. Combination of IFG and IGT marks a more advanced disturbance of glycemic homeostasis [5].

The central mechanism that is responsible for risks in prediabetes is endothelial dysfunction due to the elevated formation of reactive oxygen species (ROS) and advanced glycation end products (AGEs) as well as increased lipid peroxidation under hyperglycemic conditions [6].

N-acetyl cysteine (NAC), the acetylated variant of the amino acid L-cysteine, is an excellent source of sulfhydryl (SH) groups, and is converted in the body into metabolites capable of stimulating glutathione (GSH) synthesis, promoting detoxification, and acting directly as free radical scavengers [7]. It is a powerful antioxidant and a potential treatment option for diseases characterized by the generation of free oxygen radicals [8].

The objectives of this study was to evaluate the effects of NAC administration on glucose homeostasis parameters in prediabetic patients.

Methods

Study design

The current study was conducted on 50 prediabetic patients (31 males, 19 females) their ages from 30-65 year were seen in Al-Sader Teaching Hospital. The patients were diagnosed clinically by physician as having prediabetes. Criteria for the diagnosis of prediabetes and diabetes [9] is shown in table 1.

Table 1. Criteria for the diagnosis of prediabetes and diabetes

	Prediabetes	Diabetes
A1C	5.7-6.4%	≥6.5%**
FPG	100-125 mg/dL (5.6-6.9 mmol/L)	≥126 mg/dL (7.0 mmol/L)**
OGTT*	140-199 mg/dL (7.8-11.0 mmol/L)	≥200 mg/dL (11.1 mmol/L)**
RPG		≥200 mg/dL (11.1 mmol/L)***

* 2-hour plasma glucose value after a 75-g OGTT

** Confirm results with repeating testing

*** Diagnotic in patients with established symptoms of hyperglycemia

A1C: Glycated hemoglobin, FPG: Fasting plasma glucose, OGTT: oral glucose tolerance test, RPG: random plasma glucose

To have an idea about the normal values of study parameters and in order to assess how much the drug used in the study were able to normalize the abnormal parameters, other 20 patients was added to 50 patients.

Patients

This study included, 25 patients treated with dietary control and life style modifications for 12 weeks, 25 patients treated with NAC (600 mg) oral tablets twice daily plus dietary control and life style modifications for 12 weeks.

Sample collection and preparation

After 12 hours fasting, blood samples were collected from all patients and healthy subjects by venipuncture (10 mL), before starting drug treatment (as zero time) then after 12 weeks of treatment to follow up the changes in the studied parameters. Blood samples were divided into two tubes, one heparinized tube (1 mL of whole blood used for HbA1c determination) and the other part was collected in plane tube, then centrifuged at 3000 rpm for 10 min at 4 °C. After centrifugation and isolation of cellular fraction; the obtained plasma fraction was divided into

two parts in Eppendorf tubes and stored frozen until analysis performed.

Measurement of glycemic control

Fasting blood glucose Level (FBS)

Serum glucose level was evaluated using a ready-made kit for this purpose, according to the method of [10], which is based on enzymatic oxidation of glucose to form glucuronic acid and hydrogen peroxide, and the reaction of the later with phenol and formation of quinonimine was followed spectrophotometrically at 505 nm. Results were expressed as mg/dL, based on comparison with a standard glucose solution treated with same method.

Glycated Hemoglobin (HbA1c)

The Bio-Rad VARIANTT Mhemoglobin A1C program is intended for the determination of HbA1c in human whole blood using the principles of ion exchange high performance liquid chromatography (HPLC) for the automatic and accurate separation of hemoglobin A1c (HbA1c). It is fully automated assay using HPLC technology to deliver precise and accurate HbA1c results. Program offers a simple preparation followed by automatic sampling, and an analysis time of three minutes per sample. Preceding analysis, a simple preparation of the sample is required to hemolyze and remove labile A1C. Samples are first diluted with hemolytic reagent and then incubated at 18-28Co for a minimum of 30 min. The VARIANTS II dual-piston pumps deliver programmed buffer gradients of increasing ionic strength to the analytical cartridge. Prepared samples are automatically injected into analytical cartridge where the hemoglobin is separated based on their ionic interaction with the material. The separated hemoglobin then passes through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured. A chromatogram of the changes in the absorbance is plotted versus the retention time. This chromatogram helps in result interpretation [11].

Serum Insulin levels

The Demeditec insulin ELISA (enzyme-linked immunosorbent assay) is a solid phase ELISA based on sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the insulin molecule. An aliquot of patient sample containing endogenous insulin is incubated in the coated well with enzyme conjugate, which is anti-insulin antibody conjugated with Biotin. After incubation the unbound conjugate is washed off. During the second incubation step Streptavidin –Peroxidase –Enzyme complex binds to the biotin-anti-insulin-antibody. The mount of horseradish peroxidase (HRP) complex is proportional to the concentration of insulin in the sample. Having added the substrate solution, the intensity of the color developed is proportional to the concentration of Insulin in the patient sample. The result expressed as uU/mL.

Insulin resistance

The homeostatic model assessment (HOMA) model is simple and accessible measurement method for the evaluation of insulin sensitivity and consider as a model of interactions between glucose and insulin dynamics that is then used to predict fasting steady-state glucose and insulin concentrations for a wide range of possible combinations of insulin resistance and β-cell function. Both the original HOMA and the updated HOMA2 assume a feedback loop between the liver and β-cell [12,13] glucose concentrations are regulated by insulin-dependent HGP, whereas insulin levels depend on the pancreatic β-cell response to glucose concentrations. Thus, deficient β-cell function reflects a diminished response of β-cell to glucose-stimulated insulin secretion. Likewise, insulin resistance is reflected by diminished suppressive effect of insulin on glucose production. HOMA describes this glucose insulin homeostasis by a set of empirically derived nonlinear equations. The model predicts fasting steady state levels of plasma glucose and insulin for any given combination of pancreatic β-cell function and insulin sensitivity [14]. The approximating

equation for IR has been simplified and uses a fasting plasma sample in which glucose (fasting plasma glucose; FPG) and insulin (fasting plasma insulin; FPI) are measured, together with a constant. The product of FPG×FPI is an index of IR.

HOMA-IR = (glucose × insulin)/405.

Insulin concentration is reported in uU/mL and glucose in mg/dL. The constant of 405 is a normalizing factor, i.e. normal FPI of 5 uU/mL × the normal FPG of 81 mg/dL typical of a 'normal' healthy individual = 405. Therefore, for an individual with "normal" insulin sensitivity, HOMA-IR =1.

Statistical analysis

Paired Student's t test was used to compare values obtained before and after treatment administration within each group while independent sample t tests were used for between all patients and healthy subjects. Multiple comparisons were also carried out by using Analysis of variance (ANOVA) with least significant difference (LSD) post-hoc testing to compare changes in variables between groups before and after the 12 weeks' treatment period. Data are presented as mean ± Standard deviation (SD). For all statistical analyses, P<0.05 was considered statistically significant using a two-tailed test. Statistical analysis of data was performed using the Statistical Package for Social Sciences software version 16.0 [15].

Results

Comparison of patients with prediabetic and healthy subjects with respect to different parameters

In healthy group, the mean±SD for FBS, HbA1c, fasting serum insulin, insulin resistance were 83.42±9.73, 5.12±0.06, 11.14±1.23 and 2.84 ±0.71 respectively.

In patients group the mean±SD for FBS, HbA1c, fasting serum insulin, insulin resistance were 119.42±5.56, 6.14±0.09, 13.54±1.89 and 3.99±0.6 respectively. Unpaired t-test was used to compare the baseline characters between the healthy and prediabetic patients group, revealed that were significant differences in FBS, HbA1c, fasting serum insulin and insulin resistance (p<0.001) levels between both groups as shown in table 2.

Table 2. Comparison of different parameters of prediabetic patients to that of healthy subjects at baseline

Parameters	Patients		Healthy		P value
	Mean	±SD	Mean	±SD	
FBS (mg/dL)	119.42	5.56	83.42	9.73	0.001
HbA1c %	6.14	0.09	5.12	0.06	0.001
Fasting Insulin (?)	13.54	1.89	11.14	1.23	0.001
In. resistance	3.99	0.6	2.84	0.71	0.001

Effect of study treatment on (FBS, HbA1c, fasting insulin and insulin resistance)

Study treatment demonstrated a significant decrease in the FBS, HbA1c, fasting insulin and insulin resistance at the end of 12 weeks (P<0.05) compared with baseline measurements. In comparing with control group, the reductions in FBS, HbA1c, fasting insulin and in insulin resistance was significantly at week 12 of the study (P<0.05) as shown in table 3 and figure 1.

Table 3. Effect of study treatment on (Fasting blood glucose, HbA1c, fasting insulin and insulin resistance) after 12 weeks' treatments in study group and multiple comparison of the change from baseline

Parameters	Groups	Control		N-acetyl cysteine (NAC)	
		Mean	±SD	mean	±SD
FBS	Baseline	118.12	5.42	121.19	5.32
	12 weeks	107.25*	4.97	106.13*	5.14
	ΔF.B.S	-10.87	1.22	-15.06a	1.87
HbA1c	Baseline	6.22	0.084	6.19	0.092
	12 weeks	6.1*	0.081	5.63*	0.093
	ΔHbA1c	-0.09	0.022	-0.56a	0.019
Fast. In	Baseline	12.44	1.17	12.35	1.31
	12 weeks	11.09*	1.13	9.91*	0.97
	ΔFast.In	-1.35	0.14	-2.44a	0.16
In. resis	Baseline	3.61	0.34	3.69	0.34
	12 weeks	2.94*	0.33	2.59**	0.31
	Δ In. resis	-0.67	0.07	-1.1a	0.03

Fast. In: Fasting Insulin, In. resis: Insulin resistance

*=statistically significant (P<0.05) difference after 12 weeks compared with the baseline by using paired t-test

**= statistically highly significant (P<0.001) difference after 12 weeks compared with the baseline by using paired t-test

a= statistically significant (P<0.05) difference after 12 weeks compared with control group using ANOVA post hoc test or unpaired t-test

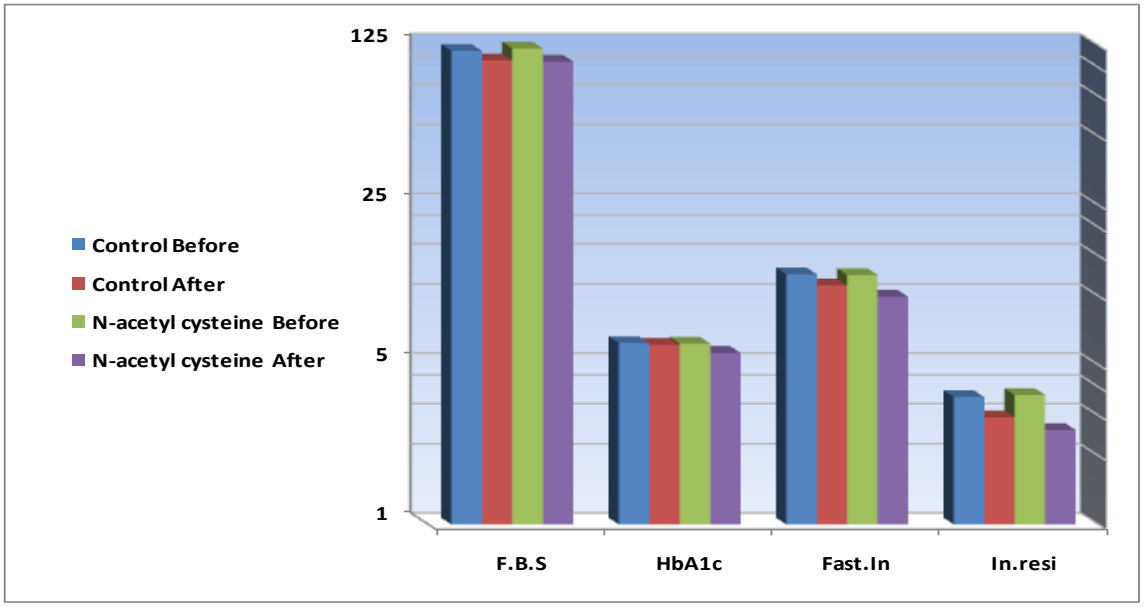


Figure 1. F.B.S, HbA1c, Fast. In and In. resi before and after 12 week of the study treatment

Discussion

Study treatment demonstrated a significant decrease in the FBS, HbA1c, fasting insulin and insulin resistance at the end of 12 weeks ($P<0.05$) compared with baseline measurements.

The NAC is the acetylated precursor of both amino acid L-cysteine and reduced glutathione. It has been shown to have proven activity on insulin secretion in pancreatic cells, as well as on the regulation of the insulin receptor in human erythrocytes [16]. The peak plasma level of NAC is attained 1 hour after an oral dose and it disappears from the plasma after 12 hours. The biological activity of NAC is attributed to its sulfhydryl group, which enhances glutathione-S-transferees activity aiding in the protection of all cells and membranes The activity of NAC on insulin secretion in pancreatic-cells, as well as on the regulation of the insulin receptor in human erythrocytes [17]. It increases the cellularlevels of reduced glutathione (GSH), an antioxidant, which has been shown to influence insulin receptor activity in vivo. Diabetics frequently experience glutathione deficiency, and NAC was shown that it improves endothelial cell function in such patients [18]. According to an intraperitoneal glucose tolerance test in mice, treatment with NAC retained glucose-stimulated insulin secretion and moderately decreased blood glucose levels. Vitamins C and E were not effective when used alone but slightly effective when used in combination with NAC [19]. Further, it is suggested that NAC might protect against oxidant-related upregulation of endothelial adhesion molecules and slow down the progression of vascular damage in non-insulin dependent diabetes [20]. The antioxidant status is poor in non-insulin dependent diabetes mellitus. NAC, as an effective radical scavenger could be a valuable adjunct treatment in diabetic patients, but further studies are required to confirm these findings. Supplementation with antioxidants as a promising complementary treatment can exert beneficial effects in diabetes [20].

According to the results presented in this study it is easy to conclude that the administration of NAC could improve glycemic control with consequent beneficial effects on oxidative stress in prediabetic patients, may be through mechanisms of up regulating peripheral tissue responses to the available insulin at receptor levels in association with potent antioxidant effects.

Acknowledgments

Authors would like to acknowledge all the staff members in the Al-Sader Teaching Hospital Misan.

Author Contribution

Dawood: performed the laboratory work. Dr. Abu Raghif: study design and statistics, Dr. Yaseen: doses calculation and final revision of manuscript.

Conflict of interest

Authors declare no conflict of interest.

Funding

There is no funding source for this research.

References

1. Bansal N. Prediabetes diagnosis and treatment: A review. World J Diabetes. 2015; 6(2): 296–303. doi: 10.4239/wjd.v6.i2.296.
2. Burns C, Sirisena I. Type 2 diabetes: etiology, epidemiology, pathogenesis, treatment. In: Ahima RS (ed). Metabolic syndrome: a comprehensive textbook. Springer; 2016. p. 601-17.
3. Hemmingsen B, Sonne DP, Metzendorf MI, et al. Insulin secretagogues for prevention or delay of type 2 diabetes mellitus and its associated complications in persons at increased risk for the development of type 2 diabetes mellitus. Cochrane Database Syst Rev. 2016; 10: CD012151. doi: 10.1002/14651858.CD012151.pub2
4. Basevi V, Di Mario S, Morciano C, et al. Comment on: American Diabetes Association. Standards of medical care in diabetes--2011. Diabetes Care 2011; 34(Suppl. 1): S11-S61. Diabetes Care. 2011; 34(5): e53; author reply e54. doi: 10.2337/dc11-0174.
5. Nathan DM, Davidson MB, DeFronzo RA, et al. Impaired fasting glucose and impaired glucose tolerance. Diabetes Care. 2007; 30(3): 753-9. doi: 10.2337/dc07-9920.
6. Yang W, Fu J, Yu M, et al. Effects of flaxseed oil on anti-oxidative system and membrane deformation of human peripheral blood erythrocytes in high glucose level. Lipids Health Dis. 2012; 11: 88. doi: 10.1186/1476-511X-11-88
7. Larsson SC, Håkansson N, Wolk A. Dietary cysteine and other amino acids and stroke incidence in

women. Stroke. 2015; 46(4): 922-6. doi: 10.1161/STROKEAHA.114.008022.
8. Shahin AY, Hassanin IM, Ismail AM, et al. Effect of oral N-acetyl cysteine on recurrent preterm labor following treatment for bacterial vaginosis. Int J Gynaecol Obstet. 2009; 104(1): 44-8. doi: 10.1016/j.ijgo.2008.08.026.
9. Zou D, Ye Y, Zou N, et al. Standards of medical care in diabetes 2017: Section 11. Older adults. cross-sectional survey in Guilin. China J Diabetes Investig. 2017; 8(2): 188-194. doi: 10.1111/jdi.12549.
10. Barham D, Trinder P. An improved colour reagent for the determination of blood glucose by the oxidase system. Analyst. 1972; 97(151): 142-5.
11. Mayer TK, Freedman ZR. Protein glycosylation in diabetes mellitus: a review of laboratory measurements and of their clinical utility. Clin Chim Acta. 1983; 127(2): 147-84.
12. Matthews DR, Hosker JP, Rudenski AS, et al. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985; 28(7): 412-9. doi: http://dx.doi.org/10.1007/BF00280883
13. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. Diabetes Care. 2004; 27(6): 1487-95. doi: http://dx.doi.org/10.2337/diacare.27.6.1487.
14. Muniyappa R, Lee S, Chen H, et al. Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. Am J Physiol Endocrinol Metab. 2008; 294(1): E15-26. doi: 10.1152/ajpendo.00645.2007
15. Arbuckle JL, Wothke W. Amos 6.0 user's guide. Chicago, IL: SPSS. 2005.
16. Lanzone A, Fulghesu AM, Andreani CL, et al. Insulin secretion in polycystic ovarian disease: effect of ovarian suppression by GnRH agonist. Hum Reprod. 1990; 5(2): 143-9. doi: http://dx.doi.org/10.1093/oxfordjournals.humrep.a137058.
17. Bouzakri K, Karlsson HK, Vestergaard H, et al. IRS-1 serine phosphorylation and insulin resistance in skeletal muscle from pancreas transplant recipients. Diabetes. 2006; 55(3): 785-91. doi: http://dx.doi.org/10.2337/diabetes.55.03.06.db05-0796.
18. Pieper GM, Dembny K, Siebeneich W. Long-term treatment in vivo with NOX-101, a scavenger of nitric oxide, prevents diabetes-induced endothelial dysfunction. Diabetologia. 1998; 41(10): 1220-6. doi: 10.1007/s001250051055.
19. Kaneto H, Kajimoto Y, Miyagawa J, et al. Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. Diabetes. 1999; 48(12): 2398-406. doi: http://dx.doi.org/10.2337/diabetes.48.12.2398.
20. Ozkilic AC, Cengiz M, Ozaydin A, et al. The role of N-acetylcysteine treatment on anti-oxidative status in patients with type II diabetes mellitus. J Basic Clin Physiol Pharmacol. 2006; 17(4): 245-54.

Correspondence to Muntadher H. Dawood
E-mail: mntzrhnwn2@gmail.com
Received Dec. 20th 2017
Accepted Jan. 23rd 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Deficiency of Serum 25-hydroxyvitamin D in Patients with Breast Cancer in Iraq

Taha H.T. Al-Saigh *FICMS*

Dep.t of Surgery, Nineveh college of Medicine, University of Nineveh, Iraq

Abstract

Background Many studies defined the association between vitamin D and breast cancer. The relation between human epidermal growth factor receptor 2 (Her2/nue), estrogen or progesterone receptors and vitamin D in breast cancer patients was not clear, since few studies was conducted.

Objective To find out the association of deficiency of vitamin D and breast cancer. The effect of Her2/neu, estrogen, and progesterone receptors on vitamin D in the breast cancer patients was also studied.

Methods Forty patients with benign breast lump as a control group and 40 patients with breast cancer early diagnosed were included in the study. Blood samples (5 mL) were taken from the control and patient groups and analyzed for serum 25-hydroxyvitamin D by using chemiluminescent immunoassay technology. Serum cancer antigen (CA15-3) was measured by using monoclonal antibodies against CA15-3. HER2/neu, estrogen and progesterone receptors were determined in breast cancer patients by immunochemical method.

Results Serum vitamin D in the breast cancer patients before surgery was significantly lower ($p \leq 0.05$) than that in the control group. Preoperatively, serum CA15-3 in Her2/neu positive patients was significantly ($p \leq 0.05$) higher than that in Her2/neu negative patients. Postoperatively, serum CA15-3 in Her2/neu positive patients was not significantly different from that in Her2/neu negative patients. Serum vitamin D, after surgery, in Her2/neu positive patients was not significantly different from that in Her2/neu negative patients. Serum vitamin D was not significantly different in estrogen and progesterone positive patients from that in estrogen and progesterone negative patients, respectively.

Conclusion Severe deficiency of vitamin D was noticed in breast cancer patients in Iraq, and mild deficiency in benign subjects. Serum CA15-3 was higher in positive Her2/neu than negative patients before operation. Her2/neu, estrogen and progesterone positive receptors have no effect on serum vitamin D level in breast cancer patients.

Keywords Vitamin D, breast cancer, CA15-3, Her2/neu

Citation Al-Saigh THT. Deficiency of serum 25-hydroxyvitamin D in patients with breast cancer in Iraq. Iraqi JMS. 2018; 16(4): 400-404. doi: 10.22578/IJMS.16.4.7

List of abbreviations: None

Introduction

The association of vitamin D with calcium hemostasis is elucidated for decades, vitamin D also plays a role on other disease states such as metabolic syndrome, type 2 diabetes, and systemic hypertension [1]. Serum vitamin D deficiency was significantly associated to colorectal, breast and prostate cancer risk [2]. Relation of breast cancer risk and vitamin D status has been defined in many studies but the true association is still not understood and needs further research [3,4]. Preclinical and some

clinical studies strongly suggest that vitamin D deficiency increased risk of developing cancer and that avoiding deficiency and adding vitamin D supplements outcome reduced risk of breast cancer [5]. Accordingly, it is recommended to maintain vitamin D store in breast cancer survivors [6]. However, high 25-hydroxyvitamin D was weakly associated with low risk of breast cancer [7]. Further studies are needed to clarify the potential role of vitamin D and breast cancer risk [8].

High hypovitaminosis D was found in many countries including USA, China, and Iran [9-11]. In Europe vitamin D deficiency was less prominent than other countries [12]. The indoor life and the dressing style play an important role for vitamin D deficiency in Iraq, even with the sunny weather during most of the year. Therefore, measurement of serum vitamin D should be considered and made regularly.

Few studies were managed to find out the relation of vitamin D and human epidermal growth factor receptor 2 (Her2/neu) [13]. Vitamin D supplementation improved the life of breast cancer patients with Her2/neu positive [14].

This work was conducted in order to evaluate the association of vitamin D and breast cancer. The effect of Her2/neu, estrogen and progesterone positive receptors on serum vitamin D level in the breast cancer patients was also studied.

Methods

This study was conducted at Nineveh Medical Center and Al-Jammhory Teaching Hospital, Mosul, Iraq during the period from February 2013 to Jun 2013. Eighty patients were divided into two groups according to their presentation into: 40 patients with benign breast lump with mean age 34.45 ± SD: 10.45 years and 40 patients with breast cancer early diagnosed with mean age 44.85 ± SD: 10.73 years. Blood samples (5 mL) were taken from the patients before surgery and from the control subjects and analyzed for serum 25-hydroxyvitamin D by chemiluminescent immunoassay technology (Liaison instrument, DiaSorin Company, Germany). Kit by serum cancer antigen (CA15-3) was also measured in the breast cancer patients by immunochemical method by using monoclonal antibodies against CA15-3 (Minividas, USA, Kids by Biomerieux Company, France). HER2/neu, estrogen and progesterone receptors were determined in breast cancer patients by immunochemical method (Dako Company, Denmark).

Data are presented as mean ± SD and were analyzed by using non-paired t-test. p values ≤ 0.05 were considered significant. Statistical analysis was performed by using SPSS version 16.

Results

The breast cancer patients suffered from severe deficiency in serum vitamin D, while the control (benign breast lump) suffered from mild deficiency. This classification of severe and moderate is according to the American College of Cardiology [1]. Serum vitamin D before surgery in the breast cancer patients was significantly ($p \leq 0.05$) lower than that in the benign breast lump subject (Table 1).

Table 1. Serum vitamin D in patients with breast cancer and in subjects with benign breast lump

Variables	Benign breast lump subjects (n=40)	Breast cancer patients (n=40)	p-value
Vitamin D ng/mL	18.39 ± 4.11	7.31 ± 2.93	≤ 0.05

Preoperatively, serum CA15-3 in Her2/neu positive patients was significantly ($p \leq 0.05$) higher than that in Her2/neu negative patients. Postoperatively, serum CA15-3 in Her2/neu positive patients was not significantly different from that in Her2/neu negative patients. Serum vitamin D in Her2/neu positive patients was not significantly different from that in Her2/neu negative patients (Table 2).

Serum vitamin D was not significantly different in estrogen and progesterone positive patients from that in estrogen and progesterone negative patients, respectively (Data not shown).

Table 2. Serum cancer antigen 15-3 pre-operative and post-operative, vitamin D in patients with positive human epidermal receptor and negative human epidermal receptor in breast cancer

Variables	HER2/neu positive N=24	HER2/neu negative N=16	p–value
CA 15-3 pre-operative	48.42 ± 16.74	41.88 ± 10.3	≤ 0.05
CA 15-3 post-operative	27.33 ± 11.43	23.19 ± 7.74	N S
Vitamin D ng/mL	7.64 ± 3.42	6.82 ± 2.01	N S

Discussion

In the present study, serum vitamin D in the breast cancer patients before surgery was significantly lower than that in the control subjects. The results are consistent with other workers [15]. Vitamin D deficiency is correlated with poor outcome of patients with breast cancer [7]. In addition, high vitamin D levels correlated with low tumor size and high better survivals [16].

In the present study, breast cancer patients suffered from severe deficiency in serum vitamin D, while the control group (benign breast) suffered from mild deficiency.

Alipour et al. found that severe vitamin D deficiency produces three folds' increase in the risk of breast cancer patients, the median serum vitamin D level was higher in the control subjects, lower in benign mass and the lowest in breast cancer patients [17].

In this study, CA15-3 in Her2/neu positive patients was higher than that in the negative patients. Hashim [18] also noticed high CA15-3 in Her2/neu positive patients. Serum Her2/neu and CA15-3 were useful marker for aggressiveness of breast cancer [19]. Serum CA15-3 level was significantly higher in the breast cancer patients compared with the control group, and this level dropped after surgery [19].

No significant difference between Her2/neu positive and negative for the present serum vitamin D. The 25-hydroxyvitamin D concentration was inversely associated with prognosis of patients with cancer estrogen positive but not with Her2/neu positive [20]. Furthermore, in disease free survival of breast cancer women, vitamin D deficiency had negative correlation related to Her2/neu receptor expression [21].

No significant difference between estrogen or progesterone positive or negative receptors for serum vitamin D was noticed in this work. Cell culture strongly support and in vivo data in mice that vitamin D would play a beneficial role in prevention of estrogen positive breast cancer [22]. Calcitriol suppresses estrogen expression and estrogen mediated signaling [23]. Vitamin D down regulate estrogen receptor and thereby attenuates estrogen signaling in breast cancer [24]. However, deficiency of vitamin D levels were shown to be a risk factor estrogen negative tumor [25].

In conclusion, severe deficiency of vitamin D was noticed in breast cancer patients in Iraq, and mild deficiency in benign subjects. Serum CA15-3 was higher in positive Her2/neu than

negative patients before operation. No relationship was measured between positive receptors of Her2/neu, estrogen or progesterone, and serum vitamin D in breast cancer patients.

Acknowledgments
To laboratory team in Al-Jammhoy Hostital.

Conflict of interest
None.

Funding
This work was supported by Nineveh College of Medicine, University of Mosul.

References
1. Lavie CJ, Lee JH, Milani RV. Vitamin D and cardiovascular disease. J Am Coll Cardiol. 2011; 58(15): 1547-56. doi: 10.1016/j.jacc.2011.07.008.
2. Ordóñez Mena JM, Brenner H. Vitamin D and cancer: an overview on epidemiological studies. Adv Exp Med Biol. 2014; 810: 17-32.
3. Lopes N, Sousa B, Martins D, et al. Alteration in vitamin D signaling and metabolic pathways in breast cancer progression: A study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. BMC Cancer. 2010; 10: 483. doi: 10.1186/1471-2407-10-483.
4. Shao T, Klein P, Grossbard ML. Vitamin D and Breast Cancer. Oncologist 2012; 17(1): 36-45. doi: 10.1634/theoncologist.2011-0278.
5. Feldman D, Krishnan AV, Swami S, et al. The role of vitamin D in reducing cancer risk and progression. Nat Rev Cancer. 2014; 14(5): 345-57. doi: 10.1038/nrc3691.
6. Hines SL, Jorn HK, Thompson KM, et al. Breast cancer survivors and vitamin D: A review. Nutrition. 2009; 26(3): 255-62. doi: 10.1016/j.nut.2009.08.020.
7. Kim HJ, Lee YM, Ko BS, et al. Vitamin D deficiency is correlated with poor outcomes in patients with luminal-type breast cancer. Ann Surg Oncol. 2011; 18(7): 1830-6. doi: 10.1245/s10434-010-1465-6.
8. Yin L, Grandi N, Raum E, et al. Meta-analysis: serum vitamin D and breast risk. Eur J Cancer. 2010; 46(12): 2196-205. doi: 10.1016/j.ejca.2010.03.037.
9. Forrest KY, Stuhldreher WL. Prevalence and correlates of vitamin D deficiency in USA adults. Nutr Res. 2011; 31(1): 48-54. doi: 10.1016/j.nutres.2010.12.001.
10. Shi L, Nechuta S, Gao YT, et al. Correlates of 25-hydroxyvitamin D among Chinese breast cancer patients. PLoS One. 2014; 9(1): e86467. doi: 10.1371/journal.pone.0086467.
11. Jamshidinaeini Y, Akbari ME, Abdollahi M, et al. Vitamin D status and risk of breast cancer in Iranian women: a case-control study. J Am Coll Nutr. 2016; 35(7): 639-46. doi: 10.1080/07315724.2015.1127786.
12. Spiro A. Buttriss JL. Vitamin D: an overview of vitamin D status and intake in Europe. Nutr Bull. 2014; 39(4): 322-50. doi: 10.1111/nbu.12108.
13. Zhang X, Harbeck N, Jeschke U, et al. Influence of vitamin D signaling on hormone receptor status and HER2 expression in breast cancer. J Cancer Res Clin Oncol. 2017; 143(7): 1107-22. doi: 10.1007/s00432-016-2325-y.
14. Zeichner SB, Koru-Sengul T, Shah N, et al. Improved clinical outcomes associated with vitamin D supplementation during adjuvant chemotherapy in patients with HER2+ nonmetastatic breast cancer. Clin Breast Cancer. 2015; 15(1): e1-11. doi: 10.1016/j.clbc.2014.08.001.
15. Yousef FM, Jacobs ET, Kang PT, et al. Vitamin D status and breast cancer in Saudi Arabian women: case control study. Am J Clin Nutr. 2013; 98(1): 105-10. doi: 10.3945/ajcn.112.054445.
16. Hatse S, Lambrechts D, Verstuyf A, et al. Vitamin D status at breast cancer diagnosis: correlation with tumor characteristics, disease outcome, and genetic determinants of vitamin D insufficiency. Carcinogenesis. 2012; 33(7): 1319-26. doi: 10.1093/carcin/bgs187.
17. Alipour S, Hadji M, Hosseini L, et al. Levels of serum 25-hydroxy-vitamin d in benign and malignant breast masses. Asian Pac J Cancer Prev. 2014; 15(1): 129-32. doi: http://dx.doi.org/10.7314/APJCP.2014.15.1.129.
18. Hashim ZM. The significant of CA15-3 in breast cancer patients and its relationship to HER-2 receptor status. Int J Immunopathol Pharmacol. 2014: 27(1): 45-51. doi: 10.1177/039463201402700107.
19. Samy N, Ragab HM, Al Maksoud NA, et al. Prognostic significance of serum Her2/neu, BCL2, CA15-3, CEA in breast cancer patients: a short follow up. Cancer Biomark. 2010; 6(1): 63-72. doi: 10.3233/CBM-2009-0119.
20. Kim Y, Je, Y. Vitamin D intake, blood 25(OH)D levels, and breast cancer risk or mortality: A meta-analysis. Br. J. Cancer. 2014; 110(11): 2772-84. doi: 10.1038/bjc.2014.175.
21. Ismail A, El-Awady R, Mohamed G, et al. Prognostic significance of serum vitamin D levels in Egyptian females with breast cancer. Asian Pac J Cancer Prev. 2018; 19(2): 571-6. doi: 10.22034/APJCP.2018.19.2.571.
22. Krishnan AV, Swami S, Feldman D. The potential therapeutic benefits of vitamin D in the treatment of estrogen receptor positive breast cancer. Steroid. 2012; 77(11): 1107-12. doi: 10.1016/j.steroids.2012.06.005.

23. Swami S, Krishnan AV, Peng L, et al. Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements. Endocr Relat Cancer. 2013; 20(4): 565-77. doi: 10.1530/ERC-12-0281.

24. Krishnan AV, Swami S, Feldman D. Vitamin D and breast cancer: inhibition of estrogen synthesis and signaling. J Steroid Biochem Mol Biol. 2010; 121(1-2): 343-8. doi: 10.1016/j.jsbmb.2010.02.009.

25. de Sousa Almeida-Filho B, De Luca Vespoli H, Pessoa EC, et al. Vitamin D deficiency is associated with poor breast cancer prognostic features in postmenopausal women. J Steroid Biochem Mol Biol. 2017; 174: 284-9. doi: 10.1016/j.jsbmb.2017.10.009.

E-mail: t.alsaigh@yahoo.com

Received Jan. 23rd 2018
Accepted Jun.14th 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Extraction, Purification and Therapeutic Use of Bacteriophage Endolysin against Multi-Drug Resistant *Pseudomonas aeruginosa*: An In-vitro and In-vivo Study

Mohammed R. Ali[1] *PhD*, **Shurooq R. Kadhim[2]** *PhD*, **Ahmed S. Abdulamir[1]** *PhD*

[1]Dep.t of Microbiology, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [2]Dept. of Microbiology, College of Pharmacy, Al-Mustansiriya University, Baghdad, Iraq

Abstract

Background Persistent infection with multidrug resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) represents a real problem for health care providers. Bacteriophage lytic enzymes or lysins are highly evolved molecules that have been specifically developed by phages to quickly and efficiently allow their progeny to be released from the host bacterium while destructing that bacterium.

Objective Isolation of endolysin from P. *aeruginosa* bacteriophages, and administering them systematically in vivo lab animal and measuring their therapeutic efficacy as well as evaluation of their biosafety.

Methods This study was performed from March 2015 – August 2017, during which 50 bacteriological samples of *P. aeruginosa* were collected, and examined for their antibiogram, then bacteriophage cocktails were done for 5 resistant strains of them. Endolysins were extracted from their corresponding bacteriophages and characterized. The enzymatic and antibacterial activities as well in vivo therapeutic efficiency of these enzymes were investigated.

Results This study showed that the extracted endolysin from these bacteriophages was effective in treating laboratory mice from bacteremia with *P. aeruginosa* and saving their lives when injected intraperitoneal.

Conclusion Endolysin can be extracted directly from their bacteriophages and used effectively in proper doses to treat bacteremia in mice.

Keywords *Pseudomonas aeruginosa*, bacteriophage, endolysin, MDR

Citation Ali MR, Kadhim SR, Abdulamir AS. Extraction, purification and therapeutic use of bacteriophage endolysin against multi-drug resistant *Pseudomonas aeruginosa*: An in-vitro and in-vivo study. Iraqi JMS. 2018; 16(4): 405-412. doi: 10.22578/IJMS.16.4.8

List of abbreviations: EDTA = Ethylenediaminetetra acetic acid, LB = Luria–Bertani broth, MDR = Multi-drug resistant, OD = Optical density, P = Pseudomonas, PBS = Phosphate buffer saline, pP = Phage to *Pseudomonas aeruginosa*

Introduction

Bacterial infections are responsible for significant morbidity and mortality in clinical settings [(1)]. Many infections that would have been cured easily by antibiotics in the past now are resistant, resulting in sicker patients and longer hospitalizations. The endolysins of the bacteriophages are highly evolved molecules that have been specifically developed by phages to quickly and efficiently allow their progeny to be released from the host bacterium. These enzymes damage the bacterial cell wall's integrity by hydrolyzing the four major bonds in its peptidoglycan component [(2)]. The endolysins that have been characterized are amidases; usually do not have signal sequences to translocate them

through the cytoplasmic membrane and cleave their substrate in the peptidoglycan. Instead, the endolysins' translocation is controlled by a second phage gene products called holins [3]. During the bacteriophages development in the infected bacterium, endolysin accumulates in the cytoplasm until bacteriophage maturation. During a specific time genetically controlled, holin molecules, which inserted in the cytoplasmic membrane are activated, resulting in the formation of pores so the preformed endolysin in the cytoplasm can access the peptidoglycan, there-by causing cell lysis and the release of the new bacteriophages [4]. Bacteriophages are able to lyse their targeted bacterial hosts, a fact that has been known for almost a century, and since the late 1910s bacteriophages have been used to prevent and treat human and animal diseases of bacterial origin. However, bacteriophage-encoded enzymes have only newly begun to be used for various applications; e.g.: reducing bacterial contamination in dairy products and the preparation of bacterial vaccines [5,6].

The current study aimed to isolate endolysin from *P. aeruginosa* bacteriophages, and evaluate their therapeutic efficacy as well as their biosafety in lab animals.

Methods

Samples of bacteria were collected from Al-Imamein Al-Kadhimein Medical City. A total of 50 different *P. aeruginosa* isolates were collected from Bacteriology laboratory. The specimens were collected from hospitalized patients and outpatients suffering from severe urinary tract infection, otitis media, skin infection, and septicemia. The specimens were cultured in screw universal tubes containing nutrient broth wrapped by parafilm or by using sterile swabs; both were put in ice bags and were transferred at the same day to the laboratory of Microbiology Department in the College of Medicine, Al-Nahrain University to be subcultured on nutrient agar or stored in refrigerator at 4 0C for 24 h [7].

Different crude samples for phage isolation were obtained from different regions in Baghdad including sewage (30-40 ml), waste water (30-40 ml), feces of sheep (20 gm), chicken litter (15-20 gm), swab from surgical lounges during the period from January 2015 to June 2015. The samples were put in clean test tubes wrapped by parafilm in ice bag and transported to the laboratory in the same day. Each sample was divided into two aliquots; one was stored in the refrigerator at 40 °C until be used and the other was worked at the same day.

Stored specimens were plated on nutrient agar by streaking method. *P. aeruginosa* formed smooth round colonies with a fluorescent greenish color on nutrient agar. Single colonies of *P. aeruginosa* from a growing stock were re-cultured by ABC streaking on nutrient agar plates in order to isolate single discrete colonies. Then, a battery of diagnostic approaches was pursued for the diagnosis of Pseudomonas bacteria including oxidase test, Gram staining, microscopical examination and Luria broth. This media was prepared according to the manufacturing instructions and sterilized by autoclaving at 121 °C for 15 min. Diagnosis of the isolated bacteria relied first on oxidase test. Oxidase positive isolates suggests *Pseudomonas species*, microscopic examination (rod shaped bacteria), Gram staining (Gram-negative rods) confirm the presence of *P. aeruginosa*. The bacteria were stocked in Luria–Bertani broth containing glycerol (30% v⁄v) and preserved at -20 °C.

Primary phages are those phages that were isolated from environmental specimens when were mixed with target bacteria. The procedure of isolating and propagating primary phages was done according to the methodology conducted in a patent concerned with phage isolation [8].

Virulent phages were screened by phage spotting test on a nutrient-agar. Phage spotting can be used to provide a first approximation of the ability of a phage to lyse certain bacterial isolates. The formation of clear zones suggests the presence of lytic phages [9].

The therapeutic effect of extracted endolysin was evaluated by using three groups of mice (2 months' age, weighed 25±1.5 g/mice) for each

bacterium (*P. aeruginosa*), each group was composed of 5 mice.

The first two groups of mice received intraperitoneal (IP) injections of 400 µl aliquots of bacterial suspension at concentration 108 CFU/ml, one of these groups was treated with 0.4 ml of 20 µg/ml of Pseudomonas specific endolysin, which was injected 3h after the bacterial challenge in order to evaluate the ability of administered endolysin to rescue the tested bacteremic mice from the inevitable fate of death by the bacterial infection (9). On the other hand, the third group received only IP injection with 0.4 ml of 20 µg/ml of Pseudomonas specific endolysin without bacterial infection.

After disinfecting the area of injection by 70% alcohol, the first and second groups of mice were injected IP with 108 CFU/ml of the bacterial isolate. Then, every hour interval, the mice of control, test, and endolysin only groups were monitored for their health and physical activities and timely health score was recorded. According to (Biswas) [10], certain health scoring system was used as shown in table (1).

Table 1. The health scoring system of bacteremic mice

Health signs	Health level
Normal and unremarkable condition	5
Slight illness(lethargy and ruffled fur)	4
Moderate illness(severe lethargy, ruffled fur, and hunched back)	3
Sever illness (severe lethargy, ruffled fur, hunched back, and exudative accumulation around partially closed eyes)	2
Moribund state	1
Death	0

Results

In this study, three phages active against *P. aeruginosa* were isolated and purified. All of the isolated phages formed visible plaques in the early stage when tested on bacterial lawn of specific MDRs P. aeruginosa. They were isolated directly from environment by showing lysis on bacterial lawns as shown in figure (1).

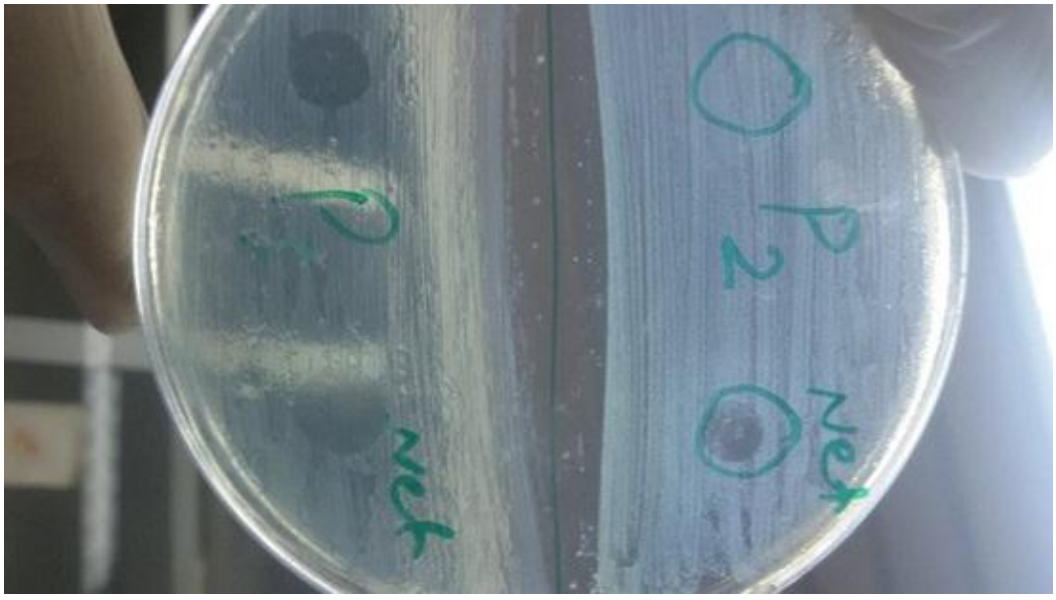


Figure 1. Phage spot assay of bacteriophage to Pseudomonas aeruginosa

Characteristics of these phages were determined by the diameter, clarity/turbidity, margin cut, and shape of their plaques. The size of plaques ranged between 1.5-2.6 mm. The morphological characteristics of the plaques are shown in table (2).

Table 2. The plaque characteristics of the isolated phages to MDR Pseudomonas aeruginosa bacteria

Bacteriophage isolates	Plaques Size (mm)	Margin cut	Plaques clarity	Plaques shape
pP1	1.5	Irregular	Clear	Circular
pP2	2.6	Irregular	Semi-clear	Oval
pP3	1.8	Irregular	Semi-clear	Circular

pP: phage to *Pseudomonas aeruginosa*

Endolysin was successfully extracted from all the three *P. aeruginosa* bacteriophages using sephadex G100 column chromatography. After sephadex G100 chromatography, each bacteriophage lysate gave eight aliquots of 0.5 ml eluted fluid fractions in Eppendrof tubes, one of them showed positive result on corresponding bacterial lawns as shown in table (3).

Table 3. The fraction that endolysin like activity was found for each bacteriophage

Bacteriophage	Endolysin positive tube
pP1	2nd
pP2	3rd
pP3	2nd

pP: phage to *Pseudomonas aeruginosa*

The optical density of bacterial broth for each bacteria was measured initially once at zero time, just before the addition of corresponding endolysin. After the addition of the purified endolysin, three days after their extraction, with the concentrations (25, 40, 20 µg/ml), the optical density was measured every five minutes till one hour. The optical density of the tested bacterial broth was obviously decreasing with time as shown in table (4 a and b).

The result of this experiment showed that the health score of the first group (five mice injected with bacteria alone) started to decrease after two hours, afterwards the health score declined progressively. They died after eight hours. On the other hand, the second group (five mice injected with bacteria and endolysin) lived for more than four weeks with full physical activity and the third group (five mice injected with endolysin alone) also lived for more than four weeks with full physical activity.as shown in tables (5) and (6).

Table 4a. Changes of optical density of bacterial broth with endolysin addition

Time (min.)	OD of P1*	OD of P2	OD of P3
0	1.34	1.34	1.34
5	1.3	1.32	1.29
10	1.25	1.27	1.24
15	1.21	1.24	1.16
20	1.12	1.19	1.09
25	1.05	1.14	1.02
30	0.96	1.07	0.94
35	0.85	0.98	0.85
40	0.76	0.92	0.74
45	0.64	0.83	0.63
50	0.56	0.74	0.5
55	0.52	0.65	0.46
60	0.49	0.56	0.43
Δ OD/ min	0.014	0.013	0.015

*: net OD of P1-3 after deducting OD of the broth only (0.46)
OD: optical density, P: Pseudomonas aeruginosa

Table 4b. Pace of OD change every 5 minutes

Time (min.)	Δ OD of P1	Δ OD of P2	Δ OD of P3
5	0.04	0.02	0.05
10	0.05	0.05	0.05
15	0.04	0.03	0.08
20	0.09	0.05	0.07
25	0.07	0.05	0.07
30	0.09	0.07	0.08
35	0.11	0.09	0.09
40	0.09	0.06	0.11
45	0.12	0.09	0.11
50	0.08	0.09	0.13
55	0.04	0.09	0.04
60	0.03	0.09	0.03

Table 5. Health score of mice injected with bacteria alone or with endolysin

Hours	Health scores bacteremic group 1	Median of health scores of bacteremic group 1	Health scores of test group 2 (bacteria + endolysin)	Median of health scores of test group 2 (bacteria + endolysin)	P value (Mann Whitney test)
0 h	5,5,5,5,5	5	5,5,5,5,5	5	0.920
2 h	3,3,4,3,2	3	3,4,3,3,4	3	0.465
4 h	3,2,3,3,1	3	5,5,4,4,5	5	0.012
6 h	1,1,1,1,0	1	5,5,5,5,5	5	0.012
8 h	0,0,0,0,0	0	5,5,5,5,5	5	0.012

Table 6. Health Score of mice Injected with endolysin alone or nothing

Hours	Health scores endolysin only group 3	Median of health scores of endolysin only group 3	Health scores of healthy group 4	Median of health scores of healthy group 4	P value (Mann Whitney test)
0 h	5,5,5,5,5	5	5,5,5,5,5	5	0.920
2 h	5,5,5,5,5	5	5,5,5,4,5	5	0.674
4 h	5,5,5,5,5	5	5,5,4,4,5	5	0.347
6 h	5,5,5,5,5	5	5,5,5,5,5	5	0.920
8 h	5,5,5,5,5	5	5,5,5,5,5	5	0.920

Discussion

Phage lysin therapy is a possible alternative to antibiotics for the treatment of bacterial infections. Indeed, it has proven to be medically superior to antibiotic therapy [12,13]. The results of the current research support this notion, as phage lysin was shown to be highly efficacious against infections caused by inoculation with antibiotic-resistant *P. aeruginosa*.

In this study, three phages for different *P. aeruginosa* were isolated and characterized. The phages demonstrated high lytic activity underscoring its great potential to treat infections with these bacteria. The experiments presented here revealed that a single intraperitoneal injection of the corresponding lysin rescued mice from death due to *P. aeruginosa* even when bacteremia was already well established, and it was observed that effective protection was achieved in mice when a single dose of a specific endolysin was administered two hours after inoculation of *P. aeruginosa*.

The results obtained in this experiments are encouraging, since these findings and those of others support the development in a near future of modified procedures to improve the use of lysins in a more efficient way. For example, intravenous administration of the enzymes may confer a superior protection to animals when used later after the challenge. Most interestingly, a single intraperitoneal injection of endolysin was sufficient for a complete cure of mice. provides a rapid and specific lytic activity, making these proteins very promising candidates in current antimicrobial therapies [14].

This study shows that bacteriophage lysins from *P. aeruginosa* can be used to efficiently reduce the bacterial burden of these bacteria, which are multidrug resistant, both in vitro and

in vivo, and this study emphasizes the potential therapeutic role of phage lysins for treatment of Gram-negative bacterial infections.

Animal studies have generally supported the utility and safety of endolysin phage therapy against bacterial pathogens, such as P. aeruginosa [15].

The route of endolysin phage administration was particularly important to the efficacy of the treatment. Intraperitoneal route provides significant protection similar to that of intravenous route. It was stated that endolysin for *P. aeruginosa* phages administered by the IP route are distributed, in high titers, more rapidly and delivered for a more sustained period of time to all of the tissues [15].

Actually using a severe model of sepsis that leads to 100% lethality of mice in less than 24 h is a difficult challenge for every imaginable antibacterial agent. Two hours after bacterial injection, health score of mice began to decline progressively; A single injection of endolysin, rescued 100% of MDR *P. aeruginosa* septicemic mice. Thus, this study showed that endolysin administered in a single dose 2 h post bacterial infection resulted in 100% survival of treated mice when compared to the control group.

This study concluded that endolysin can be extracted directly from their bacteriophages and used by injection of mice with bacteremia with the proper dose of the extracted endolysin of the corresponding bacteriophages which was effective in all of them.

Acknowledgments

Special thanks for the staff members of Microbiology Department, College of Medicine, Al-Nahrain University for their continuous support, and the Research Unit staff in the College of Medicine, Al-Nahrain University for help in extraction, purification and characterization of the endolysins of the corresponding bacteriophages.

Author Contribution

Dr. Abdulamir: made the research design and the protocol for bacteriophages formulation. Dr. Kadhim helped in research design with extraction of the endolysin. Dr. Ali collected the bacterial and bacteriophage samples with their preparation and isolation and the endolysin extraction with its characterization, and he reviewed and wrote this article.

Conflict of interest

No conflicts of interest.

Funding

Self-funding.

References

1. Wise R. The relentless rise of resistance. J Antimicrob Chemother. 2004; 54(2): 306-10. doi: 10.1093/jac/dkh340.
2. Young R. Bacteriophage lysis: mechanism and regulation. Microbiol Rev. 1992; 56(3): 430-81.
3. Wang IN, Smith DL, Young R. The protein clocks of bacteriophage infections. Annu Rev Microbiol. 2000; 54: 799-825. doi: 10.1146/annurev.micro.54.1.799.
4. Szostak MP, Hensel A, Eko FO, et al. Bacterial ghosts: non-living candidate vaccines. J Biotechnol. 1996; 44(1-3): 161-70. doi: 10.1016/0168-1656(95)00123-9.
5. Gaeng S, Scherer S, Neve H, et al. Gene cloning and expression and secretion of Listeria monocytogenes bacteriophage-lytic enzymes in Lactococcus lactis. Appl Environ Microbiol. 2000; 66(7): 2951-8.
6. Bauer AW, Kirby WM, Sherris JC, et al. Antibiotic susceptibility testing by a standardized single disc method. Am J Clin Pathol. 1966; 45(4): 493-6.
7. Budzik JM, Rosche WA, Rietsch A, et al. Isolation and P. aeruginosa CRISPR/Cas and Bacteriophage Resistance a generalized transducing phage for Pseudomonas aeruginosa strains PAO1 and PA14. J Bacteriol. 2004; 186: 3270-3. doi: 10.1128/JB.186.10.3270-3273.2004
8. Jassim SA, Abdualamir AS, Abu baker F. Methods for bacteriophage design in "international application published under the patent cooperation (PCT)" (W.I.P. organization, ed.), 2010. Vol.WO2010064044 A1.
9. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: From the natural environment to infectious diseases. Nat Rev Microbiol. 2004; 2(2): 95-108. DOI: 10.1038/nrmicro821.
10. Biswas B, Adhya S, Washart P, et al. Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant Enterococcus faecium. Infect Immun. 2002; 70(1): 204-10.
11. Donovan D. M. Bacteriophage and peptidoglycan degrading enzymes with antimicrobial applications. Recent Pat Biotechnol. 2007; 1(2): 113-22.
12. Gu J, Xu W, Lei L, et al. LysGH15, a novel bacteriophage lysin, protects a murine bacteremia model efficiently against lethal methicillin-resistant Staphylococcus aureus infection. J Clin Microbiol. 2011 Jan;49(1):111-7. doi: 10.1128/JCM.01144-10.

13. Schuch R, Nelson D, Fischetti VA. A bacteriolytic agent that detects and kills Bacillus anthracis. Nature. 2002; 418(6900): 884-9. DOI: 10.1038/nature01026
14. McVay CS, Velásquez M, Fralick JA. Phage therapy of Pseudomonas aeruginosa infection in a mouse burn wound model. Antimicrob Agents Chemother. 2007; 51(6): 1934-8. doi: 10.1128/AAC.01028-06
15. Soothill JS. Bacteriophage prevents destruction of skin grafts by Pseudomonas aeruginosa. Burns. 1994; 20(3): 209-11.

Correspondence to Dr. Mohammed R. Ali
E-mail: dr_mohamadrazak@colmed-alnahrain.edu.iq
dr_mohamadrazak@yahoo.com
Received Feb. 1st 2018
Accepted Apr. 5th 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Comparing Dialysis Patients According to Estimated Glomerular Filtration Rate at Dialysis Initiation and Five Years Outcome

Jwad A. A. Al-Joher *MMedSc Nephrology*

[1]Al-Yarmouk Teaching Hospital, Baghdad, Iraq

Abstract

Background It is not clear on which level of estimated glomerular filtration rate to start dialysis until now.

Objective To compare between patient groups according to estimated glomerular filtration rate at dialysis initiation.

Methods Eighty-seven Patients are selected randomly from a list of patients admitted for dialysis (Hemodialysis or Peritoneal dialysis) at Northern General Hospital - Sheffield Kidney Institute from 1st of January to 31st of December 2000. Patients have been categorized into two groups. First group includes 45 patients started dialysis with glomerular filtration rate less than 5 ml/min/1.73m^2. Second group includes 42 patients started dialysis with glomerular filtration rate from 5-10 ml/min/1.73m^2. Patients have been followed up for five years retrospectively until 31st December 2005.

Results The study showed males are the predominant gender. White Caucasian patients are majority of patients. The mean age is 56 years. About half of patients are middle aged (45-65 years) and are overweight or obese. This study shows no difference between patients who started dialysis early (estimated glomerular filtration rate 5-10 ml/min/1.73m^2) versus late (estimated glomerular filtration rate below 5 ml/min/1.73m^2) in term of mortality and morbidity throughout five years of follow up.

Conclusion This study showed no justification to decide on time to initiate dialysis based solely on estimated glomerular filtration rate level.

Keywords Chronic kidney disease, estimated glomerular filtration rate, hemodialysis, peritoneal dialysis, end stage renal disease, renal replacement therapy

Citation Al-Joher JAA. Comparing dialysis patients according to estimated glomerular filtration rate at dialysis initiation and five years outcome. Iraqi JMS. 2018; 16(4): 413-423. doi: 10.22578/IJMS.16.4.9

List of abbreviations: CANUSA study = Canada-USA multicenter study of peritoneal dialysis adequacy, CKD = Chronic kidney disease, CPD = Chronic peritoneal dialysis, DM = Diabetes mellitus, eGFR = estimated Glomerular Filtration Rate, ESRD = End stage renal disease, HD = Hemodialysis, KDIGO = Kidney disease: Improving global outcomes, Kt/V = K dialyzer clearance of urea, t dialysis time, V volume of distribution of urea, MD = Maintenance dialysis, MDRD = Modification of diet in renal disease, MHD = Maintenance hemodialysis, NHANES = National Health and Nutrition Examination survey, NKF-K/DOQI = The National Kidney Foundation Kidney Disease Outcomes, PD = Peritoneal dialysis, PTH = Parathyroid hormone, RRT = Renal replacement therapy, SD = Standard deviation

Introduction

Chronic kidney disease (CKD) is a worldwide public health problem. There were estimated to be over 37,800 adult patients receiving renal replacement therapy (RRT) in the UK at the end of 2004. Haemodialysis (HD) was the very first modality of RRT in 71.0% of patients, peritoneal dialysis (PD) in 26.5% and preemptive transplant in 2.3%, which compares with 58% starting HD in 1998 [(1)].

The glomerular filtration rate (GFR) is equal to the sum of the filtration rates in all of the functioning nephrons; thus, the GFR gives a rough measure of the number of functioning nephrons. A reduction in GFR implies either progression of the underlying disease or the development of a superimposed and often reversible problem, such as decreased renal perfusion due to volume depletion [(2)].

Estimated GFR (mL/min/1.73 m^2)
= 186 X (SCr)-1.154 X (Age)-0.203 X (0.742 if female) X (1.210 if African-American)
= exp(5.228-1.154Xln(SCr)-0.203 X ln(AGE)-0.299 if female) + (0.192 if African-American))
Based on data from NHANES III, the the National Kidney Foundation (NKF)-Dialysis Outcome Quality Initiative (NKF-K/DOQI) CKD Guidelines workgroup estimated GFR using the equation derived from the MDRD Study, which factors in age, gender, race, and serum creatinine level [3,4].

The timing of starting a dialysis modality may be due to a number of factors. Most commonly it is the increasing presence of symptoms that triggers the commencement of dialysis, and generally this will correspond to a decline in kidney function measured by GFR. Some patients will develop sever fluid retention unresponsive to diuretic therapy or electrolyte disturbances, particularly hyperkaliemia, that requires dialysis to start in the absence of symptoms. The other trigger is decline in eGFR even in the absence of symptoms. However, this may not always be accepted by patients who do not feel unwell [5].

Early guidelines outlined by the NKF-K/DOQI recommended that renal replacement therapy be considered when GFR declined below 10.5 ml/min/1.73 m^2, and definitely implemented if there was unintentional weight loss, a decrease in normalized protein intake, or there were clinical signs or symptoms of uremia [6].

The NKF 2006 update of clinical practice guidelines highlights the need for nephrologists to evaluate the risks and benefits of dialysis initiation when patients reach stage 5 CKD (estimated GFR < 15 ml/min/1.73 m^2) while acknowledging the importance of individual patient factors and their influence on the timing of dialysis. In Canada, clinical practice guidelines recommend dialysis initiation at a GFR < 12 ml/min/1.73 m^2 if there is evidence of uremia or malnutrition or at a GFR <6 ml/min whether symptomatic or not [7].

Despite these guidelines, there appears to be a wide variation in the level of renal function at hemodialysis initiation and many patients continue to start dialysis at very low levels of predicted GFR. For example, Obrador et al. reported a mean predicted GFR of 7.1 ml/min/1.73 m^2 among new dialysis starts in the United States from April 1995 through September 1997, and as many as 23% were considered late starts as defined by a GFR of <5 mL/min/1.73m^2 [8].

On the other hand, it is becoming increasingly clear from observational registry data from the United States that patients with comorbidities initiate dialysis therapy at higher levels of estimated GFR [9].

In 2003, mean estimated GFR at the initiation of dialysis therapy was 9.8 mL/min/1.73 m^2. This mean value reflects lower average values (~7 to 9 mL/min/1.73 m^2) for young and middle-aged adults and higher average values (~10 to 10.5 mL/min/1.73 m^2) for children and elderly patients. Average GFR at initiation has increased in all age groups since 1995; it has increased most in the oldest patients [10].

It is difficult to make a recommendation for initiating RRT based solely on a specific level of GFR. Several studies concluded that there is no statistically significant association between renal function at the time of initiation of RRT and subsequent mortality [12-15]. However, others suggested that worse kidney function at initiation of RRT is associated with increased mortality or morbidity [11-16].

The purpose of this study, to resolve the dilemma of when to start dialysis to patients with end stage kidney disease. This study might help to give answer to controversial opinions on level of eGFR to initiate patient on dialysis modality when starting dialysis early have suggested that it may improve nutrition with subsequent decrease in hospitalization, mortality, and costs. On the other hand, early initiation of dialysis poses ongoing diet and fluid restrictions, imposes limits on travel, impacts on patient and family quality of life, while exposing the individual to complications of dialysis earlier than may be necessary.

Methods

The study is to compare between patient's groups according to initial eGFR (MDRD calculation) in term of followings points:

- Patients' demography
- Retrospectively follow up of patients for 5 years to study dialysis outcome in term of mortality, anemia, bone biochemistry, lipid, Blood pressure, Kt/V (K dialyzer clearance of urea, t dialysis time, V volume of distribution of urea), and albumin as nutrition status marker with body mass index (BMI).
- Survival of patients and its association with covariates of study for each group of patients
- Hospitalization in form of number of admissions to hospital.

Patients

Patients are selected randomly from a list of patients admitted to dialysis (HD or PD) at Northern General Hospital - Sheffield Kidney Institute from 1st of January to 31st of December 2000. The list of patients (provided by Sheffield Kidney Institute Academic office) is randomly sorted by patients' hospital code numbers, which consists of six digital numbers. Patients should have been on any dialysis modalities for more than 90 days to be considered to have chronic disease and not acute phase of kidney disease. Any patients who have had dialysis for more than three months before 2000 were excluded. Ninety patients were selected by simple random selection, only 3 patients have initiated dialysis with eGFR more than 10 ml/min/m^2 and they were excluded from this study because of their small number. Eighty-seven patients were divided into two groups according to baseline eGFR. Group 1, consists of 45 patients with baseline eGFR less than 5 ml/min/1.73 m^2. Group 2 are 42 patients with baseline estimated GFR 5-10 ml/min/1.73 m^2. Patients have been followed up for five years retrospectively until 31st of December 2005.

Data collection

Approval to access patient's information was obtained. Username and password were provided by Northern General Hospital – Sheffield Kidney Institute Academic office. All patients' data were accessed through Proton program, which installed on computer systems at Health Sciences Library, Samuel Fox House and Study Room 4, Coleridge House at Northern General Hospital. Retrospectively, data were collected. The list of patients was provided by Sheffield Kidney Institute to patients admitted for dialysis in year 2000. The list contains patients' code numbers in form of six digits (xxxxxx). Randomly selected patient's code number, entered into Proton program to access patient's information.

Using Excel office program for Windows helps to enter data, and organizes work. Age of patients is calculated which represents age in years from date of birth to date of dialysis.

Patients' demographic parameters (age in years, gender as male or female, and ethnicity) are obtained. BMI is calculated from weight of patient in Kilogram divided by square height of patient in meter.

eGFR is recorded at time starting dialysis. MDRD equation is used to eGFR. Serum creatinine at day of dialysis, age of patient, gender and race are all recorded and entered in MDRD equation to calculate estimated GFR.

Underlying cause of ESRD is recorded. It includes diabetic nephropathy, glomerulonephritis, hypertension, renovascular disease, cystic disease, obstructive uropathy and others like lupus nephritis, Alport disease, and analgesic nephropathy…etc. Uncertain underlying causes is also recorded.

Type of dialysis modalities patients started on either HD or PD is recorded. Hemodialysis machine type is Fresenius 2008K, and FX800 dialyzer are used. During following up patients, mortality, switch of modality and change of renal replacement therapy into transplantation are recorded with the date of each event to calculate years each patient spends on dialysis. Moreover, date of referral to Sheffield Kidney

institute is recorded down and period from first referral to date of dialysis is calculated.

Comorbidity, which includes cardiovascular disease, cerebrovascular accident, cancer, access line complications and diabetes are all recorded. Patients with smoking history are considered as a smoker regardless of quantity of smoking. Number of admission and causes of admission to hospital is recorded for five years since started dialysis.

In both groups, mean value for variables were recorded for each patient on each year and for five years. The followings are variables that were taken:

- Hemoglobin level in g/dl is taken as indicator for anemia.
- Albumin in g/l nutritional status.
- Serum calcium level in mmol/l, parathyroid hormone (PTH) in ng/l, Phosphate in mmol/L are taken as indicator for bone disease.
- Kt/V is recorded reflecting adequacy of dialysis for both HD and PD.
- Systolic and Diastolic blood pressure
- HbA1c percentage for diabetic patients
- Serum cholesterol and triglyceride in mmol/l

Statistical analysis

The IBM Statistical Package for the Social Sciences (SPSS) program version 15 (installed on Microsoft Windows operating system) is used for statistical analysis of data. Parametric independent simple t-test is used to compare between mean values. Cross tables are formed and Chi square test is used to study difference between the two groups for categorical data. Continuous variables are changed into binary variable (0 and 1). The followings are cutoff points used based on Kidney Disease Improving Global Outcomes (KDIGO) guidelines (www.kdigo.org) [17]:

Patients have Hemoglobin below 11 g/dl assigned as 1, and 0 for 11 and above. Patients have albumin less than 30 g/L assigned as 1, and 0 to albumin level 30 or more. Calcium more than 2.6 mmol/l is considered as high, more than 1.8 mmol/l for phosphate and more than 300 ng/L for PTH. Patients on HD with Kt/v with 1.2 and more are achieved KDIGO target. In other hand, 2 is target for PD patients. Blood pressure above 130/80 is considered as high value. HbA1c more than or equal to 7% is taken as poor control for diabetic patients. More than 5 mmol/l for cholesterol and 1.69 mmol/l for triglycerides. Cox Regression is used to study covariates with years of survival with P value of less than 0.05 is considered significant. Hospitalization studied using number of admissions with linear Regression model. P value less than 0.05 is considered significant. Linear Regression is used to study number of admission to hospital and covariates

Results

Ninety patients are fulfilled criteria of the study, 3 patients have baseline eGFR >10, these patients were excluded from statistical analysis due to small number. Eighty-Seven patients are divided into two groups according to Baseline estimated GFR. Group 1 with eGFR less than 5 ml/min/1.73 m^2 consists of 45 patients who represent 52%. Group 2 with eGFR between 5-10 ml/min/1.73 m^2 consists of 42 patients (48%).

Patients' demography

Two groups did not show any significant difference in age of patients. Males are predominant in both groups and represent 68%, while females are 32%. Statistical analysis shows no significant difference in gender between two groups. White Caucasians are a majority of patients and represents 94%. There are no significant differences between the two groups. There are no significant differences in BMI and number of smokers between the two groups (Table 1).

Underlying cause of ESRD

Diabetic nephropathy was the major cause of ESRD over all, and predominantly in group 2 as it shown in table 2.

Table 1. Patients' demography

	Group 1	Group 2	Total	P value
Number of patients	45	42	87	
Mean Age	56.71	56.8		
18- 44 years	9 (10%)	8 (10%)	17 (20%)	0.97
45-65 years	22 (25%)	19 (22%)	41 (47%)	0.84
>65 years	14 (16%)	15 (17%)	29 (33%)	0.63
Male	28 (32%)	31 (36%)	59 (68%)	0.62
Female	17 (19%)	11 (13%)	28 (32%)	0.91
Caucasian	42 (48%)	40 (46%)	82 (94%)	0.66
Asian	3 (3%)	2 (3%)	5 (6%)	0.78
Mean BMI	25.434	25.718		
Underweight (<18.5)	2 (2%)	2 (2%)	4 (5%)	0.71
Normal (18.5-24.9)	22 (25%)	22 (25%)	44 (50%)	0.82
Overweight (25-29.9)	12 (13%)	10 (12%)	22 (25%)	0.6
Obese >30	9 (10%)	8 (10%)	17 (20%)	0.94
Smoker	10 (12%)	10 (12%)	20 (24%)	0.86

Table 2. Underlying causes of ESRD in the two groups

Underlying cause	Group 1 No. (%)	Group 2 No. (%)	Total
Diabetic nephropathy	2 (4%)	16 (17%)	18 (21%)
Glomerulonephritis	7 (8%)	1 (1%)	8 (9%)
Hypertension	3 (4%)	4 (4%)	7 (8%)
Renovascular disease	2 (2%)	1 (1%)	3 (3%)
Cystic disease	5 (5%)	4 (5%)	9 (10%)
Obstructive uropathy	4 (5%)	1 (1%)	5 (6%)
Uncertain	14 (16%)	6 (7%)	20 (23%)
Others	8 (10%)	9 (10%)	17 (20%)

Dialysis modality

From both groups (Group 1 and Group 2), 49% of patients are on HD and 51% patients are on PD. In group 1, there are 22 (25%) patients on HD, while 23 (26%) patients are on PD. In Group 2, 21 (24%) patients are on HD and 21 (24%) patients are on PD. There are no significant differences in dialysis modalities between both groups (Pearson chi-square significant=0.918).

Fate of patients

During five years of follow up, 31% of patients died, 18% have had transplanted kidney and 21% of them changed dialysis modality from PD to HD. Mean survival is 3.6±1.6 SD years for group1 and 3.3±1.7 SD years for group2. Comparing mean values of survival years showed no statistic significant (P value=0.398). During the first year, from date of dialysis, four (4%) patients died from group 1, and five (5%) from group 2. In the second year, three (3%) patients died from group 1, and four (4%) from group 2. In the third year, one (1%) patient

died from group 1 and 3 (3%) from group2. In the fourth year, 4 (4%) patients died from group1 while 3 (3%) patients died from group 2. Statistical analysis did not show any significant differences in number of deaths during the first, second, third and fourth year of follow up (Pearson Chi-square significant are 0.694, 0.282, 0.280, and 0.235 respectively).

There were no deaths recorded during the fifth year. There were no significant differences in the number of patients that have had a transplanted kidney and patients who changed PD into HD between group 1 and 2 (Pearson Chi-Square significant are 0.131, and 0.371 respectively) (Table 3).

Table 3. Fate of patients in form of mortality, transplantation and switch modality

Fate	Group 1 No. (%)	Group 2 No. (%)	Total
Mortality	12 (14%)	15 (17%)	27 (31%)
Transplanted	11 (12%)	5 (6%)	16 (18%)
Switch modality	11 (12%)	7 (9%)	18 (21%)

Comorbidity

Thirty percent of patients have Diabetes Mellitus (Figure 1), 30% of patients have history of myocardial infarction or ischemic heart disease, 6% of them have cancer. One patient has renal tumor as a primary cause of ESRD, other four patients have extra renal tumors. Seventeen percent of patients have had a stroke.

Anemia

Statistical analysis shows significant differences between the two groups in the first year of follow up. Group 1 is significantly lower in mean Hemoglobin than group 2 in the first year (Independent simple t test =0.19). The rest of the follow up years did not show any statistical significance in mean values of serum hemoglobin between the two groups (independent simple t test are 0.599, 0.77, 0.81, 0.83 in second, third, fourth and fifth year respectively) (Table 4).

Calcium, hosphate, and parathyroid hormone:

For Mean Serum Calcium level, statistical analysis shows no difference between group 1 and 2 on each year of follow up (P values 0.7, 0.5, 0.9, 0.8, 0.6 respectively). Mean Serum Phosphate and PTH again no difference between the two groups on each year of follow up (P values for phosphate are 0.07, 0.3, 0.2, 0.7, and 0.6 and for PTH are 0.4, 0.2, 0.2, 0.09, 0.2 respectively).

Cholesterol and triglyceride

Comparing the two groups, P values for Mean Cholesterol on each year of follow up are 0.2, 0.7, 0.3, 0.5, 0.3. P values for Mean Triglyceride are 0.4, 0.2, 0.1, 0.2 and 0.09. Mean Cholesterol and Triglyceride do not show statistic significant difference between group 1 and 2. Forty-eight %, 32%, 33%, 16%, 11% of patients have Cholesterol above target and 69%, 73%, 68%, 63%, 47% of patients have Triglyceride above target on each year of follow up respectively.

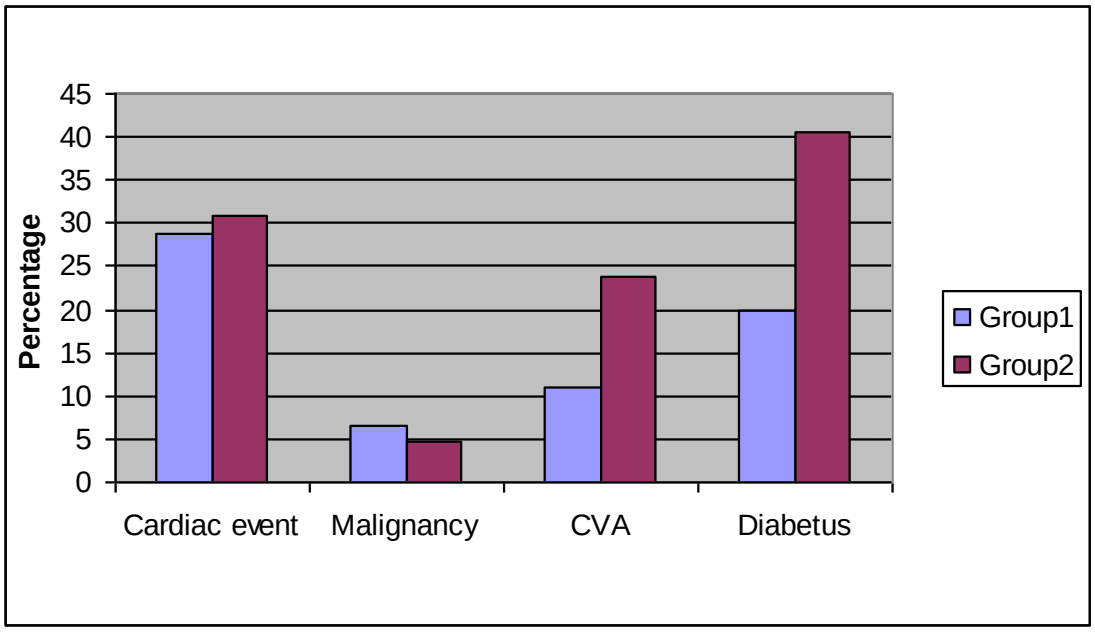


Figure 1. Comorbidity; cardiac events (myocardiac infarction, ischemic heart disease), cancer, CVA (Cerebrovascular accident), and diabetes in both groups

Table 4. Mean value for Serum Hemoglobin on each year of follow up

Year	Mean Hb g/dl		P value
	Group 1	Group 2	
1	9.6	10.2	0.19
2	10.9	11	0.599
3	11.3	11.2	0.77
4	11.4	11.3	0.81
5	11.5	11.6	0.83

Albumin

Statistical analysis shows no significant differences in mean Albumin between the two groups in all years except the third year when statistical analysis showed a significant difference (P value in each year of follow up as following:0.3, 0.5, 0.01, 0.1, 0.3). Sixteen %, 14%, 24%, 29%, 31% of patients have Albumin level below the target during each year of follow up respectively.

Systolic and diastolic blood pressure:

No statistic significances between the two groups in Mean systolic and diastolic blood pressure during the five years (P value 0.6, 0.2, 0.2, 0.9, 0.8 for systolic, 0.4, 0.2, 0.9, 0.2, 0.1 for diastolic). 82%, 68%, 77%, 79%, 74% of patients have systolic blood pressure higher than the target (130 mmHg) during the first, second, third, fourth, and fifth year respectively. 35%, 51%, 46%, 30%, 36% of patients have diastolic blood pressure above the target (80mmHg) during years of follow up respectively.

Hb1Ac

Statistical analysis shows significant difference in mean HbA1c% during first, second and

fourth year. Group 2 significantly have HbA1c% higher than group 1 in these years (P value 0.025, 0.041, and 0.02 in first, second and fourth year) while no statistic significant in third and fourth year (P value=0.2 each). Eleven %, 10%, 9%, 4% and 4% of patients have uncontrolled diabetes level during first, second, third, fourth and fifth year respectively.

Kt/V

Forty-five percent of patients achieve Kt/V target in the first year. 62%, 60%, 80% and 80% of patients achieve Kt/V target in second, third, fourth and fifth year respectively. No statistical significance between the two groups in the number of patients achieving Kt/v target during five years (Pearson Chi square=0.072, 0.410, 0.934, 0.945, 0.631)

Survival

Cox Regression is used to study covariates with years of survival. Age shows statistic significant covariates with years of survival in both groups. BMI shows significant with group1. All other covariates show no statistical significance with survival of patients in both groups (Table 5).

Table 5. Covariates in both groups and survival (Cox Regression P values)

Covariates	Group 1 (P value)	Group 2 (P value)
Age	0.03	0.026
BMI	0.034	0.99
Gender	0.3	0.2
Smoking	0.9	0.7
Dialysis modalities	0.4	0.9
Cardiovascular disease	0.5	0.1
Cancer	0.5	0.3
Cerebrovascular accident	0.07	0.4
Albumin	0.5	0.2
Calcium	0.3	0.6
Phosphate	0.4	0.9
PTH	0.4	0.2
Cholesterol	0.8	0.2
Triglyceride	0.9	0.3
Hemoglobin	0.8	0.7
Systolic blood pressure	0.3	0.2
Diastolic blood pressure	0.9	0.3
Diabetes	0.7	0.6
Years from referral to dialysis	0.1	0.3
Kt/V	0.8	0.5

Hospitalization:

Mean number of admissions to hospital for group 1 is 3.4±3.6 SD during five years and it is 3.1±3 SD. Independent simple t-test shows no significance between mean values of number of admission between the two groups (P=0.623). Linear Regression is used to study number of admission to hospital and covariates. Statistical analysis shows smoking is a significant covariate in number of admission in group 1. Patients admitted for peritonitis at least once have significant statistical analysis in association with the number of admission in group 2. All other covariates do not show significance with number of admission of patients for both groups (Table 6).

Table 6. P values of number of admissions and covariates in linear regression analysis

Covariates	Group 1 (P values)	Group 2 (P values)
Age	0.1	0.2
Gender	0.9	0.5
BMI	0.9	0.3
Smoking	0.04	0.3
Dialysis Modalities	0.2	0.1
Cardiovascular disease	0.6	0.1
Cancer	0.6	0.5
Cerebrovascular accident	0.3	0.08
Peritonitis	0.07	0.000
Access line complication	0.09	0.8
Calcium	0.3	0.8
Phosphate	0.5	0.5
PTH	0.5	0.6
Diabetes	0.9	0.2
Cholesterol	0.5	0.3
Triglyceride	0.2	0.5
Systolic blood pressure	0.9	0.7
Diastolic blood pressure	0.7	0.5
Kt/v	0.2	0.056
Years from referral to dialysis	0.1	0.1

Discussion

In this study, about two third of patients are males and it is in the same proportion in the two groups. In addition, gender is not a predictor factor for survival or number of admissions for both groups. Majority of patients are white Caucasian patients represent 94%. Small number of other ethnic group would not help as comparison group with white Caucasian group. Hence, ethnicity has not been studied as predictor for survival or number of admissions to hospital.

About half of patients are between 45-65 years old, and one third of patients are more than 65 years old. Age is a predictor for survival of patients in both groups. It is not surprising that increase in age would increase risk of death and that was a predictor in both groups.

Natures of underlying causes that lead to end stage renal disease are variable. About twenty percent of patients' cause of renal disease is diabetic nephropathy. Diabetic nephropathy is highest underlying cause of end stage renal disease. Therefore, helping diabetic patients by preventing them from getting into this level of renal injury might save patients and health system from devastating complications, preserve quality of life and enormous money spend. Diabetic patients in group 2 were higher in number than group 1 and that might explain higher deaths in group 2 although it was not statistically significant. Moreover, glycemic control was worse in group 2 than group 1 predominantly in first year, and it could be explained by renal residual function and insulin clearance. The lower estimated GFR results in lower insulin clearance and hence lower glucose levels. Therefore, group 1 (eGFR lower than 5 ml/min/1.73 m^2) had lower glucose levels than group 2.

Still underlying cause for a large number of patients is unidentifiable and that may give impression of late presentation with the disease when biopsy study would be useless. Smoking found as a covariate that predict number of admissions to hospital in group 1

but not group 2. Though, Smokers are equally distributed in both groups and represent 23% of all patients.

Dialysis modalities are not a predictor for survival or number of admissions to hospital. Half of patients on HD and other half on PD and same proportions are applied to each group. Patients on hemodialysis would have access line complications like infection, bleeding, clotting or obstruction of line, on other hands, patients on peritoneal dialysis are reliable for peritonitis, exit site infection, drain problem or leakage of catheter and hernia. One of the drawbacks of this study is that it did not show differences in number of admissions in associated with dialysis modality. Access line complications are not a predictor for number of admissions in both groups. On the other hand, peritonitis is a predictor for number of admissions in group 2 only but not in group 1. Number of admissions is not enough to reflect hospitalization. This Study should have conducted duration of admission and cost for admissions and compare that between the two groups.

Despite of no statistic difference in number of deaths between the two groups of patients in each year of follow up, Lead time bias should have been considered in this study when patients' residual renal function might affect morbidity and mortality. Variety in residual renal function should have been taken in account in this study (i.e., would appear to increase survival of those individuals starting dialysis with more residual renal function).

Regarding hemoglobin level, improvements in the level of hemoglobin are shown in the next year with half of patients whom have hemoglobin below the target level. This can be explained by starting the patients on erythropoietin stimulating agents and Iron therapy which they were not exposed to them previously. Patients tend to present with lower hemoglobin level in group 1 in comparison with group 2 in first year of dialysis. Again patients with delay presentation would have lower level of Hemoglobin in comparison to the ones started dialysis and treated earlier.

The IDEAL (Imitating Dialysis Early and Late) study (Cooper et al, 2010) was landmark randomized controlled trial. This trial studied patients with early dialysis (eGFR 10-15 ml/min/1.73 m^2) versus late dialysis (eGFR 5-7 ml/min/1.73 m^2) [18]. There was no advantage in the early dialysis regarding mortality and morbidity in similar way to this study but the difference was the early dialysis group were with higher eGFR. Still the results were similar though.

Conversely, multiple retrospective analyses from multiple cohorts demonstrate that patients who start dialysis at lower eGFRs have better survival, compared to those who start with higher levels eGFRs (Sawhney et al, 2009; Wright et al, 2010; Clark et al, 2011) [19,20].

This study concluded that it could not support or reject the hypothesis of early initiation of dialysis when all patients started dialysis with eGFR equal or below 10 ml/min/m^2 and not above this level. However, Patients started dialysis later with eGFR below showed worse anemia level than those with early initiation (eGFR between 5-10), which might give impression that early initiation of dialysis would avoid later complications. Since prolong history of anemia has dramatic effect on the heart and causing left ventricular hypertrophy. However, this tragedy can be definitely avoided by early correction of anemia and not necessarily an indication to start dialysis earlier per say.

On other hand, the group with earlier initiation of dialysis has worse glucose control than the ones started dialysis later and this is related to insulin clearance. However, this finding did not show death difference statically but the number of death were slightly higher though in the group with worse glycemic control.

With no significant difference in morbidity or mortality in patients with the group started dialysis early (eGFR 5-10) and whom started later (eGFR below 5), throughout the five years of follow up, there is no justification to decide on time to initiate dialysis based solely on eGFR level.

Statistical analysis has shown smoking in group 1 is the only predicator for number of admissions to hospital, while peritonitis is the predicator for group 2.

Acknowledgments
Thanks to Mrs. Denis Smith, secretary at Northern General Hospital - Renal Unit, and to Miss Jean Russell for her help in statistical analysis.

Conflict of interest
No conflict of interest.

Funding
Self-funded.

References
1. Ansell D, Feest T, Rao R, et al (eds). The Renal Association UK Renal Registry. Eighth Annual Report. 2005.
2. Stevens LA, Levey AS. Measurement of kidney function. In: Singh AK (ed). Medical Clinics of North America. Philadelphia: W.B. Saunders; 2005; p. 457.
3. Levey, A, Bosch, J, Lewis, J, et al. A more accurate method to estimate glomerular filtration rate from serum creatinine: A new prediction equation. Ann Intern Med. 1999, 130(6): 461-70. doi: http://dx.doi.org/10.7326/0003-4819-130-6-199903160-00002.
4. Kausz, AT, Obrador, GT, Arora, P, et al. Late initiation of dialysis among women and ethnic minorities in the United States. J Am Soc Nephrol 2000; 11(12): 2351-7.
5. Levey AS, Coresh J, Balk E, et al. National Kidney Foundation practice guidelines for chronic kidney disease: Evaluation, classification, and stratification. Ann Intern Med. 2003; 139(2): 137-47. doi: 10.7326/0003-4819-139-2-200307150-00013.
6. National Kidney Foundation. K/DOQI clinical practice guidelines for peritoneal dialysis adequacy. Am J Kid Dis. 1997, 30 (3, Suppl 2): S134-6.
7. Churchill DN, Blake PG, Jindal KK, et al. Clinical practice guidelines for initiation of dialysis. J Am Soc Nephrol. 1999;10 Suppl 13: S289-91.
8. Obrador GT, Arora P, Kausz AT, et al. Level of renal function at the initiation of dialysis in the U.S. end-stage renal disease population. Kidney Int. 1999; 56(6): 2227-35. doi: 10.1038/sj.ki.4491163.
9. U.S. Renal Data System. USRDS 2005 Annual Data Report: Atlas of End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases. Bethesda, MD.
10. Tattersall J, Greenwood R, Farrington K. Urea kinetics and when to commence dialysis. Am J Nephrol. 1995; 15(4):283-9. doi: 10.1159/000168850
11. US Renal Data System: USRDS 2004 Annual Data Report. The National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2004.
12. Ellis PA, Reddy V, Bari N, et al. Late referral of end-stage renal failure. QJM. 1998; 91(11): 727-32.
13. Ifudu O, Dawood M, Homel P, et al. Timing of initiation of uremia therapy and survival in patients with progressive renal disease. Am J Nephrol. 1998; 18(3): 193-8. doi: 10.1159/000013336
14. Roubicek C, Brunet P, Huiart L, et al: Timing of nephrology referral: Influence on mortality and morbidity. Am J Kidney Dis. 2000; 36(1): 35-41. DOI: 10.1053/ajkd.2000.8241.
15. Sesso R, Belasco AG. Late diagnosis of chronic renal failure and mortality on maintenance dialysis. Nephrol Dial Transplant. 1996; 11(12): 2417-20.
16. Fink JC, Burdick RA, Kurth SJ, et al. Significance of serum creatinine values in new end-stage renal disease patients. Am J Kidney Dis. 1999; 34(4): 694-701. doi: 10.1016/S0272-6386(99)70395-1.
17. Kidney Disease; Improving Global Outcomes Clinical Practice Guidelines: Official Journal of the International Society of Nephrology (Kidney International). 2012; 2 (Suppl 1): 2012.
18. Cooper BA, Branley P, Bulfone L, et al. A randomized controlled trial of early versus late initiation of dialysis. N Engl J Med. 2010; 363(7), 609-19. doi: 10.1056/NEJMoa1000552.
19. Sawhney S, Djurdjev O, Simpson K, et al. Survival and dialysis initiation; comparing British Columbia and Scotland registries. Nephrol Dial Transplant. 2009; 24(10): 3186-92. doi: 10.1093/ndt/gfp189.
20. Clark WF, Na Y, Rosansky SJ, et al. Association between estimated glomerular filtration rate at initiation of dialysis and mortality. CMAJ 2011; 183(1): 47-53. doi:10.1503/cmaj.100349

E-mail: jwad78@gmail.com
Received Mar. 18th 2018
Accepted Jun. 26th 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Hodgkin Disease in Children

Sawsan S. Abbas[1] *CABP*, **Ruaa H. Ali**[2] *FICMS*

[1]Dept. of Pediatrics, College of Medicine, Al-Nahrain University, Baghdad, Iraq, [2] Dept. of Pediatrics, Al-Imamein Al-Kadhimein Medical City, Baghdad, Iraq

Abstract

Background Hodgkin lymphoma (HL) is a malignant process involving the lympho-reticular system that accounts for 6% of childhood cancers. Infectious agents may be involved, such as human herpes virus 6, cytomegalovirus, and Epstein-Barr virus (EBV). Infection with EBV confers a 4-fold higher risk of developing HL.

Objective To have an idea about the mode of presentation, pathological subtypes, treatment applied and outcome in one center in our country.

Methods A retrospective study was done whereby twenty cases were collected from the Pediatric Oncology Clinic in Al-Imamein AL-Kadhimein Medical City over a period of ten years from the first of January 2007 – end of December 2016. Information was taken from the patient's records in the Pediatric Oncology Clinic including age at presentation, sex, physical finding, histopathological subtypes, staging , treatment applied and outcome.

Results Among the studied group, 14 cases (70%) were males and 6 cases (30%) were females, male: female ratio equal to 2.3:1, rang of age was between 5-12 years with a peak age at presentation was between 5-7 years, 12 cases (60%). The initial presentation was an enlarged cervical lymph node in 19 of them (95%), histopathologically, most of the patients had mixed cellularity subtype, 9 cases (45%), stage II and stage III comprise the majority of cases 9 cases (45%), 6 cases (30%) respectively, B symptoms were reported in 7 cases (35%). Chemotherapy was the mainstay of treatment with good response and remission, 17 cases (85%). Three cases refractory to treatment (15%) with no response, no death was reported.

Conclusion Male predominance was noticed with younger age group at presentation. The majority presented with enlarged cervical lymph node. Although mixed cellularity was encountered in most of the patients, the response to chemotherapy was good. Rituximab can be used along with chemotherapy for refractory cases and for lymphocyte predominant Hodgkin lymphoma.

Keywords Hodgkin Disease , Children

Citation Abbas SA, Ali RH. Hodgkin disease in Children. Iraqi JMS. 2018; 16(4): 424-429. doi: 10.22578/IJMS.16.4.10

List of abbreviations: ABVD = Adriamycin, Bleomycin, Vinblastine, Dacarbazine, ABVP = Adriamycin, Bleomycin, Vinblastine, Predinsolone, CD = Cluster of differentiation, Chlvpp = Chlorambucil, Vinblastine, Procarbazin, Prednisolone, cHL = classic Hodgkin lymphoma, EBV = Epstein-Barr virus, HL = Hodgkin lymphoma, L&H = Lymphocytic and histiocytic, LP-HL = Lymphocyte predominance Hodgkin lymphoma, LPHD = Lymphocyte predominance Hodgkin disease, REAL = Revised European American lymphoma, UKCCSG: United Kingdome Children's Cancer Study group, WHO = World Health Organization

Introduction

Hodgkin lymphoma (HL) is a malignant process involving the lympho-reticular system that accounts for 6% of childhood cancers [(1)]. Infectious agents may be involved, such as human herpes virus 6, cytomegalovirus, and Epstein-Barr virus (EBV). Infection with EBV confers a 4-fold higher risk

of developing HL [1]. A significant male-to-female ratio of 3:1 is observed in children younger than 10 years. In older children and adults, the male-to-female ratio is about 1:1 [2]. The REAL/WHO classification recognizes a basic distinction between lymphocyte predominance HL (LP-HL) and classic HL (cHL), reflecting the differences in clinical presentation and behavior, morphology, phenotype, and molecular features. cHL has been classified into four subtypes: lymphocyte rich, nodular sclerosing, mixed cellularity, and lymphocyte depleted [3]. Treatment in children evolved from extended- field radiation therapy to the use of multi- agent chemotherapy [1]. CD20 is detected in the malignant lymphocytic and histiocytic (L&H) cells of almost all Hodgkin disease of lymphocyte predominance (LPHD) type, the neoplastic Reed Sternberg cells of classical Hodgkin disease also express CD20 with a reported frequency ranging from less than 5% to more than 50% of tumors [4]. Onconging clinical trials report encouraging results with the use of CD20 antibody (rituximab) particularly in lymphocyte predominant Hodgkin lymphoma [1,5] where trials in relapsed disease have shown an overall response rate of 94% [1]. Other trials, was rituximab as a single agent in refractory or recurrent cHL, rituximab plus gemcitabine in refractory or recurrent cHL, and rituximab plus ABVD in newly diagnosed cHL [6]. CD20 is involved in the regulation of human B-cell growth and differentiation [6]. Rituximab targets the surface proteins of malignant Hodgkin cells and Reed Sternberg cells and eliminates the CD20 reactive B lymphocytes which support the Hodgkin Reed Sternberg cells in whom CD20 is also positive thus depriving the malignant cells of survival and causing cell death [6].

The objective of this study was to have an idea about the mode of presentation, histopathological subtypes, treatment applied and outcome in one center in our country.

Methods

A retrospective descriptive study was done where by twenty cases (17 cases with initial remission and response to treatment, 3 cases refractory to treatment, non with relapse) were collected from the Pediatric Oncology Clinic in Al-Imamein AL-Kadhimein Medical City over a period of ten years from the first of January 2007 – end of December 2016. Information was taken from the patient's record in the Pediatric Oncology Clinic including age at presentation, sex, physical finding, histopathological subtypes, staging, treatment applied and outcome. Work up done including history looking for B symptoms as fever, night sweats and weight loss, investigations recorded including complete blood picture, liver function test, renal function test, chest X-ray, ultrasound and bone marrow examination; the diagnosis was established by lymph node excisional biopsy or fine needle aspirate, staging was done according to the Ann Arbor staging system [7]. Chemotherapy was the mainstay of treatment; favorable response is considered when there is (50%) or greater reduction in a measurable lymph node [8]. Numerical data and percentage were estimated and tabulation were done using Microsoft Excel program.

Results

Among the studied group 14 cases (70%) were males and 6 cases (30%) were females, male: female ratio equal to 2.3:1, age range was between 5-12 years with 12 cases (60%) between 5-7 years as it is shown in table (1).

The initial presentation was an enlarged cervical lymph node in 19 cases (95%) as it is shown in table (2).

Histopathological findings are shown in table (3), which showed that most of the patients had mixed cellularity subtype; 9 cases (45%).

Staging is shown in table (4), stage II and stage III comprise the majority of cases, 9 cases (45%) and 6 cases (30%) respectively. B symptoms were reported in 7 cases (35%). Chemotherapy was the mainstay of treatment even in stage I as these patients had the mixed cellularity

subtype, as per the Hodgkin's disease UKCCSG protocol and the parents refused radiotherapy, ABVD (Adriamycin, Bleomycin, Vinblastine, Dacarbazine) was used in 18 cases (90%) with ABVP protocol (Adriamycin, Bleomycin, Vinblastine, Predinsolone) in 2 cases (10%).

Table 1. Age at presentation and sex distribution

Age at presentation / years	Male		Female	
	No.	%	No.	%
< 5	/	/	/	/
5-7	9	45	3	15
8-10	4	20	2	10
>10	1	5	1	5
Total	14	70	6	30

Table 2. The initial presentation of patients

Initial presentation	No.	%
Cervical lymph node enlargement	19	95
Axillary lymph node enlargement	0	0
Inguinal lymph node enlargement	0	0
Parotid mass	1	5
Pallor	3	15
Hepatomegaly	3	15
Splenomegaly	3	15
Mediastinal mass	1	5
Para aortic lymph nodes	3	15
Bone marrow involvement	0	0
Total	20	100

Note: Some patients had more than one physical finding as initial presentation

Table 3. Histopathological sub types

Histopathological sub types	No.	%
Mixed cellularity	9	45
Lymphocyte predominant	7	35
Nodular sclerosis	4	20
Lymphocyte depletion	/	/
Total	20	100

Table 4. Staging of the disease at diagnosis

Stage	No.	%
I	5	25
II	9	45
III	6	30
IV	0	0
Total	20	100

No patient received radiotherapy as it is shown in table (5), 17 cases (85%) showed good response with complete remission and cure, three cases (15%) were refractory to the initial treatment with no response as shown in table (6). Refractory cases received alternate ABVD and ChIVPP (Chlorambucil, Vinblastine, Procarbazin, prednisolone) but they were resistant to treatment, review of the histological subtype revealed lymphocyte predominant subtype and positive CD20, so rituximab was added, the response to rituximab was good with remission and cure noticed as it is shown in table (5).

The outcome of the treatment is shown in table (6), 17 cases (85%) showed complete remission with good response to treatment and were cured, 3 cases (15%) were refractory to treatment, they responded well after rituximab was added, underwent complete remission and cured, no one relapsed and no death reported.

Table 5. Treatment applied for the patients with Hodgkin disease

	Protocol applied	No.	%
Newly diagnosed	ABVD	18	90
	ABVP	2	10
	Radiotherapy	0	0
Refractory to initial treatment	Alternate ABVD and ChVIPP + Rituximab*	3	15
Total		20	100

*Rituximab added to refractory cases with positive CD20

Note: Total patients were 20, 3 of them were refractory and received new protocol of chemotherapy plus rituximab

Discussion

In this study, the age of the patients ranges between (5-12) years with a peak age at presentation of (5-7) years, which is nearly equal to a study done in Iran, 2002 (5-9 year) [9] but younger than the French study (4-16 year), 2003 [10] as well as a study done in Korea, 2012 (more than 16 years) [11].

Males were affected more than females, which goes with that reported by studies done in USA, Iran, France [1,9,10] and that reported by the National Cancer Institute [12].

Cervical lymph node involvement is reported in (95 %) of cases, which goes with studies done in USA and Bulgaria respectively [1,13].

Table 6. Outcome of the disease according to the stage of the disease

Stage	No. of patients with favorable response and complete remission	No. of patients who refuse treatment	No. of refractory cases (received rituximab)	No. of relapsed cases	No. of patient lost to follow up	No. of death
I	5	0	0	0	0	0
II	8	0	1	0	0	0
III	4	0	2	0	0	0
IV	0	0	0	0	0	0
Total	17	0	3	0	0	0
%	85	0	15	0	0	0

The most common histopathological subtype was mixed cellularity (45%) indicating more severe and more aggressive disease, this pattern is similar to that reported in other countries. It was (66.7%) in South Iran [9], (50%) in South Taiwan [14], (28%) in Jordan [15], (25%) in the United Kingdom [16]. Where in Jordan and United Kingdome the most common histopathological subtype was nodular sclerosis, (49%, 66%) respectively [15,16].

Most of the patients were stage II and III (75%) compared with other studies stage II and III were (65.8%) in Iran [9] while in the French study the majority (89%) were stage I and II [10] and in the Istanbul study (54%) were stage I–II and (46%) were stage III-IV [17]. This may be explained by the parents' awareness when noticing abnormal and unusual lymph node enlargement and early referral by doctors to the oncology centers. Thirty-five percent had B symptoms, which is less than studies done in Iran (57%) [9] and Istanbul (46%) [17]. This may be explained by the younger age group of our patients as older patients are more likely to have B symptoms at presentation [9].

Radiation therapy was not used as parents refused in some cases, or the early appointments were not available of note, current children oncology group trials investigated whether radiation therapy can be eliminated altogether in patients who have good rapid and early response to pre-radiation induction chemotherapy [1].

Chemotherapy was not regularly available; the parents need to buy some of them from private pharmacies.

Chemotherapy was the mainstay of treatment with complete remission and response reported in (85%) of cases. This can be explained by the use of ABVD and ABVP protocols both of which were found to be very effective chemotherapy for the treatment of Hodgkin lymphoma [1,7]. Alternate ABVD, ChIVPP are good combination for refractory cases [7] but because of resistance to treatment Rituximab was added, particularly after the cases were found to be of lymphocyte predominant subtype with positive CD20 [1]. In this study the response to treatment after adding rituximab in refractory cases was good in term of remission and disappearance of the mass in the three refractory cases (15%). In comparison with studies done elsewhere, Oki showed promising early results with rituximab. [6] while King et al showed that in patients with advanced-stage (III-IV) disease, there was no significant difference between rituximab and non-rituximab therapies [18].

This study concluded that male predominance was noticed with younger age group at presentation. The majority presented with enlarged cervical lymph node. Although mixed cellularity was encountered in most of the patients, the response to chemotherapy was good. Rituximab can be used along with

chemotherapy for refractory cases and for lymphocyte predominant Hodgkin lymphoma.

Acknowledgments

To all children with malignancy who fight for their survival.

Author Contribution

Both authors contributed to this manuscript. They coordinated study recruitment, implementation and progress of this study and helped with data interpretation and manuscript organization and editing.

Conflict of interest

The authors have no conflict of interest.

Funding

Self-funding.

References

1. Hochberg J, Giulino-Roth L, Cairo MS. Hodgkin lymphoma. In: Kleigman RM, Stanton BF, Schor NF (eds). Nelson Textbook of Pediatrics. 20th ed. Philadelphia: Elsevier, WB Saunders company; 2016. p. 2445-7.
2. Percy CL, Smith MA, Linet M, et al. Lymphomas and reticuloendothelial neoplasms. In: Ries LA, Smith MA, Gurney JG, et al. (eds). Cancer incidence and survival among children and adolescents: United States SEER Program 1975-1995. Bethesda, Md: National Cancer Institute, SEER Program, 1999. p. 35-50.
3. Piccaluga PP, Agostinelli C, Gazzola A, et al. Pathobiology of Hodgkin lymphoma. Adv Hematol. 2011; 2011: 920898. doi: 10.1155/2011/920898.
4. Raj JA, Bhuvaneshwari. Diagnosis of Hodgkin disease. Int J Sci Res. 2017; 6(6): 1895-7.
5. Shanker AG, Kirkwood AA, Hall GW, et al. Childhood and adolescent lymphocyte predominant Hodgkin lymphoma: A review of clinical outcome based on histopathological variant. Br J Haematol. 2015; 171(2): 254-262. doi: 10.1111/bjh.13540.
6. Oki Y, Younes A. Does rituximab have a place in treating classic Hodgkin lymphoma? Curr Hematol Malig Rep. 2010; 5(3): 135-9. doi: 10.1007/s11899-010-0052-z.
7. United Kingdom Children's Cancer Study Group, Hodgkin disease protocol. 1999. p. 1-18.
8. Moghbel MC, Kostakoglu L, Zukotynski K, et al. Response assessment criteria and their applications in lymphoma: Part 1. J Nucl Med. 2016; 57(6): 928-35. doi: 10.2967/jnumed.115.166280.
9. Karimi M, Yarmohammadi H, Ghavanini AA, et al. Epidemiological surveillance of pediatric Hodgkin disease in South Iran. Med Sci Monit. 2002; 8(8): CR572-5.
10. Pellegrino B, Terrier Lancomb MJ, Obelin O, et al. Lymphocyte predominant Hodgkin lymphoma in children, therapeutic abstention after initial lymph node resection, A study of French Society of Pediatric Oncology. J Clin Oncol. 2003; 21(15), 2948-52. doi: 10.1200/JCO.2003.01.079.
11. Won YW, Kwon JH, Lee SI, et al. Clinical features and outcomes of Hodgkin lymphoma in Korea: Consortium for Improving Survival of Lymphoma (CISL). Ann Hematol. 2012; 91(2): 223-33. doi: 10.1007/s00277-011-1297-x.
12. Ries LA, Kosary CL, Hankey BF, et al. SEER Cancer Statistics Review 1973-1995. Bethesda, Md: National Cancer Institute, 1998. Last accessed August 30, 2017.
13. Petkova M, Stanimirov P, Videnova L. Hodgkin disease in children - a clinical case. Int J Sci Res. 2017; 6(11): 810-813.
14. Chuang SS, Lin CN, Li CY. Malignant lymphoma in South Taiwan according to Revised European – American classification of lymphoid neoplasm. J. Cancer. 2000: 89(7): 1586-92. doi: https://doi.org/10.1002/1097-0142(20001001)89:7<1586::AID-CNCR24>3.0.CO;2-1.
15. Sughayer MA, Haddad HA, Al-Yousef RA, et al. Epstein–Barr virus and Hodgkin lymphoma in Jordan, Hematol Oncol Stem Cell Ther. 2014; 7(2): 85-9. doi: 10.1016/j.hemonc.2013.
16. Nachman GB, Spoto R, Herzog P, et al. Randomized comparison of low dose involved field radiotherapy and no radiotherapy in children with Hodgkin disease who achieve a complete response to chemotherapy. J Clin Oncol. 2002; 20(18): 3765-71. doi: 10.1200/JCO.2002.12.007.
17. Kebudi R, Buyukkapu SB, Gorgun O, et al. Risk adapted treatment in childhood Hodgkin lymphoma: outcome and changing epidemiologic features in 25 years. Blood. 2016; 128: 4158.
18. King MT, Donaldson SS, link MP, et al. Management of lymphocyte predominant Hodgkin lymphoma in modern era. Int J Radiat Oncol Biol Phys. 2015; 92(1): 67-75. doi: 10.1016/j.ijrobp.2015.02.001.

Correspondence to Dr. Sawsan S. Abbas
E-mail: sawsansati@yahoo.com
saw-195@colmed-alnahrain.edu.iq
Received May 15th 2018
Accepted Sep. 27th 2018

Iraqi JMS

Published by Al-Nahrain College of Medicine
P-ISSN 1681-6579
E-ISSN 2224-4719
Email: iraqijms@colmed-alnahrain.edu.iq
http://www.colmed-alnahrain.edu.iq
http://www.iraqijms.net
Iraqi JMS 2018; Vol. 16(4)

Detection of Some Biofilm Genes Related with Multidrug-Resistant in *Acinobacter baumannii* Isolated from Clinical Isolates

Sura S. Talib *MSc*, **Thanaa R. Abdulrahman**[1] *PhD*, **Shatha H. Ali**[2] *PhD*

[1]Dept. of Microbiology, [2]Dept. of Pediatrics, College of Medicine, Al-Nahrain University, Baghdad, Iraq

Abstract

Background *Acinetobacter baumannii* (*A. baumanii*) has recently emerged as a major pathogen causing nosocomial infections in patients admitted to intensive care units with a surprisingly rapid acquisition of antibiotic resistance.

Objective To study the rate of occurrence of *A. baumanii* in different clinical samples and to investigate the association between biofilm formation and presence of ompA and bap genes in multi-drug resistance isolates.

Methods A total of 150 clinical samples were collected from (blood, sputum, urine, wound swab) during a period from the first of October 2017 to the end of March 2018 from Al-Imamein Al-Kadhimein City, Central Teaching Hospital of Pediatrics, Welfare Children Protection in Medical City and Al-Yarmouk Teaching Hospital and tested against 14 antibiotics by disc diffusion method. Quantitative microtiter plate assay was done for detection of biofilm formation. Polymerase chain reaction (PCR) was performed to detect ompA and bap genes.

Results There were 75 *A. baumanii* isolated from different clinical samples as follows: 41 from blood, 13 from wound, 12 from sputum and 9 from urine. The results of antimicrobial susceptibility test showed, high rate of resistance to Aztronem (94.7%) followed by Cefotaxime (89.3%), Cefepim (86.7%), Meropinem (86.7%), Ceftriaxone (86.7%), Ceftazidime (85%), Gentamicin (85%), and Piperacillin (82.7%) respectively. Moderate - to - low rate of resistance to Ciprofloxacin (78%), Impenim (46.7%), Levofloxacin (46%), Amikacin (44%), Tigacycline (42.3%) and Colistin (44%). The detection of biofilm formation showed that (52%) of isolate produce biofilm and the prevalence of ompA gene was 86.7% while the prevalence of Bap-gene was 34.7%.

Conclusion High frequency of *A. baumannii* infection was observed in different hospitals in Baghdad. More than half of the isolates were biofilm producer and there is highly significant association between the presence of bap gene and the biofilm formation but not with ompA gene.

Keywords *Acinetobacter baumannii*, ompA, Bap, MD

Citation Talib SS, Abdulrahman TR, Ali SH. Detection of some biofilm genes related with multidrug-resistant in *Acinobacter baumannii* isolated from clinical isolates. Iraqi JMS. 2018; 16(4): 430-438. doi: 10.22578/IJMS.16.4.11

List of abbreviations: Bap = Biofilm associated protein, bp = Base pair/ base pairs, CFU = Colony forming unit, CLSI = Clinical & Laboratory Standards Institute, DNA = Deoxy Ribo Nucleic Acid, MDR = Multi drug resistant, OMPA = Outer membrane protein A, PCR = Polymerase chain reaction

Introduction

Acinetobacter baumannii (*A. baumanii*) is a Gram-negative bacterium related to hospital-acquired infection, especially in intensive care unit (ICU) where it causes bacteremia, pneumonia, meningitis, urinary tract infection and wound infection [(1)]. Serious infections caused by *A. baumanii* are usually cured by imipenem as an efficient drug of choice. However, reports of imipenem-resistant *A. baumannii* strains have been increasing significantly over recent years and these isolates are often multidrug-resistant (MDR) [(2)]. One of the major factor involved in bacterial resistance to antimicrobial, chronic infections or survival in varying environments is

the ability to form biofilms. Biofilms are complex mixtures of microbes which are predominantly attached to hard surfaces. They are often enclosed by thick polysaccharide layer which makes them resistant to antibiotics and thus very hard to eliminate [3]. On the other hand, sensitivity to different antibiotics as well as microbial metabolism due to biofilm formation will be reduced. This is attributable to lack of food in the biofilm depth. Slower metabolism and antibiotic resistance lead to bacterial dissemination which can create a quick critical situation [4]. There is a variety of virulence determinants involved in biofilm formation of *A. baumannii*. This bacterium produces a molecule called the biofilm-associated protein (Bap), which is encoded by the bap gene [5].

BAP contributes to the initiation of biofilm production after *A. baumannii* attaches to a particular surface [6]. Bap is a member of a group of surface proteins, which have high molecular mass with tandem repeats of domains involved in intercellular adhesion. Bap is a large protein (854 kDa) characterized in a bloodstream isolate of *A. baumannii* and Loehfelm et al., 2008 [7] suggested the development and thickness of the mature biofilm structure was associated with this protein.

OmpA is a porin and one of the major protein in the outer membrane with a molecular mass of 38 kDa. Data have shown that this protein plays a role in biofilm formation on plastic, and also in the interaction of human epithelial cells and *Candida albicans* filaments [8].

The objectives of this study was to study the rate of occurrence of *A. baumanii* in different clinical samples and to investigate the association between biofilm formation and presence of OMPA and BAP genes in MDR isolates.

Methods

A total of 150 patients were enrolled in this study. Different clinical samples (blood, sputum, urine, wound swab) were collected during a period from the first of October 2017 to the end of March 2018 from Al-Imamein Al-Kadhimein Medical City, Central Teaching Hospital of Pediatric, Welfare Children Protection in Medical City and Al-Yarmouk Teaching Hospital/Baghdad. Bacteria were isolated and identified by ordinary methods according to morphological characteristics on cultures and by biochemical tests in the laboratories of Microbiology Department, College of Medicine, Al-Nahrain University. A loopful of pure colony was used to confirm identification by the phenotypic VITEK-2 Systems method.

Antibiotic susceptibility testing

Seventy-five of *A. baumannii* isolates were tested for their susceptibility to fourteen antimicrobial agents include Aztronem, Cefotaxime, Ceftriaxone, Ceftazidime, Ciprofloxacin, Levofloxacin, Tigacycline, Peperacillin, Colistin, Gentamicin, Amikacin, Cefepim, Imipenem and Meropinem in accordance to Clinical & Laboratory Standards Institute (2016) recommendations.

Detection of biofilm formation.

Quantitative microtiter plate assays for biofilm formation were performed according to Brossard and Campagnari (2012) [9] as follows: A 100 µl of *A. baumannii* broth turbidity equal to 0.5 McFarland and an equal volume of Luria Bertain (LB) broth supplemented with 20% glucose were added to each well in 96-well polystyrene microtiter plates. The plates were incubated overnight at 37 °C. The cultures were softly removed. The wells were washed three times with phosphate buffered saline. The adherent cells were fixed with absolute methanol for 10 min, stained with 0.4 % crystal violet for 15 min, washed three times with sterile distilled water and then allowed for air-dried. The plates were filled with 250 µl of 33 % acetic acid and waited for 15 min. The reading of OD at 595 nm absorbance was determined.

Chromosomal DNA of *A. baumannii* isolates was extracted using genomic DNA extraction

kit (Promega, USA). The extracted DNA used as a template in Polymerase chain reaction (PCR).
Specific primer sequences of OmpA, and Bap were amplified by PCR, table (1). Twenty-five microlitter of PCR mixture reaction were prepared according to the program published by Badmasti et al. in 2015 [10].

Table 1. Sequences and products of ompA and Bap genes

Genes		Nucleotide sequences (5' ⟶ 3')	Products bp	GenBank References
OmpA	F	GTTAAAGGCGACGTAGACG	578	Accession number AY485227
	R	CCAGTGTTATCTGTGTGACC		
Bap	F	ATGCCTGAGATACAAATTAT	1449	Accession number KR080550.1.
	R	GTCAATCGTAAAGGTAACG		

The primers diluted by adding nuclease free water depending on the manufacture companies' instructions
The master mix contents were thawed before use at room temperature, and the PCR master mix was prepared on a separate biohazard safety cabinet with wearing hand gloves at all times to avoid contamination.
2.0 µl of each forward and revers specific primers was mixed with 12.5 µl PCR master mix tubes, 3 µl of DNA template was added then the volumes were completed to 25 µl by nuclease free water.
A 25 µl of the PCR mixture were spun down with a mini centrifuge.
The tubes were placed on the PCR machine and the PCR program with the cycling conditions was installed according to, the Cleaver Scientific Thermal Cyclers TC32/80 was used for all PCR amplification reactions.
Initial denaturation step at 95 °C for 60 seconds, denaturation step 94 °C, annealing step 50 °C, extension 72 °C for 60 seconds and final extension 72 °C for 3 minutes, total of cycles 22 cycle.
A 7 µL of each amplified sequence and 100 bp ladder resolved by electrophoresis according to Sambrook and Russell (2001) [11]. The products were visualized in UV Tran- illuminator (LKB, Sweden).

Results

There were 75 *A. baumanii* isolates from different clinical samples as follows: 41 from blood, 13 from wound, 12 from sputum and 9 from urine. This study includes 44 males and 31 females. The percentage was 58.7% and 41.3% of males and females respectively.

Antibiotics by disc-diffusion method

The results of antimicrobial susceptibility test are showed in table (2).

Detection of biofilm formation in *A. baumannii* isolates and its association with antibiotic resistance

The current study showed that (52%) of *A. baumannii* isolates were produced biofilm while (48.0%) not biofilm producer.
All biofilm producer isolates were resistant to four or more antibiotics of different classes, which considered as Multidrug resistance (MDR). This study showed that, out of 39 biofilm producer there were 12 isolates sensitive to tigacyclin and 10 isolates sensitive to colistin, in addition to one isolate was sensitive to peperacillin. Statistically there is significant association between biofilm and antibiotic resistance (Table 3).

Table 2. Susceptibility of *A. baumannii* to different antibiotics using disc-diffusion method

Antibiotic disc	Sensitive	Resistance	Intermediate
Amikacin	25 (33.3%)	33 (44.0%)	17 (22.7%)
Aztronem	4 (5.3%)	71 (94.7%)	0 (0.0%)
Ceftriaxone	10 (13.3%)	65 (86.7%)	0 (0.0%)
Ceftazidime	11 (14.7%)	64 (85.0%)	0 (0.0%)
Cefepim	10 (13.3%)	65 (86.7%)	0 (0.0%)
Cefotaxime	8 (10.7%)	67 (89.3%)	0 (0.0%)
Colistin	36 (48.0%)	31 (41.0%)	8 (10.7%)
Ciprofloxacin	12 (16.0%)	59 (78.0%)	4 (5.3%)
Levofloxacin	31 (41.0%)	35 (46.0%)	9 (12.0%)
Impenim	32 (42.7%)	35 (46.7%)	8 (10.7%)
Meropinem	9 (12.0%)	65 (86.7%)	1 (1.3%)
Tigacycline	35 (46.7%)	31 (41.3%)	9 (12.0%)
Gentamicin	6 (8.0%)	64 (85.0%)	5 (6.7%)
Piperacillin	13 (17.3%)	62 (82.7%)	-

Gene amplification

By PCR essay, the amplification of ompA and Bap genes in chromosomal DNA of *A. bamannii* was produced an amplicon of size 578 bp and 1449bp respectively (Figures 1 and 2). The present results also showed that, the prevalence of ompA gene was 86.7% in DNA of isolates, while the prevalence of Bap-gene was 34.7%.

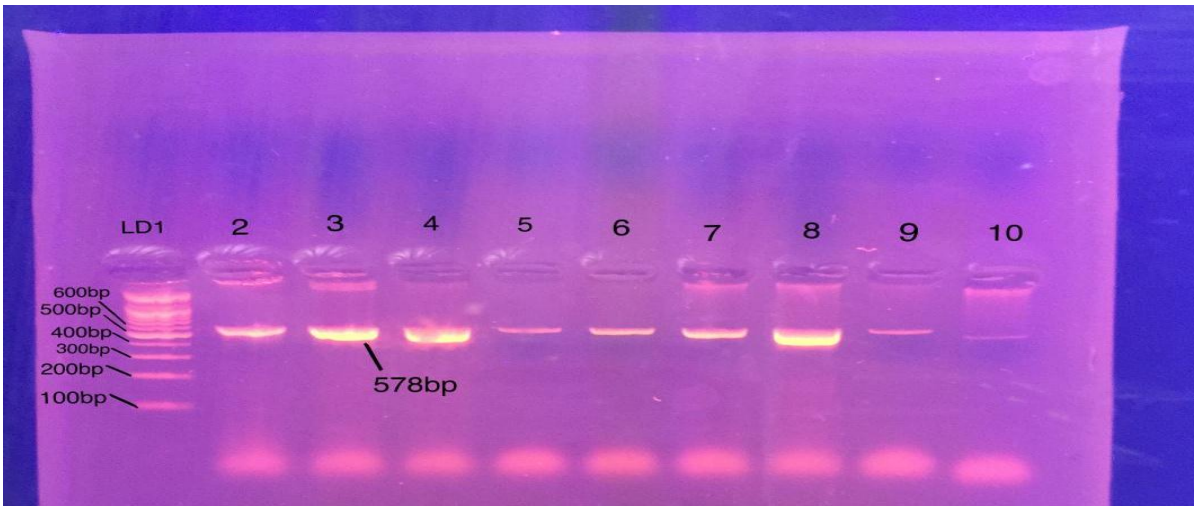


Figure 1. Gel electrophoresis of amplified ompA gene(578bp). Lane 1: 100 bp ladder. Lanes 2-10: Clinical isolates showing positive result (1.5% agarose, 7 v/cm^2, 1.5 hrs)

Table 3. The association between biofilm and antibiotic resistance

Antibiotic		Biofilm		P value
		Negative	Positive	
AK	I	8	0	0.000
	R	9	39	
	S	19	0	
AZT	R	32	39	0.004
	S	4	0	
CRO	R	27	39	0.001
	S	9	0	
CAZ	R	26	39	0.000
	S	10	0	
FEP	R	27	39	0.001
	S	9	0	
CTX	R	28	39	0.002
	S	8	0	
CO	I	5	0	0.000
	R	11	29	
	S	20	10	
CIP	I	2	0	0.002
	R	28	39	
	S	6	0	
LEVO	I	3	0	0.000
	R	13	39	
	S	20	0	
IMP	I	5	0	0.000
	R	11	39	
	S	20	0	
MEM	R	32	39	0.004
	S	4	0	
TG	I	3	0	0.005
	R	16	27	
	S	17	12	
GEN	I	4	0	0.003
	R	28	39	
	S	4	0	
PIP	R	27	38	0.005
	S	9	1	

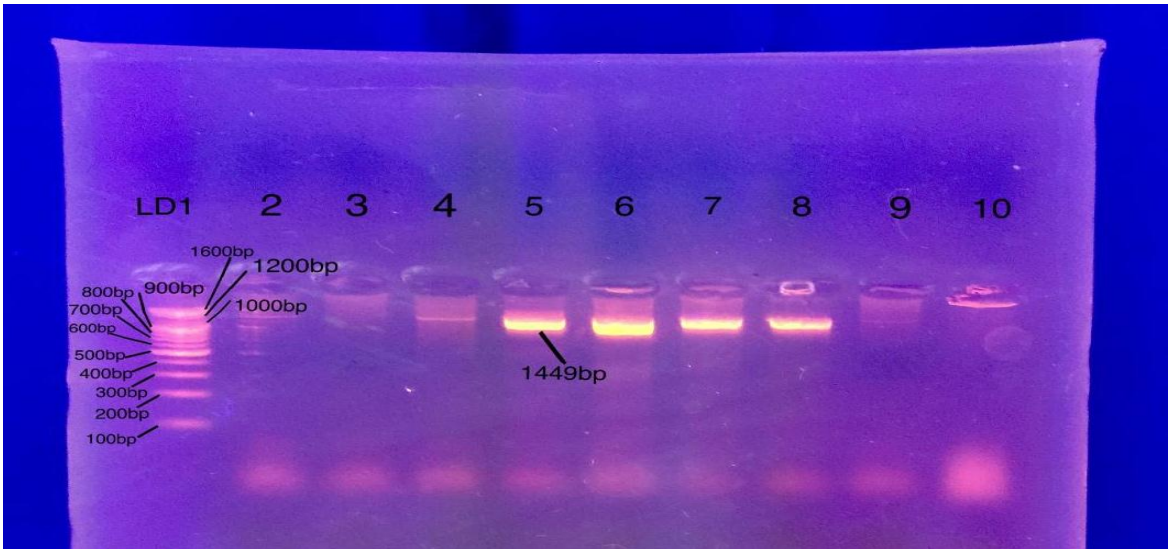


Figure 2. Gel electrophoresis of amplified Bap-gene (1449 bp) in genomic DNA using PCR with specific primers Lanes 4-8: Clinical isolates showing positive result. (1.5% agarose, 7 v/cm^2, 1.5 hrs)

Association between biofilm formation and presence of ompA and bap genes

Out of 39 isolates that produce biofilm there were 92.3% possessed ompA gene while out of 36 isolates that non biofilm producer there were 80.6% of isolates possessed ompA. Statistically there is no significant association between biofilm and presence of ompA.

On the other hand, the present study found that out of 26 isolates that possessed Bap gene there is 64% of isolates were biofilm producer and only 2.8% non-biofilm producer, statistically there is significant association between the presence of Bab gene and biofilm formation as shown in table (4).

Table 4. Association between biofilm and presence of ompA and Bap genes

Biofilm	omp A		Bap	
	Negative	Positive	Negative	Positive
Negative	7 (19.4%)	29 (80.6%)	35 (97.2%)	1 (2.8%)
Positive	3 (7.7%)	36 (92.3%)	14 (35.9%)	25 (64.0%)
Total	10 (13.3%)	65 (86.7%)	49 (65.0%)	26 (34.7.0%)
P value	0.124 * Ns		0.001* S	

*Ns: non-significant

* S: significant

Discussion

This study revealed wide distribution of 75 (52.0%) *A. baumannii* among Iraqi patients than other microorganism with different illness. These results were agreed with the study conducted in Baghdad by Al-Kadhmi, 2018 [12] who reported that *A. baumannii* isolated more than other bacteria. The present study also exhibited that the percentage of isolates were (58.7%) in males and (29.03%) in females. This study agreed with study in Iran [13] reported that, (67%) of *A. baumannii* was in males and (32.6%) in female. Also study in Palestine [14] was reported that (62.5%) in male and (37.5%) in female, while the current result disagreed with study done in Iraq [15] was

reported that, *A. baumannii* in males (42%) less than in females (58%). The percentage of *A. baumannii* infection in males more than in females because males were more exposed to the wars than female.

The result of this study showed that 100% of isolates are MDR when tested by standard disk diffusion method. The most result of antibiotic sensitivity that are the bacterial lowest resistance to colistin (44%) and tigecyclin (42.3%). This result is in agreement with study in USA by Qureshi et al., 2015 [16] who reported that about (50%) of isolates were resistant to colistin. While this result disagreed with the study done in Thailand (2012), who reported that (97%) of the isolates were sensitive to colistin [17]. A study in Saudi Arabia showed that (3.9%) of isolated resistance to colistin by Al-mously et al., 2013 [18]. Because this treatment is of little use, so there is little resistance to it.

In this study a very high percentage resistance of *A. baumannii* to antibiotic, similar findings showed in Thailand by Thummeepak et al., 2016 [19] who found that *A. baumannii* clinical isolates developed (82.2%) of resistance to ceftazidime, (78.7%) to meropenem, (54%) to amikacin and (84%) to ciprofloxacin. Also, study by Ghajavand 2015 [13] who reported that resistance percentage (53%) to amikacin, (52%) of isolates resistance to impenim, (43%) to amikacin, (79%) to piperacillin, (86%) to ceftazidime and (87%) to ciprofloxacin.

The existence of β-lactamases, which is the most predominate mechanism of β-lactam resistance. These enzymes, at least partly, hydrolyze carbapenems along with extra β-lactams [20]. Recently a new prolonged spectrum AmpC enzyme was recognized in *A. baumannii*, which able to hydrolyze aztronam, cefipime and ceftazidime [21].

In this study among 75 clinical isolates (52%) of studied *A. baumannii* showed biofilm formation ability. This finding agreed with study conducted by Azizi *et al*., 2016 [22] who found that (63%) of clinical *A. baumannii* isolates were positive for biofilm formation. Another study in Iran 2014 reported that (66.6%) of isolated produce biofilm [6]. Also study by Rodríguez-Baño *et al*., [23] who reported that Fifty-six (63%) of isolates formed biofilm.

The differences in biofilm formation among clinical isolates, in association with the epidemicity of strains and the severity of infections [24].

The current result showed that (86.7%) percentage of *A. baumannii* isolates have ompA gene, this result agreed with study conducted in Thailand [19] they found that (84%) of *A. baumannii* were harbored ompA gene, while other study in Iran reported that all isolates of *A. baumannii* (100%) had ompA gene [10,22].

ompA has been exhibited to play a role in a numerous of interactions with the host during infection, involved adherence/invasion to epithelial cells, initiation of apoptosis in host cells and differentiation of host immune cells [25].

This present study showed that the highest percentage of ompA gene were in males (55.4%) than in females (44.6%) but there is not denoting any major connection between the sex of the patients infected with *A. baumannii* and their chances of harboring ompA gene, this because the gene found in the same species bacteria which infect both female and male.

According to the PCR result the highest percentage of positive isolates to ompA gene (53.8%) was in blood, while the lowest percentage (12.3%) was in urine.

This study noted that the presence of ompA gene had no significant association with the presence of biofilm in clinical isolates, this result agreed with other result [19] reported that there were no significant differences between biofilm producer and non-biofilm producer in the presence of ompA gene.

The current result showed that (34.7%) percentage of *A. baumannii* isolates have Bap gene. Which agreed with study conducted in Iran 2015 [10] who reported that (30%) of *A. baumannii* isolates positive for Bap gene while Fallah *et al*., 2017 [26] who found that (92%) of clinical isolates of *A. baumannii* positive to Bap gene.

This study was found that, significant association between presence of Bap gene and

biofilm formation. The result of this study in agreement with the study [26] who reported that there was strong association between the presence of Bap gene and biofilm formation in *A. baumannii* isolates. While disagreed with the study [19] who reported that no significant association between presence of Bap gene and biofilm formation. A study has shown that Bap plays a role in static biofilm maturation and maintenance, increasing biofilm thickness on glass surfaces [7].

From data presented in this study, we concluded that, biofilm formation is influenced by Bap genes, the ability of *A. baumannii* to construct or form biofilms could cause a high level of antibiotic resistance and survival properties. *A. baumannii* isolates are capable to form biofilms might be selected under antibiotic pressure, or conversely, *A. baumannii* might acquire resistance to multiple drugs from the biofilm communities.

Acknowledgments

The authors express their deepest gratitude to all staff of Microbiology Department, College of Medicine, Al- Nahrain University and staff of Laboratory Department of Children Welfare Hospital and Al- Imamein Al-Kadhimein Medical city for their endless cooperation by equipment and experiences.

Author Contribution

Talib: Preparation, performing and doing the tests of the research. Dr. Abd Al-Rahman: Interpretation the results done under her supervision, Dr. Ali: help in collection of samples.

Conflict of interest

The authors have no conflict of interest.

Funding

Self-funding.

References

1. Munoz-Price LS, Weinstein RA. Acinetobacter infection. N Engl J Med. 2008; 358(2): 1271-81. doi: 10.1056/NEJMra070741.
2. Perez F, Hujer AM, Hujer KM, et al. Global challenge of multidrug-resistant Acinetobacter baumanni. Antimicrob. Agents Chemother. 2007; 51: 3471-84. doi: 10.1128/AAC.01464-06.
3. Imane M, Hafida H, Samia B, et al. Biofilm formation by Acinetobacter baumannii isolated from medical devices at the intensive care unit of the University Hospital of Tlemcen (Algeria). African J Microbiol Res. 2014; 8(3): 270-6. doi: 10.5897/AJMR2013.6288.
4. Hassan A, Usman J, Kaleem F, et al. Evaluation of different detection methods of biofilm formation in the clinical isolates. Braz J Infect Dis. 2011; 15(4), 305-11. doi: https://doi.org/10.1016/S1413-8670(11)70197-0.
5. McConnell MJ, Actis L, Pachón J. Acinetobacter baumannii: Human infections, factors contributing to pathogenesis and animal models. FEMS Microbiol Rev. 2013; 37(2): 130-55. doi: 10.1111/j.1574-6976.2012.00344.x.
6. Babapour E, Haddadi A, Mirnejad R, et al. Biofilm formation in clinical isolates of nosocomial Acinetobacter baumannii and its relationship with multidrug resistance. Asian Pac J Trop Biomed. 2016; 6(6): 528-33. doi: https://doi.org/10.1016/j.apjtb.2016.04.006.
7. Loehfelm TW, Luke NR, Campagnari AA. Identification and characterization of an Acinetobacter baumannii biofilm-associated protein. J Bacteriol. 2008 Feb;190(3):1036-44. doi: 10.1128/JB.01416-07.
8. Gaddy JA, Tomaras AP, Actis LA. The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. Infect Immun. 2009; 77(8): 3150-60. doi: 10.1128/IAI.00096-09.
9. Brossard KA, Campagnari AA. The Acinetobacter baumannii biofilm-associated protein plays a role in adherence to human epithelial cells. Infect Immun. 2012; 80(1): 228-33. doi: 10.1128/IAI.05913-11.
10. Badmasti F, Siadat SD, Bouzari S, et al. Molecular detection of genes related to biofilm formation in multidrug-resistant Acinetobacter baumannii isolated from clinical settings. J Med Microbiol. 2015; 64(Pt 5): 559-64. doi: 10.1099/jmm.0.000058.
11. Sambrook J, Russell DW. Molecular cloning: a laboratory manual Cold Spring Harbor. New York, USA, Cold Spring Harbor Laboratory Press; 2001.
12. Al-Kadhmy IMS, Ali ANM, Salman IMA, et al. Molecular characterization of Acinetobacter baumannii isolated from Iraqi hospital environment. New Microbes New Infect. 2017; 21: 51-7. doi: 10.1016/j.nmni.2017.10.010.
13. Ghajavand H, Esfahani BN, Havaei SA, et al. Molecular identification of Acinetobacter baumannii isolated from intensive care units and their antimicrobial resistance patterns. Adv Biomed Res. 2015; 4: 110. doi: 10.4103/2277-9175.157826.
14. Al Jarousha AM, El Jadba AH, Al Afifi AS, et al. Nosocomial multidrug-resistant Acinetobacter baumannii in the neonatal intensive care unit in Gaza City, Palestine. Int J Infect Dis. 2009; 13(5): 623-8. doi: 10.1016/j.ijid.2008.08.029.

15. Babakir-Mina M, Rashid K, Beg SS, et al. "Epidemiological Characteristics and Antibiotic Resistance of Acinetobacter baumannii Isolated from Burn Patients. EC Microbiology. 2017; 7.4: 112-20.
16. Qureshi ZA, Hittle LE, O'Hara JA, et al. Colistin-resistant acinetobacter baumannii: Beyond carbapenem resistance. Clin Infect Dis. 2015; 60(9): 1295-303. doi: 10.1093/cid/civ048.
17. Tunyapanit W, Pruekprasert P, Laoprasopwattana K, et al. Antimicrobial susceptibility of Acinetobacter baumannii isolated from hospital patients. ScienceAsia. 2014; 40: 28-34. doi: 10.2306/scienceasia1513-1874.2014.40.028.
18. Al-Mously N, Hakawi A, Author C. Acinetobacter baumannii bloodstream infections in a tertiary hospital: Antimicrobial resistance surveillance. Int J Infect Control. 2013; 9: 2–2. doi: 10.3396/ijic.v9i2.012.13.
19. Thummeepak R, Kongthai P, Leungtongkam U, et al. Distribution of virulence genes involved in, biofilm formation in multi-drug resistant acinetobacter Baumannii clinical isolates. Int Microbiol. 2016; 19(2): 121-9. doi: 10.2436/20.1501.01.270.
20. Jain R, Danziger LH. Multidrug-resistant Acinetobacter infections: An emerging challenge to clinicians. Ann Pharmacother. 2004; 38(9): 1449-59. doi: 10.1345/aph.1D592
21. Rodríguez-Martínez, J. M., Nordmann, P., Ronco, E. & Poirel, L. Extended-spectrum cephalosporinase in Acinetobacter baumannii. Antimicrob Agents Chemother. 2010; 54: 3484-8. doi: 10.1128/AAC.00050-10.
22. Azizi O, Shahcheraghi F, Salimizand H, et al. Molecular analysis and expression of bap gene in biofilm-forming multi-drug-resistant Acinetobacter baumannii. Rep Biochem Mol Biol. 2016; 5(1): 62-72.
23. Rodríguez-Baño J, Martí S, Soto S, et al. Biofilm formation in Acinetobacter baumannii: Associated features and clinical implications. Clin Microbiol Infect. 2008; 14(3):276-8. doi: 10.1111/j.1469-0691.2007.01916.x.
24. Longo F, Vuotto C, Donelli G.. Biofilm formation in Acinetobacter baumannii. New Microbiol. 2014; 37(2): 119-27.
25. Choi CH, Lee JS, Lee YC, et al. Acinetobacter baumannii invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. BMC Microbiol. 2008; 8: 216. doi: 10.1186/1471-2180-8-216.
26. Fallah A, Rezaee MA, Hasani A. Frequency of bap and cpaA virulence genes in drug resistant clinical isolates of acinetobacter baumannii and their role in biofilm formation. Iran J Basic Med Sci. 2017; 20(8): 849-855. doi: 10.22038/IJBMS.2017.9105.

Correspondence to Sura S. Talib
E-mail: sura.saadtalib@yahoo.com
Received Sept. 2nd 2018
Accepted Nov. 18th 2018

المجلد السادس عشر، العدد الرابع، 1440 هـ، 2018م
DOI: 10.22578/IJMS.16.4.

المجلة العراقية للعلوم الطبية

عنوان المراسلات إلى المجلة العراقية للعلوم الطبية، صندوق بريد 70044 بغداد، العراق. تلفون (7717516090 964+).

رقم الإيداع في دار الكتب والوثائق ببغداد 709 لسنة 2000

Iraqi Journal of Medical Sciences

IRAQI JMS

المجلة العراقية للعلوم الطبية

Volume 16, Number 4 , 2018
October - December

P- ISSN 1681-6579
E- ISSN 2224-4719

Contents

مطبعة الفرح – بغداد ٠٧٩٠١٣٥٧٧٥١