

Original Research Article

<https://doi.org/10.20546/ijcmas.2017.608.258>

Assessment the Effects of Low Power Laser Light on Some Virulent Factors of *Klebsiella pneumoniae* Isolated from Respiratory Tract Infections

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ABSTRACT

One of the most opportunistic and nosocomial infections causative agent is *K. pneumoniae*, its tendency to be chronic and often display multidrug-resistance phenotypes that are commonly caused by the presence of Extended-spectrum β -lactamases or carbapenemases, making it difficult to choose appropriate antibiotics for treatment. Opportunistic *K. pneumoniae* mostly affects those with compromised immune systems or who are weakened by other infections. Its colonization can be made further infection in the human especially in the urinary tract, and respiratory tract. Thus this study was a goal to assess the effect of low power laser light on the virulence factors of *Klebsiella pneumoniae*. The results showed 17.9% were characterized as *Klebsiella pneumoniae* from 151 cases of respiratory tract infections. Antimicrobial sensitivity test showed elevation in the resistance of these isolates for most usable antimicrobial agents, the most resistant isolate was selected. Another selection was done depending on the type of laser dye, the result of using methylene blue dye at 650 nm. Showed the numbers of the colony were varied according to the conc. of dye, they were direct decreases due to the decreasing of dye conc. with time. Meanwhile, it became smaller with rough texture when cultured on MacConkey agar, and microscopically the bacterial capsules showed slight thickness around the bacteria in compared with the control isolate. As well as there was changing in the degree of glucose fermentation. On the other hand, the antimicrobial sensitivity results showed, the exposed isolate was changed the sensitivity condition to resistance for both of Ciprofloxacin (CIP) and Tobramycin (TN). While using another laser dyes (Rhodamine110 and Anthocyanin) at 532 nm. There was variation in the colony size and texture. Meanwhile microscopically, the bacterial capsules elucidated slight thickness around the bacteria in compared with the control isolate. In addition to the changing in some biochemical tests, as well as the antimicrobial sensitivity results showed heterogeneities toward several antimicrobial agents. On the other hand, the results of biofilm formation showed a negative result for all tests.

Keywords

Klebsiella pneumoniae,
Low power laser
light, Methylene
blue, Rhodamine
dyes and
Virulent factors.

Article Info

Accepted:
19 June 2017
Available Online:
10 August 2017

Introduction

Klebsiella pneumoniae, is a member of the family Enterobacteriaceae, rod-shaped, Gram-negative, lactose-fermenting bacilli with a luxurious capsule. Typical *K. pneumoniae* is an opportunistic pathogen that is widely

found in the mouth, skin, and intestines as normal flora, epidemiologically play a role in the hospital as nosocomial pathogen through contaminating medical devices (Podschun *et al.*, 1998). Nosocomial infections caused by

K. pneumoniae tend to be chronic due to the two following major reasons: *K. pneumoniae* biofilms formed in vivo protect the pathogen from attacks of the host immune responses and antibiotics (Jagnow *et al.*, 2003), and often display multidrug-resistance phenotypes that are commonly caused by the presence of Extended-spectrum β -lactamases or carbapenemases, making it difficult to choose appropriate antibiotics for treatment (Paterson *et al.*, 2004; Munoz-Price *et al.*, 2013). Opportunistic *K. pneumoniae* mostly affects those with compromised immune systems or who are weakened by other infections. Its colonization can be further found in the urinary tract, respiratory tract, gastrointestinal tract and blood. At least 78 capsular (K antigen) serotypes have been recognized for *K. pneumoniae* (Pan *et al.*, 2008; Hsu *et al.*, 2013).

A few serotypes (including predominantly K1 and K2) have a unique hypermucoviscous (hypervirulent) phenotype due to increased production of capsule polysaccharide (CPS). Hypervirulent *K. pneumoniae* is highly invasive and may cause life-threatening and often community-acquired infections, such as pyogenic liver abscess, meningitis, necrotizing fasciitis, endophthalmitis and severe pneumonia (Shon *et al.*, 2012). This bacteria has many virulence factors as a whole make it pathogenicity, included Capsule (Steward *et al.*, 2001). In addition to the different types of adhesions or colonization factors like Pili (fimbriae) such as Type I pili or mannose-sensitive Klebsiella hemagglutination (MS/K-HA), Type III Pili or mannose-resistant Klebsiella hemagglutination (MR/K-HA) (Podschun *et al.*, 1991), both can contribute to biofilm formation (Podschun *et al.*, 1991; Podschun *et al.*, 1998). Some of this bacteria can produce a thick layer of extracellular biofilm as a virulence factor that helps the organism attach to living or abiotic surfaces, and preventing

the effects of antimicrobial agents (Vuotto *et al.*, 2014) and siderophore production (Brooks *et al.*, 2001).

The laser "Light Amplification by Stimulation Emission of Radiation" its concept is a particle of light with energy of a particular frequency could stimulate electrons to emit radiation of light of the same frequency. This phenomenon is the key to the operation of the laser (Saunders *et al.*, 1980). In the life science, medical science and even in nuclear fusion, many research projects are being carried out using laser (Shimoda, 1984). Different researchers have documented the effect of this technique on microorganisms. This technique was applied by using different types of dyes, called laser dyes, its organic compounds that relax radioactively after optical excitation, emitting in the visible or infrared range (Alsalhi *et al.*, 2007). Most laser dyes operate with materials that belong to the class of xanthenes derivatives especially Rhodamine dyes. They cover the wavelength region from 500-700 nm and generally very efficient (Balaji *et al.*, 2011). Because of multidrug resistance in Gram-negative bacteria, particularly Enterobacteriaceae such as *E. coli* and *K. pneumoniae*, has become one of the biggest global concerns. Infections with these bacteria lead to prolonged hospital admissions and higher mortality rates, and a worldwide using the laser, this study was concerned with the effect of low power laser light on some virulence factors, and biochemical activities of pathogenic *K. pneumoniae* isolated from respiratory tract infection.

Materials and Methods

Samples collection

Samples of (151) episodes of lower respiratory tract infection were collected from Baghdad/Iraq (Central teaching of Pediatric,

Al-Yarmouk Teaching, and Baghdad medical city) hospital, during a period of November /2016 – February/2017. The specimens were cultured on MaCconkey agar (LAB M, U. K.) and incubated for 18-24h at 37 C.

Identification of the isolates

Specimens isolates were identified depending on colony morphology, Microscopic examination, biochemical tests elaborated by Epi20E (bio-merieux/France), chromo agar as confirmatory tests and Congo red agar prepared from (5.2g of B.H.I.A., 5g of sucrose, 0.08g of Congo red in 100ml D.W).

Antimicrobial sensitivity test

Antimicrobial sensitivity test was done for each isolate characterized as *Klebsiella pneumonia* by using 10 different types of antimicrobial disks, included (Erythromycin, Ciprofloxacin, Tobramycin, Methicillin, Gentamycin, Imipenem, Cloxacillin, Trimethoprim, Ampicillin, and Streptomycin) (Mast Diagnostics, Mast Group Ltd., Merseyside, U.K.) which are deemed as the most local usable antimicrobial agents for variable types of infection.

The isolate that appeared resistant for most usable antimicrobial disks was selected for further experiments.

Primary selection by using low power laser light

Activation and preparation of bacterial isolate

Activation of selected isolate for 18h using brain heart infusion broth (HIMEDIA/India), subsequently adjusted the dilution of the isolate in comparison with MaCconkey agar 1×10^8 bacteria/ml.

Calculation and preparation of laser dye

Methylene blue was selected as laser dye for primary bacterial excitation. According to the (Von Bathen *et al.*, 2014), the amount of methylene blue was calculated and prepared at a concentration of 10^{-4} (stock solution dye) depending on the following equations.

$$C = \text{Wt.} \times \text{Mw.} \times V. / 1000$$

Serial dilutions (10^{-5} and 10^{-6}) were prepared from the stock one according to the following equation. $C_1 \times V_1 = C_2 \times V_2$

Preparation of the isolate/ dye mixture

Three copies for each methylene blue dye concentration ($10^{-6}, 10^{-5}, 10^{-4}$) were prepared tubes, each one included 100µl of usable conc., and then aliquot of 1000µl from 10^8 isolate suspension transferred to these tubes, subsequently each tube was plotted to the low power laser light (CW Diode Laser/4.5 volt D.C, 800 mA) at 650nm for different time (1,2 and 3)min. after a while, a loop full was taken and cultured on Mueller-Hinton agar and MaC conkey agar. Depending on the number of bacterial growth after exposure, a series of experiments were done to obtain the best dilution and amount for isolate suspension and methylene blue dye that affected on the bacterial virulence factors (number of the colony, morphology, biochemical activities, biofilm and capsular formation, and sensitivity to antimicrobial agents).

Finally selection by using low power laser light

According to primary experiment, the best concentration for both isolate suspension and laser dye conc. were obtained with determining the suitable time for plotting to low power laser light, that affected on the

bacterial virulence factors with less bacterial lethality.

Thus 500µl of 10^7 CFU isolate suspension with 100µl of 10^{-4} methylene blue dye was used and mixed, and then plotted to low power laser light at 650nm for 2 min. After a while, a loop full was taken and cultured on (MacConkey, Congo red) agar and Chromo agar as a confirmatory test. This experiment was done for the first day, and then by using the same exposed isolate, the experiment was repeated five times for five days subsequently.

Other experiments were done by using wavelength 532 nm. (DPSS Laser /Diode Pumped Solid state laser), for different laser organic dyes Rhodamine 6G (Lambda Physik, USA), Rhodamine 110 (Lambda Physik, USA) and Anthocyanin (locally prepared from a plant source).The same steps for the previous experiment were carried out for each dye.

The result of plotted selected colony was elaborated to antimicrobial sensitivity test and Epi 20E for all sixth passages in comparison to virulent isolate.

Results and Discussion

Identification bacterial isolates

Several tests such as microscopically, colony morphology and biochemicals were made to identify bacterial isolates. According to (Holt *et al.*, 1994), the characteristic parameters showed, 27 (17.9%) were *Klebsiella pneumoniae* from 151 total number of respiratory specimens, through a Gram-negative, non-motile, small straight rods and arranged singly or in pairs under light microscope, in addition to a mucoid texture, large size, round regular colonies and lactose fermenter on MacConkey agar (Figure 1), and

a positive result as *Klebsiella pneumonia* for API 20E (Fig. 3) and confirmatory test by chromo Agar (Fig. 2). Furthermore, the results of biofilm formation on Congo red showed a negative result (Fig. 4).

Antimicrobial sensitivity test

Depending on (C.L.S.I, 2015) guideline, antimicrobial sensitivity test was done for 27 *Klebsiella pneumoniae* isolates. The results appeared increasing in resistance to antimicrobial agents as shown in table 1, elucidated that the resistance to Cloxacillin, Erythromycin, Methicillin, trimethoprim, Streptomycin, Tobramycin, Ampicillin, Gentamicin, Ciprofloxacin, and Imipenim were (100, 85.2, 66.6, 62.9, 55.5, 48.1, 44.4, 33.3, 33.3, 22.2)% respectively.

The isolates that appeared resistance for most usable antimicrobial disks was selected for further laser experiments (Fig. 5).

The primary selection by using low power laser light with methylene blue dye

The results of the primary selection by using 10^8 CFU isolate suspension with different conc. of methylene blue dye, shown according to the bacterial growth (the no. of colonies and its morphology), the numbers of the colony were varied according to the conc. of dye, they were direct decreases due to the decreasing of dye conc. with time table 2.

The diluents of 10^{-4} of Methylene Blue dye with 2 minutes exposure was selected as a reason of the colony diameter (2mm) which reflects the bacterial (CFU) ability to grow. In spite of the no. of the colony in this conc. which was represented more than other usable conc., therefore the further experiments were done with less bacterial conc. hence we selected 10^7 CFU

Finally selection by using low power laser light

The suitable conditions were applied, the results showed changing in colony size, it becomes smaller than the control with rough texture when cultured on MacConkey agar (Fig. 6), and microscopically appearance, the bacterial capsules showed slight thickness around the bacteria in compared with the control isolate. Biochemical changing occurred in the degree of glucose fermentation, it varies in its color from yellow to brown for all 6th passages (Fig. 9). As well as the antimicrobial sensitivity results showed several variations in the degree of sensitivity, the exposed isolate showed changing of sensitivity against both of Ciprofloxacin (CIP) and Tobramycin (TN) to resistant conditions (Table 3), this result may as (Piatti *et al.*, 2008) reported that, a contrary relation between virulence and resistance to quinolones has been established in some studies. Quinolones and fluoroquinolones resistant strains show reduced virulence and, while the susceptible strains are more virulent. Thus a number of virulence factors carried, and the presence or absence of specific virulence factors, as well as their expression, may occur under certain conditions. While resistance to aminoglycosides and its relation with virulence is still unknown as (Horcajada *et al.*, 2005) mentioned, the resistance to some members of aminoglycoside agents was associated with a reduction in the prevalence of fimH which is the main structure in type 1 fimbriae that related with the first step of bacterial colonization. In addition to an increase in iutA prevalence, in comparison to susceptible isolates. On the other hand, the results of biofilm formation showed a negative result for all passages which was represented no changes was occurred in compared with control one.

The results of other experiments were done by using wavelength 532 nm. for another two organic laser dyes (Rhodamin 110 and Anthocyanin). The results of exposure by using rhodamin 110 dye showed changing in colony size, it becomes larger than the control with a smooth texture, when cultured on MacConkey agar (Fig. 7), and microscopically the bacterial capsules appeared slight thickness around the bacteria in compared with the control isolate. In addition to the changing in the some biochemical tests such as ADH (Arginine dehydrogenase) and ODC (Ornithine decarboxylase) tests, it varies from the negative result (yellow color) in the control and for the fifth passages to a positive result (red color) in the last 6th passage (Fig. 10). As well as the antimicrobial sensitivity results showed several variations occurred in the degree of sensitivity, the exposed isolate showed indolent variation for SAM, its changed from resistance to a sense one in the 1st passage, and throughout 2ed, 4th, and 6th passages the isolate became intermediates. the conversion of the exposed isolate from resistant to intermediate or sensitive for these types of beta-lactam antimicrobial agents may due to some genetic changes were occurred that lead to counteract resistance to β -lactams included several mechanisms including modification of penicillin-binding proteins, loss of porins, production of β -lactamases, or over expression of efflux pumps as reported Whereas the isolate converted to intermediate to streptomycin (S) in the 3rd and 4th passages while to sensitive in the 6th passage, and the changing from intermediate for Gentamycin (GM) to sensitivity in 2ed passages. Because there are different mechanisms that made bacterial cell converted to resistant to these types of antimicrobial agents such as modification of the 16S RNA of bacterial 30S ribosomal subunit by mutation or methylation of the aminoglycoside binding site.

Table.1 The number and percentage of *Klebsiella pneumoniae* isolates sensitivity toward antimicrobial agents

Antibiotic	Resistant %(no.)	Intermediate%(no.)	Sensitive%(no.)
Gentamycin	33.3 (9)	0	66.7 (18)
Imipenime	22.2 (6)	11.1 (3)	66.6 (18)
Ciprofloxacin	33.3(9)	14.8 (4)	51.9 (14)
Methicilline	66.6 (18)	14.8 (4)	18.6 (5)
Trimethoprim	62.9 (17)	14.8 (4)	22.2 (6)
Streptomycine	55.5 (15)	18.5 (5)	26 (7)
Cloxaciline	100 (27)	0	0
Erythromycin	85.2 (23)	3.7 (1)	11.1 (3)
Tobramycine	48.1 (13)	2.7 (1)	48.1 (13)
Ampiciline	44.4% (12)	14.8% (4)	40.8 (11)

Table.2 The number of colonies with different methylene blue dye conc. according to the time

1ml of 10 ⁸ bacterial suspension with 0.1ml of 10 ⁻⁴ M.B	1 min >561	2 min > 438	3 min > 432
1ml of 10 ⁸ bacterial suspension with 0.1ml of 10 ⁻⁵ M.B	1 min >506	2 min >493	3 min >296
1ml of 10 ⁸ bacterial suspension with 0.1ml of 10 ⁻⁶ M.B	1 min >420	2 min >350	3 min >250

Table.3 Antimicrobial sensitivity test for isolate suspension with the methylene blue dye that exposure to low power laser light at 650nm for 2 min

Antibiotic	control	1Pass.	2Pass.	3Pass.	4Pass.	5Pass.	6Pass.
IMI	3.1	2.1	2.1	1.9	2.0	2.3	2.0
CIP	3.0	0	0	0	<1.0	1.1	1.0
GM	1.3	1.6	1.8	1.6	1.6	1.9	1.6
CX	0	0	0	0	0	0	0
TS	0	0	0	0	0	0	0
MEC	1.6	1.6	2.0	1.5	1.6	1.9	1.6
E	0	1.0	0	0	0	1.0	<1.0
TN	1.8	0	0	0	0	0	<1.0
SAM	1.0	0	0	0	0	0	0
S	0	0	1.1	1.0	1.0	1.0	<1.0

Table.4 Antimicrobial sensitivity test for isolate suspension with Rhodamine 110 dye that exposure to low power laser light at 532nm for 2 min

Antibiotic	Control	1pass.	2pass.	3pass.	4pass.	5pass.	6pass.
IMI	3.1	3.0	3.0	3.2	3.0	3.1	3.1
CIP	3.0	3.0	2.9	3.0	2.9	3.0	2.8
GM	1.3	1.5	1.6	1.6	1.5	1.5	1.5
CX	0	0	0	0	0	0	0
TS	0	0	0	0	0	0	0
MEC	1.6	1.7	1.6	1.8	0	0	0
E	1.0	0	0	0	0	0	0
TN	1.8	1.8	2.0	2.0	1.7	2.0	1.8
SAM	1.0	1.4	1.4	1.8	1.6	1.6	1.6
S	0	1.3	1.2	1.3	1.3	1.3	1.4

Fig.1&2 Growth of *K. pneumoniae* on MacConkey agar and *K. pneumoniae* on chromoagar



Fig.3 *Klebsiella pneumoniae* for EPI 20E



Fig.4&5 Antimicrobial sensitivity test of *K. pneumoniae* and *K. pneumoniae* on Congo red agar



Fig.6 Exposed isolate on MacConkey agar by using methylene blue as laser dye

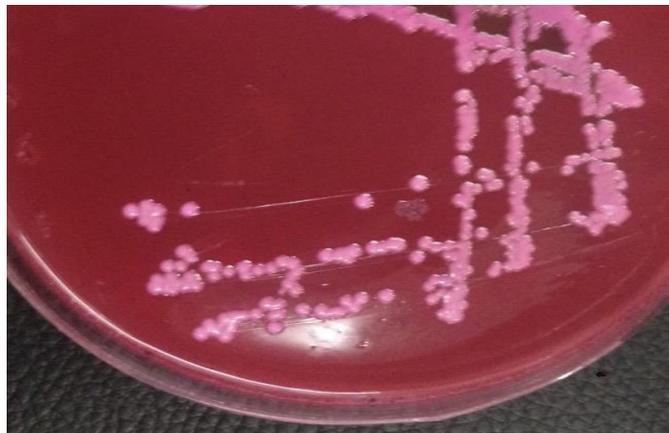


Fig.7&8 Exposed isolate on MacConkey agar by using isolate on MacConkey agar and exposed Rhodamine 110 as laser dye by using anthocyanine as laser dye



Table.5 Antimicrobial sensitivity test for isolate suspension with Anthocyanine dye that exposure to low power laser light at 532nm for 2 min

Antibiotic	Control	1pass.	2pass.	3pass.	4pass.	5pass.	6pass.
IMI	3.1	3.0	3.0	3.2	3.0	3.1	3.1
CIP	3.0	3.0	2.9	3.0	2.9	3.0	2.8
GM	1.3	1.5	1.6	1.6	1.5	1.5	1.5
CX	0	0	0	0	0	0	0
TS	0	0	0	0	0	0	0
MEC	1.6	1.7	1.6	1.8	0	0	0
E	1.0	0	0	0	0	0	0
TN	1.8	1.8	2.0	2.0	1.7	2.0	1.8
SAM	1.0	1.4	1.4	1.8	1.6	1.6	1.6
S	0	1.3	1.2	1.3	1.3	1.3	1.4

Fig.9 The Epi 20E to exposed isolate by using methylene blue as laser dye compared with control



Fig.10 The Epi 20E to exposed isolate by using Rhodamine 110 as laser dye compared with control



There is a decrease of the intracellular concentration of the antibiotic resulting from the occurrence of modifications in the bacterial outer membrane; increase of the activity of active efflux systems; decrease of the drug transport into the cell; and by enzymatic deactivation of aminoglycosides as (Talbot, 2013) mentioned, thus we suggested that the resistance to aminoglycosides may be retired due to exposed to laser (Table 4). On the other hand, the results of biofilm formation showed a negative result for all passages. The results of the exposure to low power laser light at 532nm with using other organic dye (anthocyanine) were showed as that occurred with the Rhodamine 110 experiments, for morphologic and microscopic characteristics (Fig. 8).

As well as about antimicrobial sensitivity results also showed several variations especially with GM which converted from intermediate to sensitive, while about S, the exposed isolate was changed from resistance to

intermediate. Whereas the exposed isolate showed changing the sensitivity for (MEC) to resistance in the last 3 passages, resistant bacteria generations was associated in most cases, either directly or indirectly with decreased virulence and fitness, genetic events also associated with the evolution of pathogen on virulence and fitness costs, in gram-negative, β -lactamases production was the most prevalent mechanism of β -lactam resistance, followed by permeability alterations, with extrusion by efflux pumps, and lesser extent penicillin binding protein (PBP) alterations as (Alejandro *et al.*, 2013) reported, thus we suggested that the plotted of pathogenic *K. pneumoniae* to laser may make cell wall/cell membrane alteration. Whereas the gradual changing from resist of (SAM) to sensitive in 3rd, 4th and 5th, and 6th (Table 5). On the other hand, As other experiments, the results of biofilm formation showed a negative result for all passages which was represented no changes was occurred in compared.

It can be concluded that the use of low power laser light with different organic dyes had an effect on bacterial virulence factors, and need further developed studies to improve these finding.

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How to cite this article:

Diana Fadhil Al-Saadi, Mouruj A. Al-Aubydi and Layla M. Al-Ameri. 2017. Assessment the Effects of Low Power Laser Light on Some Virulent Factors of *Klebsiella pneumoniae* Isolated from Respiratory Tract Infections. *Int.J.Curr.Microbiol.App.Sci.* 6(8): 2179-2190. doi: <https://doi.org/10.20546/ijcmas.2017.608.258>