Historical Research Letter
ISSN 2224-3178 (Paper) ISSN 2225-0964 (Online)
Vol.30, 2016

A Study on the Effect of Ethidium_Bromide on Virulence Factors (Protease and Biofilm Formation) by Klebsiella Pneamoniae Isolated from Different Clinical Sources

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Abstract
About 145 isolate of *Klebsiell*a species were isolated from different clinical sources for6 month ago from (1-9-2014)to(1-2-2015).these isolates were identified morphologically and biochemically and Api 20 kit,thus there was only 22isolates were identified asKlebsiella pneumoniae (15.1%) Protease activity were tested by measuring the diameter of cleared around wells containing bacterial cells harvested from 18 h of incubation at37c on skim milk agar medium or by (wells method).The results showed that k4 is the higher isolate of protease production(18mm in diameter)while k2 showed a lowest protease activity of (12mm in diameter).Biofilm Formation for 22 Klebsiella isolates were tested on congo-red agar mediumand the results showed that all the isolates have (100%) activity by forming dark colonies on congo-red medium In our study, first ttempt was made on the effect of ethidium bromide on the virulence factors of pathogenic bacteria (Klebsiella pneumoniae)at concentrations (10^{-1} to 10^{-6})that have an effect at which the 22 isolates of (K.p)loss the protease activity and biofilm formationat these concentrations while little and normal activities were observed at concentrations (10^{-7} to 10^{-10}) of ethidium bromide on the 22 studied isolates of K. pneumoniae .Also, the results of Agarose –gel electrophoresisof both (normal case)Klebsiella pneumoniae k4 and cured isolates showed the presence ofchromosomal and plasmid DNA bands in the normal case while only chromosomal DNA bands occure with the Klebsiella isolates treated with Ethidium –Bromide at concentrations of (10^{-2} to 10^{-4}).
Keywords: Ethidium –Bromide – Klebsiella – Protease –Biofilm .

Introduction

The genus *Klebsiella pneumoniae is* ;a gram negative ;non fermentive bacillus ;has emerged as a major pathogen among noscomial infections which is related to possessing anti phagocytosis capsule (Levinon;2004;Umhe etal;2006).*Klebsiella* species are routinely found in the Human nose ,mouth and gastrointestinal tract as normal flora ;however they can also behave as opportunistic human pathogens (Einsten,2000;Abbot,2003;Vincent,2004). *Klebsiella* infections can involve ;pneumonia ,urinary tract infection,septicemia,diarrhea and soft tissues infections (Kaye,et al ;2000).There are many factors contribute to pathogenicity of *Klebsiella pneumoniae* especially protease production and biofilm formation (in addition capsule antigen ,ability of adhesion ,sideroghores and others).Aprotease(or proteimase)in any enzyme that performs proteolysis,that is,begins protein catabolism by hydrolysisof the peptide bands that link amino acids together in a poly peptide chain.Proteaseshave evolve multiple times,and different classesof protease can perform the same reaction by compeletly different catalytic mechanisms Proteases can be found in animals,plants,bacteria,archaea, and viruses .They can attack the immunoglobulines and immune cells(Nehad,T.A,2003;Senior,1999) .Another important virulence factor contributing to the *K.pneumoniae* pathogenesis in clinical settings is the biofilm mode of growth involved in chronic as well as in acute infections (Schaber et al ;2007).Biofilm are formed from individual free-floating (planktonic) cells and are defined as an exopolysaccharied-surrounded bacterial complex on the biotic or a biotic surfaces (Hoiby et al 2001).Bacterial cells in the biofilm often display a variety of phenotypic differences from those in the planktonic culture .These include some phenotypic changes such asmotility,production ofextacellular polysaccharide and increased resistance to antibiotic and host defence system (Ceri et al,1999;Tarkkanen et al ,1997; Rollin and Joseph,2000) .This study was viewed on the effect of Ethidium –Bromide on these two virulence factors (protease and biofilm formation) in which it is (et-br) an interkylating agent commonly used as a fluorescent agent (nucleic acid stain),in molecular biology Laboratories for techniques such as agarose gel electrophoresis ,whev exposed to ultraviolet,it will fluoresce with an orange color ,intensifying almost 20fold after binding to DNAunder the name (homidine). Ethidium –bromide may be a mutagen, a carcinogen, or a teratogen , although this depend on the organism exposed and the circumstances of exposure. (Sambrook et al 1989).

Materials and Method

Isolation and Identification of Bacterial Isolates

The present study included (145)samples from different clinical sites during 6 months included :98 urine,14 stool,7 blood,5 ear swap ,13 wound ,2 burn swap,4 throat swap,2 skin swap . These clinical samples were collected from the main three hospitals in Baghdad – Iraq (Al kindy ,Al kadhamiyaa, Al –yarmoq) in addition

to general health Laboratries . Bacterial isolates were identified to the level of subspecies using the Traditional biochemical and morphological test described by (Baron et al ,1999) and then confirmed using rapid identification systems (Api 20 E) as recommended by the manufacture(Biomeriex _France)

Protease Activity
Protease activity were measured before and after ethidium bromide by measuring the diameter of lysis area after growing of (18-36 h) of incubation at 37^0c on skim milk agar media for all isolates,using (wells method) (Barron and Fine gold ,1994).

Biofilm Formation
The bacterial activity for biofilm formation were measured by culturing on congo –agar media,in which cells of 18 h to 36h of incubation at 37 c , dark ends of growing determined the biofilm production (Todar,2007).This activity tested before and after addition of ethidium bromide to nutrient agar medium at concentrations(10^{-1} to 10^{-10}) (Baron and Fingold1999)

DNA –Analysis
Chromosomal)and plasmid DNA was extracted from cultured cells using the alkaline –SDS method described by Kirby et al ,1995.

Results and Discussion
Isolation and Identification of Bacterial Isolates
Results of morphological and biochemical characterization tests revealed that a total of 22 isolates were belonged to Klebsiella pneunmoniae

Protease production and Biofilm Formation by *Klebsiella* isolates
The production of extracellular protease from a number of pathogenic bacteria represent one of the most important virulence factors have a wide spread of interesting field for studying , the results of this study showed that *Klebsiella pneumoniae* 4was the highest activity (18 mm indiameter) by measuring the diameter of lysis area on skim milk agar media (or by wells method) while the k2 was the lowest protease activity (12mm in diameter) as shown in table (1) and figure (1).

These findings are in agreement with results obtained by Nehad (2003) who found similar and higher results of protease from Proteus mirabilis ,in some genetic studies of protease production from different bacteria found that it is chromosomally determined (Pons *et al* ,2004;Wassif *et al1995*).there are many factors affect protease production such as the time of incubation ,the presence of inhancers ,metals .in addition ,protease can cleavage the immunoglobulins and many immune cells .(Nehad,2003)

ACTIVITY / ISOLATE NO.	PROTEASE ACTIVITY DIAMETER OF LYSIS AREA IN (mm)	BIOFILM FORMATION BY(CRA)
K1	14	100
K2	12	100
K3	16	100
K4	18	100
K5	14	100
K6	14	100
K7	14	100
K8	14	100
K9	14	100
K10	16	100
K11	14	100
K12	14	100
K13	17	100
K14	14	100
K15	15	100
K16	14	100
K17	16	100
K18	12	100
K19	14	100
K20	13	100
K21	11	100
K22		
K(Control)	14	100

Table (1) showed both the protease activity of 6 K.pneumoniae isolates detected by measuring the

diameter of lysis area after 18h of incubation at 37^0C on skim milk agar media and biofilm formation after incubation at 37^0C on congo agar media.

Incontrast,our results showed that allthe 22 *Klebsiella* isolates showed complete biofilm formation(100%)as in table (1) by forming deep darkng colonies on congo-red agar medium figure(3).These results are agreement with that of Podschan *et al* (2000)which they can isolate ahigh frequency of Germany clinical isolates that have similar virulence factors activity like capsule production ,siderophores ,resistance to serum and biofilm formation(Podschan et al,2000).Also a high Beta-TEM-59-lactamaseresistance and other s including biofilm formation by *K. oxytoca* were isolated at

(26%)from different European clinical sources.(Bermuds *et al*,1999)

The Effect of Ethidium –Bromide on both Protease and Biofilm Formation by Klebsiella pneumoniae isolates

The results were shown in table (2) represent that the mutagenic agent (ethidium-bromide) have an effect at concentrations (stock solution,to 10^{-6})at which all 22 isolates loss the protease activity and biofilm formation as in figure (3),(4) while little and normal activities were observed at concentrations(10^{-7} to 10^{-10}) as in table (2).These findings are in agreement with the results obtained by many researchers(;Kafaf,2000) who found many of antibiotic resistance were plasmid determined and affected after curing experiments .Incontrast ,many results revealed the chromosomally determined abilities such as Actinorhodin-like substance production by *Streptomyces* IQ45(Nehad,1998)

Jones *et al* (1990) isolate many *Proteus mirabilus* mutants affect their virulence factors .

BACTERIAL ACTIVITY / ETHIDIUM BROMIDE SOLUTION	PROTEASE ACTIVITY	BIOFILM FORMATION
Stock solution	No-activity	No-activity
10^{-1}	No-activity	No-activity
10^{-2}	No-activity	No-activity
10^{-3}	No-activity	No-activity
10^{-4}	No-activity	No-activity
10^{-5}	No-activity	No-activity
10^{-6}	No-activity	No-activity
10^{-7}	Little (10mm)	Normal darking (100%)
10^{-8}	Normal (12mm)	Normal darking (100%)
10^{-9}	Normal(14mm)	Normal darking (100%)
10^{-10}	Normal(14mm)	Normal darking (100%)

Table (2) show the effect of Ethedium bromide concentration on both protease and biofilm formation by K.pneumoniae isolates .

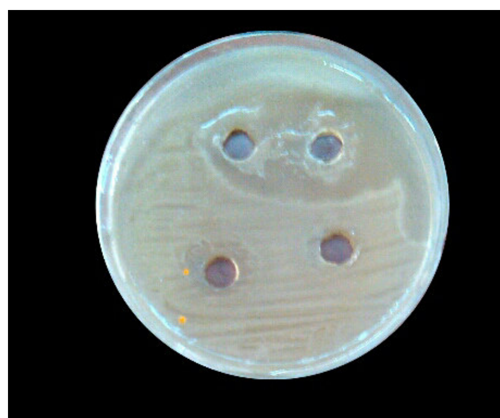


Figure (1) showed the protease activity (12 mm) in diameter of K. pneumonia (K4) measured by wells method after 18 h of incubation at 37°C

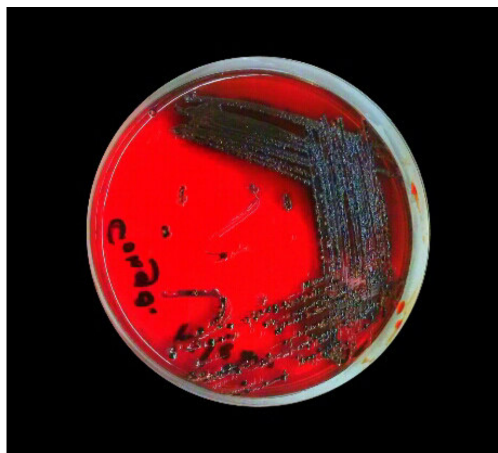


Figure (2) showed the biofilm activity of K.pneumoniae (K6) measured by congo-red agar method (CRA)

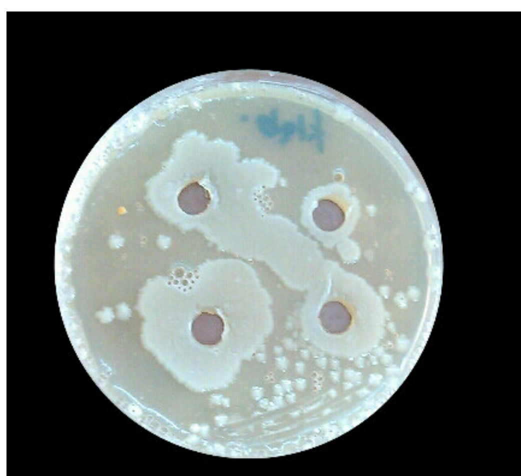


Figure (3) showed the absence of protease activity after growing on medium containing Et-Br concentrations (0 to 10^{-7})

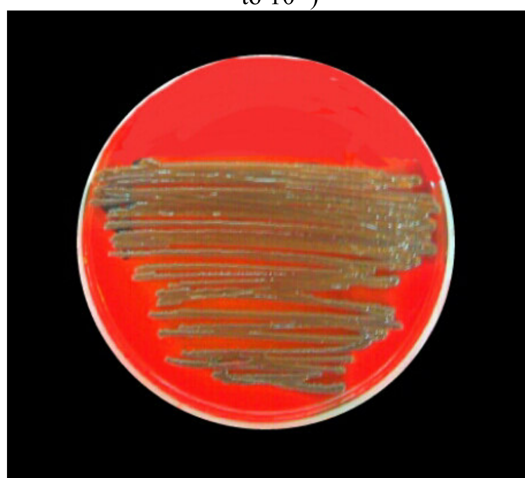
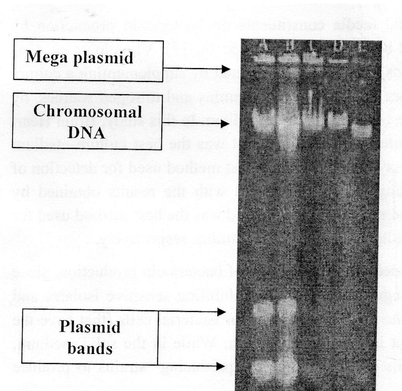


Figure (4) showed the absence of biofilm activity (0%) after growing on medium containing Et-Br concentration (10^{-4}).

DNA –Analysis

The results of DNA –analysis of both normal and cured isolates showed the prescence of chromosomal and plasmids bands in (normal case) while only chromosomal bands observed in *Klebsiella* isolates treated with Ethidium –Bromide at concentrations (10 and 10) as in figure (5) ,the absence of plasmids was correlated with the absence of potease production and biofilm formation by *Klebsiella* isolates as mentioned below which explain the fact of their genetics ,they may be ,in most probable plasmids determined neither than

chromosome .Finally ,our results showed that Ehidium-Bromide have an effect on the virulence factors especially protease and biofilm formation due to the mutagenic effect on the specific genes of their production by *Klebsiella* isolates under this study.



Figure(5) : Agaros gel electrophoresis of chromosomal and plasmid DNA isolated from K pneumoniae K for (line A,B) normal and curing isolates treated with Eth. Bromide at constrations (10^{-2} and 10^{-4}) panel (C,D,E)

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