Effect of Acridine Orange on Pathogenicity of *E. coli* Isolated from Urinary Tract Infection Patients

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Abstract

A total of (108) isolate from Urinary tract infection (UTI) patients, only (47) isolate gave a positive result (43.51%) after a number of morphological and biochemical characterization. Our result showed only 28 isolate have the ability for protease production and haemolysin production (59.5%), and *E. coli* no.6 was the highest protease production isolate (24mm of lysis area) while the *E. coli* no.18 was lowest protease production (12 mm of lysis area). After incubtion on skim milk agar medium at 37°C for 18h and with (100%) haemolysin activity for all 28 isolates after growing on blood agar medium. In our study, first attempt made on the effect of acridine orange (0.1%) as a curing agent on virulence factors of pathogenic bacteria (both protease and haemolysin productin) by highly producer *E. coli* no.6 at concentrations (0, 10^{-1} to 10^{-10}) and the results showed that *E. coli* no.6 loss the protease and haemolysin productin at concentration of (10^{-1} to 10^{-4}) while little and normal activities were observed at concentration of (10^{-5} to 10^{-10}) of acridine orange. Also, this study was investigate the agarose - gel electrophoresis of both cured and normal cells and the results showed the presence of both chromosomal and plasmid bands in the normal cells and only the presence of chromosomal band for the cured cells *E. coli* treated with acridine orange at concentrations of (10^{-2} to 10^{-4}).

Key words: *Acridine orange, E. coli, Protease, Haemolysin*

Introduction

Urinary tract infection (UTI) could be defined as the presence of microorganism in property collected specimen of Urine bacteriuria more than 10^5 bacteria per ml of urine [1]. The symptoms of UTI are frequent urination, flank, pain, dysuria, bumming while urination and some time fever In general bacterial infection of urinary tract are the commonest cause of both community acquired and nosocomial infection in patients admitted to hospital occurred Secondly after respiratory tract infection [2].

The infection are a common source of morbidity as much as twelve per 1000 consolation in general practice are on account of them [3]

The Genus *Esherichia*

The genus Escherichia is related to the family Enterobacteriacae, is a gram-negative, facultative anaerobic , rod-shaped , coli form bacterium that is commonly found in the lower intestine of warm-blooded organisms (endotherms) *E. coli* and other facultative anaerobes constitute about of gut flora, [4] and fecal oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination. A growing body of research, though, has examined environmentally persistent *E. coli* which can survive for extended periods outside of a host.

Uropathogenic *E. coli* (UPEC) is one of the main causes of urinary tract infections.[5] It is part of the normal flora in the gut and can be introduced in many ways. In particular for females, the direction of wiping after defecation (wiping back to front) can lead to fecal contamination of the urogenital orifices. Anal intercourse can also introduce this bacterium into the male urethra, and in switching from anal to vaginal intercourse, the male can also introduce UPEC to the female urogenital system [5].

Hemolysis can be identified by their ability to lyse red blood cells in vitro [6]. One way hemolysin lyses erythrocytes is by forming pores in phospholipids bilayer. Other hemolysis lyse erythrocytes by hydrolyzing the phospholipids in the bilayer. Hemolysin is normally secreted by the bacteria in a water-resoluble way. These monomers diffuse to the target cells and are attached to them by specific receivers. After this is already done, they oligomerize, creating ring-shaped heptamer complexes. Hemolysin can be segregated by many different kinds of bacteria such as *Staphylococcus aureus*, *Escherichia coli* or *Vibrio parahemolyticus* among other pathogens. We can take a look at the bacterium *Staphylococcus aureus* to study more precisely the formation of these pores. *Staphylococcus aureus* is a pathogen that causes many infectious diseases such as pneumonia and sepsis. This ring-shaped complex we'll take a look at, is called staphylococcal alpha-hemolysin pore. In nature, what happens with these pathogens is that, in its fight for resources, bacterium.pores. *Staphylococcus aureus* is a pathogen that causes many infectious diseases such as pneumonia and sepsis. This ring-shaped complex we'll take a look at, is called staphylococcal alpha-hemolysin pore [6].

Protease

Are extracellular proteolytic enzyme produced by several species 01 pathogenic bacteria, both gram positive and gram negative. Several line of evidence suggest that the enzymes play a role in bacterial virulence Especially, by *E.coli*, gonorrhea , bacterial meningitis ,and upper respiratory infections this enzyme can be detected quantitatively by ELISA and by measuring the lysis aria after plating on skim milk agar media [7].

Acridine Orange

Acridine Orange (AO) is an organic compound used in nucleic acid selective fluorescent cationic dye useful for cell cycle determination at 502nm &emission maximum shifts to 460nm (blue). A.O will also enter acidic compartment such as lysosomes & become pronated & sequestered in this low pH conditions . the dye will emit orange light Thus Acridine orange can be used to identify engulfed apoptotic cell , because it will fleuoresce up on engulfment the dye is often used in epifluorescence microscopy & cytometry analysis of cellular physiology &cell cycle status [8].

Materials and Methods

Sample collection

Fourty seven bacterial samples used in this study were obtained from midstream urine of U.T.I patients.

Bacterial Diagnosis

Initial diagnosis depending on gram reaction and morphological characteristics of the colonies based on bacterial growth on macConkey agar , blood agar and EMB (Eosine methylene blue medium). , as well as the number of biochemical test and VITEK2 compact system)(Biomeriex –France).

Acridine orange A.O.

In this part of study , the highly *E. coli* protease producer were used for studying the effect of curing agent A.O. concentration of (0, 10^{-1} to 10^{-10}) by dissolving 0.1 mg of acridine orange in 10ml D.W. then the above concentration were prepared and added to a plate of skim milk agar and blood agar seeded with alawn of nutrient broth(18-24 h) culture of *E. coli* no.6. Then the protease production and haemolysin production were measured at each concentration [9].

Protease production [10]

Protease activity were measured before and after acridine orange by measuring the diameter of lysis area(in mm) after growing of isolates of (18-36h) of incubation at 37°C on skim milk agar media of all isolates using well methods.

Haemolysin production [10]

Haemolysin production were measured before and after acridine orange by measuring the lysis activity of isolate after growing on blood agar medium of (18-36h) of incubation at 37°C by spreading method.

DNA profile by gel electrophoresis

Bacterial plasmid DNA was extracted from cultured cells using the alkaline SDS method with promega DNA kit described by as follow [11]:

25ml culture was centrifuged, resuspended the pellet in 0.5ml of lysozyme solution containing (0.3M NaOH, 2% SDS) mix and incubate at 70°C for 15min,

add 0.08ml of acid phenol/chloroform (1:1) and mix gently, separate phases by centrifugation at 1000xg for 10 min and transfer the upper aquous phase to a new Eppendroff tube containing 0.07M sodium acetate and 0.7ml isopropanol, centrifuge again for 2min, the pellet dissolve in 0.05ml TE buffer, Stored at 4°C and samples were electrophorated using 0.7% agarose with 5V/cm for 2h, after ending of electrophoresis process, the gel was exposed to U.V light with 340nm to observe plasmid bands.

Results and Discussion

The present study included (47) samples from U.T.I. patients with different ages and sexes from different hospitals even laboratories. Bacterial isolates were identified to the level of subspecies using the traditional biochemical and morphological test described by [10] and then confirmed by VITEK2 compact system. So, the results revealed that all (47) isolates were belonged to *E.coli* and only(28) isolate showed the ability for protease and haemolysin production(59.5%).

Protease production and Haemolysin Formation by *E. coli* 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 *E. coli* no.6 was the highest activity (24 mm in diameter) by measuring the diameter of lysis area on skim milk agar media (or by wells method) while *E. coli* no.18 the was the lowest protease activity (12mm in diameter) as shown in table (1) and figure (1), figure (2).

Figure 1: The haemolysin production by *E.coli* on blood agar media

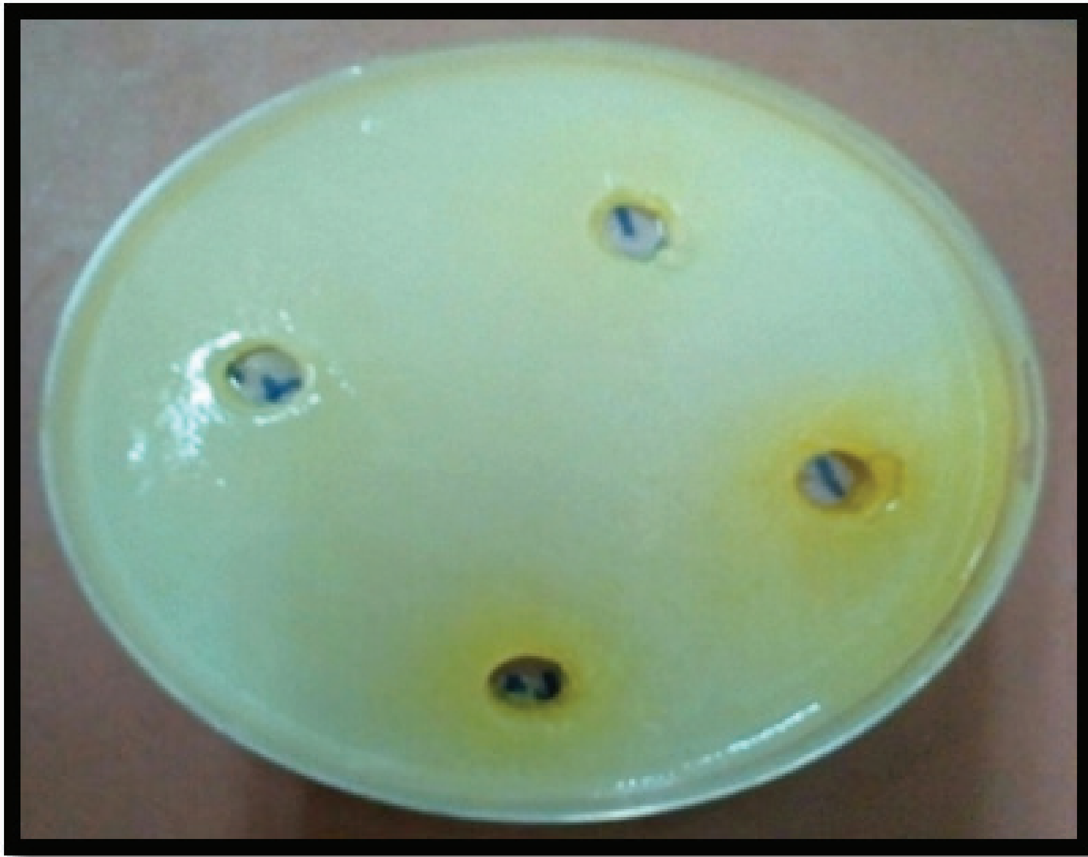

Figure 2: The protease production by *E.coli* on skim milk agar media

These findings are in agreement with results obtained by Taher (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 [12,13]. There are many factors affect protease production such as the time of incubation, the presence of enhancers, metals. in addition, protease can cleavage the immunoglobulin and many immune cells [14].

In contrast, our results showed that all the 28 *E. coli* isolates showed complete haemolysin formation (100%) as in table (1) and figure (2). These results are agreement with that of Podschan *et al* (2000) which they can isolate a high frequency of Germany clinical isolates that have similar virulence factors activity like capsule production, siderophores, resistance to serum and protease production [15]. Also a high Beta-TEM-59-lactamaseresistance and others including protease and haemolysin production by K. Oxytoca were isolated at (26%) from different European clinical sources [16].

Table 1: Showed both the protease activity and haemolysin production of 28 *E. coli* isolates

Activity Isolation No.	Protease activity diameter of lysis area (mm)	haemolysin production
1	18	100%
2	16	100%
3	16	100%

***Cont...* Table 1: Showed both the protease activity and haemolysin production of 28 *E. coli* isolates**

4	14	100%
5	16	100%
6	24	100%
7	18	100%
8	20	100%
9	16	100%
10	16	100%
11	20	100%
12	20	100%
13	14	100%
14	14	100%
15	16	100%
16	18	100%
17	16	100%
18	12	100%
19	14	100%
20	14	100%
21	14	100%
22	16	100%
23	16	100%
24	16	100%
25	18	100%
26	18	100%
27	20	100%
28	16	100%

The Effect of acridine-orange on both Protease and haemolysin production by *E. coli* isolates

The results were shown in table (2) represent that the mutagenic agent (acridine-orange) have an effect at concentrations (stock solution, to 10^6) at which all 28 isolates loss the protease activity and haemolysin production, while little and normal activities were observed at concentrations (10^{-5} to 10^{-10}) as in table (2). These findings are in agreement with the results obtained by many researchers [17] who found many of antibiotic resistance were plasmid determined and affected after curing experiments. In contrast, many results revealed the chromosomally determined abilities such as Actinorhodin-like substance production by *Streptomyces* IQ45 [17]. Jones *et al* (1990) isolate many *Proteus mirabilus* mutants affect their virulence factors [18].

Table 2: Show the effect of acridine-orange concentration on both protease and haemolysin production by *E.coli* isolates

Bacterial Activivty Acridine orange solution	Protease production	haemolysin production
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	Little (10mm)	100%
10-6	Normal(14mm)	100%
10-7	Normal 18mm	100%
10-8	Normal 22mm	100%
10-9	Normal 24mm	100%
10-10	Normal 24mm	100%

DNA profile by gel-electrophoresis

The results of DNA profile by gel-electrophoresis of both normal and cured isolates showed the prescence of chromosomal and plasmids DNA bands (520 & 950 bp) in (6, 7) isolates respectively in (normal case) while only chromosomal bands observed in *E. coli* isolates 8 which treated with acridine-orange at concentrations (10^{-2} and 10^{-4}) as in figure (3), the absence of plasmids was correlated with the absence of protease production and haemolysin formation by *E.coli* isolates as mentioned below which explain the fact of their genetics, they may be, in most probable plasmids determined neither than chromosome.

Finally, the obtained results in this research showed that acridine-orange have an effect on the virulence factors especially protease and haemolysin formation due to the mutagenic effect on the specific genes of their production by *E. coli* isolates under this study.

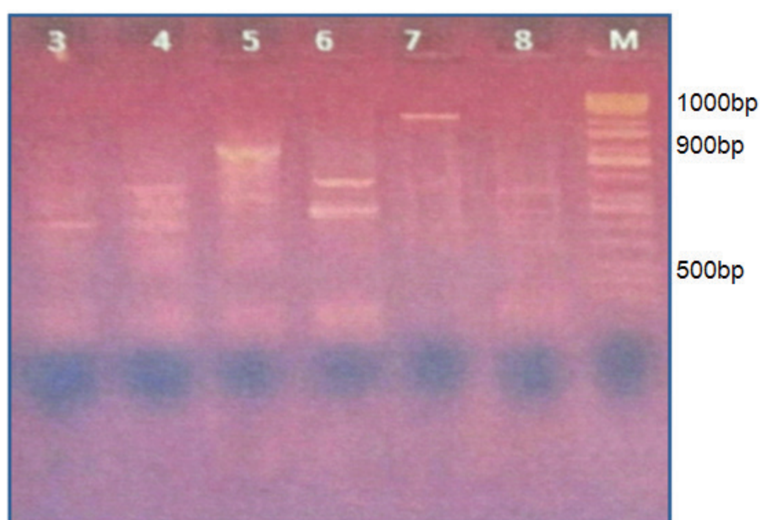

Figure 3: Agarose gel electrophoresis of both chromosomal and plasmid DNA isolated from *E.*coli iraqi isolates.lane 6,7 represent normal isolate, lane 8 represent treated isolate with acridine orange at concentration (10^{-2} and 10^{-4}) , M represent a marker.

Another study showed the presence of one mega plasmid band of *Bacillus thuringenisis* after 1.30h of electrophoresis while, there is a complex plasmid profile with different molecular weight occure within the same species, This reason may related to the differences of plasmid properties between strains related to the same species or even subspecies level [19].

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