Staphylococcal nuclease removes *Escherichia coli* and *Klebsiella pneumoniae* previously adhered to uroepithelial cells

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Abstract

Twenty-seven *S. aureus* isolates were obtained from patients referring various hospitals in Baghdad. Only 17 isolates produced DNase. SNase was extracted and purified from *Saphylococcus aureus* 3 isolate since it produces the largest zone of clearance on DNase agar. Nevertheless, only those phenotypically-producer of DNase harboured *nuc* gene. Present study revealed that the crude enzyme had a specific activity of 50.66 unit/mg; while it reached 241 unit/mg after ion exchange chromatography using carboxymethyl cellulose column. SDS-PAGE showed a single sharp band with an approximately 16.8 kDa molecular weight. A matter indicates that the enzyme is consistently pure. Results proved that SNase was able to significantly ($P< 0.05$) reduce the number of the uropathogens; *Escherichia coli* and *Klebsiella pneumoniae* attached to the uroepithelial cells.

Keywords: *Staphylococcus nuclease, E.coli, K.pneumoniae*, uroepithelial

ازالة الايشيركية القولونية و الكليبسيلا الرئوية الملتصقة سلفا على الخلايا الطلائية البولية بالانزيم حال الدنا العنقودي

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الخلاصة

تم الحصول على 27 عزلة من بكتريا المكورات العنقودية الذهبية من مرضى يعودون مختلف المستشفيات في بغداد. فقط 17 عينة استطاعت انتاج حال الدنا .استخلص انزيم حال الدنا العنقودي من العزلة رقم 3 الاكثر انتاجا. تبين انه فقط تلك العزلات التي اعطت نتيجة موجبة على اكار الدناز كانت تحمل جين حال الدنا .كشفت الدراسة الحالية بأن الانزيم الخام امتلك فعالية نوعية 50.66 وحدةاملغم بينما وصلت الى 241 وحدةاملغم بعد كروموتوغرافيا التبادل الايوني باستعمال انبوب الكاربوكسي مثيل سيليلوز .ظهر من خلال تقنية الرحلان الكهربائي و جود حزمة واحدة واضحة بوزن جزيئي يقرب من 16.8 كيلودالتون، وقد عد هذا دليلا على نقاوة الانزيم المستخلص .برهنت النتائج على ان حال الدنا العنقودي قادر وبشكل ملحوظ ($P< 0.05$) على اختزال عدد ممرضات المجاري البولية (الكليبسيلا الرئوية و الايشيركية القولونية) الملتصقة بالخلايا الطلائية البولية.

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Introduction

Staphylococcus aureus is a persistent human pathogen that is responsible for a range of diseases that vary widely in clinical presentation and severity. The pathogenesis of *S. aureus* infection is a complex process involving a diverse array of secreted and surface-associated virulence determinants that are coordinately expressed at different stages of infection [1].

Escherichia coli is a genetically diverse species that causes a variety of infections which fulfill many or all of the proposed criteria for biofilm-associated infections [2].

Klebsiella pneumoniae is an opportunistic pathogen responsible for a wide range of nosocomial infections. One important factor associated with virulence in *K. pneumoniae* is its capacity to adhere to surfaces and form biofilms [3].

Extracellular DNA (eDNA) plays a significant role in bacterial adhesion, however, there is no definite proof on a cause-and-effect relationship between DNA release and adhesion. In rare cases eDNA has been shown to inhibit bacterial settlement. Therefore, it is possible that nuclease mediated eDNA degradation would therefore promote adhesion. Consequently, it is not clear whether microbial nucleases contribute to the bacterial adhesion in clinically relevant situations [4].

To address this, the preventive activity of SNase on the adherence of each of *K. pneumoniae* and *E. coli* on uroepithelial cell were investigated.

Materials and Methods

S. aureus, E. coli and *K. pneumoniae* isolation and identification

One hundred sixty-three specimens were collected over a period of a month between August and December 2013. Specimens comprised anterior nares swabs (n= 18), sputum (n= 29), mid-stream urine (n= 91), burns swabs (n= 13) and blood (n= 12) from patients referring Al-Yarmouk teaching Hospital, medical teaching hospitals, and Al-Kadhimiyah teaching hospital in Baghdad, Iraq.

In regard to *S. aureus* isolation, all specimens were directly inoculated or Mannitol Salt Agar (MSA) and incubated at 37°C for 24 hr. Same specimens were used for the isolation of *E. coli* and *K. pneumoniae*. A loopful of a given specimen was streaked on the surface of MacConkey agar plate, followed by incubation overnight at 37°C.

Identification of suspected colonies was assessed by biochemical test and microscopic properties [5,6]. Identification of all isolates was confirmed by api and VITEC2 systems. All isolates were stored at -40°C in freezer vials containing 15% glycerol for long term preservation.

DNase test

All *S. aureus* isolates were cultured in trypticase soy broth (TSB) overnight in a concentration comparable to McFraland standard no. 0.5. Afterward, all tubes were centrifuged at 8000 ×g for 15 min. The resultant supernatant was taken, boiled for 15 min and cooled down at 4°C. Afterward, 50µl of supernatant was poured in wells punched in DNase agar and incubated overnight. Subsequently, the plate was flooded with 1N HCl and left for 5 min. A zone of clearance around the well indicated a positive result; which were measured by an aid of metric ruler [7].

Purification of *S. aureus* SNase

The method described by Ohsaka *et al.* [8] was followed with some modifications for the purification of SNase from *S. aures* isolate. The isolate; which developed the largest zone of clearance on DNase agar, was cultured in 500 ml of TSB at 37°C for 24 hr. Subsequently, supernatant was obtained, heated in a boiling water for 20 min, cooled down and diluted with 0.1 M Tris-HCl buffer, containing 0.1 M EDTA (pH 7.5). After heating and cooling to 4°C, ammonium sulphate powder was added to attain 60%-75% saturation. The obtained solution was dialyzed (< 14 kDa) against 0.01M sodium acetate buffer pH 6.5 for 24 hours at 4°C with continuous stirring and then concentrated by sucrose. Thereafter, it was neutralized with 3 M NH_4OH. Ethanol (96%) was precooled in a freezer and added to attain 50% concentration while the mixture was stirred in an ice bath. Stirring was continued for 30 minutes. The precipitate was collected by centrifugation at 4°C and was dissolved in a minimum amount of 0.1 M Tris-HCl buffer, pH 9.0.

The carboxymethyl cellulose was packed in a column (3 × 13 cm) and equilibrated with 0.1M sodium acetate buffer, pH 6.5. The sample applied was the solution from ethanol precipitation (5 ml). Elution with 0.5M sodium acetate buffer, pH 6.5, containing 0.1 through 0.5M NaCl solutions was started. Subsequently, 100 fractions (20 fractions per concentration) had been collected. The flow rate was a drop per 5 seconds, 3 ml per tube; temperature, 10°C. After that, A_{280} and SDNase activity was measured for each fraction.

Nuclease Assay

Escherichia coli DNA was extracted by Presto™ Mini gDNA Bacteria Kit (Geneaid Corporation\ Korea). A volume of 2.5 µl of the sample was incubated with 7.5 µl *E. coli* DNA (1 mg/ml) and 40 µl DNase buffer (0.01 M $CaCl_2$, 0.1 M Tris HCl; pH 8) for 60 min at 37°C. The nuclease reaction was stopped with 12.5 µl of 0.33 M EDTA (pH 8.0). One unit of activity was expressed as change in absorbance at 260nm per minute of 1.0 [8]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique was performed according to Laemmili protocol [9] using 15% resolving gel.

Detection of *nuc* by PCR

All *S. aureus* isolates were submitted to *nuc* gene detection using PCR technique following the procedure described by Poulsen *et al*. [10].

One ml of phosphate-buffered saline (PBS) is transferred to a 1.5-ml Eppendorf tube. Using a disposable inoculation loop, a loop full of bacteria is picked from a plate and transferred to the Eppendorf tube, centrifuged at 14000 rpm for 5 min. Supernatant was discarded and the pellet was resuspended in 100 µl TE 10:1. The suspension was boiled for 5-10 minutes and transferred directly to ice. The lysed DNA was diluted 10 fold in TE 10:1.

nuc1 (5'-TCAGCAAATGCATCACAAACAG-3') and nuc2 (5'-CGTAAATGCACTTGCTTCAGG-3') primers (Bionear, Korea) were dissolved in sterile distilled water (D.W.) to give a final concentration of 100 pmol/ µl as recommended by provider and stored in a freezer until use. Two microliters of each primer, 50 ng of DNA extracted from each *S. aureus* isolates and deionized D.W. were added to PCR premix tubes (Bionear, Korea) to reach 20µl as a final volume. The PCR profile was: denaturation for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C, and finally elongation for 2 min at 72°C.

Detection of SNase activity by gel electrophoresis

Purified SNase (20 U/ml) was added to the DNase buffer and incubated with the *E. coli* heat treated DNA in one tube and non-treated DNA in another tube (75 ng as a final concentration). Following incubation for 10 min at room temperature, reactions were stopped by the addition of 0.1 volume of 10% SDS and directly applied this solution to 1% agarose gel [11].

Impact of purified SNase on the adherence of *E. coli* or *K. pneumoniae* on epithelial cell

Uroepithelial cells were collected from healthy young female, washed three times with phosphate buffered saline. Overnight cultures of the *E. coli* or *K. pneumoniae* to be tested were suspended to 10^8 cfu/ml in phosphate buffered saline which is comparable to McFarland 0.5. Equal volumes of the bacterial suspensions, uroepithelial cells, and purified SNase were mixed and incubated at 37°C for 30 min. Afterward, the suspensions were washed thrice with 10 ml of phosphate buffered saline. Cells were retained by centrifugation (1000 ×g for 10 min.), placed on microscope slides, fixed with methanol, and Gram stained. The assays were started within one hour of the collection of the uroepithelial cells. A negative control contained bacterial cells and uroepithelial cells without SNase. The number of attached bacteria was calculated in 40 random uroepithelial cells [12].

Statistical methods

All assays were performed in triplicate and mean values ± SD, ANOVA, and t-test were calculated using Microsoft Office Excel 2010. All values were deemed significantly different when $P < 0.05$; however, they were considered highly significant $P < 0.001$.

Results and Discussion

Purification of SNase

Depending on the results presented in table- 1, *S. aureus* S3 was chosen for SNase extraction since that it accomplished the highest zone of clearance. Supernatant of this isolate culture was boiled to achieve the inhibition of other enzymes activities and remaining bacterial cell. Tang *et al*., [13] indicated that the nucleases secreted by strains still showed functional activity after 30 min at 121°C. Present study revealed that the supernatant (crude enzyme) had a specific activity of 50.66 unit/mg (table-2).

Table 1- Diameter of the zones of clearance developed by *S. aureus* on DNase agar.

SD	**Mean* (mm)**	**Isolate code**	**SD**	**Mean* (mm)**	**Isolate code**
0.05	1.13	S15	0.05	1.26	S1
0	0	S16	0.05	1.16	S2
0.11	1.06	S17	0.05	1.53	S3
0.05	1.13	S18		0	S4
0	0	S19	0.20	1.26	S5
0.11	1.13	S20	0	1.2	S6
	0	S21		0	S7
0.11	1.13	S22	0	1.2	S8
0.05	1.16	S23	0.1	1.1	S9
0	0	S24		0	S10
	0	S25	0.11	1.16	S11
0	1.2	S26		0	S12
0.05	0.96	S27		0	S13
			0.05	1.13	S14

*mean of triplicate. SD= standard deviation. P= 1.3×10^{-40}, $LSD_{0.05}$= 0.12

Table 2- Summary of purification procedure

Purification step	Volume (ml)	Protein Concentration (mg)	Activity (AU/ml)	Total Activity (AU)	Specific Activity (AU/ mg)	Fold Purification	% Yield
Crude	500	0.49003	24.83	12415	50.67036712	1	100
Ammonium sulphate, 60-70%	45	0.23107	18.18	818.1	78.677	1.55	6.58
Ethanol, 0-50%	10	0.09561	11.9	119	124.463	2.45	0.95
CM cellulose	6	0.03187	7.71	46.26	241.920	4.77	0.37

*CM= carboxymethyl

When EDTA is added to the buffer containing Tris HCl, a loss of about 10% nuclease activity will result; at the same time, however, about 98% of acid phosphatase, and about 95% of alkaline phosphatase were eliminated. The extracted protein is recrystallized and thus recovered by gradually warming the cold solution to room temperature. This method has the added advantages that the extraction media may be buffered or stabilizing agents be added to retain the maximum enzyme activity. The alkaline phosphatase is activated not only by Mg^{++} but also, to a lesser degree, by Ca^{++}. This makes it a particularly dangerous

contaminant of nuclease. The alkaline phosphatase is much more thermolabile than the nuclease. This property is utilized to remove the alkaline phosphatase. The removal of acid phosphatase by thermal destruction is not very efficient. Much more efficient separation of the acid phosphatase is achieved by chromatography [14].

Precipitation by ammonium sulphate

Results presented in table -2 revealed that precipitation with ammonium sulphate raised the purification fold of SNase by 1.522 with a specific activity up to 78.675 unit/mg.

Saturation degree of 60% and 75% was used in this study to precipitate SNase in accordance to Sulkowski and Laskowski [15]. Given that Protease is precipitated at 90% saturation [16] and a lipase non-producer was used; therefore, no acid phosphates, alkaline phosphatase (eliminated at first step), protease and lipase will contaminate the extract.

In the protocol described by Sulkowski and Laskowski [15], trichloroacetic acid (TCA) helped in precipitating proteins and enzymes that have molecular weight nearly similar to the nuclease. Nevertheless, TCA precipitation of proteins is commonly used to concentrate protein samples or remove contaminants, including salts and detergents, prior to SDS-PAGE. TCA precipitation denatures the protein, so it should not be used if the protein must remain in its folded state (e.g., if a biochemical activity of the protein is wanted to be measured) [16]. Consequently, this step was eliminated.

Ion exchange column chromatography

The final step in purification was ion exchange chromatography by carboxymethyl cellulose column, 100 fractions were collected, each of which contained 3 ml of elution. Enzyme activity was calculated for each collected fraction. However, the maximum activity for enzyme was seen in fractions number 83 and 84 as it illustrated in figure -1. A specific activity of pooled 83 and 84 fractions was 241 unit/mg. Sulkowski and Laskowski [15] achieved a potency (potency = activity / A280) of 237 with a CM cellulose column; whereas Ohsaka *et al*., [14] used DEAE cellulose column reached a potency of 7.58.

Two weak exchangers can be used for protein separation are carboxymethylcellulose (CM-cellulose) and diethylaminoethyl-cellulose (DEAE-cellulose). CM-cellulose is negatively charged, so it is a weak cation exchanger. DEAE-cellulose is positively charged at neutral pH and so DEAE-cellulose is a weak anion exchanger [18]. In an initial attempt to identify the nuclease, Kiedrowski *et al*., [18] indicated that the nuclease is cationic. Taking together, it can be realized the reason behind the high efficiency of CM cellulose over DEAE cellulose.

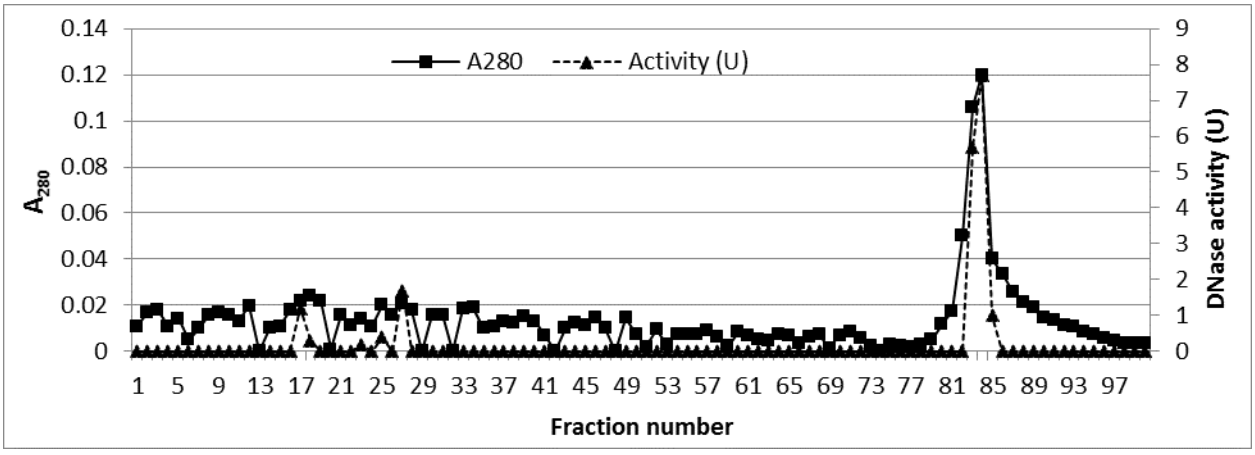

Figure 1- Elution pattern of SNase from a CM-cellulose column (3 × 13 cm), the gel was equilibrated with 0.1M sodium acetate buffer, pH 6.5. Elution was started with 0.5M sodium acetate buffer, pH 6.5, containing 0.1 through 0.5M NaCl solutions. Subsequently, 100 fractions (20 fractions per concentration) had been collected. The flow rate was a drop per 5 seconds, 3 ml per tube; temperature, 10°C.

Detection of SNase activity by gel electrophoresis

To determine if purified enzyme is the SNase its activity to degrade DNA by gel electrophoresis technique was checked. A single band appeared referred to untreated DNA; however, a smear was seen in regard to SNase treated DNA whether it is a single (heat treated) or double stranded (figure -2).

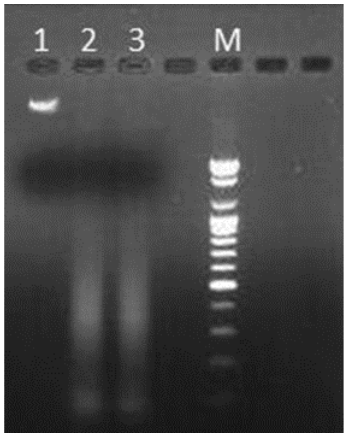

Figure 2-Degradation of DNA derived from *E. coli* isolate by SNase. Shown are representative samples of untreated DNA (lane 1), heated DNA (lane 2), and non-heated DNA (lane 3). Lane M represents 100 pb DNA ladder. Visualized by 1% agarose analysis at 5 V/cm for 1 hr, stained with ethidium bromide and visualized on a UV transilluminator documentation system.

SDS-PAGE of SNase

Staphylococcus aureus DNase was assayed by SDS-PAGE in order to check the SNase purity. Single sharp band has been seen in the polyacrylamide gel as it is shown in figure- 3 a matter indicates that the enzyme is homogenously pure. Using a standard curve illustrated in (figure -4), the molecular weight was estimated about 16.8 kDa, accordingly; we determined the presence of extracellular enzyme SNase.

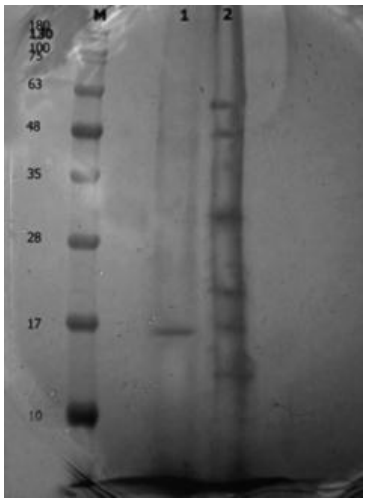

Figure 3- SDS-PAGE analysis of *S. aureus* SNase. M: Protein marker, lane 1: Pure SNase, lane 2: crude preparation (culture supernatant).

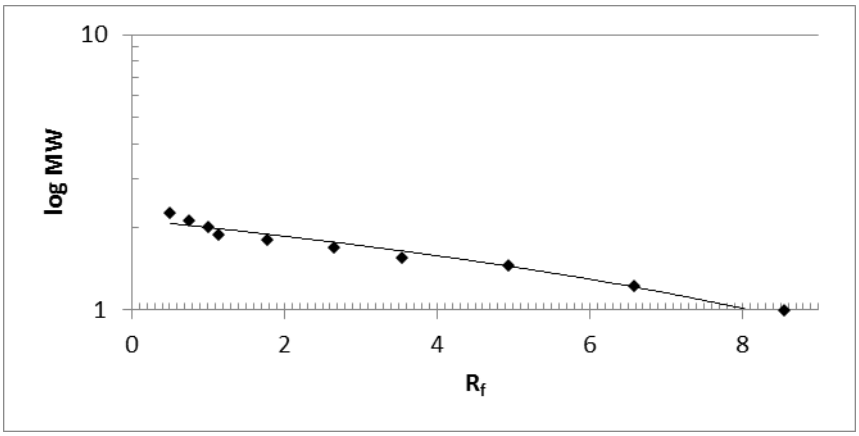

Figure 4- A representative standard curve (trendline) for molecular weight estimation. Protein marker standard with molecular weight range from 10 kDa to 180 kDa were separated in 15% SDS-PAGE.

The findings from in vitro expression and mutagenesis studies together indicated that two types of thermostable nucleases, encoded by two different genes, coexisted in *S. aureus*, designated as SNase and TNase [18]. Recently, Gabriel *et al*., [16] reported that SNase enzyme was produced freely in the medium but TNase remain anchored to the bacterial surface. Therefore, it can be concluded that the extracted enzyme in the present study is no doubt SNase.

Detection of *nuc* gene by polymerase chain reaction

Only isolates that gave positive results on DNase agar harboured *nuc* gene (figure -5).

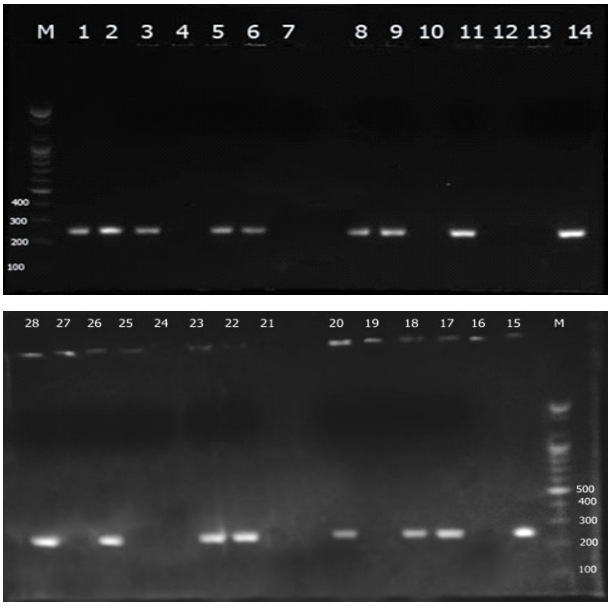

Figure 5-Visualization of *nuc* gene by 1.5% agarose gel analysis. The shown bands are representative of PCR products (255 pb) amplified from *S. aureus* isolates (lanes 1 - 27), negative control (lane 28), lane M represents 100 bp DNA ladder.

Impact of SNase on uropathogenic bacteria adherence on uroepithelial cell

Results revealed that SNase was able to significantly ($P< 0.05$) reduce the number of the uropathogens; *E. coli* and *K. pneumoniae* attached to the uroepithelial cells (figure- 6, tables -3 and 4).

Bacterial adhesion to epithelial cells may thus be of importance in the pathogenesis of urinary tract infections. Uropathogenic *E. coli* (UPEC) colonizes the perineum, crosses the urethra to the bladder, and occasionally ascends to the kidney. Adherence of UPEC to urinary tract mucosa is mediated mainly by bacterial surface fimbriae such as type 1 or P. [19].

In a preliminary report, it was suggested that the clinical expression of urinary tract infections as symptomatic or asymptomatic might be related to the ability of the bacteria to adhere to the mucous surfaces of the urinary tract *E. coli* bacteria isolated from the urine of patients with acute pyelonephritis were shown to adhere in significantly larger numbers to human uroepithelial cells *in vitro* than did bacteria from patients with asymptomatic bacteriuria [20].

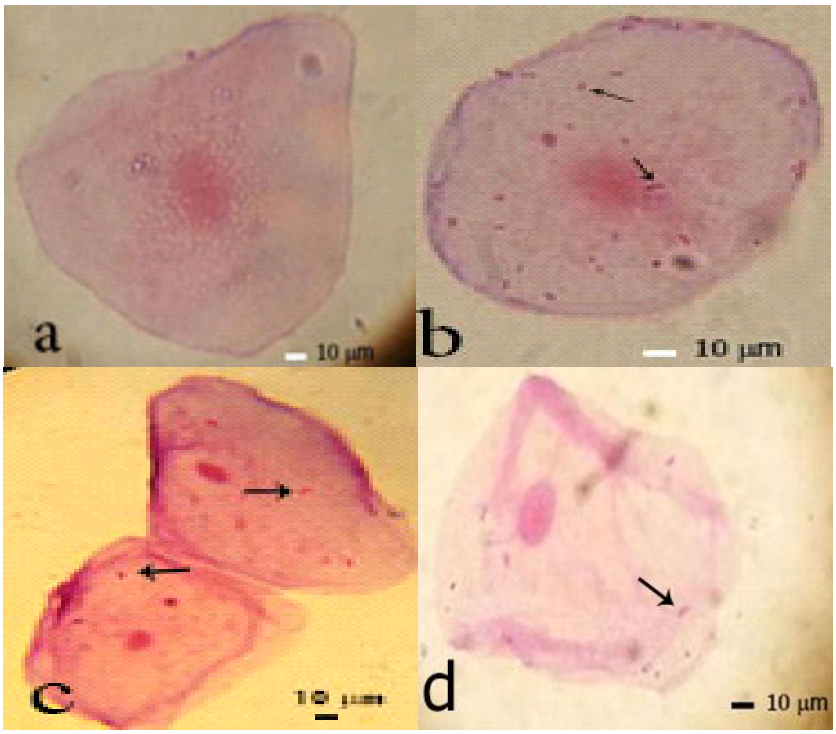

Figure 6- SNase blocked adhesion of uropathogen on uroepithelial cell. a) Negative control. b) Positive control. c) SNase with *E. coli*. d) SNase with *K. pneumoniae*. Arrows signify the attached bacteria. Gram stain. 1000X.

Table 3- Impact of SNase on adhesion of *E. coli* on uroepithelial cells

P value	After treatment		Before treatment		isolate code
	SD	Mean	SD	Mean*	
0.000373	2.081666	3.666667	3.05505	32.33333	E1
0.000594	1.527525	6.333333	2	22	E2
0.004845	2.516611	4.333333	4.50925	25.66667	E3
0.000101	2.081666	9.33333	1.527525	36.33333	E4
0.029873	1.527525	5.333333	4.041452	16.33333	E5
0.000292	1.527525	4.666667	2.645751	35	E6
0.001047	2	6	3.05505	27.66667	E7
0.003112	2	5	4.041452	28.66667	E8
0.005995	1.527525	6.333333	4.041452	27.66667	E9
0.007765	1.154701	2.333333	2.516611	13.33333	E10
0.001071	1.527525	2.666667	2.081666	16.66667	E11
0.001303	2	4	3.785939	32.66667	E12
0.000279	1.527525	3.666667	2.516611	31.66667	E13
0.003901	1.527525	4.333333	2.516611	16.66667	E14
0.001628	2.516611	4.666667	3.05505	22.66667	E15
0.003404	2.081666	4.666667	4.725816	33.33333	E16
0.000313	1.527525	4.333333	2	23	E17
0.000611	1.527525	4.666667	2.516611	26.66667	E18
0.00073	2.309401	3.666667	2.081666	20.66667	E19
0.00087	1.527525	4.333333	3.21455	34.66667	E20
0.00028	3.21455	6.666667	2.516611	37.33333	E21
0.0018	1.732051	3	3.785939	31.33333	E22
0.000768	1.527525	2.666667	2.645751	25	E23
0.003046	2.645751	3	3.605551	21	E24
0.001383	1	4	2.645751	29	E25
0.002117	1.154701	1.666667	2.081666	14.66667	E26

*measured by bacterial cell/epithelial cell, SD= standard deviation

Table 4- Impact of SNase on adhesion of *K. pneumoniae* on uroepithelial cells.

P value	After treatment		Before treatment		isolate
	SD	Mean	SD	Mean*	code
0.001342	0.57735	3.666667	2.516611	35.33333	K1
0.000136	1.154701	1.666667	2.081666	33.33333	K2
0.000215	1.527525	4.333333	2	25	K3
0.00472	2.081666	3.666667	2.081666	13.33333	K4
0.000298	2	3	2.081666	22.66667	K5
0.000231	1.527525	3.666667	2.516611	33.33333	K6
0.000331	3.05505	6.666667	2.081666	36.33333	K7
0.000613	2	3	2.081666	19.33333	K8
0.001365	3.605551	5	2	31	K9
0.00032	2.645751	4	2.081666	28.33333	K10
0.000317	1.527525	4.666667	2.081666	24.33333	K11
0.000161	1.154701	3.666667	2.081666	33.66667	K12
0.000228	2	6	1.527525	26.33333	K13
0.021576	1.527525	6.333333	2.516611	13.33333	K14
0.011045	1.527525	3.333333	3.05505	14.66667	K15
0.000317	1.527525	6.666667	2.081666	26.33333	K16
0.003808	0.57735	2.666667	1.527525	12.33333	K17
0.001311	1.732051	3	2.516611	19.33333	K18
0.000316	2.081666	4.666667	2.516611	27.66667	K19
0.000452	2	4	2.081666	21.66667	K20
0.000513	2	5	3	31	K21
0.000732	2.516611	4.333333	3.05505	26.66667	K22
0.009119	2.645751	5	3.511885	17.66667	K23
0.000155	2.645751	5	2	35	K24

*measured by bacterial cell/epithelial cell, SD= standard deviation

Maximal adhesive ability was always seen after termination of the logarithmic growth phase. The most rapid increase in adhesion was observed during the first 60 min of incubation [21]. However, Koontz [17] obtained good adherence during 30 min of incubation.

Once attached, bacteria may penetrate, activate the defense mechanisms of the host, or multiply on the cell surface until the epithelial cell is discharged and new adhesion must take place. The normally slow turnover of bladder epithelium is likely to be faster during infection. Epithelial cells in the urine sediment of infected patients are, thus, likely to have bacteria attached to the surface [22].

Poulsen *et al*., [10] reported that eDNA commonly promote adhesion and biofilm formation by bacteria. What's more, Das *et al*., [23] stated that the presence of eDNA on bacterial cell surfaces enhances adhesion and surface aggregation due to the involvement of acid-base interactions. eDNA also creates thermodynamically favorable conditions for bacterial adhesion to hydrophobic surfaces, whereas adhesion to a hydrophilic surface is mediated predominantly by thermodynamically favorable conditions for surface aggregation of adhering bacteria. In addition, its binding with Ca^{2+} at biologically relevant concentrations was shown further increase in bacterial aggregation via cationic bridging.

Consequently, SNase showed a marked ability to remove previously adhered uropathogens; *E. coli* and *K. pneumoniae* to uroepithelial cells more likely due to degradation of eDNA; hence, weaken the interaction between cells and pathogen.

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