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ORIGINAL ARTICLE

Detection of Some Virulence Factors and Pyelonephritis–Associated Pilus (pap) Encoding Operon Gene in Uropathogenic *Escherichia coli*

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Key words:

UTI,
Virulence factors of *E.coli*,
pap gene

ABSTRACT

Background: *Urinary tract infection is one of the most common bacterial infections caused by E.coli that have virulence properties including the expression of specific adhesions, toxins such as haemolysin , also the serum resistance, gelatinase production and The P fimbriae which considered an essential virulence factor causing pyelonephritis and encoded by The pyelonephritis–associated pilus (pap) operon.* ***Objectives:*** *This work aimed to detect the association of some virulence factors of uropathogenic Escherichia coli (UPEC) strains: cell surface hydrophobicity, haemolysin production, serum resistance, gelatinase production, extended spectrum β lactamase production and pap adhesion encoding operon gene which is responsible for adhesion of E.coli to uroepithelium.* ***Methodology:*** *This work was carried out on 80 patients (27males and 53 females, their ages ranged from 15 to 60 years old) attending the Outpatient Clinic of Urology Department of Benha University Hospital suffering from urinary tract infection (UTI). 80 Urine samples (patients group) and 20 stool samples (control group) were subjected for isolation and identification of UPEC and commensal E.coli respectively. Antibiogram by disc diffusion method, detection of some virulence factors and pap gene by PCR were done for all isolated E.coli strains.* ***Results:*** *UPEC was the most common isolated bacteria 50(62.5%). 33 (66%) of UPEC strains show resistance to ampicillin (10 µg), 45 (90%) of UPEC strains show sensitivity to amikacin (30 µg). In commensal E.coli strains: 12(60%) strains show resistance to ampicillin(10 µg) while 20 (100%) strains were sensitivity to gentamycin (10 µg). 23 (46%) of UPEC strains were hydrophobic, 12 (24%) strains were haemolysin producers, 31 (62%) strains were serum resistant, 1(2%) strain liquefied gelatin and 26 (52%)strains were extended spectrum β lactamase production (ESBL).In commensal E.coli strains: 9 (45%) strains were hydrophobic, 3 (15%) strains were haemolysin producers, 11 (55%) strains were serum resistant, no (0%) strain liquefied gelatin and 8 (40%)strains were ESBL. In UPEC; 36(72%) strains had PAP gene while 12(60 %) strains of commensal E.coli had PAP gene.* ***Conclusion:*** *It can be concluded that pap gene plays an important role in virulence of UPEC.*

INTRODUCTION

UTI is one of the most common bacterial infections, and is defined as colonization of a pathogen occurring anywhere along the urinary tract: kidney, ureter, bladder, and urethra[1, 2]. *E.coli* is by far the most common cause of UTI accounting for 80 - 90% of all UTIs seen among ambulatory populations. These *E. coli* are named uropathogenic *E. coli* (*UPEC*) and have virulence properties that are associated with infection in the normal urinary tract including the expression of specific adhesions, toxins such as haemolysin , also the serum resistance and gelatinase production[3]. The P fimbriae is considered as an essential virulence factor causing pyelonephritis. The pyelonephritis–associated pilus (pap) operon encodes for the P fimbriae adhesion which has been shown to mediate attachment to specific cell surface glycopeptides present throughout the urinary tract. They facilitate colonization and invasion of the renal parenchyma[4]. Treatment of E.coli infections

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is increasingly becoming difficult because of the multidrug resistance exhibited by the organism. The incidence of ESBL producing strains of E.coli among clinical isolates has been steadily increasing resulting in limitation of therapeutic options[5].This work aimed to detect the association of some virulence factors of *UPEC* strains: cell surface hydrophobicity, haemolysin production, serum resistance, gelatinase production, ESBL production and pap adhesion encoding operon gene which is responsible for adhesion of *E.coli* to uroepithelium.

METHODOLOGY

Patients:

This work was carried out in Microbiology and Immunology Department, Benha Faculty of Medicine in the period from January 2014 to March 2015 on eighty patients attending the Outpatient Clinic of Urology Department of Benha University Hospital suffering from UTI. They were 27 males and 53 females and their age ranged from 15 to 60 years old. Twenty healthy individuals matched for age and sex with the patients group were enrolled in the study as control group.

Samples

Clean catch mid stream urine samples were collected from UTI patients in sterile screw capped containers and twenty stool samples as a source of commensal *E.coli* were collected from the control group.

The collected urine and stool samples were subjected for the following:

1. **Isolation and identification of *E.Coli strains*:**
   Urine and stool samples were cultured on MacConkey agar *(Oxoid),* lactose fermenting colonies *were identified by* biochemical reaction tests for identification of *E.coli.*
2. **Antibiotic susceptibility testing for isolated E.coli:**
   Using Muller Hinton agar *(Oxoid)* and antibiotic discs *(Bioanalyse)* with antimicrobial content matching Clinical Laboratory Standard recommendations [6] including ampicillin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), cefotaxime (30 µg) and ceftazidime (30 µg).
3. **Detection of some virulence factors for isolated E.coli:**
   a. **Cell surface hydrophobicity:** was detected by salt aggregation test (SAT) [7]. The isolated *E.coli* strains grown on MacConkey agar plates were inoculated into 1 ml of Phosphate buffer saline (PBS) pH 6.8, the turbidity was matched with McFarland 7 turbidity that get a colony count of $5x10^9$ colonies/ml. Different concentrations of ammonium sulphate namely 0.4 molar, 1.0 molar, 1.25 molar, 1.4 molar and 2.0 molar concentrations were prepared. One loopful of the isolated *E. coli* strains suspension was mixed with a drop of ammonium sulphate solution of different molarity on a glass slide with rotation and observed for aggregation for 1 min. *E. coli* strains show aggregation in 1.25 molar solution or less is considered hydrophobic.
   b. **Haemolysin production*:*** The isolated *E.coli* strains were inoculated onto 5% sheep blood agar and incubated overnight at 35ºC. Haemolysin production was detected by presence of a zone of complete haemolysis around the colony.
   c. **Serum resistance:** One loopful of the isolated *E. coli* strains grown on nutrient agar at 37ºC for 24h was dissolved in 1ml of Hanks balanced salt solution (HBSS) (tube 1). 50µl from tube (1) was added to 50µl of serum (tube 2) and incubated at 37ºC for 180 min. 10 µl from each tube(tube1 and tube 2) was withdrawn, cultured on blood agar plates and incubated at 37ºC for 18h. The viable count on blood agar plates cultured from tube 2 were detected and compared with blood agar plate cultured from tube 1. Bacteria were termed serum sensitive if the count dropped to 1% on blood agar cultured from serum compared to blood agar cultured from HBSS, while it termed serum resistant if the count was ≥ 90.
   d. **Gelatin liquefaction:** Tested by gelatinase production using gelatin agar tubes.
   e. **Extended spectrum β lactamase production*:*** Tested by using antibiotic discs:-
      - Cefotaxime (30µg) and Ceftazidime (30µg) *(Bioanalyse).*
      - Combined discs of Cefotaxime+ clavulanic acid (30ug/10µg) and Ceftazidime + clavulanic acid (30ug/10µg) *(MAST. Endomedex Co.).*

   Using standard disc diffusion method according to criteria recommended by NCCLS. An inhibition zone of ≤ 22mm for ceftazidime and ≤ 27mm for cefotaxime indicates probable ESBL producing strain.
4. **Detection of pap gene by PCR:**
   DNA extraction using Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721. The purified DNA was stored at -20°C till used in amplification step. DNA amplification carried out by using master mix *(Thermo Scientific).* Dream Taq Green PCR Master Mix (2X) #K1081 and Primers *(Biosearch technologies, USA).*
   Sequence of primer for pap gene:
   Pap F: 5`-GACGGCTGTACTGCAGGGTGTGGCG – 3`
   Pap R: 5`- ATATCCTTTCTGCAGGGATGCAATA – 3`

Table 1: PCR cycle for amplification of pap gene:

Step	*Temperature, °C*	*Time*	*Number of cycles*
Initial denaturation	95°C	3min	1
Denaturation	95°C	30 sec	25-40
Annealing	65°C	30 sec	
Automated fluorescent extension	72°C	21 sec	
Final extension	72°C	10 min	1

Then detection of 328bp amplified product of pap gene by agarose gel electrophoresis was carried out according to[8].

RESULTS

Out of 80 cultured urine samples 73 strains (91.25%) were isolated: These were 50(62.5%) *E.coli,* 11*(*13.8%) *Enterococci,* 4*(*5.0%) *Klebsiella aerogenes,* 3*(*3.7%) *Pseudomonas aeruginosa,* 3(3.7%) *Proteus mirabilis* and 2(2.5%) *Citrobacter* while 7 urine samples show no growth as shown in figure 1.

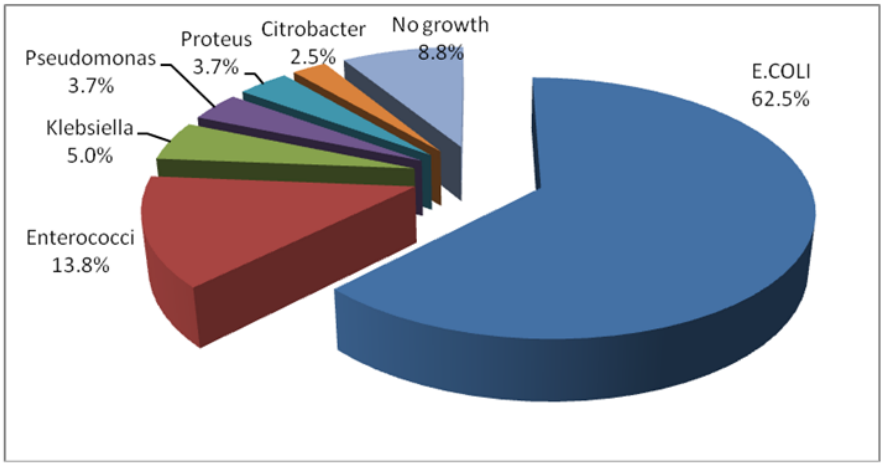

Fig. 1: Percentage and type of organisms isolated from urine samples of UTI patients.

Antibiotic sensitivity test of *UPEC* strains showed that out of 50 isolated uropathogenic strains: 33(66%) strains showed resistance to ampicillin(10µg), 45 (90%) strains showed sensitivity to amikacin (30µg). 34(68%) strains showed sensitivity to ciprofloxacin (5ug). 40 (80%) strains showed sensitivity to gentamycin(10g). For cefotaxime (30µg), 17 (34%) strains were resistant, and 24 (48%) strains were sensitive. 42(84%) strains showed resistance to ceftazidime(30µg). Antibiotic sensitivity test of commensal strains (control) showed that out of 20 isolated commensal *E.coli* strains: 12(60%) strains showed resistance to ampicillin (10µg). 17(85%) strains showed sensitivity to amikacin (30µg). 18(90%) strains showed sensitivity to ciprofloxacin (5µg). All isolated strains (100%) showed sensitivity to gentamycin (10µg). 12(60%) strains showed sensitivity to cefotaxime (30µg). 16(80%) strains showed resistance to ceftazidime (30µg).

The isolated uropathogenic and commensal *E.coli* strains showed resistance or intermediate sensitivity to Cefotaxime (30µg) or Ceftazidime (30µg) were subjected to test of ESBL production using Cefotaxime /clavulanic acid 30/10ug and Ceftazidime /clavulanic acid 30 /10µg discs. The results showed that the strains that give inhibition zone diameter when combined discs used ≥ 5mm of diameter of inhibition zone when cefotaxime 30µg and ceftazidime(30µg) discs used were termed as ESBL producer.26 (52%) of *UPEC* strains were ESPL producers and 8 (40%) of commensal *E.coli* strains were ESBL producers. Comparison between antibiotic sensitivity pattern of the isolated *UPEC* and commensal *E.coli* strains revealed a significant statistical value to ciprofloxacin and gentamycin (P-value <0.05) while there is insignificant statistical value (P-value > 0.05) between *UPEC* and commensal *E.coli* strains.

Table 2: Comparison between antibiotic sensitivity pattern of isolated uropathogenic and commensal E.coli strains:

		Antibiotic sensitivity pattern of isolated *UPEC* and commensal *E.coli* strains		
Antibiotic	**isolated *E.coli* strains**	**Resistant**	**Intermediate sensitivity**	**Sensitive**
Ampicillin (10 µg)	Uropathogenic	33 (66%)		17 (34%)
	Commensal	12 (60%)		8 (40%)
p-value		>0.05*		>0.05*
Amikacin (30 µg)	Uropathogenic	3(6%)	2 (4%)	45 (90%)
	Commensal	3(15%)		17 (85%)
p-value		>0.05*		>0.05*
Ciprofloxacin (5 µg)	Urine	16 (32%)		34 (68%)
	Stool	2 (10%)		18 (90%)
p-value		<0.05**		<0.05**
Gentamycin (10 µg)	Uropathogenic	8 (16%)	2 (4%)	40 (80%)
	Commensal			20 (100%)
p-value				<0.05**
Cefotaxime (30 µg)	Uropathogenic	17 (34%)	9 (18%)	24 (48%)
	Commensal	5 (25%)	3 (15%)	12 (60%)
p-value		>0.05*	>0.05*	>0.05*
Ceftazidime (30 µg)	Uropathogenic	42 (84%)	8 (16%)	
	Commensal	16 (80%)	4 (20%)	
p-value		>0.05*	>0.05*	

* Insignificant (P value >0.05) **Significant (P value <0.05)

Virulence factors of UPEC and commensal *E.coli:*

For *UPEC*,23 (46%) strains were hydrophobic, 12 (24%) strains were haemolysin producers, 31 (62%) strains were serum resistant, 1(2%) strain liquefied gelatin and 26 (52%) were ESBL. There is insignificant statistical difference between the isolated positive and negative cell surface hydrophobic *UPEC* strains. There is high significant statistical difference (P-value <0.001) between the isolated positive and negative haemolysin and gelatinase *UPEC* producing strains. There is significant statistical difference between the serum resistant and sensitive strains. The control commensal strains 9 (45%) were hydrophobic, 3 (15%) were haemolysin producers, 11 (55%) were serum resistant, no (0%) liquefied gelatin and 8 (40%) were ESBL producers. There is insignificant statistical difference (P-value>0.05) between the isolated positive and negative commensal *E.coli* strains as regard cell surface hydrophobicity and serum resistance. There is high significant statistical difference (P-value<0.001) between the isolated positive and negative haemolysin commensal *E.coli* producing strains. The comparison between virulence factors of the isolated uropathogenic and commensal *E.coli* strains revealed insignificant statistical difference (P value >0.05) in cell surface hydrophobicity, haemolysin production, serum resistance, gelatinase test production and ESBL production.

Table 3: Comparison between virulence factors of isolated uropathogenic and commensal *E.coli* strains:

Virulance factors	***Result***	***UPEC***	***Commenseal E.coli***	***p-value***
		No. (%)	**No. (%)**	
Cell surface hydrophobicity	+ve	23(46%)	9(45%)	>0.05
	-ve	27(54%)	11(55%)	*NS
Haemolysin production	+ve	12(24%)	3(15%)	>0.05
	-ve	38(76%)	17(85%)	*NS
Serum resistance	Resistant	31(62%)	11(55%)	>0.05
	Sensitive	19(38%)	9(45%)	*NS
Gelatinase test production	+ve	1(2%)	0(0%)	>0.05
	-ve	49(98%)	20(100%)	*NS
ESBL production		26(52%)	8(40%)	>0.05
				*NS

*NS: Non significant

The isolated *E.coli* strains subjected to PCR for detection of pap gene revealed that 36(72%) and 12(60 %) of *UPEC* and commensal *E.coli* strains had pap gene respectively.

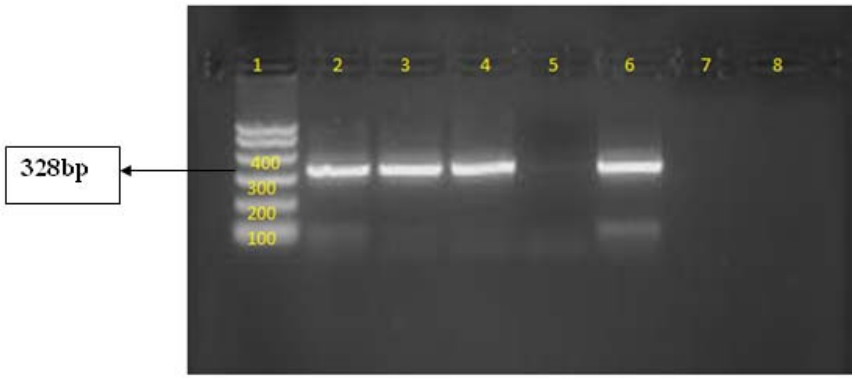

Fig. 2: Lane (1) marker, lane 2,3,4,6 positive for *E.coli* pap gene while lane 5, 7, 8 negative for *E.coli* pap gene

DISCUSSION

UTI is a prevalent public health problem that varies from cystitis to pyelonephritis. The major pathogen associated with this infection is *E. coli*. This infection depends on the virulence factors of the infecting strains and on the susceptibility of the host, especially if there is an associated urological anomaly [9,10]

This study aimed to detect the association of some virulence factors of *UPEC* strains and pap adhesion encoding operon gene which is responsible for adhesion of *E.coli* to uroepithelium.

In this study out of 80 cultured urine samples 73(91.25%) show bacterial growth. They include 50 (62.5%) *E.coli* strains. These results agree with Raksha et al., and Ranjan et al., [7, 11] who found that out of 220 urinary isolates, 151 (68.6%) were *UPEC*. Also Khawcharoenporn et al[12] found that *E. coli* remains the most common pathogen in UTIs; *E.coli* were 323 (72%) out of 431 UTI patients. In this study the isolated *UPEC* strains were subjected to antibiotic sensitivity test and the results revealed that 66% and 84% were resistant to ampicillin (10 µg) and ceftazidime (30 µg) respectively. while 90%, 68%, 80% and 48%were sensitive to amikacin (30ug), ciprofloxacin (5ug), gentamycin (10µg) and cefotaxime (30µg). Sharma et al[13] found that *E.coli* resistance occured to commonly used antibiotics such as ampicillin,ciprofloxacin, co-trimoxazole, cefotaxime, gentamicin, amikacin and netillin. The presence of multidrug resistance may be related to the dissemination of antibiotic resistance among hospital isolates of *E. coli*. Among aminoglycosides, netillin was found to have an edge over gentamicin and amikacin. Maximum number of isolates (76.9%) were resistant to ampicillin and the lowest (42.8%) to netillin. Kausar et al [14] reported that the majority of *E.coli* isolates 92% were sensitive to Amikacin. The maximum resistance was recorded for Ampicillin and Nalidixic acid (91.5%) and (93%) respectively. Oliveira et al[15] reported in their study that 59% of *UPEC* strains resistant to one or more antimicrobials. The most frequent antimicrobial resistance were found against ampicillin (51%); this in agreement with Houdouin et al and Talan et al[16,17] who found that ampicillin resistance ranging from 30 to 58% The second most frequent resistance observed in their work was to trimethoprim/sulfamethoxazole (44%) while the resistance to ciprofloxacin was found in 13% of strains. These results show that resistance to the most frequently used antimicrobials agents are found in a high percentage of *UPEC* isolates; a cause for concern since this reduces the first-line alternatives for therapy. A high level of resistance was found due to antimicrobials frequently used for UTI treatment.

In this study the isolated commensal *E.coli* strains were subjected to antibiotic sensitivity test; 85%, 90%, 100% and 60% were sensitive to Amikacin (30µg), Ciprofloxacin (5µg), Gentamicin (10µg) and Cefotaxime (30µg) respectively. 60% and 80%were resistant to ampicillin(10µg) and Ceftazidime (30µg) respectively. This result agrees with Qin et al [18] who reported that most of the intestinal commensal isolates in their study were susceptible to all the tested antimicrobial agents.

This study showed that 26 (52%) out of 50*UPEC* and 8 (40%) out of 20 commensal *E.coli* strains were ESBL producers. This result is in agreement with Sharma et al[13] who reported that out of 75 isolates resistant to cefotaxime (30µg), 70 (93.4%) were ESBL producers; they were positive by confirmatory test for ESBL and observed that the high rate of ESBL production by *E. coli* may be due to indiscriminate use of cephalosporins. Mukherjee et al [19] reported that the ESBL phenotype confirmation test was performed on 28 isolates which were resistant to either all three third generation cephalosporins (ceftriaxone, ceftazidime, cefotaxime) or any one as was revealed by the disc diffusion technique. 18(64.3%) out of 28 cephalosporin

resistant isolates were ESBL producers; the zone of inhibition increased by >5mm when it was tested in the presence of a cephalosporin containing disk and drug inhibitor combination discs respectively. Moreover, the ESBL confirmatory test must always be performed with both the ceftazidime/clavulanate discs compared with cefotaxime/clavulanate discs combinations, as using one combination may give negative results.

In this study the isolated *UPEC* strains were tested for presence of some virulence factors like cell surface hydrophobicity, haemolysin production, serum resistance, gelatinase production and ESβL production; 46% were hydrophobic, 24% were haemolysin producers, 62% were serum resistant, 2% liquified gelatin and 52% were ESBL producers. As regards the control commensal *E.coli* strains 45% were hydrophobic, 15% were haemolysin producers. 55% were serum resistant, 100% were negative for gelatin liquefaction and 40% were ESBL producers. Sharma et al [13] reported that haemolysin production is associated with pathogenicity of *E. coli*, especially the more severe forms of UTI as 23.7% of isolates of *E. coli* produced haemolysin. It has been suggested that colonization with haemolytic strains of *E. coli* is more likely to develop into urinary tract infections. Haemolysis, though not essential for establishment of acute pyelonephritis, may contribute to tissue injury, survival in renal parenchyma and entry into blood stream. They found that surface hydrophobicity is another important virulence factor of *E. coli* that causes extraintestinal infections, 33.4% of *UPEC* isolates were hydrophobic. The high hydrophobicity of the bacterial cell surface promotes their adherence to various surfaces like mucosal epithelial cells. Also they reported that 86.8% of *UPEC* isolates were resistant to serum bactericidal activity. While other studies such as. Raksha et al [7] reported that serum resistance was detected in 32.7% of *E. coli* isolated from urine.

Kausar et al[14] reported that out of 200 *E. coli* isolates 160(80%) had one or more virulence factors. Haemolysin production was observed in 42 (21%) of uroisolates, while 99(49.5%) isolates were serum resistant. It has been suggested that capsular antigen of *E.coli* plays an important role in virulence of bacteria conferring serum resistance and inhibiting phagocytosis. Ranjan et al[11] reported out of the 220 UTI cases; 91 (41.36%), 58 (26.36%) and 72 (32.72%) E.coli strains were hemolyetic, cell-surface hydrophobic and resistant to serum respectively. *Baby et al* [20] reported that among 300 *UPEC* isolates 63.5 % showed resistance to the bactericidal action of the serum, which coincides with the present result where 62% of *UPEC* isolates were serum resistant. Also Mittal et al[21] in their study reported 47.4%, 59%, 67.5% and 61% of their *UPEC* were: hemolysin producers, serum resistant, gelatinase producers and had cell surface hydrophobicity.

In this study it was found that 72% of the isolated *UPEC* strains and 60 % of the isolated commensal *E.coli* strains had pap gene. Duriez1 et al [22] reported that only 11.3% of their isolated commensal *E.coli* strains had pap gene, while *Fathollahi1 et al* [8] detected pap gene in 61% of their isolated *UPEC* strains. Qin et al [18] reported that pap gene was positive in 28% (20/70) of their *UPEC* isolates and 5% (2/41) of the intestinal commensal isolates, while Firoozeh et al[23] reported that pap gene was found in 52 (34.6%) of isolated *UPEC* of patients suffered from pyelonephritis and cystitis. Zaki and Elewa[24] reported that numerous virulence factors contribute to the pathogenicity of *E. coli* in UTI. The virulence factors are the results of different genes which can be detected by PCR method as they found pap gene in 63.7% of children suffered from UTI in Egypt while Neamati et al[25] reported that virulence genes were detected in 126 (84%) *UPEC* isolates. The PCR results identified pap gene was found in (16.6%) of the isolates.

CONCLUSIONS

The virulence factors of *UPEC* will increase the degree of the pathogenesis of the organism. Pap gene plays an important role in virulence of UPEC. Further studies for understanding of interaction of different virulence factors and their genetic role.

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