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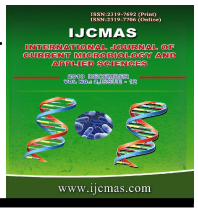

Original Research Article

Killing of αlpha-hemolytic and non-hemolytic Escherichia coli strains from paediatric patients in human serum and polymorphonuclear leucocytes

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K e y w o r d s

Escherichia coli; alpha hemolysin; intracellular killing; serum bactericidal assay.

A B S T R A C T

To assess serum bactericidal activity, intracellular killing in polymorphonuclear leucocytes, antibiotic susceptibility patterns and clinical profile in infections caused by serologically classified α-hemolytic and non-hemolytic *Escherichia coli.* To compare the same parameters in wild type *E coli* and isogenically derived strains characterized by lowered production of α-hemolysin. Alpha-hemolysin was quantified and its role was assessed for the resistance to the bactericidal activity of human serum and intracellular killing in polymorphonuclear leucocytes. Both α-hemolytic and non-hemolytic *E coli* were assessed for susceptibility to 10% and 100% human serum and for bactericidal activity of PMNL using acridine orange staining method, antibiotic susceptibility was performed using Kirby Bauer disc diffusion technique and all strains were serologically classified. Isogenic mutant derivatives were developed by using 3 µg/ml of rifampicin. Our study showed that α-hemolytic strains were significantly more resistant to the bactericidal activity of 10% and 100% human serum ($p<0.0001$) and to intracellular killing in polymorphonuclear leucocytes than non-hemolytic strains. The isogenic strains producing less α -hemolysin were similarly found to be more susceptible to bactericidal activity of human serum and PMNL killing. It was observed that certain serotypes were more commonly associated with α -hemolytic *E. coli* e.g. 02, 04, 08, 018. The α -hemolytic strains were also associated with significant resistance to amoxicillin, cephalexin and gentamicin . Thus, we conclude that α-hemolysin may be another virulence factor which helps *E coli* to establish invasive infection by counteracting the bactericidal activity of human serum and intracellular killing by polymorphonuclear leucocytes.

Introduction

Escherichia coli continues to be one of the dominating pathogens causing septicemia and meningitis during the first year of life. The progressively increasing prevalence

and number of fatalities from infections caused by *Escherichia coli* have resulted in attempts to delineate factors of importance in the pathogenesis of such infections. Clinical studies have shown that the virulence factors of *Escherichia coli* like production of bacterial adhesins, certain O and K antigens (Evans, Jr. D et al,1981) , expression of the siderophore aerobactin, production of colicin V (Siegfried L, 1994), production of thermolabile and thermostable enterotoxins, verocytotoxin, cytotoxic necrotising factor (Caprioli A et al, 1987; Donnenberg MS and Welch RA, 1996), α -hemolysin and resistance to bactericidal activity of human serum and intracellular killing in polymorphonuclear leucocytes play a role in the pathogenesis (Siegfried L et al, 1992; Fatima N et al, 2012).

This study was designed to assess the degree to which α -hemolysin may add to the virulence of *E. coli.* We quantified α -hemolysin production and evaluated its role in the resistance of *Escherichia coli* isolates to the bactericidal activity of human serum and intracellular killing in polymorphonuclear leucocytes. Virulence of derived isogenic strains with reduced α -hemolytic activity was compared to wild type α -hemolytic *E coli.*

Materials and Methods

A total of 103 isolates of *Escherichia coli* were studied. Of these, 53 isolates of *Escherichia coli* were from patients with extraintestinal infections, (35 cases of urinary tract infection, 11 cases of septicemia and 7 cases of meningitis). The remaining 50 isolates of *Escherichia coli* were taken from patients with intestinal infections. The cases varied from those of acute onset to those with a duration of a few months. The relevant specimens were accompanied with a detailed clinical history.

Quantification of α-hemolysin production

The test was a modification of the method of Siegfried et al, 1992. The strains to be tested were first subcultured onto 10% sheep blood agar and incubated at 37°C for 18-24 hours. The next day these strains were inoculated into Todd Hewitt broth which contained 10mM calcium chloride. This was again incubated overnight at 37°C. The following day, 125 µl of the Todd Hewitt broth was introduced into 5ml of fresh Todd Hewitt broth and incubated in a water bath at 37°C for 4 hours with occasional agitation. Supernates were collected after centrifugation at 10,000 r.p.m. at 4° C. The pH was adjusted to 7.0-7.4 with IN NaOH and the supernates were assayed for hemolytic activity in polystyrene microtitration plates. Two fold dilutions (in 100-µl volumes) of culture supernatant were made in tris-buffered saline (TBS), pH 7.4, containing 0.01M calcium chloride. To these dilutions 50 µl of fresh 1% washed sheep erythrocytes were added and the mixture was incubated at 37°C for 2 hours with occasional shaking. After incubation was complete, the number of unlysed erythrocytes was determined using the Neubauer haemocytometer, and the alpha-hemolytic activities of a strain was defined as the highest dilution of the culture supernatant that showed 50% hemolysis of the erythrocyte suspension used. The reciprocal of the dilution was considered the HU50/ml.

Elimination of hemolysin production:

Three strains which showed hemolysis of sheep RBC in the assay described above,

in dilutions 1:4 onwards were selected to study the effect of elimination of hemolysin production on the serum bactericidal assay and intracellular killing in polymorphonuclear leucocytes (Mitchell IG and Kenworthy R, 1997). The selected stains of alpha-hemolytic *Escherichia coli* were grown overnight in nutrient broth at 37°C, diluted to a concentration of 10^5/ml and 0.1 ml of this suspension was added to 10ml of nutrient broth containing 3µg/ml of rifampicin or more. Cultures were sampled after 24 hours and if necessary after 48 hours. They were diluted in phosphate buffered saline and plated onto blood agar to yield separate colonies. If colonies with altered hemolytic activity were not found, a few drops of the culture were transferred to fresh nutrient broth with rifampicin and the procedure was repeated.

Serum Bactericidal Assay

Serum for the assay was collected from healthy human volunteers who had not received any antibiotic treatment for at least one month. Blood was allowed to stand undisturbed for 15 minutes at room temperature. After centrifugation, the serum was removed, fractionated into small volumes and stored at-20°C until needed.

Overnight cultures of *Escherichia coli* which were grown at 37°C on blood agar were harvested. The cell suspensions were adjusted in Hank's balanced salt solution (HBSS) to contain 2.5 x 10^6 cfu/ml. Microplates were employed for incubation of bacteria with serum. For each strain 0.05 ml of culture suspension was incubated with 0.05 ml of 10% and 100% serum. The control well contained 0.05 ml of HBSS instead of serum. The microplates were sealed with adhesive tape to prevent evaporation and rotated for 180 minutes. Samples (10µl) were withdrawn after incubation for 60, 120 and 180 minutes at 37°C and spread on 10% sheep blood agar plates. The plates were incubated for 18 hours at 37°C and the viable count was determined.

Susceptibility of bacteria to serum was estimated by the method advocated by Benge et al, 1992. Strains were termed serum sensitive if the viable count dropped to 1% of the initial value and resistant if >90% of organisms survived after 180 minutes. Strains that gave results between these values were considered of intermediate sensitivity.

Intracellular killing in polymorphonuclear leucocytes

The intracellular killing of bacteria by human polymorphonuclear leucocytes (PMNL) was determined microscopically with a slightly modified acridine orange staining method (Siegfried L et al, 1992). Fresh, unheparinized human blood (0.2ml) was placed on a sterile coverslip in a humidified plastic chamber and allowed to clot for 60 minutes at 37°C Coagulated blood was removed by rinsing the coverslips gently in warm (37°C) HBSS. A PMNL monolayer was thus formed on the coverslip. Bacteria washed once in HBSS were pre-opsonised in 100% serum at 37°C (in a concentration of $3xl0^7$cfu/ml) for 15 minutes were then placed on the PMNL monolayer. After incubation for 60 minutes, the slides were removed, air dried and stained with acridine orange (1mg/ml) for 1 minute. In this assay, intracellular bacteria fluoresce green when viable and red if non viable. The viability of organisms in a total of 100 PMNL was estimated. The percentage killed was the percentage of intracellular organisms that were non viable (red).

Serotyping

Serological identification was carried out at Central Research Institute, Kasauli (India) by the slide agglutination technique using the specific antisera.

Antibiogram

Antibiotic susceptibility testing of all the strains of *E. coli* was done by the Kirby Bauer disc diffusion technique for the following antibiotics: netilmicin 10µg, gentamicin 10 µg, amikacin 10 µg, amoxicillin 20 µg, cephalexin 30 µg, nalidixic acid 30 µg, ciprofloxacin 5 µg, gatifloxacin 5 µg, cefotaxime 30 µg, cefoperazone 75 µg, cefoperazone-sulbactam 75/75 µg. ESBL detection was done by double disc synergy method (Rizvi M et al, 2009).

Statistical Analysis

The chi square test was used for statistical comparison of the proportion of serum resistant isolates in α-hemolytic versus non-hemolytic *E coli.* Differences in intracellular killing (α -hemolytic versus non-hemolytic isolates) were estimated by student's *t test* (two sample analysis). Statistical comparison of results in wild type strains and isogenic derivatives was made by Student's paired *t* test. Values of $p<0.05$ were regarded as significant.

Result and Discussion

Quantification of α–Hemolysin

Fifty-six α-hemolytic and 47 non-hemolytic *Escherichia coli* isolates were studied. The majority 35 (66%) of α -hemolytic *E coli* were of extra-intestinal origin and the remaining 21 (44%) were of intestinal origin. The α -hemolytic isolates were quantified, with the HU50/ml ranging from 4-512 as shown in Table 1. As seen, significantly higher titres were observed in isolates of extra-intestinal origin.

Serum Bactericidal Activity

A significantly greater number of α-hemolytic *E coli* were resistant to both 10% and 100% human serum. All 56(100%) α -hemolytic isolates were resistant to 10% human serum and 35 isolates (63%) were resistant to 100% human serum which was significantly higher as compared to 29(62%) and 10 (21%) of non-hemolytic isolates by 10% and 100% human serum respectively($p<0.0001$)(Table2).

Susceptibility to Bactericidal Activity of Polymorphonuclear leucoyctes (PMNL)

The resistance of α-hemolytic *Escherichia coli* to intracellular killing by PMNL was significantly greater than of non-hemolytic *E coli.* The percentage mean killing of α-hemolytic *E coli* was 71.02 + 12.28 (range: 51-92) as against 81.99 ± 11.47 (range : 58-97) of non-hemolytic *E coli*($p>0.001$) (Table 2).

Susceptibility to antibiotics

α-hemolytic *E coli* were significantly resistant to amoxicillin (88%), cephalexin (82%) cefoperazone (75%), cefotaxime 72% and gentamicin (57%) as against non-hemolytic *E coli* (Table 2 and 3). ESBLs were also significantly associated with α-hemolytic *E.coli.* There was no significant difference in the resistance pattern of α-hemolytic and non- hemolytic *E coli* against netilmicin, ciprofloxacin and nalidixic acid although α-hemolytic *E. coli* were generally more resistant to them.

Characteristics of Isogenic Strains

The non-hemolytic isogenic variants were more susceptible to serum bactericidal activities and to intracellular killing in PMNL as is seen in Table 3.

Serotyping

A total of 30 different 0 serotypes were found in the *E. coli* isolates in the present study. Ten strains were found to be rough and 6 were untypable. 57 isolates (53.3%) out of a total of 103 strains were clustered in eleven O serotypes: 02, 04, 06, 07, 08, 018, 020, 026, 075, 0131, 0152. Out of these 9% strains belonged to 06 serotype, 8% to 018, 7% to 08, 6% to 075 and 5% each to 04 and 026. Among these 89% of 06, 83% of 075, 75% of 02 and 018 and 71% of 08 were α-hemolytic. Serum resistance was higher in serotypes 06, 018, and 075 i.e. 78%, 63% and 67% respectively (Table 4). Intracellular killing in polymorphonuclear leucocytes was least of serotypes 02 and 04.

Clinical Profile

Distribution of α-hemolytic strains of *E. coli* in urinary tract infections (UTI), septicemia, meningitis and diarrhoea patients is given in Figure1. Alpha-hemolytic *E. coli* were significantly associated with healthy patients as against non-hemolytic strains which were associated with debilitated patients. Patients infected with α-hemolytic *E. coli* had a longer duration of stay in hospital; diarrhoea due to α- hemolytic *E. coli* was associated with severe dehydration. It was observed that patients infected with α-hemolytic *E. coli* had a graver clinical picture with four infants expiring as compared to non-hemolytic *E. coli* infection. Patients with preexisting diseases like leukaemia, thalassemia nephrotic syndrome, congenital abnormalities or with clinical protein energy malnutrition (grade III or IV) were commonly infected by the less virulent non-hemolytic *E. coli.*

The role of α-hemolysin in resistance of E *coli* to human serum and intracellular killing in PMNL is not fully known, α-hemolysin associated with certain O antigens, is largely found in *E. coli* causing extra-intestinal infections and is infrequently isolated from healthy people (Siegfried L, 1995). In this study serum susceptibility and intracellular killing in polymorphonuclear leucocytes (PMNL) of α-hemolytic and non-hemolytic *Escherichia coli* were compared.

Many reports have shown that there are several virulence determinants at the cell wall surface that may play a role in the resistance of E *coli* strains to the bactericidal activity of human serum(Roberts IS et al, 1989; Stawski G et al, 1985;Timmis KM et al, 1990). These virulence factors may act independently or their action may be complementary to each other (Mishra M et al, 2001). Our study suggests that α-hemolysin could be an important determinant in enhancing the serum resistance of *E. coli.* This is clearly indicated by the significantly higher serum resistance seen in α-hemolytic isolates than in non-hemolytic *E. coli.* Siegfried et al, 1992 have also suggested a possible role of α-hemolysin in serum resistance.

Serum resistance is one of the survival strategies of pathogenic organisms to overcome human defense mechanisms (Dharmadhikari SM and Peshwe SA, 2009).This premise is further corroborated by the significantly higher resistance found in wild type α-hemolytic isolates than in isogenic derivatives with reduced production of α-hemolysin.

Table.1 Quantification of alpha-hemolysin in intestinal and extra-intestinal isolates of *E coli*

Titre	*HU50/ml*	Number of Strains	E.I.I. No (%)	I.I. No. (%)
1:2	0	0	0	0
1:4	4	5	2(4)	3(6)
1:8	8	9	4(44)	5(55.6)
1:16	16	14	9(64.2)	5(35.8)
1:32	32	7	4(57)	3(43)
1:64	64	9	7(77.7)	2(23.3)
1:128	128	6	4(66.6)	2(33.4)
1:256	256	5	4(80)	1(20)
1:512	512	1	1(100)	0

HU: Hemolytic unit
E.I.I: Extra-intestinal isolates
I.I: Intestinal isolates

Table.2 Distribution of virulence characters in 56 alpha hemolytic and 45 non hemolytic *E. coli* isolates

Character	α-hemolytic E. coli No. (%)	Non-hemolytic E. coli no. (%)	P. Value
Serotypes 02, 04, 06, 07, 08, 018, 055, 075	34(60.7)	14(29.7)	<0.001
Serum resistance			
10% serum	56(100)	29(62)	<0.001
100% serum	35(63)	10(21)	<0.0001
Mean Intracellular Killing by PMNL	Mean 71.02±12.28	81.19±11.47	<0.001
Range	51-92	58-97	
Resistance to amoxicillin	99(88)	22(47)	<0.01
Resistance to cephalexin	46(82)	19(40)	<0.01
Resistance to gentamicin	32(57)	15(32)	<0.02
Resistance to cefoperazone	42(75)	18 (38.2)	<0.01
Resistance to cefotaxime	40(72)	17(36)	0.01
ESBL production	37(66)	20(42.5)	0.02

PMNL: Polymorphonuclear leucocyte

Table.3 Characteristics of three pairs of isogenic *E. Coli* strains

S. No	Source of stain	Titre	serotype	Antibiotic Susceptibility Pattern						Serum susceptibility			PMNL Killing
				Ax	Cf	G	N	Cefp	Cipro	S	IS	R	
1	UTI												
	AH^+	128	06	R	R	R	S	S	S	-	-	+	64%
	AH^-	<2	06	R	R	R	S	S	S	+	-	-	81%
2	UTI												
	AH^+	64	018	R	R	S	R	S	S	-	-	+	71%
	AH^-	<2	018	R	R	S	R	S	S	+	-	-	81%
3	UTI												
	AH^+	16	055	S	R	S	S	S	S	-	-	+	79%
	AH^-	<2	055	S	R	S	S	S	S	-	-	=	88%

AH^+ : α-hemolytic E. coli
AH : isogenically derived E. coli with reduced □-hemolysin
Ax : Amoxicillin; Cf : cephalexin, G : Gentamicin; N : Netilmicin;
Cefo : Cefotaxime; Cipro : Ciprofloxacin
PMNL : Polymorphonuclear leucocyte

In the present study, a lesser number of a hemolytic *E. coli* were killed after phagocytosis than non-hemoytic *E. coli.* There were, however, considerable differences in intracellular killing among the different α-hemolytic isolates studied. Thus, the α-hemolytic extra-intestinal isolates which generally produced a higher level of α-hemolysin were killed in lesser numbers than α-hemolytic intestinal isolates which produced lower levels of α-hemolysin. Proposed mechanism to explain this observation have stressed that hemolysis liberates hemoglobin which appears to provide a nutritional boost for *E coli.* Moreover, α-hemolytic *E. coli* have been reported to be toxic to phagocytes and this cytotoxic effect corresponds with titres of α -hemolysin production (Gadeberg OV and Orskov, 1984; Gadeberg OV et al, 1989). To further elucidate the role of α-hemolysin in intracellular killing, three sets of isogenic strains with no or minimal α-hemolysin production were compared with the wild type strains. The results demonstrate that the wild α-hemolytic *E. coli* were significantly more resistant to intra-cellular killing than the isogenic stains with reduced α -hemolysin production. These results support those of Siegfried et al (1992), who found lesser intra-cellular killing of wild type α-hemolytic *E. coli* than their non-hemolytic derivatives.

Alpha-hemolysin is known to be associated with certain O antigens. Hughes et al (1982), reported that as many as 50% of *E. coli* strains of groups 018 and 075 were hemolytic. They further suggested an association of α-hemolysin production and serum resistance with serogroups 06, 018 and 075. Siegfried et al (1995) suggested a similar association with O groups 6 and 55. Our results showed that α-hemolysin production and serum resistance were associated with 0 groups 06, 018, 075 and to a lesser extent to 02 and 022, the latter

two not having been reported earlier. We studied antibiotic susceptibility patterns of α-hemolytic *E. coli* in depth. We found a statistically significant difference in the resistance pattern of α-hemolytic and non-hemolytic isolates in relation to amoxicillin, cephalexin, cefoperazone and cefotaxime ($p<0.01$) and gentamicin ($p<0.01$). Significant ESBL production was associated with α-hemolytic *E.coli* $P<0.02$. The α-hemolytic strains were also more resistant to gatifloxacin and ciprofloxacin. However, the three isogenically derived non-hemolytic strains had the same antibiotic susceptibility pattern as the wild strains. Thus antibiotic resistance appears to be an independent association. We further analyzed α-hemolysin in relation to the clinical picture of the patients.

61% of *E. coli* isolated from cases of meningitis and septicemia and 68.6% *E coli* from urinary tract infection were α-hemolytic. It was observed that patients infected with α-hemolytic *E coli* had a graver clinical picture when compared with patients who had non-hemolytic *E coli* infection.

Four infants infected with a-hemolytic *E. coli* expired. Patients with pre-existing diseases were more commonly infected by the less virulent non-hemolytic *E. coli.* Roantree et al (1960) have also indicated that bacterial factors play a significant role in meningitis and septicemia, particularly in normal infants.

Taking into account the results obtained in the present study and of the previous studies, we suggest that α-hemolysin contributes to the resistance of *E coli* to the bactericidal activity of serum and to intracellular killing in PMNL.

Figure.1 Clinical profile of patients with α-hemolytic and non-hemolytic *E coli* infection

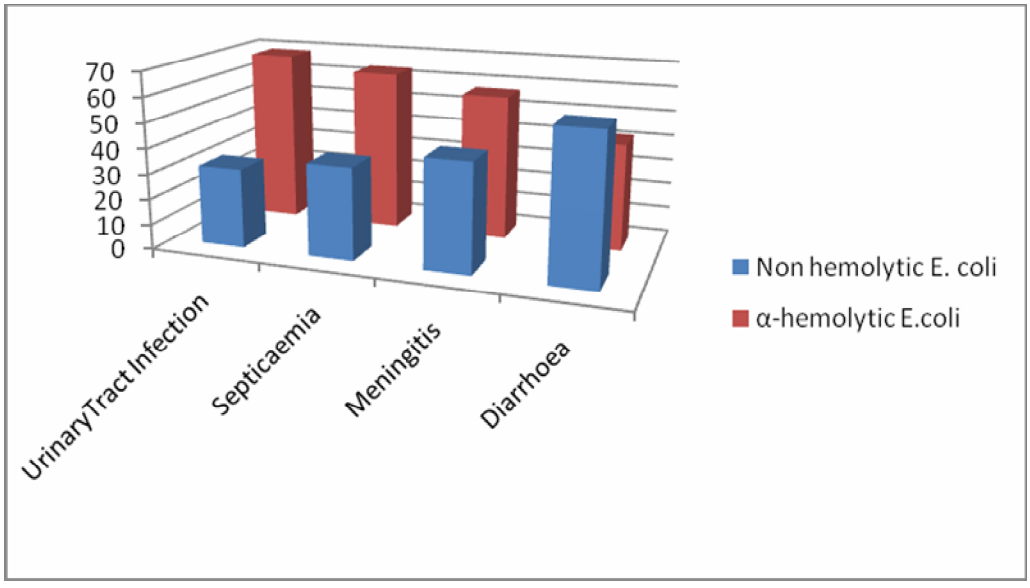

Table.4 Association of *E coli* O serogroups with isolate origin, α –hemolysin production and serum resistance

Serotype	Total	Strain Origin		α Hly(%)	S.R. (%)
02	4	E E	2 2	2 (75) 2	2 (50) 0
04	5	E D	4 1	3 (60) 0	2 (40) 0
06	9	E D	6 3	6 (89) 2	5 (77.7) 2
07	4	E D	6 3	1 (50) 1	1 (25) 0
08	7	E D	5 2	4 (71) 1	3 (57) 1
018	8	E D	6 2	4 (75) 2	4 (62.5) 1
020	4	E D	2 2	2 (50) 0	2 (50) 0
075	6	E E	3 3	3 (83) 2	3 (66.6) 0
Other O antigenic Type	35	E D	10 25	6 (20) 1	3 (11.42) 1
Untypable	6	E D	2 4	2 (66) 2	0 (33) 2
Rough	10	E D	9 1	4 (70) 3	1 (30) 1

E : Extra intestinal; D : Diarrhoea; α-Hly: α-hemolytic E. coli; S.R. : serum resistant E. coli

The enhanced serum resistance in α-hemolytic *E. coli* was explained by Bhakdi et al(1989) who suggested that the protective lipoproteins, serum albumin and other as yet unknown plasma factors are neutralised by α-hemolysin, thus, giving α-hemolytic *E. coli* a survival advantage. α-hemolysin appears to play a distinct role in morbidity and to some extent mortality of healthy normal risk infants.

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