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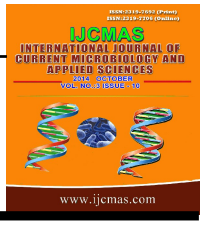

Original Research Article

Phenotypic profiling of *Escherichia coli* strains associated with infertility

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Keywords

Male infertility, male genital tract, *Escherichia coli* , asymptomatic infection

A B S T R A C T

Escherichia coli is usually considered to be an opportunistic pathogen which constitutes a large portion of the normal intestinal flora of humans. This organism can, however, contaminate, colonize, and subsequently cause intestinal and extraintestinal infections ranging from gastroenteritis, urinary tract infections, neonatal meningitis, septicemia and peritonitis (Doughari *et al*., 2011). Because of its clinical significance *E. coli* has been the subject of numerous investigations in an attempt to define those virulence factors which allow it to initiate and sustain infections. It is now believed that virulence in *E. coli* is multifactorial and that certain properties are associated primarily with virulent strains. *E. coli* probably represents the most frequently isolated microorganism in genitourinary infections (Liu *et al*., 2002) and appears to affect different sites of the male reproductive tract. Several reports describe sperm agglutination and sperm immobilization by *E. coli* which in turn leads to infertility. However the virulence markers which have been associated with those *E. coli* which have role in infertility have yet to be identified. So the present study was undertaken to determine differences among *E. coli* strains for their sperm agglutinating and sperm immobilizing activity on the basis of phenotypically expressed virulence factors.

Introduction

Infections of the male genitourinary tract represent a significant health care problem and account for almost 15% of cases of male infertility (Keck *et al*., 1998). Semen cultures from the male partners of infertile couples revealed a wide range and high frequency (5-45%) of bacterial presence (Toth 1981). Among bacterial species that interact with spermatozoa are well-known causative pathogens of genitourinary infections such as *Escherichia coli, Ureaplasma urealyticum, Mycoplasma hominis,* and *Chlamydia trachomatis* (Liu *et al*., 2002). *E. coli* is isolated frequently from seminal and prostatic fluids in patients with prostatitis and epididymitis (Weidner *et al*., 1991). The etiological role of these infections in male infertility has been paid attention in recent years. These infectious processes may lead to deterioration of spermatogenesis, impairment of sperm function and obstruction of seminal tract. Several reports describe sperm agglutination and immobilization by *E. coli*. Paulson and Polakoski (1977) investigated the mechanism of how *E. coli* immobilizes

spermatozoa and they reported a factor, apparently excreted by the bacteria which immobilizes spermatozoa without agglutinating it. However, Diemer *et al.* (1996) reported that *E. coli* inhibits sperm motility by directly adhering to and agglutinating spermatozoa. Therefore urogenital infections are considered as significant cause of male infertility (Weidner *et al*., 1991).

Materials and methods

Microorganism

Among 14 *Escherichia coli* strains used in the present study, 8 were isolated from semen samples of males attending infertility clinic at Government Multi Speciality Hospital (GMSH) Sector 16, Chandigarh and 6 standard strains viz. MTCC 3222, MTCC 4296, MTCC 4315, MTCC 433, MTCC 2991, MTCC 1687 were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh.

Isolation of Microorganism from the Ejaculates of Infertile Males

Semen samples were obtained from males attending infertility clinic at Government Multispeciality Hospital, Sector 16, Chandigarh, by masturbation into a sterile wide mouth beaker. Then the samples were streaked immediately on blood agar and Mac conkey plates separately; the plates were incubated aerobically at 37˚C for 24–48 h and observed for the bacterial growth. The isolates were subjected to various tests of identification according to the characteristics laid down in the Bergey's Manual of Determinative Bacteriology (Bergey *et al*., 1994).

Grouping of strains on the basis of interaction with human spermatozoa

Equal volume of semen sample (40 x 10^6 spermatozoa/ml) and cell cultures (1 x 10^6 cells/ml) were mixed and incubated at 37˚C for 30 min, 1, 2 and 4 h. One drop of each was placed on a glass slide covered with a cover slip and observed for sperm agglutination and sperm immobilization of spermatozoa at 400X magnification under light microscope. On the basis of this interaction, all the strains were categorized in 3 groups

(a) sperm agglutinating
(b) sperm immobilizing
(c) Non sperm agglutinating / Non sperm immobilizing strains.

Screening of various virulence factors of *E. coli*

Haemolysin production

Haemolysin was detected qualitatively by the presence of a zone of lysis around the colony on Blood agar plate (Raksha *et al*., 2003). To further confirm, in vitro estimation of cell free and cell bound haemolysin was carried out and results were expressed in terms of haemoglobin released per ml.

Haemagglutination activity

The haemagglutination was detected by clumping of erythrocytes by fimbriae of bacteria in the presence of D-mannose. This test was carried out as per the direct bacterial haemagglutination test - slide method and mannose-sensitive and mannose-resistant haemagglutination tests (Duguid *et al*., 1979).

Serum bactericidal assay

250 µl of each bacterial cell suspension was mixed with 500 µl of fresh human serum and incubated at 37˚C. Aliquots of the sample were withdrawn at 0 h, 1.5 h, 3 h and plated on nutrient agar (NA) plates after appropriate dilutions to obtain viable counts. Strains were termed serum sensitive if the viable count dropped to 1% of the initial value and resistant if >90% of organism survived after 3 h incubation period (Seigfried *et al*., 1996).

Siderophore estimation (Enterochelin)

Overnight grown cultures of *E. coli* were centrifuged. To 1 ml of cell free supernatant, 1 ml of 0.5 N HCl, 1 ml of nitrite molybdate reagent (prepared by dissolving 10gm of sodium nitrite and 10 gm of sodium molybdate in 100 ml distilled water) and 1 ml of 1N NaOH was added. Final volume was made to 5 ml with distilled water and absorbance was recorded at 510 nm immediately.

Outer membrane protein profiling
Extraction of OMPs

E. coli strains grown at 37˚C for overnight were centrifuged and pellet was washed twice with Tris HCl buffer (10 mM, pH 7.6) containing 5 mM $MgCl_2$ which was further suspended in 10 ml sonication buffer containing 2 mM phenylmethyl-sulfonylfluoride (PMSF).

Sonication was done using B. Braun Labsonic 2000 sonicator (10 cycles of 30 each). The sonicate was centrifuged at 12000 rpm for 30 min and the supernatant (source of OMPs) was collected in sterile tube. Protein content was estimated by standard method (Lowry *et al*., 1951).

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

Outer membrane protein pattern was observed on SDS-PAGE (Lammeli *et al*., 1970). Here 12% gel was prepared and accordingly SDS was added. Sample i.e. the purified protein was first denatured and then loaded onto the gel along with the standard protein markers viz. myocin (rabbit muscle), phosphorylase b, BSA, ovalbumin and carbonic anhydrase. After the gel was run, staining with coomassie blue was done and molecular weight was estimated.

Antimicrobial susceptibility pattern (antibiogram)

Antibiotic sensitivity testing was performed for all *E. coli* strains by Kirby Bauer's disc diffusion method using ampicillin (10mcg), cefotaxime (30 mcg), cephalothin (30mcg), co-trimoxazole (25mcg), gentamycin (10mcg), nalidixic acid (30mcg), nitrofurantoin (300mcg) and norfloxacin (10mcg).

Serotyping

Serotyping of *E. coli strains* was done at Central Research Institute, Kasauli, Himachal Pradesh.

Transmission electron microscopy

Washed cells of 48 h grown *E. coli* strains were suspended in sterile distilled water and stained with 1% PTA dye for analysis of fimbriae using transmission electron microscope.

Plasmid profiling

Plasmid profiling was done by conventional method of plasmid isolation and agrose gel

electrophoresis with flouroscent ethidium bromide (Birnboim *et al*., 1983).

Results and discussion

A total of 14 *Escherichia coli* strains were used in the present study. Initially all the strains were identified as *Escherichia coli* using Gram staining, growth on Mac Conkey's agar and Eosin Methylene Blue agar and were further confirmed by biochemical characterization. They were grouped on the basis of their interaction with human spermatozoa.

From the results (Table 1) it was observed that 5 out of 8 clinical isolates and 3 out of 6 standard strains showed specific sperm agglutinating activity and remaining 3 out of 8 isolates and two out of six standard strains showed sperm immobilizing activity, whereas only 1 standard strain which is fecal isolate had no effect on human spermatozoa and was used as a control strain (Table 1).

Based on the presence of agglutinating or immobilizing activity, the strains were divided into 3 groups i.e. agglutinating strains, immobilizing strains, and those which had no effect on human spermatozoa (control) and were further screened for their phenotypic characteristics.

The association of hemolytic *E. coli* with extraintestinal disease of humans has been documented by several investigations. Haemolysin provides *E. coli* with possible selective advantage by releasing iron from lysed erythrocytes and enhances pathogenicity by destroying phagocytic and epithelial cells (Kaper *et al*., 2002, Wullt 2002). In the present study, all the strains tested showed haemolysis on blood agar plates. However, when in vitro estimation of cell free and cell bound haemolysin was carried out, it was seen that cell free haemolysin production was observed in 7 (Isolate 1, 5, 6, 7, and MTCC433, 4296, 2991) out of the 8 isolates (87%) and cell bound haemolysin production in 5 (Isolates 1, 2, 5, 6,and MTCC 2991) out of the 8 isolates (62.5%) of sperm agglutinating group. In case of sperm immobilizing group, all the strains viz. Isolates 3, 4, 8 and MTCC 3222, 4315 showed the presence of both cell free and cell bound haemolysin. However, in fecal strain only cell free haemolysin production was seen as it lacked the expression of cell bound haemolysin.

Colonization is the first and the foremost step in pathogenesis of a disease. The type I fimbriae and P fimbriae are widely prevalent and are probably involved in colonization of lower and upper urinary tract respectively (Kaper *et al*., 2002, Wullt 2002). The presence of type 1 fimbriae can be assessed by mannose – sensitivite haemagglutination (MSHA) (Brauner *et al*., 1990, Duguid *et al*., 1979) whereas phenotypic expression of P fimbriae can be detected by Mannose – resistant haemagglutination of human erythrocytes. In our study, all the strains were found negative for both mannose sensitive and mannose resistant haemagglutination.

Bacteria are killed by normal serum through the lytic activity of the complement system. It has been suggested that capsular antigen of *E. coli* plays an important role in virulence of bacteria conferring serum resistance and inhibiting phagocytosis (Seigfried *et al*., 1996). In our study, both agglutinating and immobilizing strains were serum resistant showing their robust nature for survival. However, fecal strain was found to be serum sensitive.

The presence of a single factor rarely makes an organism virulent; a combination of factors will determine if a bacterium can cause infection (Dobrindt, 2005).

Table 1: Categorization of different *E. coli* strains on the basis of their sperm agglutinating / immobilizing activity

Sperm agglutinating strains	**Sperm immobilizing strains**	**Non agglutinating/non immobilizing strain. (Control)**
Isolate 1	Isolate 3	MTCC 1687 (fecal strain)
Isolate 2	Isolate 4	
Isolate 5	Isolate 8	
Isolate 6	MTCC 3222	
Isolate 7	MTCC 4315	
MTCC 2991		
MTCC 433		
MTCC 4296		

Table 2: Serotyping

Sperm agglutinating strains:	**Sperm immobilizing strains:**	**Fecal strain**
Isolate 1 : Rough Isolate 2 : Rough Isolate 6 : O8 Isolate 7 : O8 MTCC 4315 : O2 MTCC 2991 : O7 MTCC 433 : O25	Isolate 3 : O8 Isolate 8 : O8 MTCC 3222 : U.T.	MTCC 1687 : Rough

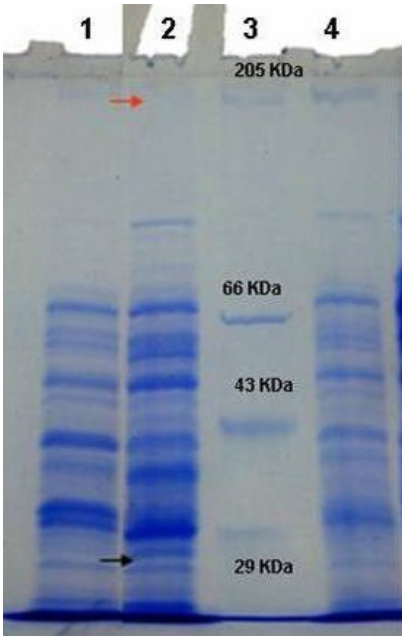

Figure 1: Outer membrane protein profiling. Lane 1: 40 µg protein of MTCC 2991(sperm agglutinating strain); Lane 2: 40 µg protein of MTCC 1687 (fecal strain); Lane 3: Marker; Lane 4: 40 µg protein of MTCC 4315 (sperm immobilizing strain).

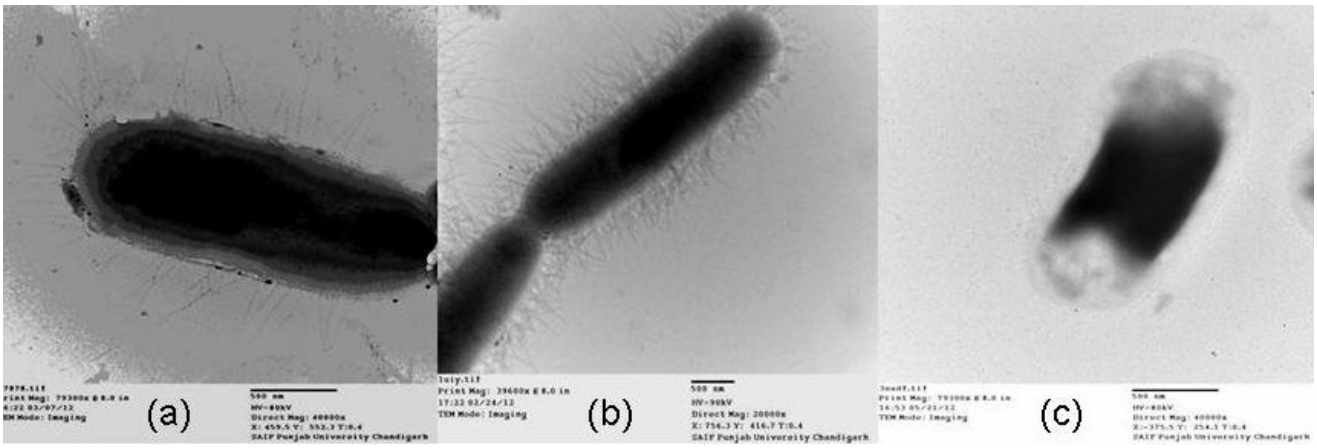

Figure 2: Transmission electromicrograph of (a) sperm agglutinating, (b) sperm immobilizing and (c) fecal strain

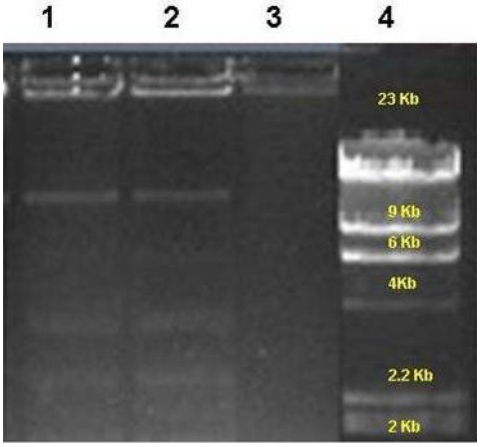

Figure 3: Agrose gel electrophoresis stained with ethidium bromide depicting plasmids isolated from different strains. Lane 1: Fecal strain; Lane 2: sperm agglutinating strain (Isolate 2); Lane 3: sperm immobilizing strain (Isolate 3); Lane 4: Marker.

Moreover, the determining factor is not simply the presence or absence of virulence associated genes, but also their levels of expression, which can vary between pathogenic and non pathogenic isolates (Pitout, 2012). For example, iron supply for many microbes plays a decisive role in the infection process and acquiring iron from the environment has a significant effect on the establishment of infection in the host, and microbial pathogens have evolved different mechanisms to overcome iron restriction (Dale *et al*., 2004). Our results also reinforce this phenomenon in Enterochelin production (Phenolate compounds) which is expressed in terms of siderophore units. Out of 8 strains, having sperm agglutinating property, 3 strains (Isolate 7, MTCC 2991 and MTCC 433) showed significant siderophore production (0.1 - 0.2 units) as compared to remaining strains. In case of sperm immobilizing strains, Isolate 8 showed maximum siderophore production (0.41 units) as compared to other strains and in case of fecal strain it was found to be insignificant (0.03 units).

Further, an investigation was done to verify the differences in OMPs expression. The

bacterial outer membrane represents the critical interface between the microorganism and its environment (Christopher *et al*., 2007). For pathogenic bacteria, the proteins localized to the outer membrane directly interact with components of the host and can act as adhesins or receptors to facilitate colonization and mediate acquisition of nutrients and macromolecules from this restrictive niche (Cullen *et al*., 2004, Odenbreit *et al*., 1999). In the present study, a difference in the protein profile was observed as the fecal strain lacked the expression of an 205 kDa OMP which was present in both sperm agglutinating and immobilizing strain. Alternatively, fecal strain also showed the expression of an additional 29 kDa OMP which was absent in remaining two groups. However, no apparent change was seen among the profiles of sperm agglutinating and immobilizing strains. Therefore it can be suggested that the outer membrane protein composition of strains may vary, and that all strains of *E. coli* do not have the same polypeptides present in their outer membranes (Fig 1).

Serotyping aims at the determination of surface antigens by using a defined set of surface antigens and polyclonal or monoclonal antibodies. *E. coli* species consist of various serotypes ranging from highly pathogenic to non pathogenic strains. It has also been noted in a number of studies that certain serotypes are far more likely to be associated with some of the virulence factors (Bettelheim *et al*., 2003). Frequency of different serotypes among sperm agglutinating, sperm immobilizing and non agglutinating/non immobilizing strains was analysed and it was found that serotype O8 was isolated with maximum frequency followed by rough, 02, 07 and O25. It has been suggested from previous studies that serotypes like O1, 02, O4, O6, O7, O8, O25, O68, and 075 are most prevalent to infect the urinary tract (Vosti *et al*., 1964, Grunberg 1969., Reid *et al*., 1987, Roberts *et al*., 1979). In the present study, 62.5% of sperm agglutinating and 100 % of sperm immobilizing strains were found to be associated with urinary tract infections where as fecal strain was found to be of rough serotype O25 (Table 2).

Upon studying the antibiogram profile of *E. coli* strains it was found that out of 8 sperm agglutinating strains, all were highly sensitive to CoTrimoxazole, 6(75%) to Nitrofurantoin and gentamycin, 5(62.5%) to ampicillin and cephalothin, 4(50%) to Norfloxacin and Nalidixic acid, 3(37.5%) to Cefotaxime. However, only 1(12.5%) showed intermediate sensitivity to Gentamycin, Nitrofurantoin and Naldixic acid and 3(37.5%) to Cefotaxime.

Out of 5 sperm immobilizing strains; all were highly sensitive to CoTrimoxazole, 3(60%) to gentamycin, 2(40%) to Cephalothin, 1(20%) to Norfloxacin and 3(60%) showed intermediate sensitivity to Nitrofurantoin, 2(40%) to Cefotaxime and gentamycin.

Fecal strain was highly sensitive to CoTrimoxazole, Naldixic acid and Norfloxacin and intermediate sensitive to Ampicillin, Cefotaxime, Cephalothin and Nitrofurantoin and was resistant to gentamycin.

Transmission electron microscopic studies were done to analyse the presence or absence of fimbriae on *E. coli.* In the present study both the sperm agglutinating as well as sperm immobilizing strain showed the expression of fimbriae while fecal strain which had no effect on human spermatozoa was found to be afimbriated (Fig 2). However, the type of fimbriae could not be

established as these strains lacked the haemagglutination activity. Therefore it has been proposed that the strains may possess other type of fimbriae such as Dr, X or S fimbriae. The expression of fimbriae may therefore be implicated in the sperm agglutinating and sperm immobilizing properties of *E. coli* as non-fimbriated fecal strain failed to exhibit an effect on spermatozoa. These results are in concordance with the observations of Monga and Roberts (Monga *et al*., 1996) who suggested the presence of type 1 and P fimbriae on *E. coli.*

On studying the plasmid profiles it was found that both sperm agglutinating and fecal strain carry a detectable plasmid of apparent molecular weight of 4Kb where as sperm immobilizing strain lacked the expression (Fig 3).

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