American Journal of Infectious Diseases 4 (2): 117-123, 2008
ISSN 1553-6203

Urinary Tract Infection-A Survey of Local Population

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Abstract: Urinary Tract Infection, commonly known as UTI, affects as many as 50% women at least once during their lifetime. All individuals are susceptible to Urinary Tract Infection (UTI); however the prevalence of infection differs with age, sex and certain predisposing factors. With the above background in mind we conducted a survey of the local population (women of 3 age groups) to compare the urinary tract microbial community of control individuals with the UTI positive patients. Attempts were made to identify pathogens through Serum Bactericidal Antibody Response. 200 urine samples collected from control as well as UTI patients were randomly inoculated in parallel on four varieties of chromogenic agar plates and the Colony Forming Units (CFU) per milliliters (ml) of each microbe was determined. The serum bactericidal antibody assay was performed to demonstrate the presence of serum antibodies with bactericidal activity against the bacterium found in urine. However, with further experimental analysis, this bactericidal activity was found to be non specific and a similar percentage of bacteriolysis was observed incase of the control population also. 10^4-10^5 CFU mL^{-1} was the demarcating value between normal and pathological samples in asymptomatic cases. A significant variation was also noted in the microbial profile of various age groups. *E. coli* is the most prevalent pathogen in the post menopausal group. 15 different bacterial isolates were obtained of which the 16S rDNA sequence of the 6 novel ones are available in GenBank. The control and patient population showed a clear cut variation in the percentage of urinary tract microbes.

Key words: Urinary tract infection, serum bactericidal antibody response, CFU, urine microbial flora

INTRODUCTION

Urine located within the urinary tract, excluding the distal region of the urethra is considered sterile in healthy individuals, as indicated by the absence of cultivable bacterial cells. UTI describes a condition in which there are micro organisms established and multiplying within the urinary tract. It is most often due to bacteria (95%), but may also include fungal and viral infection[1].

On the basis of the work done by Kass, 10^5 colony forming units of a single species per milliliters in a clean catch midstream sample of urine is considered as significant bacteriuria[2,3]. While this threshold still holds in asymptomatic patients, in many cases a lower threshold is considered significant for symptomatic patients. The largest group of patients with UTI is adult women[4,5]. Women are more susceptible to UTI because a woman's urethra is short, allowing quick access of bacteria to the bladder. Also a woman's urethral opening is near sources of bacteria from the anus and vagina. The incidence increases with age and sexual activity[6,7]. Rates of infection are high in postmenopausal women, because of bladder or uterine prolapse causing incomplete bladder emptying; loss of estrogen with attendant changes in vaginal flora, loss of Lactobacilli, which allows periurethral colonization with gram negative aerobes, such as *E. coli*[8,9,10]. Catheterization is also associated with a very high incidence of UTI[11].

It has already been demonstrated that circulating antibodies found in human serum represent a protective immune response which may limit bacterial invasion of the urinary tract by the antibodies acting as opsonins to increase phagocytosis. Serum antibodies found in UTI positive patients have bactericidal activity against the bacterium. Bacteria are killed by infected human serum (with circulating antibodies) through the lytic activity of the complement system[12].

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Chromogenic substrates have been widely used in culture media for the detection, isolation, identification and enumeration of microorganisms in clinical samples. Chromogenic media incorporates enzymatic methods using specific chromogenic substrates added to primary selective and nonselective media. Colourless substrates produce characteristic colours when cleaved by organism-specific enzymes[13].

A survey was conducted of women from in and around Kolkata. A regular supply of urine samples was obtained from the pathological centres of Peerless Hospital as well as Nilratan Sarkar Medical College and Hospital (N.R.S.M.C.H.). The subjects were divided into three age groups. A total of 200 samples were screened, out of which 150 were of control individuals and 50 were pathological samples. The control or normal samples were also collected from members of the university and their family. The objective of the study was to compare the variation in the urine microbial flora among normal and infected women population within this geographical location.

MATERIALS AND METHODS

Study cohorts: The study subjects included six cohorts of women. The first three cohorts were of a group of healthy women, free of urinary tract infection. The subjects were divided into three age groups: first cohort-age group of less than 1-19 years, second cohort 20-45 years, third cohort 46 years onwards i.e. the postmenopausal group. The remaining three cohorts were of women with urinary tract infection. They were also similarly divided into three age groups (fourth, fifth and sixth cohort).

The clinical history of each subject was recorded. Incase of the control group population the informed consent of the subject was obtained. For the test group population which mainly consisted of UTI positive hospitalized patients and samples obtained from pathological centres, the informed consent of the subject could not be obtained, because the study did not involve direct interviews. These were samples for routine examination from hospitalized patients. This study was approved by the Bioethical Committee of West Bengal University of Technology.

The urine and serum samples from two UTI positive catheterized patients from cohort five and six were analyzed for serum bactericidal antibody assay. This test was repeated thrice in triplicate. Eight control subjects were also analyzed.

Media used: Four types of commercially available chromogenic media (selective and differential) were used for the enumeration of urinary tract microbes, namely Hicrome UTI Agar (M1353), Pseudomonas Agar(M120), Hicrome Kleb Selective Agar Base (M1573), Klebsiella Selective supplement (FD225), Hicrome Aureus Agar Base (M1468) and Egg Yolk Tellurite Emulsion (FD046) as per the manufacturers protocol (Himedia). Hicrome UTI Agar is a differential medium that can be used for presumptive identification of *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis* and *Candida albicans*. When the chromogenic substrate is cleaved by enzyme ß-glucosidase possessed by *Enterococci*, it produces blue coloured colonies. *E. coli* produces pink coloured colonies due to the presence of the enzyme ß D-galactosidase while *Proteus sp.* due to the presence of tryptophan deaminase activity produces brown colonies[14]. Hicrome Klebsiella Agar and Hicrome Aureus Agar were selective media. *Klebsiella sp.* showed typical bluish pink coloured colonies while *Staphylococcus aureus* shows typical black coloured colonies with a zone of clearing around it, which was due to lecithinase activity in the egg yolk media[15].

Isolation of UTI causing microbes: Early morning midstream urine samples were collected in sterile 15ml falcon tubes. 50 µL of urine sample from each of the subjects was spread on the four different types of hicrome agar plates. The plates were incubated overnight at 37ºC under inverted condition. The aureus media plates were incubated for 48 h. Next day the numbers of different coloured colonies were counted and the CFU mL^{-1} was determined. 15 different coloured colonies were isolated. Each isolate was identified through 16S rDNA based molecular characterization.

Serum bactericidal antibody assay: A modification of the technique described by David L. Smalley and Donald D. Ourth (Smalley and Ourth, 1979), was used to perform the bactericidal assay. Urine samples from two catheterized UTI positive patients from N.R.S.M.C.H. were spread on hicrome agar plates. The bacterial strains whose CFU mL^{-1} was found to be more than 10^5 were isolated and cultured separately. Ten fold serial dilutions (10^{-1}-10^{-10}) of each of the bacterial strains were made in sterile 0.85% NaCl. Fresh sera were obtained from the individual patients. 200 µL of each bacterial dilution and 200 µL of the respective patient's serum were pipetted into eppendorfs with each dilution in a separate marked tube. Control A had only 200 µL of bacterial dilution and 200 µL of sterile 0.85% NaCl.

Fresh serum was also collected from a non-UTI healthy individual whose urine was culture negative. Control B had 200 µL of bacterial dilution and 200 µL of serum from the culture negative non-UTI subjects (serum samples from 8 clinically non UTI subjects were analysed). The test as well as control tubes were incubated at 37ºC water bath for one hour. 50 µL of each dilution was then plated on Luria Bertanni (L.B.) agar plates and this experiment was done in triplicate. The entire test was repeated five times. The plates were incubated overnight at 37ºC.

Next day, the number of colonies of the test and control plate was counted. The number of colonies from the control plate was compared with the number of colonies in the test plate at each culture dilution to obtain the percentage of bacteriolysis. Test plate counts were considered to be positive for serum bactericidal activity at the dilution at which 50% or more of the bacterial colony growth was inhibited when compared with control plates of the same dilution. To verify bacterial lysis versus agglutination as the reason for colony growth inhibition, the reaction mixture was observed under 40X bright field Axiostar Plus fluorescence microscope from Zeiss after crystal violet staining.

Statistical analysis: t-test was performed to check if there was significant variation in the microbial percentage of control and patient population at 0.05 probability and the respective degree of freedom.

RESULTS

Isolation of UTI causing microbes: The specificity of the media was verified through 16S rDNA based molecular analysis of the 15 isolates. The media was found to be specific upto the genus level i.e. some of the colonies isolated from Hicrome Aureus agar plates, meant to be specific for isolating only *Staphylococcus aureus* were found to be *Staphylococcus haemolyticus* (data not shown). The novel sequences are available at GenBank with the following Accession Numbers, EF644491-EF644496.

Comparison of colony counts In CFU mL^{-1} of different microbes in control and patient population: In majority of the cases, under asymptomatic conditions, 10^4-10^5 seems to be the demarcating value between normal and pathological samples (Fig. 1). In case of each of the urinary tract microbes, we find that the percentage population of control individual declines with increase in CFU mL^{-1}. The case is just the reverse in patient population.

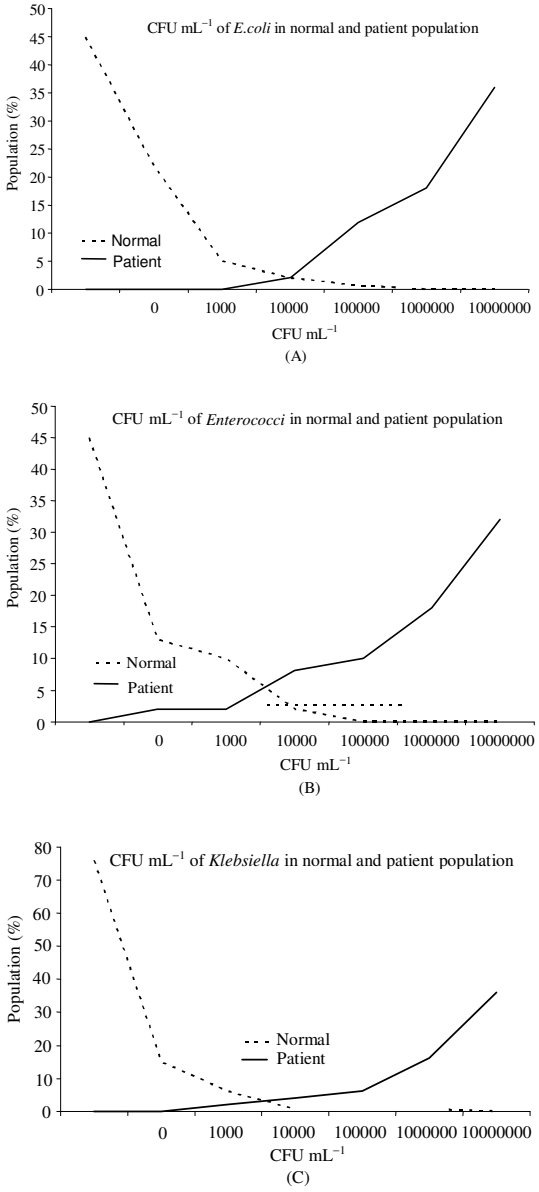


Fig. 1: Comparison of colony counts in CFU mL^{-1} in control and patient population of (A) *E. coli*, (B) *Enterococci* and (C) *Klebsiella*

The starting point of the patient graph almost merges with the end point for the control group. The percentage population increases steadily with CFU mL^{-1} in patient group.

Comparison of urinary rract microbes in different age groups: Figure 2 reveals a significant amount of variation in the microbial profile of women among various age groups. In normal 0-19 years age group the

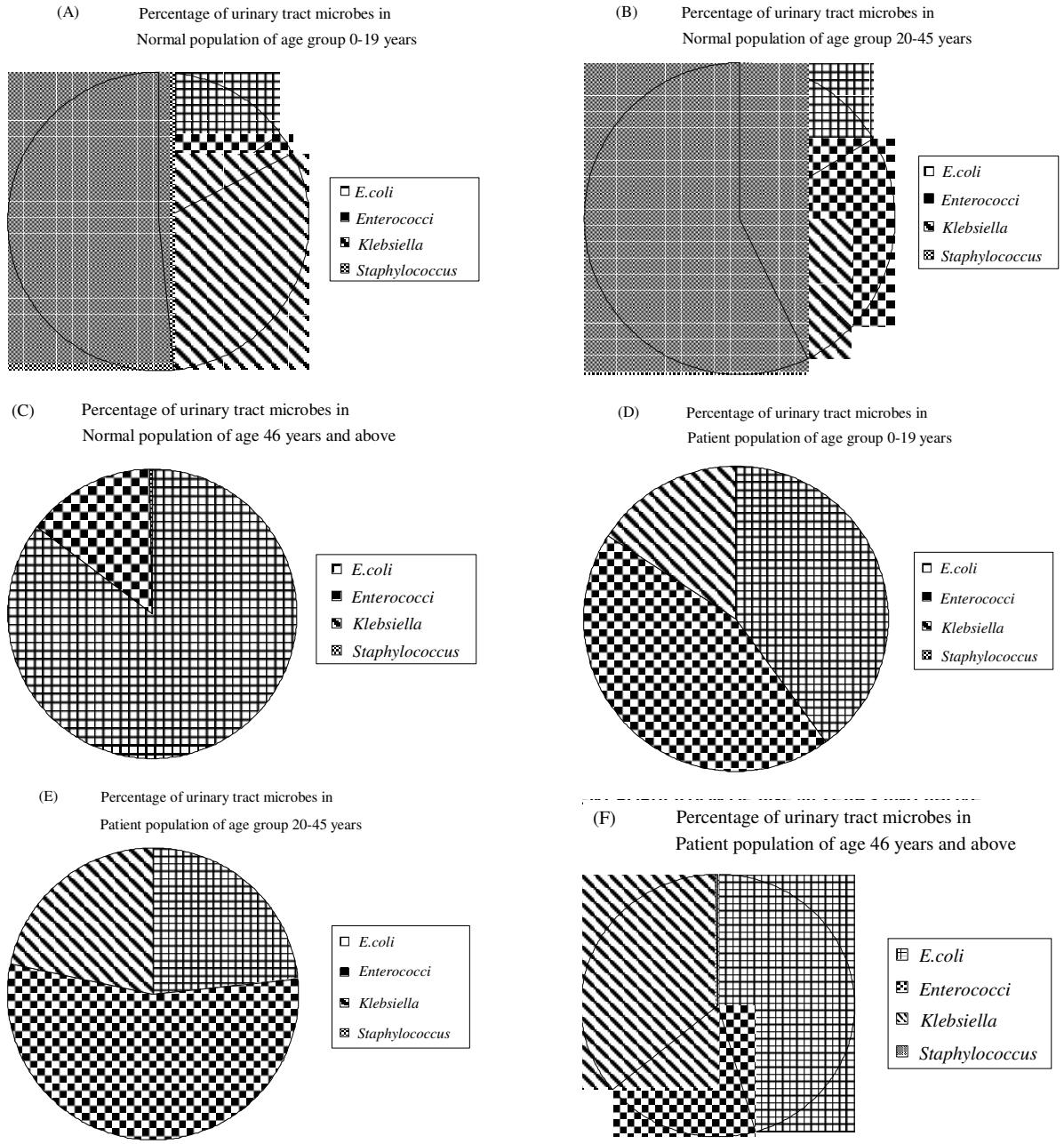


Fig. 2: Comparison of relative abundance of urinary tract microbes in (A) Control population of 0-19 years, (B) Control population of 20- 45 years, (C) Control population of 46 years and above, (D) Patient population of 0-19 years, (E) Patient population of 20-45 years and (F) Patient population of 46 years and above

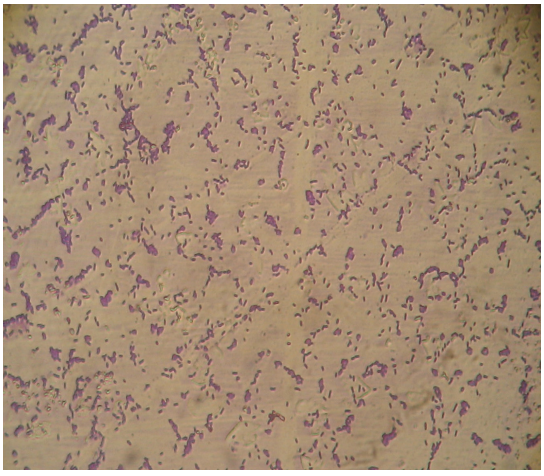
percentage of *Staphyloccus* is maximum followed closely by *Klebsiella*, *E.coli* and *Enterococci*; in normal 20-45 years age group also we notice almost a similar trend with slight decrease in *Klebsiella* percentage compensated by increase in Enterococci (Fig. 2A and B). While incase of the patient population of the same age groups, we observe an increasing occurrence of *E.coli* and *Enterococci* with almost no significant detectable *Staphylococcus*. Incidence of occurrence of UTI by *Enterococci* is more (55%) followed by *E.coli* (23%) in sexually active young females (as observed in Fig. 2E) while in younger age group *Enterococci* (44%)

Table 1: Results of serum bactericidal antibody assay at 10^{-5} dilution with three different bacterial isolates for two UTI positive patients 200

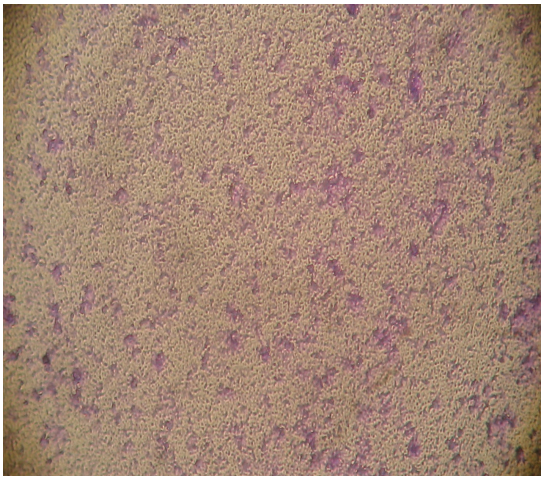
Patient I	Strain	CFU mL^{-1} in test plate	CFU mL^{-1} in control A plate	(%) of bacteriolysis in test plate
	E. coli O157 H7	40×10^5	1380×10^5	97.1
	Pseudomonas	60×10^5	2200×10^5	97.2
	Klebsiella	180×10^5	4820×10^5	96.2
Patient II	Strain	CFU mL^{-1} in test plate	CFU mL^{-1} in control A plate	(%) of bacteriolysis in test plate
	E. coli O157 H7	700×10^5	3240×10^5	78.39
	Pseudomonas	2280×10^5	6160×10^5	62.98
	Klebsiella	3200×10^5	9700×10^5	67.0

Table 2: Results of serum bactericidal activity in control B plates

Subjects	CFU mL^{-1} in control B plate with *E. coli* from patient I	(%) of bacteriolysis	CFU mL^{-1} in control B plate with *E. coli* from patient II	(%) of bacteriolysis
1	1040×10^5	24.63	60×10^5	98.14
2	320×10^5	76.81	100×10^5	96.91
3	700×10^5	49.27	40×10^5	98.76
4	300×10^5	78.26	0	100
5	1080×10^5	21.73	0	100
6	560×10^5	59.42	0	100
7	100×10^5	92.75	60×10^5	98.14
8	140×10^5	89.95	80×10^5	97.53



(A)



(B)

Fig. 3: (A) Control a slide showing intact rod shaped *E.coli* 0157 H7 cells and (B) Test slide showing cell lysis due to the bactericidal effect of serum antibodies under 40X bright field of Axiostar Plus fluorescence microscope from Zeiss. Both were incubated for the same time at the same temperature. The difference was only the addition of serum for B as compared to A

and *E. coli* (40%) are almost equally prevalent in infected cases (Fig. 2D). In the postmenopausal group of normal as well as patient population *E.coli* is the most predominant microbe in the urinary flora; about 85%, followed by *Enterococci* in the normal population (Fig. 2C).

Result of serum bactericidal assay: The test plate counts were considered to be positive for serum bactericidal activity at 10^{-5} dilution, since more than 50% of bacterial colony growth was inhibited as compared to the control plates at the same dilution (Table 1). When the bacterial dilutions incubated with serum was observed under a microscope, there was clear evidence of cell lysis (Fig. 3). In case of Control B, results as expected were not obtained. There was inhibition in cell growth in clinically non urinary tract infected subjects. Eight subjects analyzed were found to inhibit the growth of *E.coli* 0157 H7 isolated from both the patients (Table 2). Incase of subject 8 this test was

repeated after 4 months (after the subject underwent antibiotic treatment with complete course of Ofloxacin with Ornidazole) and the percentage of bacteriolysis, which was 89.95% drastically diminished to 27.23%. Another point to be noted was that the subject had the recent history of immunization with Anti Rubella (R-VAC from Serum Institute of India) and Hepatitis B (Engerix B GlaxoSmithKline, Belgium) vaccine. Another subject (subject 9-data not shown) was analyzed who did not undergo any antibiotic treatment for years and had no history of immunization after childhood. In this case there was no bacteriolysis at all.

Statistical analysis: For each of the microbes the calculated value was greater than the tabulated value (for *E.coli*, t = 2.11>2 at d = 62, for *Enterococci*, t = 2.09>2 at d = 68, for *Pseudomonas*, t = 5.79>2 at d = 70, for *Klebsiella*, t = 2.93>2 at d = 62, for *Staphylococcus* t = 2.30>2.02 at d = 51). Since the calculated t-value exceeds the tabulated value, the means are significantly different at that probability, i.e. the test was found to be significant for the variation in microbial population among the control and patient group.

DISCUSSION

The two curves (representing the control and patient population microbial counts) in the graphical diagram of Fig. 1 intersect at the range of 10^4-10^5 CFU mL^{-1} which is considered as the region of transition and the demarcating value between normal and pathological samples. However this range cannot be considered as the absolute indicator of UTI, as some symptomatic patients have CFU mL^{-1} less than 10^4. The normal and patient population graphs show a clearcut variation in the colony counts and the percentage population of *E.coli*, *Enterococci* and *Klebsiella*. The reason for the high *Enterococci* percentage in patient population (Fig. 2) is due to the spreading of infection mainly through an ascending route by the fecal reservoir through the urethra to the bladder.

The postmenopausal group, both incase of normal and patient population, seems to reflect a true picture of the literature survey which states *E.coli* is the most predominant microbe in urine in postmenopausal women due to the absence of *Lactobacilli*. In case of infection the picture changes slightly with a slight reduction in the percentage of *E. coli* as compared to normal followed by simultaneous increase in the population of *Klebsiella* (Fig. 2F). To verify the efficiency of the serum antibody assay, serum from two infected individuals were incubated with bacterial culture and significant reduction in growth due to circulating antibodies was observed. To prove that the reduction in colony count was due to bacteriolysis and not due to any bacteriostatic mechanism, the bacterial serum mixture was observed under the microscope after incubation, post crystal violet staining (Fig. 3).

Cell debris in the test sample indicates a positive bactericidal effect. Intact cells in Control A slides illustrate an expected absence of bactericidal effect. But, this cannot be considered as a full proof assay to detect the exact pathogen as there was a significant amount of reduction in Control B plates where bacterial dilution was incubated with non-UTI subject's serum. Thus, this bactericidal activity observed in control group (subject 1-8) might be due to some partially non-specific interaction between the circulating serum antibodies and bacterial antigen. These antibodies appear either due to immunization or some other infection. The observation of decrease in percentage of lysis after antibiotic treatment incase of subject 8 points towards the second cause of inhibition i.e. preexisting (throat) infection.

On the other hand no inhibition in case of subject 9 but still partial inhibition in case of subject 8 (after antibiotic treatment) points towards the possible role of immunization towards generation of circulating antibody which nonspecifically bind to the bacteria and causes the cell lysis. In all the subjects except 7 there is a notable difference in the percentage of bacteriolysis for the two different strains of *E.coli*. It might be that the percentage inhibition is more in that particular *E.coli* strain which is more frequently found as compared to a rear variety. These facts lead to the understanding that most likely a partially non-specific inhibition operates in these cases, where the circulating antibody binds to each and every type of antigen on the bacterial cell surface.

ACKNOWLEDGEMENT

The authors would like to acknowledge the financial support of Department of Biotechnology, India; West Bengal University of Technology, India; Department of Science and Technology, India under the DST Fast Track Scheme as well as Department of Atomic Energy, India under the Board of Research in Nuclear Sciences scheme. They would like to thank Bioinormatics Infrastructure Facility (DBT, GOI supported) of West Bengal University of Technology for its computational facility.

Authors from would also like to acknowledge the assistance of Dr S Das of Pearless Hospital during sample collection and the members of the University as well as their family who volunteered to be a part of this study.

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