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Research paper

Salient bactericidal activity of blood serum protein complement against *Escherichia coli* and *Shigella flexneri* in urban and slum population of Bangladesh : a comparative study

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Abstract:

Studies over the years evinced that complement participates in nearly every step of the immune reaction justifiably deserve a central position in the immunological research. The last several years have been observed as an enormous expansion in our understanding of the details of complement biochemistry and in our appreciation of the role of complement proteins in biological phenomena. This observation particularly calls for the in vitro examination of complement proteins activity, derived from blood serum of a defined group of urban and slum people against *E. coli* and *Shigella flexneri,* which are a major cause of large scale epidemics and thousands of sporadic cases of gastrointestinal illness. In this exertion we attempt to make a comparative analysis on complement mediated killing of clinical isolates of *E. coli* and *S. flexneri* derived by serum collected from urban and slum population. Hence the slum population showed more effective complement mediated killing in comparison to urban population. Moreover complement plays a central role in the installation of the adaptive innate immune response in clearence of *E. coli* and *S. flexneri in vitro* from both population which is statistically significant.

Keywords: *Blood serum, urban, rural, complement mediated lysis.*

1. Introduction

The intestinal microflora is a multifaceted ecosystem containing over 400 bacterial species. However, anaerobes outnumber facultative anaerobes which are sparsed in the stomach and upper intestine, but luxuriant in the lower bowel. Intestinal bacteria are a crucial component of the enterohepatic circulation in which metabolites that are conjugated in the liver and excreted in the bile are deconjugated in the intestine by bacterial enzymes, then absorbed across the mucosa and returned to the liver in the portal circulation. [Finegold S., 1994]. Many drugs and endogenous compounds undergo enterohepatic circulation. The flora also plays a role in fiber digestion and synthesizes certain vitamins. Enterotoxin-Mediated Diarrheas caused by enterotoxigenic bacteria, such as *Vibrio cholerae* and enterotoxigenic *Escherichia coli* strains, colonize the upper bowel and cause watery diarrhea by producing an enterotoxin that stimulates mucosal cells to secrete fluid via an increase in intracellular AMP. Invasive Diarrheas caused by invasive bacteria, such as *Shigella* and *Campylobacter*, penetrate the intestinal mucosa. A bloody, mucoid diarrheal stool with inflammatory exudate is produced. [Gorbach L.S., 1996]. The toxin has the ability to inhibit protein synthesis which leads to the death of cells. This killing of cells leads to a breakdown of the lining and to hemorrhage. The first response is commonly a bloody diarrhea. The toxin also disrupts small blood vessels, mainly the glomerulus which leads to kidney failure and the development of the often hemolytic uremic syndrome. The toxin has effect on lungs and as well as the nervous system. The bacterium *Shigella flexneri* is a causative agent of shigellosis, which is a severe infection of the colonic epithelium. It is primarily transmitted between hosts via the fecal-oral route to its infective site in the colon. [Gore AL. *et al*., 2010]. Shiga toxin–producing *E. coli* (STEC) cause approximately 100,000 illnesses, 3,000 hospitalizations, and 90 deaths annually in the United States (CDC, 2009; Mead et al., 1999). *E. coli* cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination (Feng et al., 2002). But persistent *E. coli* has demonstrated that they can survive in the environment, outside of a host for extended periods (Thompson, 2007). Being a versatile functional element of immune system, over the years it is now well accepted that complement is a complex innate immune surveillance system, playing a key role in host homeostasis, inflammation, and in the defense against pathogens. Complement cascade is activated immediately after encountering the pathogen amd following generation of pathogen specific antibodies, complement contributes in the clearance of immune complexes and pathogen elimination. Studies over the years demonstrated that complement takes part in nearly every step of the immune reaction and that it deserves a central position in the immunological research. [Merle NS *et al*, *2015*]. The objective of this study is to compare and investigate the bactericidal activity of human blood serum, collected from urban and slum based population, against *Escherichia coli* and *Shigella flexneri*. This comparative study will help to analyze whether the susceptibility of *S.flexneri* and

E. coli to complement activity of human blood serum is greater in the urban or slum based population of Bangladesh. This type of work is rare in Bangladesh and therefore it will improve our understanding about the exposure of different population groups to this microorganism and their response against it. The study will also help us to determine the possible reasons behind any difference that is observed.

2. Materials and methods

2.1 Bacterial strains

The research survey was carried out in the Microbiology Specialized Research Laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Bangladesh. Strain of *Escherichia coli* and *Shigella flexneri* were obtained from the Microbiology Specialized Research Laboratory of the Department of Mathematics and Natural Sciences, BRAC University, Bangladesh. Bacteria used for estimation of complement sensitivity were always in the logarithmic growth phase. From an overnight culture in nutrient broth, the organisms were then harvested by centrifugation, washed in water and adjusted to the concentration required by optical density.

2.2 Subjects

The serum samples used in the analysis were collected from two different locations: for rural population survey serum samples were collected from BRAC University, Mohakhali, Dhaka and for slum population survey samples were collected from TNT slum, Mohakhali, Dhaka . Before collecting blood, each of the volunteers was asked to fill out a questionnaire. A completed questionnaire would provide information about name, age, sex, blood group, occupation, present clinical symptoms, vaccination, donor ship and food habit about the volunteer and also other information such as place and date of collection. Venous blood was drawn aseptically and allowed to clot, and the serum was separated by centrifugation. Individuals from whom serum was obtained had not received any antimicrobial therapy during the preceding 2 weeks.

This survey was done to help understand the body condition of the volunteer and its effect on the individual's serum activity. The place of collection is the most important piece of information which helped, in comparing the two different population (urban and slum area) based on their life style. The study was approved by the local ethic board and written informed consent was obtained from all population from two panels before samples were taken.

2.3 Bactericidal assay of human serum against *Escherichia coli* and *Shigella flexneri*

The complement components present in blood serum aqueous layer can be isolated from clotted blood. Serum is an environment in which bacterial cells should not exist. The serum complement system provides innate defense against microbial infections. It consists of atleast 35 proteins, mostly in pre-activated enzymatic forms. [Bugla-Ploskońska, G. et al., 2009]. The activity of complement components is modulated by a system of regulatory proteins that prevent tissue damage as a result of inadvertent binding of activated complement components to host cells or spontaneous activation of complement components in plasma. A general precaution should be taken during the collection and storing of samples for complement analysis. Because some complement components are heat-labile, serum samples, if not used immediately, should be stored at −70°C to optimize the preservation of complement proteins in functionally active forms.Each and every work of serum collection was done with extra caution to eliminate unwanted contamination and errors.

3. Results

Identification of the bacteria was done for reconfirmation. *S. flexneri* and *E. coli* were reconfirmed by streaking on a selective media and by performing biochemical tests.

3.1 Serum activity analysis

The following relevant diagrams Figure 3.1 and 3.2 and Figure 3.3 and 3.4 show the activity of serums collected from both urban and slum area, against *E. coli* and *S. flexneri* at two different times. The 0 minute result usually shows a high count due to the lack of time which the serum needs to act upon the bacteria. But thereafter, as the serum starts to inhibit the growth of the bacteria, the 180 minutes result shows a noticeable decline in colony count for both organisms.

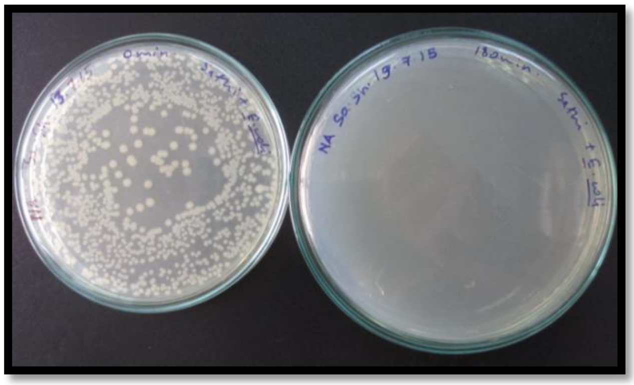


Fig. 3.1: Serum activity against *E. coli* from urban population, BRAC University

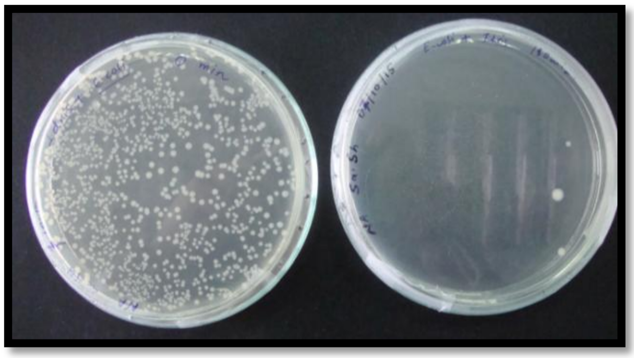


Fig. 3.2: Serum activity against *E. coli* from slum population, T&T Slum

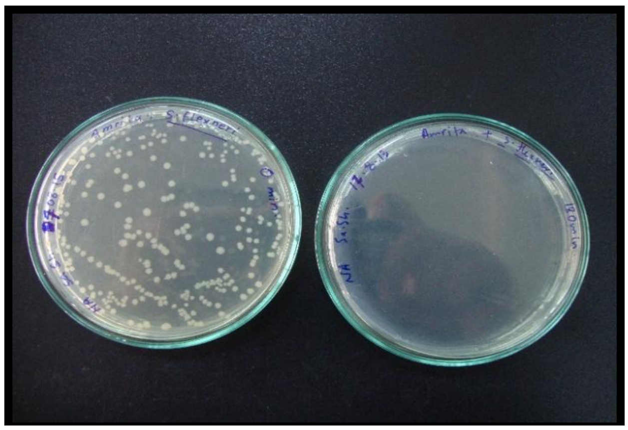


Fig. 3.3: Serum activity against *S. flexneri* from urban population, BRAC University

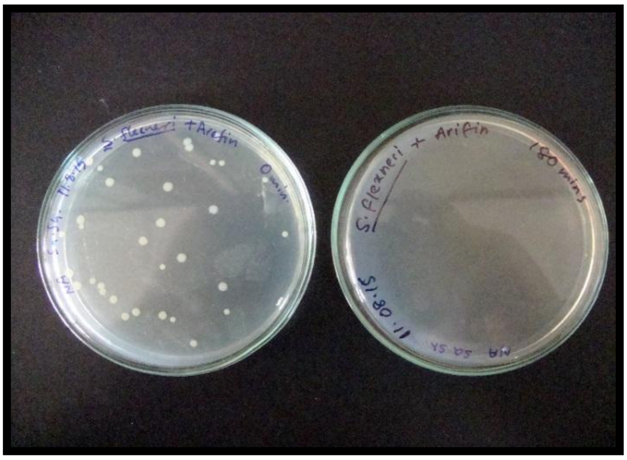


Fig. 3.4: Serum activity against *S. flexneri* from slum population, T&T Slum

3.2 Statistical Analysis

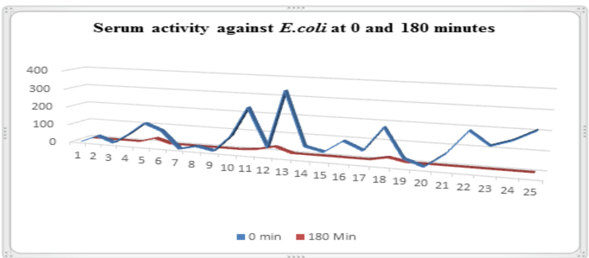
The statistical analysis was performed using an independent samples t-test comparing the means for two variables. The significance level for a given hypothesis test is a value for which a P-value less than or equal to 0.05 is considered statistically significant.

Overall, this survey serves the purpose of aiding, strengthening and supporting the research procedure and thus establishing an organized, data based result.

3.2.1 Data analysis of the serum activiy of bloody diarrhea causing *E. coli* at 0 minute and 180 minutes

Referred to Figure 3.1 and 3.2 , the complement proteins present in sera samples of slum population were successful in eliminating *E. coli* after 180 minutes of incubation *in vitro*. This result also coinside with the result of sera activity against *E. coli* in urban population (Figure 3.5 A and B) . This finding was corroborated in subsequent statistical t-test work where we tried to find evidence of a significant difference between means (2-sample **t**-test) of time required for complement functional activity which was found signifinat (Referred to table 3.2.1.1 and 3.2.1.2).

A



B

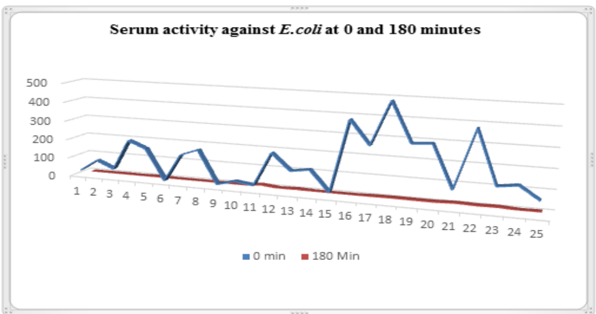


Fig. 3.5*:* Serum activity against *E.coli* at 0 and 180 minutes ; (A) In slum population the complement proteins were successful in clearance of *E.coli* which was also observed in urban people (B)

Table 3.2.1.1: Summary output for *E.coli* in slum population t-Test: Two-sample assuming equal variances

	0 min	**180 Min**
Mean	99.08	3.52
Variance	8219.576667	77.59333333
Observations	25	25
Pooled Variance	4148.585	
Hypothesized Mean Difference	0	
df	48	
t Stat	5.24543067	
P(T<=t) one-tail	1.73884E-06	
t Critical one-tail	1.677224196	
P(T<=t) two-tail	3.47769E-06	
t Critical two-tail	2.010634758	

Summary output

Since we set our $alpha$ value at 0.05, if the $p\ value$ is less than 0.05, then we say that there is a statistically significant difference between the means of the two trials.

In this case $p = 3.47769 \times 10^{-6}$, which is less than 0.05, so the difference is significant.

Table 3.2.1.2: Summary output for *E.coli* in urban population t-Test: Two-sample assuming equal variances

	0 min	**180 Min**
Mean	165.56	2
Variance	17807.59	7.25
Observations	25	25
Pooled Variance	8907.42	
Hypothesized Mean Difference	0	
df	48	
t Stat	6.127116572	
P(T<=t) one-tail	8.01497E-08	
t Critical one-tail	1.677224196	
P(T<=t) two-tail	1.60299E-07	
t Critical two-tail	2.010634758	

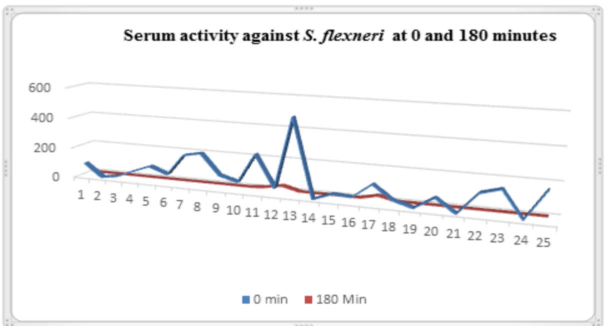
Summary output

Since we set our $alpha$ value at 0.05, if the $p\ value$ is less than 0.05, then we say that there is a statistically significant difference between the means of the two trials.
In this case $p = 1.60299 \times 10^{-7}$, which is less than 0.05, so the difference is significant.

3.2.2 Data interpretation of the complement activiy of bloody diarrhea causing *S. flexneri* at 0 minute and 180 minutes

Referred to Figure 3.3 and 3.4 , the complemnt proteins present in sera samples of slum population were successful in eliminating *S.flexneri* after 180 minutes of incubation *in vitro*. This result also synchronized with the result of sera activity against *S. flexneri* in urban population (Figure 3.6 A and B). The subsequent statistical t-test work also evidated the result significance where complement functional activity was validated (Referred to table 3.2.2.1 and 3.2.2.2).

A



B

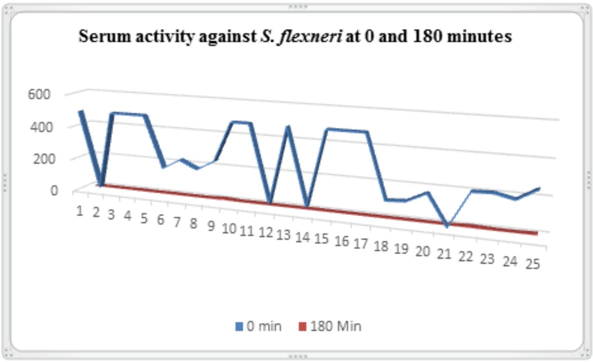


Fig. 3.6: Complement activity against *S. flexneri* at 0 and 180 minutes ; (A) In slum population the complement proteins were efficacious in clearance of *S. flexneri* which was also seen in urban people (B)

Table 3.2.2.1: Summary output for *S. flexneri* in slum population t-Test: Two-sample assuming equal variances

	0 minute	180 minutes
Mean	105	2.72
Variance	12241.25	66.12666667
Observations	25	25
Pooled Variance	6153.688333	
Hypothesized Mean Difference	0	
df	48	
t Stat	4.609756417	
P(T<=t) one-tail	1.50124E-05	
t Critical one-tail	1.677224196	
P(T<=t) two-tail	3.00248E-05	
t Critical two-tail	2.010634758	

Summary output

Since we set our $alpha$ value at 0.05, if the $p\ value$ is less than 0.05, then we say that there is a statistically significant difference between the means of the two trials.
In this case $p = 3.00248 \times 10^{-5}$, which is less than 0.05, so the difference is significant.

Table 3.2.2.2: Summary output for *S. flexneri* in slum population t-Test: Two-sample assuming equal variances

	0 minute	180 minutes
Mean	300.36	0.72
Variance	32327.24	2.543333333
Observations	25	25
Pooled Variance	16164.89167	
Hypothesized Mean Difference	0	
df	48	
t Stat	8.332367105	
P(T<=t) one-tail	3.4799E-11	
t Critical one-tail	1.677224196	
P(T<=t) two-tail	6.9598E-11	
t Critical two-tail	2.010634758	

Summary output

Since we set our $alpha$ value at 0.05, if the $p\ value$ is less than 0.05, then we say that there is a statistically significant difference between the means of the two trials.
In this case $p = 6.9598 \times 10^{-11}$, which is less than 0.05, so the difference is significant.

4. Discussion

Major contributions of this paper are the identification of importantance of diet and lifestyle factors that modify the natural immune response against *Escherichia coli* and *Shigella flexneri* , two prominant organisms responsible for gastrointestinal disorders. This study calls for the reporting of the comparative complement activity of blood serum against *E. coli* and *S. flexneri* in urban and slum population. The two population groups are considered due to their notable contrasting lifestyle pattern. The slum based population live in overcrowded condition and often lack access to basic sanitation and safe drinking water. This might cause them to have a high exposure to the these two pathogens and exhibit higher resistance against it compared to the urban population.

Bangladesh being a densely populated, small developing country, a large portion of the population is living below the poverty line. Naturally this part of the population is more exposed to these organisms than those who are well off. It can be assumed that people who are living in slums would be more exposed to *E. coli* and *S. flexneri* than those who do not.

In pathology, complement can be a friend or a foe. It acts as a friend as it plays a central role in the homeostasis and the installation of the adaptive innate immune response by its supporting role in the clearance of apoptotic and necrotic cells, and its importance in the polarization of T lymphocytes and the humoral response by the cooperation with the B-lymphocytes. Nevertheless, it can be also platy an antagonistic role, when pathogens seize complement regulators to protect themselves from the immune system. This pivotal job for the correct functioning of the organism is illustrated by the fact that both complement deficiencies and complement overactivation are alliance with severe life threatening diseases. While generally considered to be confined to the effector side of immune reactions, recent evidence indicates that the complement system also directly recognizes and is triggered by a number of bacteria and viruses as well as virus-infected cells in the absence of antibody. The improvement of our understanding of the role of complement in health and disease opens up the possibility to use complement modulating drugs in the clinical practice. [Merle NS *et al, 2015*]. This study is reporting if the different lifestyle of the two sections of the population (one living in urban area, the other in slum area) of Bangladesh has any effect on the complement inhibition activity against *E. coli* and *S. flexneri*. Since experimental evidence [Durack, D. T., and P. B. Beeson. 1977] and clinical observations [Elgefors, B., and S. Olling. 1978] suggest that serum resistance is an important virulence factor against gram-negative bacteria.

The comparative serum activity for both population can be obtained from the line charts from the result section at at 0 and 180 minutes. In the study, it was observed that the growth of *S. flexneri* at both 0 and 180 minutes was higher for serum samples collected from urban area compared to that collected from slum area which also coinside with the average complement activity against *E. coli* at 0 and 180 minutes among urban and slum area. Statistical analysis showed good correlation for both poulation. According to the t-test the result is statistically significant at 0 and 180 minutes where the functional integrity of complement was evidated.

The differences in living condition or life style do affect the complement arbitrated inhibition activity of human serum. Both the urban and slum population more or less has been exposed to *E. coli* and *S. flexneri* but the slum population has adapted to the organism since they are more exposed to it through poor living condition. The complement system of their serum has been able to dismantle more of the organisms. Thus the slum residents are able to show greater resistance than that of urban against *E. coli* and *S. flexneri*. The residents of slums can be more exposed to various potentially pathogen organisms due to an underprivileged lifestyle compare to the people who live in the urban area. This exposure may actually aid the slum people in having a better resistance. We conclude that the pathogenic strain of *E. coli* and

S. flexneri are more susceptible to human serum from slum individuals than urban population.

The pooling of data from two studies allowed us to evaluate associations with risk, mortality, and lifestyle factors that could mediate susceptibility risk. Although we used statistical method to adjust for the associations observed among our candidate populations, associations could still be chance findings that need replication in other similar populations.

5. Conclusion

Relative interpretation of the data showing complement mediated lysis of *E.coli* and *S. flexneri* clearly illustrtes the experiments conducted *in vitro* with the sera of urban and slum population are statistically significant revaling the successful immune response of complemnt proteins against these two oranisms.Nevertheless, the result also showed significant activity of complement proteins from slum people against these two organisms was more satisfactory than urban population.

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