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BACTERICIDAL ACTIVITY OF HUMAN SERA AGAINST *SALMONELLA TYPHI* AND *SALMONELLA PARATYPHI* A, B, C.
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BACTERICIDAL ACTIVITY OF HUMAN SERA AGAINST *SALMONELLA TYPHI* AND *SALMONELLA PARATYPHI* A, B,C.

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ABSTRACT

***Objectives*: To determine the sensitivity of *Salmonella typhi* and *paratyphi* A,B,C, to normal human blood serum; and assess the role of blood groups (ABO system), Complement and Immunoglobulin in the resistance or susceptibility of *Salmonella typhi* and *Salmonella paratyphi* A,B,C infections.**
***Design:* Cross sectional study.**
***Subjects*: Ninety-six apparently healthy males and females volunteers, aged 18-24 years.**
***Main outcome measures:* Resistance of *Salmonella typhi* and *Salmonella paratyphi* A,B,C infections may be blood group and immune status dependent.**
***Results*: Blood group B was most resistant to *Salmonella typhi* and *Salmonella paratyphi* A,B,C while blood group O showed least resistance (51.9%) and (22.2%) for *Salmonella typhi* and *Salmonella paratyphi* A,B,C. There was no difference in resistance pattern when blood was pooled in respect to their group types. Age or sex of the blood donors had no effect on the bactericidal activity of the sera.**
Conclusion*: Blood group is an important factor in the susceptibility or resistance of an individual to S*almonella typhi* and *paratyphi* infections. Individuals of blood group O are likely to be more susceptible to infections caused by S*almonella typhi* and *paratyphi A,B,C.

INTRODUCTION

Human serum is considered an important host defence mechanism against invasive diseases caused by Gram-negative bacteria(1-3). The bactericidal and bacteriolytic activities of serum have been described and the relative roles of complement and antibody established(4,5). It has been reported that there are some associations between blood groups with particular diseases and that blood groups of individuals could determine resistance or susceptibility to infectious agents(6,7).

The association of blood groups with diseases was thought to concern the relation of the antigen of the infecting organisms with those of the blood group(8). For instance, if an organism carries an antigen resembling that of blood group A, then a group A- person being unable to make an anti-A antibody would be in a more disadvantaged state than a group O-person who already had such an antibody. But the results were not the case(9). It has been reported that syphilis is markedly present in blood group-A people. The most notable feature is that in the treatment of syphilis, a positive Wasserman serological reaction of the serum is on the average much more persistent in A and B blood group persons than O-blood group person(7). Also, meningitis is marked by a considerably raised group A-frequency(9). Studies have also shown that malaria seems to be associated with blood group A individuals, but other factors, for example, glucose 6- phosphate dehydrogenase deficiency and sickle cell may be involved in resistance to malaria(7).

Previous studies have revealed that natural *Salmonella* bactericidal antibody is present in the serum of most adults, while *Salmonella* species isolated from patients with disseminated *Salmonella* infection were found to be serum resistant(10). Some workers(6,10), reported that typhoid and paratyphoid infections tend to be associated with a raised blood group-O frequency, but only in the case of paratyphoid does this reach the conventional level of significance. Thus persons with S-S haemoglobin are exceedingly susceptible to *Salmonella* infections, particularly osteomyelitis; and persons with sickle cell trait (A-S) may be more susceptible than normal individuals(10). It has been established that besides natural antibodies in the serum of apparently normal human beings, there are other factors such as antitoxins, antiviral antibodies, opsonins and antilysins, which play a role in the bactericidal activity of human serum(11,12). The complement system, which exists in the blood, is a major amplification and effectors mechanism of humoral immune responses; and it has been reported that complement

protein is essential for killing susceptible Gram-negative bacteria(13). Bactericidal antibody and terminal complements are indispensable factors for protection against diseases caused by the Gram-negative bacteria(13). In addition to complement, lysozyme, calcium (Ca^{2+}) and magnesium (Mg^{2+}) are important components of extra-cellular fluids that are required for killing Gram-negative bacteria. Calcium and magnesium ions are essential for initiation of the classical complement sequence(6). It has also been established that immunoglobulins (IgG, IgM) antibodies were able to promote killing of some gram-negative bacteria *in vitro* in the presence of complement(11) but immunoglobulin-M fraction was proved to be bactericidal(14).

Salmonella species have been incriminated in several disease states(15-17); and due to the high incidence of typhoid and paratyphoid fevers and the sporadic nature of *Salmonella* infections, it has become necessary to review the role of resistance factors to *Salmonella* species. Although reports on the baseline *Salmonella* antibody titre have been documented(18-20), the role played by A,B,O blood system, complement and immunoglobulins in the bactericidal activity of normal human serum against *Salmonella typhi* and *Salmonella paratyphi* A,B,C has not been documented. This study attempts to determine the sensitivity of *Salmonella typhi* and *Salmonella paratyphi* A,B,C to normal human serum and the role of complement system and immunoglobulin in bactericidal effect of blood serum.

MATERIALS AND METHODS

Bacterial strains: Isolates of *Salmonella* species used were obtained from the University of Benin Teaching Hospital, Benin City and Central Hospital Ubiaja, all in Edo State of Nigeria. These were clinical isolates from urine, blood and stool of patients who were attending clinics. Isolates were further confirmed following standard laboratory procedures as described(21-22). A standard strain of *Salmonella spp.* (ATTC 13076) was used as control.

Human serum and salmonella antibody screening: Blood samples were collected from a total of ninety six adult male and female volunteers age range 18-42 years, who were not on any antibiotics for the past eight months before this study. The blood groups of individual donors were determined using commercially-prepared antisera (Laboratory Diagnostic Products Ltd) as previously described(3,5,18). Altogether, there were 41 females and 55 males, consisting of 20, 16, 6 and 54 of A, B, AB, and O blood groups, respectively. The blood was screened for presence of antibody to *Salmonella spp* using the agglutination test method of whole organisms as the antigen. Briefly, the colony of the organism was emulsified in 0.05 ml normal saline and an equal volume of the serum was added. The mixture was mixed and rocked for few minutes to detect visible agglutination. Those that showed no agglutination had no detectable antibody against *Salmonella spp.* and so were considered suitable for the study. Serum not immediately used was stored frozen at -4°C in screw cap vials. Serum used as control was heat-inactivated at 56°C for 30 minutes in water bath(21).

Preparation of bacterial suspension for bactericidal assays: Strains of *Salmonella typhi* and *Salmonella paratyphi* A, B, C were grown on blood agar plates. Cells were harvested and spun at 6000g, washed twice and the pellets re-suspended in distilled water to give a cell count of approximately $1x10^2$ CFU as described(3,4,21). Briefly, the opacity of the bacterial suspension was matched with a standard set of opacity tube (Brown's tube) containing varied concentration of Barium Sulphate ($BaSO_4$). Each standard opacity tube was numbered and the equivalent number of organisms/ml was listed for tube 1. Tube 2 was equivalent to twice the number of tube 1, tube 3 was thrice tube 1. Each tube had varying numbers for different organisms. Matching was performed using a tube with the same bore and thickness of glass as the standard tubes.

Performance of bacterial assay: The serum bactericidal test was performed in a microtitre system using a disposable U-well trays(1,3,5). Before use the sera were sterilised using a 0.45ul membrane filter as described by Baker *et al*(23). The sera were doubly diluted from 1-2 to 1-256; each dilution was performed in triplicates. The test mixture contained 50ul fresh test serum and 50ul final dilution of the organism (100 ul). The trays were sealed and incubated at 37°C on gravity shaker for 60mins. A sterilised standard wire loops were then used to deliver a drop of mixture from each well unto blood agar plates. The plates were incubated aerobically for 18hours at 37°C and the colony count was obtained as earlier described(1,3,5). Briefly, the culture plates were opened and a magnifying hand lens was raised above it and looking through the lens count was made .

Determination of the role of complement system: The role of complement in serum bactericidal activity against *Salmonella typhi* and *paratyphi* A, B,C was performed. Source of the complement for the bactericidal assay was Guinea-pig serum. The blood was obtained by cardiac puncture. The blood was allowed to clot at room temperature for about three hours and the serum was separated from erythrocytes by centrifugation at 2000Xg for 15mins at 4°C. The supernatant was drawn into sterile bijou bottles. Serum was divided into two portions; the portion not used immediately was stored at -4°C in screw cap vials, while the serum portion used for the test was heat-inactivated at 56°C for 30minutes.

To determine the role of the complement system, the mixture which contained 50ul heat-inactivated serum, 50ul guinea-pig serum and 50ul final dilution of test organisms was added to 50 ul 10 mMol Mg^{2+}-EGTA (Ethelene glycol tetra Acetic acid) and 10 mMol EDTA (Ethlene Diamine Tetra Acetic acid) in U-well trays. Trays were then incubated at 37°C for 60 minutes as described by Blasser *et al*(2).

Determination of the role of immunoglobulin: The role of immunoglobulin was determined using the method described by Gary *et al*(15). Sera were treated with 2- mercapto-ethanol by adding 50ul dilution of organism to 50ul of normal saline and 50ul of 2-mercapto-ethanol in a U-well tray and trays incubated at 37°C for 60 minutes.

Interpretation of the bactericidal test: Significant bactericidal activity was defined as a 50% reduction in cfu when compared with the heat inactivated serum control. This was obtained by using the formula of Skirrow(24).

$$\text{Percent killed} = \frac{\text{No. of cfu(fresh serum)} \times 100}{\text{No. of cfu inactivated}}$$

Statistical analysis: The Fisher exact test of probability for small numbers was used to test probability and the Student t-test was used to compare normally distributed population.

RESULTS

The results showed that *Salmonella typhi* and *Salmonella paratyphi* A,B,C were highly sensitive to normal human sera from blood group B. The percentage sensitivity was 93.8% for S. *typhi* and 62.5% for *S. paratyphi* A, B, C (Table 1). This was followed by blood group A, 80% and 60% for *S. typhi* and *S.paratyphi* A,B,C respectively. *Salmonella typhi* was sensitive to four (66.7%) of the six AB blood group while only two (33.3%) *S. paratyphi* A,B,C were sensitive. The results indicated that blood group O-sera recorded the least sensitivity of 51.9% and 22.2% for *S. typhi* and *S. paratyphi* A,B,C respectively. It was also shown that sera bactericidal effect is lower with *S.paratyphi* than with S. *typhi* (Figure l).

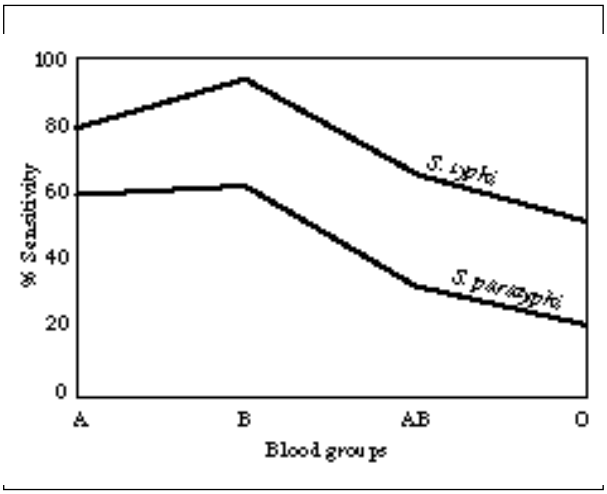
Table 1

Percentage sensitivity of S. typhi and S. paratyphi A,B,C to different blood groups

Blood group	Total No. of donors	*S. typhi* No. of sensitive strains	*S. paratyphi* A,B,C No. of sensitive strains
A	20	16 (80%)	12 (60%)
B	16	15 (93.75%)	10 (62.5%)
AB	6	4 (66.7%)	2 (33.3%)
0	54	28 (65.9%)	12 (22.2%)
Total	96	63 (65.2%)	36 (37.5%)

Figure 1

Percentage of sensitivity of S. typhi and S. paratyphi A,B,C to different blood groups



When sera samples were pooled in respect to their types and experimented, the result was similar to that of individual sera. Sensitivity of *S. typhi* and *S. paratyphi* were highest for blood group B (80% and 71%) and least for blood group O (50% and 20%) respectively (Figures 2 and 3). The sex and age of subjects showed no effect on the sensitivity as result patterns were the same for pooled and individual sera. Preliminary screening of blood samples for evidence of antibody to *Salmonella* species was negative.

Figure 2

Sensitivity of pooled blood groups (A,B,AB,O) against salmonella typhi

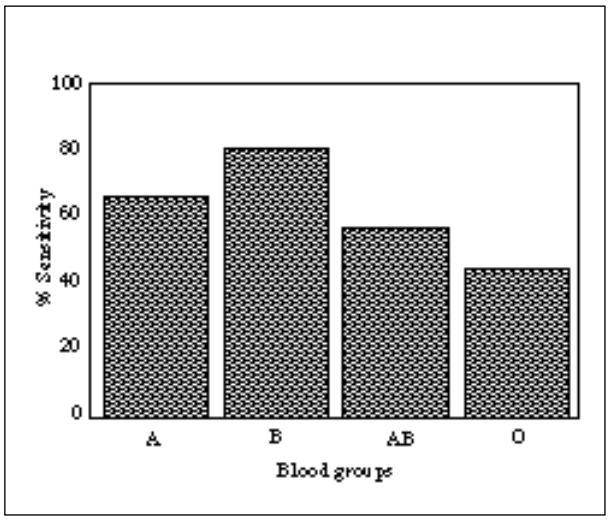
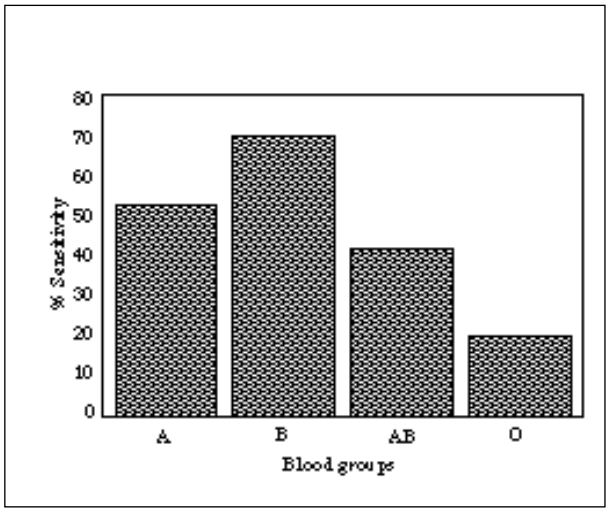


Figure 3

Sensitivity of pooled blood groups serum against salmonella paratyphi A,B,C



Role of complement in bactericidal activity of serum to salmonella species: When *Salmonella typhi* and *Salmonella paratyphi* A,B,C were incubated with individual serum of blood groups, and pooled serum of same blood groups, results showed that the organisms were serum sensitive. However, when the sera were heated to inactivate the complement, the organisms became resistant with virtually no killing effect. But with the

addition of external source of complement (Guinea pig sera), the sera regained sensitivity (Table 2). There was no significant difference between the pooled sera and individual sera ($p > 0.01$). When serum was treated with EDTA there was no killing effect, but treatment of serum with Mg^{2+}-EGTA caused slight bactericidal effect.

Table 2

Bactericidal effect of normal human serum and decomplemented serum on Salmonella species

Treatment	Serum sensitivity of all groups tested	Remarks
Pooled normal human serum	>75% killing	S^s
Pooled decomplemented normal human serum	< 50% killing	S^r
Pooled decomplemented + complemented	< 75% killing	S^s
Pooled normal human serum treated with EDTA	<50% killing	S^r
Pooled normal human serum treated with Mg^{2+}-EGTA	Slight bactericidal action	< 50%
Control	No killing	S^r

Key
S^s: Serum sensitivity
S^r: Serum resistant

Table 3

Bactericidal titre of human serum treated with 2- mercapto-ethanol

Serum tested	Bactericidal titre	
	S. typhi	*S.paratyphi* ABC
Individual serum	1024	512
Pooled serum	512	256
Individual serum treated With 2-mercapto-ethanol	<2	<2
Pooled serum treated with 2- mercapto-ethanol	<2	<2

Role of immunoglobulin: Addition of 2-mercapto-ethanol reduced the killing potential of antibody. Bactericidal titre of serum dropped from 1024 and 512 to <2 for *Salmonella typhi* and *Salmonella paratyphi* A,B,C, respectively (Table 3).

DISCUSSION

The bactericidal activity of normal human sera to *Salmonella typhi* and *Salmonella paratyphi* ABC was investigated. Results obtained revealed that the most prevalent isolates of *Salmonella* species in this locality are serum sensitive. *Salmonella typhi* was extremely sensitive to blood group A and B sera (93.8% and 80%), respectively. *Salmonella paratyphi* ABC, showed 62.5% and 60% sensitivity to blood group A and B sera. This result is in agreement with previous studies(3,5). These authors reported that the sensitivity or resistance of bacterial isolates to natural serum bactericidal activity could serve as an important epidemiological marker in the study of bacteria infections. In the present study, group O blood sera were least active to the *Salmonella* species studied, 51.9% for *Salmonella typhi* and 22.2% for *Salmonella paratyphi.* This suggests that the species, particularly *Salmonella paratyphi* ABC may resist natural bactericidal action of blood sera of group O individuals, which implies that individuals of blood group O could be more susceptible to infections caused by these species of *Salmonella.*

When serum samples were pooled with respect to their blood groups, group B sera still showed the highest percentage killing effect to the organisms. Percentage sensitivity was higher with individual serum than pooled sera. This may be due to the varied surface antigenic make up and the immunological properties that exist between individuals as suggested(6). Statistically, the difference between individual serum and pooled sera was not significant ($p > 0.01$). Age and sex of donors showed no effect on the reaction pattern of the sera as shown by the pooled sera test, implying that there is no age limitation to bactericidal activity of human serum. Serum concentration was graduated and used to test for sensitivity; the result pattern was the same irrespective of the dilution. This indicates absolute resistance of blood group sera to *Salmonella typhi* and *paratyphi* A,B,C.

The results of this study indicate that antibody alone did not reduce viable count of the organisms, which implies that killing of S*almonella* species by normal human serum is complement-dependent as reported by previous workers(25,26). The present study showed that treatment of serum with Mg^{2+}-EGTA, which inhibits complement activities, produced slight bactericidal action and treatment of serum with EDTA reduced killing effect, which showed that part of the killing was mediated by complement.

It was shown that presence of antibody enhances total bactericidal activity and is crucial in the *in-vivo* host defence against S*almonella* species(27), but study has demonstrated that only IgM serum fraction has proved to be bactericidal(15). The reducing agent mercapto-ethanol inhibits IgM antibody(15). Treatment of whole serum with mercapto-ethanol to determine immunoglobulin class responsible for the bactericidal activity, showed decrease in bactericidal activity to a titre as low as <2, which shows that IgM mediates the bactericidal activity of serum. *Salmonella* bactericidal activity of normal human sera is a function of IgM as determined by its susceptibility to mercapto-ethanol. This result is in agreement with previous reports(10,28).

This study reveals that complement determines the serum sensitivities to *Salmonella* infection as decomplemented human serum resulted in virtually no killing of the organisms. Also killing of *Salmonella typhi* and *Salmonella paratyphi* A,B,C is dependent on the presence of IgM antibody. Blood group O, which constitutes the highest number in the population, is the least active blood group to *Salmonella* species. The study may explain why certain individuals are susceptible to *Salmonella* typhoid fevers than others. The results have

provided useful information in determining individuals sensitivity or resistance to infection caused by *Salmonella typhi* and *Salmonella paratyphi* A,B,C. Since it has been reported that vaccine is not adequate protective measure against *Salmonella* infections, strict hygienic precautions should be adapted to avoid ingestion of the organism.

REFERENCES

1. Vostil, K. L. and Randa, E. Sensitivity of Serologically classified strains of *Escherichia coli. Amer. J. Med. Sci.* 1980; **259**:114-119.
2. Blasser, M.J, Smith, P.F. and Kohler, P.F. Susceptibility of Campylobacter isolates to the bactericidal activity of human serum. *J. Infect. Dis.* 1985; **151**:227-235.
3. Obi, C.L. and Coker, A.O. Natural bactericidal action of human serum against strains of *Campylobacter jejuni* isolated in Lagos, Nigeria. *Cent. Afr. Med. J.* 1989; **35**: 444-448.
4. Friendlander, A.M. DNA release as a direct measure of Microbial Killing: Serum bactericidal activity, J. *Immun.* 1975; **115**: 1404-1407.
5. Rice, P.A, cormack, W.M. and Kasper, D.L. Natural serum bactericidal activity against *Neisseria gonorrhoeae* isolates from disseminated locally invasive and uncomplicated disease. *J. Immun.* 1980; **124**: 2105-2109.
6. Morrison, D.C. and Kline, L. F. Activation of the Classical and properdin Pathways of Complement by bactericidal Iypopolysaccharides (LPS). *J. Immunol.* 1977; **118**:362-368.
7. Agbonlahor, D.E, Obi, C.L, Esumeh, F.I., Ajanaku, O. Obi, H.H, Ekundayo, A.O. and Igumbor, E.O. Association of ABO blood groups and malaria parasitaemia among students of Edo State University Ekpoma, Nigeria. *J. Med. Lab. Sci.* 1993; **3**: 18-21.
8. Michael, J.G, Whitby, J.L and Landy, M. Studies on 'natural' antibody to Gram negative bacteria. *J. Exp. Med.* 1962; **115**: 131 - 146.
9. Athreya, B.H. and Corriell, L.L. Relation of blood group to infection. *Amer. J. Epidem.* 1967; **86**: 292-303.
10. Anyiwo, C.E, Obi, C.L. and Nnajia, A. Waning significant of anti-treptolysin O (ASO) titre in diagnosing Streptococcal infection in Lagos, Nigeria. *East Afr. Med. J.* 1989; **66**:636-640.
11. Obi, C.L. and Cooker. Production of Antisera against Campylobacter spp. in Lagos, Nigeria. *Cent. Afr. J. Med.* 1988; **34**:139-141.
12. Davis, S.D. and Wedgwood, R. J. Kinetics of the bactericidal action of Normal serum on Gram- negative bacteria. *J. Immunology.* 1965; **95**: 75-79.
13. Gregory, T.S., Alan, L.L., Brenda, L.S., Bonnie, D. and Thomas, F. L. Activation of Complement of the surface of cells infected by human immunodeficiency virus. *J. Immuno.* 1990; **118**:362-368.
14. Shinya, I., Kazuyoshi, N., Shiegeyaki, E., Keijiro,K. and Akara, M. Activation of the alternative complement pathway by water-insoluble glucans of *Streptococcus mutans:* The reaction between their chemical structures and activating potencies. *J. Immun.* 1976; **117**:1256- 1260.
15. Gary, K.S., Hans, D.O. and Thomas, M.B. Immunoglobulin class responsible for gonococcal bactericidal activity of normal serum. *J. Immun.* 1979: **122**;1771-1779
16. Sappha Ivan and Winter J.W. Clinical manifestations of *Salmonella* in man. *N. Eng. J. Med.* 1957; **256**: 1128-1132.
17. Tassios, P.T., Markogiannakis, A. Vatopoulos, E.K., Vatopoulos, A., Katasanikou,E., Velonkis, E.M., Krimastinou, J.K. and Legakis, M. Molecular epidemiology of antibiotic resistance of *Salmonella enteritis* during a 7-year period in Greece. *J. Clin. Microbiol.* 1997; **35**: 1316-1321.
18. Brodie, J. Antibodies and the Aberdeen typhoid outbreak of 1964: The Widal reaction. *J. Hyg.* 1977; **79**: 161-180.
19. Igumbor, E.O., Ekundayo, A.O, Azeez, M.M and Oyego, E.O. Baseline titre of *Salmonella* antibodies in Ekpoma area of Edo state, Nigeria. *Nig. J. Microbiol.* 1998; **12**: 9-12.
20. Mohammed, I., Gashau, W. and Ghikwem, J.O. Determination by Widal agglutination of the baseline titre for the diagnosis of typhoid fevers in two Nigerian States. *Scand. J. Immun.* 1993; **36**: 153-156.
21. Craven, D.E., Shenk, T. and Frash, C.E. Natural bactericidal activity of human serum against *Neisseria meningitides*, isolation of different serogroups and serotypes. *Infect. Immunol.* 1982; **37**: 132-137.
22. Association of pathologists. Broad sheet 58, October 1967. The isolation and identification of Salmonella from publishing manger, *J. Clin. Pathol.* 1967; **58**: 1-20.
23. Baker, F.J., Breach, M.R., Leighton, I. And Taylor, P. Sterilisation: In: *Medical Microbiology Techniques*, Vol. 1. Butterworths, London, Boston, pp. 45, 1980.
24. Skirrow, M.B. *Campylobacter enteritis.* A new disease. *Brit. Med. J.* 1975; **21**: 9-11.
25. Heddle, R.J. and Rowley, D. The anti-bactericidal properties of dog Ig^A, Ig^M and IgG antibodies to *Vibrio cholerae*. *J. Brit. Soc. Immun.* 1975; **29**: 179-208.
26. Schoolnik, G. K., Buchanan, T.M. and Holmes, K.K. Gonococcal causing disseminated gonococcal infections and resistant to the bactericidal action of natural human sera. *J. Clin. Invest.* 1976; **58**:1163.
27. Gordon, D.L. Serum bactericidal activity against *Haemophilus influenzae. Pathology.* 1988; **20**:124-129.
28. Cohen, T.R., Kellogg, D.S. jr. and Norms, L.C. Serum antibody response in experimental human gonorrhoea immunoglobulins. *Brit. J. Ven. Dis.* 1969; **45**:325.