

SEROPREVALENCE AND DISTRIBUTION OF ANTIBODIES TO VIBRIO CHOLERAE SOMATIC ANTIGENS IN SERA AND BREASTMILKS OF PARTURIENT WOMEN IN CALABAR, CROSS RIVER STATE, NIGERIA

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ABSTRACT

Seroprevalence of somatic vibro antibodies in sera and breastmilks of parturient women in Calabar were investigated. Serum and breastmilk samples from 335 parturient women who delivered at the university of Calabar Teaching Hospital between March 1998 and March 1999 and single serum samples from 239 randomly selected non pregnant women of child bearing age were evaluated for *Vibrio cholera* antibodies. The agglutinating antibodies were determined using direct agglutination technique in a 96-well W.H.O Perspex (microtitre) plate while the vibriocidal antibodies were detected using the immune bacteriolysis method. In all, 104(31.5%) and 51(22.2%) of the parturients and non-parturients had significant (>1:160) agglutinating *Vibrio cholera* antibodies in their serum samples. This shows a highly significant difference ($p < 0.01$). The prevalence rates of significant vibriocidal antibodies in parturient and non-parturient women were 54(16.1%) and 16(6.9%) respectively. There was also high significant difference ($p < 0.01$) among the two groups. The prevalence rates of agglutinating and vibriocidal antibodies in the breastmilk of parturient women were 14(4.2%) and 18(5.4%) respectively. It can therefore be concluded from the result of these study that large percentage of parturient women in Calabar possess natural immunity against cholera which may be probably due to their continued natural exposure to *V. cholerae* antigens.

KEYWORDS: Seroprevalence, Somatic, Vibro antibodies, Breastmilk, Parturient women

INTRODUCTION

Cholera is an acute disease of the intestine characterized by vomiting and frequent painless diarrhea (rice water stool). Cholera could develop to dehydration haemoconcentration shock and death of the patient with no prompt medical attention (Holmgren, 1981,

Jauda *et al.*, 1988). Cholera which is a gastrointestinal disease has for decades been associated with toxigenic *Vibrio cholera* sero-group 01. *V. cholera* 01 strains do not produce toxin but cause gastroenteritis in humans (Morris *et al* 1984). *V. cholera* 01 occurs as two



biotypes, the classical and the Eltor biotypes (Glass *et al.*, 1991). Cholera is normally known as a disease of the lower socio-economic groups living in slums with poor sanitary conditions. However cholera can spread very rapidly especially through water and infected food and in dwellers of high density communities where sanitation and the supply of drinking water are defective (Udo 1993., Siddique *et al.*, 1992). Utsalo *et al.*, 1992 identified Calabar as a cholera endemic region and reported that the inhabitants are vulnerable to acquisition of the infection probably through the ingestion of contaminated water and seafoods. In 1991, an increased number of clinically diagnosed cholera cases in the emergency unit of University of Calabar Teaching Hospital (UTCH) was noted (Eko *et al.*, 1994). In developed and developing countries the first six months of life, infants are protected against many infections through the action of transplacentally acquired maternal antibodies. However, protection against cholera during infancy is chiefly through the intestinal microflora and local antibodies which are derived from mother's milk (Gerrard, 1974). Breast feeding is responsible for establishing a lactobacillus bifidus flora in the gut which limits the growth of colonies of intestinal pathogens (Wesh and May, 1979). Breastmilk contains large amount of secretory IgA and lesser amounts of IgG and IgM (Tomasi, 1972).

Serum, milk or colostrums samples collected from mothers living in certain endemic parts of the world showed the presence of antibodies directly against the somatic or toxin antigens of *V. cholera* 01 (Holmgren *et al.*, 1976.,

Svennerholm *et al.*, 1984). This immunity was probably acquired by their continued natural exposure to *V. cholera* 01 antigens. Moreover, the presence of such antibodies in clostrum has a practical significance in inducing protection against cholera. This may be achieved through breast feeding of infants by other living in an endemic area. The advancement in medicine and delivery of drugs to rural community has adversely reduced the scourges and spread of cholera in developing countries. The reason is that at present effective treatment measures are available which can save practically all infected persons who reports for treatment within the first six hours of symptoms. Levine *et al.*, (1979) and Levine (1980) reported that clinical cholera provides solid protection for at least ten weeks against re-challenge with either the homologous or the heterologous serotype. The assessment of the duration as well as the quality of the immunity stimulated by natural infection is critical for directing the development of cholera vaccines.

The insurgence of cholera is mostly found in communities with inadequate natural resources such as good water supply, food etc. attention has been turned towards the development of oral vaccines that can stimulate intestinal immunity more efficiently. These efforts have now provided several oral vaccines bases on either non-living bacterial antigens or live attenuated mutants of *V.cholera* 01 (Kaper, 1989). The most advanced of these new oral vaccines consist of combination of inactivated whole bacterial cells (W-C) together with the purified B-subunit component of cholera toxin. It has been tested in

large scale field trials and shown to confer, without any side effects, a long lasting protection against cholera as well as diarrhea caused by enterotoxigenic *E.coli* (Clemens *et al.*, 1988).

The most outstanding characteristics of *V. cholera* is the ability of virulent strains to produce potent enterotoxin responsible for the watery diarrhoeas observed in affected patients (Almeida *et al.*, 1990). Cholera toxin is one of the most important enterotoxins in terms of resultant effect on the health of the patient. The target tissue for cholera toxin is the epithelium of the small intestines. The clinical manifestations of cholera begins in an average of 2 to 3 days after ingestion of the bacilli, with the abrupt onset of watery diarrhea and vomiting. The resulting severe fluid and electrolyte loss can lead to dehydration, metabolic acidosis (bicarbonate loss), hypokalemia shock (potassium loss), with cardiac arrhythmia and renal failure (Janda *et al.*, 1988). The mortality rate is 60% in untreated patients but less than 1% in patients promptly treated to replace loss fluids and electrolytes. Treatment with antibiotic therapy, although of secondary value, can reduce exotoxin production and more rapidly eliminate the organism. Docycline or tetracycline is the drugs furazolidone is used for pregnant women, and trimethoprim-sulfamethoxazole has been reported to be resistant to trimethoprim-sulfamethoxazole (Chesbrough, 1992). This study is aimed at evaluating the prevalent characteristics of *V. cholera* vibriocidal and agglutinating antibodies in the sera and breastmilks of parturient women in Calabar.

MATERIALS AND METHODS

Sample collection

Three hundred and thirty-five serum and breastmilk samples were collected from parturient women who delivered at term in the labour wards of the UTCH, Calabar. The samples were taken 1-2 days after normal delivery. Questionnaires were administered to subjects to obtain additional useful information. Blood samples were collected by standard venepuncture method and introduced into clean, dry bottles and allowed to clot at room temperature and sera separated by centrifuging at 3000rpm for 10mins according to the method of Obi and Coker (1989). With the aid of Pasteur pipettes, the sera were then transferred to small tubes with good stoppers and stored at -20°C until use. Breastmilk samples were obtained as soon as the beginning of milk secretion was detected. Collection was done by manual expression into sterile containers after cleaning the nipple area with methylated spirit and discarding the first two drops. Each breastmilk sample was centrifuged at 4000rpm for 10mins and the clear middle layer (between the fat layer at the top and pellet at the bottom) carefully withdrawn and stored at -20°C until use. Two hundred and thirty blood samples were used as control, there were sent to the laboratory for other investigations but not cholera.

Bacterial strains

The following *Vibrio cholera* strains SVC 302, SVC 729 AND SVC 925 were pooled and used as antigens while strains 569B served as a reference. The characteristics of the isolates. The stock cultures were maintained on nutrient agar slants at 4°C .



Complement

Pooled non-vibriocidal Guinea pig serum was used as complement. Individual Guinea-pig sera were pre-tested for naturally occurring vibriocidal activity against *V.cholerae* at the same concentration that was used in the tests. Four healthy guinea pigs were killed, the blood collected and allowed to clot. The clotted blood was spun at 6000rpm for 10mins to obtain the serum. The serum was distributed in 2ml amounts or units and stored at frozen temperature until use. Under this condition the activity was retained for at least 6 months. The complement did not contain any preservative.

Agglutination antibody test

A microtiter agglutination test method amenable to assaying small volume serum samples was used as described by Benenson et al., (1968) instead of the tube dilution method. The microtechnique was carried out in sterile microtitre U-well plates with slight modifications.

Serum preparation

The sera were not inactivated at 56⁰C for 30 minutes and also did not contain any preservative, since the later might cause lysis of the rather light antigen preparation.

Control sera

Sera of unexposed healthy adults were pooled for a negative reference control while sera from cholera patients were pooled for a positive reference control.

Antigen preparation

One loopful of growth from a 16 to 18 hr (overnight) culture of the test strains on nutrient agar was inoculated into 100ml of nutrient broth in a 250ml Erlenmeyer flask. The flask culture was incubated at 37⁰C without shaking for 6 hr or until

the opacity was equivalent to 0.5 Mcfarland standard containing approximately 10⁴ organisms/ml. the antigen was then ready for use or placed in ice-water bath for up to 4 hr. fresh antigen suspension were used for each batch of tests.

Vibriocidal antibody test

This test depends on the bacteriocidal effect of O antibody on *V. cholera* in the presence of complement. The method employed was that reported by Beneson et al., (1968) and Cash et al., (1974) with slight modifications. The serum were used without prior inactivation since an excess of complement was required in the reaction mixture.

Antigen preparation

The afternoon before the test, the same pooled local strains of *V.cholerae* 01 Eltor Ogawa and the reference strain 569B were separately inoculated onto brain heart infusion agar slants and incubated at 37⁰C overnight (16-18 hr). Uninoculated BH Agar slants were placed in the incubator to prewarm. The pre-warmed BH1 agar slants were inoculated with a loopful from the overnight culture and then incubated at 37⁰C for 4 hrs. chilled phosphate buffered saline (PBS, pH 7.3) was used to wash the growth from the slants. The antigen suspension was further diluted in chilled PBS until the opacity was equivalent to 0.5 Mcfarland standard containing approximately 10⁴ organisms/ml. the suspension was promptly mixed with complement. Fresh antigen suspensions were used for each batch of tests.

Complement

Guinea pig complement was diluted 1:5 in chilled PBS (pH 7.3). equal volumes of the diluted complement was added to



equal volumes of antigen suspension. The complement-antigen mixture was maintained in an ice-waterbath and used within 30mins.

Statistical analysis

The data generated in the study were treated statistically using Chi-square and correlation coefficient analysis to determine their significance and possible relationships.

RESULTS

Distribution of serum agglutinins

Vibrio cholerae agglutinins prevalence in the serum of parturient women and age-match control. Ages of the parturient women and control subjects ranged from 16-40yrs with mean values of 28.6 and 30.0yrs respectively. It was observed that 191 parturient women and 78 non-parturients had agglutinating antibodies of various titres in their serum samples. In both subject populations, women in the 26-30yrs age group showed the highest prevalence of vibrio agglutinating antibodies; 108(32.2%) for parturient women and 30(13.0) for controls. The lowest prevalence rate of 0.3% and 0.4% occurred among the parturient and control subjects within the age groups of 36-40yr and 16-20 yr respectively. It was observed that the prevalence rates for parturient women and the controls were significantly ($p < 0.01$) high.

Serum agglutinating antibody titre profiles

It was observed that out of the 191 parturient women that had detectable agglutinating antibodies in their serum samples, 3(1.6%), 27(14.1%), 30(15.7%) and 44(23.0%) had titres of 1/1280, 1/640, 1/320, and 1/160 respectively. Among the lower titres of 1/20, 1/40, and 1/80 were detected in 8(4.2%),

27(14.1%), and 52(27.2%) subjects respectively. Among the 78 agglutinating antibody positive non-parturient women, 23(29.5%), 20(25.6%) and 8(10.3%) had titres of 1/640, 1/320 and 1/160 respectively. Lower titres of 1/20, 1/40 and 1/80 were also detected in 6(7.9%), 10(12.8%) and 11(14.1) respectively.

Serum Vibriocidal antibodies

The result obtained showed that 115 out of 335 parturient women had detectable levels of vibriocidal antibodies in their serum samples, while 43 in the control had detectable levels of vibriocidal antibodies. However, the highest prevalence rates of 18.2% and 8.3% in both parturient and non-parturient women respectively, occurred among subjects in 26-30 years age group. Prevalence rates among the parturient women were lowest (0.9%), and 0.3% for those in the age groups of 16-20 years and 36-40 years respectively. Among the non-parturient women, the lowest prevalence rates similarly occurred in those in the youngest (0.4%) and oldest (6.5%) age groups.

Serum vibriocidal antibody titre profiles

It was observed that 115 parturient women had detectable levels of vibriocidal antibodies of 1(0.8%), 19(16.9%) and 34(29.6%) with titres of 1/280, 1/320 and 1/160 respectively. Lower vibriocidal antibody titres of 1/20, 1/40 and 1/80 were associated with 5(4.4%), 12(10.4%) and 44(38.3%) of these subjects. Vibriocidal antibody profiles among 43 non-parturient women were distributed as follows: 1(2.3%), 5(11.6%), 21(48.8%), 12(27.9%) and 4(9.3%) had titres of 1/20, 1/40, 1/80, 1/160 and 1/320 respectively. A titre of



1/80 was the most prevalent and occurred in sera of 44 (38.3%) parturient and 21(48.8%) non-parturient women. A total of 54(47%) parturient and 16(37.2%) control subjects with vibriocidal antibodies had serum titres of 1/160.

***V. cholera* agglutinins in serum and breastmilk**

A total of 104 of parturient women had significant titres ($\geq 1/160$) of *V. cholera* agglutinating antibodies in their serum samples. A total of 67(20.0%) parturient women had detectable levels of agglutinating *V. cholera* antibodies in their breastmilk samples. The most prevalent antibody titre in both serum and breastmilk was 1/80 accounting for 52 (15.5%) and 34(10.5%) of these samples respectively. A significant positive correlation was obtained ($r=0.75$) when the prevalence rates of agglutinating antibodies in serum and breastmilk were compared. However, the agglutinating titres were generally higher in serum than breastmilk samples.

Vibriocidal antibodies in sera and breastmilks

A titre of 1/160 was adopted for significant vibriocidal antibody in this study, a total of 54 (16.1%) women had significant titres in their serum samples. Only 18(5.4%) women had significant levels of vibriocidal antibodies in their breastmilk samples of a total of 32(9.6%) women who had detectable levels of such antibodies in their breastmilk samples as follows: 1(0.3%), 4(1.2%), 9(2.7%), 16(4.8%) and 2(0.6%), with titre of 1/20, 1/40, 1/80, 1/160 and 1/320 respectively. The most prevalent serum vibriocidal antibody titre was 1/80 (13.3%) while a titre of 1/160(4.8%) was the most prevalent in breastmilk. There was a positive correlation in the distribution of serum vibriocidal and breastmilk vibriocidal antibodies ($r = 0.83$).



Table 1: Age- specific distribution of Vibrio Cholerae agglutinin titres profiles of parturient and non parturient subjects

Age group (years)	Number of parturients with agglutinin titres								Number of non- parturient with agglutinin titres							
	No. of subject	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	No.of subjects	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
16-20	5	-	-	1	-	1	1	-	7	-	-	-	-	-	1	-
21-25	45	1	5	9	7	6	3	-	46	-	2	3	2	7	4	-
26-30	193	4	18	28	24	15	16	3	78	2	5	4	1	9	9	-
31-35	87	3	4	14	12	8	7	-	45	3	2	1	1	3	4	-
36-40	5	-	-	-	1	-	-	-	54	1	1	3	4	1	5	-

Table 2: Age- specific distribution of serum vibriocidal antibody titre profile of parturient and non-parturient subjects

Age group (years)	Number of parturients with vibriocidal antibody titres								Number of controls with vibriocidal antibody titres							
	No. of subject	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	No.of subjects	1/20	1/40	1/80	1/160	1/320	1/640	1/1280
16-20	5	1	-	1	1	-	-	-	7	-	-	1	-	-	-	-
21-25	45	3	-	7	8	-	-	-	46	1	2	8	2	1	-	-
26-30	193	-	8	22	17	14	-	-	78	-	2	8	7	2	-	-
31-35	87	-	4	14	8	5	-	-	45	-	1	2	2	1	-	-
36-40	5	1	-	-	-	-	-	1	54	-	-	2	1	-	-	-

DISCUSSION

Cholera a serious gastrointestinal tract infection with high morbidity and mortality particularly in the third world countries is caused by *Vibrio cholera* 01. Clinical infection is usually accompanied by the development of protective antibodies. Post-infection immune-protection has been reported to be superior to that provided by use of vaccines and may last for three years after infection (Levine et al., 1981). The results of this study clearly demonstrate the presence of appreciable quantities of agglutination antibodies to the somatic antigens of *V.cholerae* in a large percentage (57%) of serum samples collected from parturient women. Interestingly, only 33.8% of agglutinating antibodies were detectable in the serum samples collected from non-parturient women. However, 104(31.5%) of parturients and 51(22.2%) of non-parturients had significant titres ($\geq 1:160$) of agglutinating antibody in their serum samples. Significant difference ($p < 0.01$) in the prevalence of high titre antibody between the two subject group was observed. No obvious reason for this observation is immediately known, but it is possible that pregnant women because of their uncontrolled or indiscriminate and unhygienic eating habits are more exposed and susceptible to *Vibrio cholera* or related antigen endemic in Calabar.

Chaicumpa and Rowley (1972) had associated some level of immunity to cholera with the presence of agglutinating antibodies. They reported that the agglutination of vibrios by antibody prevents the adherence of the

bacteria to the intestinal wall of infant mice and thereby protects the animals against experimental cholera. In an interesting study Schrank and Verwey (1976) reported the agglutination (chumping) of cholera vibrios in the intestinal lumen of immunized rabbits. The authors interpreted these findings to imply that agglutination of vibrios, mechanically prevented these bacteria from penetrating the mucus layer and in this manner protected against experimental cholera. It seems reasonable therefore, to suggest that the high titres of agglutinating antibodies recorded in this study may in a way correlate with some levels of protection against *V.cholerae*.

The results obtained also shows that none of the subjects gave any history of cholera earlier or vaccination against it in recent years. Therefore, this result present possible differences in the degree of subclinical exposure to these antigens. These results agrees with the report of Majumdar *et al.*, (1982) who examined Indian and Swedish women in a similar study and found that antibodies to *V. cholera* somatic antigens were more prevalent among Indian and Swedish women. Holmgren *et al.*, (1989) had reported the presence of antibodies directed against the somatic or toxin antigens of *V.cholerae* in breastmilk samples collected from mothers living in certain endemic parts of the world. The result also revealed the presence of detectable levels of agglutinating vibrio antibodies in only 20% breastmilk samples. Significant breastmilk agglutinins titres ($\geq 1:160$) were recorded in only 4.2% of samples.



This variation in titre level was probably a reflection of the degree and period of their contact with the antigens. A strong positive correlation ($r = 0.8$) between serum and breastmilk agglutinin titre levels was also observed. However, it was noted that three serum samples from parturient women had significantly high titre (1/1280) without a corresponding high breastmilk titre levels. This probably was an indication of a very recent contact or infection in which case there was absence or low quantities of antibody transferred to the breastmilk and hence none was detected.

The vibriocidal antibody test has been extensively used for seroepidemiological surveys, and the acquisition of significant levels of antibody, either through natural exposure or as a result of vaccine has been correlated with apparent immunity to cholera on a community population based study and in volunteer challenge studies (Wachsmuth et al., 1980). In other words, the acquisition of significant levels of vibriocidal antibodies is an index of protection against cholera. Also, the vibriocidal test is significantly more sensitive than the agglutination test and detects basically IgG class whereas agglutination non-differentially detects both IgM & IgG (Feeley, 1965).

The results also revealed that 34.5% of parturient women had detectable levels of vibriocidal antibodies in their serum samples with 16.1% showing significant titres. In contrast, 18.7% of non-parturient women were positive for vibriocidal antibodies with 6.9% having significant titres. Significant difference ($p < 0.01$) in the prevalence and titres of antibodies was

found in the two subject groups. Similarly, 9.6% of parturients showed detectable levels of vibriocidal antibodies in their breastmilk samples with 5.4% having significant titres. The reason for this observation is not known, but it is possible that the mechanism or reactions for the conversion and transfer of vibriocidal antibodies to breastmilk is slow. This result is again in accordance with earlier observation by Majumdar *et al.*, (1982). However, a strong positive correlation ($r = 0.83$) was observed between serum and breastmilk vibriocidal antibody levels or titres. The results also revealed that 4.2% of parturient women had significant levels of breastmilk agglutinins whereas 5.4% showed significant vibriocidal antibodies in their breastmilk samples. This observation supports earlier reports by Levine et al., (1981) and Snyder *et al.*, (1981) that agglutinating antibodies are relatively short lived than vibriocidal antibodies. However, there was a close ($r = 0.6$) correlation between breastmilk agglutinating and breastmilk vibriocidal antibody levels or titres.

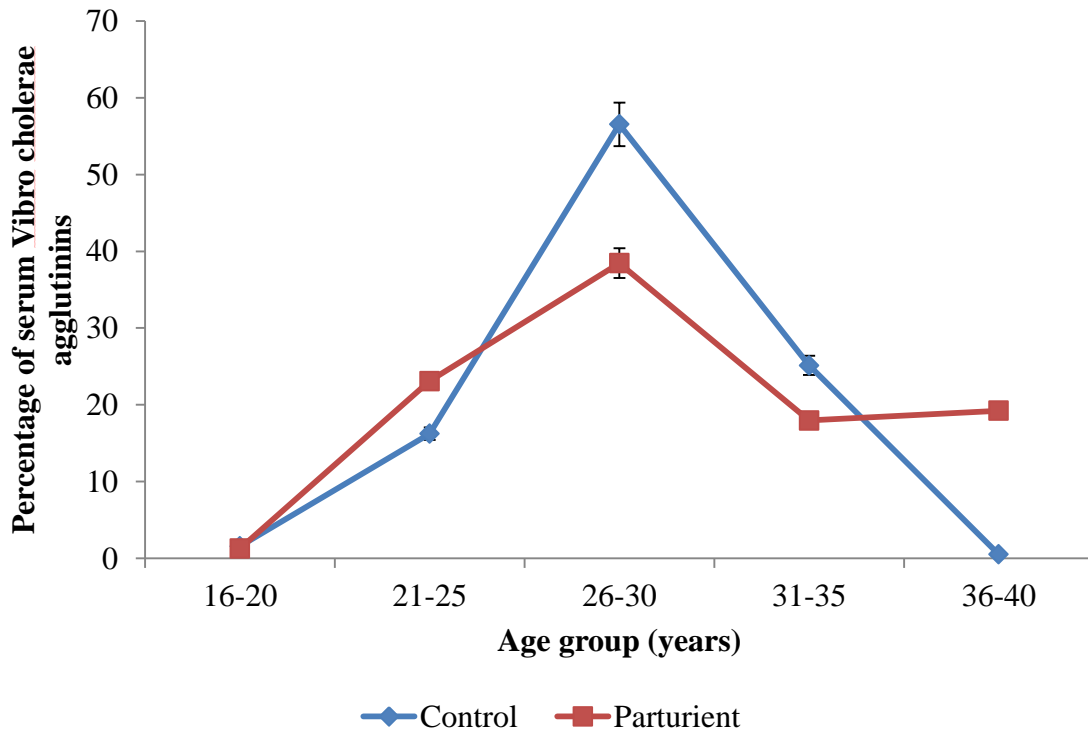
The agglutination and vibriocidal tests can be expected to demonstrate a rise in antibody in sera from 90 to 95% of patients with bacteriologically confirmed cases of cholera (Wachsmuth *et al.*, 1980). A comparison of the serum agglutinins and serum vibriocidal antibodies results probably reflects exposure to O antigens of only *V. cholera* without cross reactions with related organisms. This is also supported by the observed absolute or perfect correlation ($r = 1.0$) between these two results. Authors who have made such a comparison have found a very high, perfect or near perfect correlation

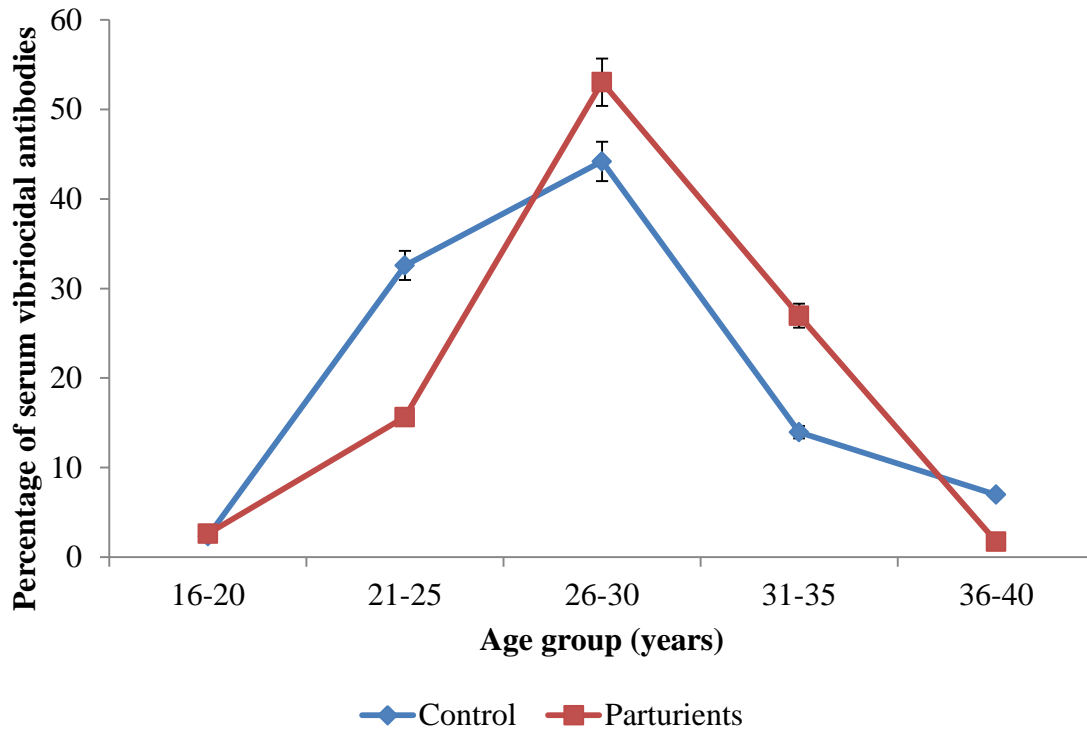


between the results of living vibrio agglutination tests and those of vibriocidal tests in detecting diagnostic rises in antibody level (Wachsmuth *et al.*, 1980).

Although most serum vibriocidal antibody is directed against lipopolysaccharide (LPS), antibodies against protein antigen also exist. Serum vibriocidal antibody is not by itself considered to be the mediator of protection but is rather regarded as a

marker for the presence of intestinal antibodies against critical antigens on the bacterial surface (Attridge and Rowley, 1983). However, this study point to the existence of naturally acquired immunity against *Vibrio cholera* in a large percentage of parturient women in Calabar, provided the antibody response in the mammary secretion is a reflection of the gut associated immunity.





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