

A Serological Survey for Cholera Antibodies in Rural East Pakistan

1. The Distribution of Antibody in the Control Population of a Cholera-vaccine Field-trial Area and the Relation of Antibody Titre to the Pattern of Endemic Cholera *

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Controlled cholera vaccine field trials were held in Matlab Bazar in rural East Pakistan in 1963 and 1964. In July–September 1965, a serological survey for cholera antibodies was carried out on a random sample of the field-trial population. Results are given for the control group only, as representative of the pattern found in an endemic cholera area. Only 2% of the blood samples from children under 10 years of age were found to have detectable agglutinating antibody (titres of 1:20 or more), while the proportion in the age-group over 30 years was 27%–30%. Tests for vibriocidal antibody showed that none of the blood samples from children under 1 year of age contained detectable antibody, while 87%–90% of those from adults over 30 years did so. The rise in the geometric mean vibriocidal titre showed an almost linear correlation with a sharp fall in the cholera case rate with age. Since a similar survey in a non-endemic area (Czechoslovakia) did not reveal any rise in antibody titre with age, and since cholera epidemics in areas without recent experience of the disease are characterized by a higher incidence rate in adults than in children, it is suggested that the antibody titres give a measure of the level of immunity in a population.

Since 1963 the Pakistan–SEATO Cholera Research Laboratory has conducted two controlled cholera-vaccine trials in Matlab Bazar Thana in rural East Pakistan (Oseasohn et al., 1965; Benenson, Mosley et al., 1968). In November–December 1963, cholera vaccine found on the basis of laboratory tests to be highly antigenic and a typhoid vaccine control were

administered randomly to 14 064 persons in 23 villages. In September–November 1964, the same cholera vaccine, a tetanus-toxoid control and a purified Ogawa antigen preparation were given randomly to an additional 25 267 persons living in 35 other villages. The surveillance for cholera in these 58 villages has been maintained for over 2 years by daily house-to-house visiting. Rectal swab cultures have been obtained from all villagers with an acute diarrhoeal illness, and treatment has been given in a field hospital. These trials have confirmed the efficacy of the cholera vaccine in reducing the cholera frequency, even after 2 years, as compared with the controls, while the purified Ogawa antigen has had only a minimal (but still statistically significant) effect.

In the summer of 1965 a random-sample serological survey was conducted in this field-trial area to determine the antibody pattern in the population living in this endemic cholera area. In addition, the survey was designed to determine whether it was

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possible to detect differences in antibody level between the cholera-vaccinated and control populations. Finally, because reliable data on the incidence of cholera in the population were available it was possible to examine the relationship between the antibody level in the population and the cholera frequency.

This paper examines the results of serological tests on 609 persons from the control (typhoid-vaccine and tetanus-toxoid) population; these results may be taken as reflecting the antibody pattern in an endemic cholera area. For comparison with a non-endemic area, Dr Karel Zacek of the Institute of Epidemiology and Microbiology, Prague, provided sera from the WHO Serum Reference Board at that Institute, taken from a random-sample survey in Czechoslovakia. The second paper in this series (Mosley et al., 1968)¹ compares the antibody titres in the vaccinated groups with those for the controls.

MATERIALS AND METHODS

Blood and serum samples

Six hundred and nine blood samples were obtained at random from the control population in the Matlab vaccine-field-trial area between 2 July and 6 October 1965 according to the methods described below. Since the samples were drawn from a population inoculated either 1 or 2 years previously, there were no samples from infants under the age of 1 year. For data on this age-group, blood was obtained from 32 infants hospitalized with non-cholera illnesses at the Matlab field hospital between September 1965 and June 1966.

Two hundred and twenty-six sera were received from Czechoslovakia. These had been selected at random for each age-group from a larger collection comprising approximately 1000 specimens collected in 1966 by the Institute of Epidemiology and Microbiology, Prague, for the primary purpose of a poliovirus antibody survey in one of the ten administrative regions of Czechoslovakia. The samples were collected from normal individuals selected at random and in proportion to population density from both urban and rural areas.

Sampling procedure

Individual census cards were available for the entire population of 39 331 individuals included in the Matlab vaccine trials. With the aid of these

cards, the population was stratified into 40 groups according to vaccine status, age (0-4 years, 5-14 years, 15-29 years and over 30 years) and sex. From each of these strata, 50 cards were selected with the use of a random-number table. This was done in 2 steps: initially, one-tenth of each group was selected on the basis of the last digit of the census number and then 50 persons were selected from this 10 % sample on the basis of the next-to-the-last digit of the census number. This method of sampling ensured a cross-sectional representation of every village throughout the trial area and avoided familial aggregation in the sample. The 2000 persons selected for the survey were listed by census number, name, age, and sex to ensure identification in the field.

The sample size was calculated to allow for up to 40 % non-participation because of death or migration since the vaccination campaign, or refusals. It is assumed that failure to obtain a specimen for one of the above reasons would not affect a comparison between vaccine groups, since the non-participants should be distributed equally in all groups.

Collection of specimens

All specimens were collected by 1 physician assisted by a nurse. Finger-tip blood was collected in a 50- μ l capillary tube² and immediately diluted 1:10 in 0.45 ml of sterile saline in a screw-capped vial. The specimen was labelled with the census number, dated and placed in a portable icebox. At the time of collection of the specimen, the age of the individual was confirmed, and the history of cholera vaccination was checked.

The specimens were submitted to the laboratory, identified only by the census number, so that the age, sex, and vaccine group were unknown to the technicians. Upon receipt in the laboratory, the diluted plasma was separated from the cells and held in the deep-freeze until the titrations were performed. Titrations for vibriocidal and agglutinating antibodies against both Ogawa and Inaba organisms were done by microtechniques developed in this laboratory (Benenson, Saad & Paul, 1968; Benenson, Saad & Mosley, 1968), using an initial dilution of the original blood sample of 1:20. Specimens were titrated in random groups of 36 so that day-to-day variations in the test results were eliminated as a factor in the survey. Appropriate controls were run

¹ See the paper on p. 335 of this issue.

² Microcaps, Drummond Scientific Co., Broomall, Pa., USA.

daily with known positive sera. All specimens were titrated for vibriocidal antibody. Agglutinating titres were determined for 65% of the specimens.

Statistical methods

The calculation of geometric mean titres was turned into an arithmetic averaging process by assigning the value of 0 (zero) to titres of less than 1: 20, 1 to titres of 1: 20, 2 to titres of 1: 40, 3 to titres of 1: 80, 4 to titres of 1: 160, etc.

RESULTS

Table 1 gives the age distribution of the control population at the time of vaccination in 1963 and 1964. Since an analysis of the results has revealed that sex is not a factor either in the serological survey or in the cholera experience of the populations, the distribution by sex will not be included in any of the tables. Specimens were collected from 609 persons in the control population. This represents 76.1% of the 800 persons in the original sample. The highest percentage of collections (85%) was made in the 0-4-year age-group. The lowest percentage of collections (60%) was made in the 15-29-year age-group, primarily because of the large proportion of young men away at work in this group. Blood was obtained from every individual who was available.

Table 2 gives the proportion of blood samples from each age-group in the control population with

TABLE 1
AGE DISTRIBUTION OF SURVEY POPULATION

Age-group (years)	No. in group		
	Typhoid-vaccine group ^a	Tetanus-toxoid group ^b	Total
0-4	1 288	1 588	2 876
5-9	1 546	1 793	3 339
10-14	868	1 105	1 973
15-29	1 242	1 426	2 668
≥ 30	2 154	2 541	4 695
Total	7 098	8 453	15 551

^a This group formed the control group in the Matlab vaccine field trial in 1963, when a typhoid vaccine was used as the control vaccine.

^b This group formed the control group in the Matlab vaccine field trial in 1964, when tetanus toxoid was used as the control vaccine.

antibody titres of 1: 20 or greater. (The age-groups given in Tables 2, 3, 4 and in Fig. 1 are based on the age given by the individual at the time that the blood sample was collected. This would differ by 1-3 years from the age at the time of vaccination. In the tables which involve analysis of cholera frequencies, the age at the time of census is used.) Table 2 demonstrates that there is a progressive increase with age in the proportion of the population with detectable

TABLE 2
PERCENTAGE OF BLOOD SAMPLES FROM THE SURVEY POPULATION WITH AGGLUTINATING AND VIBRIOCIDAL ANTIBODY TITRES OF 1: 20 OR GREATER, BY AGE-GROUP AND SEROTYPE

Age-group ^a (years)	Agglutinating antibody			Vibriocidal antibody		
	No. tested	Percentage positive		No. tested	Percentage positive	
		Ogawa	Inaba		Ogawa	Inaba
< 1 ^b	32	0.0	0.0	32	0.0	0.0
1-2	30	0.0	0.0	50	14.0	18.0
3-4	48	0.0	2.0	75	32.0	21.4
5-9	50	2.0	2.0	115	57.4	45.2
10-14	44	6.8	4.5	87	73.6	65.5
15-29	72	26.4	18.0	127	85.8	80.3
≥ 30	90	30.0	26.7	155	87.1	90.3

^a Age at the time of the serological survey.

^b The under-1-year group represents infants admitted with non-cholera diarrhoea to the Matlab field hospital.

antibody. Only 2% of the blood samples from children under 10 years had detectable agglutinating antibody, while in the age-group over 30 years 27%–30% of the blood samples had an agglutinating titre of 1:20 or greater. The vibriocidal test, which has proved to be consistently more sensitive, revealed that none of the blood samples from the children under 1 year of age had vibriocidal titres of 1:20 or greater, while in the age-group over 30 years, 87%–90% of the samples had detectable vibriocidal titres.

The over-all level of agglutinating antibody in the blood samples from the control population was quite low—only 4% of the specimens tested had a titre of 1:40 or greater against either the Ogawa or the Inaba bacterial suspension. These samples were all from persons in the age-group over 15 years. The over-all level of vibriocidal antibody was much higher. This is shown, by age-group, in Fig. 1. This figure illustrates the shift in the distribution of vibriocidal titres to higher levels with age, so that in children under the age of 10 years, the majority of the blood samples tested had a titre of less than 1:20, in the age-group 10–14 years the distri-

bution of titres revealed a second peak at 1:40, and at ages over 30 years the distribution of titres revealed a single peak at 1:80. Fig. 1 also shows that in this population the vibriocidal titres against Ogawa and Inaba suspensions closely paralleled one another. This was true not only for the population as a whole but also for the individual blood samples tested, where there was a good correlation between the titres against Ogawa and Inaba suspensions ($r = 0.76$; $P < 0.001$).

This increase in antibody titre with age could be due to at least three factors: repeated exposure to cholera infections, repeated cholera vaccinations, or a non-specific serological reaction, possibly due to cross-reacting antibodies from other bacteria. To examine the role of cholera vaccine in producing this antibody pattern, each individual, or in the case of children the parent, was asked at the time the specimen was obtained if he had ever received cholera vaccination in the past. It was found that the proportion of the population giving a history of cholera vaccination increased with age from 2% in those under the age of 3 years to 75% of those over 30 years (Table 3). This increase might seem at first

FIG. 1
PERCENTAGE DISTRIBUTION OF VIBRIOCIDAL TITRES IN THE CONTROL POPULATIONS,
BY AGE-GROUP AND SEROTYPE

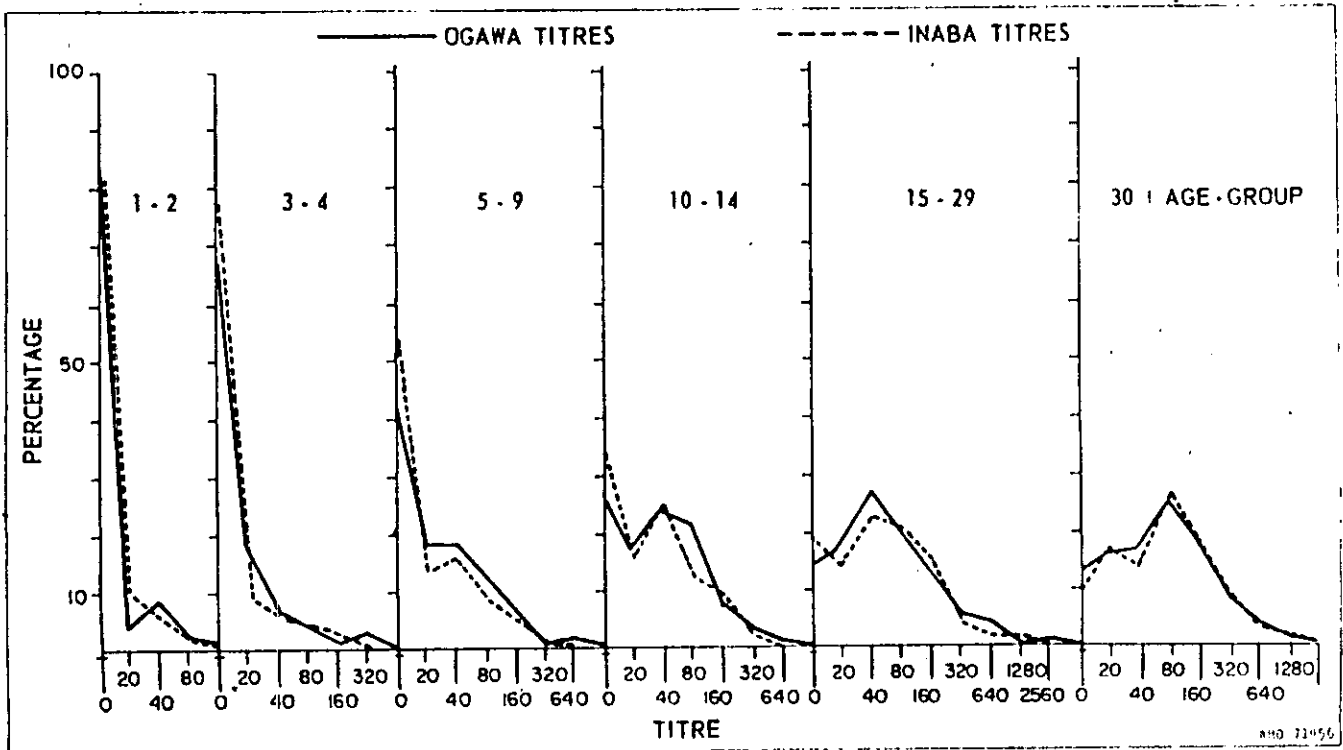


TABLE 3
PERCENTAGE OF BLOOD SAMPLES WITH VIBRIOCIDAL TITRES OF 1:20
OR GREATER, BY AGE-GROUP AND HISTORY OF CHOLERA VACCINATION

Age-group ^a (years)	Previously vaccinated against cholera			Not previously vaccinated against cholera		
	No. tested	Percentage vibriocidal		No. tested	Percentage vibriocidal	
		Ogawa	Inaba		Ogawa	Inaba
< 1 ^b	0	—	—	32	0.0	0.0
1-2	1	0.0	0.0	46	8.7	13.0
3-4	10	67.0	30.0	63	23.6	20.6
5-6	11	54.5	63.6	45	53.3	40.0
7-9	20	65.0	45.0	36	61.1	50.0
10-14	53	69.8	66.1	29	79.3	58.6
15-29	87	86.2	81.6	38	84.0	76.3
≥ 30	117	88.8	90.5	34	79.4	88.2

^a Age at the time of the serological survey.

^b The under-1-year group represents infants admitted with non-cholera diarrhoea to the Matlab field hospital.

sight to parallel the increase in antibody titres with age. The detailed analysis given in Table 3 reveals, however, that the proportion of individuals with detectable vibriocidal antibody is unrelated to the history of cholera vaccination. Persons who stated that they had not been vaccinated against cholera showed more or less the same rise in antibody titres with age as those giving a positive vaccination history.

To examine the possibility that the titres were due to a non-specific antibody, 226 sera collected in a survey in Czechoslovakia were titrated using the

same techniques. Table 4 reveals that in this population, taken to represent that of a non-endemic area, the majority of the sera in every age-group had no detectable antibody at a 1:20 dilution. The positive sera had low titres; only 1 had a vibriocidal titre of 1:160, and 3 of 1:80. The 4 individuals concerned stated that they had not been vaccinated against cholera or travelled to a cholera-affected area.

Thanks to the intensive surveillance for cholera among the participants in the Matlab vaccine trial since 1963, the incidence of cholera was accurately

TABLE 4
PERCENTAGE OF CZECHOSLOVAK SERUM SAMPLES WITH AGGLUTINATING
AND VIBRIOCIDAL ANTIBODY TITRES OF 1:20 OR GREATER,
BY AGE-GROUP AND SEROTYPE

Age-group (years)	No. tested	Agglutinating antibody		Vibriocidal antibody	
		Percentage positive		Percentage positive	
		Ogawa	Inaba	Ogawa	Inaba
0-2	44	0.0	0.0	2.3	0.0
3-4	32	0.0	0.0	3.1	3.1
5-9	34	0.0	0.0	0.0	5.9
10-14	36	2.8	0.0	11.1	0.0
15-29	39	0.0	0.0	12.8	7.7
≥ 30	38	2.6	2.6	5.3	5.3

TABLE 5
CHOLERA CASES AND CASE RATE PER 10 000 PERSONS, BY AGE-GROUP AND SEROTYPE,
OCTOBER 1964-JUNE 1966

Age-group (years)	Population	Ogawa cholera		Inaba cholera	
		Cases	Rate per 10 000	Cases	Rate per 10 000
0-4	2 876	6	20.9	49	170.4
5-9	3 339	4	12.0	31	92.8
10-29	4 641	4	8.6	16	34.5
≥ 30	4 695	1	2.1	6	12.8
Total	15 551	15	9.6	102	65.6

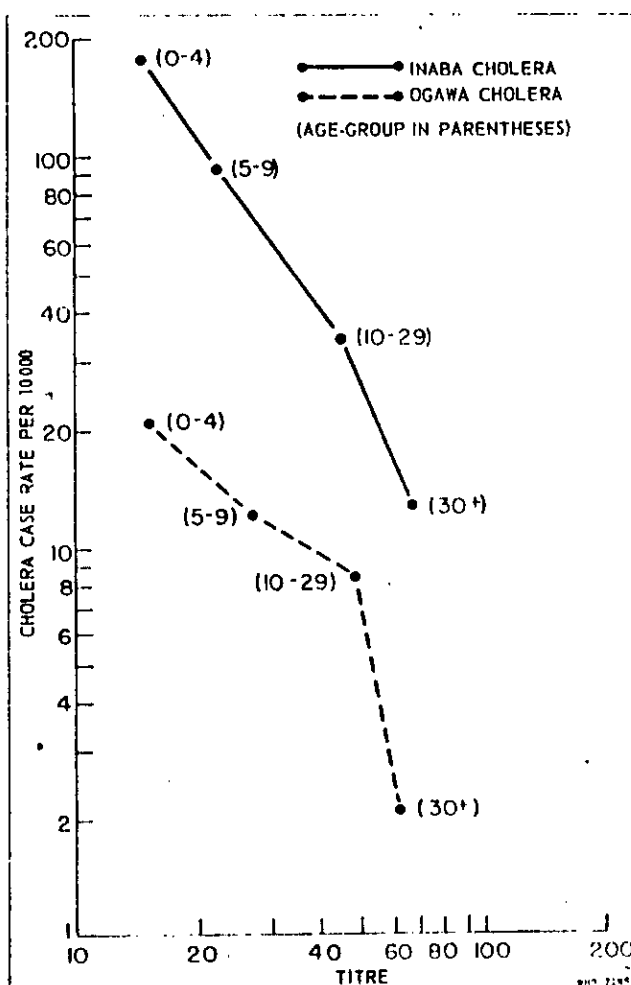
known. Thus, it was possible to examine the relationship between the level of immunity in the population and the cholera case rate. Since the serological survey was taken between 2 epidemic seasons, the cholera incidences for the 9 months preceding and following the survey have been combined. Table 5 gives the cholera case rates by age and serotype of the infecting organism for the period from October 1964 to June 1966. The majority of the cases were due to the Inaba serotype. The table shows that the case rates were highest in children under 5 years, and fell steadily with age.

In order to examine the relationship between the falling cholera case rate and the rising antibody level with age, the geometric mean vibriocidal titres were calculated for the serological samples from the survey population corresponding to the age-groups given in Table 5. These data, presented in Table 6, reveal a rise in the geometric mean vibriocidal titre with age. Fig. 2 shows the almost linear correlation between the fall in case rate with age (shown on a logarithmic scale) and the increase in the geometric mean titre.

TABLE 6. GEOMETRIC MEAN VIBRIOCIDAL TITRES FOR SAMPLES FROM SURVEY POPULATION, BY AGE-GROUP

Age-group (years)	No. examined	Geometric mean vibriocidal titre	
		Ogawa	Inaba
0-4	171	15	14
5-9	99	27	22
10-29	187	49	45
≥ 30	152	63	66

FIG. 2
RELATIONSHIP BETWEEN CHOLERA CASE RATE AND
GEOMETRIC MEAN VIBRIOCIDAL TITRE,
OCTOBER 1964-JUNE 1966, FOR OGAWA
AND INABA CHOLERA



DISCUSSION

The role of immunity, and of vaccination, in the protection of man from cholera has been the subject of considerable debate, as amply surveyed by Pollitzer (1959). One line of indirect evidence indicating the importance of acquired immunity is cited by Burnet (1962), who noted that adults show higher case rates than children in epidemic cholera while in endemic cholera the reverse is true. The data presented in this paper confirm the latter part of this observation. There are numerous studies on epidemic cholera documenting higher case rates in adults, the most recent ones in Taiwan, the Philippines and Thailand (Yen, 1964; Dizon et al., 1965; Siddhichai & Grayston, 1960). The joint Philippine-Japan-WHO Cholera Vaccine Field Trial conducted in Negros Occidental Province, Republic of the Philippines, in 1964, provides data from a non-endemic area¹ which should be comparable with the case rates from East Pakistan given in this report, since surveillance for cases was carried out both in the field and in the hospital and all cases were bacteriologically confirmed (Philippines Cholera Committee, 1965). In the control population in the Philippine trial the case rate in adults over the age of 45 years was 1.3 times higher than in children under the age of 5 years.

The present serological survey has revealed that in East Pakistan there is a rise in the level of cir-

culating antibody with age. Data from a similar serological survey in Czechoslovakia revealed that this rise was not found in a non-endemic area. The cause of this rise cannot be given with certainty. While a similar rise was found in the proportion of persons receiving vaccination with age, individuals who denied ever receiving cholera vaccine had the same antibody levels as those with a history of cholera vaccination. This suggests natural infection with *Vibrio cholerae* as the factor in producing the antibody level found in this survey. This hypothesis would be consistent with the incidence rate of almost 1% per year for clinical cholera in the 0-9-year age-group found in this population and the estimate based on family studies by this laboratory that the case:infection ratio for cholera may be about 1:3 or 1:4 (Oseasohn et al., 1966).

The impressive relationship between the rise in antibody levels and the fall in case rates with age suggests that circulating antibody provides a measure of immunity in the population; however, this does not necessarily imply that the vibriocidal antibody *per se* has a protective effect. The possibility that the rising antibody titres and the falling case rates may be independent variables which are both correlated with age must also be considered. Data have been presented to indicate that both of these patterns occur only in an endemic cholera area, suggesting that there may be a cause-and-effect relationship. Additional data supporting the relationship between antibody titre and protection will be given in the second paper in this series (Mosley et al., 1968).

¹ At the time of writing, Negros Occidental may be characterized as an endemic area; in 1964, the endemic pattern had not been fully established.

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RÉSUMÉ

En 1963 et 1964, on a procédé dans la population de Matlab Bazar (localité rurale du Pakistan oriental) à des essais contrôlés de vaccin anticholérique. De juillet à septembre 1965, on a fait une enquête sérologique pour rechercher et titrer les anticorps anticholériques dans un

échantillon aléatoire de la population qui avait été soumise à l'essai. Ces titrages ont été faits par des microtechniques qui ont permis d'employer des échantillons de sang prélevé au bout du doigt. Le présent rapport ne donne de résultats que pour le groupe témoin.

les données ainsi recueillies étant représentatives de la manière dont se présente le choléra dans une zone d'endémicité.

On a constaté que la proportion de la population ayant des anticorps décelables (avec des titres d'au moins 1:20) augmentait progressivement avec l'âge. Des échantillons de sang prélevés sur des enfants de moins de 10 ans, 2% seulement contenaient des anticorps agglutinants décelables, alors que dans le groupe d'âge de plus de 30 ans 27 à 30% des échantillons présentaient un titre d'agglutinine égal ou supérieur à 1:20. La recherche des anticorps vibriocides, qui s'est révélée être toujours plus sensible, a mis en évidence le fait qu'aucun des échantillons de sang prélevés sur des enfants de moins d'un an n'avait de titre d'anticorps vibriocides égal ou supérieur

à 1:20 alors que 87 à 90% des sujets du groupe d'âge de plus de 30 ans avaient des titres d'au moins 1:20. On a constaté une corrélation presque linéaire entre l'augmentation de la moyenne géométrique du titre d'anticorps vibriocides et la chute brusque du taux d'incidence cholérique avec l'âge.

Etant donné qu'une enquête similaire, faite dans une zone de non-endémicité (Tchécoslovaquie), n'a révélé aucune augmentation du titre d'anticorps avec l'âge, et que les épidémies de choléra qui surviennent dans des zones où il n'y a pas eu récemment de cas de cette maladie se caractérisent par un taux d'incidence plus élevé chez les adultes que chez les enfants, on pense que l'étude des titres d'anticorps permet d'évaluer le niveau d'immunité d'une population.

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