J. Hyg., Camb. (1967), **65**, 135
With 1 *plate*
Printed in Great Britain

Attenuation of virulence in *Vibrio cholerae*

By K. BHASKARAN and V. B. SINHA
Department of Microbiology, Central Drug Research Institute, Lucknow, India

(*Received* 18 *November* 1966)

Results of recent field trials have exposed the limitations of present-day cholera vaccines for conferring immunity to man (Cvjetanovic, 1966). A summary of the results obtained in different field trials is given in Table 7. As these vaccines were made up of mixtures of killed cells of the two antigenic types, Inaba and Ogawa, of *Vibrio cholerae* or of *V. el Tor*, there is speculation whether live vaccines may prove superior (Panse & Dutta, 1964). There is also a revival of the concept, originally put forward by Besredka (1927), that local immunity in the intestinal tract might play an important part in immunity to cholera, which is predominantly an intestinal infection without systemic invasion (Burrows, Elliott & Havens, 1947; Freter, 1956, 1965; Mukerjee, 1963).

The development of any live vaccine, whether for oral or parenteral administration, should necessarily involve a detailed study of attenuation of virulence. The attenuated nature of certain strains of *V. el Tor*, isolated from the natural waters of the Middle East and India, has been described (Mukerjee, 1963). The present paper describes the isolation and study of an attenuated mutant of *V. cholerae*. The results and the current evidence which warrants trials of live cholera vaccine will be discussed.

MATERIALS AND METHODS

Characterization of strains

From *Vibrio cholerae*, strain 162/p, a purine-requiring attenuated mutant (strain A11) was initially isolated with the aid of N-methyl-N′-nitro-N-nitrosoguanidine (NTG). This mutant was further characterized with additional nutritional deficiencies and streptomycin resistance, to yield strain C14–S5, which was employed in this study along with the parent strain 162/p. The sequence of mutant isolations and the markers of each can be seen in Table 1. In other respects, all the strains were typical of *V. cholerae*, O Group I (Gardner & Venkatraman, 1935), possessing the following characters: gram negative curved motile rods, liquifying gelatin, fermenting mannose and saccharose but not arabinose (Group I, Heiberg, 1935), non-haemolytic to sheep cells, sensitive to Group IV phage (Mukerjee, 1961) and not agglutinating sheep red cells (Barua & Mukherjee, 1963). The colonies formed by the attenuated mutants on nutrient agar were much smaller than those of the parent strain, 162/p, and were therefore designated dwarf colony types (see Table 1).

For agglutination and agglutinin absorption tests, an Inaba type mutant of 162/p (strain 162/p-IN) was isolated by the technique of Shrivastava & White (1947), and a rough mutant (strain A69) by treatment with NTG.

All strains were maintained on nutrient agar slants in the refrigerator at 4°C. For the experiments, transfers were made on the same medium and incubated overnight at 37°C. This was used as seed for inoculating nutrient broth when required. All cultures were grown at 37°C.

Table 1. Vibrio cholerae, *strain* 162/*p*, *and its attenuated mutants*

Strain designation	Markers	Origin	Technique employed for isolation
162/p	Prototroph, Ogawa, virulent	162 (pur⁻) (wild type)	Selection
A11	pur⁻, Ogawa, dwarf colony type*, attenuated	Mutant of 162/p	NTG
B16	pur⁻, nic⁻, Ogawa, dwarf colony type*, attenuated	Mutant of A11	NTG
C14	pur⁻, (ser⁻), nic⁻, Ogawa dwarf colony type*, attenuated	Mutant of B16	NTG
C14–S5	pur⁻, (ser⁻), nic⁻, str-r, Ogawa, dwarf colony type*, attenuated	Mutant of C14	Selection

pur⁻, Requirement for purine (satisfied by hypoxanthine); nic⁻, requirement for nicotinic acid; (ser⁻), requirement for serine (partial); str-r, resistance to streptomycin (500 μg/ml.); NTG, treatment with N-methyl-N′-nitro-N-nitrosoguanidine.

* Dwarf colonies (after 24 hr. incubation at 37°C.) had an average diameter of 1·57 mm. on nutrient agar and 2·63 mm. on brain heart infusion agar (Difco): colony size of the parent strain (162/p) were 3·45 mm., and 4·1 mm. respectively on these media.

Isolation of mutants by treatment with N-methyl-N′nitro-N-nitrosoguanidine (NTG)

Ten ml. of a 4 hr. nutrient culture of the strain were centrifuged and the bacterial deposit was suspended in 1 ml. of physiological saline (pH 6·0). To this (with a viable count of $3–5 \times 10^9$ organisms), 0·1 ml. of a 0·4 % freshly prepared aqueous solution of NTG (Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A.) was added and incubated for 30 min. Later, 10 ml. of broth were added and the culture was centrifuged. The deposit was resuspended in 10 ml. of fresh nutrient broth and incubated for 4 hr. The culture thus obtained was seeded on nutrient agar plates, so as to give discrete colonies after 24 hr. incubation. Colonies thus isolated were tested for mutant characters.

Culture media

Nutrient broth contained, per litre of distilled water: peptone (Oxoid), 10 g.; yeast extract (Oxoid), 2·5 g.; and NaCl, 5 g. It was adjusted to pH 8·5 before sterilization. For nutrient agar, nutrient broth was solidified with 1 % agar powder (Oxoid, no. 3).

Minimal medium contained per litre of distilled water: K_2HPO_4, 7 g.; KH_2PO_4, 3 g.; NaCl, 5 g.; $Na_3C_6H_5O_7 . 2H_2O$, 0·5 g.; $(NH_4)_2SO_4$, 1 g.; $MgSO_4 . 7H_2O$, 0·1 g.; and DL-methionine, 0·1 g. The pH of the medium was 7·2. Sterile 50 % glucose solution was added to give a final concentration of 0·1 % before use. The medium was solidified, when required, by the addition of 1 % Agar powder (Oxoid, no. 3).

Other experimental details are described in the text where required.

RESULTS

Agglutination and agglutinin absorption tests

As this study was intended to demonstrate attenuation of virulence in *V. cholerae*, while retaining full antigenicity, it was essential to prove that the observed differences between parent and mutant strains did not result from smooth to rough mutations in the organism. Rough mutants of *V. cholerae* are known to be avirulent to animals, and a comparative virulence study of rough and attenuated mutants (strains A 69 and A 11 respectively) of 162/p was described briefly elsewhere (Bhaskaran & Sinha, 1966).

The procedure employed was to carry out agglutination and agglutinin absorption tests to detect the presence of rough O antigens in the strains under study. O antisera were obtained from rabbits, immunized with heat-killed suspensions of 162/p, C 14–S 5 and A 69 (rough), and their agglutinin titres were determined before and after absorption with homologous and heterologous strains.

For preparing the antisera, adult male rabbits (1·5–2·0 kg.) received six 1 ml. intravenous doses of heat killed suspensions of overnight nutrient agar cultures (approximately 5×10^9 organisms) over a period of 11 days. The animals were bled on the 17th day and sera, after separation, were stored at 4° C. without preservative. For titration of agglutinins the sera were diluted in saline, and saline suspensions of live cultures were used as antigens. The cell-serum mixtures were kept at 37° C. for 4 hr. and overnight at 4° C. before reading the results. In these tests, sodium chloride concentration was maintained at 0·85 %, except in tests with the rough strain when the salt concentration was reduced to 0·5 % to prevent autoagglutination. Absorption of agglutinins was done by treating 0·1 ml. of each serum with 2·4 ml. of a dense live suspension in 0·85 % saline of the absorbing strain at 37° C. for 4 hr. and overnight at 4° C.

The titres before and after absorption are given in Table 2. It will be seen that O antisera prepared with 162/p and C 14–S 5 did not agglutinate the rough mutant (A 69) not were their agglutinins removed by it. Likewise, O antisera to A 69 neither agglutinated nor were absorbed by 162/p or C 14–S 5. Owing to incomplete absorption, a low residual titre was seen even after absorption with homologous strains in some cases. This does not however, influence the fact that the rough antigen was not detectable in 162/p and C 14–S 5, which were therefore typical smooth strains. As absorption with 162/p-IN did not significantly lower the titre of sera prepared with 162/p and C 14–S 5, the latter strains belonged to the Ogawa type. This is consistent with the present knowledge of O antigenic structure of *V. cholerae* which possess a common O antigen, and minor type-specific O antigens corresponding to Ogawa and Inaba types (Gardner & Venkatraman, 1935).

Mouse virulence

Although mice do not display the typical pathological manifestations of cholera, they succumb to intraperitoneal infection with *V. cholerae*. The infective dose may be considerably reduced if the organisms are suspended in mucin (Griffitts, 1942). This technique thus permits quantitative measurements of virulence of different

strains, and high mouse virulence is generally preferred in strains of *V. cholerae* (and *V. el Tor*) selected for use as vaccines in man.

In spite of the artificial nature of this experimental model, which may not be relevant to the disease in man or immunity to it (Panse, Jhala & Dutta, 1964), it seemed worth while to carry out experiments with 162/p and C14–S5 and deter-

Table 2. *O agglutinin titres in immunized rabbits and agglutinin absorption tests*

Serum no.	Absorbed with	O agglutinin titre (Ogawa) 162/p	(Ogawa) C14–S5	(Inaba) 162/p-IN	(Rough) A69
1		5,000*	10,000*	500*	0*
	(Ogawa) 162/p	125	250	0	—
	(Ogawa) C14–S5	250	250	0	—
	(Inaba) 162/p-IN	2,000	5,000	0	—
	(Rough) A69	2,000	5,000	200	—
2		10,000*	10,000*	500*	0*
	(Ogawa) 162/p	250	250	0	—
	(Ogawa) C14–S5	250	250	0	—
	(Inaba) 162/p-IN	2,000	10,000	0	—
	(Rough) A69	5,000	10,000	500	—
3		2,000*	5,000*	500*	0*
	(Ogawa) 162/p	50	125	0	—
	(Ogawa) C14–S5	125	250	0	—
	(Inaba) 162/p-IN	1,000	2,000	0	—
	(Rough) A69	1,000	5,000	500	—
4		2,000*	5,000*	200*	0*
	(Ogawa) 162/p	0	50	0	—
	(Ogawa) C14–S5	50	125	0	—
	(Inaba) 162/p-IN	1,000	2,000	0	—
	(Rough) A69	1,000	2,000	200	—
5		0*	0*	0*	500*
	(Ogawa) 162/p	—	—	—	500
	(Ogawa) C14–S5	—	—	—	500
	(Inaba) 162/p-IN	—	—	—	500
	(Rough) A69	—	—	—	0
6		0*	0*	0*	1,000*
	(Ogawa) 162/p	—	—	—	1,000
	(Ogawa) C14–S5	—	—	—	1,000
	(Inaba) 162/p-IN	—	—	—	1,000
	(Rough) A69	—	—	—	50

* Titre of unabsorbed serum; 0, titre less than 50.

Serum nos. 1 and 2 derived from rabbits immunized with heat-killed suspensions of 162/p; serum no. 3 and 4 derived from rabbits immunized with heat-killed suspensions of C14–S5; serum no. 5 and 6 derived from rabbits immunized with heat-killed suspensions of A69.

mine how virulent they were to mice. Swiss mice (of either sex), 14–16 g. in weight, reared in this Institute, were distributed in groups of ten, each group receiving 0·5 ml. of graded decimal dilutions of a 4 hr. nutrient broth culture of the strain under study. All dilutions were made in nutrient broth, except that intended for

intraperitoneal inoculation of mice, for which an aqueous suspension of 5 % mucin (Granular mucin, Type 1701-W, Wilson Laboratories, Chicago 9, Illinois, U.S.A.), adjusted to pH 7·2 after sterilization, was used as diluent.

Table 3. *Mouse virulence of strain* 162/*p and its attenuated mutant C*14–*S*5

	162/p		C14–S5	
Dose (0·5 ml.)	Test no. 1 ($2·6 \times 10^8$/ml.)	Test no. 2 ($4·6 \times 10^8$/ml.)	Test no. 1 ($5·4 \times 10^8$/ml.)	Test no. 2 ($2·2 \times 10^8$/ml.)
10^{-1}	—	—	7/10	5/10
10^{-2}	—	—	1/10	1/10
10^{-3}	—	—	1/10	0/10
10^{-4}	—	—	—	—
10^{-5}	10/10	—	—	—
10^{-6}	9/10	10/10	—	—
10^{-7}	7/10	10/10	—	—
LD 50*	Less than 13 organisms	Less than 23 organisms	10^7 organisms	$1·8 \times 10^7$ organisms

Figures in parentheses indicate viable counts of cultures employed. Numerator represents no. of deaths (in 48 hr.) and denominator the no. of animals in the group.

* Determined by the method of Reed & Muench (1938).

Table 3 records the results of this study. The LD 50 of C14–S5 was indeed very high (10^7 organisms and $1·8 \times 10^7$ organisms in two experiments), while 162/p proved to be highly virulent, the LD 50 being < 13 and < 23 organisms in two experiments. Although vibrio strains with extremely high mouse virulence (with an LD 50 of 1·69 and 1·90 organisms) have been reported (Pesigan, 1965), there does not appear to be any reference to a smooth strain of *V. cholerae* having such low mouse virulence as observed with C14–S5. In earlier experiments (Bhaskaran & Sinha, 1966), the LD 50 of 162/p was estimated at about 111 organisms, and this was probably due to the use of a batch of mucin of different manufacture.

Virulence studies in adult and infant rabbits

The rabbit intestine is known to be suitable for the study of enteropathogenicity of *V. cholerae*, and infant rabbits, 10 to 16 days old, are particularly susceptible to intra-intestinal infection with *V. cholerae* or their toxic products (Dutta & Habbu, 1955; Oza & Dutta, 1963). Oral infection (and intoxication) is also possible *(Feeley, 1965; Finklestein, Norris & Dutta, 1964). Diarrhoea starts 18–24 hr. after* intra-intestinal infection and the animal shows signs of dehydration. Death occurs in 36–48 hr. and autopsy reveals a characteristic picture, in which a massive distension of the caecum with fluid resembling the rice-water stool of cholera in man is a constant finding.

Rabbits more than a month old are not susceptible to an experimental infection of this kind. Diarrhoea is not produced and the animal shows no abnormality. In these animals, however, if the organism is introduced in closed ileal loops of about 4 in. in length, created by ligatures at the ends, and the animal examined 24 hr. after infection, the loop presents a distended appearance due to the accumu-

lation of 14–20 ml. of fluid which is sometimes blood-stained (De & Chatterji, 1953). Choleragenic toxins also produce a similar effect (De, Ghose & Chandra, 1962; Finkelstein, 1965*b*). As many as four ileal loops may be infected in an animal, under ether anaesthesia after preliminary sedation with 'Intraval' Sodium (May and Baker Ltd., Dagenham, England).

These two experimental models were employed with advantage for the study of enteropathogenicity of 162/p and C14–S5. Adult rabbits (1·0–1·5 kg.) were employed for the ileal loop studies. Ten-day-old infant rabbits (albino), weighing between 100 and 130 g., were used for intra-intestinal infection which was done under ether anaesthesia by direct introduction of the organisms into the lumen of the small intestines of the animal after making an abdominal opening which was subsequently sutured. In these experiments, the dose employed for infection was 1 ml. of a 4 hr. nutrient broth culture, with a viable count of $3–5 \times 10^8$ organisms.

Table 4. *Experimental infection of adult and infant* (10 *day old*) *rabbits with strain* 162/*p and its attenuated mutant C*14–*S*5

	Adult rabbits			Infant rabbits		
Strain	No. of animals	No. of ileal loops infected	No. showing positive reaction	No. of animals infected	No. of animals exhibiting diarrhoea	No. of animals with fluid distension of caecum
162/p	17	25	21	15	12	14
C14–S5	8	22	0	15	0	0

Table 4 reveals the differences in enteropathogenicity observed between 162/p and C14–S5. Strain 162/p uniformly gave rise to ileal loop distension in adult rabbits, with the exception of four loops in two different animals, and C14–S5 gave consistently negative results. In many experiments, both the strains were used for infecting different loops in the same animal, and the contrast was striking (see Plate 1).

Of the 15 infant rabbits infected with 162/p, diarrhoea was observed in 12 animals after 24–40 hr. and autopsy performed on all the animals at the end of this period revealed the characteristic distension of caecum in 14 of them. Only 1 showed no signs of disease.

Similar experiments were performed with C14–S5, after this strain had been passaged through two infant rabbits in series, as such passages are known to enhance the virulence of *V. cholerae* strains to these animals (Dutta & Habbu, 1955). In the absence of diarrhoea or caecal distension, the passaged strain was reisolated by seeding the intestinal contents on nutrient agar containing streptomycin (500 μg./ml.). However, none of the 15 infant rabbits infected with C14–S5 (passaged strain) showed any evidence of diarrhoea. All the animals were killed after 24–40 hr. and autopsy revealed a normal appearance of the gut.

Chick embryo virulence

Gardner, Lyles, Lankford & Hagens (1963) showed that the 13 day chick embryo was well suited for the study of virulence of *V. cholerae* and that only smooth strains were virulent. Rough and smooth-rough (SR) variants were avirulent to the chick embryo. If deaths were scored 24 hr. after allantoic inoculation, reproducible LD 50 titrations of virulence of different strains were possible. Compared to *V. cholerae*, strains of *V. el Tor* showed an extremely high virulence (Finkelstein, 1964). Factors determining the resistance of older chick embryos to allantoic infection with *V. cholerae* have also been elucidated (Finkelstein & Ramm, 1962).

It was therefore expected that the difference in virulence observed between 162/p and C14–S5 in mouse and rabbit would be further confirmed in the chick embryo. Twelve-day-old White Leghorn eggs were inoculated allantoically with 0·1 ml. of decimal dilutions in saline of 4 hr. broth cultures of the strains. The infected eggs were incubated at 37° C. and examined 24 hr. later for viability. Results are shown in Table 5.

Table 5. *Chick embryo virulence of strain* 162/*p and its attenuated mutant* C14–S5

	Experiment no. 1		Experiment No. 2		Experiment no. 3	
Dose (0·1 ml.)	162/p ($2{\cdot}33 \times 10^8$/ml.)	C14–S5 ($2{\cdot}26 \times 10^8$/ml.)	162/p (2×10^8/ml.)	C14–S5 (3×10^8/ml.)	162/p ($2{\cdot}8 \times 10^8$/ml.)	C14–S5 ($5{\cdot}7 \times 10^8$/ml.)
10^{-1}	n.d.	1/8	n.d.	1/8	n.d.	2/8
10^{-2}	7/8	1/8	6/8	0/8	8/8	1/8
10^{-3}	4/8	0/8	7/8	0/8	6/8	1/8
10^{-4}	5/8	n.d.	7/8	n.d.	6/8	n.d.
10^{-5}	1/8	n.d.	4/8	n.d.	5/8	n.d.

Figures in parentheses indicate viable counts of the cultures employed. Numerator represents deaths and denominator the total number of embryos infected. n.d., not done.

The difference in virulence of the two strains was confirmed. But a precise dose/effect relationship was not obvious, especially with strain 162/p, and this was possibly owing to the use of 12-day instead of 13-day embryos, or to a slight variation in the age of the embryos at the time of supply. As each of the three experiments recorded in Table 5 was done with a single lot of eggs received on the day of the experiment, and distributed at random into groups of 8, it is extremely unlikely that any shift to virulence with C14–S5 will be observed even with stricter control of the age of the embryos in this test.

Population changes of 162/*p and* C14–S5 in vivo

It would be logical to expect that the ability of 162/p to produce fluid distension of ileal loops of adult rabbits, cholera-like disease in infant rabbits, and death of mice and chick embryos, might be due to the active multiplication of the organism *in vivo*, perhaps associated with the release of toxic factors. In the same way, the uniform pattern of avirulence of C14–S5 in the four different models may be an

indication of the inability of this strain to multiply in the tissues of the animal to an extent required for causing the pathological changes, or of its non-toxicity. Its inability to multiply is suggested by the peculiar dwarf colony character of C14–S5 which reflected its slow rate of growth. Although precise growth studies have not yet been carried out, the growth of C14–S5 in fluid media like nutrient broth is much slower than 162/p especially from small inocula. If this is due to different optimal conditions for the growth of C14–S5, the specific requirements are yet obscure.

With a view to studying the *in vivo* growth of 162/p and C14–S5 in the experimental models investigated, viable counts were performed 24 hr. after infection and were compared with the initial numbers employed for setting up the infection. Such estimates were made in closed ileal loops of adult rabbits, and in the intestinal tract of 10-day-old infant rabbits (Table 6).

Table 6. *Growth and/or survival of strain* 162/*p and its attenuated mutant C*14–*S*5 *in the ileal loops of adult rabbits and in the intestinal tract of infant* (10-*day-old*) *rabbits*

Experiment	Strain	Viable count: Infecting dose	Viable count: 24 hr. after infection	Ratio final count initial count
Ileal loop (adult rabbits)	162/p (test no. 2)	$5{\cdot}6 \times 10^{8}$	$8{\cdot}3 \times 10^{9}$	14·82
	162/p (test no. 2)	$4{\cdot}4 \times 10^{8}$	$1{\cdot}9 \times 10^{10}$	43·18
	C14–S5 (test no. 1)	$5{\cdot}4 \times 10^{8}$	$2{\cdot}0 \times 10^{9}$	3·70
	C14–S5 (test no. 2)	$4{\cdot}9 \times 10^{8}$	$7{\cdot}9 \times 10^{7}$	0·16
Intra-intestinal infection (infant rabbits)	162/p (test no. 1)	$2{\cdot}23 \times 10^{8}$	$6{\cdot}0 \times 10^{10}$	269·10
	162/p (test no. 2)	$2{\cdot}23 \times 10^{8}$	$3{\cdot}0 \times 10^{11}$	1345·00
	C14–S5 (test no. 1)	$2{\cdot}15 \times 10^{8}$	$7{\cdot}4 \times 10^{7}$	0·34
	C14–S5 (test no. 2)	$2{\cdot}15 \times 10^{8}$	$7{\cdot}0 \times 10^{7}$	0·33

As expected, experiments with 162/p gave rise to pathological reactions in both, whereas with C14–S5 they were absent. For the counts, each ileal loop was dissected out and disintegrated in a liquidiser (Kenwood Manf. Co. Ltd., Woking, England). The material thus obtained was suspended in 100 ml. of nutrient broth, and dilutions of this in broth were seeded on nutrient agar plates and the colonies counted after 24 hr. at 37° C. From the counts obtained, an approximate estimate of the total number of viable organisms of the test strain was made. In the case of infant rabbits, the entire gut (small and large intestines) was removed and disintegrated and total viable counts were performed as described above.

Strain 162/p so far outgrew the normal intestinal flora that these caused no difficulty in the colony counts, but with C14–S5, which did not multiply so freely,

counts had to be performed on nutrient agar containing streptomycin (500 μg./ml.) to inhibit the normal flora.

Table 6 shows the wide divergence between the two strains. The number of viable organisms of 162/p, 24 hr. after infection, was considerably in excess of the input, especially in the gut of infant rabbits. With C14–S5, the final counts were generally less, except in one animal, which registered a small rise. As non-viable cells were automatically excluded from these estimates, the final counts provided only an approximate estimate of the extent of bacterial growth. Even though C14–S5 did not register a significant rise in numbers during the 24 hr. period, the final counts varied from one-fifth to one-third of the input in three animals, which showed the absence of any rapid clearing mechanisms in the host. As infant rabbits, infected with C14–S5, did not pass any faecal matter during the 24 hr. period, this mode of elimination of the organism was excluded, but with 162/p there must have been loss through diarrhoea occurring in the infant rabbits. These considerations do not, of course, apply to the closed ileal loops in adult rabbits.

DISCUSSION

In this study, the term virulence, as applied to *V. cholerae*, is used in the general sense to mean the ability to cause either death by rapid proliferation or characteristic pathological manifestations in experimental animals, regardless of any toxic factors. In fact, a variety of toxic factors may be demonstrated in *V. cholerae* and differentiated from one another by their association with different parts of the cell, dialysability, and various toxicities demonstrable in mice, chick embryos, adult and infant rabbits, cell culture lines and anurian epithelium (Burrows, 1965). More is now known regarding the choleragenic toxin (choleragen), which has been identified as a protein and which is probably released during active proliferation of *V. cholerae* under optimal conditions. Of particular interest is the observation that neither endotoxin nor mucinase play any significant role in the pathogenesis of cholera. These advances are the result of some noteworthy research in this field by Finkelstein and his colleagues (Finkelstein *et al.* 1964; Finkelstein, 1965*a*, *b*; Finkelstein, Atthasampunna, Chulasamaya & Charunmethee, 1966; Finkelstein, Sobocinski, Atthasampunna & Charunmethee, 1966).

While Mukherjee (1963) identified apathogenic strains of *V. el Tor*, with a certain degree of residual virulence, in natural isolates from water sources, we have in this work used the chemical mutagen, N-methyl-N′-nitro-N-nitrosoguanidine, for the isolation of an attenuated mutant of *V. cholerae*, and subsequent labelling with marked characters. A variety of other mutants have also been isolated with this agent, as well as with ethyl methane sulphonate (Bhaskaran, Sinha & Iyer, 1966).

The attenuated mutant C14–S5 is a slow growing strain, giving rise to dwarf colonies on nutrient agar, a frailty that possibly accounts for its non-pathogenicity to mouse, rabbit and chick embryo. The capacity of this strain to elaborate the choleragenic toxin and other toxicities during growth remains to be studied. Antigenic analysis excluded the possibility of roughness in the strain. Further,

C14–S5 is as effective as wild type strains when employed as live vaccine in mouse protection tests (Bhaskaran & Sinha, unpublished observations) carried out as recommended by Pittman & Feeley (1965).

The stability of the attenuated mutant, C14–S5, and its predecessors (A11, B16 and C14) seems to be well established. These strains have been under study for over 6 months, during which a number of transfers have been carried out on nutrient media and a variety of animal experiments have been performed. At all times, the dwarf colony character and attenuation have remained a constant feature of these mutants. Whether the genetic determinants of virulence and normal growth characteristics can be transferred to the attenuated mutants by conjugation with wild type *V. cholerae* strains is not known. With the recognition of a fertility factor in *V. cholerae* (Bhaskaran, 1960, 1964) such studies should be possible.

Recent field trials have shown that killed cholera vaccines are of low efficacy in man, with the exception of a few which however caused severe reactions (see Table 7). In the laboratory there is convincing evidence to suggest that live vaccines may be more effective. Panse *et al.* (1964) showed that infant rabbits, in which experimental cholera can be produced, were passively immunized against the disease by intraperitoneal administration of antisera derived from rabbits immunized with live vibrios. In contrast, antisera obtained with heat-killed and formalin-killed suspensions of the organism were ineffective. This observation was further supported by the finding that the offspring of female rabbits immunized with live vibrios were resistant to experimental infections, whereas female rabbits immunized with killed suspensions of the organism gave birth to susceptible infants (Panse & Dutta, 1964). Feeley (1965) made similar observations and showed that live vibrio antisera, administered intraperitoneally, protected infant rabbits against oral infection with a highly virulent strain of *V. cholerae*. He also observed that the protective antibodies appeared in the later stages of a multiple immunization schedule in rabbits, which synchronised with the preponderance of agglutinin activity in 7S immunoglobulins over that detectable in the 19S factor, the former potentially capable of diffusing more rapidly into the extra-vascular space. In this study as well as in those of Finkelstein (1965*a*) it was emphasized that the protective antibody (antibacterial or antitoxic) should cross the intestinal barrier and appear free in the gut for effective immunity.

The titre of circulating antibody (agglutinating and vibriocidal) seems to bear no relationship to immunity in man, as cases of cholera in the acute phase have shown high antibody titres (Mukerjee, 1963; Finkelstein, Powell, Woodrow & Krevans, 1965). This, considered with the possibility that the effective site of immunity is the intestinal tract, should restore faith in local immune mechanisms, probably mediated by coproantibody (Burrows *et al.* 1947). Freter (1962) and Freter & Gangarosa (1963), using refined methods for estimating coproantibody, showed in human volunteers that these are produced with killed cultures administered orally with a greater regularity than is observed after parenteral immunization. For induction and maintenance of a strong coproantibody response, repeated doses of oral vaccine were required. It was suggested, however, that for reducing

Table 7. *Provisional results obtained in controlled field trials of cholera vaccines*

(Data reproduced from Cvjetanovic (1966), with the permission of the World Health Organization.)

Place, year (project reference)	Vaccines	Strains used	Method of preparation	Side reactions	Maximum efficacy (%)	Duration of immunity	Predominant epidemic strain
Phillipines, 1964–65* (WHO-Phillipines-Japan)	1. Agar-grown *V. cholerae* (fluid)	Ogawa 41† Inaba 35A3†	Phenol	±	63	4 months	El Tor vibrio, Ogawa
	2. Agar-grown El Tor vibrio (fluid)	Ogawa, local Inaba, local	Phenol	±	63	4(–6§) months	
	3. Agar-grown *V. cholerae*, oil adjuvant	Ogawa 41† Inaba 35A3†	Formalin	Very strong	67	More than 1 year	
Calcutta, 1964‡ (WHO-ICMR)	1. Agar-grown *V. cholerae*, freeze-dried	Ogawa 41† Inaba 35A3†	Formalin	±	55§	3 months§	El Tor vibrio, Ogawa
	2. Agar-grown *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	33§	?	
	3. Agar-grown *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	20§	?	
	4. Fluid cultured *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	0§	—	
Calcutta, 1965 (WHO-ICMR)	1. Agar-grown *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	40§	Less than 6 months	El Tor vibrio, Ogawa
	2. Fluid cultured *V. cholerae* (fluid)	Ogawa, local Inaba, local	Phenol	±	6§	Less than 6 months	
Dacca, 1963–65** (Pakistan-SEATO)	1. Agar-grown *V. cholerae* (fluid) with high antigen content	Ogawa 41†	Heat-killed	Strong	79	More than 1·5 years	*V. cholerae* Inaba

(fluid) indicates the vaccines prepared according to the Requirements for Biological Substances (*WHO techn. Rep. Ser.* 1959, No. 179).

± Uncertain or weak and small reactions.

* Phillippines Cholera Committee (1965). *Bull. Wld Hlth Org.* **32**, 603.

† Reference strains used for mouse protection tests by United States NIH.

‡ Taneja, B. L. (1965). *Proceedings of the Cholera Research Symposium*, p. 373. Washington, D.C.; United States Government Printing Office.

§ Statistically no significance as compared with control group (95% confidence limit).

** Oseasohn, R. O., Benenson, A. S. & Fahimuddin, Md. (1965). *Proceedings of the Cholera Research Symposium*, p. 362. Washington, D.C.: United States Government Printing Office.

the doses of oral vaccine attenuated strains capable of extensive proliferation in the gut may be employed (Freter, 1965). Alternatively, it was proposed that parenteral vaccine may be used to induce coproantibody production followed by spaced doses of oral vaccine to maintain their production.

The ideal cholera vaccine may be one that will be effective in inhibiting the proliferation of the pathogen in the gut, thereby suppressing the release of the choleragenic toxin. The point at issue is whether a suitable vaccine can be discovered which, when administered parenterally, will result in the release of protective antibodies in the lumen of the gut or whether their synthesis can only be achieved by a local antigenic stimulus as by oral vaccines. The success achieved by Haffkine with live vaccines administered parenterally in man (for review, see Cvjetanovic, 1965), as well as the experiments on passive immunization with live vibrio antisera referred to earlier, should justify further research with parenterally administered live vaccines in man, side by side with similar studies with oral live vaccines. The effective route of immunization and the choice of vaccine will be resolved only when the nature of the protective antibodies and means of detecting them are understood.

It is imperative that live cholera vaccines, intended for trial in man, should be stable attenuated strains. The findings recorded in this paper show that such mutants of *V. cholerae* may be isolated in the laboratory and with mutational techniques may be marked for specific nutritional requirements and antibiotic resistance which would permit their easy identification and quick differentiation from wild type vibrio strains. In the absence of such markers, it would be difficult to identify fluctuations in virulence of the attenuated strains occurring either in course of time or during oral vaccine trials in man. The range of markers is capable of further extension which will be very useful especially if they are in contrast to specific fermentations and phage sensitivities generally identified in *V. cholerae*.

SUMMARY

This paper describes the study of a mutant of *Vibrio cholerae* which is shown to be attenuated by its avirulence to mouse, rabbit and chick embryo. This mutant character is stable, and the avirulence of this strain probably results from its inability to multiply actively in the tissues of the infected animals. The need for the study of such attenuated strains as live vaccines in man is discussed, and certain aspects of immunity to cholera are reviewed.

This work was supported by grants from the Indian Council of Medical Research and the World Health Organization. Dr K. Bhaskaran wishes to express his appreciation to Dr B. Cvjetanovic (Chief, Bacterial Diseases, World Health Organization, Geneva) for many stimulating discussions on the subject of cholera vaccines and controlled field trials. He is also grateful to Dr F. Jacob (Pasteur Institute, Paris) for technical hints on the use of N-methyl-N′-nitro-N-nitrosoguanidine for mutant isolations in bacteria. The authors are very grateful to Dr M. L. Dhar (Director, Central Drug Research Institute, Lucknow) for his interest and support.

REFERENCES

Barua, D. & Mukherjee, A. C. (1963). Direct bacterial haemagglutination test for differentiating *El Tor* vibrios from *V. cholerae*. *Bull. Calcutta Sch. trop. Med. Hyg.* **11**, 85.

Besredka, A. (1927). *Local Immunization*, Baltimore: Williams and Wilkins Co.

Bhaskaran, K. (1960). Recombination of characters between mutant stocks of *Vibrio cholerae*, strain 162. *J. gen. Microbiol.* **23**, 47.

Bhaskaran, K. (1964). Segregation of genetic factors during recombination in *Vibrio cholerae*, strain 162. *Bull. Wld Hlth Org.* **30**, 845.

Bhaskaran, K. & Sinha, V. B. (1966). Attenuation of virulence in *Vibrio cholerae*. *WHO/Cholera Information* 66.6, p. 13.

Bhaskaran, K., Sinha, V. B. & Iyer, S. S. (1966). Study of mutations in *Vibrio cholerae* and *Vibrio el Tor*. *WHO/Cholera Information*/66.6, p. 5.

Burrows, W. (1965). Cholera vibrio toxicities. *Proceedings of the Cholera Research Symposium*, p. 120. Washington, D.C.: United States Government Printing Office.

Burrows, W., Elliott, M. E. & Havens, I. (1947). Studies on immunity to Asiatic Cholera. IV. The excretion of coproantibody in experimental enteric Cholera in the guinea-pig. *J. infect. Dis.* **81**, 261.

Cvjetanovic, B. (1965). Earlier field studies of the effectiveness of Cholera vaccines. *Proceedings of the Cholera Research Symposium*, p. 355. Washington, D.C.: United States Government Printing Office.

Cvjetanovic, B. (1966). WHO activities in the field of cholera. In *Training Course on Cholera WHO/Cholera*/66·5, Calcutta, 7–31 March 1966. Geneva: World Health Organization.

De, S. N. & Chatterji, D. N. (1953). An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. *J. Path. Bact.* **66**, 559.

De, S. N., Ghose, M. L. & Chandra, J. (1962). Further observation on Cholera enterotoxin. *Trans. T. Soc. trop. Med. Hyg.* **56**, 241.

Dutta, N. K. & Habbu, M. K. (1955). Experimental Cholera in infant rabbits: a method for chemotherapeutic investigation. *Br. J. Pharmac. Chemother.* **10**, 153.

Feeley, J. C. (1965). Passive protective activity of antisera in infant rabbits infected orally with *Vibrio cholerae*. *Proceedings of the Cholera Research Symposium*, p. 231. Washington, D.C.: United States Government Printing Office.

Finkelstein, R. A. (1964). Virulence of El Tor vibrios for chick embryos. *Nature, Lond.* **202**, 609.

Finkelstein, R. A. (1965*a*). Immunological aspects of experimental Cholera. *Proceedings of the Cholera Research Symposium*, p. 58. Washington, D.C.: United States Government Printing Office.

Finkelstein, R. A. (1965*b*). Observations on the nature and mode of action of the choleragenic products of cholera vibrios. *Proceedings of the Cholera Research Symposium*, p. 264. Washington, D.C.: United States Government Printing Office.

Finkelstein, R. A., Atthasampunna, P., Chulasamaya, M. & Charunmethee, P. (1966). Pathogenesis of experimental cholera: biologic activities of purified procholeragen A. *J. Immun.* **96**, 440.

Finkelstein, R. A., Norris, T. H. & Dutta, N. K. (1964). Pathogenesis of experimental cholera in infant rabbits. I. Observations on the intra-intestinal infection and experimental cholera produced with cell-free products. *J. infect Dis.* **114**, 203.

Finkelstein, R. A., Powell, C. J., Woodrow, J. C. & Krevans, J. R. (1965). Serological responses in man to a single small dose of cholera vaccine with special reference to the lack of influence of ABO blood groups on natural antibody or immunological responsiveness. *Bull. Johns Hopkins Hosp.* **116**, 152.

Finkelstein, R. A. & Ramm, G. M. (1962). Effect of age on susceptibility to experimental cholera in embryonated eggs. *J. infect. Dis.* **111**, 239.

Finkelstein, R. A., Sobocinski, P. Z., Atthasampunna, P. & Charunmethee, P. (1966). Pathogenesis of experimental cholera: identification of choleragen (procholeragen A) by disc immunoelectrophoresis and its differentiation from cholera mucinase. *J. immun.* **97**, 25.

Freter, R. (1956). Coproantibody and bacterial antagonism as protective factors in experimental cholera. *J. exp. Med.* **104**, 419.

Freter, R. (1962). Detection of coproantibody and its formation after parenteral and oral immunization of human volunteers. *J. infect. Dis.* **111**, 37.

FRETER, R. (1965). Coproantibody and oral vaccines. *Proceedings of the Cholera Research Symposium*, p. 222. Washington, D.C.: United States Government Printing Office.

FRETER, R. & GANGAROSA, E. J. (1963). Oral immunization and production of coproantibody in human volunteers. *J. Immun.* **91**, 724.

GARDNER, A. D., & VENKATRAMAN, K. V. (1935). The antigens of the cholera group of vibrios. *J. Hyg. Camb.* **35**, 262.

GARDNER, E. W., LYLES, S. T., LANKFORD, C. E. & HAGENS, S. J. (1963). A comparison of the virulence of *Vibrio cholerae* strains for the embryonated egg. *J. infect. Dis.* **112**, 264.

GRIFFITTS, J. J. (1942). The use of mucin in experimental infections of mice with *Vibrio cholerae*. *Publ. Hlth Rep., Wash.* **57**, 707.

HEIBERG, B. (1935). *On the classification of* Vibrio cholerae *and Cholera-like vibrios.* Copenhagen: Arnold Busck.

MUKERJEE, S. (1961). Diagnostic uses of cholera bacteriophages. *J. Hyg., Camb.* **59**, 109.

MUKERJEE, S. (1963). Preliminary studies on the development of a live oral vaccine for anti-cholera immunization. *Bull. Wld Hlth Org.* **29**, 753.

OZA, N. B. & DUTTA, N. K. (1963). Experimental cholera produced by toxin prepared by ultrasonic disintegration of *V. comma*. *J. Bact.* **85**, 497.

PANSE, M. V., & DUTTA, N. K. (1964). Cholera vaccines and placental transmission of antibodies. *J. Immun.* **93**, 243.

PANSE, M. V., JHALA, H. I. & DUTTA, N. K. (1964). Passive immunity in experimental cholera. *J. infect. Dis.* **114**, 26.

PESIGAN, T. P. (1965). Cholera and El Tor vaccines used in the controlled field trials in the Philippines by the joint Philippine-Japan-WHO Cholera Committee. *Proceedings of the Cholera Research Symposium*, p. 171. Washington, D.C.: United States Government Printing Office.

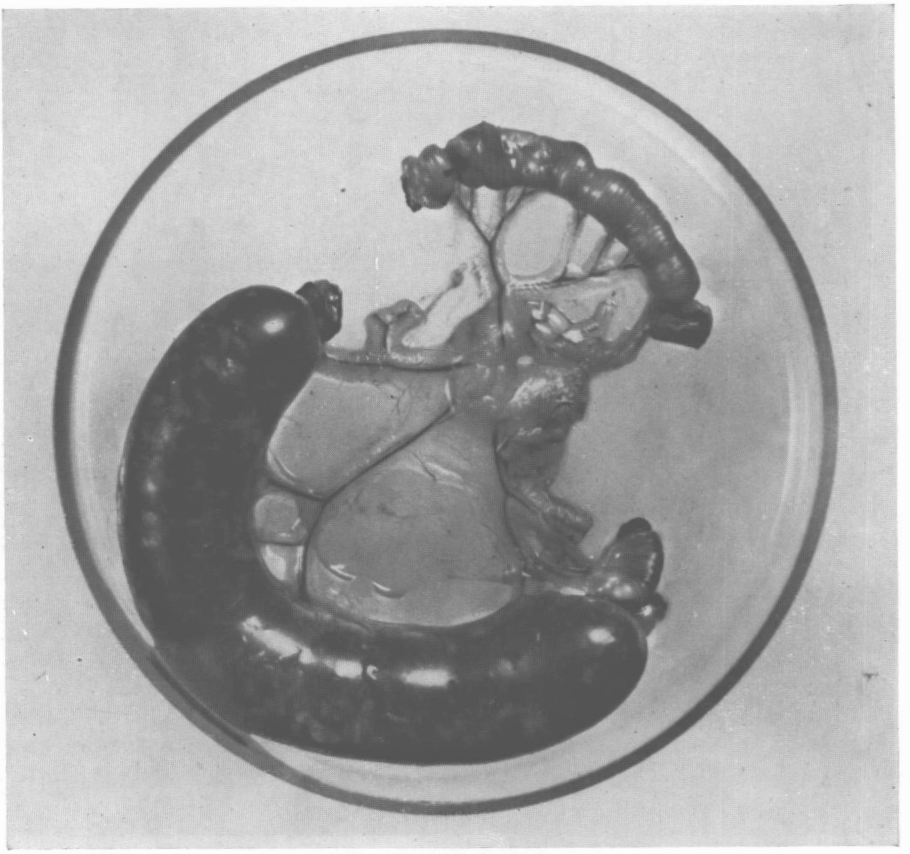
PITTMAN, M. & FEELEY, J. C. (1965). Laboratory assay of Cholera vaccine potency. *Proceedings of the Cholera Research Symposium*, p. 163. Washington, D.C.: United States Government Printing Office.

REED, L. J. & MUENCH, H. (1938). A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* **27**, 493.

SHRIVASTAVA, D. L. & WHITE, P. B. (1947). Note of the relationship of the so-called Ogawa and Inaba types of *Vibrio cholerae*. *Indian J. med. Res.* **36**, 409.

EXPLANATION OF PLATE

Two ileal loops of an adult rabbit, showing the difference between the effects of virulent and attenuated strains of *Vibrio cholerae* introduced into the lumen of the gut.



K. BHASKARAN AND V. B. SINHA

(*Facing p.* 148)

https://doi.org/10.1017/S0022172400045642 Published online by Cambridge University Press