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REVIEW

The utility of human challenge studies in vaccine development: lessons learned from cholera

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Abstract: Experiments in which virulent infectious organisms are administered to healthy adult volunteers with the intent to deliberately induce infection have been practiced for centuries. Many useful applications have developed from these experiments such as the provision of evidence of microbial pathogenicity and the identification of key virulence factors. Challenge studies have also played an important role in the evaluation of preliminary efficacy of potential vaccine candidates. Over the past 40 years, these experimental human challenge studies have found particular utility with regards to the development of both living and nonliving attenuated cholera vaccines. This review highlights some of the important contributions made by these challenge studies to cholera vaccine research.

Keywords: virulent infectious organisms, human challenge studies, cholera, vaccine research

Challenge studies

Volunteer challenge studies involve the intentional induction of infection by the administration of virulent organisms to healthy, consenting volunteers under carefully controlled conditions. Challenge studies may at first seem to be a direct violation of one of the sacred maxims of the Hippocratic oath, "I will keep them from harm …," promised by physicians across the world. These studies, however, can be ethically justified when there is a compelling rationale to investigate infections that are self-limited or that can be easily and fully treated.[1] The studies must be conducted by competent investigators who abide by rigorously developed protocols with meticulous attention to safety. Volunteers must be fully informed of the risks and anticipated discomforts and freely provide consent before being allowed to participate.[1] In the appropriate setting, challenge studies can save time, money, and resources, and have proven to be a valuable tool in recent vaccine development.

Challenge studies can be applied to prove microbial pathogenicity, confirm host factors that contribute to the acquisition of infection and the severity of disease, define microbial virulence factors, and identify potential vaccine candidates capable of inducing protective immunity.[2] Perhaps one of the most useful applications of challenge studies, though, is the assessment of preliminary vaccine efficacy.[3] Challenge models can prevent the unnecessary exposure of thousands of human subjects in large Phase III field trials by eliminating vaccines that do not demonstrate preliminary evidence of protection. In addition, challenge studies can be used to refine the formulation and schedule of a vaccine that will be further evaluated in field trials.[2]

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Limitations

Results from challenge studies may not always be fully generalizable and careful consideration is needed before extrapolating data obtained from these studies, as demonstrated by cholera vaccine challenge studies. The challenge population, which has traditionally consisted of healthy adults from developed countries, may have many differences from the population at risk for natural disease, which usually consists of children residing in developing countries where the infection of interest is endemic, nutrition may be suboptimal, and coinfection with other intestinal bacteria and parasites is common. Indeed, there has been a recent call for the need to study strategies to overcome this "intestinal barrier," the poorly understood phenomenon of diminished responses to oral vaccines seen in populations from developing countries.[4]

Another limitation of challenge studies is that they are often designed to assess short-term protection.[2] This may be suitable for vaccines that are being developed for use predominantly in travelers, but may be less applicable when the intent is for use in endemic areas where long-term immunity is the desired goal. The experimental challenge model is often modified by increasing the virulence of the strain, inoculum, or vehicle in which it is administered to manipulate outcomes such as the attack rate of illness in volunteers. Hence, experimental infection may differ from natural infection.[2] For all of these reasons, challenge studies still require correlation through the conduct of large field trials in the population for which the vaccine is ultimately being developed. In addition to the evaluation of efficacy, large field trials also allow for continued evaluation of the safety of candidate vaccines in a more natural setting.

Challenge studies and cholera

Volunteer challenge studies with *Vibrio cholerae* have been a useful way to study many aspects of cholera. Challenge studies involving cholera date from 1892, with the first recorded intentional infection in humans with *V. cholerae* in an attempt to fulfill Koch's postulates.[5] Over the past 40 years, challenge studies have served as a unique research tool with many useful applications in the development of cholera vaccines.

Cholera

Cholera is an acute gastrointestinal illness caused by the ingestion of food or water contaminated with the Gram-negative bacillus *V. cholerae*. Infection is associated with profuse, toxin-mediated, watery diarrhea and can result in rapid and fatal dehydration if untreated. There are over 200 serogroups of cholera, based on the polysaccharide O-antigen. Epidemic cholera is associated with the O1 and, more recently, O139 serogroups. The O1 serogroup is further classified by biotype, classical or El Tor, and within this biotype by serotype, Ogawa or Inaba.[6] The 2009 World Health Organization annual cholera report identified 221,226 cases of cholera in 45 countries, resulting in 4946 deaths with a case fatality rate of 2.24%; but the disease burden is grossly underreported and the true incidence is more likely to be in the millions.[7] Cholera is endemic in Asia and Africa. Since the early 19th century, there have been seven world pandemics with *V. cholerae* O1 of the El Tor biotype. The seventh pandemic is still ongoing. In 1993, epidemic cholera due to serogroup O139 was reported in India and Bangladesh and spread rapidly raising the concern of an eighth pandemic. However, the incidence quickly fell and infection was only seen in South East Asian countries. Attention is currently being paid to an ongoing outbreak of *V. cholerae* serogroup O1 of the El Tor biotype in Haiti, which began in October 2010 with an initial reported 7% case fatality rate, one of the highest recorded in recent history.[8]

The diarrhea of cholera is caused by cholera toxin. This toxin is composed of one A subunit and five B subunit polypeptide chains. It acts on the target intestinal cell through activation of adenylate cyclase, leading to increased intracellular levels of cyclic adenosine monophosphate (cAMP) with subsequent decreases in luminal sodium uptake and increases in chloride and bicarbonate export. More detailed information regarding the microbiology, epidemiology, pathogenesis and clinical features of cholera can be found in the excellent review by Kaper et al.[9]

Treatment

The mainstay of cholera treatment involves rehydration and, in some cases, the use of antibiotics. Parenteral cholera vaccines have been available since 1885 and were first evaluated by Jaime Ferrán, but are no longer used.[10,11] After decades of work, the search for an ideal cholera vaccine is still ongoing. Two types of oral cholera vaccines are currently available: Dukoral® (Crucell-SBL Vaccines, Stockholm, Sweden) is a monovalent-killed whole-cell *V. cholerae* O1 vaccine (classical and El Tor, Inaba and Ogawa) with recombinant cholera toxin B subunit. It can be administered to adults and children over the age of 2 years, and has been used mostly for travelers to developing countries. An efficacy of up to 90% after two doses has been demonstrated in the first 6 months, falling to 60% after 2 years; mORCVAX (National Institute of Hygiene and Epidemiology, Vietnam) and Shancol (Shantha

Biotechnics, Hyderabad, India) are closely related bivalent vaccines that contain O1- and O139-killed whole cells but no cholera toxin B subunit. These can be used in adults and children over the age of 1 year. A booster is recommended 2 years after the primary vaccination. Protective efficacy after two doses was shown to be about 60% but remained at about 50% 3–5 years after vaccination. Use has been in the endemic setting. An oral live attenuated vaccine (CVD 103-HgR) was licensed in the 1990s in several countries but is no longer available.[12]

Widespread use of the currently available vaccines has been limited by cost, incomplete protection, difficulty predicting when and where epidemics will occur, and distribution barriers to populations that would likely benefit the most from vaccination in endemic settings. The current cholera outbreak in Haiti is a stark reminder of how devastating cholera epidemics can be when access to safe drinking water is limited and adequate sanitation has been compromised, highlighting the need for effective prevention strategies to control this infection under emergency situations.

Studying cholera

There are several challenges that make studying cholera difficult. Humans are the only natural host of *V. cholerae*. Despite the use of a passive protection model in young mice[13] and rabbit models,[14] animal models that accurately reflect human immune response to immunization and challenge are lacking. In addition, there is no absolute marker of vaccine efficacy. Both antibacterial and antitoxin immunity exist and act synergistically. The serum vibriocidal antibody is the best correlate of antibacterial immunity currently available. These antibodies mediate bacterial killing in vitro, in the presence of complement. They are primarily directed to the *V. cholerae* lipopolysaccharide (LPS)[15] but may also recognize outer membrane proteins.[16] Epidemiologic studies have shown that vibriocidal antibody titers increase with age in endemic areas and the risk of disease is inversely proportional to titer. These antibodies do not seem to provide protective immunity, but rather are a marker for intestinal mucosal immunity. Secretory immune globulin A intestinal antibodies are thought to mediate the actual protection. Antitoxin titers do not correlate well with protection.[9]

Challenge models

Early challenge models

Field trials performed in Bangladesh, India, and the Philippines during the 1960s established the protection of the parenteral whole-cell bacterial cholera vaccine at about 60%. Protection was short lived, lasting only 3–6 months in endemic areas, and there was an unfavorable adverse event profile.[12] The search for new, more tolerable, cholera vaccines that induced better and longer protection began while parenteral vaccines fell out of favor and became unavailable. Parenteral cholera vaccines have been recently reviewed and they may have been more effective and better tolerated than realized.[17]

In challenge studies dating back to 1969, Cash et al administered classical Inaba strain 569B and classical Ogawa strain 395 in escalating doses to 111 subjects.[18] At least 10^8 colony-forming units (cfu) were required to induce diarrheal disease in humans. If administered with 2 g of sodium bicarbonate, which neutralizes gastric acid, this infecting dose could be reduced to 10^6 organisms to induce diarrhea in 80% of volunteers. This early study provided the basis for the use of 10^6 organisms administered with sodium bicarbonate in fasting volunteers in many subsequent challenge studies to evaluate cholera vaccine efficacy and immunogenicity.

A subset of these volunteers were rechallenged with classical Inaba strain 569B or classical Ogawa strain 395 and protection was compared with cohorts of volunteers vaccinated with Inaba whole-cell parenteral cholera vaccine, Inaba whole-cell vaccine administered orally with bicarbonate, parenteral toxoid vaccine, and control volunteers.[18] Twenty-one volunteers who developed clinical cholera with first infection were completely protected against diarrhea when rechallenged 4–12 months later with the homologous organism, and vibrios were recovered from only 1/21 (4.8%) volunteers. Diarrhea developed in 4/6 (66.7%) volunteers challenged with a heterologous organism and vibrios were recovered from 5/6 (83%) volunteers. Vaccine efficacy against protection of diarrhea ranged from 81% (parenteral whole-cell vaccine) to 47% (toxoid vaccine). Several lessons were derived from this group of challenge experiments. Prior clinical cholera in volunteers due to classical Inaba strain 569B conferred immunity to rechallenge with the homologous strain for up to 1 year. There was no correlation in protection between an individual's vibriocidal and antitoxin titers with infection or diarrhea. Immunity induced by whole-cell vaccine appeared adequate for use in short-term visitors but would likely lack the long-term protection needed for residents of endemic areas. Lastly, protection by infection was more complete and longer lasting than vaccination. Rationale for the continued development of cholera vaccines and a volunteer model to produce clinical illness in North American volunteers comparable to natural disease as a way to test vaccine efficacy was thus established.

A challenge model for El Tor strains, which by now had replaced classical strains in both endemic and epidemic settings, was also developed.[19] Escalating doses of 10^3 to 10^6 cfu of El Tor Inaba strain N16961 with sodium bicarbonate were given to 26 volunteers. Infection, diarrhea, and serologic responses were seen with doses as low as 10^3 vibrios, and the severity of illness was directly proportional to the inoculum size.[20] Additional challenge studies were able to show that ingestion of 10^6 El Tor Inaba vibrios with sodium bicarbonate produced similar results to ingestion with a meal and thus the bicarbonate model was able to mimic natural infection to a reasonable extent.[20]

Later challenge models

A model for El Tor Inaba strain N16961 in volunteers from Thailand has been validated. Cholera infection is endemic in Thailand and population differences such as gut flora composition and immunological background are expected compared with the North American population. Inoculation of 1.3×10^7 organisms produced a diarrheal attack rate of 90%, though clinical illness appeared milder than that seen in the prior studies with North American volunteers.[19] To improve consistency among challenge studies, a model of cholera with frozen challenge bacteria was also validated.[21]

An epidemic strain of *V. cholerae* O139 Bengal emerged in Asia in 1993. Immunity to the O1 serogroup conferred no immunity to the O139 serogroup. A model was established for challenging volunteers with the new epidemic O139 Bengal strain using freshly harvested (Al 1837) and frozen (Al 4260B) bacteria in North American volunteers. [22,23] This was followed by validation of a challenge model using the frozen 4260B strain in Thai volunteers.[24]

These early and late challenge models have been summarized in Table 1.

Lessons in pathogenicity

Challenge studies have helped to establish the role of cholera toxin in the pathogenesis of disease. The production of experimental cholera in a human volunteer was described in 1966 after Syncase cholerigen, a sterile filtrate of broth culture of *V. cholerae* classical Inaba strain 569 B, was directly introduced into the volunteer's small intestine.[5] This observation showed that diarrhea could be produced in the absence of viable cholera vibrios and suggested that a cholerigenic factor was responsible for clinical disease. Experimental cholera was later demonstrated in a dose–response fashion after purified

Table 1 Summary of cholera challenge models

Biotype/ serotype	Strain	Volunteer n	Volunteer Type	Dose range[a]	Clinical response[b] n (%)	Microbiological response[c] n (%)	Dose at which response observed	Volunteers challenged[d] (n)	Reference(s)
Serogroup O1									
Classical Inaba	569B	67	NA	10^4–10^6	42 (81)	48 (92)	10^6	52	Cash et al[18]
Classical Ogawa	395	25	NA	10^4–10^6	22 (88)	22 (88)	10^6	25	Cash et al[18]
El Tor Inaba	N16961	26	NA	10^3–10^6	9 (90)	10 (100)	10^6	10	Levine et al[31]
El Tor Inaba	N16961	26	T	10^4–10^7	10 (91)	11 (100)	10^7	11	Suntharasamai et al[19]
El Tor Inaba	N16961[e]	40	NA	10^5	34 (85)	36 (90)	10^5	40	Sack et al[21]
Serogroup O139									
	Bengal Al 1837	13	NA	10^4–10^6	7 (78)	9 (100)	10^6	9	Morris et al[23]
	0139 4260B[e]	25	NA	10^5–10^6	14 (93)	15 (100)	10^6	15	Cohen et al[22]
	0139 4260B[e]	35	T	10^4–10^7	11 (73)	15 (100)	10^7	15	Pitisuttithum et al[24]

Notes: [a]Administered with bicarbonate; [b]with diarrhea; [c]with positive stool culture; [d]volunteers challenged with dose at which response observed; [e]prepared from frozen vials.
Abbreviations: NA, North American; T, Thai; n, number.

cholera toxin was administered orally to volunteers.[25] There are probably other toxins expressed by *V. cholerae* that contribute to diarrhea, as volunteers challenged with genetically engineered strains exhibiting deletions of the CTX genes that encode for one or both cholera toxin subunits demonstrated milder forms of diarrhea.

ToxR, a regulatory protein of pathogenic *V. cholerae* O1 strains, controls the expression of cholera toxin and the expression of a rigid toxin-coregulated pilus structure known as *tcpA*. Through the administration of classical Ogawa strain 395 *toxR* and *tcpA* mutants to volunteers, it was shown that deletion of *toxR* resulted in decreased colonizing capacity and deletion of *tcpA* prohibited colonization.[24] This group of challenge studies provided evidence for the critical role of a specific pilus structure in colonization of the human intestine by *V. cholerae* and the importance of the *toxR* regulon in pathogenesis. The role of *tcpA* in colonization by *V. cholerae* O139 was also established, while another putative pilus expressed in *V. cholerae* O139 strains and O1 El Tor biotypes, the mannose-sensitive hemagglutinin (*mshA*), did not appear to assist in colonization when volunteers were given modified strains of CVD 112, a derivative of O139 strain Al 1837, altered by deletions in *tcpA* and *mshA*.[26]

Lessons in immunity

Early challenge studies in North Americans led to some interesting observations about immunity in a population that was naïve to cholera. Serologic responses and relation to clinical or bacteriological protection were assessed without being confounded by prior infection. After 19 volunteers received monthly doses of purified glutaraldehyde-treated cholera toxoid orally or enterally at doses of either 2 mg or 8 mg for a total of 3 months, 6/10 (60%) volunteers who received a 2 mg dose and 7/9 (78%) who received an 8 mg dose had a fourfold or greater rise in antitoxin titers, but this did not correlate with clinical protection. Viable vibrios were rarely cultured from the stools of rechallenged volunteers, suggesting that antibacterial, rather than antitoxic, mechanisms play an important role in immunity, perhaps through the interference of mucosal colonization.[26] Animal studies and epidemiologic studies have also provided evidence that both antibacterial and antitoxic immunity are important.[27–29]

Clinical and bacteriological protection was noted in volunteers with clinical cholera due to classical biotype strains when rechallenged with homologous and heterologous classical *V. cholerae* strains of either serotype, expanding on earlier observations.[18] Of the volunteers who received 2 mg doses of toxoid, 6/10 (60%) were challenged with 10^6 classical Inaba 569B vibrios, along with six unimmunized controls. Eight of the volunteers given the 8 mg dose and eight controls were challenged with 10^6 classical Ogawa 395 vibrios. There were no significant differences in stool volume between the vaccine and control groups. A homologous Ogawa rechallenge study was performed using four of the volunteers who developed cholera while serving as controls in the Ogawa challenge. These volunteers were rechallenged 9 weeks later with 10^6 Ogawa vibrios. Five controls were also given Ogawa vibrios. None of the rechallenged volunteers developed diarrhea or excreted vibrios. All five controls developed diarrhea and excreted vibrios. In heterologous challenge studies, seven control volunteers who developed clinical cholera with Ogawa 395 were rechallenged 10 weeks later with 10^6 Inaba 569B vibrios. Eleven of twelve (92%) controls developed cholera, but none of the rechallenged volunteers did. Vibrios were cultured from the stools of one (14%) veteran and all controls. Similarly, five volunteers who developed cholera with Inaba challenge were rechallenged 8 weeks later with 10^6 Ogawa organisms. Nine of ten (90%) controls developed diarrhea and excreted vibrios in the stool, but none of the five rechallenged volunteers developed diarrhea or excreted vibrios. Four volunteers who received 10^6 Ogawa vibrios were rechallenged 3 years later; none experienced diarrhea, compared with 4/5 (80%) control volunteers. Vibrios were cultured from the stools of 1 of the 4 (25%) rechallenged volunteers and all of the control volunteers.[30] These homologous and heterologous rechallenge studies proved it is possible to induce immunity lasting at least several years against homologous and heterologous serotypes.

Challenge studies have also provided important insight into disease severity in relation to host factors. Epidemiologic observations of increased cholera severity among people with the blood group O were confirmed through challenge studies[31] and Tacket et al went on to establish a model of South American cholera that could be used to predict field efficacy of candidate vaccines among a population with a high prevalence of blood group O.[32] Diarrhea resulting from the ingestion of *V. cholerae* was also found to be more severe in challenge volunteers with low stomach acid.[33]

Preliminary vaccine efficacy trials

A number of live and nonliving oral vaccines against *V. cholerae* have been developed and tested using the volunteer challenge method – several of these are summarized in Table 2 and will be discussed further. This list is not exhaustive, as many challenge studies have been performed with cholera vaccine candidates. Additionally, many volunteer

Table 2 Summary of cholera vaccines tested using challenge studies

Vaccine strain	Parent strain	Protective efficacy (%)	Reference(s)
Nonliving oral vaccines			
Whole vibrio	*V. cholerae* O1 Classical Inaba strain Cairo 48, Classical Ogawa strain Cairo 50, El Tor Inaba strain Phil 6973	56	Black et al[35]
Whole vibrio-B subunit	*V. cholerae* O1 Classical Inaba strain Cairo 48, Classical Ogawa strain Cairo 50, El Tor Inaba strain Phil 6973 + purified cholera toxin B subunit	64	Black et al[35]
Live attenuated oral vaccines			
Texas Star	*V. cholerae* O1 El Tor Ogawa	61	Levine et al[39]
Peru-15	*V. cholerae* O1 El Tor Inaba strain isolated in Peru in 1991	93	Cohen et al[40]
638	*V. cholerae* O1 El Tor Ogawa strain C7258	100	Garcia et al[41]
JBK70	*V. cholerae* O1 El Tor Inaba strain N16961	89	Levine et al[42]
CVD-111	*V. cholerae* O1 El Tor Ogawa strain N16117	81	Tacket et al[45]
CVD-103 HgR	*V. cholerae* O1 Classical Inaba strain 569B	65–100	Levine et al[43] Losonsky et al[51] Tacket et al[52] Tacket et al[48]
Bengal-15	*V. cholerae* O139 strain MO10	83	Coster et al[56]
CVD-112	*V. cholerae* O139 strain AI1837	84	Tacket et al[46]

Abbreviation: *V. cholerae, Vibrio cholerae.*

studies that investigate immunogenicity without subsequent challenge have been performed. Studies that highlight specific benefits or pitfalls of challenge studies have been included for illustrative purposes. Two recent Cochrane Database reviews cover the spectrum of both oral and parenteral cholera vaccines.[17,34]

Nonliving oral vaccines

The immune response and protective efficacy of two oral nonliving cholera vaccines were tested in volunteers.[35] One of the vaccines contained heat-killed classical Inaba and Ogawa strains and formalin-treated El Tor Inaba (whole vibrio vaccine). The other contained the same whole vibrios plus purified subunit B of the cholera toxin (whole vibrio-B subunit). The vaccines were administered orally at 2-week intervals for a total of three doses. There was no reactogenicity reported in North American volunteers following immunization with either vaccine. There was a significant rise in serum vibriocidal antibody titers in 10/14 (71%) volunteers who received the whole vibrio vaccine and 17/19 (89%) volunteers who received the whole vibrio-B subunit vaccine. Four weeks after completing immunization, volunteers were challenged with El Tor Inaba strain N16961. Protective efficacy was 56% in volunteers who received whole vibrio vaccine and 64% in those that received whole vibrio-B subunit.[35] These results were validated in a field efficacy trial in Bangladesh in which the whole vibrio and whole vibrio-B subunit vaccines elicited 58% and 85% protection, respectively, 6 months after vaccination.[36] Levels of protection elicited by the whole vibrio and whole vibrio-B subunit vaccines at 12 months were 53% and 62%, respectively.[37] Unfortunately, the efficacy in children aged $\leq$5 years of age was 31% for whole vibrio and 38% for whole vibrio-B subunit at 12 months after vaccination.[37]

These studies highlight the utility of challenge studies prior to initiating large-scale field trials in endemic areas, while also illustrating the differences in short-term protection measured during challenge studies and the long-term protection desired of a vaccine for use in endemic regions. Additionally, the difference in sustained efficacy between adult volunteers and young children is shown. These preliminary studies led to further efficacy trials and eventual licensure of the whole vibrio-B subunit vaccine as Dukoral. Other volunteer studies of nonliving oral vaccines in multiple countries have been reviewed.[38]

Live attenuated oral vaccines

V. cholerae O1

Texas Star, a derivative of El Tor Ogawa strain 3083, was attenuated using chemical mutagenesis with nitrosoguanidine. This strain produces the B but not the A subunit of cholera toxin. The vaccine was given at doses of $10^5 - 5 \times 10^{10}$ organisms as either one or two doses 1 week apart. Sixteen of the 68 (24%) vaccinees developed diarrhea following vaccination that was not dose dependent. All doses induced serum vibriocidal antibodies in 63/68 vaccinees (93%), but antitoxin antibodies were elicited in only 11/42 (26%) and 9/26 (35%) after one or two doses, respectively. Eight vaccinees that received a single dose of either 10^8 or 10^{10} organisms and four unvaccinated controls were challenged with 10^6 El Tor Ogawa strain 3083 organisms 4–6 weeks after vaccination. None of the control volunteers developed diarrhea after challenge, although they excreted the strain and had serological responses, suggesting the 3083 strain had diminished pathogenicity. Vaccinees who received a single dose of 5×10^{10} Texas Star organisms were challenged with 10^6 virulent El Tor Ogawa strain E7946 organisms to determine the efficacy against challenge with a homologous serotype. To determine whether Texas Star was protective against challenge with El Tor vibrios of heterologous serotype (Inaba), volunteers that received two doses of 10^9 or 2×10^{10} Texas Star organisms were challenged with 10^6 El Tor Inaba strain N16961 5–7 weeks after completion of vaccination. Texas Star had an overall efficacy of 61% against homologous or heterologous serotypes in challenge models that induced clinical disease in 70%–80% of control volunteers. Although this vaccine elicited vibriocidal antibody responses and was moderately protective against homologous and heterologous challenge, the random nature of nitrosoguanidine mutagenesis makes identification of the precise genetic mechanisms of attenuation difficult to establish. Without knowledge of the genetic mechanisms of attenuation, reversion to virulence is a theoretical possibility.[39] Due to these uncertainties, the vaccine has not been pursued. However, these challenge studies established that live attenuated oral cholera vaccines can elicit protection in volunteers and provided the groundwork for development of live attenuated oral cholera vaccines using recombinant DNA technology.

Recombinant DNA technology has been used to develop a number of vaccines against *V. cholerae*, many of which have been tested by volunteer challenge studies.[40–49] Vaccine strain JBK70 was derived from El Tor Inaba strain N16961 by deletion of the genes for both A and B subunits of the cholera toxin. Volunteers received 10^6, 10^8, or 10^{10} JBK70 organisms with 1/4 (25%), 2/5 (40%), and 4/5 (80%) developing diarrhea, respectively.[43] Fourfold or greater rise in vibriocidal antibody titers was observed in all 14 (100%) vaccine recipients. One month after vaccination, volunteers were challenged with parental strain N16961 and the vaccine had a protective efficacy of 89%. Despite high levels of protective efficacy, significant reactogenicity limited the further utility of this strain as a vaccine.[43] Evaluation of this vaccine in challenge studies indicated that genetically engineered strains could be designed that would confer protection; however, these studies also provided evidence that other factors besides cholera toxin significantly contribute to disease.[43]

Peru-15 is a live attenuated strain derived from El Tor Inaba strain N16961. The mechanisms of attenuation include deletion of the cholera toxin gene element, defective motility, and inability to recombine with homologous DNA. In a randomized, double blind, placebo-controlled trial, volunteers received either 2×10^8 cfu of Peru-15 or placebo (buffer alone). Following a single dose of Peru-15, 39/40 (97%) vaccinated volunteers had a fourfold or greater rise in vibriocidal antibody titers. Volunteers were challenged 3 months after vaccination with El Tor Inaba strain N16961 and Peru-15 demonstrated protective efficacy of 93% against any diarrhea and 100% against severe or moderate disease.[40]

V. cholerae 638 is a derivative of El Tor Ogawa strain C7258 attenuated by deletion of cholera toxin and disruption of the hemagglutinin/protease coding sequence by insertion of *Clostridium thermocellum* endoglucanase A gene. Following vaccination, 96% of *V. cholerae* 638 recipients demonstrated fourfold or greater rise in vibriocidal antibody titers.[41] Two challenge studies were performed one month after vaccination, the first with attenuated strain El Tor Ogawa 81. After *V. cholerae* 81 challenge, there was excretion of *V. cholerae* 81 in the feces of only 2/5 (40%) vaccinated volunteers and 5/5 (100%) controls. None of the vaccinated volunteers and 3/5 (60%) controls had diarrhea after challenge. Based on these results, a second study using challenge with virulent El Tor Ogawa 3008 was performed. The virulent strain caused diarrhea in 7/9 (78%) controls and none of the vaccine recipients. *V. cholerae* 638 was 100% protective against diarrhea in this group of 12 volunteers.[41]

Derived from wild-type El Tor Ogawa strain N16117, CVD 111 was also tested using the volunteer challenge model. The N16117 strain was used as a parent strain due to lower virulence than strain E7946, the parent of CVD 110 that was found to be overly reactogenic.[47] CVD 111 was attenuated by deletion of the virulence cassette, which includes the genes for cholera toxin (*ctxAB*), core-encoded pilus (*cep*), zonula occludens toxin (*zot*), and accessory cholera enterotoxin (*ace*). Additionally, *ctxB*, the B subunit of cholera toxin, and mercury resistance gene, were introduced. CVD 111 was given in a single oral dose and was noted to be mildly reactogenic with 3/25 (12%) volunteers developing diarrhea after vaccination. Ogawa vibriocidal antibodies were detected in 23/25 (92%) vaccinees. Thirty-five days after vaccination, volunteers were challenged with El Tor Ogawa strain 3008. Vaccine efficacy was 81% and stool volume was significantly less in vaccinees who developed diarrhea than in controls.[45] Additional volunteer trials were undertaken to determine the safety and immunogenicity of combination CVD 111 (El Tor Ogawa) with CVD 103-HgR (classical Inaba).[49,50]

CVD 103-HgR was engineered from the classical Inaba strain 569B. It is attenuated by deletion of 94% of the cholera toxin A subunit and insertion of a mercury ion resistance gene into the hemolysin A locus. Multiple studies in North American volunteers have shown excellent immunogenicity and efficacy after a single dose.[42,48,51,52] Vibriocidal antibody increases of fourfold or greater were seen in 39/43 (91%) CVD 103-HgR recipients.[52] Protective efficacy of 100% against homologous challenge has been demonstrated as early as 8 days and as late as 6 months after vaccination.[48] CVD 103-HgR is also protective against challenge with biotype-heterologous O1 El Tor Inaba or Ogawa (it has about 65% protective efficacy for as long as 3 months after immunization).[42,52] CVD 103-HgR was licensed in the 1990s for use in several countries based on the results of these challenge studies.[53]

When CVD103-HgR was tested in Indonesian children (aged 5–9 years), a tenfold higher dose was required to obtain similar seroconversion rates to those identified in North American volunteer studies.[54] Potential causes of this reduced immunogenicity in endemic areas include background intestinal immunity, which could potentially interfere with the vaccine's ability to infect and elicit vibriocidal responses, and the presence of small bowel bacterial overgrowth, which may inhibit the vaccine strain.[54,55] The role of small bowel bacterial overgrowth on vibriocidal antibody response to CVD 103-HgR was evaluated in Chilean schoolchildren and increased peak H_2 (measurement of bacterial overgrowth) was associated with decreased seroconversion.[55] Additionally, the *V. cholerae*-specific cellular antibody responses to cholera toxin and LPS for volunteers vaccinated with CVD 103-HgR followed by challenge with classical Inaba strain 569B were evaluated but did not correlate with protective immunity.[51]

V. cholerae O139

Bengal-15 is a nonmotile derivative of vaccine prototype Bengal-3. To create Bengal-3, O139 strain MO10 was attenuated by deletion of virulence genes *ctxAB*, *zot*, *ace*, and *cep* as well as disruption of *recA* and insertion of *ctxB*.[56,57] Bengal-15 was given in a single, oral dose to volunteers and was well tolerated without causing diarrhea. Vibriocidal titers were detected in 3/4 (75%) volunteers receiving Bengal-15. Challenge with O139 was performed 1 month after vaccination and Bengal-15 demonstrated protective efficacy of 83%.[56]

Attenuation of O139 strain AI1837 was performed by deletion of the genes *ctxAB*, *zot*, *ace*, and *cep* along with insertion of cholera toxin B subunit and a mercury resistance gene into the hemolysin A gene creating vaccine candidate CVD 112. To determine the optimal dose, a single oral dose of either 5×10^6 or 5×10^8 cfu CVD 112 was given to volunteers.[46] None of the volunteers who received the lower dose, and only 3/6 (50%) volunteers who received the higher dose, developed diarrhea. No systemic symptoms were reported. There were no vibriocidal antibodies detected after vaccination with either dose. Five weeks after vaccination, volunteers were challenged with O139 AI1837. CVD 112 elicited a protective efficacy of 84% despite the absence of detectable vibriocidal antibodies.[46] *V. cholerae* O139 serogroup strains differ from the O1 serogroup strains in that they produce a capsule of polymerized O-antigen molecules that are not covalently linked to the core polysaccharide.[58,59] It has been hypothesized that this capsule competitively interferes with binding of antibody to the core linked O-antigen resulting in ineffective complement fixation and decreased serum bactericidal activity.[58–60] The differences in detection of vibriocidal responses following vaccination with O139 strains with similar protective efficacy (Bengal-15 83% and CVD 112 84%) suggest that, unlike for O1 serogroup strains, vibriocidal antibodies may not be good predictors of protection against the O139 serogroup. Alternatively, differences in the vibriocidal methods used could account for the lack of responses described in some studies.[46,56] It has been shown that, unlike O1 strains, assay conditions, including diluents, level of complement, and concentration of indicator bacteria, may significantly affect O139 susceptibility to antibody and complement-mediated killing.[60] Later studies, however, also failed to correlate vibriocidal antibody titers with protection against *V. cholerae* O139, despite detection of robust responses.[61]

Hybrid vaccines

Using the *Salmonella* Typhi (*S.* Typhi) vaccine strain Ty21a as a backbone, a typhoid-cholera hybrid vaccine (EX645) was developed and tested in volunteer challenge studies. Ty21a is a live attenuated strain of *S.* Typhi that has been extensively studied.[62–65] A plasmid containing the genes for LPS O-antigen of O1 Inaba was inserted into a rifampin-resistant (to facilitate selection of vaccine strain from stool), thymidine-dependent (to maintain the plasmid) strain of Ty21a. To allow expression of the cholera O-antigen on the surface, the *rfa* region of Ty21a was replaced with the homologous region from *Escherichia coli* K-12. Volunteers received three doses (on days 0, 2, and 4) of 10^{10} viable organisms with the plasmid. Following vaccination, 6/14

(43%) EX645 recipients had a fourfold or higher rise in vibriocidal antibody against O1 Inaba. Four weeks after vaccination, challenge was performed with El Tor Inaba strain N16961. The vaccine efficacy was only 25%. Although the efficacy was not significant, these results provide valuable information indicating that response against LPS O-antigen, the only *V. cholerae* antigen in the vaccine strain, contributes to protection.[44]

Conclusion

With careful attention to detail, it is possible for experienced investigators to safely and ethically perform volunteer challenge studies that can significantly aid vaccine development. There are many ethical considerations involved in the undertaking of challenge studies. Volunteers are subjected to potential harm and discomfort with no personal benefit. As with any volunteer study, it is critical to obtain informed consent and to ascertain that the participant fully understands the implications of their consent. Furthermore, the investigators are obligated to minimize risk as well as design appropriate studies so that the information obtained contributes to the scientific community and society at large.

There are many components that are required to carry out an ethical challenge study for vaccine development. Foremost of these is a pathogen that produces a self-limited or treatable disease.[2] It is of critical importance that both the investigators and facilities for these studies allow for appropriate management of the disease, including infection control (to prevent spread of the disease outside the setting of the trial) and treatment of the induced illness and potential complications.

Cholera provides an excellent example of a model system in which valuable information has been obtained through the use of challenge studies contributing to the development of several vaccine candidates and the rejection of vaccine candidates that had excessive reactogenicity, poor immunogenicity or poor protective efficacy. Cholera possesses many of the features desired of a disease to be studied in this manner: there is a well-established model of infection that induces reproducible rates of disease, there are no long-term sequelae of disease, and treatment with hydration and antibiotics are readily available in the experimental setting.

Studies to determine the protective efficacy of the whole vibrio B subunit vaccine provide a prime example both of the utility and some of the pitfalls of vaccine challenge studies. Challenge studies in North American volunteers identified a vaccine candidate that induced sufficient protection to warrant further investigation in large field trials.[35] However, there were distinct differences in efficacy between North American and Bangladeshi volunteers.[36] Additionally, the inclusion of children in the field trials highlighted the fact that studies carried out in healthy adults are not necessarily applicable to young children in endemic areas.[37] Multiple studies involving volunteers in endemic regions supported the initial findings of challenge studies.[36–38] The live attenuated vaccine CVD-103HgR was licensed in many countries in the early 1990s and the initial licensure was based exclusively on the results of challenge studies. The safety, immunogenicity, and practicality of this single-dose vaccine were validated post-licensure in numerous studies. Specifically, the practicality of a single-dose vaccine supported the use of CVD-103HgR during outbreaks.[66–68] In Micronesia, a retrospective analysis following outbreak intervention with single-dose CVD 103-HgR found a 79.2% efficacy in the target population.[67] It is important to consider differences in the volunteer population and the target populations in endemic areas as evidenced by field trials in endemic areas that showed lower rates of seroconversion and lower efficacy than North American trials;[36,37,54,55] however, challenge studies provide a cost-effective preview of the vaccine candidate before undertaking large-scale field trials and allow for the rejection of vaccine candidates with excess reactogenicity or poor immunogenicity.[39,43]

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Miller FG, Grady C. The ethical challenge of infection-inducing challenge experiments. *Clin Infect Dis*. October 1, 2001;33(7):1028–1033.
2. Kotloff KL. Human challenge studies with infectious agents. *J Investig Med*. Feb 2003;51 Suppl 1:S6–S11.
3. Levine MM. Experimental challenge studies in the development of vaccines for infectious diseases. *Dev Biol Stand*. 1998;95:169–174.
4. Levine MM. Immunogenicity and efficacy of oral vaccines in developing countries: lessons from a live cholera vaccine. *BMC Biol*. 2010;8:129.
5. Benyajati C. Experimental cholera in humans. *Br Med J*. January 15, 1966;1(5480):140–142.
6. Kaper JHJ. Oral Cholera Vaccines. In: Levine MM DG, Good MF, Liu MA, Nabel GJ, Nataro JP, Rappuoli R, editors. *New Generation Vaccines*. 4th ed. New York: Informa Healthcare USA, Inc; 2010: 506–515.
7. Cholera, 2009. *Weekly Epidemiological Record, Vol. 85, 31* 2010; http://www.who.int/wer. Accessed April 28, 2011.
8. Farmer P, Almazor CP, Bahnsen ET, et al. Meeting Cholera's Challenge to Haiti and the World: A Joint Statement on Cholera Prevention and Care. *PLoS Negl Trop Dis*. 2011;5(5):e1145.

9. Kaper J, Morris J, Levine M. Cholera. *Clinical Microbiology Reviews*. Jan 1995;8(1):48–86.
10. Mazana Casanova JaAE, MR. Jaime Ferran and the cholera vaccine. *Inmunologia*. 1992;11(1):32–36.
11. Levine MM, Kaper JB, Herrington D, Losonsky G, Tacket C, Tall B. The current status of cholera vaccine development and experience with cholera vaccine trials in volunteers. *Southeast Asian J Trop Med Public Health*. Sep 1988;19(3):401–415.
12. Cholera Vaccines: WHO position paper. *Weekly Epidemiological Record, Vol. 85, 13* 2010; http://www.who.int/wer. Accessed April 29, 2011.
13. Bishop AL, Camilli A. Vibrio cholerae: lessons for mucosal vaccine design. *Expert Rev Vaccines*. Jan 2011;10(1):79–94.
14. Ritchie JM, Rui H, Bronson RT, Waldor MK. Back to the future: studying cholera pathogenesis using infant rabbits. *MBio*. Apr 2010;1(1).
15. Holmgren J, Svennerholm AM. Mechanisms of disease and immunity in cholera: a review. *J Infect Dis*. Aug 1977;136 Suppl:S105–S112.
16. Attridge SR, Rowley D. Prophylactic significance of the nonlipopolysaccharide antigens of Vibrio cholerae. *J Infect Dis*. Nov 1983;148(5): 931–939.
17. Graves PM, Deeks JJ, Demicheli V, Jefferson T. Vaccines for preventing cholera: killed whole cell or other subunit vaccines (injected). *Cochrane Database Syst Rev*. 2010(8):CD000974.
18. Cash RA, Music SI, Libonati JP, Craig JP, Pierce NF, Hornick RB. Response of man to infection with Vibrio cholerae. II. Protection from illness afforded by previous disease and vaccine. *J Infect Dis*. Oct 1974; 130(4):325–333.
19. Suntharasamai P, Migasena S, Vongsthongsri U, et al. Clinical and bacteriological studies of El Tor cholera after ingestion of known inocula in Thai volunteers. *Vaccine*. 1992;10(8):502–505.
20. Levine MM, Black RE, Clements ML, Nalin DR, Cisneros L, Finkelstein RA. Volunteer Studies in Development of Vaccines Against Cholera and Enterotoxigenic *Escherichia coli:* A Review. In: T. Holme JH, MH. Merson, and R. Mollby, editor. *Acute Enteric Infections in Children. New Prospects for Treatment and Prevention.* Elsevier/North-Holland Biomedical Press; 1981:443–459.
21. Sack DA, Tacket CO, Cohen MB, et al. Validation of a volunteer model of cholera with frozen bacteria as the challenge. *Infect Immun*. May 1998;66(5):1968–1972.
22. Cohen MB, Giannella RA, Losonsky GA, et al. Validation and characterization of a human volunteer challenge model for cholera by using frozen bacteria of the new Vibrio cholerae epidemic serotype, O139. *Infect Immun*. Dec 1999;67(12):6346–6349.
23. Morris JG Jr, Losonsky GE, Johnson JA, et al. Clinical and immunologic characteristics of Vibrio cholerae O139 Bengal infection in North American volunteers. *J Infect Dis*. Apr 1995;171(4):903–908.
24. Pitisuttithum P, Cohen MB, Phonrat B, et al. A human volunteer challenge model using frozen bacteria of the new epidemic serotype, V. cholerae O139 in Thai volunteers. *Vaccine*. December 12, 2001;20(5–6):920–925.
25. Levine MM, Kaper JB, Black RE, Clements ML. New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development. *Microbiol Rev*. Dec 1983;47(4):510–550.
26. Tacket CO, Taylor RK, Losonsky G, et al. Investigation of the roles of toxin-coregulated pili and mannose-sensitive hemagglutinin pili in the pathogenesis of Vibrio cholerae O139 infection. *Infect Immun*. Feb 1998;66(2):692–695.
27. Glass RI, Svennerholm AM, Khan MR, Huda S, Huq MI, Holmgren J. Seroepidemiological studies of El Tor cholera in Bangladesh: association of serum antibody levels with protection. *J Infect Dis*. Feb 1985;151(2):236–242.
28. Peterson JW. Synergistic protection against experimental cholera by immunization with cholera toxoid and vaccine. *Infect Immun*. Nov 1979;26(2):528–533.
29. Pierce NF, Cray WC Jr, Sacci JB Jr. Oral immunization of dogs with purified cholera toxin, crude cholera toxin, or B subunit: evidence for synergistic protection by antitoxic and antibacterial mechanisms. *Infect Immun*. Aug 1982;37(2):687–694.
30. Levine MM. Immunity to cholera as evaluated in volunteers. In: Ouchterlony O, Holmgren J, editors. *Cholera and Related Diarrheas*. Basel, Switzerland: S. Karger; 1980:195–203.
31. Levine MM, Nalin DR, Rennels MB, et al. Genetic susceptibility to cholera. *Ann Hum Biol*. Jul–Aug 1979;6(4):369–374.
32. Tacket CO, Losonsky G, Nataro JP, et al. Extension of the volunteer challenge model to study South American cholera in a population of volunteers predominantly with blood group antigen O. *Trans R Soc Trop Med Hyg*. Jan–Feb 1995;89(1):75–77.
33. Nalin DR, Levine MM, Rhead J, et al. Cannabis, hypochlorhydria, and cholera. *Lancet*. October 21, 1978;2(8095):859–862.
34. Sinclair D, Abba K, Zaman K, Qadri F, Graves PM. Oral vaccines for preventing cholera. *Cochrane Database Syst Rev*. March 16, 2011(3):CD008603.
35. Black RE, Levine MM, Clements ML, Young CR, Svennerholm AM, Holmgren J. Protective efficacy in humans of killed whole-vibrio oral cholera vaccine with and without the B subunit of cholera toxin. *Infect Immun*. May 1987;55(5):1116–1120.
36. Clemens JD, Sack DA, Harris JR, et al. Field trial of oral cholera vaccines in Bangladesh. *Lancet*. July 19, 1986;2(8499):124–127.
37. Clemens JD, Harris JR, Sack DA, et al. Field trial of oral cholera vaccines in Bangladesh: results of one year of follow-up. *J Infect Dis*. Jul 1988;158(1):60–69.
38. Svennerholm AM. From cholera to enterotoxigenic Eschericia coli (ETEC) vaccine development. *Indian J Med Res*. Feb 2011;133(2): 188–196.
39. Levine MM, Black RE, Clements ML, et al. Evaluation in humans of attenuated Vibrio cholerae El Tor Ogawa strain Texas Star-SR as a live oral vaccine. *Infect Immun*. Feb 1984;43(2):515–522.
40. Cohen MB, Giannella RA, Bean J, et al. Randomized, controlled human challenge study of the safety, immunogenicity, and protective efficacy of a single dose of Peru-15, a live attenuated oral cholera vaccine. *Infect Immun*. Apr 2002;70(4):1965–1970.
41. Garcia L, Jidy MD, Garcia H, et al. The vaccine candidate Vibrio cholerae 638 is protective against cholera in healthy volunteers. *Infect Immun*. May 2005;73(5):3018–3024.
42. Levine MM, Kaper JB, Herrington D, et al. Safety, immunogenicity, and efficacy of recombinant live oral cholera vaccines, CVD 103 and CVD 103-HgR. *Lancet*. August 27, 1988;2(8609):467–470.
43. Levine MM, Kaper JB, Herrington D, et al. Volunteer studies of deletion mutants of Vibrio cholerae O1 prepared by recombinant techniques. *Infect Immun*. Jan 1988;56(1):161–167.
44. Tacket CO, Forrest B, Morona R, et al. Safety, immunogenicity, and efficacy against cholera challenge in humans of a typhoid-cholera hybrid vaccine derived from Salmonella typhi Ty21a. *Infect Immun*. Jun 1990;58(6):1620–1627.
45. Tacket CO, Kotloff KL, Losonsky G, et al. Volunteer studies investigating the safety and efficacy of live oral El Tor Vibrio cholerae O1 vaccine strain CVD 111. *Am J Trop Med Hyg*. May 1997;56(5):533–537.
46. Tacket CO, Losonsky G, Nataro JP, et al. Initial clinical studies of CVD 112 Vibrio cholerae O139 live oral vaccine: safety and efficacy against experimental challenge. *J Infect Dis*. Sep 1995;172(3):883–886.
47. Tacket CO, Losonsky G, Nataro JP, et al. Safety and immunogenicity of live oral cholera vaccine candidate CVD 110, a delta ctxA delta zot delta ace derivative of El Tor Ogawa Vibrio cholerae. *J Infect Dis*. Dec 1993;168(6):1536–1540.
48. Tacket CO, Losonsky G, Nataro JP, et al. Onset and duration of protective immunity in challenged volunteers after vaccination with live oral cholera vaccine CVD 103-HgR. *J Infect Dis*. Oct 1992;166(4):837–841.
49. Taylor DN, Tacket CO, Losonsky G, et al. Evaluation of a bivalent (CVD 103-HgR/CVD 111) live oral cholera vaccine in adult volunteers from the United States and Peru. *Infect Immun*. Sep 1997;65(9):3852–3856.
50. Taylor DN, Sanchez JL, Castro JM, et al. Expanded safety and immunogenicity of a bivalent, oral, attenuated cholera vaccine, CVD 103-HgR plus CVD 111, in United States military personnel stationed in Panama. *Infect Immun*. Apr 1999;67(4):2030–2034.

51. Losonsky GA, Tacket CO, Wasserman SS, Kaper JB, Levine MM. Secondary Vibrio cholerae-specific cellular antibody responses following wild-type homologous challenge in people vaccinated with CVD 103-HgR live oral cholera vaccine: changes with time and lack of correlation with protection. *Infect Immun*. Feb 1993;61(2): 729–733.
52. Tacket CO, Cohen MB, Wasserman SS, et al. Randomized, double-blind, placebo-controlled, multicentered trial of the efficacy of a single dose of live oral cholera vaccine CVD 103-HgR in preventing cholera following challenge with Vibrio cholerae O1 El tor inaba three months after vaccination. *Infect Immun*. Dec 1999;67(12):6341–6345.
53. Hill DR, Ford L, Lalloo DG. Oral cholera vaccines: use in clinical practice. *Lancet Infect Dis*. Jun 2006;6(6):361–373.
54. Suharyono Simanjuntak C. Safety and immunogenicity of single-dose live oral cholera vaccine CVD 103-HgR in 5–9-year-old. *Lancet*. 1992;340(8821):689.
55. Lagos R, Fasano A, Wasserman SS, et al. Effect of small bowel bacterial overgrowth on the immunogenicity of single-dose live oral cholera vaccine CVD 103-HgR. *J Infect Dis*. Nov 1999;180(5): 1709–1712.
56. Coster TS, Killeen KP, Waldor MK, et al. Safety, immunogenicity, and efficacy of live attenuated Vibrio cholerae O139 vaccine prototype. *Lancet*. April 15, 1995;345(8955):949–952.
57. Waldor MK, Mekalanos JJ. Emergence of a new cholera pandemic: molecular analysis of virulence determinants in Vibrio cholerae O139 and development of a live vaccine prototype. *J Infect Dis*. Aug 1994;170(2):278–283.
58. Johnson JA, Salles CA, Panigrahi P, et al. Vibrio cholerae O139 synonym bengal is closely related to Vibrio cholerae El Tor but has important differences. *Infect Immun*. May 1994;62(5):2108–2110.
59. Waldor MK, Colwell R, Mekalanos JJ. The Vibrio cholerae O139 serogroup antigen includes an O-antigen capsule and lipopolysaccharide virulence determinants. *Proc Natl Acad Sci U S A*. November 22, 1994;91(24):11388–11392.
60. Attridge SR, Qadri F, Albert MJ, Manning PA. Susceptibility of Vibrio cholerae O139 to antibody-dependent, complement-mediated bacteriolysis. *Clin Diagn Lab Immunol*. May 2000;7(3):444–450.
61. Saha D, LaRocque RC, Khan AI, et al. Incomplete correlation of serum vibriocidal antibody titer with protection from Vibrio cholerae infection in urban Bangladesh. *J Infect Dis*. June 15, 2004;189(12): 2318–2322.
62. Ferreccio C, Levine MM, Rodriguez H, Contreras R. Comparative efficacy of two, three, or four doses of TY21a live oral typhoid vaccine in enteric-coated capsules: a field trial in an endemic area. *J Infect Dis*. Apr 1989;159(4):766–769.
63. Germanier R, Fuer E. Isolation and characterization of Gal E mutant Ty 21a of Salmonella typhi: a candidate strain for a live, oral typhoid vaccine. *J Infect Dis*. May 1975;131(5):553–558.
64. Ivanoff B, Levine MM, Lambert PH. Vaccination against typhoid fever: present status. *Bull World Health Organ*. 1994;72(6):957–971.
65. Levine MM, Taylor DN, Ferreccio C. Typhoid vaccines come of age. *Pediatr Infect Dis J*. Jun 1989;8(6):374–381.
66. Cookson S, Stamboulian D, Demonte J, et al. A cost-benefit analysis of progrommatic use of CVD 103-HgR live oral cholera vaccine in a high-risk population. *International Journal of Epidemiology*. Feb 1997;26(1):212–219.
67. Calain P, Chaine J, Johnson E, et al. Can oral cholera vaccination play a role in controlling a cholera outbreak? *Vaccine*. June 23, 2004;22(19):2444–2451.
68. Cholera, 2007. *Weekly Epidemiological Record/Health Section of the Secretariat of the League of Nations*. August 1, 2008;83(31):269–283.