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ARTICLE

Genomic correlates of variability in immune response to an oral cholera vaccine

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Cholera is endemic to many countries. Recent major outbreaks of cholera have prompted World Health Organization to recommend oral cholera vaccination as a public-health strategy. Variation in percentage of seroconversion upon cholera vaccination has been recorded across populations. Vaccine-induced responses are influenced by host genetic differences. We have investigated association between single-nucleotide polymorphic (SNP) loci in and around 296 immunologically relevant genes and total anti-lipopolysaccharide (LPS) antibody response to a killed whole-cell vaccine, comprising LPS from multiple strains of *Vibrio cholerae*. Titers derived from standard vibriocidal assays were also analyzed to gain further insights on validated SNP associations. Vaccination was administered to 1000 individuals drawn from India. Data on two independent random subsets, each comprising ~500 vaccinees, were used for discovery of genomic associations and validation, respectively. Significant associations of four SNPs and haplotypes in three genes (*MARCO, TNFAIP3* and *CXCL12*) with AR were discovered and validated, of which two in *TNFAIP3* and *CXCL12* were also significantly associated with immunity (fourfold increase in vibriocidal titers). *CXCL12* is a neutrophil and lymphocyte chemoattractant that is upregulated in response to *V. cholerae* infection. LPS in the vaccine possibly provides signals that mimic those of the live bacterium. *TNFAIP3* promotes intestinal epithelial barrier integrity and provides tight junction protein regulation; possible requirements for adequate response to the vaccine. LPS is a potent activator of innate immune responses and a ligand of *MARCO*. Variants in this gene have been found to be associated with LPS response, but not with high vibriocidal titer level.
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INTRODUCTION

Major outbreaks of cholera, leading to several thousand deaths, in Zimbabwe in 2009 and in Haiti in 2010, prompted the World Health Organization to emphasize the administration of oral cholera vaccination.[1–4] Cholera is caused by toxicogenic strains of the Gram-negative bacterium *Vibrio cholerae*. Moderate to severe watery diarrhea, that appears within 1–5 days of ingestion of contaminated food or water, rapidly leads to dehydration and, if left untreated, to death. It is a significant cause of mortality in developing countries,[5] and is still endemic in many countries, including India.[6] Unfortunately, 13% of the world's population still lacks access to safe water.[7]

V. cholerae, has two major serogroups – O1 (serotypes Inaba and Ogawa) and O139 – that are responsible for most cholera cases and differ in the lipopolysachharide (LPS) structures of the outer membrane.[8] Immunity to cholera can be acquired.[9] WHO advocates vaccination for cholera.[10] The efficacy of currently available vaccines varies across populations; protective efficacy is unlikely to last more than 3 years.[11] A single-dose cholera vaccine that induces long-term immunity for those at risk remains an unfulfilled goal.[12] Persons of blood group O are more susceptible to cholera than non-O blood group persons.[13–15] However, the association between efficacy of cholera vaccines and O blood group has been inconsistent–negative in Bangladesh,[13] positive in Indonesia[16] and no association in India.[17] Inter-individual variability in immune response to vaccines impedes the control of infectious diseases. Primary failure, as assessed by postvaccination antibody levels, of vaccines is a problem; 2–10% for measles vaccine,[18,19] 5–20% for hepatitis B vaccine[20,21] and 23–40% for typhoid vaccine.[22,23] Data are unavailable for cholera vaccines. Twin studies have shown high heritability for antibody response (AR) to measles (89%), mumps (39%), rubella (46%), hepatitis B (61–77%), oral polio (60%), tetanus (44–64%), diphtheria (49%), *Haemophilus influenzae* type B (Hib) (51%) and other vaccines.[24] Polymorphic variants in HLA and other genes–such as, *IL1B, IL4R, IL6, IL10* and *TNF*–are associated or linked with AR to several vaccines.[18,25–30] Although measles, mumps and rubella vaccines are all attenuated-live vaccines that are administered simultaneously, the responses induced by these vaccines are influenced differently by host genetics.[24] Vietnam is the only country in the world with large cholera-endemic tracts where an oral vaccine for cholera has been widely used for a long time (since 1997).[31] Considerable variation in response among vaccinees was noted in a clinical trial of this vaccine[31] conducted in a non-endemic area of Vietnam (Son La province), although >90% of vaccinees seroconverted. However, in respect of the same vaccine, only ~50% of vaccinees seroconverted in the endemic area of West Bengal, India.[17]

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Vaccine response is not wholly determined by the genetic make-up of vaccine recipients, but also by the nature of microbial antigens and adjuvants.[32] Knowledge of key immunogenetic associations can be used to design a vaccine that circumvents immunogenetic restrictions.[33] Insights into the impact of genetic variation in vaccinees on AR has been obtained for some vaccines.[24,34,35] In this study, we have investigated, for the first time, whether variation in the genomic backgrounds of vaccinees contributed to variation in immunological response to the killed whole-cell bivalent oral cholera vaccine, which has been introduced in India in 2010.

METHODS

Study participants

Individuals ($n = 1000$), unrelated at least to the first-cousin level based on family history report, aged 14 years or older, inhabiting a socio-economically depressed locality of Kolkata (formerly, Calcutta), India, were recruited using a statistical sampling design,[34] with institutional ethical approval and written individual informed consent. Exclusions included: (a) pregnant and lactating women and (b) based on self-report, individuals who had ever been diagnosed with cholera or had suffered from diarrhea or vomiting during the week preceding recruitment. The sample of 948 vaccinees, whose data passed all the quality checks (explained later), was randomly split into two half-samples (HSs); one used for association-discovery and the other for association-validation.

Vaccination and collection of blood samples

With approval of the Drug Controller General of India, a two-dose vaccine (see online Supplementary Table S1 for composition) procured from Shantha Biotechnics, Hyderabad, India, orally administered 14 days apart, was used. The vaccine was stored and transported at 4–8 °C before administration. From each vaccinee, a blood sample was collected immediately before vaccination (Day 0 sample) and 28 days postvaccination (Day 28 sample).

Assessment of antibody level

Antibody level was assessed using two different methods as described below.

Total anti-LPS AR assay. Serum anti-*V. cholerae* (Inaba 569) LPS–that is, total anti-LPS antibody–was assayed using the Bio-Plex (Bio Rad, Hercules, CA, USA) platform (hereafter referred to as the 'Bioplex assay'). *V. cholerae* O1 (Inaba 569) LPS (List Biological, Cat. No.231) was conjugated to Bio Rad beads (COOH (042)) by Solulink (San Diego, CA, USA). Day 0 and Day 28 human sera were incubated with LPS beads with anti-*V. cholerae* LPS antibody and responses were detected using a cocktail of anti-human IgG-PE, IgA-PE and IgM-PE. A pooled serum with a high anti-LPS IgG/IgA/IgM response was used to create a standard curve that was assigned an arbitrary value of anti-LPS Ig ELISA units (EU)/ml. Responses (Day 0 and Day 28) in individual subjects were compared with the standard curve to calculate anti-LPS Ig EU/ml.

Vibriocidal assay. This assay was performed with *V. cholerae* O1 Inaba (OS-418), Ogawa (MAK757) and uncapsulated O139 (MO-10T4) strains using sera collected during pre- and postvaccine trials following published methods.[36] Commercially available guinea-pig serum (Rockland, Gilbertsville, PA, USA) was used as a source of complement. The sera (100 μl) were added to 100 μl of PBS in the first well to give twofold dilution and the subsequent dilutions were made reciprocally up to 4800. Reference rabbit antiserum against *V. cholerae* O1 Inaba, Ogawa and O139 were included in each set of assay as controls. In every batch of assay, sera obtained from a healthy volunteer and a high-titer antiserum obtained from one of the volunteers in this study were included as negative and positive controls, respectively.

Candidate genes and SNPs

We selected 296 autosomal genes from immunological pathways as candidates for this association study. Two thousand four hundred and eighty-eight SNPs from these genes (Supplementary Table S2 online), including about 2-kb upstream and and 1-kb downstream regions, were chosen from the HapMap database (www.hapmap.org) using a statistical protocol[37] that maximized 'informativeness' of selected SNPs. Individuals belonging to the first HS were genotyped at all the 2488 loci. Genotypes at those loci that showed statistically significant association with AR in the first HS were determined in individuals belonging to the second HS for validation of the discovered associations. Genotyping was done using the Illumina iSCAN (San Diego, CA, USA) platform using Golden Gate assays following protocols recommended by the manufacturer.

Statistical analysis

AR to vaccination was measured as the ratio (fold-change) of Day 28 postvaccination to prevaccination (Day 0) antibody levels (EU/ml). Individuals on whom AR could not be properly assayed (5%) were removed from analyses. Curation of genotype data included removal of (a) loci with $<90\%$ call rate (2% of loci), (b) non-polymorphic loci with MAF <0.05 (12.7% of loci with call rate $>90\%$), (c) loci significantly deviant from HWE (1% of polymorphic loci), and (d) individuals with $<90\%$ of loci with valid genotype calls (0.7%). After data-curation, 948 individuals and 2036 SNPs were included for further analysis. Analysis of variance (ANOVA) was performed to test equality of mean values of *ln*AR ($\log_e$-transformation was used to induce Normality) among subgroups of vaccinees. To avoid detection of spurious associations arising from systematic ancestry differences, population stratification analysis was performed using Eigenstrat.[38] Instead of using an arbitrary threshold to classify a vaccinee as a 'responder' or a 'non-responder', we sorted the vaccinees by ascending order of their AR and grouped the vaccinees into five pentile (20 percentile) groups (PGs). It is expected that the frequency of an allele that is significantly associated with response should increase (or decrease) from low responders to high responders. Detection of allelic association was done by estimating the correlation coefficient (r) between the MAF at each locus and mean AR of vaccinees belonging to the (PGs) and testing H_0: $r = 0$ using a permutation test. The false discovery rate procedure[39] was used to identify statistically significant values of r at the 5% level of significance. As correction for multiple testing when loci are in significant linkage disequilibrium (LD) remains a major statistical problem,[40,41] for single-locus association analysis, we circumvented the problem by selecting from each gene only the highly informative and minimally associated SNPs.[37] Four hundred and sixty-nine SNPs were thus selected; data on the remaining loci were kept aside for haplotype association analysis. Each significant allelic association was revalidated by confirming that the null hypothesis that the mean *log*AR values among the genotype classes are equal was rejected, using ANOVA. The standard regression procedure was used to estimate effect size (slope, β) of each significantly associated SNP. SD of effect size was estimated by bootstrapping 100 times; that is, by randomly choosing the appropriate number of samples with replacement from the original data and estimating β from the bootstrap sample and then calculating the empirical SD of the 100 estimates of β. Haplotypes were inferred using Phase.[42] Haplotype association analysis was performed using a permutation algorithm similar to that for single SNP markers.

RESULTS

Demographic characteristics of vaccinees

No statistically significant differences in the demographic characteristics of the vaccinees (Table 1) were noted between the association discovery and validation HSs in respect of age (mean ages were 34.24 ± 0.60 years and 34.36 ± 0.58 years; $p = 0.883$), proportions of vaccinees belonging to the two genders ($p = 0.602$) or the two religious groups ($p = 0.21$).

Inter-individual variation in pre- and post-vaccination antibody levels and AR

Large inter-individual variations of pre- and postvaccination antibody levels and also of AR to vaccination (fold-change) were noted (Table 2 and Supplementary Table S3 online). Mean values between the two HSs were not significantly different (Supplementary Table S3).

Table 1 Demographic characteristics of the vaccine recipients

		Half-sample 1 (Association discovery sample)		*Half-sample 2 (Association validation sample)*	
		Gender		*Gender*	
Religion	*Age group (years)*	*Male*	*Female*	*Male*	*Female*
Muslim					
	<30	74	21	47	30
	30–49	51	37	52	31
	≥50	11	11	11	14
	Total	136	69	110	75
Hindu					
	<30	45	73	53	56
	30–49	56	52	71	71
	≥50	21	22	15	23
	Total	122	147	139	150
Total		258	216	249	225

Table 2 Mean ± SD of antibody levels and response using bioplex and vibriocidal assays

Assay	*Variable*	n	*Mean*	*SD*
Bioplex[a]	Day 0 (EU/ml)	948	22.54	37.95
	Day 28 (EU/ml)	948	76.89	114.88
	Fold change	948	5.42	12.41
	ln(Fold change)	948	1.14	0.84
Vibriocidal titer	Day 0	935	58.32	111.97
	Day 28	936	127.66	179.78
	Fold change	935	14.74	54.55

[a]Total anti-LPS antibody level.

Table 3 Comparison of antibody response estimated using the vibriocidal and bioplex assays

	Fold change–bioplex assay					
Fold change–vibriocidal assay	*<2*	*2–3*	*3–4*	*4–8*	*≥8*	*Total*
<2	123	67	18	17	9	234
2–4	100	55	34	41	29	259
4–8	39	43	18	47	16	163
8–32	35	29	28	28	39	159
≥32	26	20	13	30	31	120
Total	323	214	111	163	124	935

χ^2 value = 135.8 (d.f. = 16, $p<0.0001$).
Contingency coefficient = 0.356 ($p<0.0001$).

The correlation between baseline and Day 28 antibody levels is positive and moderate (0.449), but significant ($p<0.0001$).

Antibody response

AR-estimates obtained from the two separate assays were significantly ($p<0.0001$) correlated (Table 3). As, by its very nature, the vibriocidal assay is less quantitative than the bioplex assay, in further analyses, unless specifically mentioned, AR as measured by the bioplex assay was used. Mean values of *ln*AR for the two HSs did not differ significantly ($p=0.564$) and were, 1.128 ± 0.038 and 1.160 ± 0.039, respectively. None of the possible covariates–religion, age and gender–had a statistically significant effect on *ln*AR, either individually or interactively (p-values for main effects and interactions were all >0.05). Therefore, all data were pooled in subsequent analyses.

Population stratification

No pair of vaccinees in the first HS shared >90% of the 2036 loci with identical genotypes; i.e., there was no evidence of cryptic relatedness among the vaccinees. There was also no evidence of population stratification as all individuals formed a single cluster in the scatterplot of the first three principal components (Figure 1).

Antibody levels and response in vaccinees belonging to the five PGs

Mean values of antibody levels and response were nearly equal for each PG between the HSs (Supplementary Table S4 online), but differed significantly ($P<0.05$) among the PGs (Table 4). (Mean values of fold-change based on the vibriocidal assay were consistently higher than those based on the bioplex assay.) Mean AR increased almost linearly from PG 1 through 4, after which there was a steep increase (Figure 2), which was also observed for values based on vibriocidal assay. The steep increase of mean AR in PG-5 is primarily because of the presence of some individuals who exhibited very high AR.

Association results: discovery and validation

In the discovery HS, correlations between mean AR and MAFs in PGs were found to be statistically significant at 159 SNP loci on 93 genes (of the 296 genes considered in this study; Supplementary Table S5 online). The associations of these 159 SNPs could be validated in HS-2 only for seven SNPs that are located in and around five genes (rs17180481 and rs17180600 in the flanking region and intron, respectively, of *MARCO* (2q14.2); rs598493 in an intron of *TNFAIP3* (6q23.3); rs3012694 and rs266087 in introns of *RFX3* (9p24.2); rs266087 in an intron of *CXCL12* (10q11.21); and rs6567272 in an intron of *TNFRSF11A* (18q22.1)). Mean AR for vaccinees belonging to the three genotypes for each of these seven SNP loci differed significantly for only the four SNPs in and around *MARCO, TNFAIP3* and *CXCL12* (rs17180481: $p=0.023$, effect size $=1.8 \pm 0.3$; rs17180600: $p=0.026$, effect size $=1.6 \pm 0.3$; rs598493: $p=0.001$, effect size $=3.9 \pm 0.8$; rs266087: $p=0.014$, effect size $=2.4 \pm 0.6$), but not for the remaining three. The MAF values in PGs are provided in Figure 3 for these four SNPs.

Haplotype association

For these three genes, data on additional SNPs, which were in significant LD with the SNPs included in the association analysis, were used to reconstruct haplotypes and estimate haplotype frequencies among the vaccinees. Figure 4 provides, for *MARCO, TNFAIP3* and *CXCL12,* the frequency of the haplotype that showed the maximum association with AR by PG. The strength of association (i.e., slope of the line) with AR increased significantly for haplotypes compared with the single SNPs in these genes, indicating that these three genes are important modulators of AR to the vaccine.

Concordance between anti-LPS antibody and vibriocidal responses

For the four loci that were validated to be significantly associated with AR, the proportions of individuals who elicited fourfold or higher

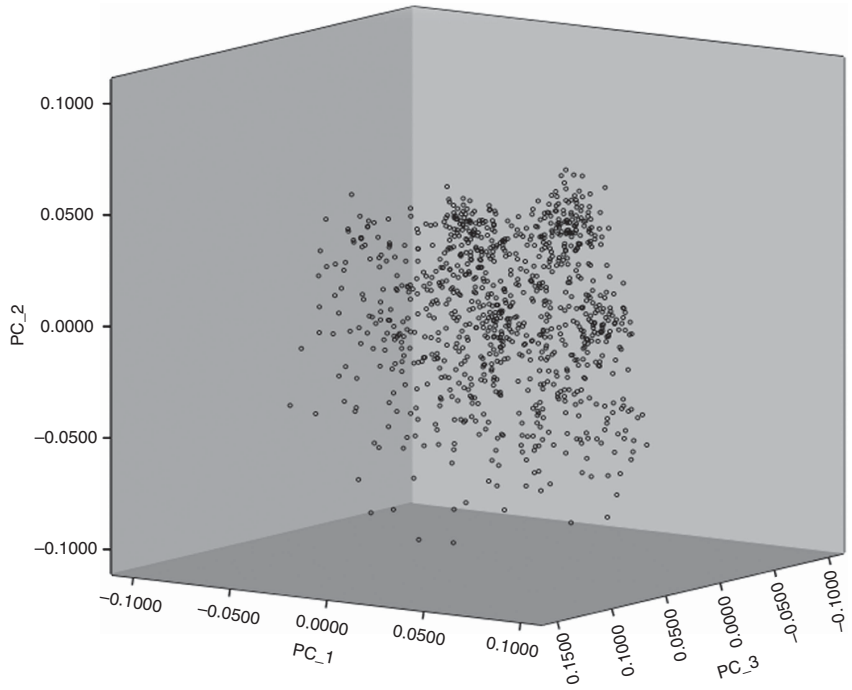

Figure 1 Scatterplot of vaccinees based on first three principal components extracted using data on 2036 SNPs show a single cluster and no evidence of population stratification.

Table 4 Means and SDs of total anti-LPS antibody levels on days 0 and 28, and antibody response, for the five pentile groups based on antibody response

Variable	*Pentile group*	n	*Mean*	*SD*
	1	190	41.58	71.51
	2	190	24.10	26.66
Baseline (Day 0)	3	190	17.92	21.03
Antibody level	4	190	17.16	17.56
	5	188	11.82	10.46
	Total	948	22.54	37.95
	1	190	52.61	86.82
	2	190	44.99	52.88
Day 28 antibody level	3	190	46.39	54.48
	4	190	72.73	77.74
	5	188	168.70	190.30
	Total	948	76.89	114.88
	1	190	1.31	0.15
	2	190	1.84	0.17
Antibody response	3	190	2.60	0.28
	4	190	4.20	0.73
	5	188	17.29	24.45
	Total	948	5.42	12.41

titer increase in the vibriocidal assay (that is likely to be suggestive of protective immunity,[43,44] although true protective immunity must be ascertained by vaccine challenge studies) significantly differed among the three genotypes at the locus only for two (rs598493 in *TNFAIP3*; $p = 0.001$ and rs266087 in *CXCL12*; $p = 0.014$) of these four

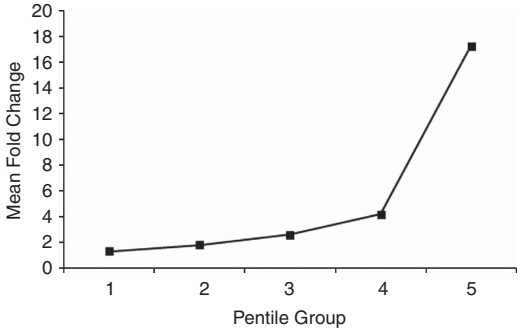

Figure 2 Mean values of antibody response of vaccinees belonging to the five pentile groups.

loci (Figure 5). Thus, although *MARCO* was found to be significantly associated with AR, it was not found to be associated with high vibriocidal titer levels.

DISCUSSION

WHO has recommended that cholera vaccination be provided in many geographical regions.[2] Protective immune response to *V. cholerae* that colonizes the intestinal mucosal surface without invasion of enterocytes[45] is believed to reside at the mucosal surface.[44] Orally administered vaccines maximize the intestinal secretory AR, and a long-lasting memory so that the primed intestinal immune system can rapidly respond to a subsequent exposure to antigen.[46] Serum vibriocidal activity, measured by a bactericidal assay requiring the fixation of complement by antibody that is bound specifically to vibrios, is used extensively as a marker of immunity to *V. cholerae*, but has limitations.[47,48] On the basis of

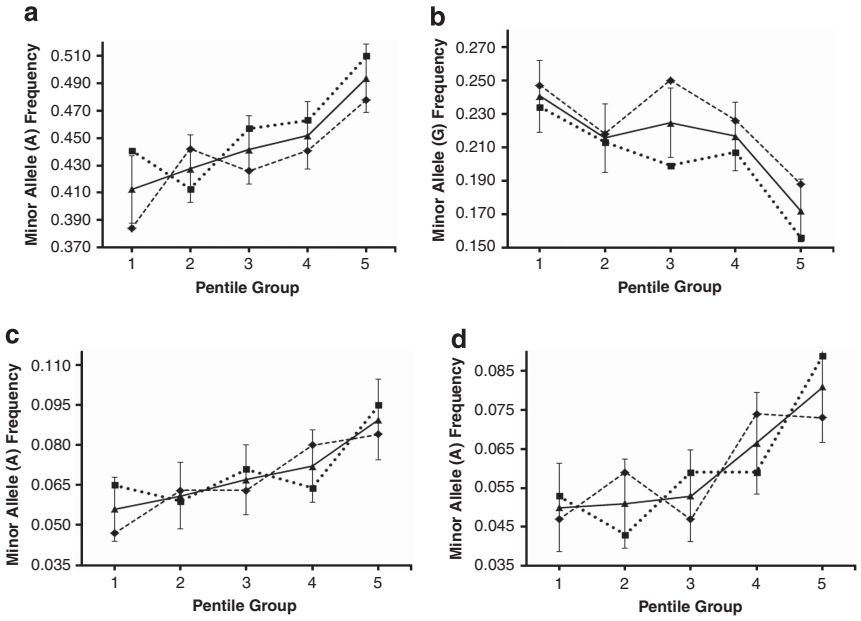

Figure 3 Minor allele frequencies at the significantly associated SNP loci in the association discovery (—◆—) and validation (---■---) half-samples, and in the pooled (—▲—) sample (±SD in the pooled sample) in PGs. (**a**) rs266087 (*CXCL12*), (**b**) rs598493 (*TNFAIP3*), (**c**) rs17180481 (*MARCO*) and (**d**) rs17180600 (*MARCO*).

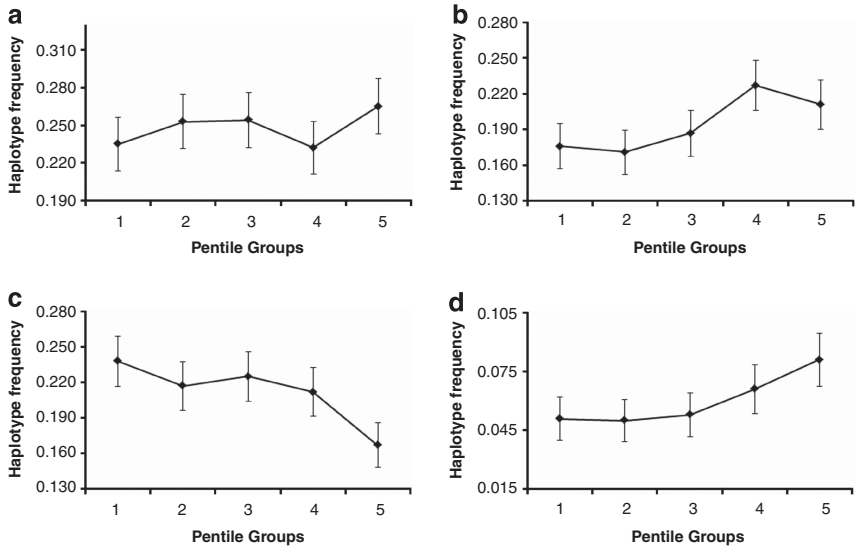

Figure 4 Frequencies of haplotypes containing the minor allele at the significantly associated SNP loci in the pooled sample PGs. (**a**) Haplotype GG**A**GCG of *CXCL12*, (**b**) Haplotype GA**A**GCG of *CXCL12*, (**c**) Haplotype GA**A**GC of *TNFAIP3*, (**d**) Haplotype **AA** of *MARCO*. Boldfaced and underlined alleles are those the minor alleles at the corresponding loci. (Another haplotype–GG**A**GC of *TNFAIP3*–had lower frequencies than those of the haplotype presented in this figure and was absent in three PGs.).

recent reccommendations[49] to measure LPS-response in multiple immunoglobulin isotypes and results[50,51] which indicate that levels of all immunoglobulin isotypes (IgG, IgM and IgA) determine the nature and extent of protection afforded by oral cholera vaccination, we have used the total anti-LPS AR and also the traditional vibriocidal assay.

Clinical trials of many oral vaccines have underscored the importance of understanding the reasons for variability in immune response to oral vaccines,[52] and the identification of genetic factors in the host linked to vaccine immunogenicity. It has been emphasized that such identification may result in adoption of 'inverse of personalized medicine' approaches to design vaccines to overcome barriers in nonresponding populations.[53] Susceptibility to cholera is significantly associated with a marker in the promoter region of *LPLUNC1* (rs11906665),[54] an innate immunity gene that likely has a role in modulating host inflammatory responses to *V. cholerae* infection.[55] We have discovered and validated that three genes–*MARCO*, *TNFAIP3* and *CXCL12*–has significant roles in modulating AR. However, *MARCO* was not significantly associated with increased vibriocidal titers. A single cell layer of the mucosal epithelium

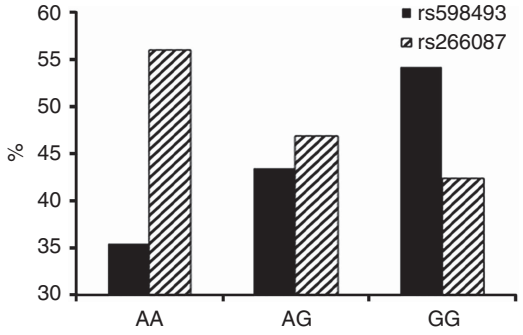

Figure 5 Percentages of vaccinees belonging to the three genotypes which elicited protective vibriocidal response (ie, seroconverted with fold-increase ≥ 4) upon vaccination for the two loci in *TNFAIP3* (rs598493) and *CXCL12* (rs266087).

essentially regulates intestinal homeostasis. This physical barrier is a prominent part of the innate immune system with damage-healing and infection-limiting properties. *CXCL12* is a homeostatic or constitutive chemokine that has a key role in healing by stimulating the activation of laminin-specific integrins.[56] *V. cholerae* infection produces strong inflammatory manifestations.[57] *CXCL12* is a neutrophil and lymphocyte chemoattractant that is upregulated in response to *V. cholerae* infection, resulting in increased recruitment of polymorphonuclear leukocytes to the site of infection.[58] The LPS in the cholera vaccine possibly provides signals that mimic those of the live bacterium, without actually causing disease. The TNF-induced protein 3 (*TNFAIP3*) is an ubiquitin-modifying enzyme and an essential negative regulator of inflammation, through the inhibition of NF-κB activation and prevention of TLR-mediated responses.[59] Tight junctions between intestinal epithelial cells mediate the permeability of the intestinal barrier. *TNFAIP3* has a role in promoting intestinal epithelial barrier integrity and in enabling maintenance of intestinal homeostasis through tight junction protein regulation;[60] possible requirements for adequate response to the whole-cell killed cholera vaccine used in this study. *TNFAIP3* has also been implicated in genome-wide studies of susceptibility of several autoimmune disorders.[61] *MARCO* is a type II transmembrane protein of the class A scavenger receptor family.[62] LPS, the major component of the vaccine used in this study, is a very potent activator of innate immune responses. LPS stimulation upregulates surface expression of *MARCO* in a dose- and time-dependent fashion.[63] *MARCO* is upregulated in bacterial infections in macrophages of most tissues; variants in this gene have crucial roles in bacterial binding.[64] No SNP in *MARCO* was found to be associated with high vibriocidal titer, possibly because different immunoglobulin isotypes are associated with anti-LPS and vibriocidal responses.[65]

The inferences of this study carried out in a cholera-endemic area, where many vaccinees possibly have prior exposure to the pathogen and consequently have high-baseline antibody levels (Table 2), may require validation in a non-endemic area. We, however, emphasize that the vaccine-response studies are more relevant and meaningful from a public-health viewpoint if conducted in endemic areas. In individuals with prior exposure to *V. cholerae*, the administered vaccine possibly acted as a booster, implying that individuals with high-baseline antibody levels are expected to show a low response (fold-change) to the vaccine, while individuals with low-baseline antibody levels are expected to show a high response. Although this feature was weakly observed (Table 2), the Day 0 and Day 28 antibody levels were significantly ($P<0.0001$) positively correlated ($r=0.449$). In this study we have used quantitative level of AR. Therefore, genomic associations with antibody fold-change detected in this study are unlikely to be affected by baseline antibody levels. However, it may be tricky is to relate the association between the level of AR and a SNP in a gene of the immune system with the level of immunological protection. In an endemic area, individuals with the lowest AR may be the best protected, while in a non-endemic area the reverse may be true. Although these issues pertaining to long-term protection remain unresolved, the genes that have shown significant association with AR are all biologically relevant. Functional studies on the markers found to be associated with AR need to be carried out, which together with the findings on *LPLUNC1*,[54,55] may provide a deep mechanistic understanding of the mode of action of the whole-cell killed oral cholera vaccine.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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