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MAJOR ARTICLE

The identification of enteric fever-specific antigens for population based serosurveillance

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Background*:* Enteric fever, caused by *Salmonella enterica* serovars Typhi and Paratyphi A, is a major public health problem in low and middle-income countries. Moderate sensitivity and scalability of current methods likely underestimate enteric fever burden. Determining the serological responses to organism-specific antigens may improve incidence measures.

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Methods: Plasma samples were collected from blood culture-confirmed enteric fever patients, blood culture-negative febrile patients over the course of three months and afebrile community controls. A panel of 17 *Salmonella* Typhi and Paratyphi A antigens was purified and used to determine antigen-specific antibody responses by indirect ELISAs.

Results: The antigen-specific longitudinal antibody responses were comparable between enteric fever patients, patients with blood culture-negative febrile controls, and afebrile community controls for most antigens. However, we found that IgG responses against STY1479 (YncE), STY1886 (CdtB), STY1498 (HlyE) and the serovar-specific O2 and O9 antigens were greatly elevated over a three-month follow up period in *S.* Typhi/*S.* Paratyphi A patients compared to controls, suggesting seroconversion.

Conclusions: We identified a set of antigens as good candidates to demonstrate enteric fever exposure. These targets can be used in combination to develop more sensitive and scalable approaches to enteric fever surveillance and generate invaluable epidemiological data for informing vaccine policies.

Keywords: enteric fever, serosurveillance, IgG antibodies, longitudinal responses

Key points

Poor enteric fever diagnostics limit our ability to estimate disease burden, which hinders vaccine introduction. We identified several protein and polysaccharide antigens that offer evidence of exposure and can aid serosurveillance studies.

Author contributions

EM: Wrote manuscript and performed analysis

LH: Wrote manuscript and performed analysis

NTVT: Wrote manuscript, ran experiments, and performed analysis

TTV: Ran experiments

CNNM: Ran experiments

ATT: Performed analysis

AK: Provided data

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PVV: Ran experiments

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THND: Ran experiments

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CMP: Performed supervision

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BACKGROUND

Enteric fever, caused by the *Salmonella enterica* serovars Typhi and Paratyphi A, is a significant health burden in many low and middle-income countries (LMICs) [1,2]. The disease caused by *S*. Typhi and *S*. Paratyphi A is clinically indistinguishable [3] and has an estimated global annual incidence of >14 million cases with >135,000 deaths [1]. The ongoing introduction of typhoid conjugate vaccines (TCV), as well as improvement of sanitation in many endemic areas has led to reduction in enteric fever incidence [1,4,5]. However, insufficient incidence data hinder accurate estimations of the disease burden in many countries, which prevents or delays vaccine implementation [6].

Enteric fever is diagnosed by isolating the infecting organism from blood, which permits antimicrobial susceptibility testing [4]. However, blood culture surveillance requires substantial laboratory infrastructure, has only moderate sensitivity, is reliant on healthcare-seeking behaviour to generate adequate incidence data and can detect only those with disease. Consequently, blood culture dramatically underestimates the actual burden of symptomatic and asymptomatic enteric fever [7–9]. A more passive approach to febrile disease surveillance, such as population-based serosurveillance can more accurately estimate the force of infection by measuring serological responses to antigens that are specific to the selected organism [8,10]. However, serosurveillance for enteric fever historically has suffered from a lack of reliable pathogen-specific antigens and is conventionally performed by measuring the serological response against the Vi polysaccharide, lipopolysaccharide O-antigen, and/or flagellar H-antigens [10–12]. Such an approach is compromised by poor-seroconversion against Vi, which is also the principal component of TCV. Furthermore, endemic communities often have high pre-existing antibodies against O- and H-antigens, which are associated with other *Salmonella* [10–14].

Here, by assessing the longitudinal antibody responses to multiple antigens in a cohort of Nepali patients presenting with febrile disease we aimed to generate new methods for enteric fever surveillance. We tested several conventional antigens in addition to various protein antigens previously identified as being potentially informative for identification of people having an immunological interaction with *S*. Typhi [14–16]. We measured IgG antibody responses over a three-month follow up period in patients with culture-confirmed *S*. Typhi and *S*. Paratyphi A infections and febrile patients with a negative blood culture.

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METHODS

Ethics

The original treatment trial from which the plasma samples originated was granted ethical approval by the Nepal Health Research Council, Kathmandu, Nepal and the Oxford Tropical Research Ethics Committee, Oxford, UK [17].

Study design and population

All plasma samples originated from an open-label, randomized, controlled trial, comparing gatifloxacin and ofloxacin for the treatment of uncomplicated enteric fever conducted at Patan Hospital, Nepal (trial registered as ISRCTN63006567) [17]. Patients were randomly assigned to a seven-day course of treatment [17]. Blood was collected in EDTA tubes on Day 1 (day of enrolment), Day 8, Day 28 (Month 1), and Day 90 (Month 3) and was immediately separated into cells and plasma. Only plasma samples from patients that presented with 72–96 hours of fever (Day 1) were analysed. Longitudinal samples were collected from 103 and 61 culture-confirmed *S.* Typhi (ST) and *S.* Paratyphi A (SPA) patients, respectively. Comparative samples were collected from 322 culture-negative febrile patients (FCN) (Table 1 and Table S1) and a single time-point plasma sample was collected from 49 afebrile, community controls (CCs).

Protein expression

Protein expression was induced in *E. coli* BL21(DE3) pLysS harbouring each of the plasmid constructs (pEK90–pEK109, Table S2) with 0.1mM isopropyl-b-D-thiogalactoside (IPTG) (Sigma Aldrich, UK) for 3 hours at 24°C, before harvesting bacteria by centrifugation (5,000 g, 4°C). For soluble proteins, pellets were resuspended by sonication in 50 mM phosphate buffer (pH 8) containing 300mM NaCl/10mM imidazole, and pelleted by centrifugation (16,000 g, 4°C, 30 minutes). Proteins were bound on nickel coated agarose beads (Ni-NTA, Invitrogen) by rocking the supernatants at 4 ^{o}C for 2 hours. The beads were loaded onto gravity flow columns (Qiagen, Germany), washed with 20mM imidazole in phosphate buffer, and proteins were eluted with 250mM imidazole in phosphate buffer. For insoluble proteins, bacterial pellets were first incubated in 8M urea (pH 7.8) in 20mM sodium phosphate/500mM NaCl. Proteins were eluted with 4M Urea (pH 3) in 20mM sodium phosphate buffer/500mM NaCl and recovered in 50mM sodium phosphate/500 mM NaCl.

Elisas

ELISAs to detect IgG in human plasma samples against selected antigens (Table 2) were performed as previously described [18]. Briefly, antigens were prepared in 50mM carbonate bicarbonate buffer at 1µg/ml for proteins and Vi, and at 15µg/ml for O2 and O9. 96 well flat-bottom ELISA plates (Nunc 2404, Thermo Scientific) were coated with 100 µl per well of each antigen overnight. Coated plates were washed and blocked with 5% fat free milk in PBS for 1 h before being washed and incubated with 100 µl per well of a 1:200 dilution of plasma at ambient temperature for 2 h. Plates were washed and incubated with 100 µl per well of alkaline phosphatase-conjugated anti-human IgG at ambient temperature for 1 hour, before

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treating with P-Nitrophenyl phosphate (SigmaFAST N1891, Sigma-Aldrich, United Kingdom) substrate for 30 minutes at ambient temperature. The absorbance was read at 405nm and 490nm using a microplate reader (Biorad). The end point absorbance was read as positive when its value was greater than that of the blank wells plus two times the standard deviation. ELISA units (EU) were expressed relative to standard curves, with best 4-parameter fit determined by a modified Hill Plot; standard curves were constructed for IgM and IgG independently. The reference standard of each ELISA was a pool of known antigen-specific antibody human plasma collected from a bank of Nepalese plasma. The standard curves were then generated from corresponding ODs of a (10-point of) 2-fold serial dilution of the plasma pool. The definition of one EU was the reciprocal of the dilution of the standard plasma that gave an OD value equal to 1 within the assay.

Data analysis

Data analyses were conducted in R software [19] (for details please refer to the supplement material). The antibody responses measured by ELISA were log transformed. The antibody responses for each time point, antigen and patient group were fitted to a loess smooth function. After analysis of normality of independent variables in the different groups (Shapiro-Wilk test) and homogeneity of the variances between the groups (Levene's test), non-parametric statistical tests were employed.

Differences between time points within the patient groups were assessed by the Friedman test followed by Wilcoxon's test for paired data with Bonferroni correction. A balanced design is necessary to conduct Friedman test, therefore we had to exclude participants without samples at each time point. Kruskal-Wallis test followed by Wilcoxon's test to calculate pairwise comparisons between group levels with Bonferroni correction were used to test the significance of the differences between the antibody titre measured at different time points within each group. The Spearman correlation coefficients were calculated to measure the correlation between the antibody responses to the different antigens at the different sampling time points. A p value <0.05 was considered statistically significant.

RESULTS

Longitudinal responses

We selected a panel of 17 antigens including Vi, O2, O9, and other protein antigens we hypothesised to show a differential response during the natural immunological history of enteric fever [14–16] (Table 2). IgG antibodies against these antigens were determined in plasma from ST, SPA, and FCN patients over the course of three months. We additionally measured the same responses in 49 afebrile community controls (CCs) as baseline measurements; these data were not considered in the longitudinal analysis. The antibody responses against each of the antigens were highly variable between individuals at all time points (Figure 1, S1, S2). The longitudinal antibody trajectories for each patient group were fitted to loess smooth functions to determine changes over time. Overall, the majority of antigen-specific IgG profiles generated comparable curves between the enteric fever (ST and

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SPA) and FCN patients and were unchanged from baseline (Figure S1 and S2), signifying that the longitudinal IgG responses against these antigens were largely uninformative.

We identified a subset of five antigens that exhibited distinct antibody dynamics between the ST/SPA and the FCN patients (Figure 1). The longitudinal IgG responses against O2, O9, and STY1498 (HlyE) generated the most distinct fitted curves and were markedly elevated in the ST and SPA groups in comparison to the FCN patients (Figure 1A-1C). Additionally, the IgG responses against STY1479 (YncE) and STY1886 (CdtB) in the SPA and ST groups were higher than in the FCN group and remained above baseline over the 3-month sampling period (Figure 1E and 1F). Notably, anti-STY1767 IgG antibodies were consistently higher in SPA patients than those in the FCN group; this was not replicated in the ST group (Figure S2E). Lastly, the antibody dynamics against Vi were not substantially different in the SPA group compared to the FCN group, while the Vi IgG trajectory elaborated an early peak in the ST group but returned rapidly to baseline (Figure 1D).

Peak antibody responses

We next disaggregated the antibody trajectories into their single time points. The antibody responses against the majority of antigens remained unchanged or slightly reduced in the FCN group over time (Figures 2, 3, S3–S5), with the notable exception of O9, which peaked on Day 8 and had declined by Month 1 (Figure 3C).

The antibody values against STY1886 (CdtB), SY1498 (HlyE), O2, and O9 in the ST and/or SPA patient groups increased significantly ($p<0.05$) from Day 1 to Day 8 and remained elevated at Month 1 (Figure 2, 3). Similarly, a significant ($p<0.05$) but less prominent increase in antibody response was also observed at Day 8 and Month 1 in comparison to Day 1 against all other antigens tested in the SPA patient group (Figure S3–S5), with the notable exception of Vi (Figure 3A). The IgG responses against STY1612 (Figure S3B), STY4539 (Figure S3C), STY1086 (Figure S4B) and STY1703 (Figure S5A) peaked at Day 8 in the ST group, with anti-STY1086 and anti-STY1703 IgG remaining high up to Month 1. Additionally, the anti-Vi IgG responses remained stable with time in the ST group and were comparable to those measured at Day 1 (Figure 3A). The majority of IgG trajectories exhibited a notable decline by Month 3, with anti-STY1479 (YncE; Figure 2A), anti-STY1498 (HlyE; Figure 2C), anti-O2 (Figure 3B), and anti-O9 (Figure 3C) having a comparable decrease in both the ST and SPA groups.

Correlation between antibody responses

Spearman correlation coefficients were calculated to determine the nature of the correlations between antibody responses on Day 8 (the peak of most antibody trajectories) within each patient group (Figure 4, S6–S9). The antibody responses to all protein antigens and Vi demonstrated a moderate to strong correlation with each other (rho>0.6) within the CC group (Figure 4A, S6). The anti-O2 and anti-O9 responses correlated less well (rho=0.4–0.7) to the majority of responses to other antigens (Figure 4A, S6). In comparison, the antibody responses in the FCN group correlated moderately with each other (rho>0.5), with the notable exceptions of anti-STY1498 (HlyE), anti-O2 and anti-O9, which correlated with each other,

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but exhibited a weak correlation to all other antigen specific responses (rho<0.5) (Figure 4B, S7).

The Day 8 antibody responses against all protein antigens and the Vi correlated moderately to strongly to each other in the SPA (Figure 4C, S8) and ST (rho>0.4) (Figure 4D, S9) groups, with the notable exception of STY1498 (HlyE; Figure 4C-D, S8, S9), which correlated poorly to all antibody responses (rho<0.2) due to its unique trajectory (Figure 1). Among SPA patients, rho values of 0.6 and 0.7 between anti-Vi and anti-STY1479 (YncE) and anti-STY1886 (CdtB) antibodies, respectively, were observed (Figure 4C). The anti-O2 and anti-O9 IgG responses correlated poorly to all other antigens and to each other in the SPA and ST groups, with rho values of 0.32 and 0.08, respectively (Figure 4C-D, S8, S9).

Longitudinal comparison of antibody titres

We lastly compared the median antibody responses between each patient group at each time point and to single time point CC and FCN plasma (Figure 5, 6, S10, S11, S12). All antibody responses, other than anti-STY1498 (HlyE; Figure 5C), anti-O2 (Figure 6B), and anti-O9 (Figure 6C) were comparable between groups on Day 1. The antibody concentrations against STY1498 (HlyE; Figure 5C), and STY1703 (Figure S12A) were significantly higher on Day 8, Month 1, and Month 3 in the ST and/or SPA groups compared to both the CC and the FCN controls ($p<0.05$ for all comparisons). The median IgG responses against STY1479 (YncE) and STY1886 (CdtB) in ST and SPA groups were higher in comparison to the FCN group on Day 8 and Month 1 (Figure 5A-B). Similarly, anti-O2 (Figure 6B) and anti-O9 (Figure 6C) IgG responses were elevated on Day 8, Month 1, and Month 3 in the ST and SPA groups. Notably, IgG against the serovar-specific O-antigen, along with anti-STY1498 (HlyE) IgG, were higher in the ST and SPA groups than the CC and FCN groups on Day 1 (Figure 5C, 6A, 6B). The median IgG values against the Vi were slightly but significantly increased on Day 8 in both SPA and ST groups compared to the FCN group, but these were comparable in convalescent samples at Month 3 (Figure 6A).

DISCUSSION

Serosurveillance for enteric fever is impaired by a lack of specific and sensitive informative antigens and methods. Here, we determined the antibody responses in enteric fever patients (blood-confirmed *S.* Typhi or *S.* Paratyphi A-infected) against 17 antigens and compared them to the responses in febrile patients with a negative blood culture and community controls (baseline). We tested protein antigens that we had previously considered as candidates for acute diagnostic markers and are largely conserved between *S.* Typhi and *S.* Paratyphi A, as well as the Vi, O2 and O9 antigens, that are commonly used to identify enteric fever patients, to identify biomarkers for enteric fever exposure.

The longitudinal antibody responses to most antigens tested were not significantly different between STY/SPA patients, FCN controls, or community controls. We identified a subset of protein antigens (STY1479 or YncE, STY1886 or CdtB, and STY1498 or HlyE) that generated distinct antibody trajectories in both STY and SPA groups compared to FCN

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patients, indicative of potential seroconversion. While antibodies against other antigens, such as STY1086, elaborated a similar increase with time, this change was small and likely of limited utility in serosurveilance. Anti-STY1767 antibodies were consistently higher in SPA compared to FCN patients, while ST patients showed such an increase only later. While anti-STY1767 antibodies in ST and SPA groups did not differ significantly, further investigations will need to determine whether responses to STY1767 can contribute to distinguishing the two infections. We additionally compared the antibody responses to Vi, O2, and O9 in enteric fever patients to febrile and afebrile controls. The early peak response observed against O9, and O2 (as well as HlyE) are likely a recall response from prior exposure to *S*. Typhi and/or *S*. Paratyphi A for these immunodominant antigens. Cross-reactivity with other Enterobacteriaceae should be considered and evaluated to ensure specificity of these antigens. In this analysis, we did see cross-reactivity between sera from *S*. Typhi and *S*. Paratyphi patients to O2 and O9 likely due to shared O12 antigen which is present in both O-antigen conformations. The O12 is also present in a number of invasive nontyphoidal *Salmonella* (NTS) and, therefore, these antigens may be less useful if used alone as a specific marker for enteric fever in populations where the burden of iNTS is high, such as Africa. Alternatively, the fact that *S*. Typhi patients generate a measurable antibody response against O2 from *S*. Paratyphi A (and vice versa for O9) may implicate anti-O12 responses, which is conserved between the two O-antigen conformations. Additionally, the baseline antibody responses against Vi in enteric fever patients suggest that anti-Vi antibodies are circulating in this community and the Vi is not suitable as a marker of enteric fever exposure. The use of Vi in this context is likely to be further hampered by ongoing TCV vaccination schemes and it is known that responses to Vi cannot distinguish between natural infection and vaccine-mediated exposure [20].

The protein antigens exhibiting the greatest potential for measuring enteric fever exposure play differing roles during infection. STY1886 (CdtB) is a component of typhoid toxin, which has a predicted role in symptom manifestation, eliminating host immune cells, and damaging host DNA [21–24]. The typhoid toxin is rarely found in non-typhoidal *Salmonella* (NTS) [25,26]. While its precise role in virulence and clinical presentation remains undefined [22], antibody responses against CdtB in enteric fever patients have been described previously [14,18,27]. STY1498 (HlyE) is a pore forming toxin which is absent from most NTS, but present in other Enterobacteriaceae including *Escherichia coli* and *Shigella* [28–30]. Like CdtB, the precise role of HlyE in virulence remains unclear; studies have suggested that HlyE promotes chronic infection, host cell invasion, and organ colonisation [40]. Antibodies against HlyE have been detected in plasma and mucosal responses in acute typhoid patients and thus HlyE has been identified elsewhere as a potential target for enteric fever serodiagnosis and serosurveillance [10,14,27,33]. Lastly, STY1479 (YncE) is a putative ATP-binding protein with unknown function but induced upon iron restriction in *E. coli* and involved in iron acquisition. In enteric fever, YncE has been identified as a potential marker for chronic carriers given its high immunoreactivity in carriers compared to acute or convalescent patients [15]. In our study, anti-YncE antibody responses were higher in enteric fever patients compared to febrile controls even in the acute stages of infection.

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This study has limitations. Even though we could determine potential seroconversion in a subset of antigen-specific responses, cross-reactivity with other Enterobacteriaceae may remain a problem. Additionally, while beyond the scope of this study, these antigens still cannot as yet distinguish between *S*. Typhi and *S*. Paratyphi A infection and development of models to predict the cause of infection is required. Given the limited sensitivity of blood culture, it is likely that some of the FCN patients were indeed enteric fever patients, and thus elevated antibody responses in this group was observed. Future studies will also need to record responses by other antibody subtypes that are relevant as already reported previously [34], as well as to include chronic carriers to confirm the use of these antigens for the purpose of identifying those individuals.

In summary, by investigating the serologic dynamics of enteric fever via determining the longitudinal responses to an array of antigens in a considerably large endemic population and comparing then to endemic febrile and healthy control groups, we identified protein antigens (i.e., HlyE, CdtB, and YncE) that are akin to findings from other groups supporting their role as discriminatory markers for detecting enteric fever patients [10,33–36], in line with them being largely specific to typhoidal *Salmonella* serovars. Further studies are underway to develop methods to distinguish between STY and SPA patients [10], as well as to optimally multiplex antigens to increase accuracy of serosurveillance and diagnostics even at early stages of infection. Whilst blood culture is a highly useful diagnostic method during symptomatic stages of enteric fever, serosurveillance is straightforward, scalable, and informative for vaccine policy. The data presented here can be used for developing mathematical inferences which can be applied to single samples (including dried blood spots assays), thereby creating a cost-effective and sensitive surveillance tool that can be made mobile and be used in endemic locations with minimal infrastructure. We conclude that the antigens and assay described here may offer a sea change in approaches to enteric fever surveillance, contributing towards the need for more of efficient and cost-effective data collection regarding enteric fever epidemiology.

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Table 1. Participant characteristics

		Community controls N=49	All patient groups N=486		Patient groups					
					FCN N=322		SPA N=61		ST N=103	
Sex	Female	NA	166	34.2%	111	34.5%	23	37.7%	32	31.1%
	Male		320	65.9%	211	65.5%	38	62.3%	71	68.9%
Age	mean (SD)	NA	17.3 (9.8)		17.9 (10.6)		16.8 (8.1)		15.6 (7.9)	
	NA		3		1		0		2	

FCN, culture-negative febrile patients; SPA, *S*. Paratyphi A-confirmed patients; ST, *S*. Typhi-confirmed patients; SD, standard deviation; NA, not available

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Table 2. *S.* Typhi/*S.* Paratyphi A antigens used in this study for serological testing

#	Ag	Gene name	Annotation
1	STY1372	pspB	Phage shock protein B
2	STY1612		Putative membrane protein – prophage associated
3	STY4539	pilL	Putative exported protein – type IV pili
4	STY0452	yajI	Putative lipoprotein – Prokaryotic homolog of protein DJ-1
5	STY0796	ybgF	Putative exported protein – Tol/pal system protein
6	STY1086		Putative lipoprotein
7	STY4190	yhjJ	Putative Zinc-protease
8	STY3208		Hypothetical protein – Unknown function
9	STY1767	nlpC	Putative lipoprotein – Endopeptidase
10	STY1498	hlyE	hemolysin
11	STY1703	ssaP	Putative secreted protein – T3SS
12	STY1522		Putative secreted protein – Choloylglycine secreted homolog
13	STY1886	cdtB	Cytolethal distending toxin subunit B homolog
14	STY1479	yncE	Possible ATP-binding protein
15	Vi		virulence antigen
16	O2		O antigens (SPA) (somatic, lipopolysaccharide)
17	O9		O antigens (ST) (somatic, lipopolysaccharide)

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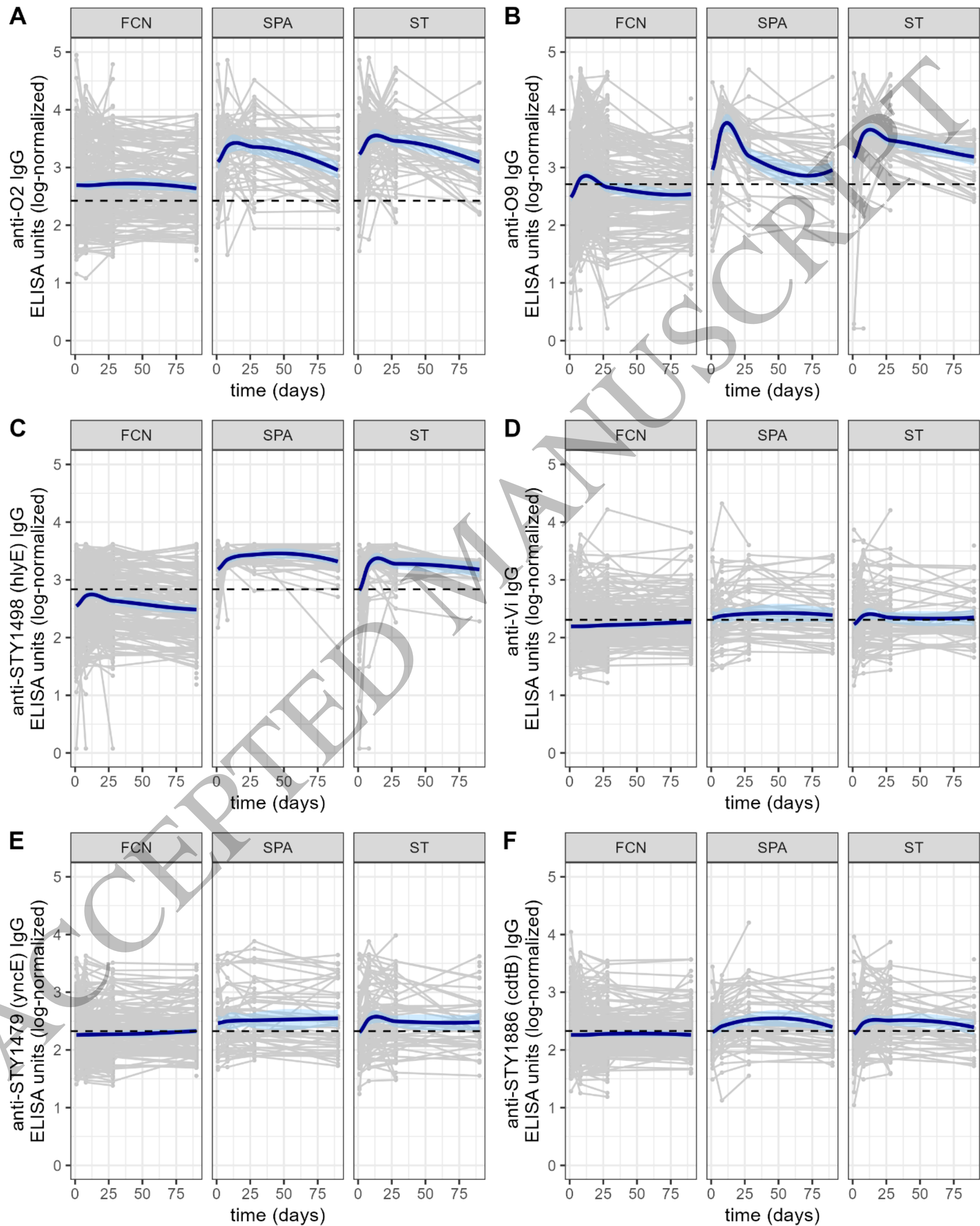
Figure 1. The longitudinal IgG responses against *S*. Typhi or *S*. Paratyphi A purified antigens in a Nepali cohort of febrile patients.



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IgG antibody responses against O2 (A), O9 (B), STY1498 (C), Vi (D), STY1479 (E), and STY1886 (F) over the course of three months in different patient groups. FCN, culture negative febrile patients, SPA, *S*. Paratyphi A confirmed patients, ST, *S*. Typhi-confirmed patients. Grey lines show antibody trajectories in individual patients and the blue line the fitted loess smooth function. Dashed lines represent the mean titre value within the control group of afebrile, community control samples for the respective antibody.

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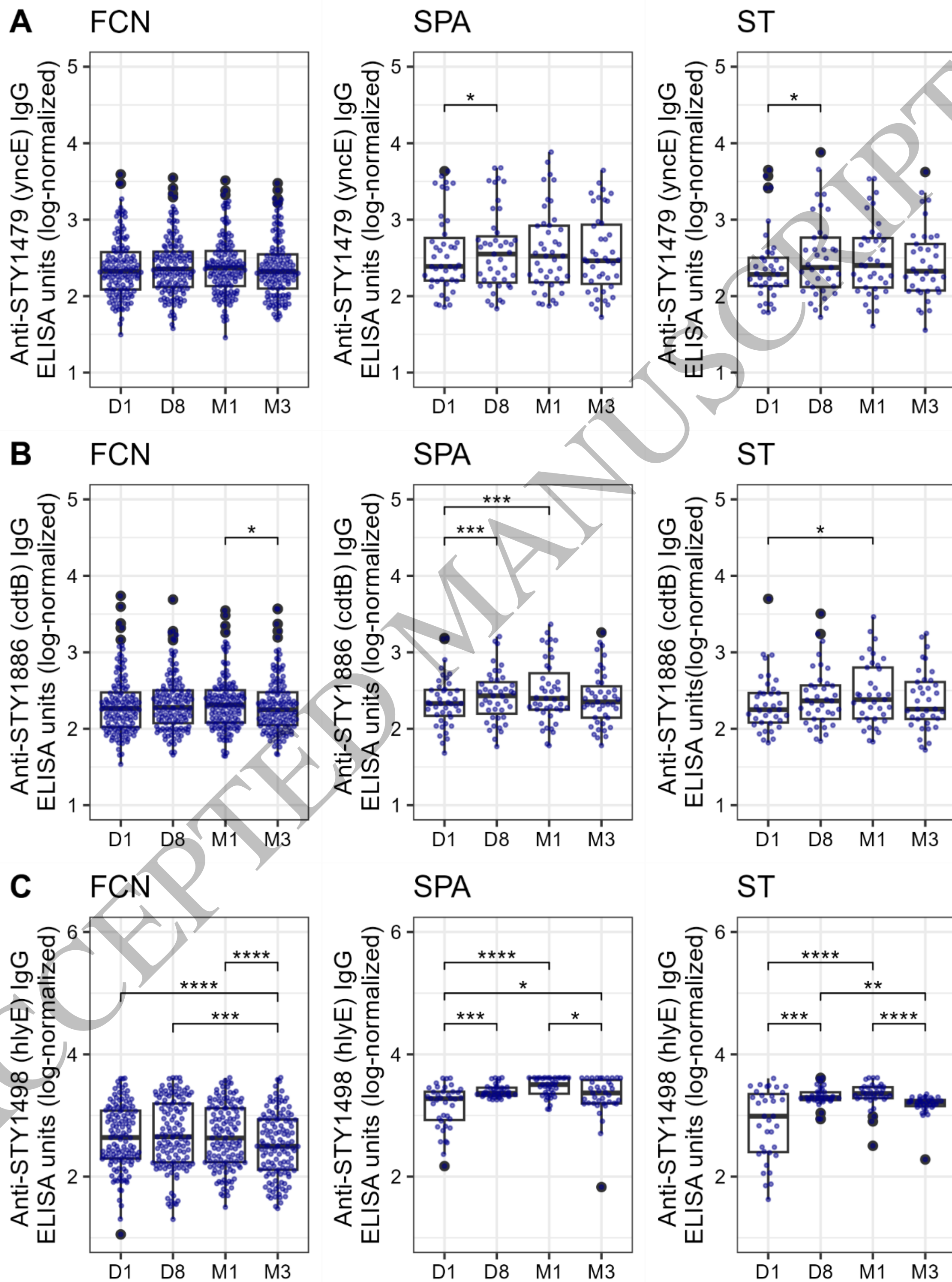
Figure 2. The distribution of serum IgG titres in a Nepali cohort of enteric fever patients and febrile culture-negative controls.



Boxplots showing IgG titres in febrile culture negative patients (FCN), *S*. Paratyphi A (SPA) or *S*. Typhi (ST) against STY1479 (A), STY1886 (B) and STY1498 (C) antigens over the course of three months. Each dot shows the

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antibody titre of an individual sample on day 1 (D1), day 8 (D8), month 1 (M1) and month 3 (M3). Differences between time points were assessed using Friedman test followed by pairwise comparisons using Wilcoxon signed-rank tests. P-values were adjusted using the Bonferroni multiple testing correction method. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

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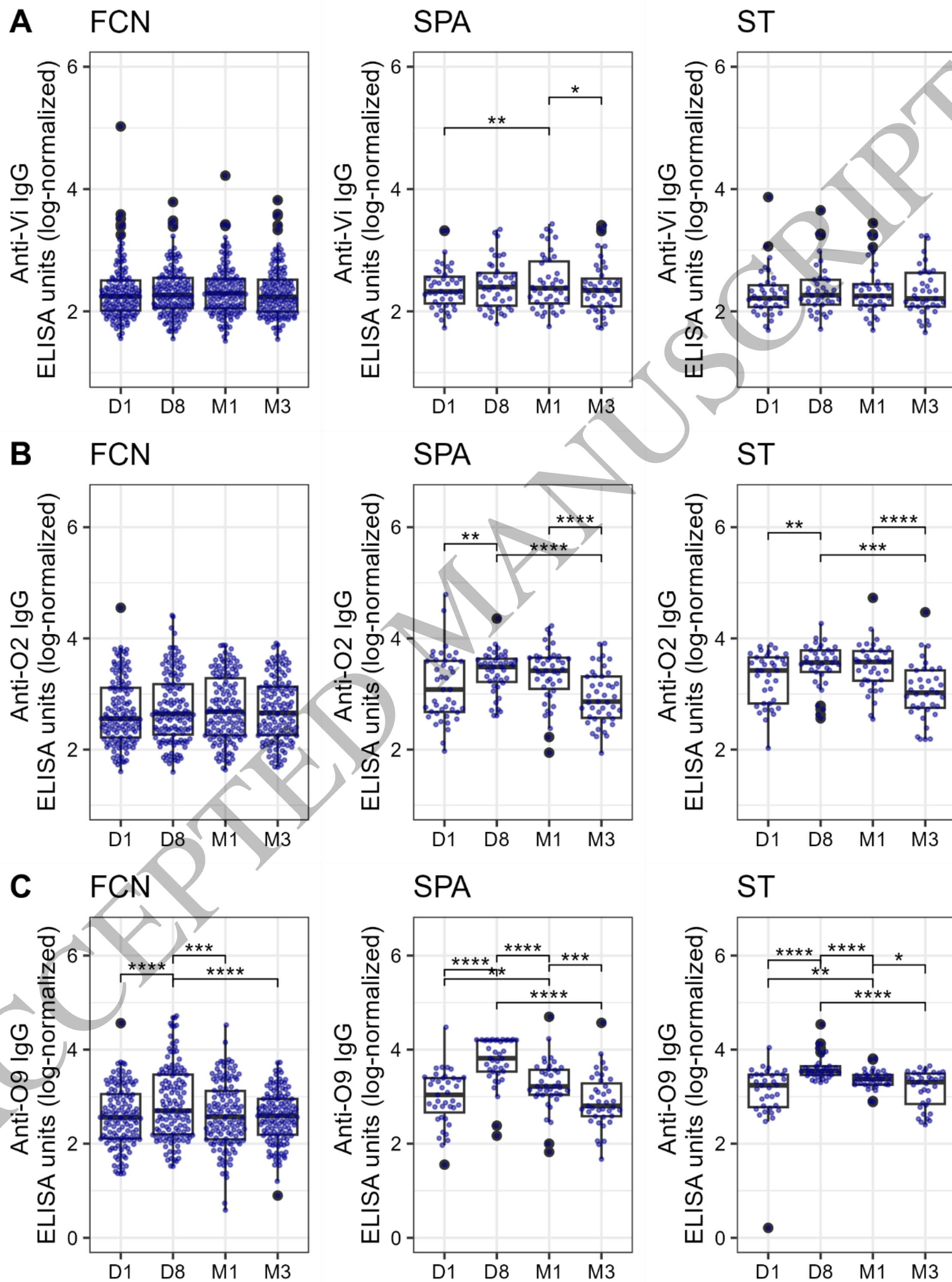
Figure 3. The distribution of serum IgG titres in a Nepali cohort of enteric fever patients and febrile culture-negative controls.



Boxplots showing IgG titres in plasma from febrile, culture-negative patients (FCN), *S*. Paratyphi A (SPA) or *S*. Typhi (ST) against Vi (A), O2 (B) and O9 (C) antigens over the course of three months. Each dot shows the

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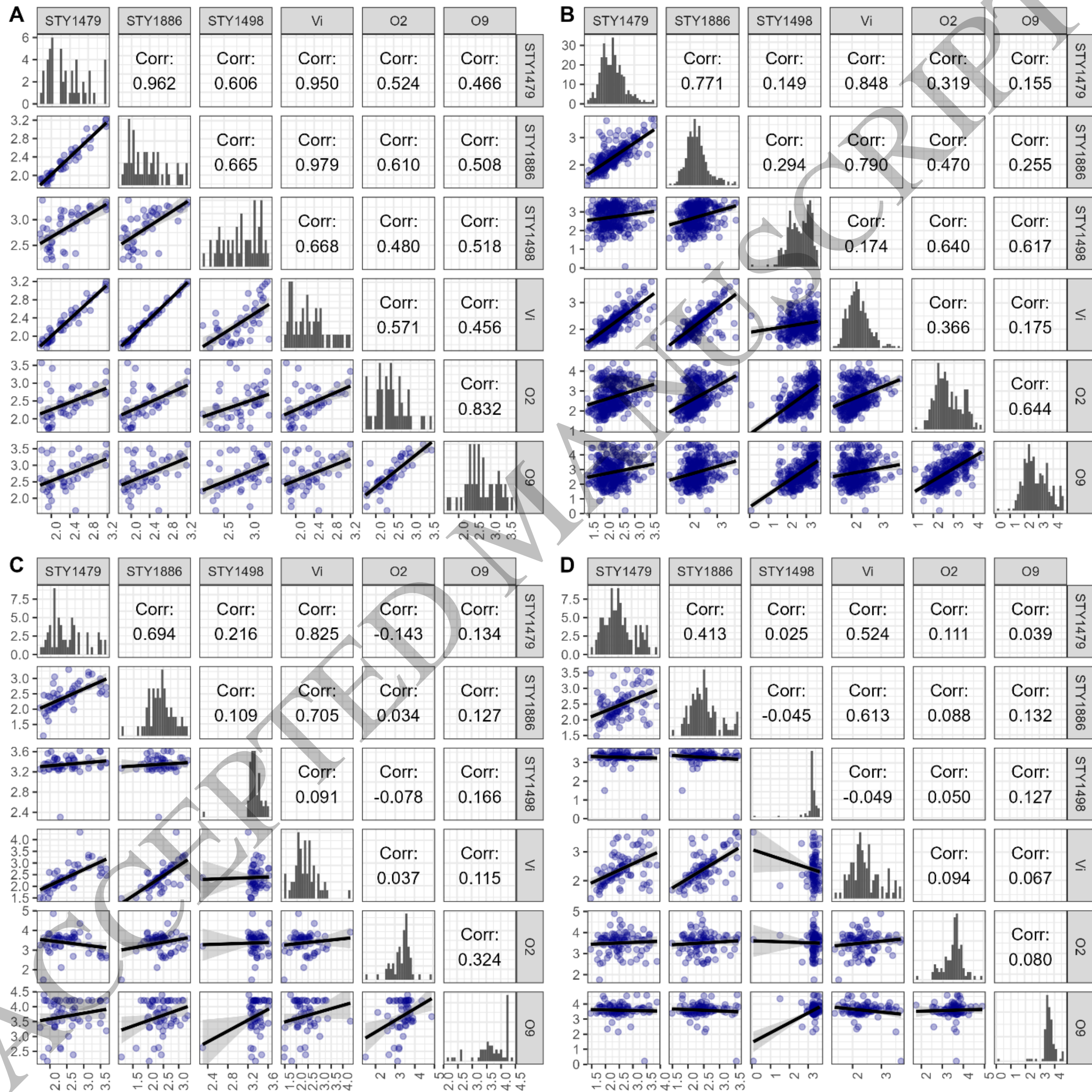
antibody titre of an individual sample on day 1 (D1), day 8 (D8), month 1 (M1) and month 3 (M3). Differences between time points were assessed using Friedman test followed by pairwise comparisons using Wilcoxon signed-rank tests. P-values were adjusted using the Bonferroni multiple testing correction method. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4. Correlation between antibody responses to selected *Salmonella* antigens on day 8.



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Spearman correlation among antigen-specific antibodies in community controls (A), from febrile, culture-negative patients (B), *S*. Paratyphi A confirmed patients (C) and *S*. Typhi confirmed patients (D). Histograms show the distribution of the of each antigen-specific IgG antibody measurement on the diagonal. Scatterplots below the diagonal represent the correlation of IgG measurements of the two antigens on a right angle to the plots. The numerals above the diagonal depict the Spearman correlation coefficient (rho) values of the mirrored plots.

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Figure 5. The distribution of serum IgG titres in a Nepali cohort of febrile patients and controls.

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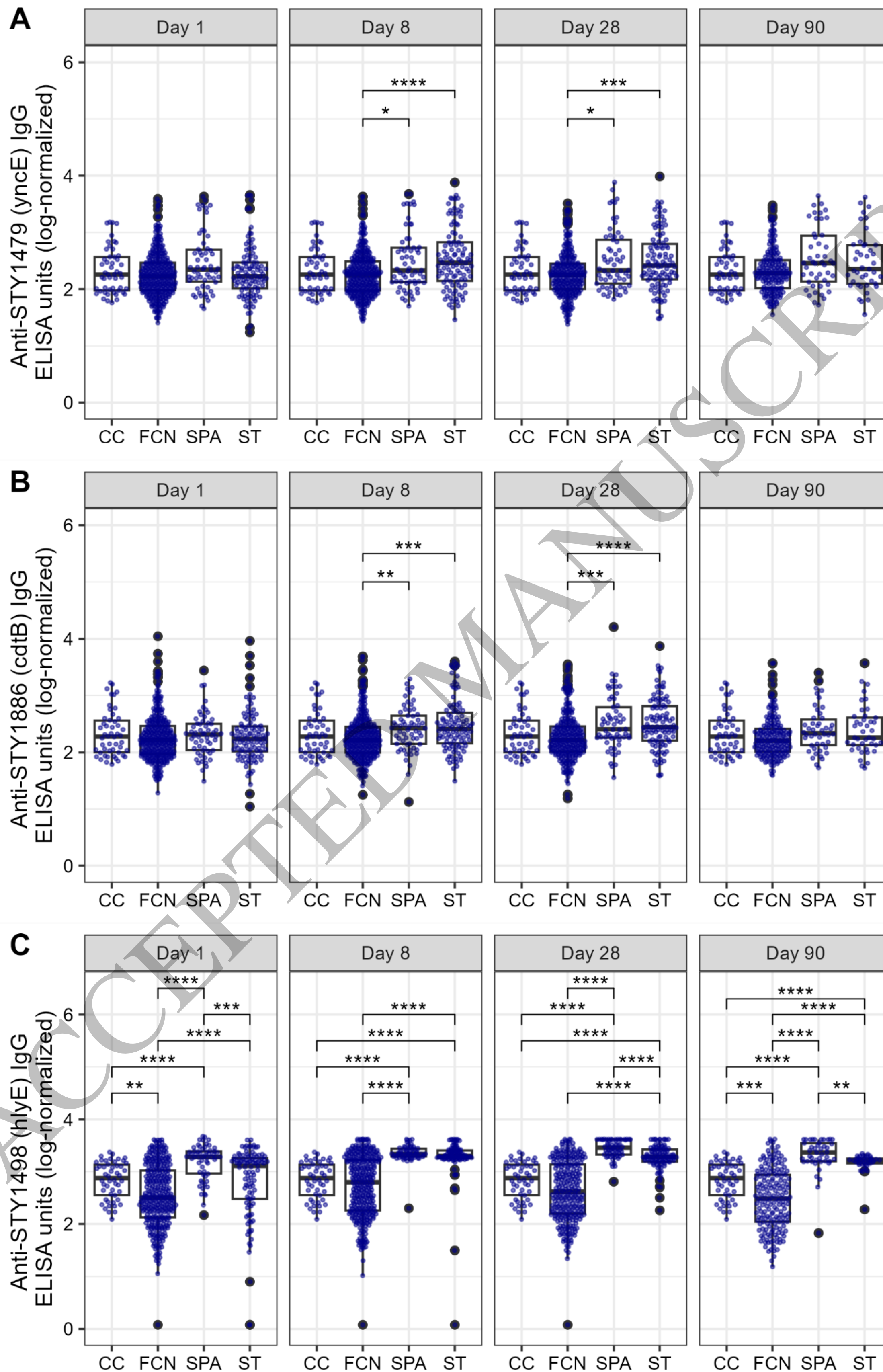

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Boxplots showing IgG titres in plasma from controls (CC), febrile, culture-negative patients (FCN) infected with an unidentified pathogen, *S*. Paratyphi A (SPA) or *S*. Typhi (ST) against STY1479 (A), STY1886 (B) and STY1498 (C) antigens over the course of three months. Differences between time points were assessed first using Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon signed-rank tests. P-values were adjusted using the Bonferroni multiple testing correction method. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6.The distribution of serum IgG titers in a Nepali cohort of febrile patients and controls.

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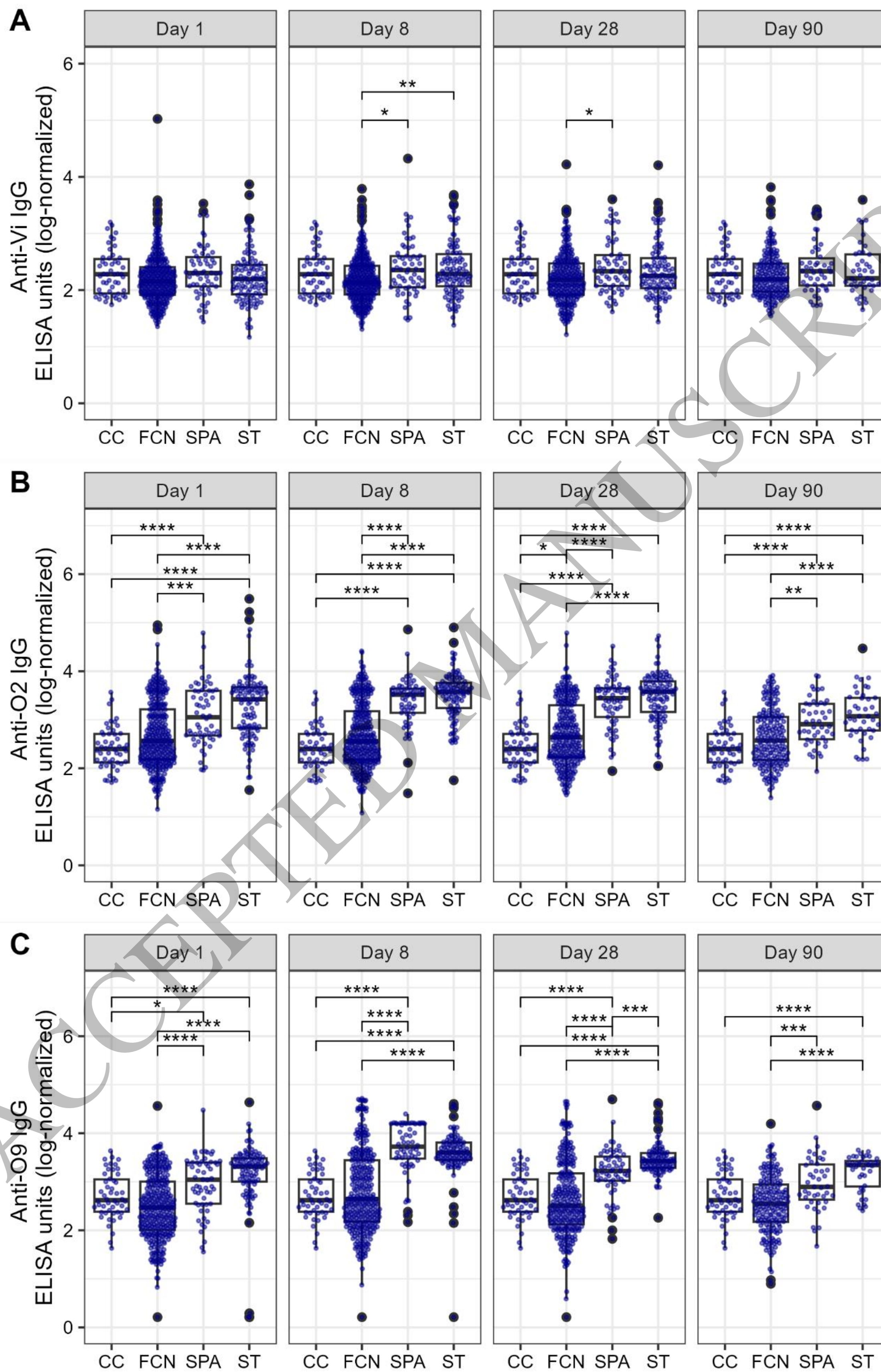

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Boxplots showing IgG titres in plasma from controls (CC), febrile patients (FCN) infected with an unidentified pathogen, *S*. Paratyphi A (SPA) or *S*. Typhi (ST) against Vi (A), O2 (B) and O9 (C) antigens over the course of three months. Differences between time points were assessed first using Kruskal-Wallis test followed by pairwise comparisons using Wilcoxon signed-rank tests. P-values were adjusted using the Bonferroni multiple testing correction method. *p<0.05, **p<0.01, ***p<0.001, ****p<0.000

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