Antibody response following scrub typhus infection: clinical cohort study

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Abstract

Objective: Scrub typhus is a common cause of fever in Asia. The antibody response to infection and its effect on subsequent infection is unclear. We studied the IgM and IgG antibody response after infection, accounting for clinical severity.

Method: We studied 197 scrub typhus patients for up to two years post-infection. Overall, 501 blood samples were analysed for scrub typhus antibodies using ELISA. IgM and IgG ELISA optical densities (OD) were analysed using quantile regression. OD values of 1.0 (IgM) and 1.5 (IgG) were used to define sero-positivity.

Results: IgM OD values fell rapidly from an initial peak after infection. 50% of cases were IgM sero-negative after 82 days. About 2 years after fever onset, 50% of cases had fitted IgG OD values of <1.5. Patients with high initial IgG OD values (≥2.5, used as a proxy for probable previous scrub typhus infection) had a more sustained IgG response than those with a low initial IgG OD, and more often presented with complications (18/36= 50% vs. 28/91= 30.8%, risk ratio= 1.63, 95%CI 1.04, 2.55, p= 0.035). This association was robust to adjusting for age (risk ratio 1.50, 95%CI 0.96, 2.33, p= 0.072).

Conclusion: Cross-sectional IgG sero-prevalence data substantially underestimate the proportion in a population ever infected with scrub typhus. A high initial IgG as a potential marker for previous scrub typhus infection may be associated with long-term IgG persistence and a higher risk of complicated scrub typhus.

Keywords: Scrub typhus, antibody, cohort

INTRODUCTION

Scrub typhus is febrile illness caused by *Orientia tsutsugamushi*, a bacterial species belonging to the genus *Orientia* (family *Rickettsiaceae*) [1]. The infection is transmitted by the larvae (chiggers) of trombiculid mites which infect mammals as incidental, dead-end hosts [2]. Scrub typhus occurs over much of tropical and subtropical Asia. The disease has recently been identified in Chile [3] and possibly East Africa [4]. In many endemic areas, scrub typhus accounts for 15% to 30% of febrile illness leading to health care use [5, 6], [7]. Scrub typhus is associated with significant mortality, estimated at 6% to 10% of untreated cases [2, 8]. Common complications include Acute Respiratory Distress Syndrome (ARDS), meningo-encephalitis, shock and renal failure [7, 9]. Complications may be avoided by early administration of antibiotics such as doxycycline or azithromycin [10]. Adverse pregnancy outcomes may occur in many pregnant women with scrub typhus [11, 12].

Epidemiological studies on scrub typhus have used sero-prevalence as a marker for the burden of infection [13, 14]. The interpretation of sero-prevalence data depends on the average duration individuals remain sero-positive after infection. For example, if infected individuals remain sero-positive for life, then sero-prevalence will reflect the proportion in a population ever having experienced an infection. A study in India suggested that on average, people may revert to IgG sero-negativity about 3 years after scrub typhus infection and to IgM sero-negativity about 10-12 months after infection [15]. Given that IgG sero-prevalence in endemic areas is often in the range of 20% to 40% [13, 14], a relatively short duration of IgG persistence of 3 years may indicate a high incidence of symptomatic or asymptomatic infection, with 20% to 40% experiencing infection within the last few years.

However, data from cross-sectional sero-surveys suggest a more complicated picture. These studies found a pronounced bimodal distribution of IgG ELISA optical density values (OD, a measure of serum antibody levels), which may be explained by long-term antibody persistence in some individuals [16, 17], possibly following repeated apparent or unapparent infection. There is also a strong increase in sero-positivity with age, suggesting a gradual accumulation of IgG antibodies to scrub typhus over a lifetime [16, 17]. However, it has also been suggested that older people may be at a higher risk of infection [16, 17], possibly due to behavioural factors, or age-related changes in skin anatomy, physiology and immunology, which could increase risk of infection after mite infestation. It is further unclear whether previous infection with scrub typhus expressed as high IgG antibody titers modifies the severity of subsequent infection. Protection against the same *Orientia tsutsugamushi* strain is thought to last for years following infection [2]. Neither partial cross-protection among different *Orientia tsutsugamushi* strains nor enhancement of infection as in dengue fever [18] has yet been convincingly demonstrated.

The present study was conducted to estimate IgG antibody persistence following scrub typhus infection to improve interpretation of population-base serological data. We further wished to estimate

IgM persistence and identify the time window during which acute scrub typhus infection can be diagnosed post-hoc. Finally, we explored whether clinical and demographic characteristics of scrub typhus patients and high initial IgG antibodies modified IgM and IgG antibody response and the clinical course.

METHODS

Study design, enrolment

The study was conducted at the Christian Medical College Vellore (CMC), a tertiary care centre in the Indian state of Tamil Nadu. It was conceived as a cohort study following up patients with scrub typhus infection over time. The study enrolled patients from two sources: (1) Patients of all ages treated as inpatients or outpatients at CMC diagnosed as scrub typhus whose enrolment occurred in two phases, between September 2015 and February 2016 (paediatric cases < 15 years only, n= 73), and between October 2018 and February 2019 (all ages, n= 85). Patients were purposively sampled based on proximity to CMC to facilitate follow up. (2) Patients of all ages treated as inpatients or outpatients at RUHSA community hospital, a secondary care centre about 40km away from the main CMC hospital (n= 40). For this cohort, enrolment occurred between August 2018 and February 2019, when all patients with fever for 3 days or longer with suspected rickettsial infection were tested for scrub typhus and spotted fever IgM/IgG.

Blood testing

Patients were followed up preferably at 1, 3, 6, 9 and 12 months, with most samples taken during the first three months after fever onset, although the actual times were usually irregular (Figure 1A). At each visit a venous blood sample was taken. After collection, blood samples were brought to CMC on the same day. Serum was separated from blood cells, divided into 3 aliquots and stored at -70°C until testing. We used enzyme-linked immunosorbent assays (ELISA) to detect IgG antibodies to *Orientia tsutsugamushi* (Scrub Typhus Detect, InBios International, Inc., Seattle, WA, USA) following the manufacturer's specifications. This ELISA uses Karp, Kato, Gilliam and TA716 recombinant proteins of the 56-kD outer membrane protein, and achieved a sensitivity and specificity of over 90% in a study from Thailand [19], and 80% sensitivity and 96% specificity in a study from South India [20]. All assays were performed using an automated ELISA analyser (Euroimmun Analyzer1, Euroimmun, Lübeck, Germany) following the protocol of the Department of Clinical Microbiology, which is an ISO15189:2012 accredited diagnostic laboratory. Quality control measures include monthly calibration of the ELISA workstation. With every run, we included an internal control and a split sample in addition to the kit controls. We applied an OD cut-off of 1.0 for IgM to suggest acute scrub typhus infection, and an IgG OD of 1.5 or higher to suggest past

infection [16]. In a subset of 17 patients, PCR was done on whole blood (buffy coat) and/or eschar samples by amplifying the 47 kDa gene. DNA extraction was performed using the DNeasy Blood and Tissue kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol.

A patient was treated as a confirmed case of scrub typhus if any of four criteria was fulfilled: (1) positive PCR for scrub typhus (n= 13); (2) increase in IgG OD of 1.0 or more from first sample to any subsequent sample within 90 days of fever (n= 63); (3) presenting with typical eschar testing negative for spotted fever (n= 107); (4) single IgM ELISA OD value of 1.0 or higher within one month of fever, with defervescence within 48 hours after initiating doxycycline or azithromycin in the absence of an alternative, plausible cause of fever (n= 176). Seven patients who were initially enrolled were excluded as they did not meet any of these criteria.

Collection of clinical data

For the CMC cohort (n= 158) clinical data were extracted from existing clinical records. The presence of an eschar was determined as documented in the records. For the RUHSA cohort (n= 40), clinical data (including eschar presence) were prospectively collected during the patient's treatment. For both cohorts, complications of scrub typhus infection were defined as follows: Acute Respiratory Distress Syndrome (ARDS) – any patient with oxygen saturation below 92% and tachypnea at any time during admission; shock – any patient with documented hypotension at presentation or during treatment, or any documented use of inotropes; kidney injury – any creatinine of 3.0 mg/dl or higher in the absence of a known, pre-existing chronic kidney disease; CNS – any focal neurological deficit, or any elevated white blood cell counts in a cerebrospinal fluid sample, or any focal or generalised seizure in an adult, or any focal or generalised seizure in a child not diagnosed as simple febrile seizure. Simple febrile seizure in children less than 6 years of age was assumed if there was no more than one generalised seizure lasting less than 15 minutes.

Statistical analysis

The sample size was determined by financial constraints. All analyses were done in STATA. We used quantile regression for modelling ELISA OD values over time, as opposed to parametric regression methods due to the skewed distribution of the data. Further, we felt that estimating OD thresholds above which a given percentage of patients would be found at a given time was of greater interest than mean OD values. As these calculations were done with the individual sample as unit of analysis, we adjusted confidence intervals for repeated measurements in the same patient using robust standard errors, following the methods proposed by Parente and Silva [21]. Models were done separately for the 10^{th}, 25^{th},

50th (median), 75th and 90th percentiles. ODs were modelled as a function of time using restricted cubic splines, with knots chosen following Harrell [22]. We explored the association between patients' characteristics (age, sex, presence of an eschar and complicated infection) and median IgM and IgG levels using median regression adjusting for time (restricted cubic splines). Similarly, we aimed at exploring the effect of high initial IgG OD on IgG and IgM OD medians, assuming that these approximate IgG antibody levels prior to the current infection. For this analysis we plotted the initial IgG OD of patients, excluding all cases without a blood sample taken within 10 days (Figure 1B). The distribution of these early IgG OD values (n= 133) shows a bimodal distribution, which for the subgroup analysis we collapsed into 2 groups (low: OD <2.5, high: OD ≥2.5). We interpreted those with an OD of 2.5 or higher as most likely representing patients with pre-existing high IgG levels who may have experienced an earlier scrub typhus infection prior to the current infection. This applied to 27.1% (36/133) of cases, a proportion similar to the IgG sero-prevalence found in cross sectional samples in the same setting [16, 17]. The risk ratios for the association between clinical variables and binary outcomes (e.g. presence of an eschar, or complications on admission or during treatment) were calculated using modified Poisson regression with robust standard errors according to Zou [23], with the individual patient as unit of analysis. Interaction was explored using likelihood ratio tests. Missing data were rare and ignored in the analysis.

Ethics

The study was approved by CMC's Institutional Review Board (CMC IRB Ref: 11726) and LSHTM's Research Ethics Committee (LSHTM Ethics Ref: 16573). Written consent was obtained from all adult participants. Written assent was obtained from minors, alongside written consent from their parents/guardians.

RESULTS

We collected 501 samples from 197 individual scrub typhus cases (Table 1). Mean follow up time was 149 days (range 3 to 838 days). About a third were 45 years or older on initial presentation; 22% were below 5 years of age. Average duration of fever prior to the first sample was 9 days. More than half were found to have an eschar. About one third developed complications, most often ARDS, followed by shock, kidney injury and CNS complications. Six people died in hospital. In about one quarter of cases only one sample was collected (Table 1).

The fitted median IgG and IgM OD values over time since fever onset are shown in Figure 2A, showing a rapid decline of IgM ODs within the first 4 months, while the ODs for IgG display an initial rise and a steep early decline which gradually flattens after 6 months. Figure 2B and 2C show the same analysis for different percentiles, restricted to the first year after fever onset. The model suggested that 50% of

patients' IgM OD values fell below 1.0 after 82 days following fever onset, with 90% of patients falling below this threshold after 231 days (Figure 2B). After one year, 50% of cases had fitted IgG OD values of 2.1 or higher, with 25% of cases showing fitted IgG OD values of 2.9 or higher (Figure 2C). At about 2 years after fever onset, 50% of cases had fitted IgG OD values of below 1.5 (Figure 2A).

Table 2 shows the association between demographic and clinical characteristics of patients and median IgM and IgG OD values, adjusted for time since fever onset using cubic splines. There was some evidence that the presence of complications and initial high IgG OD values were associated with increased IgM ODs, but these effects were reduced after adjusting for age, especially the effect of complications. Age, sex and eschar presence were not associated with IgM. Complications and initial high IgG OD were associated with increased IgG OD values, and there was some evidence that these effects remained after adjusting for age. Age and eschar were not associated with IgG OD. Females tended to have higher IgG OD values than males but this finding was much reduced after adjusting for age.

The effect of complications and high initial IgG on IgM and IgG OD over time is shown in Figure 3, confirming a somewhat stronger IgM and IgG response in patients with complicated infection (Figure 3A). Patients with a high initial IgG OD had similar IgM responses over time as those with low initial IgG OD. By contrast, those with a high initial IgG OD tended to have a slower IgG decline over time than those with a low initial IgG OD (Figure 3B).

The proportion of cases with complications tended to increase with age, except in the very young (0-4 years: 12/40 (30%), 5-14 years: 11/42 (26.2%), 15 to 44 years: 20/47 (42.3%), ≥45 years: 36/60 (60%), p for trend across categories <0.001). A high initial IgG OD (possibly a marker of prior infection) tended to be associated with a higher risk of complications (18/36= 50% vs. 28/91= 30.8%, risk ratio= 1.63, 95% CI 1.04, 2.55, p= 0.035, 6 cases of 133 cases with initial IgG value were dropped due to missing data on complications). There was some evidence that this association was robust to adjusting for age (with a quadratic term), resulting in a risk ratio of 1.50 (95% CI 0.96, 2.33, p= 0.072, one case of 127 from the unadjusted model dropped due to missing age data).

Patients with a high initial IgG OD were no more likely to have an eschar than those with a low initial IgG OD (21/35= 60.0% vs. 57/91= 62.6%, p= 0.785). Complicated infection was somewhat higher in patients with an eschar (50/107= 46.7%) than in those without an eschar (29/82= 35.4%), but statistical support for this effect was not strong (risk ratio 1.32, 95% CI 0.92, 1.89, p= 0.126). Further, the effect size differed strongly between the CMC (risk ratio 1.55, 95%CI 1.05, 2.31) and the RUHSA cohorts (risk ratio 0.67, 95%CI 0.30, 1.50, test for interaction p= 0.074). Eschar prevalence was not affected by age (0-4 years: 55.0%, 5-14 years 57.1%, 15-44 years: 57.5%, ≥45 years: 55.9%).

DISCUSSION

This serological cohort study of scrub typhus cases suggests a short persistence of IgM in the majority of cases, while about half of cases show persistent IgG antibodies beyond 2 years. IgG antibody persistence appeared to depend on the initial level of IgG. High initial IgG antibodies were associated with a 50% higher risk of complicated infection.

In view of the strong increase of IgG sero-prevalence with age observed in the same setting [16, 17], our data support the idea of a gradual build-up of IgG over repeated (symptomatic or asymptomatic) infections, rather than a higher risk of infection in older age groups. The relatively rapid decline of IgG antibodies within a few years of infection in about half of cases is in line with a previous report which found an average duration of IgG positivity of about 3 years [15]. This clearly implies that cross-sectional IgG sero-prevalence does not reflect the proportion of the population ever having been infected with scrub typhus, but substantially underestimates this risk. Cross-sectional sero-prevalence estimates may represent a mix of first-time infections within the last few years and those in the population who have experienced multiple infections during their life resulting in long-term IgG persistence. The OD cut-off of 1.5 to 1.8 previously used for IgG in our setting [16, 17] may be too conservative, as the IgG OD values of half of cases dropped below 1.5 within two years. However, a lower cut-off may increase the proportion of false positives due to cross-reactivity, highlighting the limitations of cross-sectional serological data to estimate the burden of scrub typhus in a population. Modelling studies and population-based cohort studies may shed further light on appropriate ways to interpret sero-prevalence data.

The rapid IgM decline after infection is in line with a cross sectional survey which found only 0.5% of the population in this setting being IgM positive [16]. Our results suggest that IgM antibody levels may be used to diagnose acute scrub typhus within a time window of 4-8 weeks after infection, making scrub typhus IgM ELISA a suitable diagnostic tool in large field studies.

Cases with high initial IgG antibody levels experienced a similar IgM response as those with low levels. We observed a stronger IgG antibody response in complicated than in uncomplicated cases. This may relate to the finding of high initial IgG antibodies being associated with the risk of complications. Although the risk of complications depended on age, the risk ratio for the effect of high initial IgG OD on complications was only slightly attenuated by adjusting for age. We used the initial IgG OD (measured within 10 days of fever onset) as a proxy for IgG antibody levels prior to the current infection, possibly due to an earlier scrub typhus infection (Figure 1B). If this is approximately the case, then prior infection may increase the risk of complications in subsequent infection, perhaps through antibody-dependent enhancement of infection as in dengue fever [18]. The observation of scrub typhus re-infection has so far been anecdotal. Koralur and colleagues report a case of re-infection within 14 months, but neither

infection was associated with complications [24]. The present study includes one case of a likely re-infection in a 65 year old male patient with no known comorbidities from a village with a known high incidence of scrub typhus. Two years prior to the current infection he was treated with a course of doxycycline at our hospital for uncomplicated fever with a typical eschar suggesting scrub typhus (no laboratory confirmation was done at the time). The current infection was associated with ARDS, which resolved over 48 hours after azithromycin. Blood testing showed an IgM OD of 2.7, and an IgG OD of 3.5. Similar to the case reported by Koralur, the second infection in contrast to the first was not associated with an eschar – a finding which is not in line with the overall analysis of the present study which suggested high initial IgG OD (as a proxy of prior infection) not to affect the occurrence of eschar formation. While it is too early to conclude that prior infection in fact increases the severity of subsequent infection, it seems safe to conclude that prior infection is unlikely to (cross-) protect against complicated subsequent infection. Given the substantial implication antibody-dependent enhancement of infection has on dengue fever vaccine development and vaccination policy [24, 25] studies are urgently needed to explore this issue for scrub typhus.

The presence of an eschar did not seem to affect IgM and IgG antibody response in this study. It was also unaffected by age. The overall finding of a slight increase in risk of complications in those with an eschar may be subject to selection bias which could go into either direction: Patients with an eschar may be diagnosed earlier as scrub typhus and given adequate treatment, which would cause bias suggesting a protective effect. Conversely, hospitalised patients with complications may be examined more thoroughly for an eschar, which would cause bias towards eschars being more prevalent in complicated infection. Such biases may explain diverging findings from earlier studies [26, 27] and the differences between the CMC and the RUHSA cohorts in this study.

The study is primarily limited by the purposive sampling approach, the method of case confirmation and the relatively small sample size, especially beyond one year of follow up. Enrolment was done at intervals over several years, preferably enrolling patients in reasonable proximity to the study centres. While patients enrolled at RUHSA hospital may approximate a population-based sample, those recruited at CMC, a tertiary care centre, most likely represent more severe cases. Case confirmation was made complicated by relying for 56% of cases on serology, which also served as study outcome. This may bias the study population towards those with a stronger serological response. The presence of an eschar in the absence of a positive spotted fever test as an additional criterion is not standard. Ten patients (5%) were enrolled based on this criterion alone, not meeting any of the other three criteria. We added this criterion to avoid the overwhelming majority of cases being enrolled based on serology, but there is a risk of misdiagnosis of an unrelated skin lesion as an eschar. Eschars have been observed in only about 5% of

spotted fever cases in a study from Sri Lanka [28] (which is close to the study site). In a study from our hospital, none of the 38 patients diagnosed to have spotted fever by nested PCR had an eschar [29]. Given that over 30% of undifferentiated fever cases in the study area are due to scrub typhus [7], presence of an eschar in a febrile patient is likely to have a high positive predictive value for this infection, especially during the scrub typhus season when cases were enrolled [30-32]. Overall, enrolment occurred based on a single criterion in 78 cases (40%) which is not ideal. Future studies are likely to benefit from systematically conducting additional tests such PCR and indirect immune fluorescence assays (IFA) to allow enrolment of cases being based on a combination of tests [33], which was beyond the scope of the present study.

The sample size was driven by logistics and financial constraints. Obtaining more samples beyond one year would have strengthened the analysis.

Finally, the study is limited by relying exclusively on ELISA to determine antibody levels. IFA tests that would have provided antibody titers were not done. Studies directly comparing ELISA with IFA have shown a strong correlation between ELISA OD and IFA, which however may not be linear [19]. Quantile regression as used in this analysis has the advantage of results not depending on whether OD values are transformed prior to analysis to better reflect the actual antibody concentrations.

CONCLUSION

The IgM antibody response to scrub typhus appears to be short-lived and may not depend on prior IgG antibody levels. Repeated infection may lead to IgG antibodies persisting over many years, which may explain the strong association between age and sero-prevalence [16, 17]. Cross-sectional sero-prevalence data are likely to underestimate the proportion of the population ever infected with scrub typhus. High initial IgG levels as a possible proxy for previous infection may be associated with a higher risk of complicated scrub typhus infection, a finding that may have implications for vaccine development and eventual vaccine policy.

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Table 1. Patients' characteristics

	n/N	% or mean (SD, range)
Total	197/197	100
Female gender (4 missing values)	105/193	54
Age group (years, 5 missing values)		
0-4	42/192	22
5-14	42/192	22
15-44	47/192	24
≥45	61/192	32
Duration of fever prior to first scrub typhus test (days, 13 missing values)	-	9.0 (3.9, 3 - 37)
Eschar present (8 missing values)	107/189	57
Complications		
ARDS (7 missing values)	40/190	21
Shock (7 missing values)	26/190	14
Kidney injury (7 missing values)	18/190	10
CNS complications (7 missing values)	13/190	7
Any complication (7 missing values)	65/190	34
Died during admission	6/197	3
Number of samples per patient		
1	47/197	24
2	48/197	24
3	57/197	29
4	38/197	19
5	7/197	4

Table 2. Effect of demographic and clinical characteristics on median IgM and IgG optical densities

	N[a]	Difference in OD[b]	95% CI	P value	Age-adjusted difference in OD	95% CI	P value
IgM							
Age (change per 10 year increment)	487	0.01	-0.03, 0.05	0.659			
Female sex	492	-0.01	-0.16, 0.14	0.894	-0.02	-0.19, 0.14	0.793
Eschar present	481	-0.01	-0.16, 0.14	0.916	0.01	-0.14, 0.17	0.857
Complication present	485	0.16	-0.01, 0.32	0.062	0.10	-0.07, 0.27	0.234
IgG OD ≥2.5 within 10 days of fever onset	373	0.24	-0.02, 0.50	0.065	0.11	-0.10, 0.31	0.303
IgG							
Age (change per 10 year increment)	479	0.06	0.00, 0.13	0.064			
Female sex	484	0.24	-0.10, 0.57	0.167	0.15	-0.19, 0.50	0.386
Eschar present	473	0.04	-0.33, 0.41	0.83	-0.03	-0.36, 0.30	0.841
Complication present	477	0.37	0.06, 0.69	0.019	0.31	-0.06, 0.69	0.101
IgG OD ≥2.5 within 10 days of fever onset[c]	238	0.53	0.03, 0.91	0.038	0.53	-0.04, 1.11	0.067

[a] total number of samples in model; [b] Optical density; [c] excluding IgG OD values during first 10 days after fever onset. Adjustments for age were done including a quadratic term.

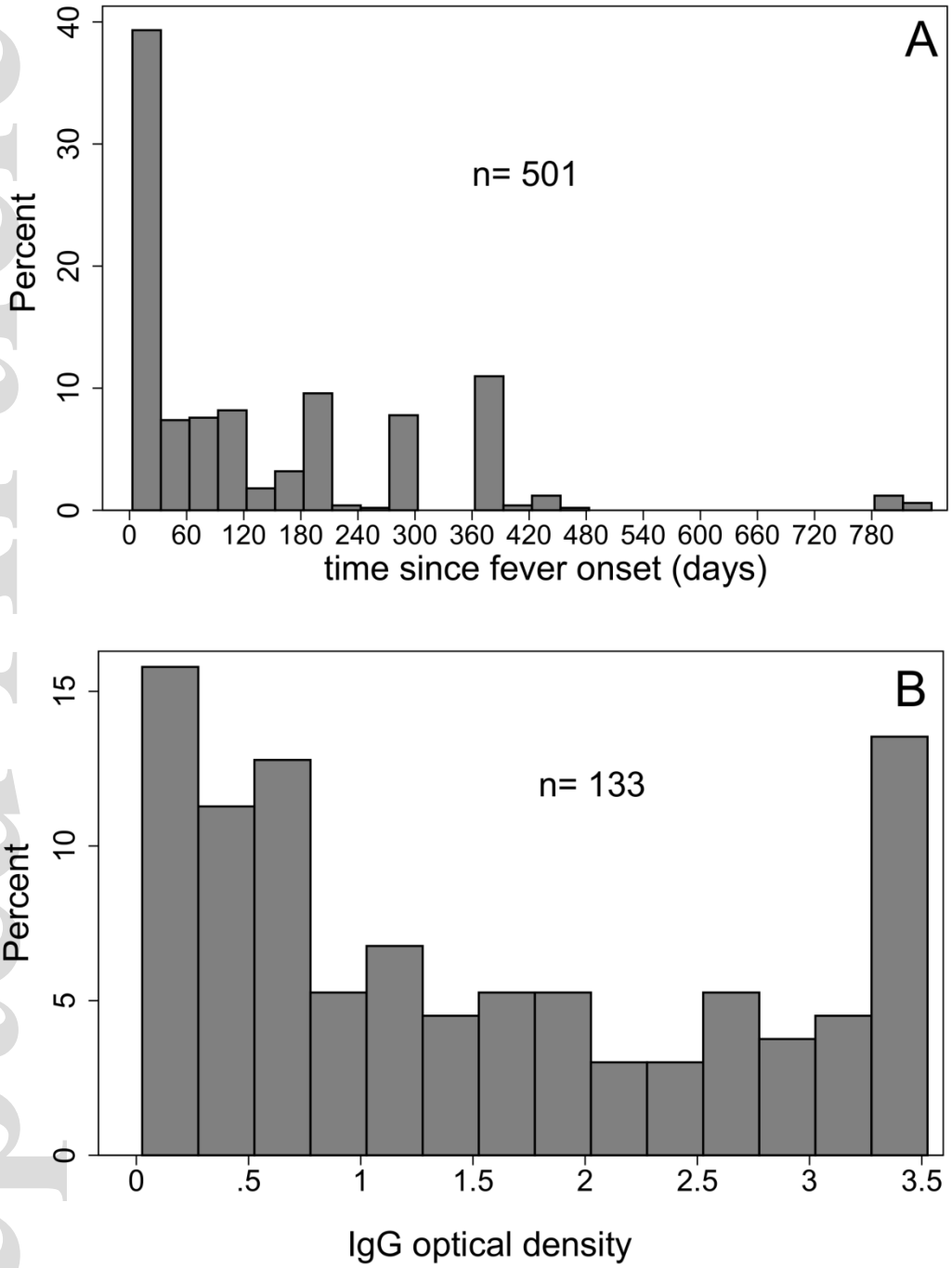

Figure 1. A) Distribution of samples over follow up period; B) distribution of initial IgG OD values (within 10 days of fever onset)

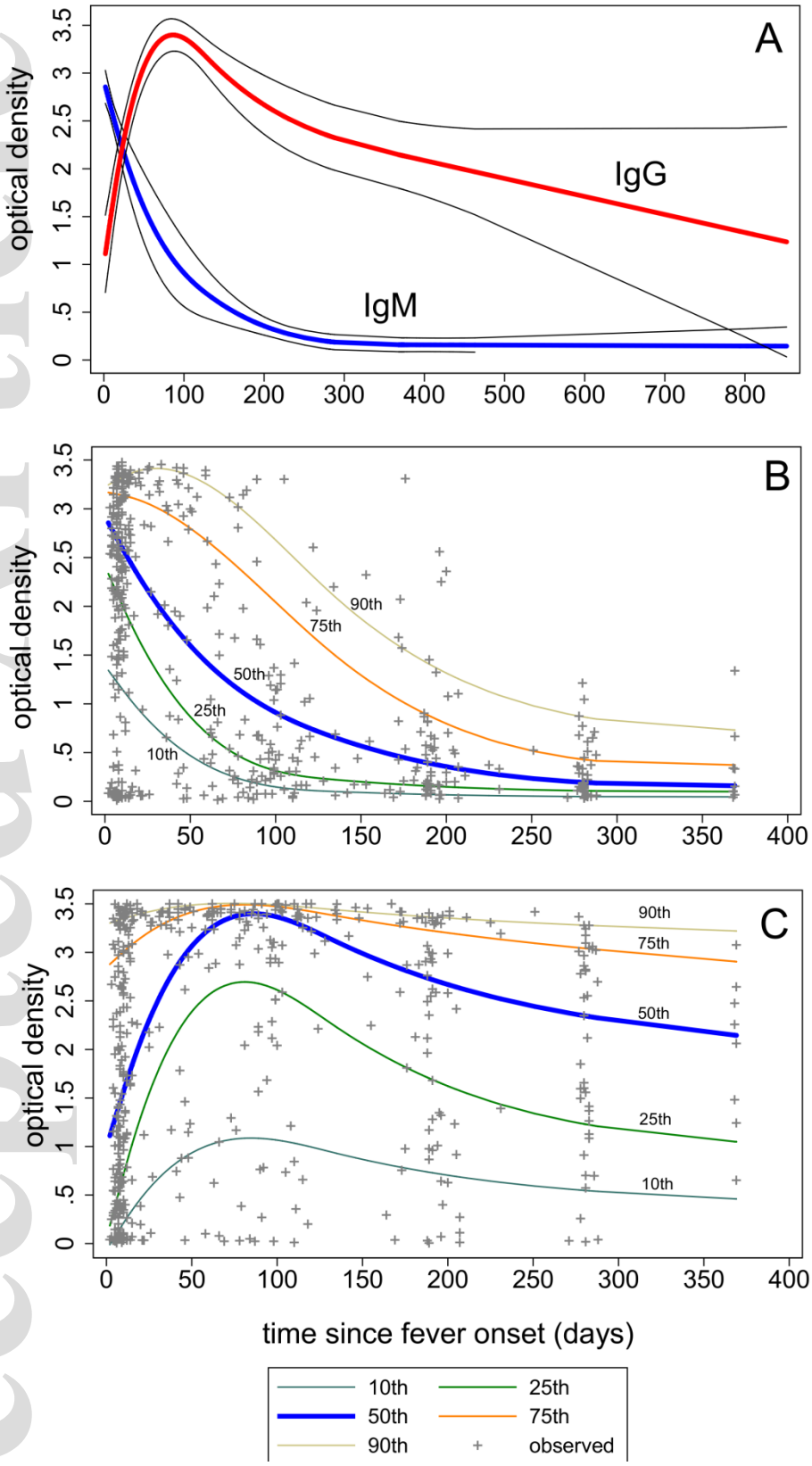

Figure 2. A) IgM and IgG optical densities since onset of fever; B) percentiles of IgM OD values; C) percentiles of IgG OD values

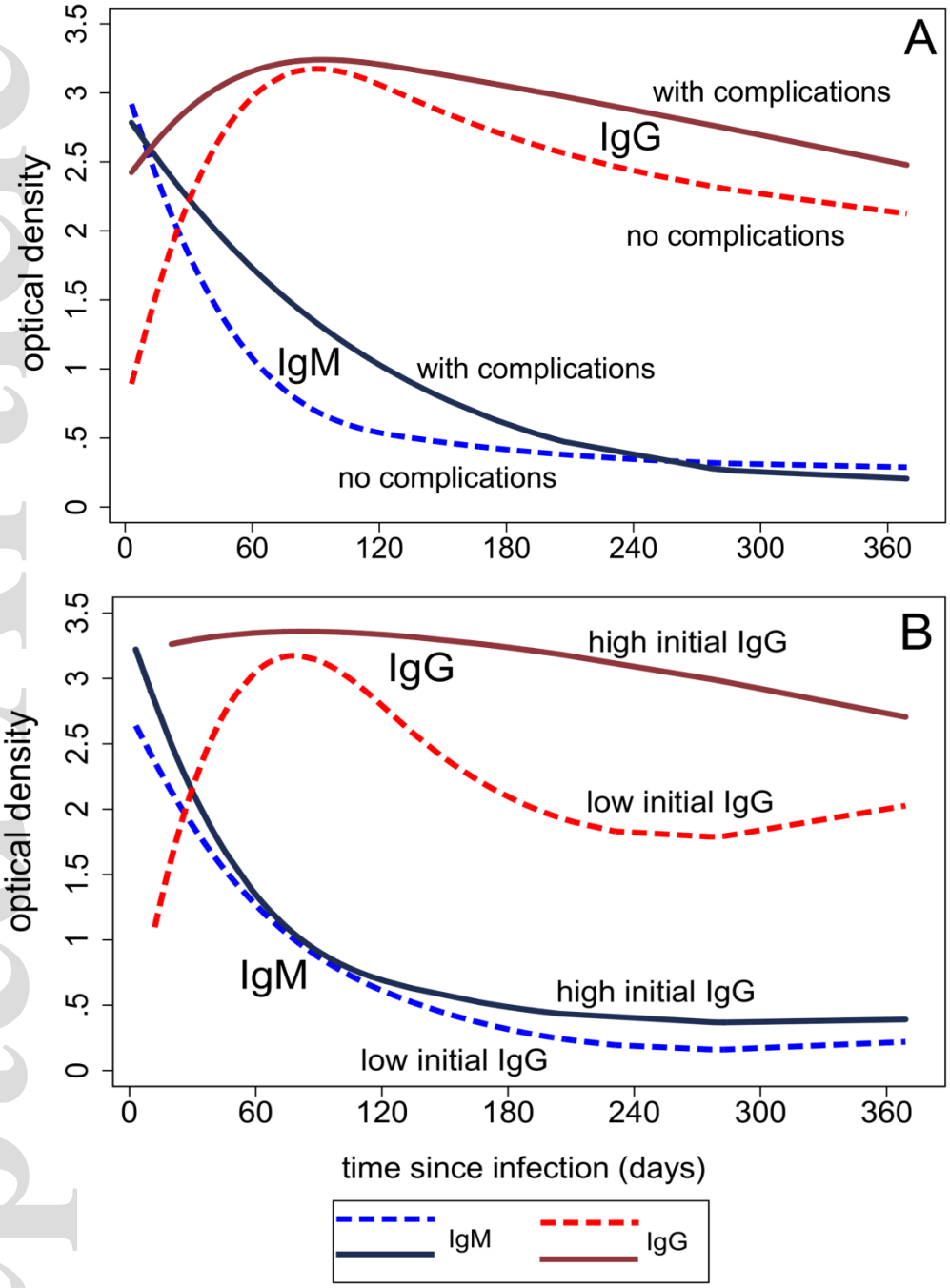

Figure 3. Subgroup analysis of IgM and IgG optical densities: A) by complicated infections vs uncomplicated infection; B) by high initial IgG OD (within first 10 days) vs low initial IgG OD. Fitted values are adjusted for age (linear and quadratic terms).