

Immune Cross-Opsonization Within *emm* Clusters Following Group A *Streptococcus* Skin Infection: Broadening the Scope of Type-Specific Immunity

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Background. Group A *Streptococcus* (GAS) skin infections are particularly prevalent in developing nations. The GAS M protein, by which strains are differentiated into >220 different *emm* types, is immunogenic and elicits protective antibodies. A major obstacle for vaccine development has been the traditional understanding that immunity following infection is restricted to a single *emm* type. However, recent evidence has led to the hypothesis of immune cross-reactivity between *emm* types.

Methods. We investigated the human serological response to GAS impetigo in Fijian schoolchildren, focusing on 3 major *emm* clusters (E4, E6, and D4). Pre- and postinfection sera were assayed by enzyme-linked immunosorbent assay with N-terminal M peptides and bactericidal assays using the infecting-type strain, *emm* cluster-related strains, and nonrelated strains.

Results. Twenty of the 53 paired sera demonstrated a ≥ 4 -fold increase in antibody titer against the infecting type. When tested against all cluster-related M peptides, we found that 9 of 17 (53%) paired sera had a ≥ 4 -fold increase in antibody titer to cluster-related strains as well. When grouped by cluster, the mean change to cluster-related *emm* types in E4 and E6 was >4-fold (5.9-fold and 19.5-fold, respectively) but for D4 was 3.8-fold. The 17 paired sera were tested in bactericidal assays against selected cluster-related and nonrelated strains. While the responses were highly variable, numerous instances of cross-reactive killing were observed.

Conclusions. These data demonstrate that M type-specific and cross-reactive immune responses occur following skin infection. The cross-reactive immune responses frequently align with *emm* clusters, raising new opportunities to design multivalent vaccines with broad coverage.

Keywords. *Streptococcus pyogenes*; *emm*-cluster; immunity; vaccine; skin infection.

Many microbial pathogens are exceptionally proficient at immune evasion, providing numerous challenges for vaccine development. Group A *Streptococcus* (GAS) is a prime example, with considerable antigenic diversity and many immune-evading virulence factors. GAS is responsible for major morbidity and mortality, particularly in developing nations, and causes at least 500 000 deaths per year worldwide [1]. GAS pharyngitis and impetigo comprise the majority of cases, and although they can be relatively mild conditions, they have the ability to lead to more severe invasive disease or to autoimmune sequela of the heart and kidneys. The highest prevalence of GAS impetigo is found in Oceania, with a median prevalence of 40.2%

[2]. Children aged <15 years are the most commonly affected by GAS impetigo [3]. Studies of the immune response following impetigo suggest a weaker response with potentially lower induction of a lasting immune response [4, 5].

Due to the high burden of disease, particularly in areas where primary healthcare is limited, there has been extensive interest in the development of a GAS vaccine. A major vaccine candidate has been the M protein, the dominant immunogenic molecule on the GAS surface [6, 7]. Early studies of a restricted number of GAS M types observed that protective immunity following GAS infection was limited to bacteria of the homologous M type. This observation was attributed to type-specific antibodies arising from epitopes on the M protein [8–10]. The gene encoding M proteins, *emm*, is the basis for sequence typing used to differentiate between strains of GAS, based on relatively minor sequence differences in the 5' regions of the genes. M protein sequence diversity exceeds 200 different *emm* types [11], and therefore a broad-spectrum vaccine against this protein has been difficult to develop. The current leading type-specific GAS vaccine candidate comprises peptides from 30 different M proteins [12].

Received 3 April 2017; editorial decision 16 June 2017; accepted 6 July 2017; published online July 19, 2017.

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Clinical Infectious Diseases® 2017;65(9):1523–31

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Preclinical studies of the 30-valent vaccine candidate found an unexpectedly high level of cross-opsonization of types not included in the vaccine (opsonization demonstrated in 39 of 49 *emm* types tested) [12, 13]. Recent studies observed substantially lower inter-M protein sequence diversity within the whole *emm* gene among strains isolated from tropical areas, in contrast to high diversity in strains recovered from high-income settings, providing a possible explanation for cross-opsonization [14–16]. Further studies of the entire *emm* gene from 1086 GAS isolates representing 175 *emm* types led to the establishment of a cluster system of typing, which groups *emm* types into 48 distinct *emm* clusters based on sequence homology and binding capacities [17]. Sixteen clusters contain 143 *emm* types and account for 90% of global GAS infections [18, 19]. There is strong evidence of shared host protein-binding capabilities within clusters, suggesting that these clusters are functionally and immunologically relevant. An experimental vaccine developed using peptides from 5 M proteins from the E4 cluster was found to induce broad opsonization of other strains within the cluster, providing further evidence that immune recognition of GAS may be a combination of “cluster-specific” and “type-specific” responses [20].

In this study, we aimed to investigate the human serologic response to GAS impetigo in an endemic setting, and to evaluate the potential relationship between *emm* clusters and cross-opsonization. We investigated the immune response to isolates belonging to 3 *emm* clusters (E4, E6, D4) that collectively account for 67 *emm* types and cause around 35% of GAS infections worldwide [21, 22].

MATERIALS AND METHODS

Study Samples

Samples were collected in a longitudinal cohort study of pharyngitis and impetigo in 457 children aged 5–15 years followed for 10 months in 3 schools in Fiji, as described previously [3, 23]. Serum samples and GAS isolates from throat and skin cultures were collected from symptomatic children as part of the study, and sera were taken at 3 predefined time points (0, 5, and 10 months), and frozen and stored at -80°C . Isolates were *emm* typed by standard methods [24]. Infections caused by bacteria from E4, E6, and D4 *emm* clusters were selected for inclusion, as these are among the most frequently recovered *emm* clusters in the Pacific region [21, 22]. Children with multiple episodes of GAS impetigo caused by different *emm* types between blood samples were excluded from the analysis.

Detection of Serum Antibodies

Enzyme-linked immunosorbent assays (ELISAs) were done to establish the antibody reactivity profile of pre- and postinfection patient sera to peptides from the M protein of (1) the infecting type, (2) heterologous strains from the same cluster as the infecting type, and (3) strains from different clusters to

the infecting type. Assays were done with modifications of the method described by Dale et al [12, 25] and Miura et al [26]. Synthetic peptides of the N-terminal 50 amino acids of M proteins were obtained commercially (GenScript, Piscataway, New Jersey) from sequences based on a representative collection of 1086 M proteins and were used to coat assay plates [11]. Pooled human sera (Sandoglobulin) were used on every plate to construct reference curves (Supplementary Methods). Pre- and postinfection samples were initially assayed against the M peptide from the infecting type only. Paired sera were diluted 2-fold serially from 1:50 to 1:3200 and added to an assay plate, coated with infecting-type M peptide. Detection of bound antibody was done using horseradish peroxidase-labeled antihuman immunoglobulin G (IgG) secondary antibody. Following incubation with substrate tetramethylbenzidine, absorbance was measured at 450 nm. Antibody titers were calculated in ELISA units from the standard curve.

Sera were included for investigation of cross-opsonization if there was sufficient sample available and they met our predefined criteria for seroconversion to the infecting peptide, which was defined as an increase in antibody titer of the postinfection sera ≥ 4 -fold of the preinfection titer. Two serum dilutions located in the log-phase of the antibody response curves, as determined from the infecting-type ELISA, were selected for each pair of pre- and postinfection sera. These sera were further assayed against M peptides from all M proteins within their *emm* cluster, as well as 6–7 M peptides from heterologous *emm* clusters (Supplementary Methods). To overcome the intrinsic variability in individual responses to a single GAS infection, we also grouped the *emm* cluster-related and the non-cluster-related responses for each *emm* cluster and compared them by *t* test.

Functional Assays

Bactericidal assays were performed with modifications of those described by Lancefield [27] and Raz et al [28]. Various streptococcal strains were cultured overnight, diluted 1:50 in 10 mL Todd Hewitt broth with 1% yeast extract (THY), and grown to an optical density (at 600 nm) of 0.2 and diluted further to $1:1 \times 10^{-4}$ before addition to assays and plating on THY agar to determine inoculum size. Heparinized whole blood was collected from healthy adult volunteers who had been prescreened by bactericidal assay to confirm nonimmune status, and added to assays within 2 hours of collection. Pre- and postinfection sera were also added to assays to a total volume of 300 μL comprising 50 μL of serum, 50 μL of bacterial culture (approximately 100 colony-forming units), and 200 μL of blood. Assays were incubated with end-over-end rotation for 3 hours at 37°C before plating on THY-triphenyl tetrazolium chloride agar and grown for 18 hours at 37°C with 5% CO_2 . Control assays, in which no sera were added, were performed concurrently, and all results were compared with the initial inoculum concentration to determine a replication factor.

Percentage killing was determined by calculating the difference between pre- and postinfection replication factors. Where there was less replication in the preinfection than postinfection serum, it was reported as “no killing” and was given a value of 0% as this could not be attributed to the presence or absence of cross-reactive antibodies. For each cluster, the infecting-type, *emm* cluster-related, and non-cluster-related bactericidal responses were grouped and compared, and the mean differences between the *emm* cluster-related and non-cluster-related strains were analyzed by *t* test. As a relatively high level of killing of the infecting strain is likely required to clearly observe cross-reactive killing, samples with $\geq 50\%$ killing of the infecting type were analyzed separately [29–31]. All GAS strains used in assays were clinical isolates collected in Pacific countries, where possible from the infection event being investigated, or as part of the cohort study [3, 32].

Ethical Approval

Ethical approval was obtained from the Fiji National Research Ethics Review Committee, the Fiji National Health Research Committee, the University of Melbourne Human Research Ethics Committee, and the Queensland Institute of Medical Research Human Research Ethics Committee. Children were enrolled only if written consent from a parent or guardian was obtained. Children aged 10 years or older were enrolled only if written assent by the child was also obtained. Blood donations from healthy adults were only taken if written assent was obtained.

RESULTS

Study Population

Samples from 53 children with a single skin infection from an *emm* type belonging to 1 of the 3 clusters were included. Of these, 20 met the seroconversion threshold for further inclusion (5 E4, 6 E6, and 9 D4) when tested against their infecting-type

peptide using ELISA, and of these, 17 samples (5 E4, 5 E6, and 7 D4) were finally included for cluster-related peptide ELISA and 17 samples (5 E4, 4 E6, and 8 D4) for bactericidal assays based on volumes of available samples (Figure 1). The median time between infection and the second blood sample was 99 days (range, 43–203 days).

Infecting-Type Enzyme-Linked Immunosorbent Assays

When antibody titers were determined for the 53 paired samples against the M peptide of their infecting type and overall, the geometric mean response to the infecting-type peptide resulted in a 4.1-fold increase (95% confidence interval [CI], 2.5- to 6.7-fold) (Figure 2). While some subjects exhibited little to no difference between pre- and postinfection samples (62% had < 4 -fold difference), others had a very large increase in antibody titer to the peptide following infection, such that the range of changes was 0.07- to 1444-fold. In each cluster, between 37% and 38% of samples had a ≥ 4 -fold increase in antibody titer to the infecting type between pre- and postinfection samples (Figure 1).

Cluster-Related Peptide Enzyme-Linked Immunosorbent Assays

In assays using multiple cluster-related and non-cluster-related antigens, samples from 17 infections caused by 12 different *emm* types were included, representing 4 from each of the 3 clusters. Responses to the cluster- and non-cluster-related strains were varied. Of the 17 paired samples, 8 (47%) had a ≥ 4 -fold increase in antibody titer to peptides from the infecting type plus at least 1 cluster-related strain (Figure 3A), whereas 7 (41%) had a ≥ 4 -fold increase in antibody titer to the peptide from the infecting type only (Figure 3B), and a further 2 (12%) had a ≥ 4 -fold increase in antibody titer to peptides from both cluster-related and non-cluster-related strains (Figure 3C). Details of all individual children are provided in the Supplementary Materials.

When pooling results for all the infections by cluster, the average fold change for cluster-related *emm* types was ≥ 4 -fold for

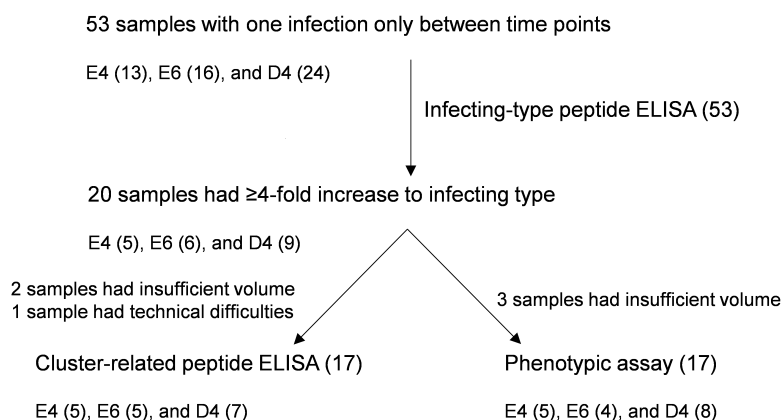


Figure 1. The numbers of samples meeting inclusion criteria for each part of the study, indicating the numbers from each cluster. Abbreviation: ELISA, enzyme-linked immunosorbent assay.

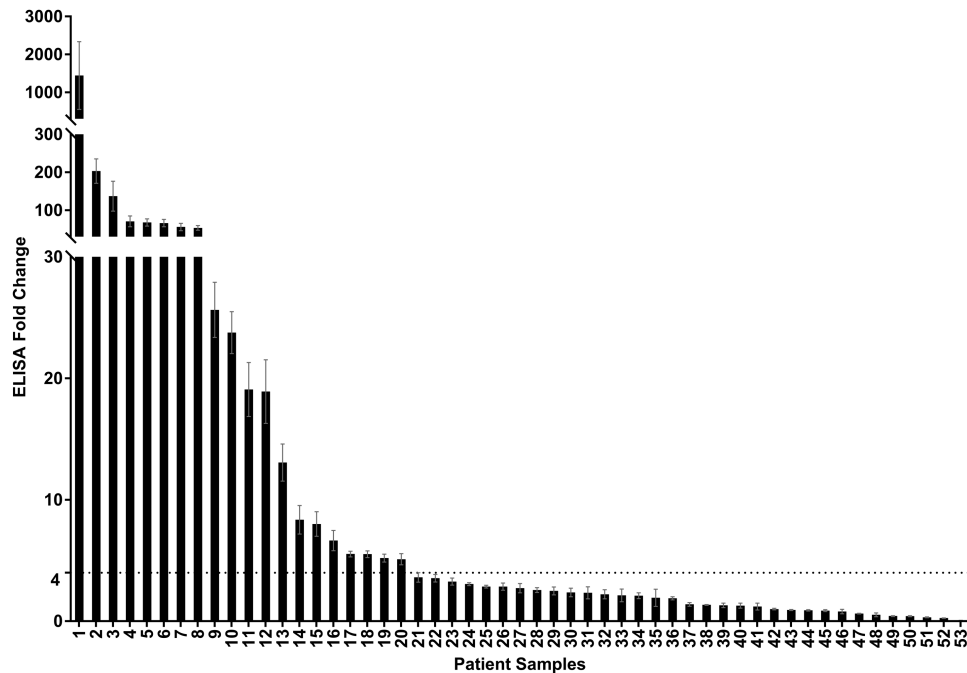


Figure 2. Change in antibody titer against infecting-type peptide. Average fold change between enzyme-linked immunosorbent assay (ELISA) titer from 53 pre- and postinfection sera to the N-terminal peptide from the M protein of the infecting group A *Streptococcus* (GAS) strain. Experiments were performed in duplicate over 7 serial dilutions and repeated, and the average values for pre- and postinfection sera were calculated from standard curves and compared. As indicated by the dotted line, the cutoff threshold for inclusion in further experiments was set at a 4-fold increase. Error bars represent the standard error of the mean.

clusters E4 and E6 (Figure 4). From these pooled analyses, the mean absolute difference in fold change between cluster-related and nonrelated for patients with infections caused by E4-cluster strains was 4.8 (95% CI, 1.5–8.0), whereas for E6 was 17.5 (95% CI, –7.1 to 42.2) and for D4 was 1.1 (95% CI, –4.9 to 7.0).

Bactericidal Assays

Sera were assayed against GAS of the infecting type and a selection of representative *emm* cluster-related and non-cluster-related strains in bactericidal assays. The magnitude of percentage killing against these selected strains was variable. In all cases, we observed some level of killing against strains other than the infecting type, which could be attributed to cross-reactive antibodies, and were sometimes consistent with ELISA results (see Supplementary Results for individual bactericidal assay and parallel ELISA data).

When analyzed by *t* test (Figure 5), the greatest difference between means of percentage killing of the cluster-related and nonrelated strains was observed in cluster E4 (27.6 [95% CI, 3.7–51.4]), compared with E6 (–5.4 [95% CI, –22.0 to 11.3]) and D4 (1.8 [95% CI, –8.6 to 12.2]).

Samples with >50% killing of the infecting type had greater cross-reactive killing against *emm* cluster-related compared with non-cluster-related strains in all 3 clusters, with this difference being most pronounced in E4 cluster samples (75% of sera

demonstrated >50% killing of cluster-related strains compared to 20% of non-cluster-related strains; Table 1).

DISCUSSION

We found that GAS impetigo elicits a systemic antibody response against the M protein, with a single episode resulting in a ≥ 4 -fold increase in anti-M protein IgG antibodies in 38% of cases. The functional nature of this antibody response is demonstrated by the fact that 8 of 17 cases had high anti-M peptide IgG antibody titers that induced >50% killing of the infecting-type strain of GAS. Furthermore, these antibodies also cross-reacted with and cross-opsonized heterologous strains.

Early studies of the serological response to superficial GAS infection suggested that pharyngitis was capable of eliciting type-specific immunity against the infecting GAS strain, but infection of the skin did not induce the same level of antibody response [4, 33]. Among the 38% of participants in our study who had a ≥ 4 -fold increase in anti-M peptide IgG antibodies, nearly half induced type-specific killing. The variable serological responses observed in this study are consistent with previous prospective studies of diagnostic markers in sera following GAS infection [34] which are typically relatively lower following skin infections compared with pharyngeal infections [4]. Antibody responses to M peptides following pharyngitis with

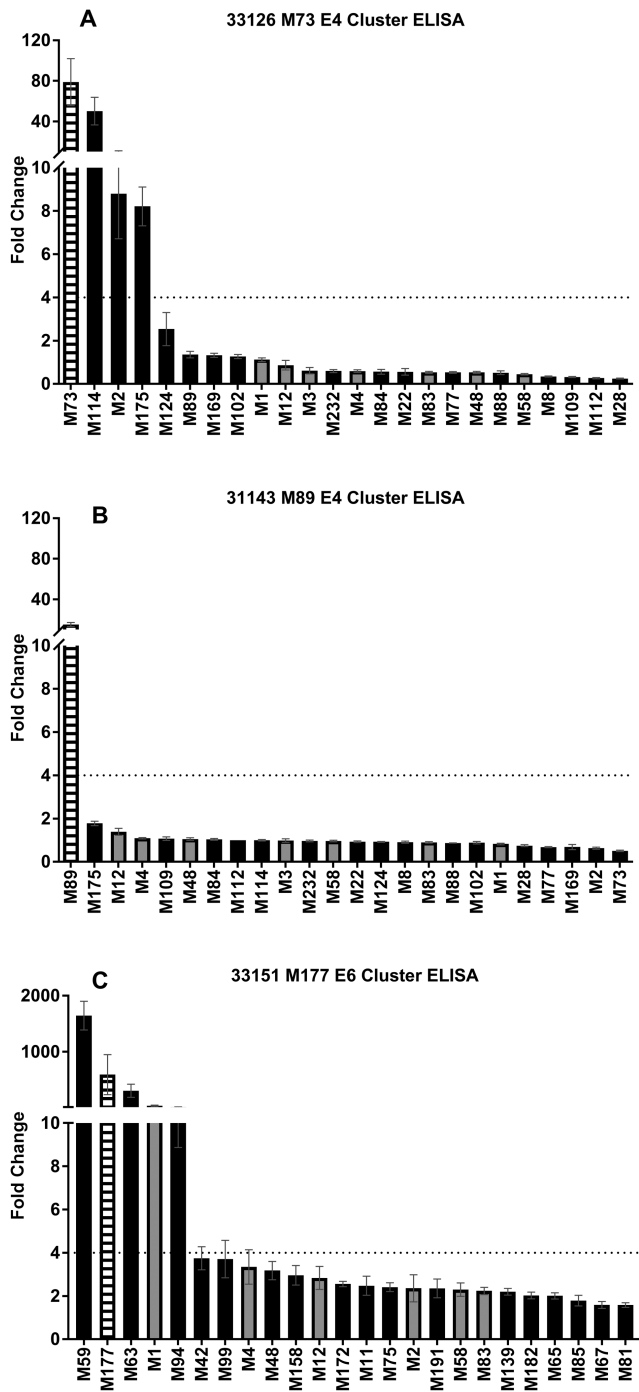


Figure 3. Representative individual antibody responses to *emm* cluster-related and non-cluster-related M peptides. The average fold change between enzyme-linked immunosorbent assay (ELISA) titer from pre- and postinfection sera from 3 individual patients to N-terminal peptides from the M protein of the infecting group A *Streptococcus* (GAS) strain, all *emm* cluster-related GAS, and 7 non-cluster-related GAS strains. The figures represent the 3 different antibody response patterns observed: high response to infecting-type and *emm* cluster-related peptides (A), high response to infecting-type peptide only (B), and high response to infecting-type, *emm* cluster-related, and non-cluster-related peptides (C). Error bars represent the standard error of the mean. White striped bars = infecting-type peptide; black bars = *emm* cluster-related peptide; gray bars = non-cluster-related peptide.

the homologous strains have also been shown to be variable, with anti-M immune responses observed in only 63% of participants in a recent study [35]. It is interesting to note that, the majority of these responses occurred following asymptomatic GAS acquisition in the pharynx, and only half of the patients with symptomatic pharyngitis had a positive immune response to the M peptide from the infecting type [35].

The *emm* cluster typing system, based on full *emm* gene sequences, provides a framework for the investigation of potential immune cross-reactivity. While the clusters are able to partially predict virulence potential based on shared ligand-binding profiles [17], there are few data relating to immunity. Bacteria from the 3 clusters investigated in our study induced differing immune responses after skin infections. Higher antibody titers were observed against cluster-related *emm* types than non-cluster-related antigens in the E6 and E4 clusters; however, this was only statistically significant for the E4 cluster. There was essentially no difference observed for strains belonging to the D4 cluster. The E4 cluster elicited high titers of cross-reactive antibodies that were capable of cross-opsonization of cluster-related *emm* types. It may be that M proteins belonging to the E6 cluster are capable of eliciting high titer antibody responses, but these antibodies have a variable cross-opsonizing capacity. M proteins belonging to the D4 cluster appear to be overall less immunogenic (average of 31% killing for the infecting strain; Figure 5C), and no difference was observed in antibody function between cluster and noncluster strains. Of note, cross-opsonization observed during preclinical studies of the 30-valent vaccine was notably lower in D4 strains than other clusters, with opsonization of 4 of 9 strains [12, 13]. The detection of antibodies that do not translate into killing may have a number of explanations. Immunity may be specific to each infection-patient pair, or influenced by other specific and conformational epitopes, or associated with an increase in specific IgG isotypes.

The existence of cross-protective immunity would suggest that multivalent vaccines have the potential to induce broader protection than type-specific immunity would predict. This hypothesis was first raised several years ago based on genetic analyses of Brazilian strains [15, 16]. It is also supported by a recent study in a rabbit model using an experimental multivalent vaccine for the E4 *emm* cluster [20]. Our study provides evidence that some cross-protection can in fact occur in vivo following clinical infection. Indeed, in one of the participants, antibody responses to the different cluster-related strains were consistent with antibody responses in rabbits to the experimental E4 vaccine [20]. While this may be the case for E4, it may not be applicable for D4 and thus a complementary antigen may be required for protection against these strains, such as, for example, the M-related protein [36]. Further investigation of antibody responses to the E6 cluster is required to fully elucidate

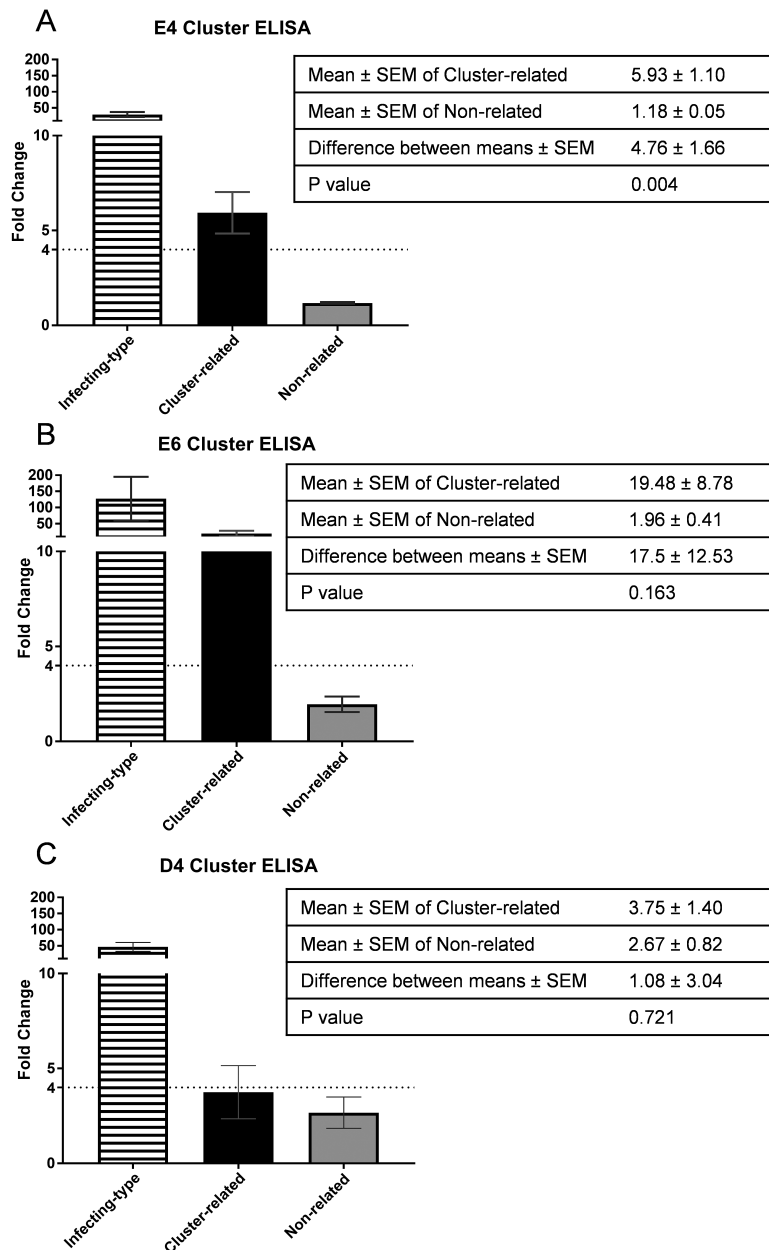


Figure 4. Grouped antibody responses to infecting-type, *emm* cluster-related, and non-cluster-related peptides. Antibody fold-changes values from paired samples were grouped according to *emm* cluster of the infecting strain. *emm* cluster-related and non-cluster-related responses were compared by *t* test to determine whether cross-reactive antibodies were more commonly raised against other M peptides within a cluster. The greatest difference was observed within cluster E6 and the least difference within cluster D4. The dotted line indicates a 4-fold increase, which was the threshold for a significant increase. Error bars represent the standard error of the mean. White striped bars = infecting-type peptide; black bars = *emm* cluster-related peptide; gray bars = non-cluster-related peptide. Abbreviations: ELISA, enzyme-linked immunosorbent assay; SEM, standard error of the mean.

the potential of a cross-protection to be exploited for vaccine development.

Epidemiological evidence suggests repeated GAS skin infection may immunize against throat infection, and also implicates a role for skin infection in the development of acute rheumatic fever [37–40]. The production of a broad immune response following impetigo is consistent with this hypothesis, but not conclusive. Follow-up studies on the duration of protection to

subsequent infection or development of autoimmune conditions is required to further investigate any potential association.

There are several limitations to this study. First, the small sample size needs to be considered when drawing conclusions. Second, samples were assayed against all cluster-related strains but only a selection of nonrelated strains. Finally, we used 50 amino acid peptides as ELISA antigens, which represent the portion of M proteins believed to be the most immunogenic but

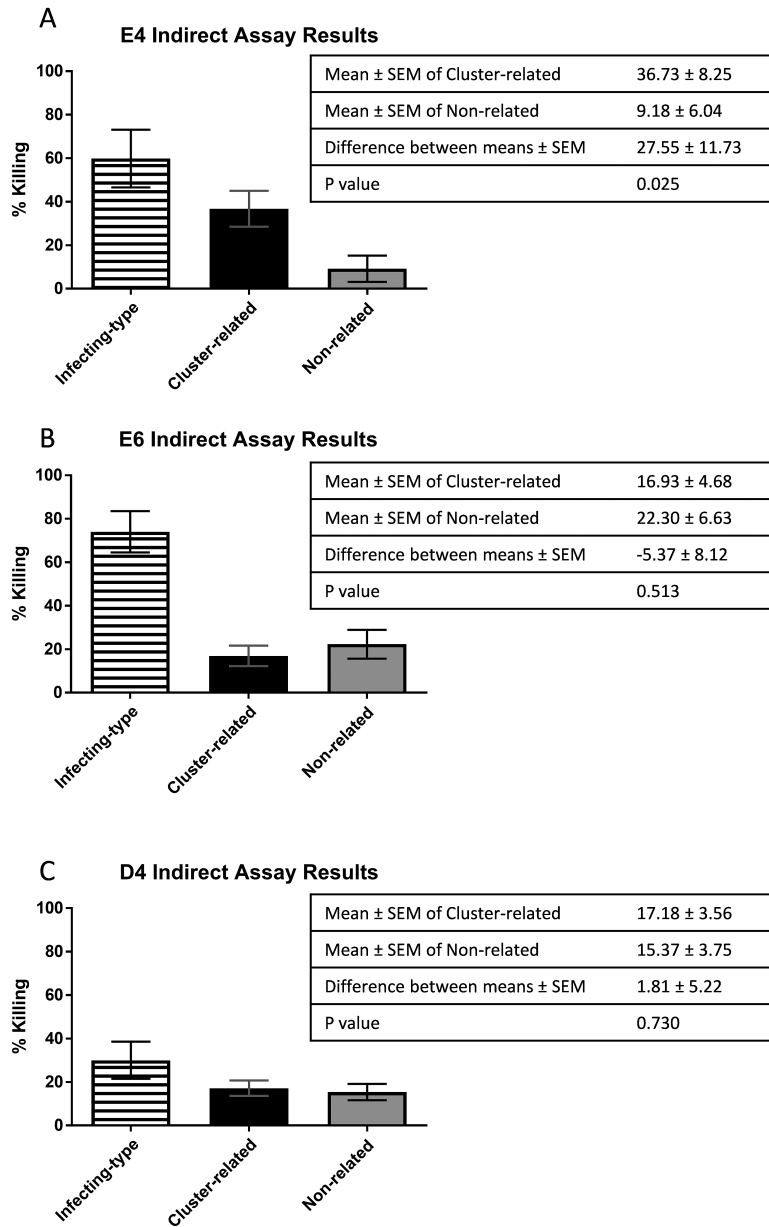


Figure 5. Grouped phenotypic responses to infecting-type, *emm* cluster-related, and non-cluster-related strains. All percentage killing results from included paired samples were grouped according to *emm* cluster of the infection. Killing of *emm* cluster-related and non-cluster-related strains were compared by *t* test to determine whether there was any difference in the overall capacity of postinfection sera to kill these strains. The greatest difference was observed within cluster E4 and there was no difference within clusters E6 and D4. White striped bars = infecting-type peptide; black bars = *emm* cluster-related peptide; gray bars = non-cluster-related peptide. Abbreviation: SEM, standard error of the mean.

Table 1. Summary of Assays With >50% Killing in Postinfection Sera

	Infecting Type			<i>emm</i> Cluster Related			Non-Cluster Related		
	Tested	Killing	%	Tested	Killing	%	Tested	Killing	%
E4	5	3	60.0	8	6	75.0	5	1	20.0
E6	4	3	75.0	6	4	66.7	6	3	50.0
D4	8	2	25.0	6	2	33.3	6	1	16.7
Total	17	8	47.1						

The samples with >50% killing of their infecting type strain were further examined for their ability to kill >50% of heterologous group A *Streptococcus* strains. In all 3 clusters, a higher proportion of the *emm* cluster-related strains had >50% killing than the non-cluster-related strains, with the greatest difference in E4.

do not account for the binding of conformational antibodies that may be highly important, particularly for cross-opsonization. Of note, the monomeric and short nature of the peptides used in our study may have led to an underestimation of the presence of cross-reactive antibodies due to loss of conformational epitopes present in the dimeric fully mature M protein.

Our study also has strengths including the use of a population at high risk of GAS impetigo for investigating the serological response to this condition. We were also able to compare the postinfection response to an internal baseline in the preinfection sera from the same patient, which allows more robust analyses than in cross-sectional studies. Furthermore, by grouping results by *emm* cluster, we were able to analyze the data at a population level rather than at the individual level, minimizing interference from the well-characterized intrinsic variation between individuals.

This study provides a population-based description of M protein immune response after GAS skin infection in an endemic setting. Our study confirms the existence of *emm*-type specific immunity, but suggests that this is an incomplete picture and that a combination of “cluster-specific” and “type-specific” responses occur. Our study suggests that cross-reactive immune responses occur following skin infection and raises hope for the development of a broadly protective multivalent vaccine.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Acknowledgments. We thank Jonathan Carapetis and Roy Robins Browne for their collaboration on this study.

Financial support. This work was supported by an National Health and Medical Research Council (NHMRC) project grant (grant number APP1051297) and the Murdoch Childrens Research Institute of Melbourne, Australia.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Title:

Immune Cross-Opsonization Within emm Clusters Following Group A Streptococcus Skin Infection: Broadening the Scope of Type-Specific Immunity

Date:

2017-11-01

Citation:

Frost, H. R., Laho, D., Sanderson-Smith, M. L., Licciardi, P., Donath, S., Curtis, N., Kado, J., Dale, J. B., Steer, A. C. & Smeesters, P. R. (2017). Immune Cross-Opsonization Within emm Clusters Following Group A Streptococcus Skin Infection: Broadening the Scope of Type-Specific Immunity. CLINICAL INFECTIOUS DISEASES, 65 (9), pp.1523-1531.
<https://doi.org/10.1093/cid/cix599>.

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