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Do mixed infections matter? Assessing virulence of mixed-clone infections in experimental human and murine malaria

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ABSTRACT

Background: Malaria parasites within an individual infection often consist of multiple strains (clonal populations) of a single species, which have the potential to interact both with one another, and with the host immune system. Several effects of these interactions have been measured in different parasite systems including competition and mutualism; however, direct observation of these effects in human malaria has been limited by sampling complexities and inherent ethical limitations.
Methods: Using multiple complementary epidemiological models, we propose a suite of analyses to more fully utilize data from challenge experiments, and re-examine historical human challenge studies with mixed-strain *Plasmodium vivax* inocula. We then compare these results with murine model systems using mixed-strain *Plasmodium yoelii* or *Plasmodium chabaudi*, to explore the utility of these methods in fully utilizing these data, including the first quantitative estimates of effect sizes for mixed-strain parasitemia. These models also provide a method to assess consistency within these animal model systems.
Results: We find that amongst a limited set of *P. vivax* (incubation time) and *P. yoelii* infections (time-to-mortality), survival times at a study population-level are intermediate between each single-clone infection, and are not dominated by the more virulent clone; in *P. vivax* relapses, mixed clone infections also show intermediate survival curves. In these infections, the results strongly suggest that highly virulent clones have their virulence attenuated by the presence of less-virulent clones. The analysis of multiple experiments with *P. chabaudi* suggests greater nuances in the interactions between strains, and that mortality and time-to-event in mixed-strain infections are both indistinguishable from single infections with the more virulent strain.
Conclusions: These divergent dynamics support earlier work that suggested drivers of virulence may differ in fundamental ways between malaria species that are reticulocyte-specific and those that readily infect all red blood cell stages which should be studied in greater detail. The effect sizes (magnitude of biological effects) from these analyses are significant, and suggest the potential for important gains in malaria control by greater incorporation of evolutionary epidemiology theory. Moreover, we suggest that using these epidemiological models may generally allow fuller use of data from experimentally challenging animal model experiments.

1. Introduction

Malaria is a major contributor to morbidity and mortality globally, with an estimated 198 million cases (95% CI: 124 to 283) and 584,000 deaths (95% CI: 367,000 to 755,000) in 2013 (World Health Organization, 2014). There are five species that generally infect humans, and the interactions between different species within a single infection can have important clinical implications (Zimmerman et al., 2004). Moreover, infections within an individual may be composed of multiple strains (clonal populations) of a single species, which may interact both with one another and with the host immune system (Alizon et al., 2011).

The impacts of these interactive parasite populations have been explored through multiple lines of research using two complementary approaches: mathematical/statistical models, and experimental animal systems. The interactions between parasite populations may lead to parasite competition or to parasite mutualism; to the evolution of virulence and drug susceptibility; and may facilitate genetic exchange within infections (Balmer and Tanner, 2011). Moreover, these assemblages of hosts and multiple parasites form an evolutionary community, and the incorporation of ecological and evolutionary theory (Alizon et al., 2009; Rigaud et al., 2010), has lead to the development of

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evolutionary epidemiology as a field in itself (Restif, 2009). However the impacts of these parasite interactions on the host are highly variable, and observed virulence in mixed-strain infections ranges along a continuum from being greater than the more virulent parasite; less than the least virulent strain; or any intermediate virulence between these extremes (Alizon et al., 2013).

In most epidemiological settings, the majority of individual *Plasmodium vivax* and *Plasmodium falciparum* infections are composed of multiple strains e.g., (Henry-Halldin et al., 2011; Juliano et al., 2010), and these infections may represent an underutilized tool to explore complex transmission dynamics (Bordes and Morand, 2009; de Araujo et al., 2012). However, the inherent difficulties of data capture across multiple spatial and temporal scales, and practical limitations in data collection from parasitized hosts in natural environments have led to suggestions for increased multidisciplinary research including the use of more diverse methodologies to triangulate findings across different specializations (Restif et al., 2012).

This work aims to address three specific questions: i) how can a broader range of analytical approaches contribute to what is known about mixed-strain infections with *Plasmodium* and within-host dynamics?; ii) are results from the limited human challenge studies consistent with what is known from murine model systems?; and iii) do these results support any specific drivers of virulence?

1.1. Strain theory and virulence

The concept of parasite 'strains' is pervasive throughout the medical and malaria literature, however, no consensus has emerged on what these clinical isolates represent (Balmer and Tanner, 2011; McKenzie et al., 2008); in general they refer to clonal or at least closely-related populations.

While results have not been entirely consistent, themes have emerged that appear to be general across parasite and host species (Alizon et al., 2013). Observational data suggest that clones may manifest with diverse clinical impacts, including differences in virulence, clinical severity, transmissibility, and both the number and spacing of hypnozoite-derived relapses in *P. vivax* infections (reviewed in McKenzie et al., 2008). Recent molecular and genetic approaches have added to this knowledge base by characterizing both inter- and intra-host infections; these studies suggest that while many strains/isolates represent a diversity of clonal populations, they generally produce stable clinical and immunological responses (McKenzie et al., 2008). Moreover, consideration of virulence metrics *across the entire duration of infection* is critical to understand the pressures that drive strain selection (Barclay et al., 2014).

Virulence in malaria infections is a composite outcome of at least three processes: specific hematological impacts; host immune response to toxins (including hemozoin and other parasitic debris); and cyto-adhesion in some parasite species including *P. falciparum* (reviewed in Mackinnon and Read, 2004; Wassmer et al., 2015). Assessments of the relative impact of these divergent processes have been the subject of many experimental studies, and new modeling approaches have been developed to disentangle the specific physiological processes behind the composite endpoint of 'virulence' (Metcalf et al., 2011). These and related studies suggest that the relative contribution of each of these facets of observed virulence can vary dramatically during the time course of an infection in a strain-specific manner (Metcalf et al., 2012).

As noted by Alizon and coworkers, theoretical models almost universally define virulence as an increase in host mortality, but in the broader parasitology literature virulence is considered to be any harm caused by a parasite that negatively impacts host fitness (Alizon et al., 2013). While a range of definitions have appeared (Casadevall and Pirofski, 2001; Poulin and Combes, 1999), herein we consider it as 'parasite populations that maximally exploit host resources' to capture the range of endpoints examined in this study, with a focus on direct parasite-induced mortality.

1.2. Previous analytical strategies

In general, previous analyses of murine systems have focused on pooled hematological or parasitological outcomes as proxies for virulence (comparing means or geometric means from all surviving animals) e.g., (Bell et al., 2006), but full multivariate analyses of outcomes or explicit consideration of times-to-events have received very limited attention. In the original analysis of Bell et al., differences between single- and mixed-strain infections were not examined; virulence was assessed by maximum anemia and parasitemia and showed broad trends, but with complex relationships between the two metrics [e.g., Fig. 1 in (Bell et al., 2006)].

Moreover, while time itself is a critical component of virulence (Day, 2002), analyses have generally focused on analysis at several (potentially arbitrary) time points. In many cases, censoring of subjects has not been considered — that is, animals are simply removed from analysis; beyond a potential for introducing biases, this greatly reduces the power of the analysis (Rothman et al., 2008). Finally, most parasitological studies report only p-values to evaluate significance, as is the norm in ecology; however, these do not allow consideration of the effect sizes. That is, statistically significant effects may not be clinically or biologically important- or as well-phrased by others, "Identifying biological importance is what all biologists are ultimately aiming for, not the identification of statistical significance." (Nakagawa and Cuthill, 2007). Although one prior study measured the statistical significance of virulence (Taylor et al., 1998), we are unaware of any prior effect size estimates.

2. Materials and methods

The human study data (Dataset I; Table 1) in this analysis was obtained during a series of rigorous challenge experiments in prison volunteers during the 1940s–50s in an antimalarial drug development program; all case-patients were malaria-naïve white males. Infections were via mosquito challenge, there was complete follow-up, and patients were protected from superinfection due to the institutional nature of this population. The study population (Table 1) includes a less-virulent historical strain from North America and a highly virulent clone from Papua New Guinea (Collins, 2013; Ungureanu et al., 1976). Full details for human challenge infections including search criteria and primary references were previously published (Lover and Coker, 2013); mixed-strain challenges can be found in (Cooper et al., 1950).

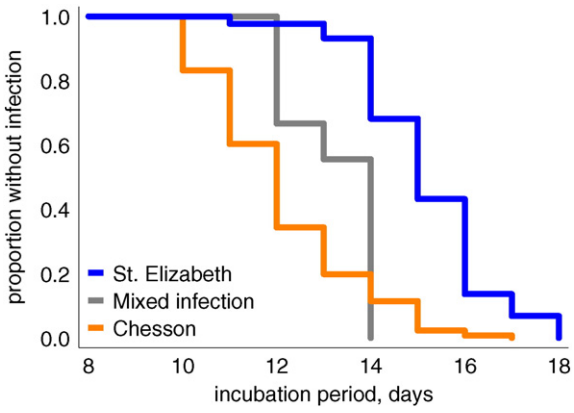

Fig. 1. Kaplan–Meier curves comparing incubation periods in single strains and mixed-strain infections in human challenge experiments with *Plasmodium vivax* (Dataset I; N = 184).