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The Effect of Reinfection and Mixed *Trypanosoma cruzi* Infections on Disease Progression in Mice

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List of DTUs — discrete typing units

PCR — polymerase chain reaction

TCDT — tissue culture derived trypomastigotes

Graphical abstract

The Effect of Reinfection and Mixed *T. cruzi* Infections on Disease Progression in Mice

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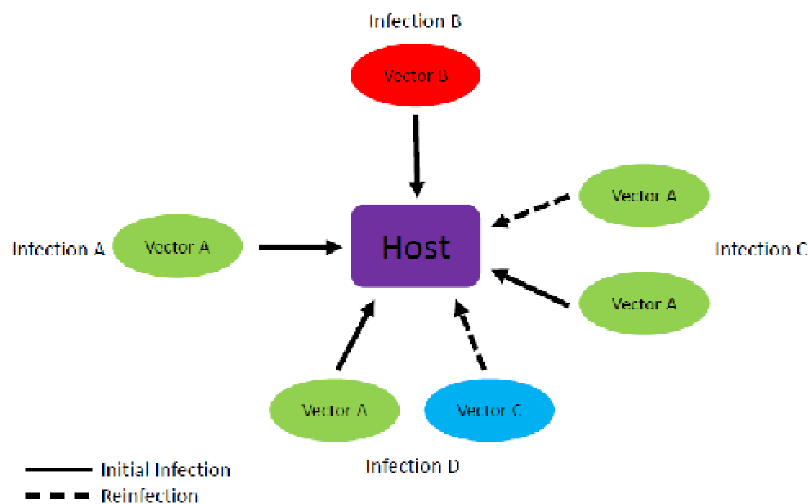
Using two phenotypically unknown isolates, this paper aims to investigate the effect of mixed *T. cruzi* infections and reinfections on murine host survivability and disease progression.



Highlights

- No differences in host survival were observed in mixed *T. cruzi* infected hosts
- Spontaneous deaths were observed following *T. cruzi* reinfection
- Compared to single infections, reinfection significantly increased mortality rate

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- Reinfection was associated with a significant reduction in host survivability

Abstract

The progression of Chagas disease (CD) varies significantly from host to host and is affected by multiple factors. In particular, mixed strain infections and reinfections have the potential to exacerbate disease progression subsequently affecting clinical management of patients with CD. Consequently, an associated reduction in therapeutic intervention and poor prognosis may occur due to this exacerbated disease state.

abbreviations

CD Chagas Disease

Keywords: reinfection; mixed infection; Chagas; disease complications; *Trypanosoma cruzi;* acute death

This study investigated the effects of mixed strain infections and reinfection with *Trypanosoma cruzi* in mice, using two isolates from different discrete typing units, TcI (C8 clone 1) and TcIV (10R26). There were no significant differences in mortality rate, body weight or body condition among mice infected with either C8 clone 1, 10R26, or a mixture of both isolates. However, the parasite was found in a significantly greater number of host organs in mice infected with a mixture of isolates, and the histopathological response to infection was significantly greater in mice infected with C8 clone 1 alone, and C8 clone 1 + 10R26 mixed infections than in mice infected with 10R26 alone.

To investigate the effects of reinfection, mice received either a double exposure to C8 clone 1; a double exposure to 10R26; exposure to C8 clone 1 followed by 10R26; or exposure to

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10R26 followed by C8 clone 1. Compared to single infection groups, mortality was significantly increased, while survival time, body weight and body condition were all significantly decreased across all reinfection groups, with no significant differences among these groups. The mortality rate over all reinfection groups was 63.6%, compared to 0% in single infection groups, however there was no evidence of a greater histopathological response to infection.

These results suggest firstly, that the C8 clone 1 isolate is more virulent than the 10R26 isolate, and secondly, that a more disseminated infection may occur with a mixture of isolates than with single isolates, although there is no evidence that mixed infections have a greater pathological effect. By contrast, reinfections do have major effects on host survivability and thus disease outcome. This confirms previous research demonstrating spontaneous deaths following reinfection, a phenomenon that to our knowledge has only been reported once before.

1.1 Introduction

Chagas Disease (CD) is a zoonotic disease of global significance, caused by infection with *Trypanosoma cruzi*. CD commonly occurs in humans and dogs, with various other mammalian and non-mammalian species acting as reservoir hosts [1, 2]. Global migration has resulted in the spread of infection, with increasing numbers of infected individuals migrating worldwide to countries non-endemic for CD [3, 4]. Historically, the global spread of *T. cruzi* transmission has been restricted by the lack of insect vectors in areas non-endemic for disease. However, there is now evidence to suggest that vectors capable of *T. cruzi* transmission may exist within these areas [2, 5-7].

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Within endemic areas, vector borne transmission remains the central mode of infection, with more than 80% of disease attributable to vectorial transmission [8] and more than 40% of sampled vectors across South America reported to be infected with *T. cruzi* [9, 10]. The domestication of triatomines, the style of housing [11] and proximity to henhouses, pigsties and animal pens, in combination with poor food hygiene, all lead to an increased risk of vectorial transmission [12]. The feeding habits of vectors within areas endemic for CD, whether that be on infected or naïve hosts, also affect the risk of host re-exposure and thus the rate of reinfection or super-infection (infection on more than one occasion) [13] and mixed infections or polyparasitism (infection with more than one genotype of the parasite) [14].

T. cruzi is genetically highly heterogeneous [15], with isolates classified into discrete typing units (DTUs) TcI through to TcVI. These classifications are based on genetic, biological and ecological characteristics, with specific correlations existing between geographical distributions and sylvatic or domestic transmission cycles [16]. The circulation of genetically different forms of *T. cruzi* within vector populations [17] means that there is likely to be a complex interaction between reinfection and mixed infections, as there are various scenarios where hosts may encounter *T. cruzi* genotypes. For example, if we consider infections with up to two different *T. cruzi* genotypes, a host may encounter a single infection with one genotype as the result of a bite from one singly infected vector; a mixed infection with two genotypes as the result of a bite from one vector infected with different genotypes; reinfection with a single genotype as the result of multiple bites from vectors singly infected with the same genotype; or reinfection with different genotypes as the result of multiple bites from vectors harbouring different genotypes (Figure 1). The complexity of

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these interactions may be extensive, with 89 different genotypes of *T. cruzi* isolated from humans in Bolivia alone [18].

How might these interactions affect the progression of CD, patient prognosis and clinical management? Reinfection can result in a more severe disease progression developing, exacerbating the consequences of infection in terms of inflammation, fibrosis and/or necrosis within host organs [19, 20]. By contrast, other studies have found an abatement of host effects with reinfection, a phenomenon described as immune protection [21]. Mixed infections may also have variable effects on disease progression, given that growth patterns and phenotypic behaviour of one genotype are potentially altered by the presence of another *T. cruzi* genotype within the host [22]. Complications associated with multiple infections may also adversely affect patient outcome and the success of therapeutic interventions [23], with genotypes varying in their sensitivity to benznidazole [24]. Additionally, the genotypes of parasites circulating in the host bloodstream can differ from those present within host organs, leading to difficulties in diagnosing mixed infections [25, 26] and the potential for underreporting. Despite these previous studies, however, we lack a clear understanding of the effects of genetic diversity in mixed infection and reinfection scenarios, or whether the order of exposure in mixed reinfections has the potential to alter disease progression depending on which genotype establishes infection first.

Mouse models for CD are commonly utilised for investigating drug efficacy and the host immune response to infection, and previous work in our laboratory has used mice to study complications to CD arising from reinfection and immune suppression. In this study, we aimed to investigate the effects of simultaneous mixed infections, and reinfections with the same or different genotypes of *T. cruzi*, on disease progression in mice using isolates of *T.*

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cruzi from two different DTUs; TcI (C8 clone 1) and TcIV (10R26). We hypothesised firstly, that simultaneous mixed infections and reinfections with these isolates would exacerbate host morbidity and mortality compared to single infections with one isolate; and further, that the impact of reinfections would be mediated by the order in which isolates were introduced into the host.

1.2 Materials and Methods

1.2.1 Experimental design

Seven treatment groups made up of either 10 or 15 Swiss mice per group were infected by intraperitoneal (i.p) injection with different combinations of *T. cruzi* isolates (Table 1). To compare the effects of single infections and mixed infections on the host, two singly infected treatment groups (one group infected with C8 clone 1 and the other infected with 10R26); and one mixed infection group (animals infected with both C8 clone 1 and 10R26) were used. In order to investigate reinfections and any effects associated with the order of exposure on the host, four treatment groups were used: C8 clone 1 infected and 10R26 reinfected; 10R26 infected and C8 clone 1 reinfected; C8 clone 1 infected and reinfected; and 10R26 infected and reinfected.

Where relevant, reinfection was carried out on day 14 by i.p. injection. Fifty thousand tissue culture derived trypomastigotes (TCDT) of the relevant isolate was utilised for each inoculant, and in the case of the mixed infection treatment group, 50,000 TCDT of each isolate was inoculated. Previous studies on these isolates in this laboratory demonstrated no significant effect of inoculant size (between 25,000 and 100,000 TCDT) on host morbidity or disease outcome (data not shown). To reduce the number of mice required, we did not utilise sham-treated control groups; previous studies have followed a similar protocol for

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intraperitoneal (i.p) injection [27-29], reinfection [20, 30, 31] and cyclophosphamide/immune suppression [32, 33], as the handling involved in these treatments is not expected to alter disease progression.

1.2.2 Parasites

The phenotypic characteristics of the two isolates that were utilised in this study are not known, but they are phylogenetically distinct (C8 clone 1 from the TcI DTU and 10R26 from the TcIV DTU) and both have relevance to CD within humans [34, 35]. The C8 clone 1 isolate was obtained from a vector within the domestic cycle, and 10R26 was isolated from a monkey in the sylvatic cycle.

Tissue culture derived trypomastigotes (TCDT) were used for the inoculation of all experimental animals. The C8 clone 1 and 10R26 clones were received as epimastigotes, shock thawed, and grown in plates containing 25 g/L Liver Infusion Tryptose medium (BD Difco, France) supplemented with 4 g/L NaCl (Sigma-Aldrich, USA), 2 g/L D-glucose (Sigma-Aldrich, USA), 0.4 g/L KCl (UniVar, New Zealand), 3.15 g/L Na_2HPO_4 (Chemsupply, Australia), 1 ml of 25 mg/ml haemin (Sigma-Aldrich, USA) prepared in 1 M NaOH (Sigma-Aldrich, USA) and 10% heat-inactivated foetal bovine serum (Sigma-Aldrich, Australia) at 27°C in 5% CO_2 to encourage metacyclogenesis. L6 cells were used to propagate TCDT for inoculation, and were grown in Roswell Park Memorial Institute-1640 medium (Sigma-Aldrich, Australia) supplemented with 10% heat-inactivated foetal bovine serum (Sigma-Aldrich, Australia) at 37°C in 5% CO_2. Following metacyclogenesis, mammalian cell monolayers were infected by the addition of metacyclic trypomastigote cultures and incubated at 37°C in 5% CO_2. After 24 hours, culture supernatants were replaced, with subsequent cultures created every 3-4 days by the addition of trypomastigotes from healthy cultures to fresh mammalian cell

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cultures. For animal work, parasites were enumerated prior to infection and adjusted to a concentration of 5 x 10^5 trypomastigotes/ml or 1 x 10^6 trypomastigotes/ml.

1.2.3 Animal monitoring and measurement

Ninety five Swiss outbred mice were received from the Animal Resources Centre (Canning Vale, Australia). During the experiment, water and chow were available *ad libitum* within a temperature controlled room on a 12 hour light/dark cycle. Enumeration of parasitaemia was carried out by fresh blood examination by microscopy of blood obtained from tail venepuncture every second day, up to 30 days post infection (p.i.). Animals were weighed (to the nearest gram) prior to infection and prior to blood sample collections. Daily monitoring was carried out prior to sampling, and noticeable changes in posture, activity, gait, respiratory patterns, hydration, body condition and hair were documented using a grading system: 0 (no obvious deviation from normal); 1 (slight possible abnormality); 2 (definite change from normal, but not marked); or 3 (a gross change from normal). The sum of these scores generated a body condition score for each animal per monitored day over the experimental period. An acute reduction in body condition was observed in a subset of reinfected animals during the experiment; these animals were immediately euthanized and counted as 'mortalities' for analysis. Cardiac punctures were carried out where possible and tissue samples taken from these 'mortalities'. On day 30, all remaining mice were euthanized, cardiac punctures carried out and tissue samples taken. Tissue samples harvested included colon, heart, both kidneys, liver, skeletal muscle and spleen. Organ samples were divided in two portions, with one portion fixed and stored in 100% ethanol for PCR analysis, and the other fixed and stored in 10% formalin for histological analysis.

1.2.4 Histopathology

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Formalin fixed samples of kidneys, skeletal muscle, heart, liver, spleen and colon were sectioned (4µm thick) and stained with Hematoxylin and Eosin. Slides and tissues were evaluated and analyzed in a single-blind study carried out by one pathologist, based on the method of Gruendling et al. [36]. For each of the tissues evaluated, the parameters included tissue parasitism, inflammatory process (intensity, type, and distribution), and tissue reaction (necrosis, haemorrhage). Tissue parasitism was evaluated at 400x magnification based on the presence or absence of amastigote nests and classified as: 0 (absent); 1 (mild, 1-5 nests); 2 (moderate, 6-10 nests); or 3 (severe, >10 nests). The inflammatory process was evaluated for intensity (number of inflammatory cells at 400x magnification) and classified as: 1 (mild, 10-25 cells); 2 (moderate, 26-50 cells); or 3 (severe, >50 cells). Tissues were additionally evaluated for necrosis and haemorrhage, and were classified as 1 (mild, 0-30% affected); 2 (moderate, 30-55% affected) or 3 (severe, >55% affected). A total pathology score was created for each animal by summing scores for inflammatory response, necrosis and haemorrhage over all tissues.

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1.2.5 PCR and DNA sequencing

DNA extraction was carried out as per kit instructions (Qiagen, Germany) and DNA was stored at -20°C until analysis was carried out. Positive and negative controls were utilised in each DNA extraction and PCR reaction. Initially, PCR was carried out using *Trypanosoma cruzi* specific primers (TCZF 5' GCT CTT GCC CAC AMG GGT GC 3' and TCZR 5' CCA AGC AGC GGA TAG TTC AGG 3') that detect an 188bp fragment of DNA. Each reaction tube contained 10x reaction buffer (670mM Tris-HCl, 166mM $(NH_4)_2SO_4$, 4.5% Triton X-100, 2 mg/ml Gelatin), 0.04% cresol red, 2mM $MgCl_2$, 1.25µM of each primer, 0.5µM of each dNTP and 0.5U Taq polymerase.

The generic primer set used to amplify *T. cruzi* satellite DNA was used to confirm the presence or absence of *T. cruzi* DNA within different tissues, with bands of expected size (188bp) selected, purified and prepared for sequencing submission. Bands were cut from electrophoresis gels and DNA from the bands was extracted [37]. DNA was purified using Agencourt AMPure PCR Purification system (manufacturer's instructions) and sequenced using an ABI Prism™ Terminator Cycle Sequencing kit (Applied Bio-systems, USA) on an Applied-Biosystem 3730 DNA Analyzer. Sequences for all bands of the expected size were confirmed as *T. cruzi* satellite DNA by BLAST search.

In order to differentiate between the two isolates within mixed infections, amplification of the intergenic region of the *T. cruzi* mini-exon genes was carried out using TCC (5' CCCCCCTCCCAGGCCACACTG 3'), TCI (5' GTGTCCGCCACCTCCTTCGGGCC 3') and TCII (5' CCTGCAGGCACACGTGTGTGTG 3') primers as previously published [38], and amplification of the 24Sα rDNA region was carried out using D71 (5' AAGGTGCGTCGACAGTGTGG 3') and D72 (5' TTTTCAGAATGGCCGAACAGT 3') primers. Each reaction tube contained 10x reaction

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buffer (670 mM Tris-HCl, 166 mM $(NH_4)_2SO_4$, 4.5% Triton X-100, 2 mg/ml gelatin), 0.04% cresol red, 0.5 mM $MgCl_2$, 0.4 µM of each primer, 0.2 mM of each dNTP and 2.5 U Taq polymerase. PCR Samples were incubated at 94°C for 5 minutes prior to undergoing 50 cycles at 94°C for 20 seconds, 60°C for 10 seconds and 72°C for 30 seconds, followed by a final elongation at 72°C for 7 minutes. Amplified bands were visualised on a 2.0% agarose gel containing 2% SYBR safe using a dark-reader trans-illuminator.

The mini-exon primer set TCC, TCI and TCII has been utilised previously to discriminate between TcI and TcIV *T. cruzi* isolates, however this primer set gave no amplification for 10R26 control DNA, a phenomenon that has previously been documented [39]. Subsequently, D71 and D72 primers [38, 40] detecting the 24Sα rDNA, were utilised and although this primer set could successfully differentiate between the TcI and TcIV DTU single controls, when the two controls were mixed only one band was detected, suggesting the preferential amplification of DNA of one of the DTUs over the other. Given this limitation, the confirmation of which of the two isolates was present within host organs within mixed infection scenarios was unable to be determined. Given the unequal distribution of parasites present within host tissues it was not feasible to determine the parasite load within host tissues and additionally the use of quantitative PCR (qPCR) was outside of the scope of these studies.

1.2.6 Data analysis

The data were analysed as two separate experiments: first, comparing the two single infection groups with the simultaneous mixed infection group; and second, comparing the single infection groups with the four different reinfection groups. Mortality rates were compared among treatments by a contingency χ^2 analysis. If a significant effect was

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identified, multiple pairwise comparisons were carried out using a Bonferroni correction, to identify which treatments differed from each other. Survival times were compared among treatments by the Kaplan–Meier method, with a χ^2 approximation to the log rank test. Final body weight, pathology score and the number of organs infected by *T. cruzi* (as determined by PCR) were compared among treatments by one-way analysis of variance, followed by Tukey's HSD test for multiple comparisons. The residuals from all of these analyses were normally distributed. The residuals from an initial analysis of body condition score were markedly non-normal, and comparisons among treatments for this variable were instead made using a non-parametric Kruskal-Wallis test, followed by pairwise Wilcoxon tests for multiple comparisons, using a Bonferroni correction.

Tissue tropism in the isolates was examined by comparing the distribution among tissues for each isolate in the two single infection groups. These data were analysed using a generalised linear model, treating presence or absence of parasites (as determined from PCR) as a binomial response variable with a logit link function, with isolate, tissue type, and their interaction as factors.

All statistical tests were carried out using the software JMP 10.0 (SAS Institute Inc., Cary, NC, USA) and survivability plots were constructed using GraphPad Prism 5.

1.3 Results

1.3.1 Single infections and mixed isolate infections

There were no significant differences in the body weight or condition score of animals assigned to the different treatment groups prior to infection. No mortalities were observed in either the C8 clone 1 or 10R26 singly infected, or C8 clone 1 + 10R26 mixed infected animals, nor did they differ in body weight or condition at the end of the experiment. There

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were significant differences among treatments, however, in pathology scores ($F_{2,37}$ = 4.67, P = 0.02), and number of organs infected ($F_{2,36}$ = 7.96, P = 0.001). Pathology scores were significantly greater for animals singly infected with C8 clone 1, or mixed infected with C8 clone 1 + 10R26, than for animals singly infected with 10R26 (Figure 1.2). The number of infected organs was greater for animals mixed infected with C8 clone 1 + 10R26 than for those singly infected with either clone (Figure 1.3 and Table 2). Although there were differences among tissues in the probability of infection (χ^2_6= 77.5, P < 0.0001) for the two single infection groups, there were no significant effects of isolate (χ^2_1= 1, P = 0) or isolate x tissue interaction (χ^2_6= 6.4, P = 0.4).

1.3.2 Single infections and reinfections

There were no significant differences in the body weight or condition score of animals assigned to the different treatment groups prior to infection. Following reinfection, spontaneous deaths were observed in all reinfection groups, with an overall mortality rate of 63.6% (Figure 1.4). There were significant differences among treatment groups in mortality (χ^2_5 = 38.97, P < 0.0001) and survival time (χ^2_5 = 29.24, P < 0.0001), with all reinfection groups differing significantly from single infection groups (P < 0.05, with the Bonferroni), but not among themselves. There were also significant differences among treatment groups in final body weight ($F_{5,74}$ = 2.79, P = 0.02), body condition (χ^2_5 = 28.38, P < 0.0001), pathology score ($F_{5,73}$ = 3.47, P = 0.01) and the number of infected organs ($F_{5,73}$ = 3.89, P = 0.003). Pairwise comparisons indicated that body weight and condition were significantly less in all reinfection groups than in single infection groups, but did not differ among themselves. Pathology scores were significantly greater for animals singly infected with C8 clone 1 than those singly infected with 10R26, but did not differ among reinfection

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groups or between reinfection and single infection groups (Figure 1.5), while the number of infected organs was greater for animals infected and reinfected with C8 clone 1 than for C8 clone 1 singly infected animals or for those infected with C8 clone 1 and reinfected with 10R26 (Figure 1.6 and Table 3).

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1.4 Discussion

The results from this study have partially confirmed our initial hypotheses. Although we found no increase in host morbidity or mortality as a result of a simultaneous mixed infection with two genetically different isolates of *T. cruzi*, there was evidence that the parasite was more widely disseminated among host tissues. Reinfection, by contrast, led to a marked increase in both morbidity and mortality, regardless of which of the two isolates were involved.

1.4.1 Effects of reinfection

Previous studies exploring reinfections have provided contrasting results. On the one hand, the generation of a parasitaemia following initial infection, with subsequent infections generating lower [21] or no parasitaemias in mice, and the observation of no significant increase in tissue damage in reinfected dogs independent of isolates utilised [41], are indicative of host immune protection. Conversely, other studies investigating reinfection have demonstrated a failure to protect mice [42], with the observation of an increased parasitaemia [30] and an increase in the severity of cardiac alterations and disease progression [20, 43]. Subsequently, these alterations may lead to death of the host [31].

The dichotomy of host effects observed following reinfection highlights the complexity of disease progression and the way in which it may be altered due to complications to infection. Much of the frustration surrounding CD research is due to the heterogeneity of symptoms and disease progression observed across the study of different *T. cruzi* strains and hosts [44]. This genetic heterogeneity means that dissimilar outcomes from reinfection experiments may reflect differences in parasite virulence or in the host immune response to the different parasite genotypes utilised. While the use of reference or highly utilised

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genotypes such as the Y [31, 43], Tulahuen [20] and Colombian strains [45] in previous reinfection studies was useful in order for comparisons to be drawn between studies, more studies involving diverse and previously uncharacterised genotypes are also required to expand our knowledge.

In the current study, all four reinfection scenarios produced a sudden and rapid deterioration in body condition 14 days p.i., sufficient to require immediate euthanasia. As far as we are aware, only one previous study has found spontaneous deaths, where unexplained deaths were acutely observed following *T. cruzi* reinfection in mice [31]. The reinfection scenarios in this study by Andrade *et al.* [31] involved the use of single Colombian infected controls, animals re-infected with two different isolates in separate inoculants (Colombian, then 21SF; or Colombian, then Y strain); and animals reinfected with three different isolates in separate inoculants (Colombian, then 21SF, then Y strain; or Colombian, then Y, then 21SF). Because there was no mixed infection or simultaneous infection baseline group, it is unclear whether the challenge of reinfection or the exposure to multiple strains was the cause of the spontaneous deaths observed, or whether it was in fact an unrelated anomaly. In the current study, we were able to determine, firstly, that simultaneous mixed infections did not produce acute morbidity and, secondly, that reinfection did produce acute morbidity, regardless of whether it involved one or two genetically different isolates of the parasite, and regardless of the order in which the isolates infected the host.

Andrade *et al*. [31] provided no explanation for the spontaneous deaths they observed, however we propose that these may have been due to cumulative effects of tissue damage by isolates that differed in their tissue tropism and pathogenicity and/or an aggravated

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hypersensitivity reaction. In the current study, the results would suggest that a hypersensitivity reaction was a more likely cause, because of the absence of any synergistic effect from infection with different isolates, either simultaneously or separately. Cardiac alterations and involvement is the leading known proximate cause of acute or sudden deaths associated with CD [46-48], and there was evidence of enhanced myocardial pathology following reinfection with three isolates by Andrade *et al*. [31]. In the current study however, there was no pathological evidence to support cardiac alterations as the cause of death. Another known cause of CD-related sudden deaths is severe meningoencephalitis [49, 50] however because brain pathology was not investigated we cannot comment on the likelihood of this occurring, nor can we rule out another unknown cause for this host response.

1.4.2 Effects of multiple infection

In contrast to results from reinfection studies, simultaneous mixed infections with the C8 clone 1 and 10R26 isolates had no observable effects on host pathology. Although no parasitaemia was observed, nor were there any differences in host survival or pathological response between mice receiving single-isolate or mixed infections, we found that mixed isolate *T. cruzi* hosts demonstrated a more disseminated infection (as determined by PCR) compared to singly infected animals. Considering the marked differences observed in tissue tropism across strains [51-53], if the tissue tropism of the infecting isolates differ, than a more disseminated infection is possible. Further, tissue tropism may be altered by parasite-parasite interactions within mixed infections [54], therefore potentially altering the dissemination of pathology. However, we found no differences in tissue tropism across the singly infected C8 clone 1 or 10R26 groups, thus not supporting this theory. There is also the

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potential for the increased dissemination of infection to be due to the increased inoculant size due to multiple exposures, however previous research carried out in our laboratory demonstrated no observable difference in the pathology, host outcome or disease progression for individuals that received higher inoculants (data not shown).

Previous research investigating *T. cruzi* experimental mixed infections is limited, however one study reported that their mixed infected group demonstrated a severe inflammatory infiltrate, compared to singly infected groups [26]. Interestingly, another study reported that within TcIII/TcV, TcIII/TcVI, and TcV/TcVI mixed infected groups, the presence of both isolates was not detected within the same host tissue [54], perhaps resulting in an overall increase in the dissemination of infection. Although we were unable to confirm which isolate was present in each host tissue, it is possible that the increase in number of organs infected with *T. cruzi* was due to competition among isolates. This presents an important area for future research. There has been much interest in within-host competition among parasite strains, particularly if those strains differ in virulence. Both theoretical [55, 56] and empirical [57] studies suggest that more virulent parasite strains will out-compete less virulent strains, although, as far as we are aware, this has not been tested in *T. cruzi*.

The results of this study highlight the significance that mixed infections may have on host survivability and disease progression in terms of the dissemination of disease throughout the host, which can affect disease outcome and the success of therapeutic interventions [23, 58]. Mixed infections also create additional complications for drug discovery work [58], and may explain contradictory results obtained from human treatments [59]. Current literature on drug efficacy and treatment outcomes for mixed (and also reinfection) scenarios are incredibly limited, given the potential ramifications to the treatment of CD.

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Further research in this area may provide useful information on the effects of mixed infection and lead to crucial improvements in treatment options.

1.4.3 Differences between isolates

Mice that were singly infected with C8 clone 1 had significantly greater mean pathology scores than those that were singly infected with 10R26. Further, animals infected and reinfected with C8 clone 1 had a significantly greater number of organs PCR positive compared to mice singly infected with C8 clone1, while 10R26 infected and reinfected animals demonstrated no such difference to mice singly infected with 10R26. These results suggest that the C8 clone 1 isolate has a greater propensity to infect and cause damage within hosts than 10R26.

Greater virulence has been associated with the presence of a parasitaemia in previous studies [28, 60, 61]. Within blood samples in the current study, C8 clone 1 singly infected animals demonstrated an absence of *T. cruzi* DNA, however *T. cruzi* DNA was detected in mixed C8 clone 1 + 10R26 infections, C8 clone 1 infected and reinfected, C8 clone1 infected and 10R26 reinfected, and 10R26 infected and C8 clone 1 reinfected scenarios. Additionally, *T. cruzi* was not detected in blood samples from animals infected with, and infected and reinfected with 10R26. Although discrimination between isolates within mixed infection scenarios was not possible, these results suggest that C8 clone 1 is more readily detected in the blood within mixed infections than 10R26, supporting the suggested greater virulence of the C8 clone 1 isolate.

1.5 Conclusions

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Reinfection with *T. cruzi* was able to alter host survival, with significantly increased pathological response and mortality rates observed within reinfected hosts compared to their singly exposed counterparts. An acute and fatal response that has only been documented once previously was observed in a large number of reinfected hosts. Simultaneous infection with two isolates (i.e. mixed infection or polyparasitism) was not associated with increased host morbidity or mortality, although it did generate a more disseminated infection, with multiply infected hosts displaying a greater number of organs containing *T. cruzi* than their singly infected counterparts. Although the host's response to these two phenomena was vastly different, both have the potential to alter the way in which disease is managed. In a clinical context, within endemic areas there is a high risk of both multiple exposures to *T. cruzi*, with a very high prevalence of infection in vectors [9, 10] and of exposure to different genotypes, with vectors commonly found to be harbouring mixed *T. cruzi* infections [17]. Although the extent to which our findings with mice can be generalised to human hosts is unclear, there is an urgent need for more investigation of the clinical consequences of reinfections and mixed infections of *T. cruzi*.

1.6 Declarations

1.6.1 Ethics approval

All experimental protocols involving the use of animals were reviewed and approved by the Murdoch University Ethics Committee (project number: 2802/15).

1.6.2 Consent for publication

Not applicable

1.6.3 Availability of data and materials

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As this work is a major component of a post-graduate research project that is currently being undertaken and not yet submitted for examination, the datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

1.6.4 Competing interests

The authors declare that they have no competing interests.

1.6.5 Funding

C.P was in receipt of a PhD scholarship from Murdoch University.

1.6.6 Author's contributions

CP contributed to experimental design, carried out animal and molecular work, and was a major contributor in writing the manuscript; AT contributed to experimental design and was a major contributor in writing the manuscript; SK assisted in carrying out animal work; AW performed the histological examination of host organs and AL contributed to experimental design, analysed and interpreted data and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

1.6.7 Acknowledgements

The parasites that were used to perform these studies were received from the London School of Hygiene and Tropical Medicine. The C8 clone 1 strain was obtained from Professor Michael A. Miles and was isolated and prepared by his research group at the London School of Hygiene and Tropical Medicine, while the 10R26 clone was also received from Professor Michael A. Miles, and originally isolated and prepared by the research group of Professor

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Michel Tibayrenc at the Institut pour Recherche et Développment, Montpellier, France. C.P would like to acknowledge Louise Pallant and Michael Lewis for their technical assistance.

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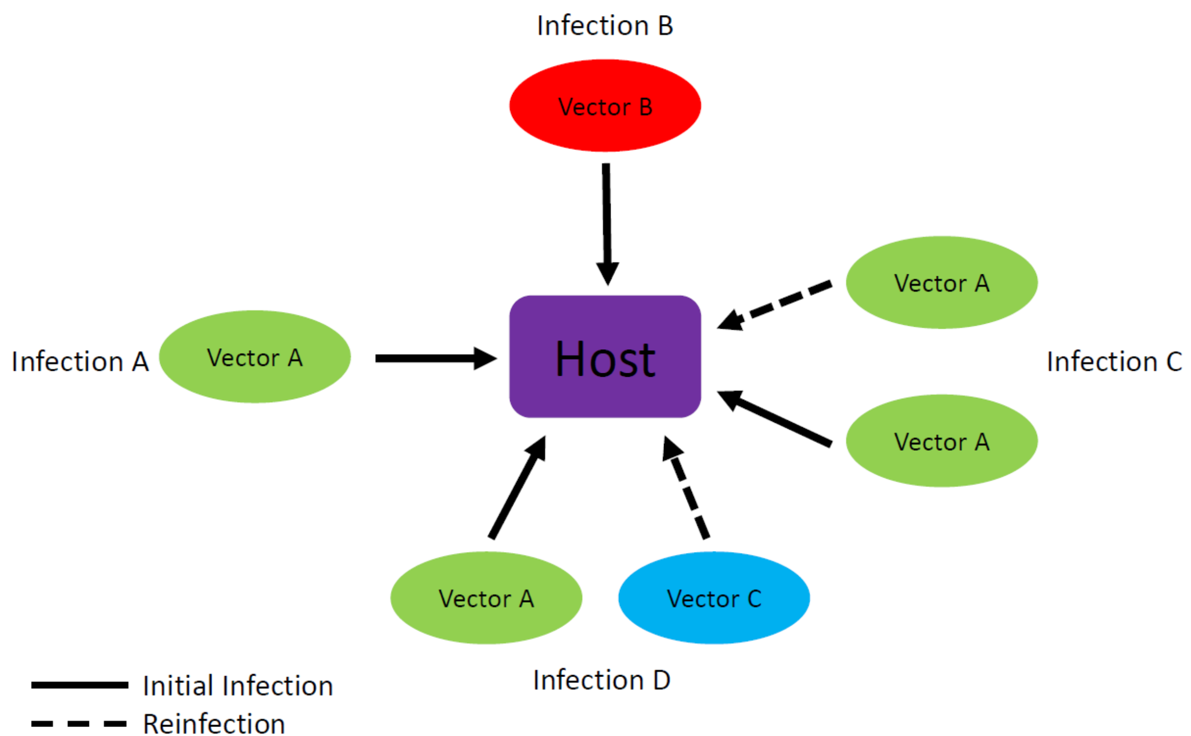

Figure 1.1: A conceptual illustration of the ways by which a host may be infected and/or reinfected when different T. cruzi genotypes are circulating in a vector population. For simplicity, four infection scenarios are illustrated, with only two genotypes and two different time frames of infection considered. A) A single infection transmitted from one vector harbouring a single parasite genotype (vector A). B) A mixed infection with genotypes 1 and 2 transmitted simultaneously from a vector harbouring both genotypes (vector B). C) Infection and reinfection with genotype 1 where the host is bitten on more than one occasion by a vector harbouring a single genotype (vector A). D) Infection with genotype 1 and reinfection with genotype 2, where the host is bitten on more than one occasion by vectors each harbouring single infections (vectors A and C).

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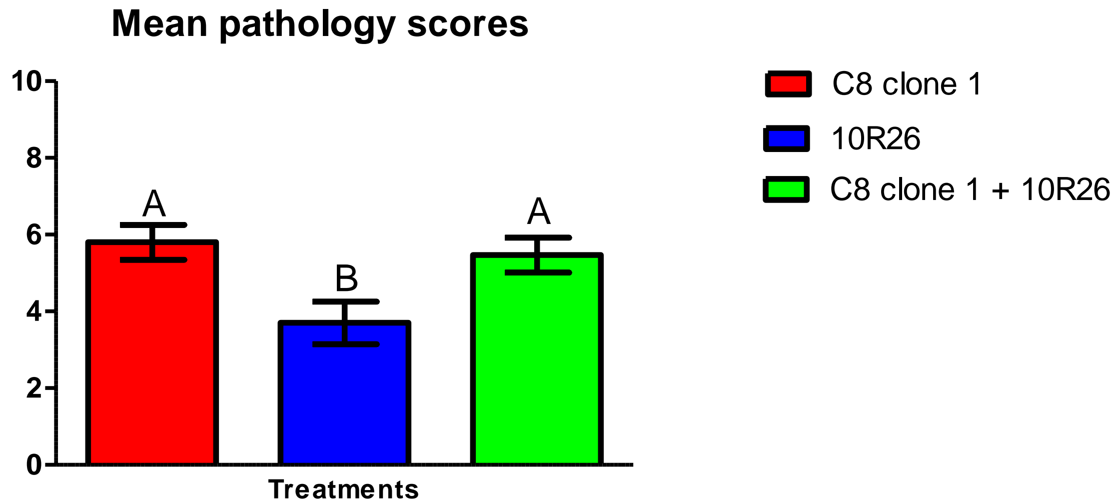

Figure 1.2: Pathology scores for animals with single and mixed infections of T. cruzi. Mean pathology scores, with standard error bars, for C8 clone 1, 10R26, and mixed C8 clone 1 + 10R26 infected animals. Treatment groups not connected by a letter are significantly different.

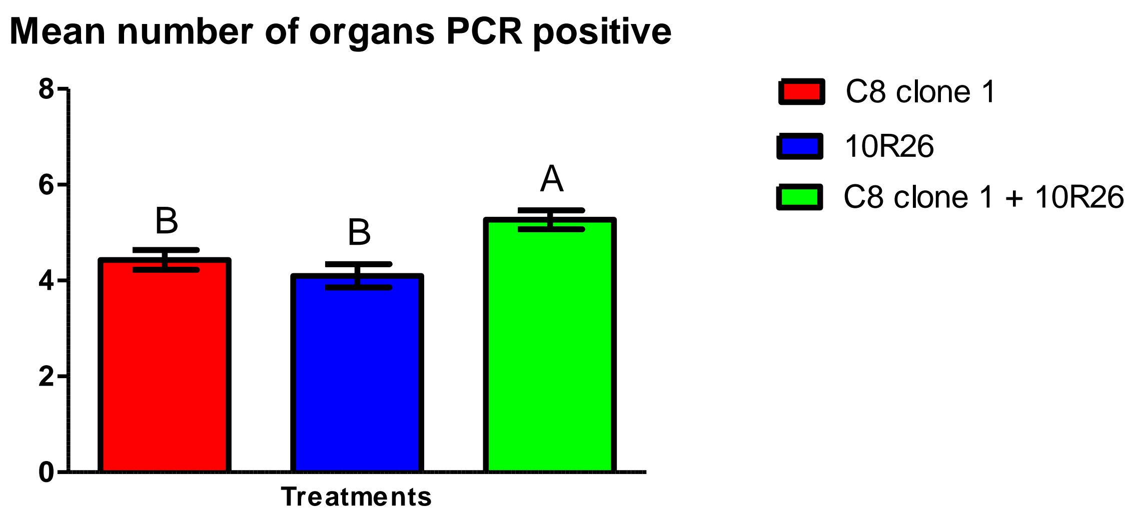

Figure 1.3: Number of organs PCR positive for animals with single and mixed infections of T.

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cruzi. T. Mean number of organs positive for *T. cruzi* DNA, with standard error bars, from C8 clone 1, 10R26 and mixed C8 clone 1 + 10R26 infected animals. Treatment groups not connected by a letter are significantly different.

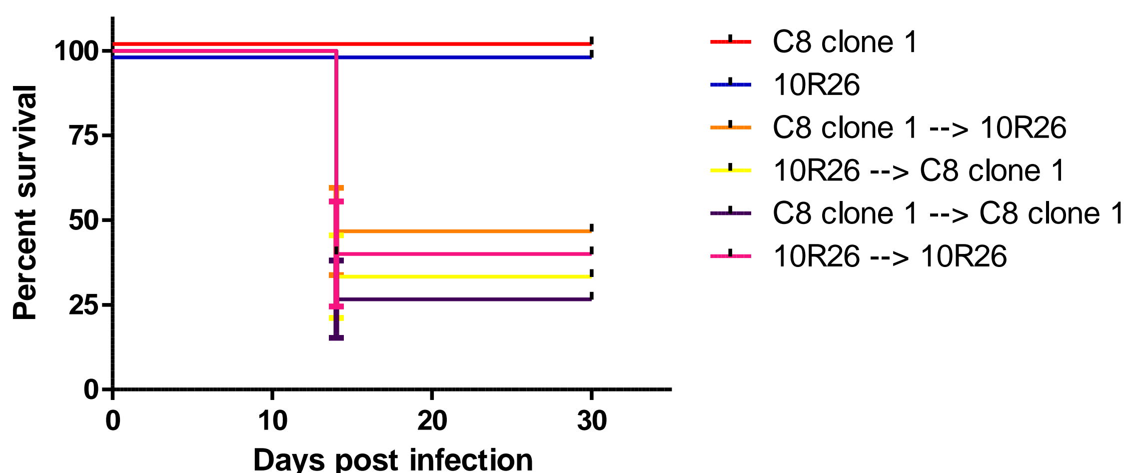

Figure 1.4: Survival rates for hosts with single infections and reinfections with different combinations of T. cruzi isolates. Percent survival for C8 clone 1 and 10R26 infected baseline groups; and mixed C8 clone 1 infected and 10R26 reinfected, 10R26 infected and C8 clone 1 reinfected, C8 clone 1 infected and reinfected, and 10R26 infected and reinfected treatment groups. Refer to Table 2 for details of infections.

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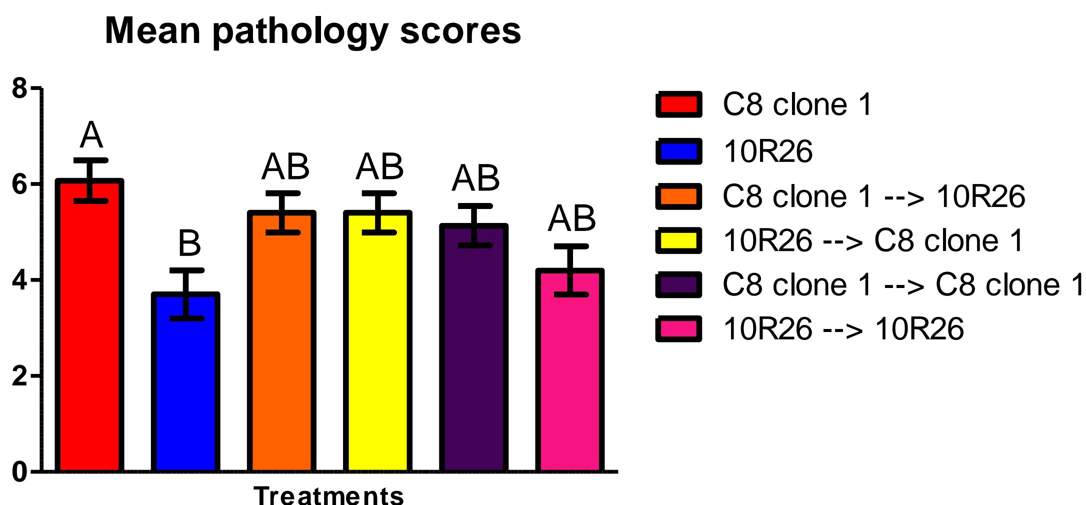

Figure 1.5: Pathology scores for animals with single infections and reinfections with different combinations of T. cruzi isolates. Mean pathology scores, with standard error bars, for C8 clone 1 and 10R26 infected baseline groups; and mixed C8 clone 1 infected and 10R26 reinfected, 10R26 infected and C8 clone 1 reinfected, C8 clone 1 infected and reinfected, and 10R26 infected and reinfected treatment groups. Treatment groups not connected by a letter are significantly different.

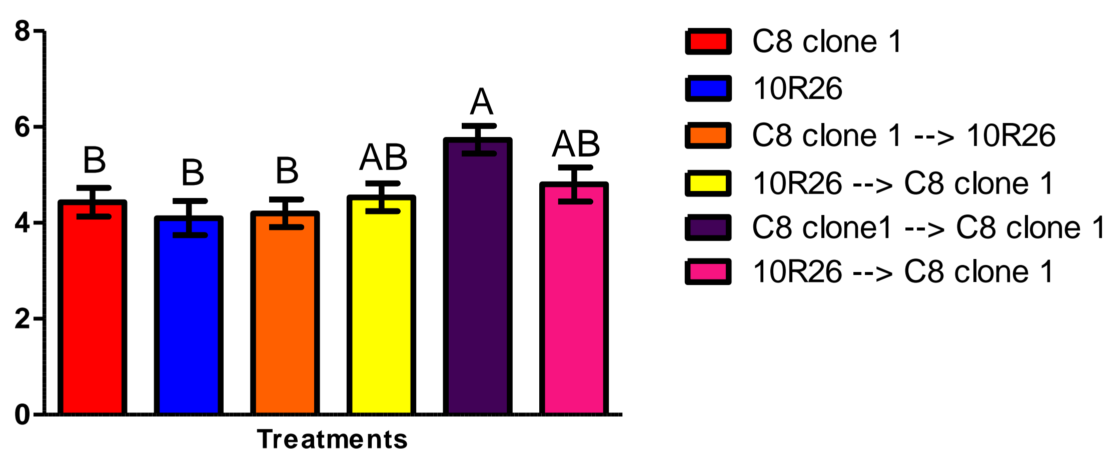

Figure 1.6: Number of organs PCR positive for animals with single infections and reinfections with different combinations of T. cruzi isolates. Mean number of organs PCR positive, with

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standard error bars, for C8 clone1, 10R26 infected baseline groups; and mixed C8 clone 1 infected and 10R26 reinfected, 10R26 infected and C8 clone 1 reinfected, C8 clone 1 infected and reinfected, and 10R26 infected and reinfected treatment groups. Treatment groups not connected by a letter are significantly different.

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Table 1: Infection combinations for the 7 treatment groups of T. cruzi infected animals. Singly, mixed and reinfected treatment groups infected with *T. cruzi*. Treatment = treatment group, N = number of mice per group.

Treatment	N	Initial infection (day 0)	Reinfection (day 14)
C8 clone 1	15	C8 clone 1	N/A
10R26	10	10R26	N/A
10R26+C8 clone 1	15	10R26 and C8 clone 1	N/A
C8 clone 1→ 10R26	15	C8 clone 1	10R26
10R26 → C8 clone 1	15	10R26	C8 clone 1
C8 clone 1 → C8 clone 1	15	C8 clone 1	C8 clone 1
10R26 → 10R26	10	10R26	10R26

Table 2: T. cruzi DNA detected within host blood and organs of mice from different treatment groups. B = blood; C= colon; H = heart; K = kidney; L = liver; SM = skeletal muscle; S = spleen; * = one exposure to *T. cruzi* infection.

Treatment group	B	C	H	K	L	SM	S
C8 clone 1 *	0/14	15/15	6/15	10/15	12/15	13/15	4/15
10R26 *	0/10	9/10	8/10	6/10	7/10	10/10	7/10
10R26 + C8 clone1 *	2/15	14/15	12/15	13/15	14/15	15/15	11/15

Table 3: T. cruzi DNA detected within host blood and organs of mice from different treatment groups. B = blood; C= colon; H = heart; K = kidney; L = liver; SM = skeletal muscle;

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S = spleen; * = one exposure; ** = two exposures to *T. cruzi* infection. For reinfected animals, blood samples were taken where possible.

Treatment group	B	C	H	K	L	SM	S
C8 clone 1 *	0/14	15/15	6/15	10/15	12/15	13/15	4/15
10R26 *	0/10	9/10	8/10	6/10	7/10	10/10	7/10
C8 Clone 1 → 10R26 **	6/13	12/15	6/15	7/15	15/15	13/15	10/15
10R26 → C8 clone 1 **	9/14	11/15	12/15	11/15	14/15	11/15	9/15
C8 clone 1 → C8 clone 1 **	2/6	15/15	14/15	14/15	15/15	14/15	14/15
10R26 → 10R26 **	0/4	8/10	8/10	6/10	8/10	9/10	9/10