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IRRADIATED *T. CRUZI* AND RESISTANT CONSOMIC ANIMALS CAN BE USEFUL IN CHAGAS DISEASE STUDIES

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ABSTRACT

Human Chagas disease is considered the most significant parasitic disease in Latin America. It is estimated that 16–18 million people are infected by *T. cruzi*. As a consequence, approximately 50,000 deaths occur every year. The acute infection usually goes unrecognized and enters into a chronic stage that persists throughout the host's life span. However, roughly 30% of infected individuals eventually will develop disease with an array of possible manifestations affecting the heart, the digestive tract, and/or the peripheral nervous system. This disease is commonly modeled in inbred mice even though mouse strains used to simulate experimental infection vary considerably. In this way, Wrightsman and Trischmann [1] [2] showed that chromosome 17 was directly involved in a *T. cruzi* resistance, showing the influence of host's genetic constitution on disease severity. Additionally, in 2003, Passos and Graefe [3] [4], working separately, quantified parasite burdens in resistant and susceptible strains and applied a backcross strategy to map the genomic *loci* linked to susceptibility and resistance in inbred mice. The genomes of the animals were scanned with microsatellite markers and the results found by these authors showed that the resistance mechanism is polygenic and is under the control of a complex network. In the particular case of Y strain, *in vivo* assays indicated that survival was related to the chromosomes 7,11,14,17 and 19. In order to evaluate the influence of each isolated chromosome as well as their interactions, we employed susceptible isogenic mice to construct consomic lineages for each one of those chromosomes. The consomic strains were injected with irradiated and native forms of Y strain *T. cruzi*, and the infectivity parameters were evaluated by quantitative methods. Radiation caused inability of trypanosomes to infect and kill mice, when these parasites were irradiated with 1 kGy of gamma rays from a ^{60}Co source. In this experiment we used 10^1, 10^2, 10^3, 10^4 and 10^5 parasites forms injected intraperitonealy, to understand the role of each of the chromosomes above on the resistance mechanisms. None of the consomic strain for the chromossomes 11, 14 and 17 survived to infection with non-irradiated parasites. However, the consomic strain for the chromossome 19; revealed that 38% of the animals survived after the injection with 10^1 forms in contrast with 10% after the injection with 10^2 forms. On the other hand, concerning the chromossome 7, 60% of the animals survived when 10^1 forms were injected, while 18% were able to control the infection with 10^2 forms. All consomic strains survived with the irradiated forms. These data could contribute with the understanding of the resistance mechanism in the Chagas disease, and suggest the importance of new experiments related with the immune response in these strains.

1. INTRODUCTION

Trypanosoma cruzi, the causative agent of Chagas disease and infects 16–18 million people throughout Central and South America. Following infection with the parasite, a hemoflagellate protozoan of the Kinetoplastida order, clinical symptoms range from negligible to acute Chagas disease that is characterized by fever, high parasitemy, and ≤ 5% mortality. The subsequent indeterminate stage may last for decades and is characterized by low parasitemy and the presence of quiescent amastigote forms in

muscle tissue. Up to 30% of the individuals in the indeterminate stage develop chronic Chagas disease. Pathologies observed in chronic chagasic patients include digestive tract anomalies (megacolon, megaesophagus) and cardiac enlargement and malfunction. If untreated, the chronic disease is often fatal. Current drug treatments are generally unsatisfactory; since available medications are highly toxic and often ineffective, particularly those used to treat the chronic stage of the disease. Many people who have tested positive are leading healthy lives with the help of their healthcare providers.

An increasing body of evidence has accumulated indicating that chronic symptoms are mediated by persistence of parasites, even when present in low or undetectable numbers. Little is known about the host genetics of *T. cruzi* infection. It is assumed that host genes are responsible for the variation of morbidity in endemic areas Graefe). In an epidemiological study, the prevalence of serum antibody to *T. cruzi* as a marker of infection was strongly influenced by genetic factors.

This variation in the resistance of humans is also found when inbred strains of mice are challenged. Inbred strains may vary from highly resistant to highly susceptible, suggesting a genetic basis for this natural resistance, but in neither the human situation nor the murine model have the factors that contribute to resistance or susceptibility been identified [5].

In some cases, resistance has been determined to be complex and under multigenic control. In other cases, resistance has been shown to be governed by a single gene or by a closely linked group of genes. In these simpler situations, it has sometimes been possible to identify a specific interaction between the infectious agent and its host as the genetic trait governing resistance. Even when resistance is governed by multiple genes, it has at times been possible to isolate a particular host-parasite interaction that is under single gene control [6].

Now, this disease is seen as a product from an interaction between two highly variable genomes: the parasite and the human genomes [7].

In this way, the difference of resistance presented by infected patients suggests that the genetic constitution of hosts has influence on the disease development and also on the surveillance of patients. This information indicates the necessity of a scientific investigation focused on the understanding of the important genes to chagas disease resistance [3].

All the strategies adopted in these studies are similar and are based on the use of programmed mates between inbred strains of mice that present different resistance or susceptible phenotypes [8].

In the particular case of *T. cruzi*, researchers have related the importance of chromosome 17 to the resistance to different *T. cruzi* strains, showing the influence of genetic constitution on disease severity [1] [2].

Recently, with the use of molecular techniques, other regions in different chromosomes have been related to *T. cruzi* resistance, showing that the mechanism of resistance to this protozoan is complex and is under a polygenic trait [3] [4].

Passos and coworkers [3] studing isogenic mice with resistant and susceptible phenotype, employing *T. cruzi* Y strain, identified a participation of chromosomes 7, 11, 14, 17 and 19 in the surveillance of high doses parasite infected animals.

Considering that those data allow the construction of consomic strains of mice that will help to unravel the importance of these chromosomes into the complex phenomenon of *T. cruzi* resistance, in the present work consomic models were used, in order to investigate their surveillance to the irradiated and non-irradiated Y strain.

2. MATERIAL AND METHODS

2.1. Animals

Inbred mice: strains of both susceptible (A/Unib) and resistant (C57BL6/Unib) phenotype mice were used..

Hybrid mice: In order to construct consomic mice a (B6 x A)F1 hybrid lineage was employed.

Consomic strains: consomic models to chromosomes 7, 11, 14, 17 and 19.

During the assays, the animals were kept into flexible isolators and into mini-isolators lodged in ventilated racks.

2.2.Consomic production

During consomic lineages production, hybrid animals (B6/UNIB x A)F1 females were backcrossed with A/Unib males. All the offspring was genotyped with polymorphic microsatellite to both lineages, in distances never higher than 10 cM for each molecular marker to each one of the five chromosomes. The AA homozygote animals were rejected and the AB heterozygote selected, in order to be matched in intercross. From these matches, the BB homozygote offspring formed the second generation of backcross. This schedule was employed for at least five generations of backcross fitted with *intercross*, in order to get a production of a consomic animal model for each one of the chromosomes of interest.

2.3. Parasites

Blood Y strain *T. cruzi* forms, were obtained from weekly passages in CBA/Unib mice.

2.4.Injections

The animals were challenged with 10^1, 10^2,10^3,10^4 or 10^5 forms of either native or 1kGy ^{60}Co gamma rays irradiated *T. cruzi*. The parasites were injected intraperitoneally in a volume of 0.1ml/animal.

2.5. Reagents

All the reagents were of analytical grade and the water was purified in a Milli-Q system.

2.6. Experimental infection of the original isogenic strains with T. cruzi

Males and females from the C57BL/6Unib and A/Unib, 10 to 12 weeks old, presenting resistant or susceptible phenotype to the parasite, respectively, were infected.

The assays were performed using 10^4 trypomastigotes/animal injected I.P.

Infection was confirmed at the 14th day after injection and mortality was followed daily for a month.

2.7. Achievement and infection of the B6AF1hybrids.

Animals from the resistant and susceptible strain were mated to generate the F1 hybrid, using C57BL/6Unib females and A/Unib males, kept in flexible isolators. The F1 progeny, after reaching 8 to 12 weeks age, was injected with 10^4 parasites. Infection and mortality were checked as described by [3].

2.8. Y strain T. cruzi trypomastigotes irradiation.

The irradiation process was done at the Center of Radiation Technology from IPEN/CNEN-SP, in a ^{60}Co source(GAMMACELL, Atomic Energy of Canada, Ltd.), in the presence of atmospheric oxygen, with a dose rate of 4,29 KGy/hour. A control

sample was kept in the same conditions, except for radiation. All the irradiated samples contained $1x10^5$ parasites.

2.9. Parasite quantification.
A 0.5µl blood sample, collected from the tail was checked under microscope (400X). The parasites were counted according to the method described by Brenner using a 43.8 correction factor [9].

3. RESULTS

3.1. Experimental infection of C57BL/6UNIBUnib and A/Unib mice strain with T. cruzi

The assays performed with different parasite amounts, injected I.P. in both the mice strains allowed us to identify the infecting dose that enables to discriminate between susceptible and resistant animals.
According to the amount of injected parasites, all the mice were examined between the 7th and the 14th day after infection, aiming to ensure the presence of the parasite.
Table 1 condensates the results for different parasite concentrations, in the two tested mice strains.
Our results confirm the existence of a significant difference between the two mice strains, when challenged with different doses of the parasite. The injection of 10^2 forms of *T. cruzi* led to 100% of mortality for the susceptible mouse strain, while the resistant one did not show any mortality.
The 10^2 trypomastigotes doses, when injected IP, enabled the discrimination between the susceptible and resistant mice populations.

TABLE 1 – Resistance pattern of the C57BL/6Unib and A/Unib, after the inoculation of different doses (10^1, 10^2, 10^3 e 10^4) of trypomastigotes.

Strain	Dose	Challenged/Resistant	% resistant
C57BL/6Unib	**10^3**	**10/10**	**100**
	10^4	**10/8**	**80**
A/Unib	**10^1**	**10/1**	**10**
	10^2	**10/0**	**0**

The C57BL/6Unib was challenged with crescent parasite doses, according to its higher resistance. The A/Unib strain received a maximum of 10^2 *T. cruzi*.

3.2.Experimental infection of the B6AF1Hybrids

After the administration of the discriminatory parasite dose (10^4 forms), the animals were examined on the 8^{th} and 9^{th} day, when infection was confirmed.
The results of this assay are shown in TABLE 2.

TABLE 2 – Parasite burden and mortality of the parental and F1 strains

	Phenotype	Parasite burden	Challenged/Resistant	(%) Resistance
A/Unib	**Susceptible**	**10^4**	**10/0**	**0**
C57BL/6Unib	**Resistant**	**10^4**	**10/8**	**80**
B6AF1	**Hybrid**	**10^4**	**10/9**	**90**
		10^5	**10/3**	**30**

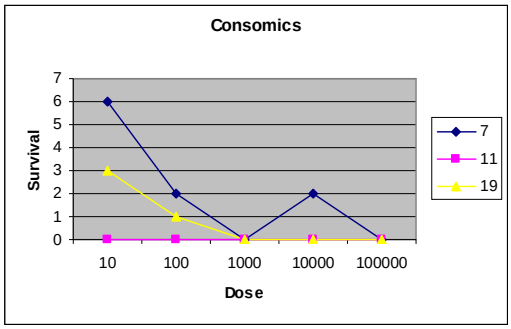
Our results indicate that when challenged with the parasite discriminating dose, the hybrid B6AF1 animals responded similarly to the original isogenic C57BL/6Unib resistant phenotype.
These data indicate that these animals could be used for the programmed mating for the generation of the consomic strains.

3.3. Experimental infection of the consomic strains

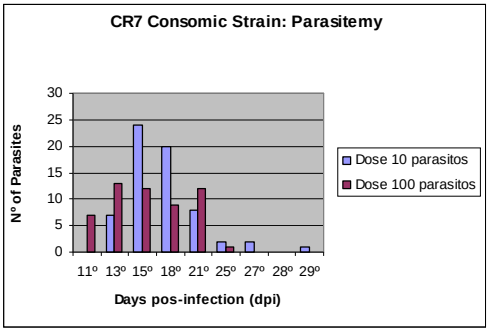
No infected consomic mice for the chromosomes 11, 14 or 17 survived.
On the other hand, the group of consomic animals for chromosome 19 showed a survival ratio of 38% when challenged with 10^1 parasites and 10% when 10^2 forms were injected.
On what refers to chromosome 7, 60% of the animals survived a challenge with 10^1 forms, while 18% were able to control infection when injected with 10^2 parasites.
Results are shown in TABLE 3:

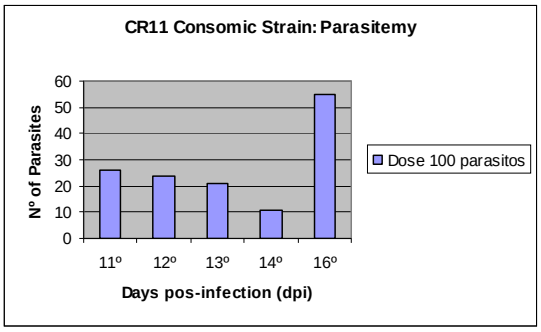
TABLE 3: challenge of the consomic strains.

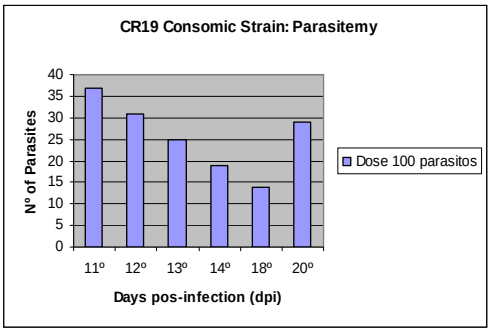
Chromosome	dose	Nº of challenged animals	Nº of survivors	% survival
7	**10^1**	**10**	**6**	**60**
	10^2	**11**	**2**	**18**
	10^3	**10**	**0**	**0**
	10^4	**10**	**2**	**20**
	10^5	**10**	**0**	**0**
19	**10^1**	**8**	**3**	**38**
	10^2	**10**	**1**	**10**
	10^3	**10**	**0**	**0**
	10^4	**10**	**0**	**0**
	10^5	**10**	**0**	**0**



3.4 Parasitemy of the consomic mice for chromosomes 7, 11 and 19.





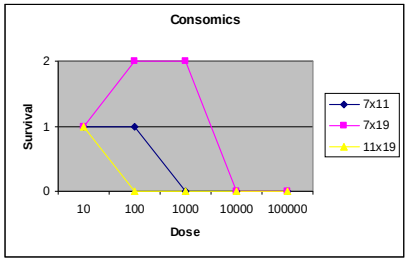


3.5. Experimental infection with the association of chromosomes 7, 11 e 19.

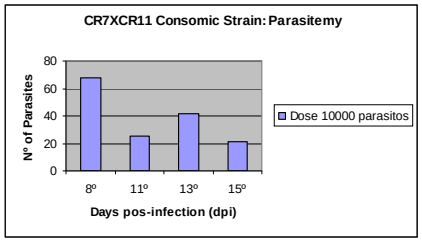
The chromosomes association was evaluated and is shown in TABLE 4.

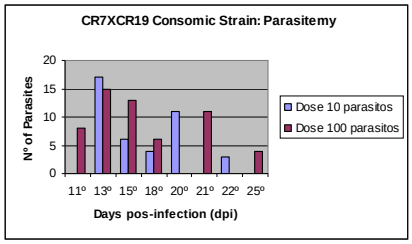
TABLE 4 – Chromossome association among Consomic strains.

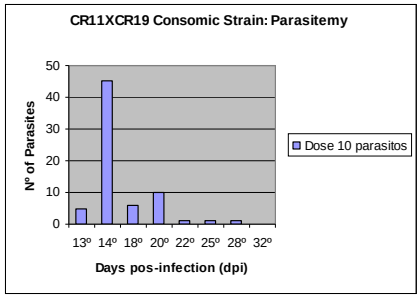
Associated Chromosome	dose	Nº challenged animals	Nº of survivors	% of survival
7 X 11	10^1	8	1	12
	10^2	10	1	10
	10^3	10	0	0
	10^4	10	0	0
	10^5	10	0	0
7 X 19	10^1			
	10^2	10	2	20
	10^3	10	2	20
	10^4	10	0	0
	10^5	10	0	0
11 X 19	10^1	8	1	12
	10^2	10	0	0
	10^3	10	0	0
	10^4	10	0	0
	10^5	10	0	0



3.6. Parasitemy observed with com chromosomes 7, 11 e 19 association.







3.7. Experimental infection with the irradiated Y strain.

The 1 kGy irradiated samples were injected in the parental resistant or susceptible isogenic strains, as well as in the consomic mice. The results are shown in tables 5, 6 and 7.
None of the animals, from any group died when challenged with the irradiated parasites.

TABELA 5 – Infection and mortality of the susceptible or resistant parental inbred strains, followed for 30 days after a challenge with 10^4 trypomastigote forms.

	A/Unib		C57Bl/6Unib	
Parasite irradiation dose	**Mortality (*)**	**Survival rate (**)**	**Mortality (*)**	**Survival rate (**)**
0 Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
100Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5(100%)
400Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
1000Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)

(*) Nº of dead / Nº injected animals (%)
(**) Nº survivors / Nº injected animals (positives %)

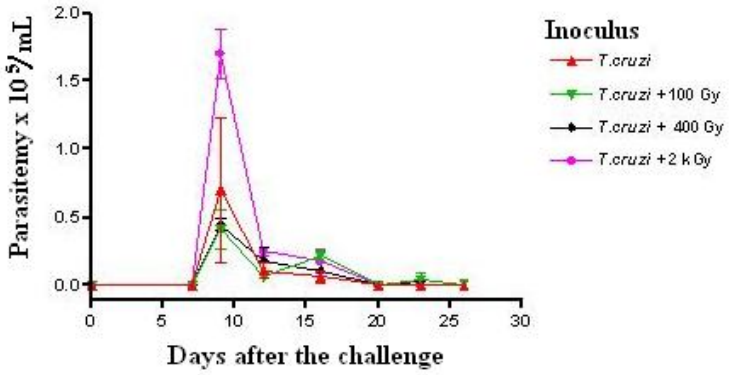


FIGURE 1. Parasitemy after the challenge with blood forms of native and irradiated *T. cruzi* in C57BL/6Unib

Table 6 – Infection and mortality of the most resistant consomic strains (chromosomes 7 and 19), followed for 30 days after a challenge with 10^4 *T. cruzi* trypomastigotes

	Chrom. 7		Chrom 19	
Parasite irradiation dose	**Mortality (*)**	**Survival rate (**)**	**Mortality (*)**	**Survival rate (**)**
0 Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
100Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
400Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
1000Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)

Table 7 – Infection and mortality of the consomic strains that did not present resistance (chromosomes 11, 14 and 17), followed for 30 days after a challenge with 10^4 *T. cruzi* trypomastigotes.

	Chrom. 11		**Chrom 14**		**Chrom. 17**	
Parasite irradiation dose	**Mortality (*)**	**Survival rate (**)**	**Mortality (*)**	**Survival rate (**)**	**Mortality (*)**	**Survival rate (**)**
0 Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
100Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
400Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)
1000Gy	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)	0/5 (0%)	5/5 (100%)

4. DISCUSSION AND CONCLUSIONS

Most of the knowledge about Chagas disease derives from assays with animal models, mostly mice. Inbred mice strains, are usually the first choice for the development of genetic investigations, since they present homozygosis of about 99% of the alleles and respond homogenously providing universal and reproducible results [3] [10].

The use of this species demonstrated that there are differences between isogenic strains after inoculation with [4].

The present work investigates the survival of mice with different genetic backgrounds which were infected with the Y strain of *T. cruzi* either in its native or irradiated form. For the achievement of a discriminatory parasite dose, different amounts of *T. cruzi* were injected, ranging from 10^1 to 10^5 forms in both C57BL/6 Unib and A/Unib mice strains.

Our results indicate a significant difference between the two strains with the C57BL/6Unib surviving doses up to 10^4 parasite forms , while the A/Unit strain did not survive, dying shortly after detection of parasitemy, with doses as low as 10^1 trypomastigotes, indicating a highly susceptible phenotype. (table1).

This approach enabled the isolation of a highly parasite-resistant population and also, allowed the identification of a discriminatory dose, defined as the dose where at least 80% of the inoculated animals survive. This dose corresponds to 10^4 trypomastigote forms, injected i.p. and discriminates between the two populations, one resistant (C57BL/6Unib) and the other susceptible (A/Unib).

To investigate the response to *T. cruzi* of a derived progeny of these parental isogenic strains, programmed mating were done and the F1 were submitted to the 10^4 parasites per animal discriminatory dose. This B6AF1 hybrid population has the characteristic of being homogeneous, enabling a single response pattern, since each half of its genetic background corresponds to the isogenic parental susceptible and resistant patterns [3] [10].

Our results revealed that the hybrid population B6AF1 behaves similarly to the resistant (C57BL/6Unib) parental strain, demonstrating that resistance follows a dominant hereditary pattern. The comparison of the hybrid animals with the susceptible parental strain (A/Unib) indicates that there is a pronounced difference between these populations, demonstrating that the resistant phenotype is being transmitted to the progeny, enabling its survival when challenged with the parasite (table 2).

These results, when compared with the findings of Graefe suggest that in the isogenic strains, recessive alleles could be responsible for the mice susceptibility to *T. cruzi* [4].

In 2003, Passos demonstrated the importance of chromosomes 7, 11, 14, 17 and 19 on the survival of mice infected with high doses of Y strain *T. cruzi* [3].

The data indicated the existence of a major chromosome involved in resistance to the parasite. To investigate the capacity of controlling the disease of these isolated chromosomes, consomic mice strains were constructed for each of these chromosomes.
The consomic strains bare only one chromosome from the donor strain, the others being preserved. The use of polymorphic DNA markers, able to identify the origin of a specific region enabled the selection of the progenitors.
In the strategy adopted for the present work, the receiving strain was A/Unib, which has a susceptible phenotype. Thus, all the chromosomes were of that strain, exception made of the chromosome of interest, which was donated by the resistant strain through programmed mating. After 5 backcross mating, the animals were challenged with parasite doses ranging from 10^1 up to 10^5, enabling to identify the relationship between the isolated chromosome and the survival of the animals.
None of the animals from the consomic strains for chromosomes 11, 14 or 17 survived, dying shortly after the infection was confirmed.
On the other hand, the chromosome 7 and 19 consomic mice were capable of controlling the infection with low parasite doses. In both strains, the resistance pattern was higher than the one observed for the parental isogenic A/Unib susceptible receptor strain. Our data also suggest that chromosome 7 has a central role on infection control, since the number of survivors is higher and the animals resist to higher parasite doses (table 3).
In parallel, the results obtained with the association of one of the chromosomes that did not manage to control infection with those that promoted resistance indicated an increase in survival rates, even with higher parasites amounts.
Furthermore, the association with a chromosome that did not induce resistance highlighted the "major" characteristic observed in the chromosome 7 consomic animals. While the association of chromosomes 7 and 11 leads to an increased resistance (10^2 parasites) with a 20 % survival ratio, the presence of chromosomes 11 and 19 in the same animals led to an ability to control infection with only 10^1 parasites in 12% of the infected mice.
On the other hand, the association between chromosomes 7 and 19, both important for resistance, provided a higher survival rate, up to 20% with 10^3 trypomastigotes.
In any of the above combinations, resistance was higher than in the parental isogenic susceptible A/Unib strain.
Additionally, the use of irradiated parasites could throw some light on important aspects of the acute Chagas´ disease control, through investigation of the immune response of mice exposed to the modified protozoan.
No mortality was observed in the animals injected with the irradiated parasites. These data corroborate previous findings by other authors that, using similar protocols, and an immunizing scheme with irradiated trypanosomes, observed that irradiation modified the parasite capacity to infect cells and promote the disease, without modifying its immunogenic properties [11].
Thus, we performed assays with consomic animal models aiming to investigate if this response could also be observed in these mice, an evidence of the importance of this model for the investigation of the immune response and resistance to the Y strain *T. cruzi*.
Our data using consomic mice infected with Y strain *T. cruzi*, in its native and irradiated forms, show that these animals are valuable tools for the understanding of resistance to this parasite in areas such as immunology, physiology and parasitology among others.

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