The hypervirulent Coxiella burnetii Guiana strain compared in silico, in vitro and in vivo to the Nine Mile and the German strain

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Running title: Virulence of Guiana and German strains

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The authors have no conflicts of interest to declare.

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ABSTRACT

Objective: Q fever epidemic outbreaks were reported in French Guiana and in the Netherlands. To determine whether the *C. burnetii* strains involved in these epidemics had a peculiar virulence pattern, we compared the pathogenicity of the Guiana and the German strain (a clone of the Netherlands strain), *in silico*, *in vitro* and *in vivo* versus the Nine Mile strain.

Method: The pan-genomes of the Guiana (Cb175), German (Z3055) and the referent Nine Mile (RSA 493) *C. burnetii* strains were compared. *In vitro,* the growth rate and the morphological presentation were compared. *In vivo* (SCID and Balb/c mice), weight loss, histological lesions, *C. burnetii* bacterial load in deep organs, and serological response were reported according to each *C. burnetii* strain studied.

Results: The Guiana strain had 77 times more missing genes and 12 times more unique genes than the German strain. The Guiana strain presented as large cell variants (LCV) and led to the most pronounced fatality rate in SCID mice (100% at 4 weeks). The German strain presented as small cell variants (SCV), and had an intermediate fatality rate (75% at 4 weeks). Both Guiana and German strains led to a significant higher serological response at two and four weeks p.i. ($p<0.05$).

Conclusion: The Guiana strain was the most virulent strain, followed by the German strain and the referent Nine Mile strain. Unique and missing genes could be implicated but further investigations are necessary to specify their role.

Keywords: Q fever, virulence, Guiana, Netherlands, outbreak, serology, *in silico*, *in vitro*, *in vivo*, *Coxiella burnetii*

INTRODUCTION

Q fever is a worldwide zoonosis caused by *Coxiella burnetii* and several outbreaks have been reported, particularly in French Guiana and the Netherlands. [1][2][3][4]. These outbreaks were associated with severe primary infection and with the circulation of a specific strain. Each of these strains was characterized by a specific multi spacer typing, i. e. MST17 for the Guiana strain, and MST33 for the strain involved in the Netherlands [1][2][3].

In French Guiana, a unique and endemic clone from the Cayenne peninsula, Cb 175, has been identified [2][5][6]. It presents the peculiarity of a 6,105 bp genome reduction coding for the type I secretion system [7]. In 2005, severe pneumonia was the most widespread clinical presentation with up to 25% of hospitalized community-acquired pneumonia cases [5].

In the Netherlands, more than 4,000 acute *C. burnetii* cases were reported between 2007 and 2011, representing the largest Q fever outbreak ever reported and responsible, to date, for more than 74 deaths [8][9][10]. The causative strain, NL3262 had 84.9% of its chromosomal components coinciding completely with those of the Z3055 strain, both belonging to the same multi spacer typing, MST33 (3)(4)(11).

Animal models have demonstrated that the strain and infection route played a role in the virulence of *C. burnetii* infection [11][12]. Based on the low infectious dose and the rapid onset of the disease, experimental studies supported the hypothesis that the Nine Mile strain was the most virulent strain and has been considered as a referent strain [12][13][14][15][16]. To determine whether the *C. burnetii* strains involved in epidemic outbreaks had a peculiar genotypic, phenotypic and virulence

pattern, we compared the Guiana strain to the German strain, which is close to the Netherlands strain, to the referent Nine Mile strain, *in silico*, *in vitro* and *in vivo*.

Material and Methods

Pan-genome analysis and phylogenetic tree

In silico analysis

The genome sequences corresponding to the three *C. burnetii* strains of interest (the Guiana, the German, the Nine Mile strains) as well as the sequence of the strain isolated during the Netherlands outbreak, i. e. NL3262, were retrieved from the NCBI database. Then, a comparative analysis of RSA 493 [Genbank: NC_002971.4], Z3055 [Genbank: NZ_LK937696], NL3262 [Genbank: NZ_CP013667] and Guiana [Genbank: HG825990.3] was performed (supplementary Material and Methods). Unique genes were defined as genes present in a single strain only, whereas missing genes were defined as absent in a strain when compared to the others strains. The phylogenetic tree was performed using PhyMl software (See supplementary Material and Methods).

In vitro experiment

Culture/bacteria

The Nine Mile strain served as reference. The Guiana strain was isolated from a patient in Cayenne who presented endocarditis [2]. The German strain was isolated from an ewe placenta from Germany [3][17]. All strains were cultured in an NSB3 laboratory and growth rate was compared as detailed in supplementary Material and Methods.

LPS comparison for the three strains

For each *C. burnetii* strain, LPS phase I and phase II were analyzed (supplementary material). The immunostained virenose of LPS phase I was visualized using a commercially available chemiluminescence kit (ECL™ Western Blotting Analysis System, GE Healthcare). Images were performed using a digital camera (Fusion FX7, Vilber Lourmat, Germany).

Morphological analysis

Transmission electronic microscopy (TEM)

Samples of L929 cells infected for 7 days were fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer and stored at 4°C for embedding (see supplementary Material and Methods). Seventy nm ultrathin sections were cut with a Leica UC7 ultramicrotome and placed onto HR25 300 Mesh Copper/Rhodium grids (TAAB, UK). Sections were contrasted with 5% uranyl acetate and 1% lead citrate and finally observed on a FEI Morgagni 268D electron microscope operated at 80 keV.

In vivo experiment

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Aix-Marseille University, France, under agreement number “C2EA-14”, and was registered by the French Ministry for Higher Education and Research under reference No. 01085.01. For each strain, 44 six-week-old Balb/c and 52 SCID male mice, weighing between 25 and 40 grams (Charles River Laboratories,

L'Arbresle, France), were housed in individual cages housing five animals each. Water and a standard diet of food were provided *ad libitum*.

Experimental design

Mice were infected with a whole-body aerosol inhalation exposure system, using a total of 10^7 phase I bacteria suspended in 5 ml of phosphate-buffered saline (PBS) and placed into a glass vial for liquid Venturi flow aerosol generation[15]. The total time of exposure was 2 h [15]. The aerosolization experiment was reproduced similarly for each strain, after decontamination. Control groups of 16 SCID and Balb/c mice received aerosolized PBS and underwent the same experimental protocol [15]. Four Balb/c and four SCID mice were euthanized immediately after aerosolization (day 0) to assess initial lung bacterial load. Subsequently, 10 Balb/c and 10 SCID mice were euthanized on days 3, 7, 14 and 28 p.i., or when limit points were present. To study the long-term results, for each of the 3 interest groups and controls, 8 additional SCID mice were infected, to be euthanized at 2 months (n=4) and 3 months (n=4) p.i.. However, in accordance with Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines, weight loss > 20% initial body weight was defined as the limit ethical endpoint at which mice were to be euthanized. Blood, lung, spleen, cervical and tracheal lymph nodes were removed at each time period (supplementary Material and Methods).

Immunofluorescence assay for C. burnetii antibodies detection in sera

Antibodies to phase I and phase II were identified in mice serum by immunofluorescence using conjugated goat anti-mouse IgG (Immunotech, Marseille, France) or anti-mouse IgM (Jackson Immunoresearch Laboratories, West Grove,

USA) as previously described [15][18]. We performed serological tests with both cognate and specific antigens.

Detection of C. burnetii DNA

DNA from the whole lung, spleen, cervical, tracheal lymph nodes and from 200 µL of blood were extracted using a QIAamp Tissue Kit (Qiagen) in 100 µL final volume as previously described [15]. The CFX96® (Biorad, France) was used to perform Quantitative real-time PCR (qPCR) using specific 16rRNA and *IS1111* probes [15].

Histological examination, immunohistochemistry and immunofluorescence

Serial sections (3-µm) of tissue specimens were obtained for routine hematoxylin-eosin-saffron (H.E.S.) staining, immunohistochemistry investigations and immunofluorescence (IF) processing (see supplementary Material and Methods). Pathological changes such as granulomas were checked from tissues as previously described [15][19].

Statistical analysis

Statistical tests were performed using the Graphpad Prism 6 ® software. When distribution was normal, variables were expressed using mean ± standard deviation or, when distribution was not normal, as medians. One-way ANOVA was used to compare many groups for normally distributed variables. A Kruskal-Wallis test by ranks was performed to compare non-normally distributed variables. A two-by-two comparison of non-parametric data was performed using a two-tailed non-parametric Mann-Whitney test.

RESULTS

In silico results

We observed that the German strain (Z3055) and the Netherlands strain (NL3262) were very similar with regard to their phylogenic distance (Figure 1A). The pan-genome for the four *C. burnetii* strains contained 1,480 core genes (Figure 1B). The number of unique genes (genes identified only in one of the 4 tested strains) was 733 for the Guiana strain versus 58, 58 and 63 for the Nine Mile, Netherlands and German strains, respectively (Supplementary Table 1). The Guiana strain genome presented 309 missing genes (genes that were present in all the other tested strains but not for this strain) versus 29, 24 and 4 for the Nine Mile, Netherlands and German strains, respectively (Supplementary Table 2-5). Most missing and unique genes identified for the Guiana strain were involved in the T4 secretion system (Supplementary Table 2-5).

In vitro experiment

Velocity growth

The respective velocity growth for each strain is reported in supplementary Figure 1. The Guiana and the Nine Mile strains grew faster.

LPS comparison

After 21 days of culture, a phase I/phase II switch was observed as attested by the loss of virenose, except for the German strain, which presented an incomplete switch to phase II (supplementary Figure 2A, columns 4, 5, 6). Regarding the silver-stained SDS PAGE (supplementary Figure 2B) some differences were identified: the Guiana

strain contained one more intense band at 20 kDa. The German strain lacked bands at 15-20 kDa and 42-72 kDa, but had an intense band at 26 kDa (supplementary Figure 2B). Moreover, each strain presented different glycoprotein profiles.

Morphological development TEM.

L929 infected cells, observed for seven days p.i. with TEM, showed mainly large cell variants (LCVs) with the Nine Mile and the Guiana strains and small cell variants (SCVs) with the German strain (Figure 2).

In vivo experiment

Clinical outcome

The infected Balb/c mice did not present any sign of discomfort nor significant body weight change throughout the experiment. At two week p.i., SCID mice infected with the Guiana strain showed signs of discomfort, prostration and a weight loss of more than 20%, requiring euthanasia. Evidence of illness occurred in the fourth week p.i. with the German strain and after five weeks p.i with the Nine Mile strain (Figure 3A-C). The fatality rate in SCIDs at four weeks p.i. was 100% with the Guiana strain versus 75% with the German strain and 0% with the Nine Mile strain, as illustrated in the survival figure 3A. Mice infected with the Guiana strain presented the highest mortality rate, conferring a hypervirulent pattern to this bacterium.

Spleen weight

For SCID mice, at two weeks p.i., the Guiana group had an increase in spleen weight significantly higher than for the other strains, whereas the German group had an increase in spleen weight, which was lower than that observed with the Guiana strain

but higher than that observed with the referent Nine Mile strain ($p<0.05$; Figure 3C). Spleen weight continued to significantly increase until four weeks p.i. to about 25 times the baseline for both the Guiana and the German strains (reaching 1.7 grams). In Balb/c mice, spleen weight changes were less important than in SCIDs, but here again, the Guiana group presented the highest maximal spleen weight (supplementary Figure 3).

Serology in Balb/c mice

A seroconversion could be detected starting two weeks p.i. with the three strains. Antibody titers were the highest for both the Guiana and the German strains at two and four weeks p.i. ($p<0.05$). (Supplementary figure 4).

Molecular detection of C. burnetii

In the lungs, the initial bacterial load was around 10^4 DNA copies (per 200µL of lung suspension) for all mice (Balb/c and SCID) regardless of the *C. burnetii* strain.
In Balb/c mice, until two weeks p.i., the bacterial load was the highest in the group infected with the Guiana strain ($p<0.05$). Thereafter, all groups showed a decrease in bacterial burden attesting to progressive clearance in non-immunosuppressed mice.
In SCID mice, globally, the levels reached higher values than in Balb/c mice, DNA copies increased until four weeks p.i., and levels were significantly higher with the Guiana and the German strains than with the Nine Mile strain(Figure 4 & supplementary Figure 5).

Histopathological findings

The earliest lesions were observed at 7 days p.i. in the lungs of Balb/c and SCID mice infected with the Guiana strain, whereas only SCID mice infected with the German strain showed lung granuloma with negative immunohistochemistry (Supplementary Figure 6-7). Pathological findings, granulomas and positive immunohistochemistry, were observed at the earliest stage of infection for the Guiana strain, followed by the German strain, then by the Nine Mile strain.

Discussion

Comparison of the three strains have shown that the Guiana was the most virulent strain in mice whereas the German strain, which is close to the strain involved in the Netherlands outbreak, has an attenuated virulence. The referent Nine Mile strain, although less virulent, remained pathogenic.

In silico analysis revealed that the genome of the Guiana strain presented 12 times more unique genes and 13 to 77 times more missing genes than the Netherlands and the German strains, respectively. Most of the unique and missing genes identified in the hypervirulent Guiana strain were genes coding for the T4 secretion system. The role of T4SS is to translocate bacterial effectors into the cytosol to facilitate bacterial survival and replication [20][21]. The T4SS system is necessary for the parasitophorous vacuole expansion, and for the prevention of apoptosis [20][21]. The specificity of T4SS in the Guiana strain deserves to be further explored.

In vitro, the Guiana strain was metabolically active, whereas the German strain presented as “dormant”. The SCVs have been described as non-replicating stationary forms, with a condensed chromatin and a unique transcriptomic profile that may contribute to *C. burnetii’s* environmental resistance [22][23]. However, the virulence of the SCV as compared to the LCV remains unknown. Regarding the different

glycoprotein profiles observed here, further investigations are necessary to elucidate their implications in the virulence mechanisms.

In vivo, the Guiana strain clearly behaved as the most virulent strain, as attested by the earlier occurrence of limit endpoint in SCID mice; the faster increase in spleen weight; the higher *C. burnetii* bacterial burden in deep organs, and the earliest evidence (one week p.i.) of pathological findings. The German strain appeared to be an intermediate between the Guiana and Nine Mile strains. A relative spleen weight strain specificity was described in Balb/c mice [24].

Some apparent discrepancies were observed. The German strain presented *in vitro* as SCVs, but produced high pathogenicity *in vivo*, whereas the Nine Mile strain presented as LCVs *in vitro* but induced delayed pathological damage. This may reflect the capacity of *C. burnetii* to adapt to its environment, as suggested by previous *in vitro* research [25].

The Guiana and the German strains both showed higher antibody response. This corroborates data observed in humans in relation to the Guiana strain. [6]. To our knowledge, no comparative study has yet focused on the serological response linked to the Netherlands strain.

Some limitations of the study need to be acknowledged. Whereas the German Z3055 strain genome is close to the NL3262 strain responsible of the Netherlands outbreaks (84% of genome similarities), further *in vivo* investigations are needed to assess the pathogenesis of the NL3262 strain. The Guiana strain we used was isolated from a patient with persistent endocarditis, whereas acute Q fever pneumonia is the main clinical presentation observed in the French Guiana. The evolution from acute Q fever

to persistent *C. burnetii* infection seems to depend on the host's response rather than on the bacterial strain virulence [1].

As a conclusion, the Guiana strain presented a peculiar pattern characterized by the association of a high number of unique and missing genes involved in the *C. burnetii* T4SS, LCV morphology, a severe clinical disease induction and an intense serological response. The German strain profile was less categorical, presented lower unique and missing genes, SCV morphology *in vitro* and an intermediate pathogenicity *in vivo*. Further investigations may be required to specify the implication of these missing genes in the virulence mechanisms.

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FIGURE LEGENDS

Figure 1.

A. Genome-based Phylogenetic tree

B. Pan-genome representation for four analyzed strains of *Coxiella burnetii*. The total number of genes for each strain is written in brackets. The number of missing genes for each strain appears in bold red.

Figure 2. Transmission electron microscopy showing L929 infected cells at seven days p.i. with the Guiana and the German strains.

High magnification identifies LCVs in L929 cells infected with the Nine Mile and the Guiana strains, in which chromatin is largely extended as compared to the German strain for which small cell variants are characterized by condensed chromatin.

Figure 3.

A. Kaplan Meier survival analysis over time (in weeks).

Significant survival differences were observed among the three strains ($p<0.001$)

B. SCID body weight over time (in weeks)

The horizontal dotted bar indicates weight loss >20% of the initial body weight. Initial weight was similar for the four groups ($p = 0.99$) of SCID mice. Body weight was significantly lower at day 28 p.i. in the Guiana group versus the other groups (*: $p < 0.05$).

C. Spleen weight over time in SCID mice (in weeks).

At two weeks p.i., spleen weight was the highest in the Guiana group (($p < 0.005$) versus the three other groups) and was higher in the German group than in the Nine

Mile group ($p < 0.05$). At four weeks p.i., spleen weight was significantly different between groups (ANOVA, $p < 0.001$). Post hoc tests showed that the Guiana and German groups had the highest values ($p < 0.001$). No significant change was observed over time in the PBS group.

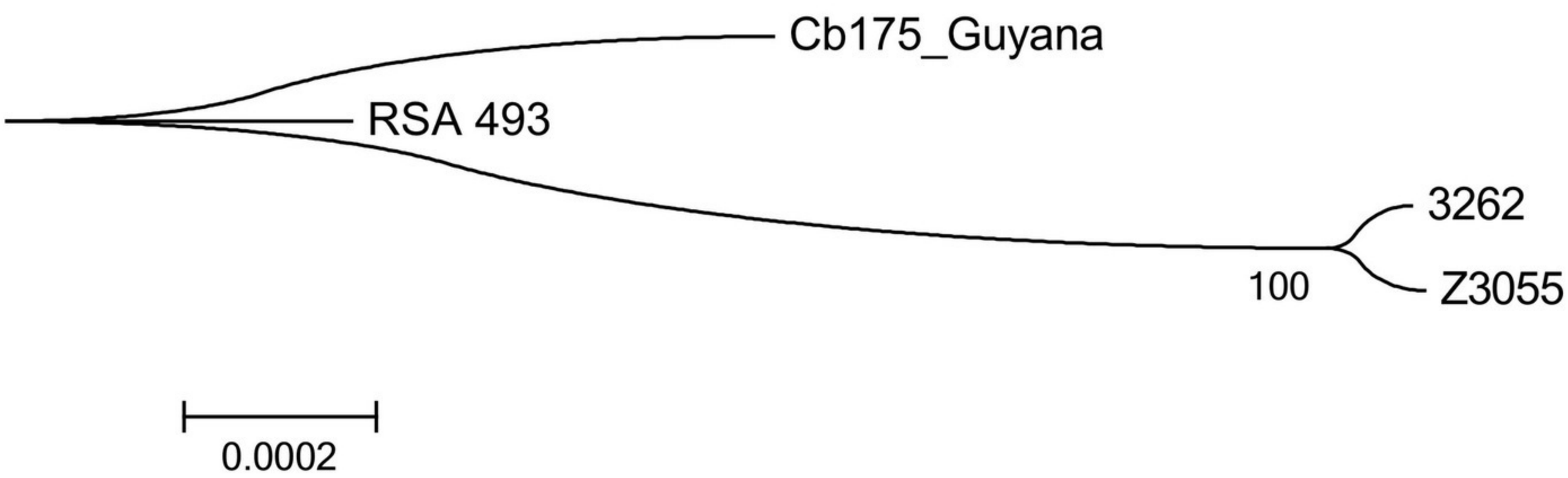
Figure 4: PCR results of *C. burnetii* detection in lung, spleen, blood and tracheal lymph nodes over time with the three different strains

The red star indicates a significant difference at a given time ($p < 0.05$) as detailed below.

In the lungs of Balb/c mice infected with both the Guiana and the German strains, the number of DNA copies was higher than with the Nine Mile strain three days p.i. (0.5 week). At one and two weeks p.i., the bacterial load was higher with the Guiana strain versus the two other strains ($p<0.05$). In SCID mice infected with the Guiana strain, the number of DNA copies was the highest at all times.

In the spleen of Balb/c mice, the number of *C. burnetii* Guiana strain DNA copies was significantly higher at one and two weeks p.i., whereas both Guiana and German strains presented higher levels of DNA copies at one and four weeks p.i. In SCID mice, both the Guiana and the German strains presented a significantly higher number of DNA copies at one, two and four weeks p.i..

A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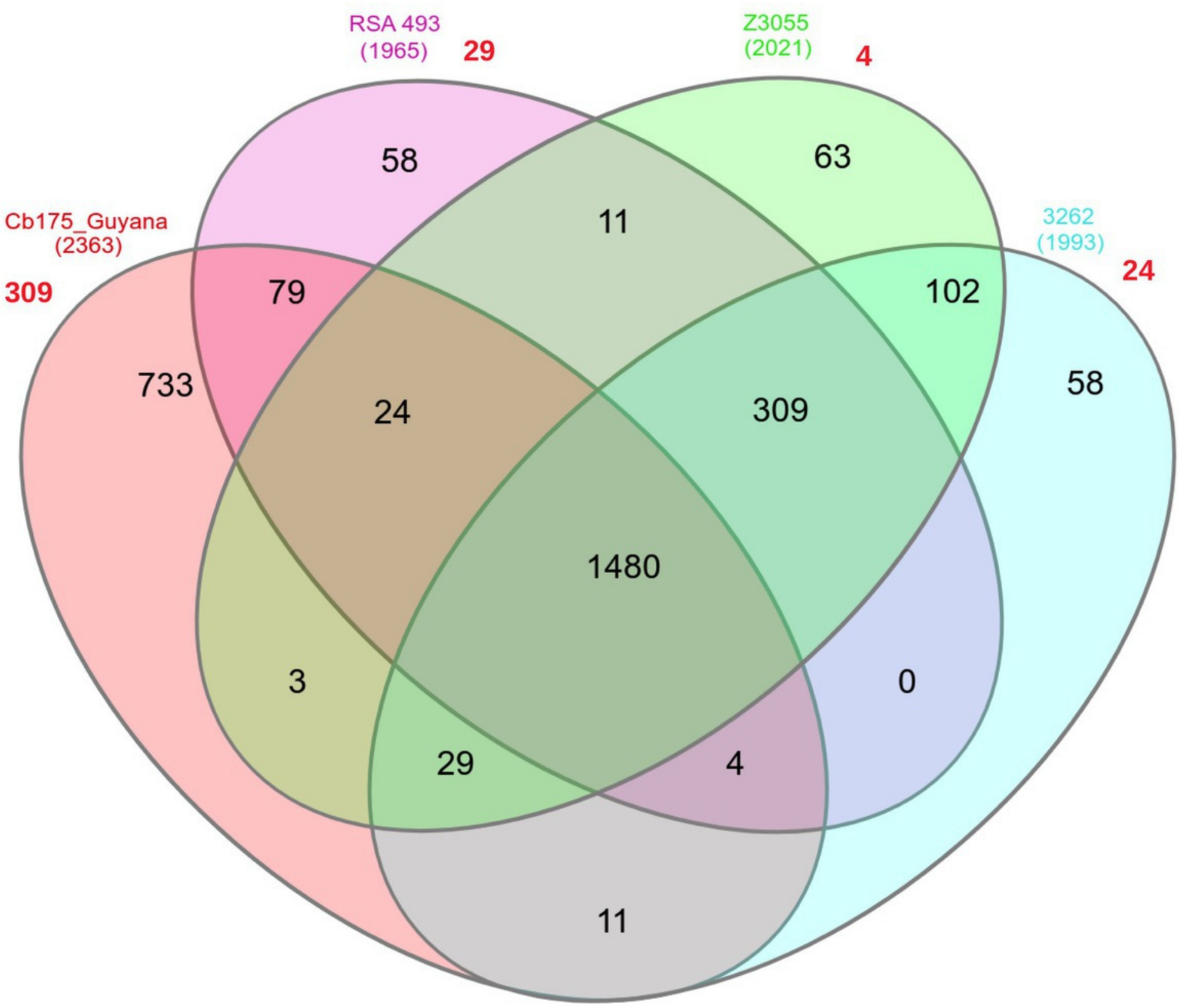
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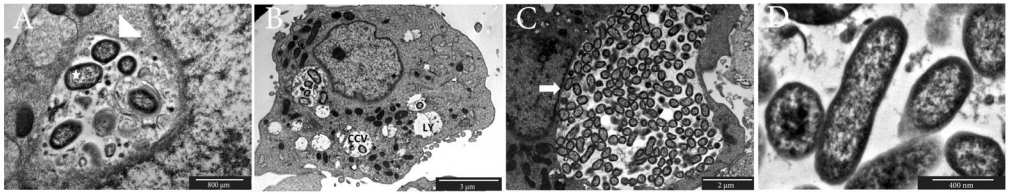
Nine Mile



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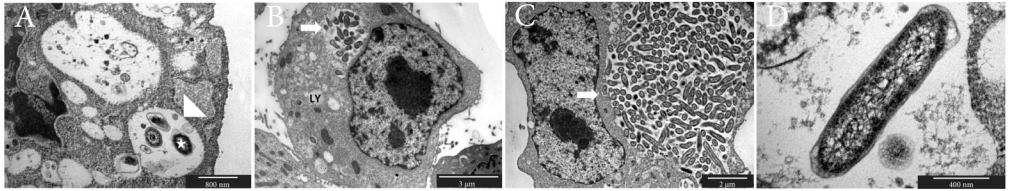
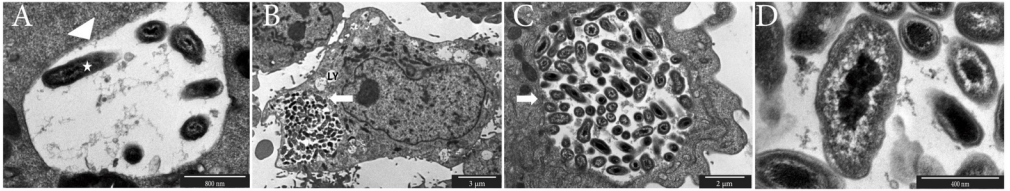
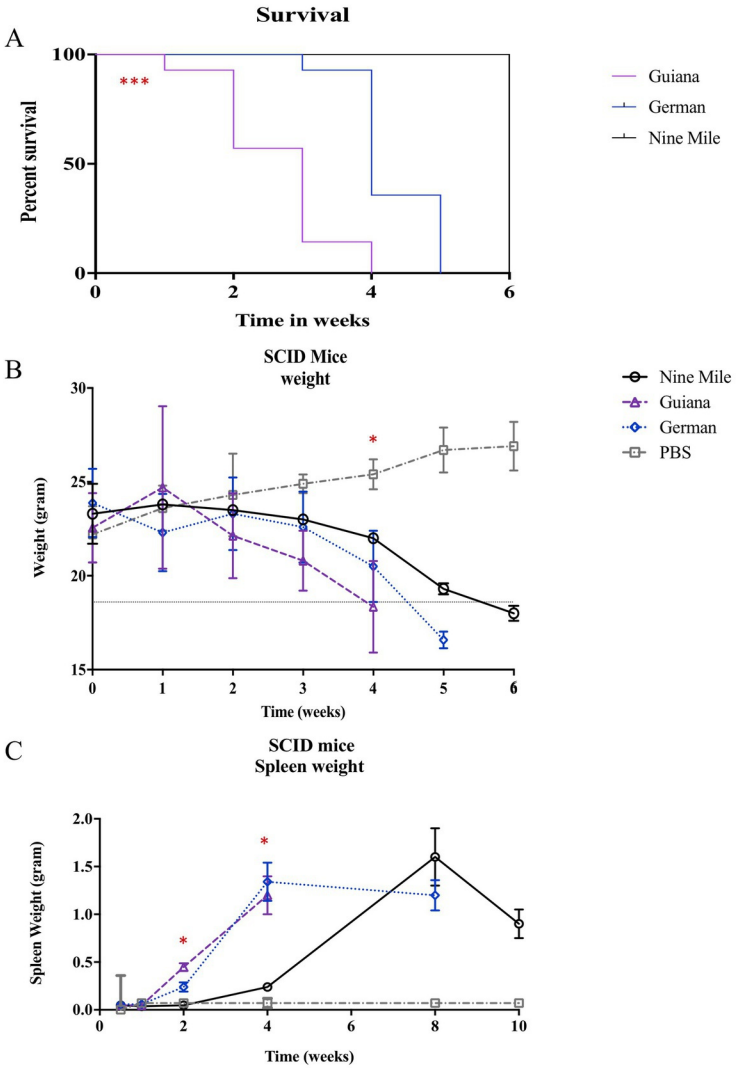
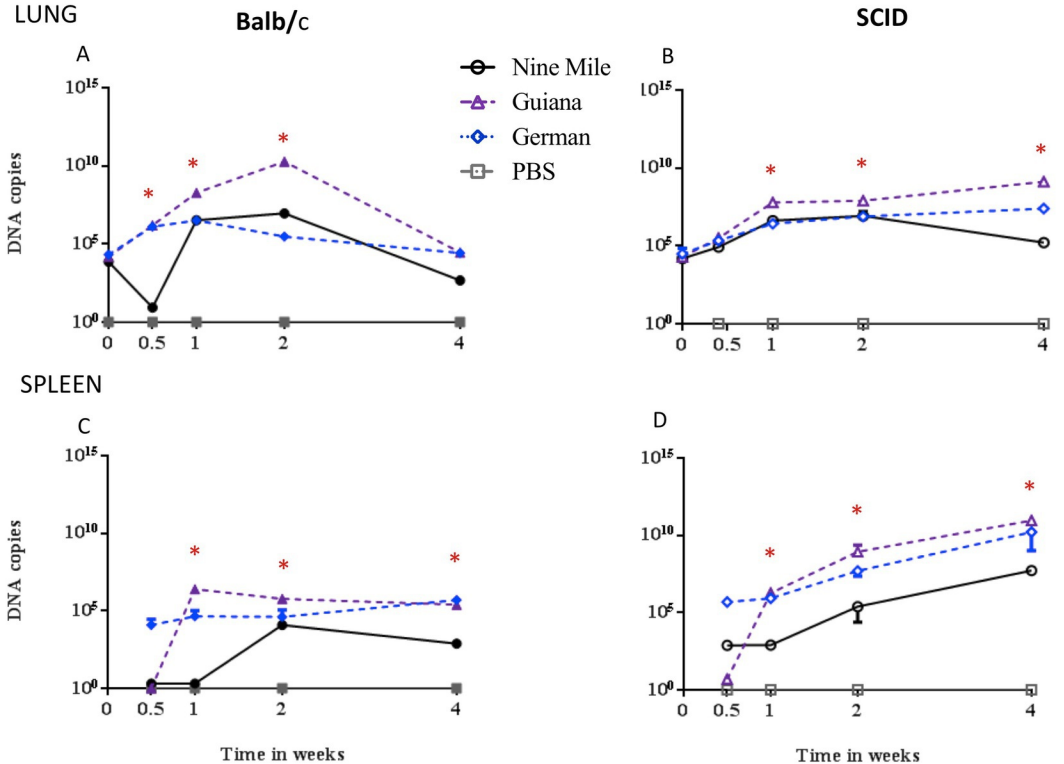


German




A
Survival
100
50
0
***
Percent survival
0
2
4
6
Time in weeks
Guiana
German
Nine Mile
B
SCID Mice
weight
30
25
20
15
*
Weight (gram)
0
1
2
3
4
5
6
Time (weeks)
Nine Mile
Guiana
German
PBS
C
SCID mice
Spleen weight
2.0
1.5
1.0
0.5
0.0
*
*
Spleen Weight (gram)
0
2
4
6
8
10
Time (weeks)


LUNG
Balb/c
SCID
A
B
C
D
Nine Mile
Guiana
German
PBS
DNA copies
SPLEEN
Time in weeks
Time in weeks