A Rodentibacter heylii strain lacking all known RTX toxin genes is highly virulent in C57BL/6 and BALB/c mice in contrast to Muribacter muris

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Research article

Keywords: Pasteurella pneumotropica, RTX toxins, bronchopneumonia, colonization

Posted Date: September 23rd, 2019

DOI: https://doi.org/10.21203/rs.2.14825/v1

Abstract

Background Rodentibacter (R.) heylii and Muribacter (M.) muris are frequently detected in laboratory rodents. Repeats in toxin (RTX) toxins are considered to be important virulent factors in R. pneumotropicus and R. heylii. As many R. heylii isolates do not carry genes encoding known RTX toxins we hypothesized that these isolates are at the most moderately virulent or even avirulent as M. muris . To test this hypothesis, we evaluated the virulence of R. heylii and M. muris strains negative for all known RTX toxin genes in experimental infections of C57BL/6 and BALB/c mice. Results Experimental intranasal infection with 10 8 colony forming units (CFU) of a pnx I-, pnx II- and pnx III-negative R. heylii strain resulted in 75% and 100% mortality of C57BL/6 and BALB/c mice, respectively. Infections of multiple internal organs such as lung and genito-urinary tract were recorded. Purulent bronchopneumonia was a common finding in lungs of early losses. Application of 10 4 CFU of the same R. heylii strain was neither associated with clinical signs nor dissemination, but with efficient colonization of the upper respiratory tract. Intranasal application of M. muris in different doses ranging from 10 4 to 10 8 CFU did not result in mortality or severe weight loss but efficient colonization and induction of systemic M. muris specific IgG in most animals. Conclusion The current study reveals high virulence of R. heylii strain SF27GVG carrying none of the known RTX toxin genes in wildtype mice. This result questions the validity of estimating virulence in the genus Rodentibacter by profiling of pnx toxin genes. Suitable colonization models for future investigations were established for R. heylii and M. muris . Application of M. muris was associated with a systemic IgG immune response and cultural detection in draining lymph nodes in most animals indicating infection and not sole colonization.

Background

Rodentibacter (R.) heylii, *R. pneumotropicus* and *Muribacter (M.) muris* are among the most frequently detected bacteria in laboratory mice [1–3]. *R. pneumotropicus* and *R. heylii* were recently reclassified from *Pasteurella (P.) pneumotropica* Biovar Jawetz and Heyl, respectively [4]. In 2009 the prevalence of *P. pneumotropica* was determined to be 13.2% and 4.0% in North America and Europe, respectively [2]. Furthermore, *R. heylii* and *R. pneumotropicus* were frequently isolated in a current study on a German Animal Research Facility [5]. Both are opportunistic pathogens colonizing efficiently the mucosal surfaces of rodents [6]. Infection of immunodeficient mice with *R. spp.* often leads to severe losses and eradication efforts in laboratory animal facilities [3]. Virulence in wildtype mice is generally regarded to be low [7]. Adhikary et al. described clonal outbreaks of *Pasteurella pneumotropica* biovar Heyl infections in mouse colonies including wildtype and immunodeficient mice [6]. The authors detected this pathogen in association with orbital and cutaneous abscesses as well as mastitis but systemic infections were not observed. However, our previous experimental study revealed systemic infections with high mortality in wild type mice after intranasal application of a *R. pneumotropicus* strain carrying three known RTX toxin genes [8]. Clinical signs observed in this experimental *R. pneumotropicus* infection were moderate to severe weight loss, conjunctivitis and dyspnoea [8].

In *Rodentibacter* species three RTX toxins encoded by *pnx* genes are described including PnxI and PnxII to be cytotoxic and haemolytic [9, 10]. Distribution of these *pnx* genes varies between *R. pneumotropicus* and *R. heylii* isolates of laboratory mice. More than 80% of the *R. pneumotropicus* isolates carry at least two *pnx* toxin genes whereas approximately 40% of the *R. heylii* isolates are negative for all known *pnx* genes [8].

Actinobacillus muris was recently reclassified as *Muribacter (M.) muris [11]*. Despite the very high sequence similarity of 96% to a type strain of *P. pneumotropica*, *M. muris* is considered avirulent.

One aim of this study was to evaluate the virulence of a *R. heylii* strain genotypically negative for all three known RTX toxin genes (*pnxI, pnxII, pnxIII*). This was conducted using comparative experimental intranasal infection of C57BL/6 and BALB/c mice essentially as described previously for a *pnxI+, pnxII+, pnxIII+ R. pneumotropicus* strain [8]. Furthermore, this study includes the first described experimental infection with *M. muris* to verify that this bacterium is apathogenic for laboratory wild type mice.

Results

Genotyping of the *R. heylii* challenge strain SF27GVG

For this study, we selected a recent *R. heylii* isolate (SF27GVG), which was PCR negative for all three known *pnx* genes encoding RTX-toxins [8]. We conducted an additional independent PCR for each *pnx* gene to verify the genotype. As shown in Fig. 1, the gene encoding the “characteristic antigen for *Rodentibacter* of laboratory origin 1“ (CARLO-1 [12]) was detected but none of the three known *pnx* genes using DNA of strain *R. heylii* SF27GVG in contrast to the *R. pneumotropicus* strain JF4Ni previously characterized in experimental infections [8].

R. heylii strain SF27GVG carrying no known *pnx* gene is highly virulent

As *R. heylii* is an opportunistic pathogen in rodents known to cause severe systemic infections in immunodeficient mice but much less so in wildtype mice, we expected only mild to moderate clinical signs after experimental infection of wildtype mice. However, intranasal application of 10^8 CFU resulted in a mortality of 100% (8/8) in BALB/c and 75% (6/8) in C67BL/6 mice due to severe weight loss above 20% or reaching other termination criteria (Fig. 2A). These mice showed unspecific signs such as ruffled coat and bended back as well as specific signs such as conjunctivitis and dyspnoea. Mortality was only observed on the first two days following experimental infection. Lungs of early euthanized mice infected with 10^8 CFU of *R. heylii* showed mild, moderate or severe catarrhal-purulent bronchopneumonia (Fig. 2B), which was not observed in control mice (Tab. 1). Within inflamed tissue larger aggregations of bacteria were found (Fig. 2C). All mice surviving the experiment until the end of the observation period including those infected with *M. muris* did not show any histopathological findings except for an interstitial pneumonia also recorded in control animals.

Moderate weight loss was observed in 3 of 4 C57BL/6 (5-11% weight loss) and 4 of 4 BALB/c mice (5-9% weight loss) infected with a lower dose of 10^6 CFU (Fig. 3). Mice infected with the lowest dose of 10^4 CFU of *R. heylii* SF27GVG did not show a weight loss exceeding 1% or any other clinical signs.

Infection with *M. muris* 694/11 did not result in any mortality. As shown in Fig. 3, after infection with 10^8 CFU per mouse, mice showed a moderate decrease in body weight with a loss of 11-13% in C57BL/6 mice and 3-8% in BALB/c mice. These mice showed mildly ruffled coat and depression.

In conclusion, *R. heylii* SF27GVG but not *M. muris* 694/11 is capable of inducing weight loss above 20% associated with purulent bronchopneumonia in C57BL/6 and BALB/c wildtype mice after intranasal application of 10^8 CFU. The known RTX toxins of the genus *Rodentibacter* are not necessary for this virulence potential.

R. heylii strain SF27GVG disseminates in various internal organs of wildtype mice

R. heylii was detected in various internal organs of early euthanized mice as specified in Fig. 4. In 3 of 6 C57BL/6 mice and 6 of 8 BALB/c mice *R. heylii* was detected in at least two internal organs including liver, spleen, kidney, genito-urinary tract and brain. Except in one C57BL/6 mouse, these mice showed dissemination of *R. heylii* to the genito-urinary tract. In the two surviving C57BL/6 mice infected with 10^8 CFU *R. heylii* was isolated from the lymphonodus (Ln.) tracheobronchialis and the genito-urinary tract. Infection with 10^6 CFU was associated with cultural detection of *R. heylii* in lungs, lymphonodi tracheobronchiales, and the genito-urinary tract but no other internal organs except for the liver of one animal (Fig. 4, Fig. 5). In contrast, a lower dose of 10^4 CFU of *R. heylii* did not resulted in detection of this pathogen in the genito-urinary tract but in the tracheo-nasal lavage with a specific bacterial load comparable to loads in animals infected with higher doses.

M. muris was identified in tracheo-nasal lavages (TNL) of all mice experimentally infected with *M. muris* (Fig. 6B). Furthermore, in 11/32 mice *M. muris* was also isolated from the genito-urinary tracts and in spleens of 4/32 mice (Fig. 6A). In contrast, *M. muris* was not detected in mock-infected mice. Based on semi-quantitative assessments the specific bacterial load of *M. muris* in the genito-urinary tract was very high in 7/32 mice.

In summary, mortality in *R. heylii* infected mice was associated with dissemination of the pathogen to internal organs. The genito-urinary tract is a main target organ of *R. heylii* and *M. muris.*

PCR-based detection of *R. heylii* and *M. muris* in swabs

PCRs for *R. heylii* and *M. muris* DNA were conducted with swabs taken post infection from the ano-genital region and the pharynx as described before [13]. Ano-genital swabs exhibited positive PCR results at 7, 14, 21 and 28 days after infection in 5/8, 7/8, 6/8 and 4/8 mice, respectively, infected with 10^4 CFU and 10^6 CFU of *R. heylii*. Surviving mice infected with 10^8 CFU of *R. heylii* exhibited positive PCR results at 21 and 28 days after infection. Furthermore, *R. heylii* DNA was detected in all pharynx swabs taken

from infected animals 28 days after experimental infection. Four of eight tested mice infected with 10^6 CFU or 10^8 CFU of *M. muris* exhibited positive PCR results in ano-genital swabs 28 days after infection. All placebo-treated mice remained PCR negative for *R. heylii* and *M. muris* throughout the experiment. In conclusion, swabs from the pharynx and ano-genital region are suitable samples for detection of *R. heylii* and *M. muris* infection using PCR.

Seroconversion of *R. heylii* and *M. muris* infected mice

As *M. muris* is considered to be apathogenic and mice infected with lower doses of *R. heylii* and *M. muris* did not show any clinical signs, we asked if these mice developed an adaptive serum IgG immune responsive against these bacteria. Therefore, an in-house whole cell extract ELISA was developed for both species (Fig. 7). Both ELISA were specific and sensitive (*R. heylii*-ELISA: sensitivity = 94.4%, specificity = 81.5%; *M. muris*-ELISA: sensitivity = 93.8%, specificity = 100%). Mice infected with *R. heylii* or *M. muris* showed increased serum IgG levels against the respective antigen 28 days after infection in comparison to phosphate buffered saline (PBS) treated mice. Specific IgG were not detected in early losses after *R. heylii* infection (Fig. 6A-B). One mouse showed elevated specific IgG levels against *M. muris* before experimental infection. Additionally, sera from mice of recent experimental infections were also investigated in the two ELISAs. None of the mice infected experimentally with *R. heylii* or *R. pneumotropicus* showed values above the cut-off in the in-house ELISA detecting IgG antibodies against *M. muris*. In contrast, 11 of 20 tested sera from *R. pneumotropicus* infected mice [8] showed positive results in the IgG anti-*R. heylii* ELISA. In summary, most of the intranasally with *R. heylii* or *M. muris* infected C57BL/6 and BALB/c wildtype mice showed specific IgG antibodies against the homologous antigen.

Discussion

In our previous study we observed unexpected high mortality in wildtype BALB/c (56%) and C57BL/6 (50%) mice after experimental intranasal infection with 10^8 CFU of a *R. pneumotropicus* strain positive for all known *pnx* genes encoding RTX toxins [8]. This was unexpected as *P. pneumotropica* was regarded to cause systemic infections only in immunodeficient mice. When expressed recombinantly, RTX toxins PnxI and PnxII are described to be haemolytic proteins, whereas PnxIII is cytotoxic and participates in hemagglutination [9, 10]. We hypothesized that the reason for the high virulence of this *R. pneumotropicus* strain is the expression of these RTX toxins and that profiling of *pnx* genes might be used to access virulence in the genus *Rodentibacter*. Noteworthy, RTX toxin genes are important virulence markers in other *Pasteurellaceae* such as *Actinobacillus pleuropneumoniae*, a pathogen causing severe pleuropneumonia in pigs [14]. To test the latter hypothesis, we investigated the virulence of a *R. heylii* strain (SF27GVG) in this study, which is in contrast to many other *R. heylii* and *R. pneumotropicus* isolates negative for *pnx*I, *pnx*II and *pnx*III [8]. In contrast to our expectations, mortality in BALB/c and C57BL/6 obtained values of 100% and 75%, respectively, using the same intranasal inoculum of 10^8 CFU as in our *R. pneumotropicus* study. Though the *R. pneumotropicus* and *R. heylii* infections were not conducted at the same time, the results are comparable as we used identical source of animals, mice

strains, housing conditions, laboratory facility, age, infection and anaesthesia protocol and health monitoring system including identical human end points. Usage of 10% serum in the Brain-Heart-Infusion medium (BHI; Becton, Dickinson and Company, France) used for cultivation of *R. heylii* and the application of contact sentinels in our study on *R. pneumotropicus* are the only two differences we are aware of. Thus, we think that the most likely explanation for the higher mortality in *R. heylii* SF27GVG infected mice is that this strain is even more virulent than *R. pneumotropicus* JF4Ni. We conclude from the results of this study that the *pnx* genes I, II and III are very questionable virulence markers in *R. heylii* and that other important virulence factors are involved in the pathogenesis of severe purulent bronchopneumonia induced in this study by *R. heylii* but not by *M. muris* intranasal infection.

We observed important differences in clearance of the bacteria in the respiratory tract between BALB/c and C57BL/6 mice. The specific bacterial load in the TNL and in the lung was substantially lower in C57BL/6 in comparison to BALB/c mice. This is very much in accordance with our previous findings on the clearance of *R. pneumotropicus* [8]. C57BL/6 mice are known to undergo a Th1-prone immune response as indicated by a higher IgG2b/IgG1 ratio and a prominent IgG2c response against *R. pneumotropicus* [8].

Interestingly, many surviving mice challenged with 10^8 and 10^6 but not 10^4 CFU of *R. heylii* carried this pathogen in the genito-urinary tract, which is in accordance with numerous studies describing field infections [15]. As mice challenged with 10^4 CFU *R. heylii* had a specific bacterial load in the tracheo-nasal lavage that is as high (BALB/c) or even higher (C57BL/6) than the load in respective mice challenged with 10^6 CFU, it is reasonable to propose that colonization of the genito-urinary tract occurred after bacteraemia and not through an ascending infection following excretion of this pathogen after nasal colonization. Noteworthy, mice infected with 10^6 but not 10^4 CFU exhibited a temporary decrease in body weight after challenge, most likely in association with the postulated bacteraemia leading to colonization of the genito-urinary tract. In contrast to *R. heylii* infection, *M. muris* was detected in numerous mice in the uro-genital tract after application of 10^4 CFU though signs of systemic infection were not observed, suggesting ascending colonization.

In contrast to *R. heylii* and *R. pneumotropicus*, *M. muris* is considered to be apathogenic. However, to the best of our knowledge *M. muris* was not used for experimental infection prior to this study. Our experimental results confirm that *M. muris* is not associated with mortality and pathological findings in wildtype BALB/c and C57BL/6 mice. However, temporary weight loss was induced after experimental infection with 10^8 CFU/mouse.

Purulent bronchopneumonia was found to be a main pathology in mice succumbing to *R. heylii* or *R. pneumotropicus* infection [8]. Biofilm formation is an important pathomechanism of bacteria causing persisting infections in the respiratory tract [16]. Noteworthy, *R. pneumotropicus* and *R. heylii* were shown to produce biofilms in contrast to *M. muris* [17]. However, the role of biofilm formation in the described models leading to death within two days after experimental infection is not clear, even more as dissemination to different internal organs was recorded. *P. pneumotropica* was shown to bind factor H

and C4BP to evade the human complement system [18]. Accordingly, complement evasion is likely to contribute to the invasive phenotype of *R. heylii* in wt mice observed in this study and which should be investigated in future studies.

Both *R. heylii* and *M. muris* were isolated from the upper respiratory tract four weeks after experimental infection even at a very low dose of 10^4 CFU. Thus, both species are very successful colonizers of the respiratory and urogenital tract after experimental application, which explains their high prevalence in animal laboratory facilities. Experimental infection is associated with IgG seroconversion even at a low dose of 10^4 CFU. This is detectable either by using whole cell extracts as antigen as shown in this study or in the case of *R. heylii* by using CARLO-1 as antigen as described previously [12]. Additionally, to demonstrating virulence in *R. heylii* this study describes a colonization model which is suitable for further studies on host-pathogen interaction and prevention measures.

Conclusions

R. heylii SF27GVG is highly virulent in wildtype BALB/c and C57BL/6 mice, though it lacks all three known *pnx* genes (*pnx*I, II and III) encoding RTX toxins. Purulent bronchopneumonia is the main pathology in intranasally infected mice. The uro-genital tract is an important target organ of *R. heylii* and *M. muris*. Experimental infection with 10^8 CFU *M. muris* 694/11does not induce mortality or high morbidity, confirming that this member of the *Pasteurellaceae* is of low virulence, at the most. *R. heylii* and *M. muris* colonize the upper respiratory tract very efficiently even after low infection doses.

Methods

Bacterial strains and culture medium

R. heylii SF27GVG (*pnx*I-, *pnx*II-, *pnx*III-) was isolated in 2017 from a mouse in a German research facility [12] and cultured overnight at 37 C on Columbia Blood Agar (Thermo Fischer scientific) or in Brain-Heart-Infusion (BHI; Becton, Dickinson and Company, France) containing 10% inactivated horse serum. *M. muris* 694/11was cultivated the same way except that BHI did not include horse serum. The strain was recently isolated from a pharynx swab of a mouse of a German research facility. Experimental infection was conducted with bacteria grown until a concentration of 10^8 CFU per millilitre was reached. Infection doses for experimental infection with 10^4 CFU, 10^6 CFU and 10^8 CFU per mouse were generated by centrifuging and diluting bacterial suspensions with PBS.

Mice

Eight-week-old female specific pathogen free BALB/c mice were obtained from Charles River laboratory (Sulzfeld, Germany) and C57BL/6 mice from Janvier Labs (Le Genest-Saint-Isle, France). Forty-eight mice per experiment with one pathogen were randomly caged in groups of four animals (24 mice of each strain). This included four infection cages per mouse strain and pathogen and two control cages. *R. heylii*

infection was carried out using two infection cages with an infection dose of 10^8 CFU, one cage with 10^6 CFU and one cage with 10^4 CFU. The *M. muris* infection was carried out using one cage of animals infected with 10^8 CFU, two cages for 10^6 CFU and one cage for 10^4 CFU. All mice were housed in sterile individually ventilated cages including HEPA filters with separate air condition and sterile bedding.

Infection, clinical examination and termination criteria

Experimental infection was performed as described previously for *R. pneumotropicus* [8]. Noteworthy, control mice received a placebo of 25 µl phosphate buffered saline without bacteria (PBS, 12.5 µl per nostril) in anaesthesia. After infection mice were weighted, clinically examined and scored every 12 hours according to the score sheet shown in Additional file 1: Table S1. Termination criteria were bleeding from orifices, paralysis, acute respiratory distress, cyanosis and weight loss of 20% or higher. Mice with a cumulative score above or equal 9 or a score above or equal 6 for 24 h were also killed for animal welfare reasons. Immediately after recording read out parameters leading to the sacrifice of a mouse, the animal was anaesthesized through intraperitoneal application of 100 mg ketamin per kg body weight and 5 mg xylazin per kg body weight. The mouse was bled by heart puncture and finally killed by cervical dislocation. Animals which did not reach these termination criteria were sacrificed 28 days after experimental infection the same way.

Sample collection and examination

First blood samples were collected from the submandibular vein seven days before infection. After centrifugation at 3900x*g* for 10 min, sera were aliquoted and stored at -20°C. Twenty-eight days post infection all surviving mice were euthanized. Samples of internal organs and tracheo-nasal lavage were collected for bacteriological and histological investigations as described previously [8]. Bacteria were identified by matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) using an extended data base. Bacterial loads were determined by semi-quantitative scoring.

Antigen preparation and ELISA

For detection of serum IgG specific for *R. heylii* and *M. muris* in ELISA, whole cell extracts were prepared as described [8]. Medium Bind microplates (Brand, Wertheim, Germany; 96-well, U-shape) were coated with 400 ng whole-cell antigen in carbonate-bicarbonate buffer (50 mmol/L, pH 9.6) for 45 min at 37 °C. Wells were washed three times (PBS, 300 µL) using a Columbus Pro ELISA washer (Tecan), blocked with Superblock (300 µL, 30 min, RT), and stored at 4 °C. ELISA was performed as described before [12]. Cut offs were determined as mean of negative controls (PBS) plus three standard deviations.

PCR

For detection of *R. heylii* and *M. muris* DNA, ano-genital and pharyngeal swaps were taken. DNA was extracted by Snooplex FastPrep Kit (GVG Genetic Monitoring GmbH, Germany) according to the

manufacturer's instructions. Specific DNA was amplified using a described PCR protocol to detect *R. heylii* and *M. muris* [13]

Detection of RTX-toxin genes and carlo-1

The infection strain *R. heylii* SF27GVG was tested for three genes encoding RTX toxins, namely *pnx*I, *pnx*II and *pnx*III. DNA of *R. pneumotropicus* JF4Ni was used as template for comparison. Two parts of each gene were amplified with primers described before (Tab. 2; [8-10]). PCR was performed with One *Taq* ® Quick-Load ® DNA Polymerase (New England BioLabs Inc.) according the manufacturer's instructions. PCR products were visualised by an 1% agarose gel with 0.01% Roti®-GelStain (Carl Roth GmbH + Co. KG). Gel electrophoresis was run for 30 minutes at 120V.

Statistical analysis

The Mann-Whitney test was performed to analyse differences between the different groups of mice. The data in the Kaplan-Meyer survival diagram were analysed with the log rank test. Probabilities were considered as follows: $p<0.05$ * (significant), $p<0.01$ ** (highly significant), $p<0.001$ ***.

Abbreviations

BHI brain heart infusion

CARLO-1 characteristic antigen for *Rodentibacter* of laboratory

origin 1

CFU colony forming units

DNA deoxyribonucleic acid

ELISA enzyme-linked immunosorbent assay

IgG immune globuline G

Ln. tracheobronialis lymphonodus tracheobronialis

MALDI matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

M. muris Muribacter muris

PBS phosphate buffered saline

PCR polymerase chain reaction

P. pneumotropica Pasteurella pneumotropica

R. pneumotropicus/heylii Rodentibacter pneumotropicus/heylii

RTX repeats in toxin

spp. species

TNL tracheonasal lavage

Declarations

Ethics approval and consent to participate

The current animal studies were registered and approved under no. TVV 17/15 and no. TVV 46/17 at the Provincial Head Office Saxony (Germany), Unit 24, Veterinary and Food Inspection (Landesdirektion Sachsen, Referat 24, Veterinärwesen und Lebensmittelüberwachung) which includes approval through the registered §15 committee for animal experiments (referring to §15 of the German Animal Protection Law). This study and handling of mice in general was conducted in strict accordance with the principles outlined in the EU Directive 2010/63/EU and German Animal Protection Law.

Consent to publish

Not applicable.

Availability of data and materials

The datasets analysed during this current study are available from the corresponding author upon a reasonable request.

Competing interests

GVG Diagnostics is a diagnostic service provider. Felix Fingas is an employee at GVG Diagnostics.

Funding

This work was financially supported by the European Funds of Regional Development (EFRE). The funding body had no role in the design of the study and collection, analysis, and interpretation of data as well as in writing the manuscript.

Authors' Contributions

CB conceived the study. CB, TG, FF, JF and SK designed the experiments. Acquisition of data was mainly conducted by SK. The clinical screenings were performed by SK and JF. LB and FF provided strains and protocols. The histopathological screenings were performed by KK. Data analysis was conducted by SK. SK and CB drafted the manuscripts. All authors critically revised the manuscript, approved the final

manuscript and agreed to be accountable for all aspects of the work.

Acknowledgements

Not applicable.

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Tables

Tab. 1: Degree of bronchopneumonia in mice intranasally infected with 10^8 CFU of *R. heylii SF27GVG* (for definition of scores see Additional File with Table A2)

		Acute – subacute catarrhal purulent bronchopneumonia		
		0	1-3	4-7
BALB/c	Control	4/4	0/4	0/4
	Losses	1/8	1/8	6/8
	Survivors	-	-	-
C57BL/6	Control	4/4	0/4	0/4
	Losses	1/6	4/6	1/6
	Survivors	2/2	0/2	0/2

Tab. 2: Pairs of primers used for detection of genes encoding RTX toxins and the gene carlo1

gene	primer pairs	length	published
pnxI	a: pnxIAF + pnxIAR	2124 bp	Fornefett et al. 2018
	b: RI2 + RI4	1248 bp	Sasaki et al. 2009
pnxII	c: pnxIIAF + pnxIIAR	4149 bp	Fornefett et al. 2018
	d: RII3 + RII4	323 bp	Sasaki et al. 2009
pnxIII	e: pnxIIIAF + pnxIIIAR	1027 bp	Fornefett et al. 2018
	f: pnxIIIA_209_f + pnxIIIA_197_r	242 bp	Sasaki et al. 2011
carlo1	CARLO-1_fd + CARLO-1_rev	951 bp	Fingas et al. 2018

Figures

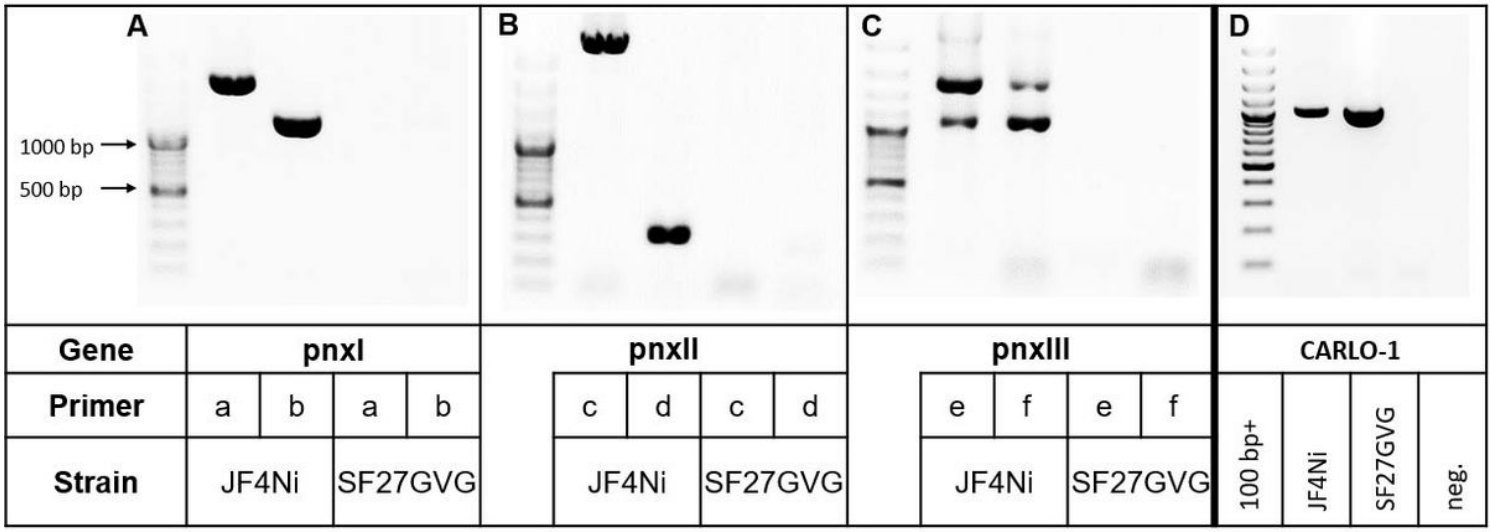

Figure 1

R. heylii SF27GVG does not contain the genes encoding the known RTX-toxins PnxI, PnxII or PnxIII Screening for genes encoding PnxI (A), PnxII (B) and PnxIII (C) by PCR was performed by amplification of two sequences for each gene. No amplicon was produced from DNA of R. heylii SF27GVG in contrast to R. pneumotropicus JF4Ni. D: The gene encoding the “characteristic antigen for Rodentibacter of laboratory origin 1“ (CARLO-1, [12]) was detected using the same DNA preparation of R. heylii SF27GVG.

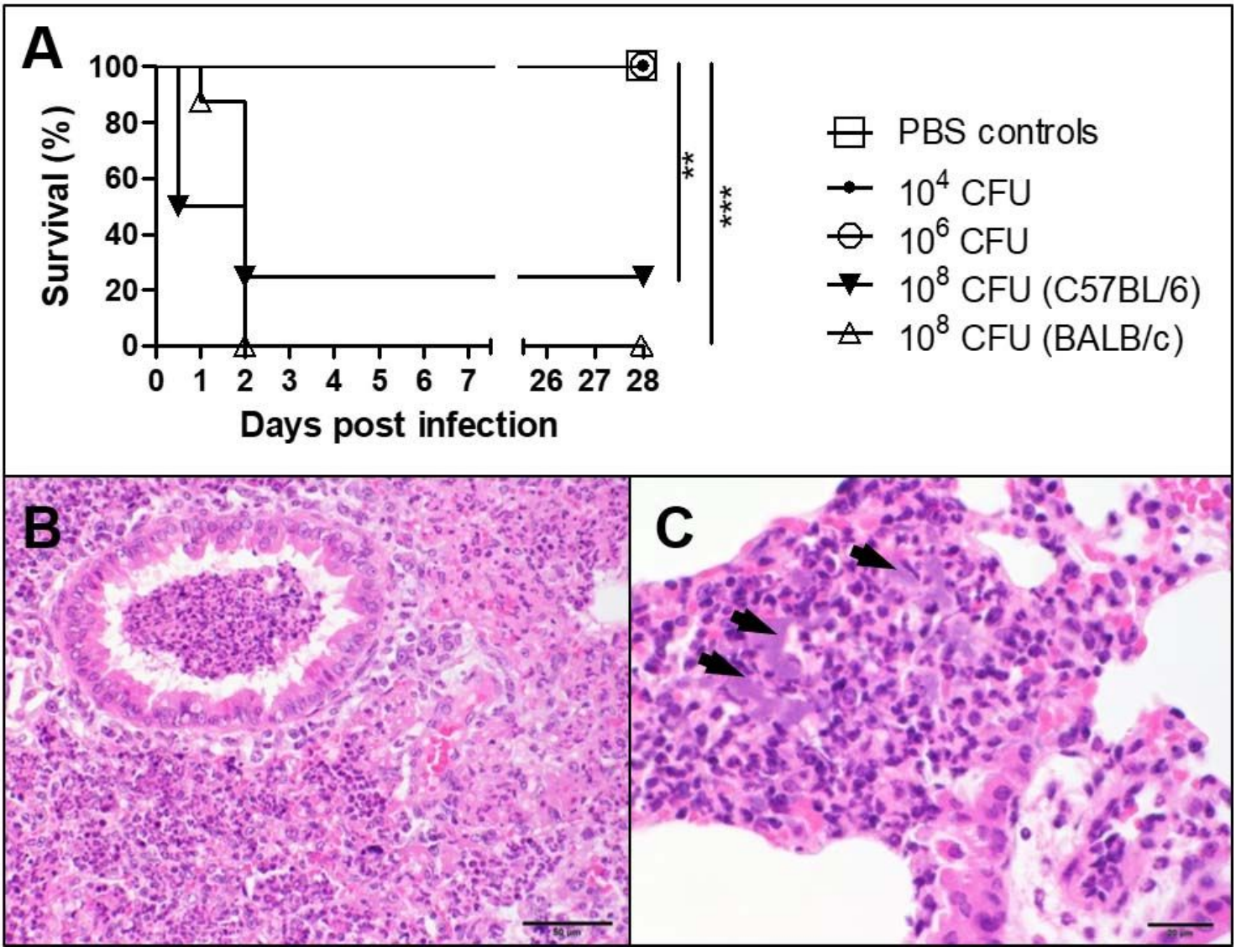

Figure 2

R. heylii SF27GVG induces high mortality and severe purulent bronchopneumonia after experimental infection Intranasal infection of C57BL/6 (▯) and BALB/C mice (▯) mice with 108 CFU R. heylii SF27GVG (pnxI-, pnxII-, pnxIII-) resulted in 75 % and 100% mortality, respectively. Mice were humanely killed after exceeding a weight loss of 20%. Experimental infection with 104 CFU/mouse (●), 106 CFU/mouse (▯) and mock-infection (□) with PBS induced no mortality in BALB/c and C57BL/6 mice (A). The Log-rank-test was used for statistical analysis. Early losses due to experimental infection with 108 CFU showed catarrhal-purulent bronchopneumonia. Alveoli, bronchioles and bronchi were infiltrated by inflammatory cells (B and C) and bacteria were detected within the lesions (arrows in C). The log rank test was used to analyse differences between the indicated groups (A). Probabilities were considered as follows: $p<0.05$ * (significant), $p<0.01$ ** (highly significant), $p<0.001$ ***.

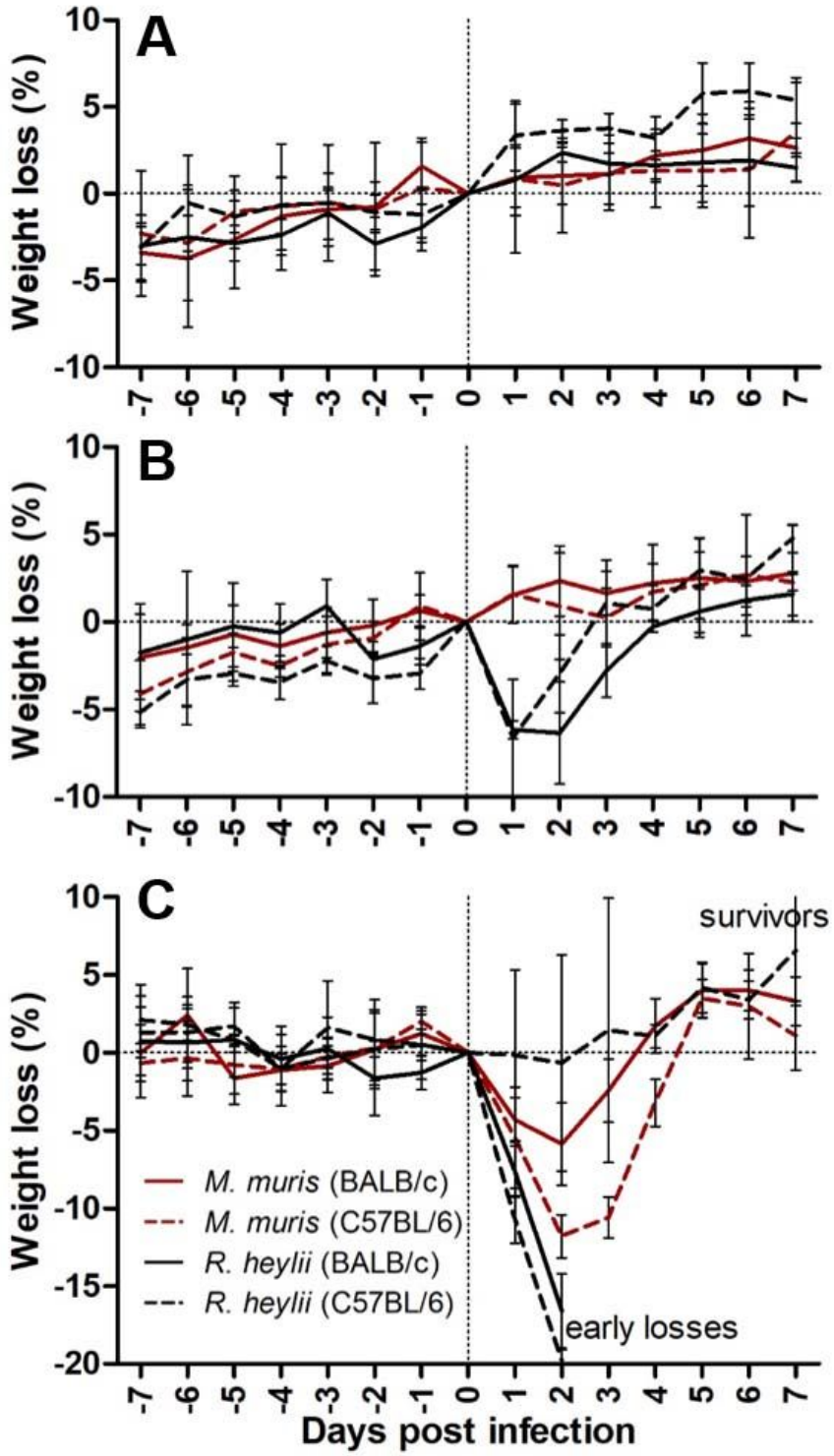

Figure 3

Weight loss in R. heylii infected mice is significantly higher than in M. muris infected mice The curves of the mean weight loss of R. heylii SF27GVG (A-C, black lines) and M. muris 694/11 (A-C, red lines) infected mice are shown. The dashed and solid lines depict C57BL/6 and BALB/c mice, respectively. Infection with 104 CFU of either strain was not associated with detectable weight loss (A). Moderate weight loss (5 to 20%) was observed after infection with 106 CFU of R. heylii SF27GVG but not with 106 CFU of M. muris

694/11 (B). One day after application, mice infected with R. heylii developed significant higher weight loss compared to mice infected with M. muris (C57BL/6: p=0.0084; BALB/c: p=0.0040). Severe weight loss (≥20%) leading to euthanasia of infected animals was only induced through application 108 CFU R. heylii SF27GVG though moderate weight loss was also recorded in mice infected with M. muris 694/11 (C). Two days after infection, BALB/c mice showed significant higher weight loss if infected with R. heylii than with M. muris (p= 0.0061). Mann-Whitney-test was used for statistical analyses. Body weight was determined every 24h and 12h before and after infection, respectively.

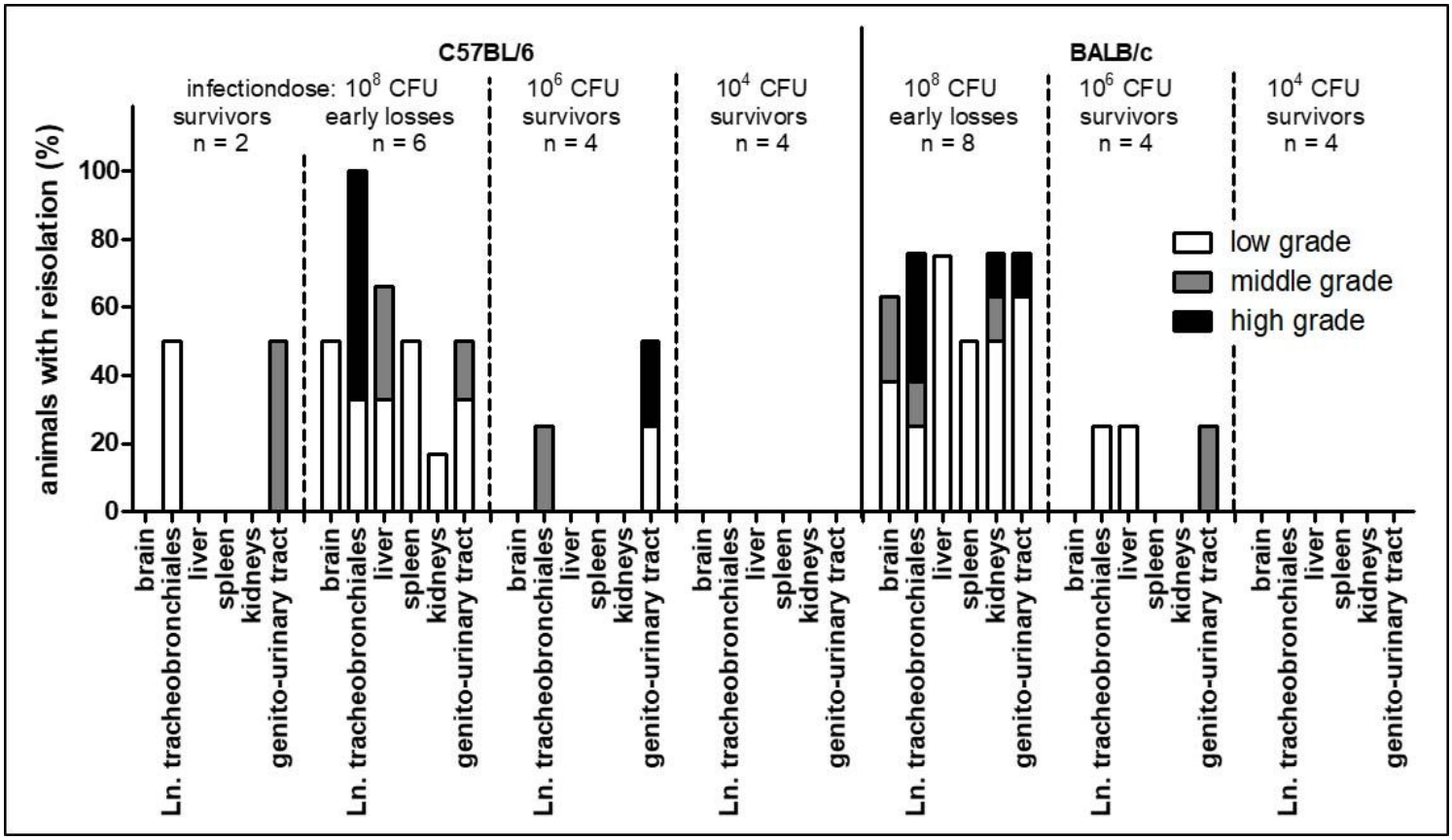

Figure 4

Intranasal infection with the pnxI-, pnxII- and pnxIII- R. heylii strain SF27GVG results in dissemination The used R. heylii challenge strain was detected through culture in numerous internal organs in early losses following experimental infection with 108 CFU R. heylii SF27GVG. Genito-urinary tract colonization was recorded in mice infected with 106 but not in mice infected with 104 CFU. The bacterial content was assessed semi-quantitatively. Black bars indicate a high grade (≥ 70 CFU); grey bars a middle grade (20-69 CFU) and white bars a low grade (<20 CFU).

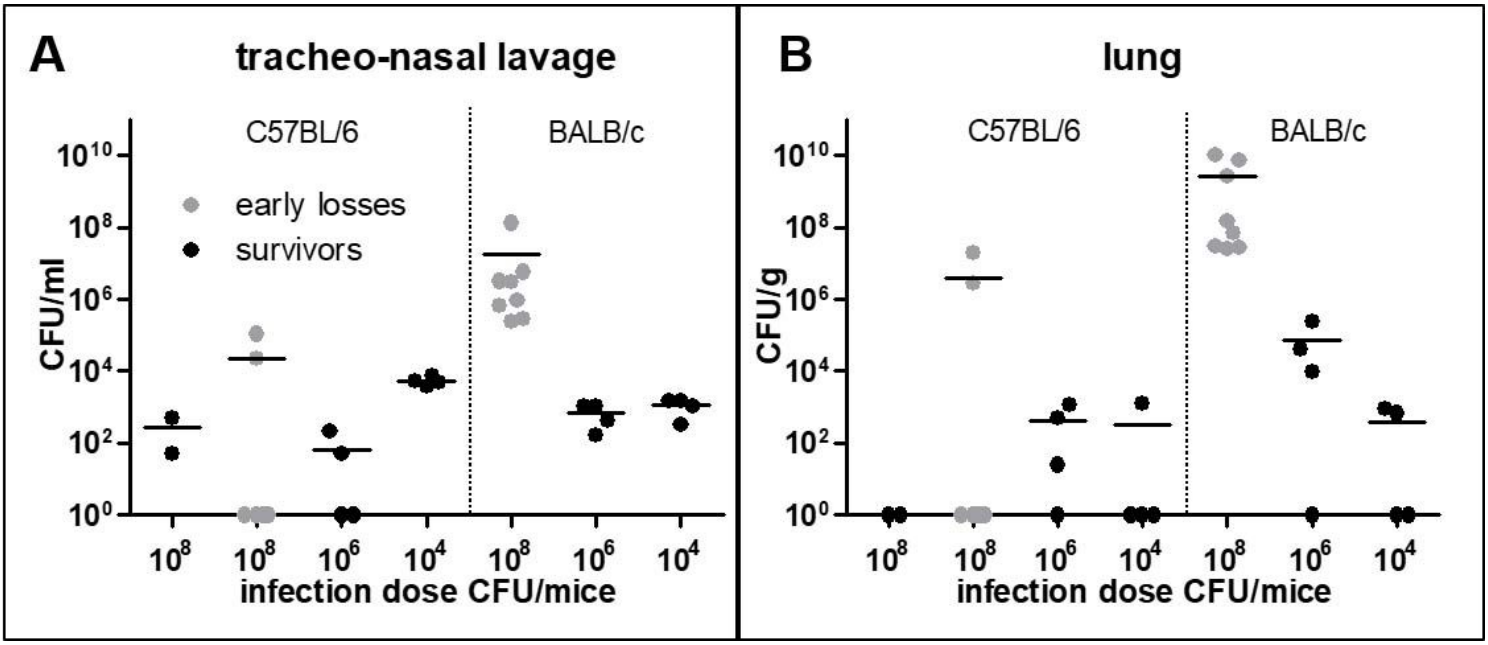

Figure 5

Load of R. heylii in the respiratory tract of experimentally infected C57BL/6 and BALB/c mice The specific bacterial content was determined quantitatively through counting of colonies of serial dilutions of tracheo-nasal lavage (A) and homogenized lung (B) after experimental infection with R. heylii SF27GVG. Mice infected with 108 CFU are divided into early losses (●) and survivors (●). Means are indicated by black lines. Differences between C57BL/6 and BALB/c mice were not significant. Mann-Whitney-test was used for statistical analyses.

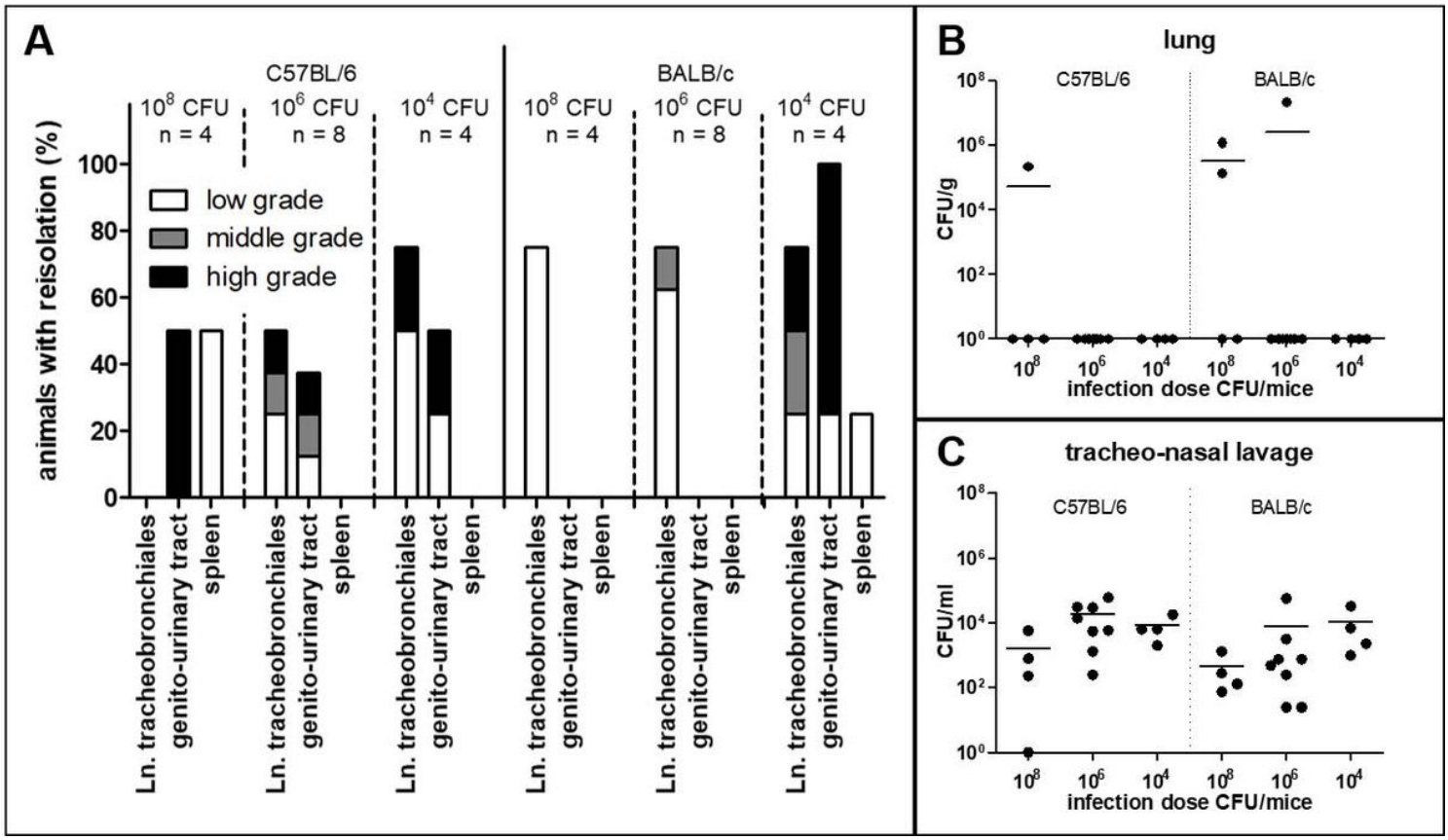

Figure 6

Intranasal application of M. muris results in colonization of the upper respiratory and genito-urinary tract Semiquantitative (A) and quantitative (B, C) assessment of the load of M. muris in the indicated tissues or samples obtained 28 days after intranasal application of the specified doses. The bacterial content was assessed semi-quantitatively in (A). Black bars indicate a high grade, grey bars a middle grade and white bars a low grade. The specific bacterial content was determined quantitatively through counting of colonies of serial dilutions of tracheo-nasal lavage (C) and homogenized lung tissue (B). M. muris was not detected in mock-infected animals (n=16). The Mann-Whitney-test was used for statistical analyses. There were no significant differences between various infection doses.

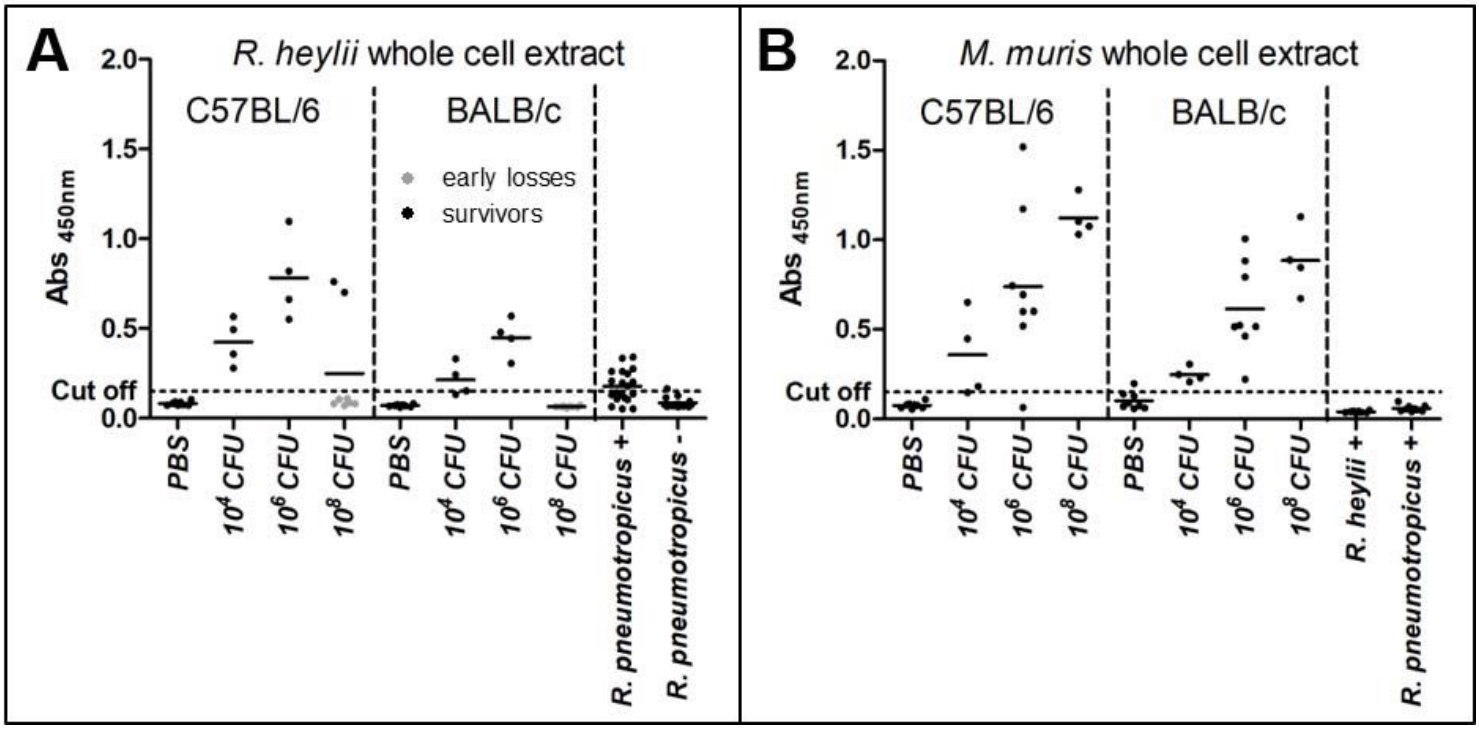

Figure 7

Experimental intranasal application of M. muris and R. heylii leads to increased specific IgG levels Serum IgG levels were determined by a specific whole-cell-extract ELISA for each bacterium. A: Black dots: survivors; grey dots: early losses. Survivors developed specific serum IgG levels after experimental infection with R. heylii SF27GVG (A, cut off: 0.14) and M. muris except in two cases (B, Cut off: 0.20), respectively. Higher infection doses resulted in higher IgG levels. Cut offs were determined as mean of negative controls (PBS) plus three standard deviations. Additionally, sera from mice of recent experimental infections are represented.

Supplementary Files

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