Brief Communication

Title: Congenic mice reveal genetic epistasis and overlapping disease loci for autoimmune diabetes and listeriosis

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Abstract

The nonobese diabetic (NOD) mouse strain serves as a genomic standard for assessing how allelic variation for insulin-dependent diabetes (*Idd*) loci affects the development of autoimmune diabetes. We previously demonstrated that C57BL/6 (B6) mice harbor a more diabetogenic allele than NOD mice for the *Idd14* locus when introduced onto the NOD genetic background. New congenic NOD mouse strains, harboring smaller B6-derived intervals on chromosome 13, now localize *Idd14* to an ~18Mb interval and reveal a new locus, *Idd31*. Notably, the B6 allele for *Idd31* confers protection against diabetes, but only in the absence of the diabetogenic B6 allele for *Idd14*, indicating genetic epistasis between these two loci. Moreover, congenic mice that are more susceptible to diabetes are more resistant to *Listeria monocytogenes* infection. This result co-localizes *Idd14* and *Listr2*, a resistance locus for listeriosis, to the same genomic interval and indicates that congenic NOD mice may also be useful for localizing resistance loci for infectious disease.

Keywords: type 1 diabetes, *Listeria monocytogenes*, *Idd14*, *Idd31*, *Listr2*, congenic NOD mice

Human studies have identified >50 loci that affect the risk for developing type 1 diabetes (T1D), but further investigation of the underlying causative alleles and gene interactions are often hindered by genetic heterogeneity, modest gene effect sizes and environmental factors (Barrett et al. 2009; Howson et al. 2012; Morahan et al. 2011; Pociot et al. 2010). The use of inbred mouse strains provides a more tractable approach for investigating disease loci. Genetic outcross studies using the NOD mouse strain have identified >30 loci (termed *Idd* loci) that affect susceptibility to autoimmune diabetes in mice (Bult et al. 2013; Driver et al. 2011). Moreover, introduction of specific genomic intervals from non-diabetes-prone strains onto the NOD genetic background (i.e. congenic NOD mice) have confirmed and localized individual *Idd* loci to small regions containing relatively few candidate genes (Driver et al. 2011). Evidence now supports both MHC and non-MHC genes (e.g. *B2m*, *Il2*, *Il21*, *Ctla4*, *Slc11a1*, *Trpv1*, *AK005651*) for which NOD mice harbor diabetes susceptibility alleles (Araki et al. 2009; Driver et al. 2011; Hamilton-Williams et al. 2001; Kissler et al. 2006; McGuire et al. 2009; Razavi et al. 2006; Tan et al. 2010; Yamanouchi et al. 2007). Congenic NOD mice have also identified genetic interactions between *Idd* loci – different combinations of alleles for *Idd* loci confer different degrees of diabetes protection or susceptibility when introduced onto the NOD genetic background (Fraser et al. 2010; Hamilton-Williams et al. 2013; Hollis-Moffatt et al. 2005; Hunter et al. 2007; Lin et al. 2013; Morin et al. 2006).

Idd14 is a member of a class of *Idd* loci for which non-diabetes-prone mouse strains harbor alleles that are more diabetogenic than NOD alleles (Brodnicki et al. 2003; Ghosh et al. 1993; McAleer et al. 1995). *Idd14* was originally located to an ~40 Mb interval on chromosome (Chr) 13 between *D13Mit61* and *D13Mit9* (McAleer et al. 1995). In a separate study, diabetic backcross progeny, generated from NOD and non-diabetes-prone B6 mice, had increased heterozygosity for polymorphic markers within this interval, suggesting that B6 mice harbor a more diabetogenic allele than NOD mice for *Idd14* (Brodnicki et al. 2003). We therefore generated a congenic NOD mouse strain (NOD.B6Idd14R0) that was homozygous for a B6-derived interval encompassing the majority of Chr13, including the linked interval defining *Idd14*. These congenic NOD mice demonstrated a significant increase in diabetes incidence compared to NOD mice (Brodnicki et al. 2003). This confirmed that B6 mice harbor a more diabetogenic allele than NOD mice for *Idd14*, but this diabetogenic effect is only observed when this B6 allele is introduced onto the NOD genetic background.

To further localize *Idd14*, we generated new congenic NOD strains that dissected the congenic interval in NOD.B6Idd14R0 mice (Table 1; henceforth, names are abbreviated, e.g., NOD.B6Idd14R0 = Idd14R0). Briefly, heterozygous Idd14R0 mice were intercrossed to generate F_2 progeny that were screened for recombination events using DNA from tail biopsies and polymorphic markers as described (Brodnicki et al. 2003). Three F_2 progeny were selected to establish new homozygous

congenic strains that have smaller congenic intervals, which either encompass (Idd14R3) or dissect (Idd14R6, Idd14R8) the originally linked interval for *Idd14* (Brodnicki et al. 2003; McAleer et al. 1995). Female cohorts for these congenic strains, as well as the Idd14R0 strain, were monitored for diabetes onset compared to NOD females. Idd14R0, Idd14R3 and Idd14R8 mice exhibited a significant increase in diabetes incidence compared to NOD mice (Fig. 1A, 1B), indicating that *Idd14* is located within the shared congenic interval (Table 1). In contrast, Idd14R6 mice had a significant decrease in diabetes incidence compared to NOD mice (Fig. 1C) and excluded this distal region on Chr13 from containing the *Idd14* locus (Table 1). These results indicate that *Idd14* localizes to an ~18 Mb interval between *D13Svi14* and *D13Mit11* (Table 1). The reduced diabetes incidence observed for Idd14R6 mice was unexpected (Fig. 1C) and points to a new *Idd* locus, termed *Idd31*, that is located within an ~48 Mb interval on the distal end of Chr13 (Table 1).

Furthermore, our new congenic NOD strains revealed genetic epistasis between *Idd14* and *Idd31*. Notably, the B6 allele for *Idd31* provides substantial protection against diabetes onset, but only in the absence of the diabetogenic B6 allele for *Idd14* (ie Idd14R6, Fig. 1C). The ability of the B6 allele for *Idd14* to mask this protective effect in Idd14R0 and Idd14R8 mice was unexpected (Fig. 1A, 1B), but such genetic epistasis between *Idd* loci is not without precedence: the diabetogenic B10 allele for *Idd5.4* suppressed the protective effect of the B10 alleles for *Idd5.2* and *Idd5.3* in congenic NOD mice for Chr1 (Hunter et al. 2007); the diabetogenic C3H allele for *Idd19* suppressed the protective effect of the C3H allele for *Idd6* in congenic NOD mice for Chr6 (Morin et al. 2006); and the diabetogenic ABH allele for *Idd21.2* suppressed the protective effect of the ABH allele for *Idd21.1* in congenic NOD mice for Chr18 (Hollis-Moffatt et al. 2005). In each of these cases, however, the diabetes incidence for the respective congenic NOD strains was no greater than NOD mice (Hollis-Moffatt et al. 2005; Hunter et al. 2007; Morin et al. 2006). In contrast, the diabetogenic B6 allele for *Idd14* not only completely masks the protective effect of the B6 resistance allele for *Idd31*, but also confers a higher diabetes incidence in Idd14R0 and Idd14R8 mice compared to NOD mice (Fig. 1A, 1B).

Given that B6 mice harbor a highly diabetogenic allele for *Idd14*, we speculated that this allele might be maintained within *Mus* species because it confers some advantage. The *Idd14* interval intriguingly overlaps *Listr2*, a resistance locus for the infectious disease listeriosis, caused by *Listeria monocytogenes* (*L. monocytogenes*). *Listr1* and *Listr2* were originally identified in an outcross between B6 and BALB/c mice, with *Listr2* mapping to a ~40 Mb interval between *D13Mit21* and *D13Mit147* on Chr13 (Boyartchuk et al. 2001). In particular, F_2 progeny harboring B6 alleles within the interval for *Listr2* were more likely to survive a *L. monocytogenes* dose that was lethal to BALB/c mice. While the *Listr1* locus on Chr5 has recently been confirmed by congenic mice (Qi et al. 2014), the protective effect of the B6 allele for the *Listr2* locus has not

been confirmed. There is a relatively broad range of sensitivities amongst inbred mouse strains for *L. monocytogenes* infection, with BALB/c and NOD mice exhibiting increased susceptibility compared to B6 mice (Boyartchuk et al. 2001; Cheers and McKenzie 1978; Usami et al. 1995). Our congenic NOD mouse strains thus enabled us to test if the B6-derived interval for *Idd14*, which increases diabetes risk, harbors the B6 resistance allele for *Listr2*.

To test for *Listr2*, cohorts of NOD, Idd14R0 and B6 mice were infected with *L. monocytogenes*. Culturing of *L. monocytogenes*, infection of mice, and measurement of organ bacterial load were performed as described (Wang et al. 2011). Mice were initially infected with ~2,500 colony forming units (CFU) of *L. monocytogenes*. This dose resulted in some infected NOD mice becoming moribund on day 3 post-infection, whereas infected B6 and Idd14R0 mice showed little to no symptoms of infection (e.g. ruffled fur, limited movement). The NOD mice were euthanized along with the B6 and Idd14R0 mice; livers and spleens were removed to measure the amount of viable *L. monocytogenes*. As expected from the observed symptoms, NOD mice had a significantly higher bacterial burden than infected Idd14R0 and B6 mice (Fig. 2A). To reduce the potential for morbidity in NOD mice, we lowered the dose to ~900 CFU and euthanized infected mice on day 3 post-infection. Although infected NOD mice have fewer symptoms for this lower dose, they still had significantly higher bacterial loads in the liver and spleen than infected B6 and Idd14R0 mice (Fig. 2B). These combined results confirm *Listr2* and demonstrated that B6 mice harbor a resistance allele for *Listr2*, which can confer increased resistance to *L. monocytogenes* infection when placed on the genetic background of a susceptible mouse strain.

Listr2 was further localized by comparing Idd14R3, Idd14R6 and Idd14R8 congenic mice for *L. monocytogenes* infection. In separate experiments, mice for these strains, along with NOD and B6 strains, were infected with *L. monocytogenes* and euthanized on day 3-4 post-infection. Both Idd14R3 and Idd14R8 had lower bacterial loads in the liver and spleen at day 3 post-infection compared to NOD mice when infected with intermediate doses to those described above (~1,100 and ~1,400 CFU respectively; Fig. 3A, 3B). Idd14R3 and Idd14R8 also exhibited little to no symptoms of infection, whereas NOD mice still exhibited symptoms at these doses, suggesting that *Listr2* is located within the shared congenic interval (Table 1). In contrast, at a lower dose (~750 CFU) that resulted in little to no symptoms in all infected mice, Idd14R6 mice had similar bacterial loads compared to NOD mice even though a slightly later time point (i.e. day 4 post-infection) was chosen to increase the chance of detecting a difference (Fig. 3C). This result excludes the distal region on Chr13 from containing the *Listr2* locus and, in combination with the results for Idd14R3 and Idd14R8, localizes *Listr2* to the same ~18 Mb interval as *Idd14* (Table 1). We do note that the B6 resistance allele for *Listr2* alone does not confer the same degree of protection to

NOD mice as observed in B6 mice (Fig 1B, 3A, 3B). This is likely due to other loci for which B6 alleles are required to recapitulate this B6 phenotype on the NOD genetic background (Garifulin and Boyartchuk 2005).

It is not yet clear whether *Idd14* and *Listr2* represent allelic variation affecting the same or different genes. However, there are genes for which allelic variation is associated with both autoimmune diabetes and infectious diseases. For example, studies using congenic NOD mice with RNA interference or knockout alleles indicate that allelic variation for *Slc11a1* (*Idd5.1*), encoding NRAMP1, provides protection against *Salmonella enterica*, but increases the risk for developing T1D (Kissler et al. 2006; Lin et al. 2013). In humans, the T1D susceptibility allele for *PTPN22* is associated with resistance to *Mycobacterium tuberculosis* (Gomez et al. 2005), but susceptibility to *Streptococcus pneumoniae* (Chapman et al. 2006), suggesting that the effect of T1D-associated alleles upon infectious disease depends on the pathogen. At present, the defined *Idd14*/*Listr2* interval is relatively large. Publicly available annotation for this interval estimates that there are 191 protein-coding genes, 26 non-coding RNA genes and 8 microRNA genes (http://www.informatics.jax.org). Sequence variation was obtained from the Sanger Institute Mouse Genomes Project (http://www.sanger.ac.uk). Comparison of NOD and B6 for this interval identified at least 43 protein-coding genes with nonsense, splice-site, or non-conservative missense mutations (Supplemental Table 1), and an even greater number of intronic and intergenic polymorphisms with unknown effects. Additional congenic mouse strains with smaller B6-derived intervals will need to be generated and tested to more precisely localize these loci and refine the list of candidate genes. From a practical point of view, testing mice for infection is typically much quicker than diabetes onset. Identifying *Listr2* may thus accelerate the discovery of *Idd14* if sequence variation for the same gene is responsible for both loci.

In summary, congenic mice enable detection of disease loci and genetic interactions that are often hidden amidst the heterogeneity present in mouse outcrosses and human association studies. Our panel of congenic NOD mouse strains uncovered a new diabetes susceptibility locus, *Idd31*, as well as genetic epistasis between this locus and *Idd14*. These congenic NOD mice also confirmed and localized *Listr2*, which overlaps the currently defined interval for *Idd14*. While NOD mice are not conventionally used in infection studies, our results indicate that congenic NOD mice, initially established to confirm and localize *Idd* loci, may prove useful for genetic studies of infectious disease.

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Figure Legends

Figure 1. Congenic NOD mouse strains bisect the original *Idd14* locus and reveal genetic epistasis. The cumulative incidence of diabetes was determined in age-matched female cohorts: (A) NOD (n = 72) and Idd14R0 (n = 64); (B) NOD (n = 58), Idd14R3 (n = 57), and Idd14R8 (n = 61); (C) NOD (n = 60) and Idd14R6 (n = 54). Congenic mouse strains were homozygous for their respective B6-derived chromosome 13 intervals (Table 1). Mice were tested once a week for elevated urinary glucose (>110 mmol/L). Three consecutive elevated urinary readings, confirmed by elevated blood glucose (>10 mmol/L), indicated the onset of diabetes. Pairwise comparisons of diabetes incidence curves were performed using the log-rank test ($P<0.003$ for NOD vs Idd14R3; $P<0.003$ for NOD vs Idd14R8). Diabetes incidence curves in panel (A) represent an independent cohort that replicates the previous finding (Brodnicki et al. 2003).

Figure 2. Congenic NOD mice confirm *Listr2*. Age-matched females were intravenously injected with *L. monocytogenes* (EGD strain) at 7 – 9 weeks of age. (A) NOD (n = 10), Idd14R0 (n = 6) and B6 (n = 5) females were infected with ~2,500 CFU and bacterial load was determined in the liver and spleen at day 3 post-infection. Horizontal lines represent the geometric mean of each group. (B) NOD (n = 5), Idd14R0 (n = 5) and B6 (n = 5) females were infected with ~900 CFU and bacterial load was determined at day 3 post-infection. One-way ANOVA with Tukey post-tests was used for statistical analyses (Adjusted P values: * < 0.05, ** < 0.01, *** < 0.001). Horizontal lines represent the geometric mean of each group. The Y-axis stops at $Log_{10} = 2$ because quantifying CFU per organ using the described method is less accurate below 100 CFU.

Figure 3. Congenic NOD mouse strains localize *Listr2*. Age-matched females were intravenously injected with *L. monocytogenes* (EGD strain) at 7 – 9 weeks of age. (A) NOD (n = 5), Idd14R3 (n = 4) and B6 (n = 4) females were infected with ~1,100 CFU and bacterial load was determined in the liver and spleen at day 3 post-infection. (B) NOD (n = 5), Idd14R8 (n = 4) and B6 (n = 4) females were infected with ~1,400 CFU and bacterial load was determined at day 3 post-infection. In both these infections, NOD mice exhibited clinical symptoms at day 3 post-infection, whereas Idd14R3, Idd14R8 and B6 mice did not. (C) NOD (n = 5), Idd14R6 (n = 5) and B6 (n = 5) females were infected with a lower dose (~750 CFU) and bacterial load was determined at day 4 post-infection. One-way ANOVA with Tukey post-tests was used for statistical analysis (Adjusted P values: * < 0.05, ** < 0.01, *** < 0.001, **** <0.0001). Horizontal lines represent the geometric mean of each group.

Table 1. Genetic intervals for congenic mouse strains

		Congenic strains[3]				
Marker[1]	~Mb[2]	R0	R3	R6	R8	
D13Mit55	9,466,732	N	N	N	N	
D13Mit3	20,523,211	N	N	N	N	
D13Svi2	26,201,552	N	N	N	N	
D13Svi3	28,749,616	B	B	N	N	
D13Svi4	30,611,687	B	B	N	N	
D13Svi5	37,454,149	B	B	N	N	
D13Mit61	41,008,627	B	B	N	N	
D13Mit139	51,863,276	B	B	N	N	
D13Svi13	53,556,122	B	B	N	N	
D13Svi14	54,828,734	B	B	N	N	
rs3693942	55,054,140	B	B	N	B	
D13Svi15	55,556,185	B	B	N	B	
D13Mit21	55,673,902	B	B	N	B	
D13Mit65	57,677,166	B	B	N	B	
D13Svi16	60,282,401	B	B	N	B	*Idd14 /*
D13Svi17	63,398,599	B	B	N	B	*Listr2*
D13Svi18	67,846,484	B	B	N	B	
D13Svi19	69,587,198	B	B	N	B	
rs13481871	71,432,597	B	B	N	B	
D13Mit11	73,132,906	B	B	B	B	
D13Svi21	76,961,640	B	B	B	B	
D13Mit9	81,241,701	B	B	B	B	
Rs13481908	82,201,419	B	B	B	B	
D13Svi24	82,269,894	B	N	B	B	
D13Svi25	83,556,000	B	N	B	B	*Idd31*
D13Mit202	91,609,236	B	N	B	B	
D13Mit147	98,359,080	B	N	B	B	
D13Mit36	100,373,415	B	N	B	B	
D13Mit290	103,638,610	B	N	B	B	
D13Mit76	111,353,478	B	N	B	B	
D13Mit151	116,341,977	B	N	B	B	
D13Mit78	119,618,032	B	N	B	B	

[1]*D13Svi* markers are available in the NCBI Probe Database (http://www.ncbi.nlm.nih.gov/probe).
[2]Genomic coordinates are from NCBI build 37 assembly, mm9.
[3]Strain names have been abbreviated (e.g. R0 = Idd14R0 = NOD.B6Idd14R0). B = C57BL/6 allele; N = NOD allele.

Figure 1

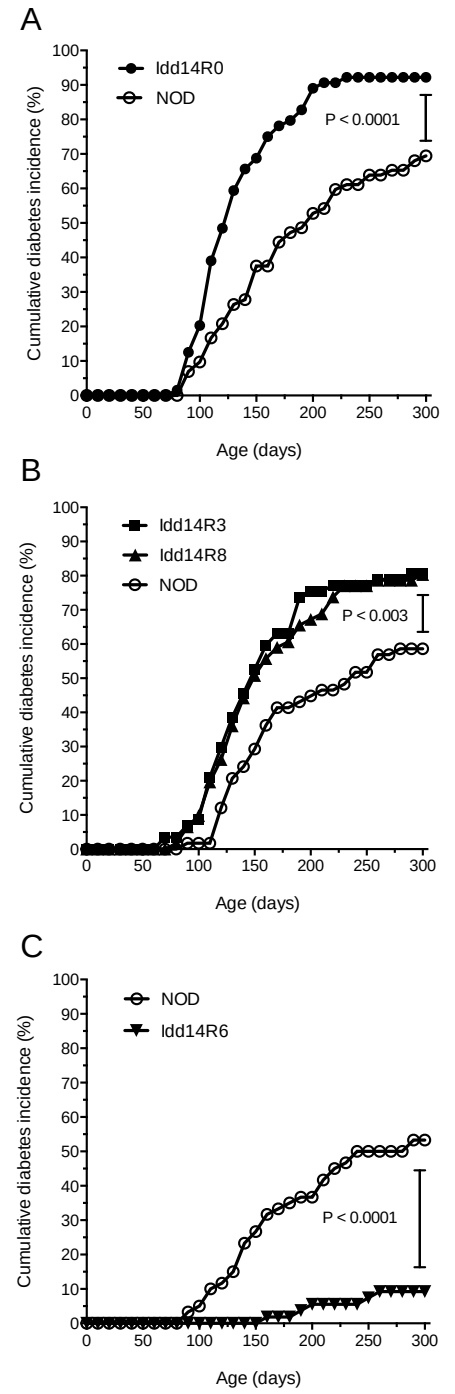

A
Idd14R0
NOD
P < 0.0001
Cumulative diabetes incidence (%)
Age (days)
B
Idd14R3
Idd14R8
NOD
P < 0.003
Cumulative diabetes incidence (%)
Age (days)
C
NOD
Idd14R6
P < 0.0001
Cumulative diabetes incidence (%)
Age (days)

Figure 2

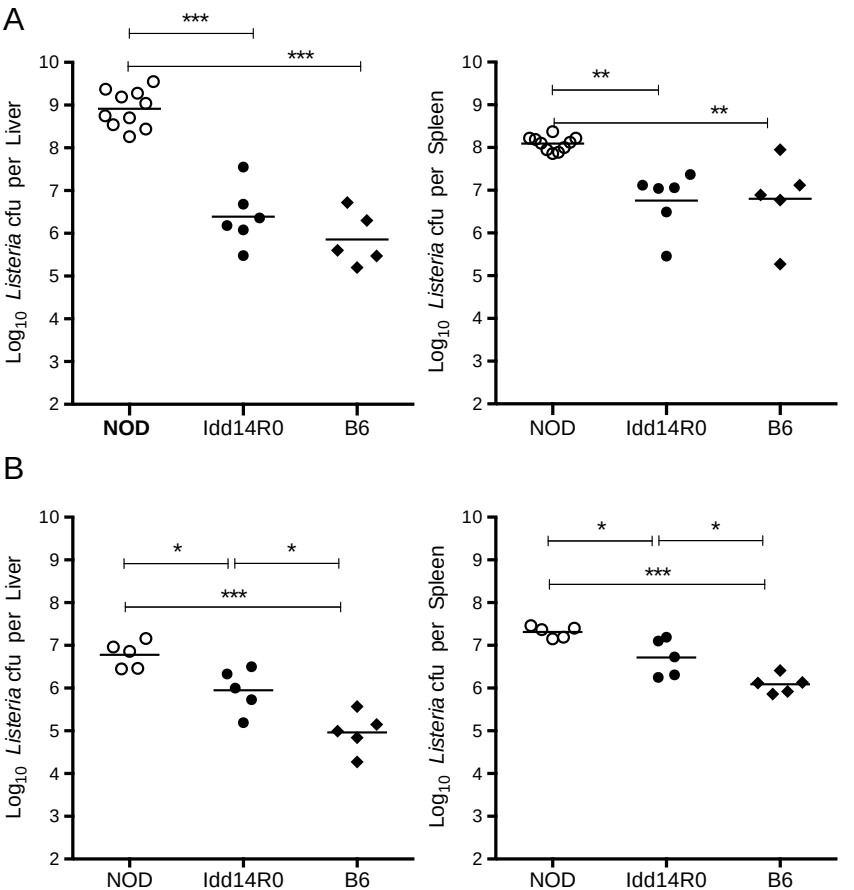
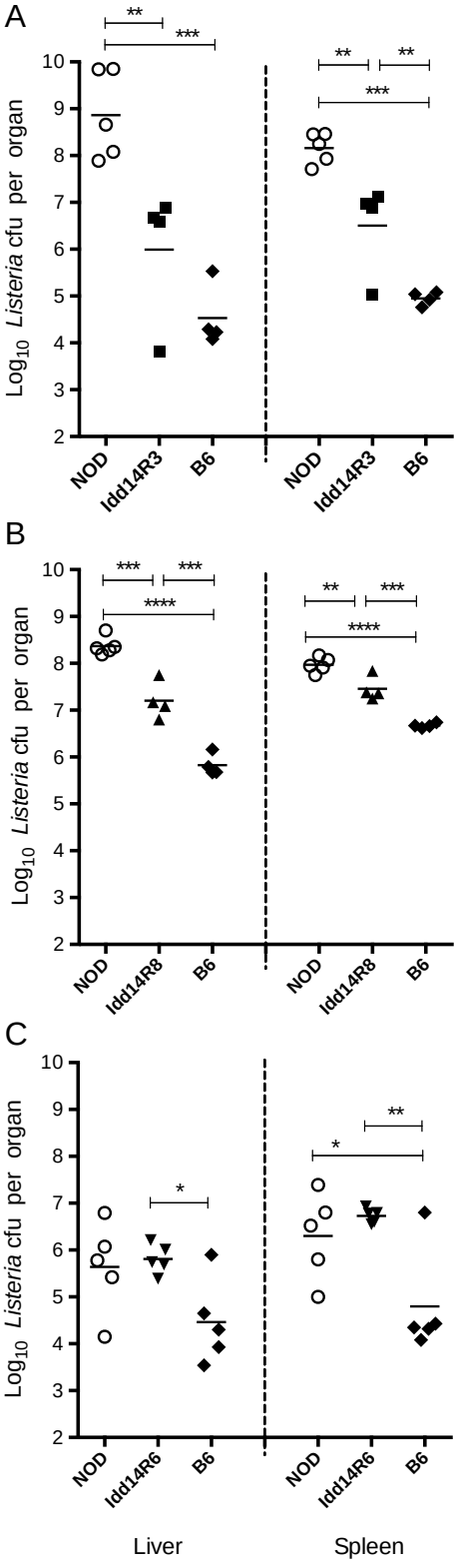

A
***
***
10
9
8
7
6
5
4
3
2
Log10 Listeria cfu per Liver
NOD
Idd14R0
B6
**
**
Log10 Listeria cfu per Spleen
NOD
Idd14R0
B6
B
*
*
***
Log10 Listeria cfu per Liver
NOD
Idd14R0
B6
*
*
***
Log10 Listeria cfu per Spleen
NOD
Idd14R0
B6

Figure 3


A
B
C
Log10 Listeria cfu per organ
NOD
Idd14R3
Idd14R8
Idd14R6
B6
Liver
Spleen

Author/s:
Wang, N; Elso, CM; Mackin, L; Mannering, SI; Strugnell, RA; Wijburg, OL; Brodnicki, TC

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