Association of bacterial infection traits with genetic variation at candidate genes for porcine disease resistance

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Summary and Implications

We predict that it may be possible to improve pig disease resistance to *Salmonella* infection by studying genes that control a piglet's initial immune response. The NRAMP1 gene controls susceptibility to multiple pathogens and acts within the macrophage. The BPI gene encodes a neutrophil protein with inhibitory/killing functions against multiple gram-negative bacteria. We investigated NRAMP1 and BPI as candidate genes for contributing to resistance in *Salmonella choleraesuis* (SC) challenge in pigs. Five NRAMP1 sequence differences (polymorphisms, SNPs) were found, while we cloned and sequenced the full-length BPI gene and identified four polymorphisms at BPI. The effects these polymorphisms have on resistance to infection were tested in two experimental disease studies. In study 1, results showed NRAMP1 and BPI genotypes were associated with decreased fecal bacterial load during infection (P values: < .0006 to < .06). Immune cell numbers were also associated with BPI genotypes. In the second study, many additional immune traits and spleen and liver bacterial counts were collected. The NRAMP1 genotypes were associated with bacterial count in liver ($P < .05$ and $P < .0006$) and with polymorphonuclear phagocytes (P values from < .003 to < .05). The BPI genotypes were significantly associated with bacteria uptake by immune cells and with bacterial counts in liver ($P<.1$) and lymphocyte response post-challenge ($P<.0001$). These data indicate NRAMP1 and/or BPI gene variation may control, in part, response to *Salmonella* infection in pigs, and that these differences could be used to identify resistant animals.

Introduction

The NRAMP1 protein plays a pivotal role in the course of early infection by *Mycobacteria*, *Leishmania*, and *Salmonella*. Mice with a mutation at *NRAMP1* proved that *NRAMP1* mutations cause susceptibility to these different pathogens. NRAMP1 protein is localized to the lysophagosomal membrane of differentiated phagocytes, indicating a role in innate (pre-adaptive) immunity. Manipulation of the *NRAMP1* gene in livestock and possibly in humans has the potential to greatly improve health and reduce dependence on antibiotics. However, manipulation at the *NRAMP1* locus must be approached with caution, as improved infectious disease immunity by *NRAMP1* gene variation was associated with hyperactive immunity and autoimmune disease.

BPI is a cationic antimicrobial protein synthesized in the primary granules of polymorphonuclear neutrophils, and which has broad anti-bacterial activity to all Gram-negative bacteria. BPI has a high affinity for endotoxin (LPS) of Gram-negative bacteria. Binding of BPI to bacteria is followed by envelope alteration, inhibition of growth and irreversible loss of viability. Neutrophils derived from newborn cord blood are deficient in BPI. Low BPI content of newborn may contribute to the increased risk of gram-negative infection. Thus BPI was studies as a candidate gene controlling immune response to bacterial challenge.

The objectives of this study:

- Identify new polymorphisms in NRAMP1 gene sequence and estimate the allele frequencies in local and commercial pigs.
- Characterize the cDNA sequence, gene structure and chromosomal location of the porcine BPI gene.
- Search for BPI polymorphisms and estimate the allele frequencies in commercial pigs.
- Evaluate the association of NRAMP1 and BPI gene variants and immune traits for *Salmonella* infection in pigs.

Materials and Methods

Experiment I: 42 piglets (3-4 from same litter; 8-19 days old) were shipped to NADC isolation facilities. Piglets were determined to be *Salmonella*-free by frequent fecal culture. Piglets were grown to 7-9 weeks of age prior to intranasal challenge with 1 billion live *S. choleraesuis* χ3246. The control group (saline inoculated) consisted of 1 piglet/litter (n = 25). The principal group (*Salmonella* infected) consisted of 2 or 3 piglets/litter (n = 59). Animals were monitored daily for temperature, clinical signs and *Salmonella* shedding (qualitative and quantitative). Pigs were necropsied at post challenge day 7 and quantitative bacteriology (most probable number) was performed on ileocecal lymph node and from fecal samples. Blood samples were drawn at specific intervals during challenge and standard complete blood counts (CBC) were performed.

Genotyping was performed for all markers using DNA prepared from blood. Phenotypic and genotype data were available on 59 challenged animals from lines A and B (15 and 44 respectively).

Experiment II: Challenged pigs were produced as part of a reference population that was bred from sires was that differed in susceptibility to salmonellosis. Briefly, sires (commercial line Y) were mated to 23 F1 gilts (crosses of commercial lines Y x Z and Z x Y). Three to nine piglets from each litter were selected for oral challenge with 8 x 10^8 *S. choleraesuis* (n=216). Piglets were challenged at 6 weeks of age. Clinical signs and fever were monitored during the six day infection period. Blood samples were taken from animals for immune trait measures one week before challenge, on the day of challenge and at necropsy. Traits of innate immunity included total leukocyte count and differential, bacterial uptake, phagocytosis and killing by neutrophils, and lymphocyte proliferation against several antigens. Six days after challenge, animals were necropsied and the amount of *Salmonella* in liver and spleen was determined by quantitative culture. Pigs were genotyped by using marker systems described above. The associations between sequence polymorphisms and phenotypes for both experiments were tested using mixed model procedures (SAS™ procedure MIXED) with a model that always included dam as a random effect and marker parameters as fixed effects. Least square means were estimated for the 3 genotype classes.

Results and Discussion

Discovery of new NRAMP1 and BPI polymorphisms and development of marker systems to detect this variation

We sequenced the beginning of the NRAMP1 gene and identified two new polymorphisms. One is detected by digestion of NRAMP1 DNA with NlaIII enzyme and the other by SmaI enzyme. The two challenge populations were genotyped with these new marker systems as well as with three other published markers (AvaII, HinfI, and MspAI1). The polymorphisms that segregated with the challenged animals and were thus potentially informative for differences in disease outcome were identified as AvaII, HinfI and NlaIII.

We cloned and sequenced the entire BPI gene and used this new sequence information to look for polymorphisms in the two challenge populations as done for the NRAMP1 markers. We identified two segregating polymorphisms, detected with AvaII and HpaII. One of these polymorphisms and the DNA test used to genotype BPI for this polymorphism is shown in Figure 1.

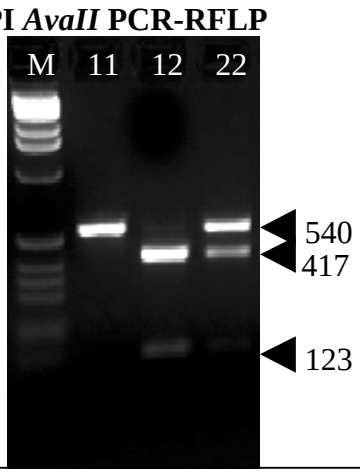

Figure 1. Gel electrophoresis showing AvaII genotyping of fragments of DNA from BPI. The first lane is the standards for size determination. The next three lanes show DNA from 11, 12 and 22 genotype animals. Allele 1 is not digested while allele 2 shows digestion to create two fragments of 417 and 123 base pairs.

Associations of NRAMP1 and BPI genotypes and measures of early immune response to infection

In the following Tables, we show some of the data indicating an association with NRAMP1 or BPI genotypes. Not all data is shown, only the most clear examples of association. For example, both AvaII and HinfI genotypes show associations with immune traits, but only the associations with AvaII are shown in Table 2.

Summary for NRAMP1

Multiple NRAMP1 genotypes are associated with specific measures of innate disease resistance such as WBC response, systemic infection and polymorphonuclear neutrophil (PMN) function. AvaII allele 2 appears to be the most favorable allele. Alleles 1 and 2 are associated with a decrease in shed bacteria (Table 1, 2) but allele 1 is associated with an increased spleen and liver bacteria, decreased PMN function and WBC response relative to allele 2 (Table 2).

Summary for BPI

The porcine complete protein coding sequence for the BPI gene was successfully cloned and characterized. Extensive polymorphism survey was identified among pig breeds and commercial lines and porcine BPI was mapped to chromosome 17 by both physical and linkage analysis (data not shown). We show that BPI AvaII is associated with fecal shedding and immune cell response during infection (Tabe 3) and demonstrate that co-segregating missense mutations, A354T and R384L (detected by HpaII digestion, not shown) are associated with altered bacterial counts in liver and in fecal shedding post-challenge (see Table 4). Our finding provides the first genetic evidence that mutations in BPI are associated with resistance/susceptibility of *Salmonella* infection in pigs.

Overall, these NRAMP1 and BPI association information may be useful for improving animal health and performance by decreasing the extent of disease and bacterial shedding during acute infections.

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Table 1. Both AvaII and NlaIII genotypes are associated with fecal bacterial shedding (Expt. I).

AvaII

	Allele substitution effects[a] (P-value)			
Trait[b]	1-all	2-all	3-all	4-all
FMPND6	-.56 (**.06**)	-.51 (.19)	.14 (.73)	.92 (.28)

NlaIII

	LSmeans (s.e.)			
Trait[b]	88	89	99	P
FMPND6	1.19 (0.15)	2.27 (0.45)	-	**.0006**

[a] These are log10 values; a difference of +1.0 is 10 fold higher bacterial counts. These comparisons were performed by combining the values for animals carrying at least one copy of the relevant allele versus animals with zero alleles. For example, for 1-all, the comparison is all animals with genotype 11, 12, 13, etc versus animals with genotype 22, 23, 33, etc.

[b] FMPND6 = mean fecal Salmonella count on day 6 after challenge.

Table 2. AvaII Genotypes Associated with Internal Bacterial Counts and Immune Cell Number and Function (Expt. II).

			Allele substitution effect*			P-values		
Trait	mean (s.e)	s.d.	1	2	3	1 vs 2	1 vs 3	2 vs 3
Spleen count	1.16 (0.09)	1.25	0.59	0.09	0	**0.007**	**0.0012**	0.56
Liver count	2.16 (0.11)	1.45	0.65	-0.02	0	**0.0006**	**0.0006**	0.91
Wbc - pre	6.63 (0.30)	3.91	-0.36	-0.23	0	0.75	0.39	0.45
Wbc – d0	7.23 (0.23)	3.01	-0.68	-0.94	0	0.49	0.08	**0.009**
Wbc - d8	10.53 (0.34)	4.1	-0.54	0.87	0	**0.04**	0.45	0.17
Wbc - pre-d0	0.57 (0.34)	4.46	-0.08	-0.84	0	0.19	0.90	0.07
Wbc – d0-d8	3.14 (0.38)	4.58	0.02	1.67	0	**0.03**	0.98	**0.02**
Wbc - pre-d8	3.56 (0.40)	4.79	-0.52	0.86	0	0.08	0.53	0.21
PMN uptake	0.54 (0.11)	1.41	-0.07	0.29	0	0.09	0.74	0.11
PMN killing	83.48 (1.57)	20.6	-6.54	1.63	0	**0.01**	**0.05**	0.59
PMN phag	3.38 (0.56)	7.3	-0.83	0.91	0	**0.03**	0.32	0.18
% PMN	73.63 (1.19)	15.6	-1.99	-6.89	0	**0.04**	0.42	**0.003**

*Allele 3 set to zero for these comparisons. These are log10 scale, so a difference of 1.0 equals a 10-fold difference in actual value.

Trait Code	Trait description-
PMN killing	Salmonella killing efficiency of Polymorphonuclear neutrophils (PMN)
Liver count	Bacterial counts in liver at one week
% PMN	percentage PMN's
PMN phag	phagocytosis efficiency of PMN's
Spleen count	Bacterial counts in spleen at one week
PMN uptake	Salmonella uptake efficiency of PMN's
Wbc – pre, d0, d8	White blood cell count pre-challenge, d0 or d8 of challenge
Wbc -pre-d0	absolute value of wbc pre –wbc d0 difference
Wbc – d0 – d8	absolute value of wbc d 0 – wbc d8 difference
Wbc – pre – d8	absolute value of wbc pre – wbc d8 difference

Table 3. BPI Ava II Genotype Association with Immune Traits (Expt. I).

Trait*	LS means by Genotype (s.e.)**			
	11	12	22	P value
tempD7	40.4 (.18) c	41.1 (.29) d	-	0.08
FMPND6	**0.44 (1.1) g**	**1.84 (1.1) h**	-	**0.006**
neut1	8.76 (.70)	8.00 (.91)	-	0.42
neut2	**7.56 (2.6) e**	**2.68 (2.8) f**	-	**0.02**
neut_diff	**-1.17 (2.8) g**	**-5.35 (2.9) h**	-	**0.008**
mono1	1.17 (.29)	1.42 (.36)	-	0.45
mono2	0.37 (.13) c	0.09 (.15) d	-	0.05
mono_diff	**1.51 (.80) e**	**-0.34 (.89) f**	-	**0.02**
wbc1	1.31 (.04)	1.25 (.05)	-	0.31
wbc2	22.7 (2.9) c	16.5 (3.4) d	-	0.06
wbc_diff	2.10 (3.5) a	-2.3 (4.1) b	-	0.21

*Traits are: TEMPD7 = temp on day 7 of challenge; FMPND6 = mean fecal Salmonella count on day 6 after challenge; (1) suffix means values before challenge; (2) suffix means values after challenge; _diff = difference in values before and after challenge; wbc:white blood cell number. Neut: neutrophil number. Mono: monocyte number.

**Significant differences (p values) for LS means are indicated as follows:
a-b: p<.30 c-d: p<.10 e-f: p<.05 g-h: p<.01 i-j: p<.005 k-l: p<.001 m-n: p<.0005 o-p: p<.0001

Table 4. BPI HpaII Genotype association with Immune Traits (Expt. II)

	LSmeans (s.e.)*		
Trait	33	34	44
liverC	2.24 (0.15) c	2.18 (0.23) c	3.33 (0.63) d
lym1	56.43 (1.01) a	59.12 (1.78) b	56.29 (5.12)
lym2	59.76 (0.96) c	58.69 (1.60) a	51.99 (4.50) d b
lym3	46.59 (1.08)	46.46 (1.88)	-
neu1	39.56 (1.05) a	36.81 (1.76) b	39.67 (4.95)
neu2	36.62 (0.90) a	37.34 (1.56) a	44.00 (4.47) b
neu3	49.10 (1.15)	48.66 (1.92)	-
SICA_pre	**210.1 (25.8) o**	**221.4 (43.6) o**	**891.8 (123) p**
uptake	**0.34 (0.14) e**	**0.91 (0.24) f**	**0.68 (0.68)**

*Significant differences (p values) for LS means are indicated as in Table 3.
SICA_pre: WBC Stimulation index in presence of Concavalin A pre-challenge.