

Identification of QTL affecting resistance/susceptibility to acute *Actinobacillus pleuropneumoniae* infection in swine

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Abstract *Actinobacillus pleuropneumoniae* is among the most important pathogens worldwide in pig production. The agent can cause severe economic losses due to decreased performance, acute or chronic pleuropneumonia and an increased incidence of death. Therapeutics cannot be used in a sustainable manner, and vaccination is not always available, but discovering more about host defence and disease mechanisms might lead to new methods of prophylaxis. The aim of the present study was to detect quantitative trait loci (QTL) associated with resistance/susceptibility to *A. pleuropneumoniae*. Under controlled conditions, 170 F2 animals of a Hampshire/Landrace family, with known differences in founder populations regarding *A. pleuropneumoniae* resistance, were challenged with an *A. pleuropneumoniae* serotype 7 aerosol

followed by a detailed clinical, radiographic, ultrasonographic, pathological and bacteriological examination. F2 pigs were genotyped with 159 microsatellite markers. Significant QTL were identified on *Sus scrofa* chromosomes (SSC) 2, 6, 12, 13, 16, 17 and 18. They explained 6–22 % of phenotypic variance. One QTL on SSC2 reached significance on a genome-wide level for five associated phenotypic traits. A multiple regression analysis revealed a combinatory effect of markers *SWR345* (SSC2) and *S0143* (SSC12) on Respiratory Health Score, Clinical Score and the occurrence of death. The results indicate the genetic background of *A. pleuropneumoniae* resistance in swine and provide new insights into the genetic architecture of resistance/susceptibility to porcine pleuropneumonia. The results will be helpful in identifying the underlying genes and mechanisms.

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Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia. It is characterised by a hemorrhagic and necrotizing pneumonia and fibrinous pleuritis (Haesebrouck et al. 1997). Peracute deaths, acute disease with severe clinical and pathologic-anatomical outcomes, chronic pleuritis in slaughter pigs and subclinical cases with declined performance (Gottschalk and Taylor 2006) all cause extensive losses in pig production worldwide (Noyes et al. 1990).

Several different strategies for combatting porcine pleuropneumonia are currently pursued, which include age-segregated rearing (Cleveland-Nielsen et al. 2002), vaccination and application of antibiotics. However, efficacy of vaccination strategies is often hampered by limited cross-serovar protection (Higgins et al. 1985; Fenwick and Henry 1994).

New subunit vaccines appear to convey better cross-protection (Haesebrouck et al. 1997), and several reports described the advantage of using a commercially available subunit vaccine containing Apx toxins and an outer membrane protein (Tumamao et al. 2004; Shao et al. 2010). However, despite vaccination, pigs were not protected against the disease, and therefore, other disease prevention measures and treatments such as antibiotics still remain essential in controlling *A. pleuropneumoniae* infections (Jirawattanapong et al. 2008; Sjölund and Wallgren Sjölund and Wallgren 2010).

Increasing use of antibiotics may provoke the emergence of antimicrobial resistant strains (White et al. 2002), and first antimicrobial resistance of *A. pleuropneumoniae* isolates has been reported (Gutiérrez-Martín et al. 2006). Thus, for future prophylaxis and control of the disease, it is essential to develop antibiotic independent measures. In addition, there is a strongly increasing demand for pork from healthy animals not subjected to treatment with antibiotic drugs (Van Oirschot 1994). Reduction of pain, suffering and damage in farmed pigs is a further serious issue that needs to be addressed with regard to swine health and infection (Reiner 2009).

In this context, the use of natural resistance and breeding selection towards more disease resistant pigs might be advantageous. Detected gene variants could be used in marker-assisted or genomic selection. Additionally, they would reveal new insights into pathogenesis and host defence, potentially inspiring new prophylactic strategies.

Mapping of quantitative trait loci (QTL) might be one approach towards the identification of relevant genes and causal polymorphisms for important traits (Haley and Andersson 1997).

Previous research has shown that differences in the immune capacity between breeds can be mapped (Edfors-

Lilja et al. 1998; Reiner 2009). However, studies of the resistance or susceptibility of pigs to infectious agents are still rare, although investigations of resistance to pseudorabies virus, *Sarcocystis miescheriana* (Reiner et al. 2002, 2007) and the porcine reproductive and respiratory syndrome virus (e.g. Lunney et al. 2011; Boddicker et al. 2012) have been conducted. Concerning *A. pleuropneumoniae*, differences in susceptibility among pigs of different populations have been shown in a detailed and comprehensive clinical study by Hoeltig et al. (2009). Gregersen et al. (2010) and Sorensen et al. (2011) mapped QTL for chronic pleuropneumonia and ventro-cranial chronic pleuritis, respectively, providing further evidence for genetic involvement in lung lesions with at least partial involvement of *A. pleuropneumoniae*.

Based on the populations characterised as distinct regarding *A. pleuropneumoniae* resistance by Hoeltig et al. (2009), the aim of the present study was to map QTL for the acute onset of pleuropneumonia in precisely characterised F2 animals under environmentally standardised conditions and to gain new insights into the genetics of disease and defence against *A. pleuropneumoniae* infection.

Material and methods

Experimental pigs

The experimental pigs used in this study emerged from a three generation family. A Hampshire boar was mated to 2 Landrace sows to produce 21 F1 animals, from which 2 F1 boars and 9 F1 sows were crossed, avoiding full-sib matings. The boar was derived from a Hampshire population that had been identified as relatively resistant to infection with the same *A. pleuropneumoniae* strain used in the present study (Hoeltig et al. 2009). The sows were taken from a population of the German Landrace that was shown to be most susceptible to *A. pleuropneumoniae* by the same recent study (Hoeltig et al. 2009). F1 animals were born at a German breeding company and then transferred to the Clinic for Small Ruminants and Swine, University of Veterinary Medicine, Foundation, Hannover, Germany. Here, 170 F2 pigs from 17 litters were born, raised and studied. Before entering the challenge study, all pigs were tested for absence of *A. pleuropneumoniae*-specific antibodies (by Apx IIA and Apx IVA ELISA, Leiner et al. 1999; Dreyfus et al. 2004). In addition, all pigs were tested negative for *Mycoplasma hyopneumoniae* and PRRSV (HerdChek, IDEXX laboratories, Westbrook, ME, USA) by antibody ELISA and for Influenza A by means of a haemagglutination inhibition test (Hoeltig et al. 2009). All F2 pigs were clinically healthy before entering the challenge study.

The piglets were weaned at the age of 4 weeks, fed with a standardised diet and housed under standardised containment level 2 conditions with exclusive access only through a gated hygiene system.

All animals were cared for in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals (approval number: 33.9-42502-04-05/919).

Infection and clinical examination

Actinobacillus pleuropneumoniae serotype 7 (Jacobsen et al. 1996; Maas et al. 2006) was obtained from the Institute for Microbiology at the University of Veterinary Medicine, Foundation, Hannover, Germany. Infection and clinical examination were established and improved in a recent project (Hoeltig et al. 2009). In short, pigs were challenged at 7 weeks with a well-introduced *A. pleuropneumoniae*-containing aerosol (Baltes et al. 2001, 2003a, b; Tonpitak et al. 2002; Jacobsen et al. 2005; Hoeltig et al. 2009). The clinical status was monitored from the day before infection (day 0) to day 7 *post infectionem* (p.i.) and consisted of: (i) an evaluation of the pigs' general appearance (posture, behaviour, feed intake, temperature, vomiting), (ii) a detailed examination of the pigs' respiratory tract (breathing noise, dyspnoea, respiratory frequency, coughing, cyanosis), and (iii) the measurement of oxygen saturation by pulse oximetry (for details, see Hoeltig et al. 2009). Parameters with two manifestations (vomiting) were scored with 0 or 4 points, parameters with three manifestations (dyspnoea) with 0, 2 or 4 points, parameters with four manifestations (breathing noise, coughing and feed intake) with 0, 1.33, 2.67 or 4 points, and the remaining parameters with five manifestations with 0, 1, 2, 3 or 4 points. The points were added and divided by 11 to consider the 11 clinical parameters shown under (i), (ii) and (iii) equally. These points produced the daily clinical score, with a score of 0 for a healthy animal and a maximum score of 4 for a severely diseased animal. Deceased animals were scored with 5 points. The clinical score for each day was calculated as the sum of the daily clinical scores from day 1 to the current day.

Additionally, ultrasonographic and radiographic examination was applied on days 0 (before infection) and 7 p.i. to evaluate lung status (for further details see Hoeltig et al. 2009). For ultrasonography, both lungs were divided into 32 areas and 0 (normal lung tissue) to 8 (no unaltered lung tissue) points were assigned per area. Radiography was carried out in two views (latero-lateral and dorso-ventral; Convix Generator 360, Fa. Picker Int., Munich, Germany, 400 mAs, 110 kV, 1,000 ms) using a shutter priority (Precimat, Fa. Picker Int.). For the radiographic score, each lung was divided into eight sections. Bronchial, alveolar and interstitial patterns, cardiac and diaphragm silhouette

and sequesters were analysed with a point range from 0 to 3. The radiographic score was the total of the scores for all observed alterations, with a score of 50 being lethal.

If a pig died between infection and necropsy, then this event was recorded

Pathological examination

On day 7 p.i., pigs were euthanized, and a pathological examination was performed. From all lungs, lesions were scored using the Lung Lesion Score (LLS) proposed by Hannan et al. (1982) (Fig. 1). In brief, areas of non-physiological consistence were recorded in a schematic porcine lung map. All seven lobes were divided into triangles. The number of damaged triangles per lobe were counted to determine the lobe's value, where 5 was the maximum score. The values of all seven lobes were added to give the LLS, with a maximum of 35.

Blood, liver and spleen samples were taken for DNA preparation

Findings from clinical, radiographic and ultrasonographic examinations and the LLS were combined to give the Respiratory Health Score (RHS) (Hoeltig et al. 2009). Based on the RHS, two groups of 50 pigs each were formed, representing highest and lowest scores, respectively (RHS*: 0/1).

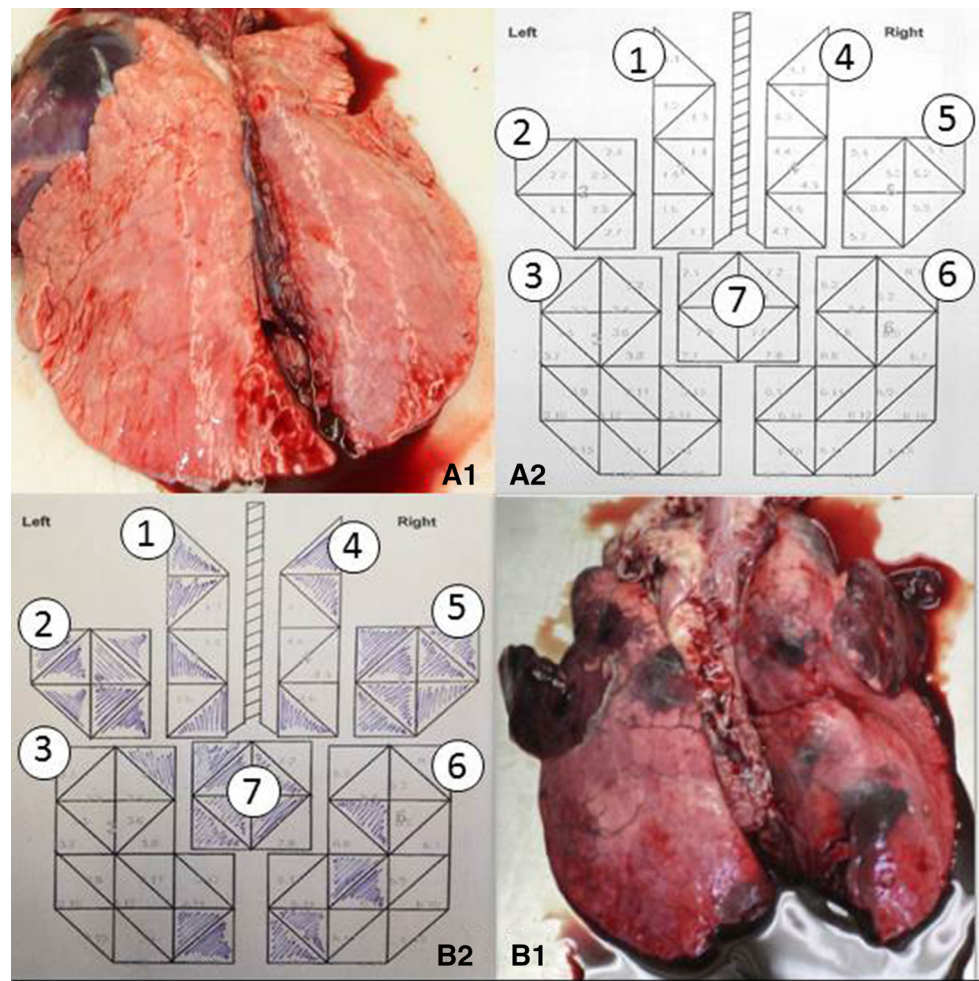
Bacteriological examination

Bacterial colonisation of the lungs was semi-quantified using the Re-isolation Score (RIS) (Maas et al. 2006). Each of the seven lung lobes, the tonsils and one lung lymph node were given score points: 0 (no growth of *A. pleuropneumoniae*), 1 (colonies growing in the swabbed area only), 2 (colonies growing in the swabbed area and in the first fractionated streak) to 3 (*A. pleuropneumoniae* colonies growing in the swabbed area and in both fractionated streaks), producing a quantitative cultural score (RIS) with values from 0–27 (summed up score of nine tissues). Tissue was taken from precisely defined positions and cultured on modified Columbia agar (Jacobsen and Nielsen 1995).

Markers and linkage map

Genomic DNA was extracted from blood, liver or spleen tissue of experimental pigs using a commercial DNA isolation kit (Analytik Jena, Germany), which proved its suitability for DNA isolation in former studies. DNA quality and quantity was measured with an Ultraspec 1100 pro photometer (GE Healthcare Europe GmbH, Freiburg, Germany).

Fig. 1 Scoring of lung lesions. **a** Picture (A.1) and Scoring Scheme (A.2) of a lung without lung lesions 7 days after infection. In the scoring scheme, the lung is divided into seven lung lobes indicated by the white numbers. Each lung lobe is schematized by different numbers of *triangles*. **b** Picture (B.1) and Scoring Scheme (B.2) of a lung with severe lesions 7 days after infection. The Scoring scheme shows *marked triangles* in accordance to the appearance of lung lesions upon the picture [Lobus accessorius (number 7) is not visible]. **c** Calculation formula of the LLS. At first a LLS is calculated for each lung lobe by the number of *marked triangles* and the number of *triangles* of the lung lobe; then, the LLS of all lung lobes are summated. The factor five in the formula determines the maximum score points of each lung lobe, and so the maximum LLS of the lung is 35



$$c \quad LLS = \sum_{i=1}^7 \left(\frac{\text{Number of marked triangles}}{\text{Number of triangles (overall)}} \times 5 \right)_i$$

Informative marker loci were selected to cover all autosomes at average intervals of 15 cM. Although genome wide 60 SNP chips have been available for the pig since 2009 (Ramos et al. 2009), we decided to work with microsatellites because they were well established in our laboratory. From 491 microsatellite markers selected from public sources (www.ncbi.nlm.nih.gov; www.usda.gov), only 155 markers were informative and used to genotype the F2 families (see Supplementary Table 1). Another four markers (*CD14*, *TNF*, *IFNAR1* and *CD40*) developed for this study were found to be associated with some candidate genes. Genetic positions of these markers were calculated with the Kosambi function of CRI-MAP, Version 2.4 (Green et al. 1990). Positions of the other markers were obtained from NCBI, UniSTS (<http://www.ncbi.nlm.nih.gov/genome/sts>), providing a much higher confidence than

those obtained from single studies. Most of the terminal markers from public sources were non-informative in this study. Therefore, the initial position of markers on SSC1, 5, 6, 7, 8, 9, 12, 14, 15, and 16 is different from 0 cM.

Statistical analysis

Phenotypic data were analysed using IBM-SPSS, Release 20.0 (IBM, Herrenberg, Germany). Means and standard deviations were calculated using the Descriptives procedure. Occurrence of death after infection was specified as a percentage of animals within groups. Significances for this binomial trait were tested with Kruskal–Wallis test for independent samples. The 50 pigs with the highest and the lowest RHS were classified as high and low responders, respectively. The remaining pigs were classified as

intermediate. Responsiveness was included into the new variable RHS*, including 1 (low responders), 2 (intermediate) and 3 (high responders).

The study was conducted consecutively with one or two litters per run. The age of piglets within the single study group was identical. Thus, the effect of age was contained in the effect of the study group. Means, standard errors and significances of clinical, pathological and microbiological traits were calculated with univariate variance analysis with RHS*, sex and study group as fixed effects, following the model:

$$y_{ijkl} = \mu + \text{RHS} *_{i} + \text{Sex}_{j} + \text{Study group}_{k} + e_{ijkl}.$$

y_{ijkl} : phenotype

μ : fixed mean

RHS*_i: fixed effect of responsiveness (−1, 0, 1)

Sex_j: fixed effect of sex (1, 2)

Study group_k: fixed effect of study group (1 to 15)

e_{ijkl} : random residual

Effects of QTL-linked markers on phenotypic traits were tested using multiple regression analysis; differences in mean values were calculated by means of the general linear model described before, with inclusion of the fixed effects of the genotypes of markers Swr345 (SSC2) and S0143 (SSC12).

QTL analysis

QTL analysis was performed using the Web-based application ‘Grid QTL’ (Seaton et al. 2006). Analyses were based on a least-square method developed for crosses between inbred and outbred lines (Haley et al. 1994). In the general linear model, the fixed effects of sex and study group were included. Chromosome-wide and genome-wide significance levels were estimated by means of permutation tests (Churchill and Doerge 1994) with 1,000 permutations. F values were calculated as average from all 18 autosomes. F values were 5.4 ($p \leq 0.05$) and 7.7 ($p \leq 0.01$) for chromosome-wide significance and 9.8 ($p \leq 0.05$) for the genome-wide significance threshold. To avoid violating the assumption that marker genotypes associated with multiple QTL are uncorrelated, the QTL analysis was performed sequentially (Holl et al. 2004). The highest QTL effect of the first run was included into the model as a background effect in the second run, and both QTL were included as background effects in the third run and so on. QTL analysis for one trait was stopped when the last run did not identify a new QTL with an F value ≥ 5.4 . Positive values with additive effects indicate that Hampshire alleles result in higher values than Landrace alleles and vice versa.

Effects of QTL-linked markers on phenotypic traits were tested by multiple regression analysis in IBM-SPSS,

Release 20.0 (IBM, Herrenberg, Germany). Instead of using the original allele length of the microsatellite markers, marker information was prepared for calculation as homozygous Hampshire derived (1), homozygous German Landrace derived (3) or heterozygous (2). Significant were only markers Swr345 (SSC2) and S0143 (SSC12). Thus, these two markers were combined to generate nine genotypes which were included in the general linear model as described before.

Results

Phenotypes

All pigs included into the study were clinically healthy before infection (day 0) with clinical scores of 0 to 0.6 (mean 0.06), sonographic scores between 0 and 2 (mean 0.12) and a radiographic score of 0. All pigs were tested free from antibodies against *A. pleuropneumoniae*, *M. hyopneumoniae*, Influenza A and PRRSV. There was neither elevated temperature, nor dyspnoea, coughing nor apathy detectable.

Upon challenge with *A. pleuropneumoniae*, clinical scores increased steadily from day 1 to day 7 p. i. Besides overall scores, the coefficients of variation increased too. Day 7 was characterised by comet tail artefacts, interruption of the pleural line, liverlike parenchyma texture, abscesses, bronchography and increasing lung opacity in sonographic and radiographic examinations, which are typical for porcine pleuropneumonia.

Due to severe clinical symptoms, five pigs died or were euthanized before day 7. Nine from 35 points and 9 from 27 points were reached in LLS, and RIS obtained during necropsy of the pigs on day 7 p.i. Although infected, some pigs did not develop scores higher than 0 in any of the parameters examined. The overall RHS varied between 0 and 66.7. Ranking scores between RHS and the other clinical parameters were between 0.345 (occurrence of death) and 0.714 (LLS) (Table 3), indicating different aspects of the disease represented by the different scores. Correlations between phenotypic traits obtained after challenge were mainly significant ($p < 0.001$). Traits obtained before infection were not correlated with traits after infection. There was a weak correlation between death because of infection (0/1) and the other scores. Contribution rates of the correlations between the RHS and the major clinical, pathological and microbiological traits varied between 41.5 and 91 % (Table 2).

Clinical, pathological and microbiological traits of the F2 pigs showed a high degree of variation (Table 1), with coefficients of variation between 0.8 and 3.6. None of the

scores obtained were normally distributed within the F2 pigs.

Score variation was significantly affected by response (low/intermediate/high) and run (14 groups of one or two litters consecutively housed and challenged), but not by sex of the piglets. A comparison of trait values in low, intermediate and high responders is given in Table 3. There were no differences between low, intermediate and high responders for all traits obtained before infection. However, the 50 low and high responders were significantly different at $p \leq 0.001$ for each of the traits obtained after infection. Scores within high responders showed a normal distribution and scores within intermediate responders showed widely normal distribution. However, scores within low responders were still highly variable.

QTL

QTL analysis mapped a total of 7 QTL, significant at least on a chromosome-wide level ($p < 0.05$), linked with the clinical score (increasing in significance from day 1 p.i. to day 7 p.i.), with the radiographic score on day 7, with the occurrence of death (0/1), and with the RHS (Table 4). The confidence interval of 14 cM was lowest in association with the clinical score 7 d.p.i. Interestingly, Hampshire alleles at this position were linked with a higher degree of susceptibility of the F2 pigs.

Clinical Score and RHS were further influenced by a QTL on SSC17; this effect appeared late after infection and again, unfavourable scores were linked with Hampshire alleles. The sonographic scores and RHS were

Table 1 Clinical, pathological and microbiological traits of the 170 F2 pigs

Trait	Mean	Standard deviation	Minimum	Maximum
Clinical Score 0 d.p.i. (0–5)	0.06	0.14	0	0.6
Clinical Score 1 d.p.i. (0–5)	0.46	0.87	0	5
Clinical Score 2 d.p.i. (0–10)	0.81	1.77	0	10
Clinical Score 3 d.p.i. (0–15)	1.11	2.72	0	15
Clinical Score 4 d.p.i. (0–20)	1.41	3.72	0	20
Clinical Score 5 d.p.i. (0–25)	1.73	4.80	0	25
Clinical Score 6 d.p.i. (0–30)	2.01	5.82	0	30
Clinical Score 7 d.p.i. (0–35)	2.28	6.85	0	35
Sonographic Score 0 d.p.i. (0–200)	0.12	0.43	0	2
Sonographic Score 7 d.p.i. (0–200)	27.6	48.4	0	200
Radiographic Score 0 d.p.i. (0–50)	0	0	0	0
Radiographic Score 7d.p.i. (0–50)	8.3	12.015	0	50
Deaths (yes/no)	0.05	0.21	0	1
LLS (0–35)	9.37	9.89	0	35
Reisolation Score (0–27)	9.37	7.74	0	27
RHS (0–100)	6.78	13.97	0	66.67

RHS Respiratory Health Score, d.p.i. days post infection

Table 2 Correlations between clinical, pathological and microbiological traits of the 170 F2 pigs

	Death	LLS	RIS	Sonographic Score 0 d.p.i.	Sonographic Score 7 d.p.i.	Radiographic Score 7 d.p.i.	Clinical Score 1 d.p.i.	Clinical Score 7 d.p.i.
LLS	0.276**							
RIS	0.320**	0.641**						
Sonographic Score 0 d.p.i.	−0.06	0.016	0.041					
Sonographic Score 7 d.p.i.	0.349**	0.694**	0.637**	0.064				
Radiographic Score 7 d.p.i.	0.349**	0.836**	0.693**	0.047	0.779**			
Clinical Score 1 d.p.i.	0.346**	0.537**	0.531**	0.027	0.567**	0.617**		
Clinical Score 7 d.p.i.	0.346**	0.615**	0.566**	0.005	0.662**	0.679**	0.849**	
RHS	0.345**	0.714**	0.644**	0.062	0.954**	0.795**	0.678**	0.808**
RHS contribution rate (%)	11.9	51.0	41.5	0.4	91.0	63.2	46.0	65.3

d.p.i. day post infection

** Significant at $p < 0.001$

Table 3 Clinical, pathological and microbiological traits of the 170 F2 pigs by low ($n = 50$), intermediate ($n = 70$) and high ($n = 50$) responders

	Low responder	Intermediate	High responder	Significance		
				Low/interm.	Low/high	interm./high
Clinical Score 0 d.p.i.						
Mean \pm SE	0.03 \pm 0.01	0.06 \pm 0.02	0.07 \pm 0.02	n.s.	n.s.	n.s.
Clinical Score 1 d.p.i.						
Mean \pm SE	0.06 \pm 0.09	0.31 \pm 0.08	1.13 \pm 0.09	<0.05	<0.001	<0.001
Clinical Score 2 d.p.i.						
Mean \pm SE	0.09 \pm 0.17	0.41 \pm 0.14	2.02 \pm 0.17	n.s.	<0.001	<0.001
Clinical Score 3 d.p.i.						
Mean \pm SE	0.12 \pm 0.25	0.47 \pm 0.21	2.76 \pm 0.25	n.s.	<0.001	<0.001
Clinical Score 4 d.p.i.						
Mean \pm SE	0.14 \pm 0.34	0.52 \pm 0.28	3.58 \pm 0.34	n.s.	<0.001	<0.001
Clinical Score 5 d.p.i.						
Mean \pm SE	0.15 \pm 0.45	0.57 \pm 0.36	4.40 \pm 0.44	n.s.	<0.001	<0.001
Clinical Score 6 d.p.i.						
Mean \pm SE	0.16 \pm 0.54	0.61 \pm 0.44	5.19 \pm 0.54	n.s.	<0.001	<0.001
Clinical Score 7 d.p.i.						
Mean \pm SE	0.18 \pm 0.65	0.64 \pm 0.53	5.95 \pm 0.64	n.s.	<0.001	<0.001
Sonographic Score 0 d.p.i.						
Mean \pm SE	0.06 \pm 0.07	0.20 \pm 0.06	0.09 \pm 0.07	n.s.	n.s.	n.s.
Sonographic Score 7 d.p.i.						
Mean \pm SE	0.30 \pm 4.15	7.57 \pm 3.39	82.67 \pm 4.13	n.s.	<0.001	<0.001
Radiographic Score 0 d.p.i.						
Mean \pm SE	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	n.s.	n.s.	n.s.
Radiographic Score 7 d.p.i.						
Mean \pm SE	0.64 \pm 1.13	4.31 \pm 0.92	19.65 \pm 1.12	<0.05	<0.001	<0.001
Death (0/1)						
Mean \pm SE	0.00 \pm 0.02	0.00 \pm 0.02	0.11 \pm 0.02	n.s.	<0.001	<0.001
LLS						
Mean \pm SE	2.84 \pm 1.11	6.57 \pm 0.91	18.58 \pm 1.10	0.01	<0.001	<0.001
RIS						
Mean \pm SE	4.73 \pm 0.86	7.34 \pm 0.70	17.75 \pm 0.86	<0.05	<0.001	<0.001
RHS						
Mean \pm SE	0.22 \pm 1.21	1.87 \pm 0.99	19.44 \pm 1.20	n.s.	<0.001	<0.001

Effects of response and run were significant by $p \leq 0.001$, except for scores obtained on day 0 p.i. Effects of sex were not significant in any of the scores

$\pm SE$ Standard error, n.s. $p > 0.05$

further linked with a QTL on SSC12. Favourable alleles came from Hampshire. Interestingly, this QTL showed linkage to the sonographic score prior to infection. Sonographic score was further linked to QTL on SSC6 and 12. The RIS was linked with two QTL on SSC16 and 18, and the LLS correlated with a QTL on SSC18. Grouping of individuals (RHS*) into low and high responders was linked with two QTL on SSC13 and 18. QTL explained between 6 and 22 % of total F2 variance. Except for the QTL on SSC2, confidence intervals were generally wide.

QTL on SSC2, 6, 12 and 17 peaked at the positions of markers *Swr345* (SSC2), *KVL3285* (SSC6), *S0143* (SSC12) and *Swn335* (SSC17). Multiple regression analyses of markers showed significant effects for *Swr345* and *S0143* on RHS, Clinical Score on day 7 and the occurrence of death. Both markers had a significant combinatory effect on all three phenotypic traits (Fig. 2a–c). The occurrence of death was linked with the homozygous presence of the unfavourable allele in one marker with at least one unfavourable allele in the other marker (heterozygous or homozygous), while in all other cases no death occurred

Table 4 QTL sorted by trait and the sequence of inclusion into QTL analysis (Seq)

Trait	Seq	SSC	cM ^a	CI ₉₅	F value	P value ^b	% ^c	A ^d	±SE	D ^e	±SE
Clinical Score 1d.p.i.	1	2	114	10–131	6.59	*	13.29	0.33	0.10	−0.10	0.13
Clinical Score 2d.p.i.	1	2	114	25–118.5	8.67	**	10.34	0.72	0.19	−0.32	0.26
Clinical Score 3d.p.i.	1	2	114	26.5–115	9.18	**	9.77	1.12	0.29	−0.52	0.40
Clinical Score 4d.p.i.	1	2	114	35.5–115.5	9.92	***	9.18	1.57	0.39	−0.79	0.54
Clinical Score 5d.p.i.	1	2	114	38–114	9.98	***	9.11	2.01	0.50	−1.04	0.69
Clinical Score 6d.p.i.	1	2	114	63–119	10.40	***	8.80	2.46	0.60	−1.33	0.84
Clinical Score 7d.p.i.	1	2	114	100–114	10.82	***	8.14	2.94	0.71	−1.65	0.98
	2	17	7	3–90	6.63	*	12.89	0.41	0.78	4.25	1.91
Sonographic Score 0 .dp.i.	1	12	20	8–106	6.32	*	14.18	0.11	0.05	−0.26	0.09
Sonographic Score 7 .dp.i.	1	6	30	13.5–109	7.23	*	12.48	1.71	5.73	37.12	9.80
	2	12	2	0–93.5	5.40	*	16.35	−15.16	7.28	−15.29	11.82
Radiographic Score 7d.p.i.	1	2	114	31–115	7.81	**	11.56	4.34	1.30	−3.25	1.81
Deaths (yes/no)	1	2	114	0–124	5.48	*	16.21	0.07	0.02	−0.44	0.03
LLS	1	18	0	0–58	6.20	*	22.16	0.18	0.99	5.23	1.49
Reisolation Score	1	16	24	0–84	6.71	*	13.36	−0.69	0.83	−4.17	1.15
	2	18	6	0–55	6.21	*	8.86	4.34	1.30	−3.25	1.81
RHS	1	2	114	83–114	10.44	***	8.65	5.69	1.41	−3.21	1.95
	2	6	28	14–109	6.49	*	13.62	0.79	1.59	9.45	2.63
	3	17	7	4–91	6.53	*	13.41	1.73	1.56	7.88	2.39
	4	12	1	0–106	5.45	*	15.68	−3.14	1.78	−4.49	2.63
RHS*	1	18	4	0–58	8.20	**	6.44	0.12	0.07	0.42	0.13
	2	13	56	0–128	7.20	*	7.07	−0.99	0.10	0.20	0.13

RHS Respiratory Health Score, RHS* two groups of pigs with the 50 highest and lowest Respiratory Health Scores; CI₉₅ 95 % confidence interval

^a Position on the chromosome

^b Significance of the QTL: * significant on a chromosome-wide level with $P \leq 0.05$; ** significant on a chromosome-wide level with $P \leq 0.01$; *** significant on a genome-wide level with $P \leq 0.05$

^c Percentage of the phenotypic variance explained by the QTL

^d Additive effects of the QTL ± standard error (SE); positive values indicate that Hampshire alleles result in higher values for the trait than Landrace alleles and vice versa

^e Dominance effects of the QTL ± SE

(Fig. 2a). The same principle was found for Clinical Score (day 7) (Fig. 2b) and RHS (Fig. 2c). Effects of the single markers and combined effects of both markers were statistically significant ($p < 0.001$) in all three phenotypic traits. The contribution ratio was 0.351. Favourable alleles of *Swr345* (SSC2) came from the German Landrace, those of *S0143* (SSC12) from Hampshire.

Discussion

Several studies confirmed the low susceptibility of a Hampshire pig population to a challenge with *A. pleuropneumoniae*. A population of German Landrace pigs has to be considered as most susceptible, as only pigs of this breeding line died or had to be euthanized due to severe clinical symptoms in preceding experiments (Hoeltig et al. 2009). We used serotype 7, but we expect similar results

upon infection with other *A. pleuropneumoniae* serotypes, because the general mechanisms of pathogenicity are species-specific. However, the genetic mechanisms responsible for the breed differences are still unknown.

The intention of the present study was to map chromosomal areas linked to the observed differences in relative susceptibility/resistance to *A. pleuropneumoniae*. The QTL have been mapped in a model of maximal accuracy of phenotypes and minimal animal numbers to address concerns of animal welfare. Founder pigs have been chosen based on the previous comparison of different pig breeds under the same experimental conditions (Hoeltig et al. 2009). Although certain populations of German Landrace proved to be most and a population of Hampshire to be least susceptible to *A. pleuropneumoniae*, segregation of resistance/susceptibility is generally given in each of the populations. Gregersen et al. (2010) investigated 7,300 slaughter pigs in the field for symptoms of the chronic

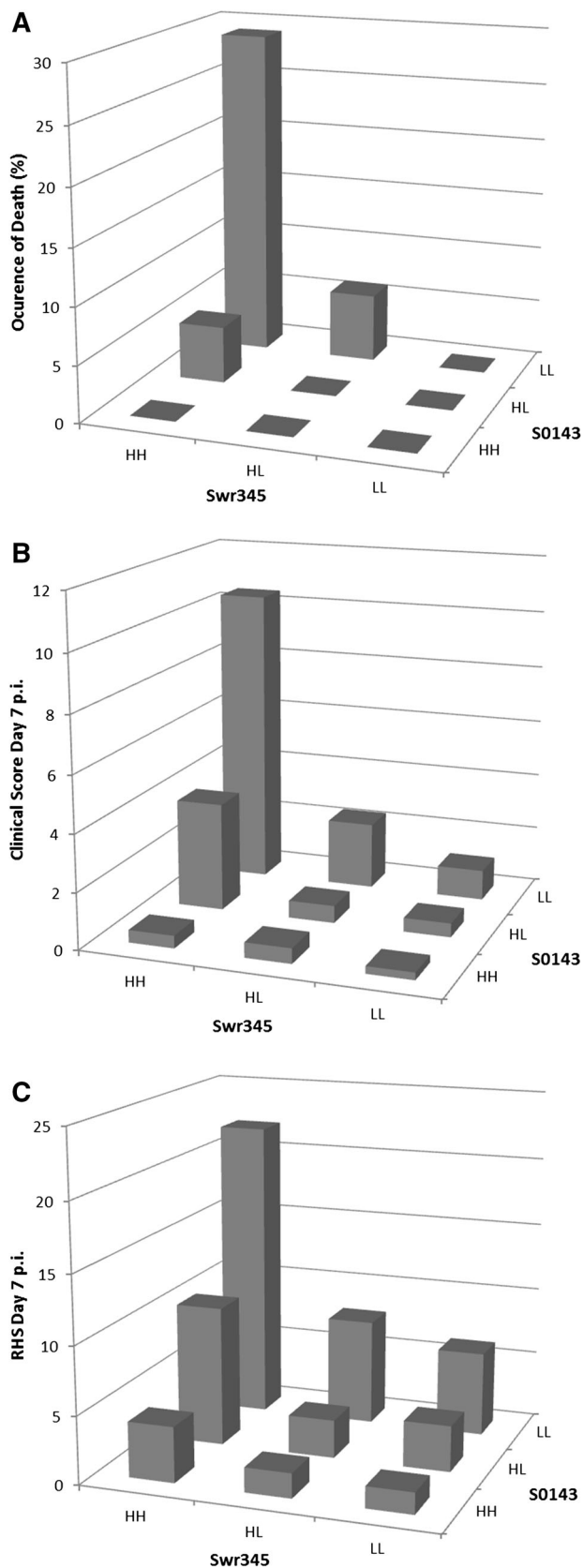


Fig. 2 Combinatory effects of markers *Swr345* and *S0143* on phenotypic traits: occurrence of death (a), Clinical Score (b), and RHS (c). *H* alleles of the Hampshire breed, *L* allele of the German Landrace. *RHS* Respiratory Health Score. Results are based on 170 pigs. Values of the y axis refer to the percentage of dead pigs, the clinical score and the RHS score, respectively, in each of the nine genotypes combined from markers *Swr345* (SSC2) and *S0143* (SSC12)

outcome of *A. pleuropneumoniae* infection, i.e. dorso-caudal chronic pleuritis. Their study revealed six QTL significant on a chromosome-wide level. The QTL on SSC2, 12, 13 and 18 were confirmed by the present study. Confidence intervals were similar in both studies. A comparison of both studies demonstrates the power of extensive individual phenotyping in QTL analysis, especially regarding traits of disease resistance. Differences in maternal immunity, immunity of the infected slaughter pigs, infection pressure in herds, infection time point and individual stressors are the environmental factors that most often interfere and lead to false classifications of an individual's resistance/susceptibility. The challenge model of the present study used the same environment, time point and dosage for infection, along with a very detailed and precisely conducted phenotyping.

Following previous study, acute lung damage appears to be decisive for the course of disease, suggesting a major role of the non-adaptive immune system in the defence of pleuropneumonia. Thus, the present experiment could be terminated on day 4 p.i. without loss of information (Hoeltig et al. 2009). We have, however, prolonged the experiment until day 7 p.i., and the data showed a steady increase in the power of the resulting QTL for the clinical score. This gradient of QTL power argues for the chance of improved mapping results, if sampling had been performed later than day 7 p.i., at least with this characteristic.

Our study shows for the first time QTL that are linked to the acute development of pleuropneumonia after standardised infection. The study detected 7 QTL located on SSC2, 6, 12, 13, 17 and 18.

The QTL on SSC2 covers the region of recently described QTL for the resistance to *Sarcocystis miescherina* (Reiner et al. 2007), pseudorabies (Reiner et al. 2002) and ventro-cranial chronic pleuritis (Sorensen et al. 2011). Candidate genes on SSC2 are interleukin 9 (*IL9*; Sorensen et al. 2011) and *CD14* (cluster of differentiation 14; Tsai et al. 2011). *IL 9* variants have been described together with human lung function variability (Aschard et al. 2009). *CD14* cooperates with *MD-2* and *TLR4* to mediate the innate immune response to bacterial lipopolysaccharides (LPS), one of the major virulence factors of *A. pleuropneumoniae* and a key response of the host defence (Cho

et al. 2005). Candidate genes for the SSC12 QTL are *STAT3*, *STAT5A*, *STAT5B* (signal transducer and activator of transcription 3, 5A and 5B). Members of the *STAT* gene family are of specific importance in immunosystem-related gene regulation (Casanova et al. 2012). The QTL on SSC13 is located in the area of the transferrin gene (*TF*). *TF* is an important factor in pleuropneumonia, depending on the disposability of iron for the bacteria (Chiers et al. 2010). The existence of *TF* variants has been described (Danilowicz et al. 2009). Different QTL involved in RHS/RHS*, mapped on the basis of 170 and 50/50 pigs, might serve as further evidence for alternative pathways in *A. pleuropneumoniae* defence in different animals. Together with the known pathophysiological effects of *TF*, these results argue, however, for the consideration of this region in further studies.

QTL on SSC8 and 14 were only detected by the group of Gregersen et al. (2010), QTL on SSC6, 16 and 17 only by the present study. This difference and the occurrence of QTL almost exclusively in singular families in the study by Gregersen et al. (2010) point towards distinct genes that might be involved in *A. pleuropneumoniae* defence.

A batch of factors involved in innate immunity has been mapped to SSC18, including *TLR9*, *IL10* and *C3C* (Wimmers et al. 2008a, b; Uddin et al. 2011). These candidate genes have been identified as significant factors modulating the innate immune system after contact with tetanus toxoid and Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) (Wimmers et al. 2008a, b). Additionally, the QTL on SSC18 overlaps with a QTL for lymphocyte numbers in a chronic disease model (Reiner et al. 2008). SNPs have been identified on SSC18 with association to CD4 + CD8-, CD4+ and CD4 + CD8+ lymphocyte subpopulation fractions (Lu et al. 2012), implicating a possible role of the adaptive arm of immunity.

The most prominent effect in the present study was that any pig which died from *A. pleuropneumoniae* infection was homozygous for one founder breed in one of the markers *Swr345* (SSC2) or *S0143* (SSC12) and carried at least one unfavourable allele in the corresponding marker. Interestingly, favourable effects regarding the outcome of *A. pleuropneumoniae* infection were linked with alleles from Hampshire in *S0143* and alleles from German Landrace in *Swr345*. This aspect adds further impact to the involvement of different genes and pathways in *A. pleuropneumoniae* defence, as discussed above. Although the founder pigs were derived from subpopulations with marked differences in susceptibility to pleuropneumonia, the actual founder pigs could not be tested individually. Thus, there might be individuals within both populations, allowing for more marked inter-population differences than those selected for the present study.

The effects of these markers are significant in the F2 population of the present study. Thus, both markers are linked with highly interesting causal gene variants that need to be defined by further studies. Because of the genetic nature of F2 crossbreds, linkage between markers and causal variants might, however, be too loose to be applied in commercial populations, without further testing.

Although the present study was based on a relatively low number of animals ($n = 170$) and markers ($n = 159$), the high accuracy of phenotypes led to a high conformity with results obtained with 7,300 pigs and a 7k porcine SNP chip (Gregersen et al. 2010) with respect to location, power and confidence intervals of the QTL. Nevertheless, some QTL might have been underestimated or even escaped from mapping because of high distances between some markers in the present study. Thus, future studies should involve available genome wide SNP chips (e.g. 60k; Ramos et al. 2009) or genotyping by sequencing (Spindel et al. 2013). Because of the relatively (SSC2) and absolutely (the other chromosomes) wide confidence intervals, fine mapping of candidate genes cannot be based solely on the results of the present study. However, combining these positional data with functional data should help to restrict functional candidate genes and lead to the discovery and use of those genes, involved in *A. pleuropneumoniae* resistance/susceptibility. While absolute resistance or susceptibility can hardly be assumed from the complexity of disease and defence mechanisms, even relative resistance would be extremely constructive for worldwide swine production. The RISs of the most resistant pigs of the present study argue for an overall reduction in the herds' infectious pressure, a major factor for the spread of infectious diseases within and between herds (Reiner 2009). Clinical scores, RHS and LLSs of the most resistant 50 pigs proof their successful defence of *A. pleuropneumoniae*. Although selecting for a unique disease resistance might also lead to susceptibility to other diseases, the knowledge of the causal gene variants and their application in future genomic selection strategies could reduce disease incidence, improve performance and product quality and, by reducing the necessity of antibiotic treatment, would improve consumer protection and animal welfare. A lot of information will be needed, before a clear picture can be drawn for a distinct resistance in the future.

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