Gene expression profile suggests that pigs (Sus scrofa) are susceptible to Anaplasma phagocytophilum but control infection

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Abstract

Background: Anaplasma phagocytophilum infects a wide variety of hosts and causes granulocytic anaplasmosis in humans, horses and dogs and tick-borne fever in ruminants. Infection with A. phagocytophilum results in the modification of host gene expression and immune response. The objective of this research was to characterize gene expression in pigs (Sus scrofa) naturally and experimentally infected with A. phagocytophilum trying to identify mechanisms that help to explain low infection prevalence in this species.

Results: For gene expression analysis in naturally infected pigs, microarray hybridization was used. The expression of differentially expressed immune response genes was analyzed by real-time RT-PCR in naturally and experimentally infected pigs. Results suggested that A. phagocytophilum infection affected cytoskeleton rearrangement and increased both innate and adaptive immune responses by up regulation of interleukin 1 receptor accessory protein-like 1 (IL1RAPL1), T-cell receptor alpha chain (TCR-alpha), thrombospondin 4 (TSP-4) and Gap junction protein alpha 1 (GJA1) genes. Higher serum levels of IL-1 beta, IL-8 and TNF-alpha in infected pigs when compared to controls supported data obtained at the mRNA level.

Conclusions: These results suggested that pigs are susceptible to A. phagocytophilum but control infection, particularly through activation of innate immune responses, phagocytosis and autophagy. This fact may account for the low infection prevalence detected in pigs in some regions and thus their low or no impact as a reservoir host for this pathogen. These results advanced our understanding of the molecular mechanisms at the host-pathogen interface and suggested a role for newly reported genes in the protection of pigs against A. phagocytophilum.

Keywords: Anaplasmosis, Genetics, Pig, Wild boar, Genomics, Immune response

Background

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is a tick-borne pathogen that infects a wide range of hosts including humans and wild and domestic animals [1,2]. A. phagocytophilum is the causative agent of human, equine and canine granulocytic anaplasmosis and tick-borne fever in ruminants [1,3,4]. In Europe, A. phagocytophilum is the most widespread tick-borne infection in animals with an increasing incidence in humans [5-10]. A. phagocytophilum is transmitted by Ixodes spp., but other tick species may subsequently also prove to be vectors [11,12]. Evidence suggests that persistent infections occur in domestic and wild ruminants, which can then serve as reservoir hosts [1,9]. The broad geographic distribution and the clinical and host tropism diversity of A. phagocytophilum strains suggest the presence of complex infection-transmission networks that may influence the epizootiology of the disease [13].

A. phagocytophilum has been reported with low prevalence in wild pigs (Sus scrofa) in the Czech Republic [14] and Slovenia [15]. Recently, 12% prevalence of was detected in wild boar in Poland [16]. In Slovenia and
Poland, the *A. phagocytophilum* gene sequences found in wild pigs were identical to that found in humans and *I. ricinus* ticks [15,16]. In Sicily, evidence suggested that *A. phagocytophilum* infection might occur in pigs [17]. In south-central Spain, where *I. ricinus* are scarce [18], *Anaplasma* spp. has not been reported in wild boar [13,19,20], although other tick species feeding on wild boar were positive for *A. phagocytophilum* DNA [12]. Recently, 165 rDNA but not p44/msp2 genotypes identical to *A. phagocytophilum* were found with low prevalence in wild boar in Japan [21] but a survey in Mississippi, United States, failed to detect pathogen DNA in feral pigs [22]. These results suggested that wild pigs might play a role in the epizootiology of *A. phagocytophilum* by serving as a natural reservoir host in some regions only.

Infection with *A. phagocytophilum* has been shown to modify the host cell gene expression. The gene expression profile has been characterized in human cells [23-28] and sheep [29] infected with *A. phagocytophilum*. As shown by recent studies in sheep [29], gene expression profile in response to *A. phagocytophilum* infection may differ between human cells and ruminant hosts. These differences may be the result of species-specific differences and/or the effect of different pathogen strains [2,29].

The objective of this study was to characterize gene expression profiles emphasizing on immune response genes in wild and domestic pigs in response to *A. phagocytophilum* using a combination of microarray hybridization and real-time RT-PCR. These results will expand current information on the mammalian host response to *A. phagocytophilum* infection and contribute to the overall understanding of the molecular mechanisms involved in pathogen infection, multiplication and persistence.

**Materials and methods**

**Experimental design and rationale**
The finding of wild pigs naturally infected with *A. phagocytophilum* in Slovenia suggested that this pathogen might also infect pigs, thus probably affecting gene expression in this species. The genes differentially expressed in response to *A. phagocytophilum* infection were first characterized in wild pigs naturally infected with *A. phagocytophilum* by microarray hybridization and real-time RT-PCR. The differentially expressed immune response genes were then further characterized in domestic pigs experimentally infected with *A. phagocytophilum* under controlled experimental conditions.

**Wild pigs and sample preparation**
Buffy coats were prepared from blood samples collected from adult (≥1 year-old) wild pig males hunter-killed during 2007 in Kočevje–Šubičeva and Kostel–Delač, Slovenia. Total DNA and RNA were extracted using MagneSil KF genomic DNA (Promega, Madison, WI, USA) and TRIzol Reagent (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA), respectively according to manufacturer’s instructions. The DNA was used to test for *A. phagocytophilum* infection using 16S rDNA and *groESL* PCRs and sequence analysis as previously reported [15]. Three of the 113 pigs analyzed tested positive for the presence of *A. phagocytophilum* DNA and were selected for further analysis. Control Buffy coats were prepared from uninfected adult wild pig males hunter-killed in south-central Spain where pigs are not infected with *Anaplasma* spp. [13,19,20]. Control animals tested negative in the *A. phagocytophilum* 16S rDNA and *groESL* PCRs. All animals tested negative for other pathogens commonly found in wild pigs such as *Mycobacterium bovis*, *Brucella suis*, Aujeszky’s Disease Virus (ADV) and porcine circovirus type 2 (PCV2).

**Microarray hybridization and analysis**
Total RNA from wild pigs was characterized using the Experion™ Automated Electrophoresis System (Bio-Rad, Hercules, CA, USA) in order to evaluate the quality and integrity of RNA preparations. One RNA sample from infected animals did not have the quality required for microarray hybridization. Therefore, two samples from infected animals were selected for microarray hybridization analysis together with three RNA samples from uninfected control animals. To obtain a comprehensive gene expression profile in response to *A. phagocytophilum* infection, the GeneChip® Porcine Genome Array was used, which contains 23,937 probe sets that interrogate approximately 23,256 transcripts from 20,201 *S. scrofa* genes (Affymetrix, Santa Clara, CA, USA; http://www.affymetrix.com/products_services/arrays/specific/porcine.affx). Two µg total RNA were labeled using the GeneChip® HT IVT Labeling Kit (Affymetrix). The images were processed with Microarray Analysis Suite 5.0 (Affymetrix). Raw expression values obtained directly from CEL files were preprocessed using the RMA method [30], a three-step process which integrates background correction, normalization and summarization of probe values. Standard quality controls based on Affymetrix original methods including average background, scale factor, number of genes called present, 3’ to 5’ ratio, and rDNA content for CEL files were assessed. All Anaplasma samples were log transformed. *A. phagocytophilum* *A. phagocytophilum* 16S rDNA and *groESL* probe sets were used as control genes for which the expression was not altered in wild pigs compared to controls. Hierarchical clustering was used to visualize the data. Values were log2 transformed and analyzed using the Student’s t-test to identify genes with significantly different expression levels between *A. phagocytophilum* infected and uninfected pigs. P-values were adjusted using the Benjamini-Hochberg method and considered significant if *p*=< 0.05.
between controls and the infected samples. Microarray data analysis was done using the free statistical language R and the libraries developed by the Bioconductor Project (www.bioconductor.org). In order to deal with the multiple testing issues derived from the fact that many tests (one per gene) were performed simultaneously, p-values were adjusted to obtain strong control over the false discovery rate using the Benjamini and Hochberg method [31]. All the microarray data were deposited at the NCBI Gene Expression Omnibus (GEO) under the platform accession number GPL3533 and the series number GSE15766.

**Sequence ontology (GO)**

Gene ontology (GO) assignments were retrieved from the GeneChip® Porcine Genome Array (Affymetrix) and verified by searching the Entrez (http://www.ncbi.nlm.nih.gov/sites/entrez) and Gene ontology (http://www.geneontology.org/) databases. The gene ontology (GO) enrichment analysis was performed with GOstats package [32]. For each GO category of interest, entries in the array were compared with results of differentially expressed genes by χ²-test (p = 0.01).

**Domestic pigs and sample preparation**

Six 9-weeks-old pathogen-free male pigs were randomly distributed into two experimental groups with three animals each, infected and uninfected. Pigs were experimentally infected with *A. phagocytophilum* by intravenous inoculation (iv) of ISE6 tick cell cultures infected with the human NY-18 isolate of *A. phagocytophilum* [33,34]. Pigs were each inoculated with one T-25 flask of *A. phagocytophilum*-infected ISE6 tick cells (11-15% infection, as determined by detection of intracellular morulae in stained cytospin cell smears; Hema-3 Stain, Fisher Scientific, Middletown, VA, USA) at days 0 and 36 of the experiment. Control pigs were inoculated with control uninfected tick cells. Uninfected and infected cultures were centrifuged at 1,000 × g for 5 min and resuspended in L-15B medium without fetal bovine serum and antibiotics in a final iv dose of 1 × 10⁷ cells/2 ml. All pigs were monitored for infection by recording clinical signs, PCR of blood samples, examination of stained blood films and by *Anaplasma* serology at days 0 (before first inoculation), 7, 15, 36 (before second inoculation), 47 and 62. At day 62, pigs were euthanized by a licensed veterinarian and subjected to gross necropsy examination. Animals were cared for in accordance with standards specified in the Guide for Care and Use of Laboratory Animals and approved by the ethical committee for animal care and experimentation (No. 10/397354.9/11).

**Detection of *A. phagocytophilum* in experimentally infected pigs by PCR**

DNA was extracted from pig blood samples using TriReagent (Sigma, St. Louis, MO, USA) following manufacturer’s recommendations. *A. phagocytophilum* infection levels were characterized by *msp4* PCR using the iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) as described previously using oligonucleotide primers MAP4AP5: 5’-ATGAAATTACAGAGAATTGCTTGTAGG-3’ and MSP4AP3: 5’-TTAATTGAAAGCAAATCTTGCTCTTATG-3’) in a 50-μl volume PCR (1.5 mM MgSO₄, 0.2 mM dNTP, 5XGoTaq reaction buffer, 5u GoTaqDNA polymerase) (Promega, Madison, WI, USA) [2]. Negative control reactions were performed with the same procedures, but adding water instead of DNA to monitor contamination of the PCR. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 kb DNA Ladder, Promega). Amplified fragments were resin purified (Invitrogen, Carlsbad, CA, USA) for sequencing both strands by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain). The *msp4* coding region was used for sequence alignment. Multiple sequence alignment was performed using the program DNA Baser (Heracle BioSoft S.R.L., Pitesti, Romania).

**Detection of anti-*A. phagocytophilum* antibodies in experimentally infected pigs by ELISA**

Serum samples were tested for IgG antibodies by means of an in-house indirect ELISA using the *A. phagocytophilum* (NY-18) recombinant MSP4 protein as antigen and protein G horseradish peroxidase as a conjugate using the protocol described by Araújo et al. [35] with some modifications. Briefly, 96-well plates (MaxiBinding, SPL Life sciences, Korea) were coated overnight at 4°C with 0.4 μg/ml of MSP4, diluted in carbonate-bicarbonate phosphate buffer. Plates were blocked for 1 hr at 37°C with 140 μl/well of a solution containing 5% skim milk with phosphate buffered saline and 0.05% Tween-20 (PBST). Sera were added directly on plate (100 μl/well) at a dilution of 1:100 in PBST and incubated for 1 hr at 37°C. Plates were then washed five times with PBST, and Protein G (Sigma Aldrich, Saint Louis, USA) was added (100 μl/well) at a dilution of 1:1,000 in PBST and incubated at 37°C for 1 hr. After five washes with PBST, the chromogen/substrate o-phenylene diamine dihydrochloride (OPD; Sigma)/H₂O₂ was added. The reaction was stopped with 50 μl/well of sulphuric acid (H₂SO₄; 3N), and the optical density (OD) was measured in a spectrophotometer at 450 nm. White-tailed deer and cattle sera positive to *Anaplasma* were included as controls. Antibody titers in experimentally infected and control pigs were expressed as the
Table 1 Primer sets and real-time PCR conditions used for analysis of differentially expressed genes

| Gene description | Genbank accession number | Upstream/downstream primer sequences (5’-3’) | PCR conditions<sup>a</sup> |
|------------------|-------------------------|---------------------------------------------|--------------------------|
| Interleukin 1 receptor accessory protein-like 1 (IL1RAPL1) | NG_008292 CN163387 | IL1-L: GTTGTCAATTTCGCAACATC IL1-R: GCCTATGGACCGATGCGTCTTA | 58°C, 30 sec/72°C, 30 sec |
| T-cell receptor alpha chain (TCR-alpha) | AB087958.1 | TcathR-L: TTCTGACCTGGAAGTCTGTG TcathR-R: GAGAAAGCCATGCTTGGT | 58°C, 30 sec/72°C, 30 sec |
| Gap junction protein alpha 1 (GJA1) | BC105461.1 CK465005 | GAP-L: TGGAATGCAAGAGAGGTTGA GAP-R: TCATAGGGGTCCAGCACTTC | 58°C, 30 sec/72°C, 30 sec |
| Thrombospondin 4 (TSP-4) | XM_001926236 BM190304 | TROMB4-L: GGGCAAGGTTTTGTTCTGA TROMB4-R: TGGATGCAAGAGAGGTTGA | 60°C, 30 sec/72°C, 30 sec |
| Beta-actin | DQ845171 | SusBetActin-L: GCACCTGGAAGCCATCGTCA SusBetActin-R: ACACGGAGTACTTGGCCTTC | 60°C, 30 sec/72°C, 30 sec |
| Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) | AF069649 | GAPDHSus-L: CCAGAACTACATCTCCGTGCTT GAPDHSus-R: GTCTCTAGTGATGCAGCAGA | 60°C, 30 sec/72°C, 30 sec |
| Cyclophilin | AY008846 | SSCYCLOPHILIN-L: AGCACTGGGGGAGAAAAGGATT SSCYCLOPHILIN-R: CTGGCAGTGGAAATGAAA | 55°C, 30 sec/72°C, 30 sec |

<sup>a</sup>PCR conditions are shown as annealing/extension in real-time RT-PCR analysis.
Results

Gene expression in pigs naturally infected with A. phagocytophilum

All infected wild pigs contained a single A. phagocytophilum 16S rDNA and groESL genotype. The 16S rDNA sequence was identical to the sequence of the USG3 strain [GenBank: AY055469] originally isolated from a dog infected by feeding infected I. scapularis ticks, as well as to strains obtained from patients diagnosed with human granulocytic anaplasmosis (HGA) [38]. The sequence of the groESL locus was identical to that identified previously in wild boar, human and I. ricinus samples in Slovenia [GenBank: AF033101 and EU246961] [15].

Of the 20,201 S. scrofa genes that were analyzed in the microarray, 942 showed significant (P < 0.05) differences between infected and control samples (936 upregulated and 6 down regulated) and 61 of them had >2 fold changes in expression in wild pigs (Table 2). Of these genes, 56 were upregulated and 5 were down regulated in infected animals (Table 2).

Gene ontology (GO) could be assigned to 32 of the differentially expressed genes (Table 2). The differentially expressed genes in wild pigs infected with A. phagocytophilum included those with cation binding, protein binding, transcription factor, enzymatic activity and receptor activity protein function involved in cell differentiation, adhesion, metabolism and structure, signaling pathway, transcription, stress, immune response and catabolic processes (Table 2). The most frequently represented protein function and biological process GO assignments were significantly overrepresented in response to infection in naturally infected pigs (Table 3). Thus, the highest GO enrichment for molecular function and biological process occurred for protein binding and signaling pathway genes, respectively (Table 3).

The immune response was among the biological processes significantly overrepresented in genes upregulated in response to A. phagocytophilum infection (Table 3). Thus, the immune response genes upregulated in response to A. phagocytophilum infection, interleukin 1 receptor accessory protein-like 1 (ILIRAPl1), T-cell receptor alpha chain (TCR-alpha), thrombospondin 4 (TSP-4) and Gap junction protein alpha 1 (GJA1), were selected for confirmation of microarray hybridization results by real-time RT-PCR. The real-time RT-PCR analysis confirmed the results of the microarray hybridization and demonstrated that the immune response genes ILIRAPl1, TCR-alpha, TSP-4 and GJA1 were upregulated in infected animals (Figure 1).

Serum IL-1 beta, IL-8 and TNF-alpha levels in pigs experimentally infected with A. phagocytophilum

Serum IL-1 beta, IL-8 and TNF-alpha levels were transiently higher in infected pigs when compared to uninfected controls (Figure 4). Significant (P < 0.05) infected to uninfected ratio for serum protein levels were obtained for IL-1 beta and IL-8 at 33 dpi and for TNF-alpha at 15 and 36 dpi (Figure 4). These protein levels were equivalent in infected animals to 3.73 ± 0.00 pg/ml (IL-1 beta), 2.18 ± 0.00 pg/ml (IL-8), 370.13 ± 0.00 pg/ml (TNF-alpha at 15 dpi) and 2.01 ± 0.00 pg/ml (TNF-alpha at 36 dpi). In uninfected control animals, protein levels at the same time points were bellow ELISA detection limits.

Discussion

Molecular evidence suggested that wild pigs could be involved in the natural cycle of A. phagocytophilum in some regions [14-16,21]. The results of sequence analyses suggested that the A. phagocytophilum strain collected at 15 (in all 3 pigs), 36 (before second inoculation in pigs No. 1 and No. 2), and 62 (in pig No. 1 only) days post-infection (dpi) in pigs inoculated with infected cells but not in control pigs. The A. phagocytophilum msp4 amplicons from pig blood were sequenced and corresponded to the NY-18 isolate sequence (Genbank accession number JQ522935). Infected and uninfected pigs did not show clinical signs or A. phagocytophilum morulae in stained blood films. Significant differences were not observed in anti-A. phagocytophilum MSP4 antibodies between pigs inoculated with infected cells and controls (P > 0.05; Figure 2). However, peaks in anti-MSP4 antibody titers were detected at 33 and 47 dpi in pigs No. 3 and No. 1, respectively (Figure 2).
| Affymetrix ID1 | Genbank accession number | Fold Change2 | SD3 | P-value4 | Description5 | GO Molecular function6 | GO Biological process7 |
|----------------|--------------------------|--------------|-----|----------|--------------|------------------------|------------------------|
| Ssc.30381.1.A1_at | CO991016 | 361.988 | 84.148 | 0.039 | Unknown | | |
| Ssc.17891.1.A1_at | CF175823 | 29.073 | 8.518 | 0.029 | Unknown | | |
| Ssc.13408.1.A1_at | BI405159 | 19.229 | 5.72 | 0.030 | Unknown | | |
| Ssc.10537.1.A1_at | BF711416 | 15.897 | 1.497 | 0.002 | Unknown | | |
| Ssc.29577.1.A1_at | CO940471 | 14.279 | 0.259 | 0.006 | Unknown | | |
| Ssc.24631.1.S1_at | CK461650 | 11.635 | 1.792 | 0.007 | Formin 1 | Protein binding | Cell adhesion |
| Ssc.31062.1.S1_at | AJ663560 | 8.047 | 1.593 | 0.021 | Unknown | | |
| Ssc.28701.1.S1_at | BG893814 | 8.008 | 0.889 | 0.015 | Sorbin and SH3 domain isoform 2, transcript variant 14 | Receptor activity | Signaling pathway |
| Ssc.29538.1.A1_at | CO941727 | 7.984 | 0.567 | 0.020 | Unknown | | |
| Ssc.10128.1.A1_at | BI399899 | 7.217 | 1.986 | 0.039 | similar to H. sapiens SIX homebox 4 | Unknown | Unknown |
| Ssc.16289.1.A1_at | U15437.1 | 5.927 | 1.945 | 0.047 | Ig heavy chain variable VDJ region | Protein binding | Immune response |
| Ssc.16269.1.S1_at | U15523.1 | 2.919 | 0.594 | 0.047 | Ig heavy chain variable VDJ region | Protein binding | Immune response |
| Ssc.19942.5.A1_x_at | U38202.1 | 2.571 | 0.398 | 0.044 | | | |
| Ssc.17872.1.A1_at | CF176409 | 5.731 | 1.428 | 0.047 | COUP transcription factor 1 (COUP-TF1) | Transcription factor | Signaling pathway |
| Ssc.31126.1.A1_at | CO94136 | 5.391 | 0.792 | 0.035 | Unknown | | |
| Ssc.29622.1.A1_at | CO942607 | 4.96 | 0.634 | 0.009 | Unknown | | |
| Ssc.17942.1.A1_at | CF176409 | 4.605 | 0.287 | 0.020 | Unknown | | |
| Ssc.31069.1.A1_s_at | BF712013 | 4.578 | 0.478 | 0.014 | DAZ interacting protein 3, zinc finger | Protein binding | Ubiquitin-dependent protein catabolic process |
| Ssc.6157.1.A1_at | BQS97772 | 3.782 | 0.555 | 0.021 | Zinc finger protein 521 | Unknown | Unknown |
| Ssc.1411.1.S1_at | BM190304 | 3.586 | 0.152 | 0.013 | Thrombospondin 4 (TSP-4) | Cation binding, protein binding | Cell adhesion |
| Ssc.7524.1.A1_at | BQS99075 | 3.397 | 0.719 | 0.033 | Sk/Dkk-1 protein precursor | Protein binding | Signaling pathway |
| Ssc.8931.1.A1_at | BQS98736 | 3.336 | 0.818 | 0.037 | Angiopoietin-like protein 2 (Angptl2) | Unknown | Signaling pathway |
| Ssc.13693.1.A1_at | BQS97772 | 3.313 | 0.589 | 0.026 | Unknown | | |
| Ssc.4707.1.A1_at | BI118246 | 3.271 | 0.917 | 0.049 | H. sapiens kit ligand (KITLG) | Protein binding | Cell adhesion |
| Ssc.13265.1.A1_at | BQS90573 | 3.236 | 0.602 | 0.029 | Unknown | | |
| Ssc.7967.1.A1_at | BQS99891 | 3.153 | 0.663 | 0.033 | Unknown | | |
| Ssc.8871.2.A1_at | CK457442 | 2.929 | 0.711 | 0.043 | Cyclin-dependent kinase inhibitor 1C (CDKN1C) | Protein binding | Signaling pathway |
| Ssc.20473.2.S1_at | CK457442 | 2.929 | 0.711 | 0.043 | Cyclin-dependent kinase inhibitor 1C (CDKN1C) | Protein binding | Signaling pathway |
| Ssc.20452.1.S1_at | BX670488 | 2.890 | 0.616 | 0.035 | Keratin associated protein 26-1 | Protein binding | Cell structure |
| Ssc.29030.1.S1_at | CO988330 | 2.838 | 0.499 | 0.032 | Unknown | | |
| Ssc.26632.1.S1_at | CN155689 | 2.813 | 0.333 | 0.030 | Tripartite motif protein 32 | Protein binding | Cell differentiation, ubiquitin-dependent protein catabolic process |
| Ssc.24221.2.A1_at | BI181166 | 2.805 | 0.302 | 0.007 | NADH-ubiquinone oxidoreductase | Enzymatic activity | Cell metabolism |

18 kDa subunit
### Table 2 Gene ontology and description of significant differentially expressed genes (P < 0.05; > 2 fold change) (Continued)

| Genbank ac | Description | log2FoldChange | Adjusted P-value | Gene Ontology | Description |
|------------|-------------|----------------|------------------|---------------|-------------|
| Ssc.428.10.S1_at | AB087975.1 | 2.767 | 0.027 | T cell receptor alpha chain (TCR-alpha) | Receptor activity, Immune response |
| Ssc.17790.1.S1_at | AB087958.1 | 2.334 | 0.031 | T cell receptor alpha chain (TCR-alpha) | Receptor activity, Immune response |
| Ssc.18884.1.A1_at | CF365209 | 2.67 | 0.008 | Unknown | Immune response |
| Ssc.25538.1.S1_at | BX918287 | 2.597 | 0.033 | Zinc finger protein 502 | Transcription factor, Transcription |
| Ssc.26587.1.A1_at | CN154795 | 2.592 | 0.030 | Unknown | Immune response |
| Ssc.20172.1.A1_at | BX676733 | 2.547 | 0.027 | Tumor endothelial marker 8 isoform 3 | Protein binding, receptor activity, Cell adhesion |
| Ssc.7090.1.A1_at | NM_214233.1 | 2.465 | 0.026 | Glutathione peroxidase 1 (GPX1) | Enzymatic activity, Stress |
| Ssc.13474.1.A1_at | BQ602423 | 2.454 | 0.022 | Unknown | Unknown |
| Ssc.8511.1.A1_at | BF703957 | 2.449 | 0.009 | Sus scrofa mRNA, clone: OVRM10011A06, expressed in ovary | Unknown, Unknown |
| Ssc.30148.1.A1_at | CO987207 | 2.341 | 0.048 | Rho-related BTB domain containing 3 (RHOTB3) | Protein binding, receptor activity, Signaling pathway, ubiquitin-dependent protein catabolic process |
| Ssc.22336.1.S1_at | CF793417 | 2.302 | 0.023 | Homeobox protein Hox-B7 (Hox-2C) | Transcription factor, protein binding, Transcription |
| Ssc.13772.5.S1_at | BI343023 | 2.264 | 0.016 | Integrin alpha-8 (ITGA8) | Cation binding, protein binding, receptor activity, Cell differentiation, Cell adhesion, Signalling pathway |
| Ssc.29167.1.A1_at | CO950916 | 2.198 | 0.030 | Rho GTPase activating protein 5 | Protein binding, Cell adhesion |
| Ssc.13363.1.A1_at | BI404946 | 2.188 | 0.038 | Ubiquitin carboxyl-terminal hydrolase 24 | Protein binding, Ubiquitin-dependent protein catabolic process |
| Ssc.17370.1.A1_at | BX665583 | 2.186 | 0.014 | Adrenergic, alpha-1B-, receptor (ADRA1B) | Protein binding, receptor activity, Signaling pathway |
| Ssc.22210.2.S1_at | CF788693 | 2.176 | 0.040 | Unknown | Unknown |
| Ssc.29565.1.A1_at | CO942018 | 2.168 | 0.025 | Unknown | Unknown |
| Ssc.19407.1.A1_at | CF359796 | 2.157 | 0.015 | Unknown | Unknown |
| Ssc.28265.1.A1_at | CN025977 | 2.143 | 0.035 | Unknown | Unknown |
| Ssc.26179.1.S1_at | BX922022 | 2.123 | 0.005 | Midnolin (MIDN) | Protein binding, Transcription |
| Ssc.4813.1.S1_at | CF789770 | 2.123 | 0.031 | Calponin 3, acidic, transcript variant 1 | Cation binding, protein binding, Unknown |
| Ssc.20453.1.S1_at | BX675824 | 2.092 | 0.012 | Laminin receptor 1 | Receptor activity, Unknown |
| Ssc.14354.1.A1_at | BQ601965 | 2.079 | 0.047 | HHEX gene for hematopoietically expressed homeobox | Transcription factor, Cell differentiation, Signalling pathway |
| Ssc.942.1.S1_at | CK465005 | 2.061 | 0.012 | Gap junction protein, alpha 1 (GJα1) | Protein binding, Cell adhesion, Signaling pathway, immune response |
| Ssc.26933.1.S1_at | CN163387 | 2.036 | 0.007 | Interleukin 1 receptor accessory protein-like 1 (IL1RAPL1) | Receptor activity, Immune response |
| Ssc.30263.1.A1_at | CO989398 | 2.032 | 0.039 | Unknown | Unknown |
| Ssc.16566.1.S1_at | BF078197 | 2.025 | 0.025 | Lactase phlorizinhydrodase | Cation binding, Cell metabolism |
| Ssc.9748.1.A1_at | BI387874 | −3.914 | 1.462 | Unknown | Unknown |
| Ssc.30189.1.A1_at | CO987781 | −4.078 | 1.800 | Pig DNA sequence from clone CH242-94D11 on chromosome 7 | Unknown, Unknown |
| Ssc.8698.1.S1_at | CN163671 | −10.246 | 0.010 | Cadherin 11, type 2, OB-cadherin (osteoblast) | Cation binding, Protein binding, Cell adhesion |
identified in wild pigs might be similar to those causing disease in dogs and humans, thus reinforcing the possible role of pigs in the epidemiology of HGA in these regions [15,38,39].

The overall effect of *A. phagocytophilum* on pig gene expression was low as only 4.7% (942/20,201) of the genes analyzed in the microarray were differentially expressed in pathogen-infected animals (P < 0.05) and only 61 genes (0.3%; 61/20,201) showed >2 fold difference between infected and control animals. Interestingly, 9 of the 61 (15%) differentially expressed genes in naturally infected pigs were related to cytoskeleton structure and function. Phagocytosis and autophagy are among the first lines of defense against bacterial infections and require a dramatic rearrangement of the cytoskeleton for internalization of invading microbes [40]. The expression of genes such as GJA1, integrin alpha-8, TSP-4, formin 1, Rho GTPase activating protein 5, keratin associated protein 26–1, calponin 3 and laminin receptor 1 was upregulated, while the expression of cadherin 11 was down regulated in *A. phagocytophilum*-infected wild pigs, thus suggesting an effect of pathogen infection on cytoskeleton rearrangement. It has been suggested that *A. phagocytophilum* affects actin reorganization to facilitate cell invasion but reduces neutrophil phagocytosis and subverts autophagy to establish intracellular infection and proliferation [41-43]. Furthermore, a recent study showed that Toll-like receptor signaling usurps components that are traditionally associated with autophagy to increase the efficiency of phagocytosis, thereby providing a link between these two microbial defense mechanisms [44]. Taken together, these results suggested that *A. phagocytophilum* infection of pigs impacted cytoskeleton rearrangement to promote phagocytosis and autophagy, thus resulting in effective pathogen clearance (Figure 5).

*A. phagocytophilum* infection has been shown to delay the apoptotic death of neutrophils [24,43,45,46]. The analysis of gene expression profile in naturally infected pigs did not show an effect on caspases 3 and 8 (CASP3/8) and the PI3K/AKT pathway, which have been linked to *A. phagocytophilum*-induced apoptosis inhibition in human neutrophils [43]. However, the activation of the Jak-STAT pathway that has been shown to occur in *A. phagocytophilum*-infected sheep and pigs may constitutes a new mechanism leading to delay in the apoptotic death of neutrophils in these species [47] (Figure 5). Reactive oxygen species (ROS) production is inhibited by *A. phagocytophilum* through modulation of NADPH oxidase assembly and/or regulation of gene expression in human cells [43], a mechanism that was not found in pigs. However, upregulation of TGF-beta in infected pigs [47] may inhibits NO production in neutrophils by suppressing STAT1 activation and accelerating iNOS protein degradation [47,48]. The effect of *A. phagocytophilum* on lipid metabolism required for pathogen infection of human neutrophils [25,43] was also not found in pigs. However, some of these discrepancies may be explained by the fact that results in pigs were obtained using RNA from Buffy coats and not

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**Table 2 Gene ontology and description of significant differentially expressed genes (P < 0.05; > 2 fold change) (Continued)**

| GO category                      | Represented on the microarray (%) | Represented among differentially expressed genes (%) |
|----------------------------------|-----------------------------------|----------------------------------|
| **Molecular function**           |                                   |                                  |
| Cation binding                   | 67 (2.4)                          | 5 (15.6)*                        |
| Protein binding                  | 14 (0.5)                          | 20 (62.5)*                       |
| Transcription factor             | 10 (0.4)                          | 5 (15.6)*                        |
| Receptor activity                | 10 (0.4)                          | 8 (25.0)*                        |
| **Biological process**           |                                   |                                  |
| Catabolic process                | 108 (3.8)                         | 3 (9.4)*                         |
| Immune response                  | 23 (0.8)                          | 4 (12.5)*                        |
| Cell adhesion                    | 20 (0.7)                          | 8 (25.0)*                        |
| Signaling pathway                | 13 (0.5)                          | 10 (31.2)*                       |
| Cell differentiation             | 7 (0.2)                           | 3 (9.4)*                         |

*Of the 20,201 *S. scrofa* genes analyzed in the microarray, 2,840 had GO assignments and were used for GO enrichment analysis.

*Of the 61 genes that showed significant (P ≤ 0.05) ≥ 2 fold changes in expression in infected wild pigs, 32 had GO assignments and were used for GO enrichment analysis. For genes with multiple GO assignments, each category was included in the analysis. For each GO category of interest, entries in the array were compared with results of differentially expressed genes by χ2-test (*α < 0.01).
purified neutrophils or cell cultures, which may produce a masking effect of other leukocyte mRNAs. Our group is interested in the characterization of the host immune response to intracellular bacteria [29,49-52]. The infection with *A. phagocytophilum* has been shown to stimulate innate immune and pro-inflammatory responses [43,45,46,53]. However, experiments in mice have shown that *A. phagocytophilum* infection may be controlled, even in the absence of innate immune effectors [54,55]. In sheep and horses, evidence suggests that *A. phagocytophilum* infection triggers innate immune responses while impairing adaptive immunity [29,56], a factor that could contribute to pathogenicity in these species.

Analysis of gene expression in naturally and experimentally infected pigs suggested that *A. phagocytophilum* infection increased innate immunity by up regulation of *IL1RAPL1*, *TSP-4* and *TCR-alpha* (Figure 5). Furthermore, kinetics of mRNA levels in experimentally infected pigs showed an early, transient up regulation of immune response genes, probably coinciding with the first bacteremia of the acute infection phase [57]. Up regulation of *IL1RAPL1* and *TSP-4* may increase the innate immune proinflammatory response through improved signal transduction and secretion of IL-1 and IL-8, respectively [58,59]. T lymphocytes use their TCR as a pattern recognition receptor to sense the presence of infection and produce after activation proinflammatory cytokines such as TNF-alpha [60]. In experimentally inoculated pigs, IL-1 beta, IL-8 and TNF-alpha serum levels were transiently higher in infected animals when compared to controls, thus corroborating...
the stimulation of proinflammatory responses suggested by gene expression studies in *A. phagocytophilum*-infected pigs (Figure 5). IL-8 secretion in response to *A. phagocytophilum* infection in human cells leads to neutrophils recruitment [43]. Although IL-1 and TNF-alpha levels have not been found to be elevated in HGA patients, higher mRNA or serum levels have been observed in horses and sheep, for which *A. phagocytophilum* is also pathogenic [61].

In vitro, *A. phagocytophilum* infection of human peripheral blood lymphocytes and monocytes induce transient mRNA expressions and protein secretion of IL-1 beta and TNF-alpha [61]. These studies suggested that although IL-8 is likely secreted by neutrophils, monocytes, rather than neutrophils, are responsible for proinflammatory IL-1 beta and TNF-alpha cytokine production [61,62]. The expression of genes involved in adaptive immunity was not impaired. In fact, the expression of *GJA1*, a member of the connexin gene family with a role in innate and adaptive immunity through the regulation of phagocytosis by macrophages and the host response to bacterial infection [63], was upregulated in infected pigs. The activation of the Jak-STAT pathway associated with *A. phagocytophilum* infection in sheep and pigs may result in immune development to aid in pathogen control [47].

The experimental infection with *A. phagocytophilum* demonstrated that pigs are susceptible to pathogen infection. The detection of bacterial DNA by PCR showed a prepatent period (calculated as the number of days from the time of pig inoculation with infected tick cells to the first day that blood samples were found to be *A. phagocytophilum* positive by PCR) of 15 days, similar to that found in sheep [64] and white-tailed deer [65] but lower than in mice [66] inoculated with *A. phagocytophilum* (NY-18) infected cells. At 36 dpi only two animals were PCR positive and by 47 dpi all animals were negative, suggesting duration of approximately 30 days for the primary bacteremia. However, although only one pig (No. 1) was PCR positive
at 62 dpi after the second inoculation, recurrent bacteremias are possible [57]. The weak antibody response detected in infected animals supports a rapid control of pathogen infection. However, similar results were obtained in sheep experimentally inoculated with *A. phagocytophilum* infected cells [64]. The pigs used in this study for microarray analysis were naturally infected with *A. phagocytophilum*. Therefore, it was not possible to establish when animals were infected. Transient up regulation of immune response genes in experimentally infected pigs suggested that naturally infected pigs were also at early infection stages. However, we cannot exclude the possibility that, if pigs become persistently infected even at low infection levels, some of the gene expression profiles described in this study in naturally infected pigs may represent the response of persistently infected animals and may differ from the response during early infection. Persistent *A. phagocytophilum* infection has been documented in sheep [57] and horses [67] and previous studies have shown differences in gene expression profiles between acutely and chronically *A. phagocytophilum*-infected sheep [29].

**Conclusions**

These results suggested that pigs are susceptible to *A. phagocytophilum* but control infection, particularly through activation of innate immune responses and cytoskeleton rearrangement to promote phagocytosis and autophagy (Figure 5). Control of *A. phagocytophilum* infection in pigs may result in infection below PCR detection levels or infection clearance, thus contributing to the low percentage of infection prevalence detected for this species in most regions, with a low or no impact as a reservoir host for this pathogen [14,15,20]. The results reported here confirmed in pigs the activation of innate and adaptive immune pathways during *A. phagocytophilum* infection reported in humans and other species (Figure 5). However, this pathogen may use other mechanisms to circumvent host-cell defenses and establish infection by downregulating other adaptive immune response genes such as IL-2 and IL-4 and delaying the

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**Figure 4** Serum IL-8, IL-1 beta and TNF-alpha levels in experimentally infected pigs. Cytokine levels were determined by ELISA in the sera from infected and uninfected control pigs and infected to uninfected average ± S.D. ratios determined. Results were compared between infected and control pigs by Student’s t-test (*P ≤ 0.05).
apoptotic death of neutrophils through activation of the Jak-STAT pathway [47]. These results further expand the existing information on the response of mammalian hosts to *A. phagocytophilum* infection and suggested a role for newly reported genes in the protection of pigs against *A. phagocytophilum*.

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
RCG performed microarray analysis and lab tests, NA, KSS, BB-B, MM and NG collected data and samples, NA, MB and JMP performed lab tests. RCG, NA, MB, JMP, CG and JF analyzed data and performed statistical analysis. JF, MKM and TA-Z conceived the study, JF designed the study. CG supervised part of study, RCG, MKM, TA-Z, CG and JF wrote the manuscript. All authors read and approved the final manuscript.

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**Figure 5** Effect of *A. phagocytophilum* infection on host cells. *A. phagocytophilum* (Ap) infection causes cytoskeleton rearrangement required for infection, but in pigs it may also promote phagocytosis and autophagy for effective pathogen clearance. Ap delays the apoptotic death of neutrophils to increase infection, but different and complementary mechanisms may operate in human and pig cells. Pathogen infection stimulates innate immune and pro-inflammatory responses in both humans and pigs. IL-8 is likely secreted by infected neutrophils but monocytes, rather than neutrophils, are probably responsible for proinflammatory IL-1 beta and TNF-alpha cytokine production. The expression of genes involved in adaptive immunity was not impaired in pigs. ROS production is inhibited by pathogen infection of human neutrophils but although this mechanism was not found in pigs, upregulation of TGF-beta1 in infected pigs may inhibits NO production by suppressing STAT1 activation and accelerating iNOS protein degradation. The effect on lipid metabolism required for pathogen infection of human neutrophils was not found in pigs. Data for human neutrophils was obtained from the recent review by Severo et al. [43].
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