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Microbiological and proteome analysis to gain insights into the pathogenesis of the highly adapted not-cultivated hemotrophic *Mycoplasma suis*

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Dedicated to

Tobias Michael Geiger

“The scientist is not a person who gives the right answers,

he is one who asks the right questions.”

Claude Lévi-Strauss, 1964

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LIST OF ABBREVIATIONS

AIHA	= autoimmune hemolytic anemia
BP	= binding protein
DE	= dimensional electrophoresis
DNA	= deoxyribonucleic acid
EC	= endothelial cell
ECF	= energy-coupling factor
EDTA	= ethylenediaminetetraacetic acid
ELISA	= enzyme-linked immunosorbent-assay
GE	= genome equivalents
GC	= gas chromatography
HM	= hemotrophic mycoplasma
HRP	= horseradish peroxidase
HspA1	= heat shock protein A1
IAP	= infectious anemia in pigs
Ig	= immune globulin
kbp	= kilo base pairs
LC	= liquid chromatography
MFS	= major facilitator superfamily
MS	= mass spectrometry
MSG1	= Glyceraldehyd-3-phosphate dehydrogenase-like protein 1
MS/MS	= tandem mass spectrometry
m/z	= mass-to-charge ratio
NMR	= nuclear magnetic resonance
P	= phosphate
PEP-PTS	= phosphoenolpyruvate phosphotransferase system
p. i.	= post infection
qPCR	= quantitative polymerase chain reaction
RBC	= red blood cell
rRNA	= ribosomal ribonucleic acid
Rt-PCR	= real-time polymerase chain reaction
TOF	= time of flight

1 SUMMARY

Bacteria belonging to the genus *Mycoplasma* occur worldwide in human beings as well as in a wide range of animals. A rather specialized group within the genus *Mycoplasma* consists of the so-called hemotrophic mycoplasmas (HMs) which are characterized by strict host specificity and uncultivability *in vitro.* This uncultivable state of HMs is a major problem and makes research a challenging task. HMs are pleomorphic bacteria which attach to the surface of red blood cells (RBCs) or also invade RBCs. Attachment as well as invasion can lead to cell deformation as well as to apoptosis of the target cells. However, the close contact between the host cell and the bacteria also seems to be essential for the specialized life cycle of HMs.

Mycoplasma suis a representing member of the HM-group induces infectious anemia in pigs (IAP). The disease is characterized by two different courses, i.e. acute infection vs. chronic infection. Especially the chronic form can cause substantial economic losses in swine production and therefore, has to be characterized in detail.

In contrast to the acute form of IAP which can be investigated by the aid of a splenectomized pig infection model, there is no established pig infection model which can be used for the analysis of the chronic IAP.

M. *suis* is connected to the surface of erythrocytes by fibrillary structures. The exact mechanism of adhesion is quite unclear up to now. Nevertheless, three surface proteins with adhesion properties (HspA1, MSG1, and α-enolase) have been investigated in detail so far. Their corresponding genes as well as recombinant gene products are used for diagnostic purposes of *M*. *suis* in clinical samples (PCR, serology). Currently, the most efficient diagnostic test is the real-time polymerase chain reaction (Rt-PCR). It enables the diagnosis of chronic as well as acute IAPs which are characterized by low or high bacterial loads, respectively.

Clinical signs of both forms can be cured by the application of tetracycline, iron dextran and glucose. However, the elimination of *M. suis* from infected hosts is not possible yet. Therefore, once infected animals act as carriers and transmitters of *M. suis* within and between herds. Nevertheless, detailed investigations on specific transmission pathways in acute as well as in chronic *M*. *suis* infections are quite rare.

The aims of this work were to establish a chronic pig infection model and to clarify unrecognized transmission pathways of *M*. *suis.* In addition, proteomics-based investigations of *M*. *suis* should be performed to improve the knowledge on the host pathogen interactions and host adaptation in IAP.

Based on the succeeded and frequently applied splenectomized *M*. *suis* pig model (acute infection model) it was possible to establish a novel non-splenectomized *M*. *suis* pig model.

This infection model enables the experimental investigation of the chronic *M*. *suis* infection. To this end, the piglets were infected intramuscularly with a highly virulent *M*. *suis* strain. Infected animals exhibited clinical signs (e.g. Morbus maculosus) including the typical cyclic course of chronic IAP.

In the next step, potential transmission pathways of *M. suis* were analyzed during acute and chronic *M*. *suis* infection based on these two pig infection models. Feces, urine, air and dust as well as nasal, vaginal and saliva excretions were collected during the course of infection and examined for *M*. *suis* DNA by Rt-PCR. For the first time it was possible to detect *M*. *suis* in urine with and without erythrocytes as well as in nasal, vaginal and saliva excretions during acute and chronic infections. These results indicate blood-independent *M*. *suis* transmission via vaginal discharge, nasal excretions, saliva, and urine.

The non-culturability limited the improvement of proteomic-based investigations of *M*. *suis*-related host-pathogen interactions. Therefore, we used modern and sophisticated proteome analysis to solve this problem. Blood samples from experimentally infected pigs at different time points of infection were investigated. For this, novel enrichment methods for *M*. *suis* proteins (especially membrane proteins) were established. These methods enabled an improved resolution of the protein expression profile of *M*. *suis* and thereby deeper insights into the pathogenesis of this microorganism. Despite of the missing cultivation system it was possible to identify more than 50% of the predicted *M*. *suis* proteins during acute infection. This identification ratio is similar to the one found in cultivable bacteria. Furthermore, the results of the proteome analysis indicate that nutrients such as glucose, hexose-6-phosphate, spermidine, putrescine, phosphate, amino acids, magnesium, potassium, sodium and iron are taken up by *M*. *suis* from the host leading to the high degree of host adaptation. Therefore, gained information on expressed *M*. *suis* proteins involved in transport are helpful in the establishment of an *in vitro* cultivation system in future. Particularly the supplementation of individual nutrients can play key functions in the media to support growth. Besides the *M*. *suis* proteome the acquired dataset firstly enables also the quantitative identification of *Sus scrofa* proteins differentially expressed during *M*. *suis* infection. This information can be used to unravel infection-relevant processes in the host in further studies.

2 ZUSAMMENFASSUNG

Bakterien der Gattung *Mycoplasma* treten sowohl bei Menschen als auch bei einer Vielzahl von Tieren auf. Eine hoch-spezialisierte Gruppe von Bakterien innerhalb der Gattung *Mycoplasma* sind die sogenannten haemotrophen Mycoplasmen (HM). Diese sind durch eine hohe Wirtsspezifität charakterisiert und können nicht unter Laborbedingungen kultiviert werden. Das Fehlen eines *in vitro*-Kultursystems erschwert die Erforschung der HM wesentlich. HM sind pleomorphe Bakterien, welche an die Oberfläche von Erythrozyten binden oder in diese eindringen. Sowohl die Anlagerung als auch die Invasion kann zu Deformationen bzw. zur Apoptose des Erythrozyten führen. Zudem scheint der enge Kontakt zwischen Wirtszelle und Bakterien für die Lebensfähigkeit der HM essentiell zu sein.

Mycoplasma suis ist ein Vertreter der HM-Gruppe, der bei Schweinen die sogenannte infektiöse Anämie (IAP) verursacht. Diese Krankheit ist sowohl durch eine akute als auch eine chronische Verlaufsform gekennzeichnet. Die akute Verlaufsform konnte anhand des splenektomierten *M*. *suis*-Models bereits sehr gut charakterisiert werden. Im Vergleich dazu existiert jedoch kein Infektionsmodel für die Analyse der chronischen Form der Anämie. Gerade diese führt jedoch zu hohen ökonomischen Verlusten in den Schweinebetrieben, deshalb ist eine genaue Charakterisierung dringend erforderlich.

Die Anlagerung von *M*. *suis* an die Oberfläche der Schweinerythrozyten findet mit Hilfe von fibrillären Strukturen statt. Der genaue Mechanismus ist derzeit noch nicht geklärt. Dennoch gelang es bereits, drei Oberflächenproteine (HspA1, MSG1 und α-enolase) mit Adhäsionseigenschaften zu identifizieren und im Detail zu untersuchen. Die korrespondierenden Gene und rekombinanten Genprodukte dienen dem diagnostischen Nachweis von *M*. *suis* in klinischen Proben (PCR, Serologie). Die derzeit effektivste Methode stellt hierbei die Echtzeit-Polymerasekettenreaktion (Rt-PCR) dar. Die Rt-PCR ermöglicht es, sowohl die chronische Verlaufsform mit geringen Bakterienzahlen als auch die akute Verlaufsform mit hohen Bakterienzahlen im Blut zu detektieren. Die auftretenden Krankheitssymptome beider Formen werden mithilfe von Tetrazyklin, Eisendextran und Glukose behandelt. Eine vollständige Eliminierung von *M. suis* aus dem Wirt wird hierbei jedoch nicht erreicht. Dies führt dazu, dass einmal infizierte Tiere lebenslang als Träger und Überträger von *M*. *suis* innerhalb und zwischen den Herden fungieren. Die genauen Übertragungswege von *M. suis* konnten bislang weder für die akute noch für die chronische Verlaufsform im Detail geklärt werden.

Ziele dieser Arbeit waren, ein Infektionsmodel im Schwein zu etablieren, dass den chronischen Verlauf der IAP darstellt, bisher unerkannte potenzielle Übertragungswege zu identifizieren und abschließend anhand Proteom-basierter Untersuchungen *M*. *suis*-

spezifische Wirts-Pathogen Interaktionen sowie die Wirtsabhängigkeit genauer zu untersuchen.

Als Ergänzung zu dem bereits sehr häufig erfolgreich verwendeten Schweinemodel (mit Splenektomie), dass die akute Verlaufsform einer natürlichen IAP widerspiegelt, gelang es uns ein neues Schweinemodel zu etablieren, dass die detaillierte Untersuchung von chronisch verlaufenden, phasenweise klinisch inapparenten *M. suis*-Infektionen erlaubt. Hierfür werden Ferkel ohne vorhergehende Splenektomie intramuskulär mit einem hochvirulenten *M. suis* Stamm infiziert. Bei den experimentell infizierten Ferkeln konnte neben Symptomen einer chronischen IAP (z.B. Morbus maculosus) auch der typische zyklische Verlauf der chronischen *M. suis*-Infektion beobachtet werden. Unter Verwendung der beiden beschriebenen Infektionsmodelle wurden potenzielle Übertagungswege von *M. suis* während der akuten bzw. der chronischen Verlaufsform der IAP untersucht. Dazu wurde von den experimentell infizierten Schweinen Kot, Harn, Nasen-, Vaginal- und Speichelsekret sowie Proben aus der Umgebung der Tiere (Wasser, Luft und Staub) gesammelt und mittels Rt-PCR auf die Anwesenheit von *M. suis*-DNA analysiert. Die Studien zeigten erstmals, dass *M. suis* offensichtlich sowohl über Harn (mit und ohne Erythrozyten) als auch über Nasen-, Vaginal- und Speichelsekret während der chronischen und der akuten Verlaufsform der Krankheit ausgeschieden wird. Somit ist klar, dass die Übertragung von *M. suis* Blut unabhängig über Körpersekrete und Harn möglich ist. Die Erforschung der Wirt-Pathogen Interaktionen und der spezifischen Anpassung von *M. suis* an seinen Wirt ist durch den nicht-kultivierbaren Status des Erregers limitiert. Um diese Probleme zu lösen, wurden moderne und hochentwickelte Proteomanalysen durchgeführt. Als Untersuchungsmaterial dienten Blutproben experimentell infizierter Schweine aus unterschiedlichen Infektionsstadien. Anhand dieser Proben gelang es, eine Anreicherungsmethode für *M. suis* Proteine (insbesondere Membranproteine) zu etablieren. Diese ermöglicht eine verbesserte Auflösung des Proteinexpressionsmusters von *M. suis* und gewährleistet tiefere Einblicke in dessen Pathogenese. So konnten trotz der Unkultivierbarkeit des Erregers ca. 50% der vorhergesagten *M. suis*-Proteine während einer akuten Phase der Infektion detektiert werden. Diese Proteinexpressionsdaten von *M. suis* sind aufgrund ihrer hohen Auflösung erstmals mit Proteomanalysen von kultivierbaren Bakterien vergleichbar. Des Weiteren zeigten die gewonnenen Daten, dass Nährstoffe wie Glukose, Hexose-6-Phosphat, Spermidin, Phosphat, Aminosäuren, Magnesium, Kalium, Natrium und Eisen, während einer *M. suis*-Infektion aus der Wirtsumgebung von *M. suis* aufgenommen werden. Die gewonnenen Erkenntnisse über die exprimierten potenziellen *M. suis*-Transportersysteme spielen insbesondere für die Entwicklung eines *in vitro*-Kultivierungssystems eine wichtige Rolle, da die Ergänzung eines essentiellen Nährstoffes in einem Wachstumsmedium zur Unterstützung des Wachstums, eine Schlüsselfunktion einnehmen kann. Neben den *M. suis*-

Proteomdaten konnten im Untersuchungsmaterial auch erstmals Daten zu unterschiedlich exprimierten *Sus scrofa* Proteinen während einer *M*. *suis* Infektion quantitativ erfasst werden. Diese Informationen können in weiteren Studien dazu genutzt werden, infektions-relevante Prozesse im Wirt besser zu verstehen.

3 INTRODUCTION

3.1 HEMOTROPHIC MYCOPLASMA

3.1.1 HISTORY

In 1928, the first anaplasma-like structures were detected by Schilling in blood smears collected from mice. The roundish particles on the surface of RBCs were named *Eperythrozoon coccoides* (Schilling 1928). Over the years Eperythrozoon-associated infections were described in different animals, e.g. sheep, goats and pigs (Moulder 1974; Zachary and Basgall 1985; Martin et al. 1988; Mason et al. 1989).

The first Eperythrozoon infections in pigs were described in 1932 in the USA (Doyle 1932). As symptoms of this new infection thin blood, anemia and hemorrhages in different organs such as liver, spleen or heart were observed (Doyle 1932; Kinsley 1932). According to the appearance of the organism in blood smears the disease was firstly named as anaplasmosis-like disease (Doyle 1932; Kinsley and Rey 1934). Due to the similarities to Anaplasma-like particles in cattles (*Eperythrozoon wenyonii*) as well as in sheep and goats (*Eperythrozoon ovis*) the pig-specific pathogen was classified as *Eperythrozoon suis* (Splitter and Williamson 1950). In Germany the first *E. suis* infection of pigs was described in 1968 during a suspicion of swine fever (Korn and Musgay 1968).

3.1.2 TAXONOMY

The taxonomic classification of HMs is still in progress. Based on 16S ribosomal ribonucleic acid (rRNA) gene sequence analysis and phenotypic characteristics, the genera *Eperythrozoon* and *Haemobartonella* out of the family *Anaplasmataceae* were transferred from the order *Rickettsiales* into the class *Mollicutes,* order *Mycoplasmatales* and family *Mycoplasmataceae* (Figure 1; Neimark et al. 2005). Since then, the organism *Eperythrozoon suis* is reclassified as *Mycoplasma suis*.

To date, in Bergey's Manual of Systematic Bacteriology (2012) HMs are taxonomically classified within the order *Mycoplasmatales*, family *Mycoplasmataceae* in an uncertain taxonomic position (incertae sedis; Uilenberg et al. 2004; Hoelzle et al. 2014).

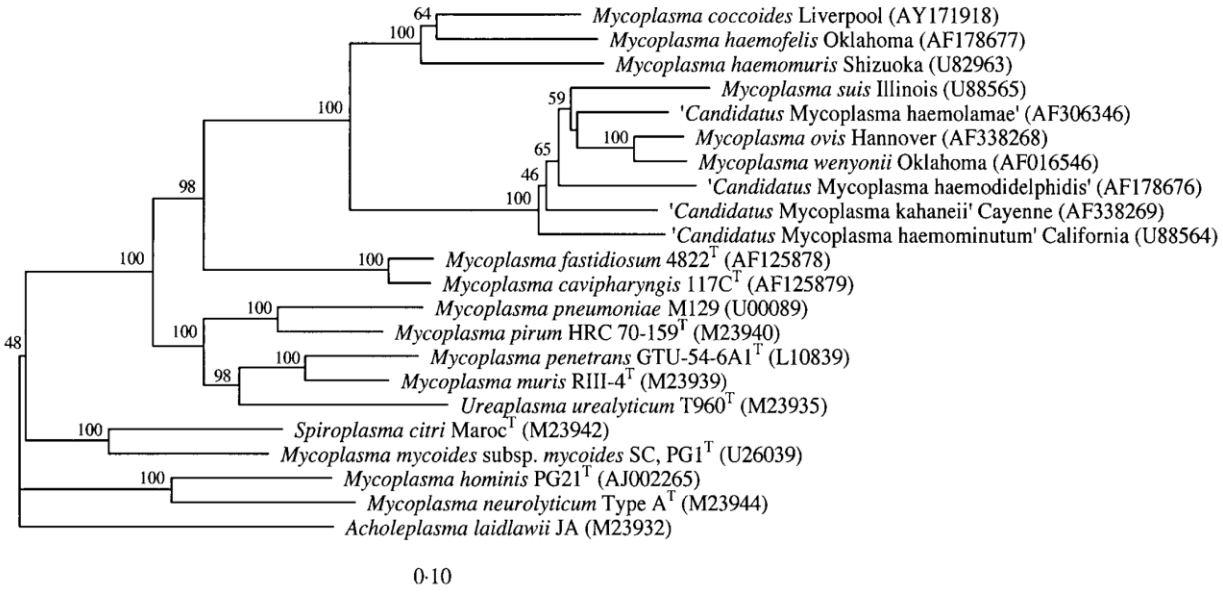

Figure 1: Phylogenetic tree of HMs based on 16S rRNA gene sequences (Neimark et al. 2005). Mapped is the relationship between *M*. *suis* and other HMs.

3.2 MYCOPLASMA SUIS

3.2.1 MICROBIOLOGICAL PROPERTIES

M. *suis* is a pleomorphic bacterium without a cell wall which is surrounded by a single membrane (Messick 2004). Electron microscopic analysis revealed that *M*. *suis* forms three different phenotypes, i.e. coccoid, discoid and ring structures (Zachary and Basgall 1985). *M*. *suis* is able to live freely in the blood plasma as well as attached individually or in chains to the surface of RBCs (Zachary and Basgall 1985). The bacteria are connected to the erythrocyte surface by fibrillary structures at a distance of approximately 30 nm (Zachary and Basgall 1985). This close contact between host cells and bacteria seems to be essential for the biology of *M*. *suis.* The unique parasitic lifestyle seems to be the main challenge for the establishment of a cultivation system for *M*. *suis* and other HMs. However, initial studies aiming on the cultivation of *M*. *suis* revealed culture persistence and nanotransformations (Schreiner et al. 2012a).

Furthermore, it is known that *M*. *suis* is capable to invade into RBCs. Invasion and attachment lead to cell deformation and apoptosis (Hoelzle et al. 2003; Groebel et al. 2009; Felder et al. 2011). However, to date the mechanisms involved in adhesion, invasion and replication of *M*. *suis* are not unraveled in detail.

Whole genome sequencing of two different *M*. *suis* strains (*M*. *suis* Illinois, *M*. *suis* KI_3806) revealed deeper insights into the biology. Their circular, double-stranded genomes are 742 kilo base pairs (kbp; *M*. *suis* Illinois) and 709 kbp (*M*. *suis* KI_3806) in size, corresponding genes encode 845 and 795 proteins, respectively (Messick et al. 2011; Oehlerking et al. 2011). According to a significant genome reduction, other parasitic mycoplasmas with comparable genome sizes lack several metabolic pathways (Pollack et al. 1997; Moran 2002). For *M*. *suis* these include genes for energy production by the pentose phosphate pathway as well as biosynthesis of amino acids i.e. the lack of the arginine biosynthesis pathway (Oehlerking et al. 2011; Felder et al. 2012). Nevertheless, *M. suis* and other mycoplasmas are self-replicating microorganisms (Messick et al. 2011; Barker et al. 2012).

3.2.2 INFECTIOUS ANEMIA IN PIGS

3.2.2.1 ETIOLOGY AND CLINICS

Chronic and acute IAP is a multi-factorial disease caused by *M*. *suis*. Physiological stress, e.g. climatic factors such as high humidity and elevated temperatures as well as stress situations for example during birth or castration may induce outbreaks of IAP (Wu et al. 2006). Infected animals suffer from acute life-threatening hemolytic anemia, mild hemolytic anemia or exhibit an asymptomatic infection depending on host susceptibility, pathogen virulence and infection dose (Heinritzi 1989; Guimaraes et al. 2011b). *M*. *suis* causes IAP in all age groups such as weaning piglets, pregnant sows and feeder pigs (Zachary and Basgall 1985; Messick 2004). In a study investigating 1176 samples derived from German post-weaning pigs representing approximately 196 pig fattening farms the prevalence of *M. suis* averaged 40,8% on pig farm level and 13.9% on single animal basis, respectively (Ritzmann et al. 2009). According to this finding, a similar study from Brazil indicated a *M. suis* prevalence of 18.2% based on single animals (Guimaraes et al. 2007). The high prevalence of *M. suis* infection in swine herds result in substantial economic losses to swine producers (Ritzmann et al. 2009).

Clinical signs of acute anemia are associated with heavy *M*. *suis* bacteremia and include fever of up to 42°C, skin pallor, icterus, hypoglycemia and cyanosis at the ear tips (Zachary and Smith 1985; Heinritzi 1990; Messick 2004). The decline of blood glucose occurs very quickly (Heinritzi 1990). During hypoglycemia animals suffer from muscular cramps and comatose conditions until they die (Heinritzi 1990).

The chronic course of IAP is signed by mild anemia, mild icterus, reduced reproduction rates and poor growth rates (Henry 1979; Gresham et al. 1994). Furthermore, allergic skin reactions, circular disorder and hemorrhage can be observed in *M*. *suis* chronic infected pigs (Henry 1979; Gresham et al. 1994). Pigs chronically infected with *M*. *suis* are predisposed to

secondary infections of the respiratory and intestinal tract (Henry 1979). These include porcine influenza, enzootic pneumonia in pigs, pasteurellosis, toxoplasmosis or porcine reproductive and respiratory syndrome (Wu et al. 2006).

3.2.2.2 EPIDEMIOLOGY AND PATHOGENESIS

M. *suis* is especially transmitted between pigs via blood-associated routes. In this connection the iatrogenic way seems to predominate. Especially contaminated surgical instruments (e.g. needles, scalpels) act as *M*. *suis* transmission pathways between pigs during vaccination or castration (Heinritzi 1990). Through bloody hierarchic encounters *M*. *suis* can be transferred among boars (Hoelzle et al. 2010). Furthermore, arthropods such as *Haematopinus suis*, *Stomoxys calcitrans* and *Aedes aegypti* are discussed in the agent transmission (Prullage et al. 1993; Messick 2004). Moreover, piglets can also be infected by the dam *in utero* or during birth (Henderson et al. 1997). The main challenge in epidemiology is associated with asymptomatically infected pigs. These animals remain unrecognized and act as carrier and transmitters within and between swine herds (Hoelzle et al. 2009).

Further information on *M*. *suis* pathogenesis are still required. Two main disease complexes are identified, i.e. anemia and hemorrhages.
In anemia, destruction of RBCs is caused by the close contact of *M*. *suis* to the surface of RBCs, invasion into the RBCs, eryptosis and the induction of autoreactive antibodies directed against RBC components, respectively (Zachary and Basgall 1985; Felder et al. 2010; Hoelzle et al. 2014).
The close parasitic contact from *M*. *suis* to the surface of RBC is possible by the expression of surface-localized membrane proteins. These proteins act as an essential juncture between the bacteria and the host. Only three *M*. *suis* surface proteins (heat shock protein A1 (HspA1), GAPDH-like protein 1 (MSG1), and α-enolase have been investigated in detail so far (Hoelzle et al. 2007b; Hoelzle et al. 2007c; Schreiner et al. 2012b). MSG1 and α-enolase are so-called “moonlight proteins” since they can perform two different functions in two different subcellular locations. Their main function is energy production by glycolysis (Hoelzle et al. 2007c; Schreiner et al. 2012b). Furthermore, α-enolase as well as MSG1 can be localized to the exterior of the cytoplasmic membrane and seem to be essential during the adhesion process (Hoelzle et al. 2007c; Schreiner et al. 2012b). Moonlight proteins could save significant amounts of energy in reproduction and growth, because the organism exhibits fewer proteins and consequently has fewer DNA which need to be replicated (Jeffery 1999).

Nevertheless, the close host-pathogen contact leads to RBC damage as well as lysis and eryptosis, respectively (Hoelzle et al. 2007c; Felder et al. 2011). Typical characteristics of eryptosis are membrane blebbing, activation of proteases and cell contractions (Felder et al. 2011). Until now, it has not been clarified by which mechanisms eryptosis is triggered during *M*. *suis* infection.

Interestingly, *M. suis* is capable of actively invading RBCs. This property seems to be important for the metabolic activity of these bacteria but nevertheless, lead also to RBC destruction (Groebel et al. 2009).

Autoreactive antibodies induced in *M*. *suis* infected pigs is a long known phenomenon in IAP (Zachary and Smith 1985; Hoelzle 2008) leading to autoimmune hemolytic anemia (AIHA; Felder et al. 2011). In IAP two different forms of AIHA are observed, i.e. cold and warm AIHA.

In the cold AIHA autoreactive antibodies bind to their target antigen at low temperatures (4°C) and in warm AIHA the target epitope is bound by the immune globulin (Ig) at body temperature (Felder et al. 2010). During both AIHAs the up-regulated autoreactive antibodies are directed against RBC components leading to erythrocyte death (Felder et al. 2010).

In detail, the RBCs undergo changes when *M*. *suis* attaches to their surface, which in turn induces IgM cold agglutinins during IAP (Zachary and Smith 1985; Hoelzle 2008). Cold agglutinins belong to the IgM isotope and are directed against carbohydrate antigens expressed on the erythrocyte surface (Zachary and Smith 1985). IgM autoantibodies exhibit maximal reactivity at 4°C. Therefore, certain body parts in which the body temperature is rather low, e.g. ears and tails are affected by blood agglutinations (Zachary and Smith 1985). Moreover, *M*. *suis* infection induce a misdirected immunoregulation and suppresses the T-lymphocyte blastogenic response (Zachary and Smith 1985). At the same time, the polyclonal B-lymphocyte activation and the production of autoreactive IgM (cold agglutinins) increase (Zachary and Smith 1985).

Warm autoreactive IgG antibodies are also formed during *M. sui*s infections. Interestingly, these autoreactive antibodies bind to the actin of RBCs which leads to erythrocyte damage (Felder et al. 2011). Additional investigations proved that these autoreactive antibodies are species-specific and are induced by a process that is known as molecular mimicry (Felder et al. 2011). So, the formation of these antibodies is induced by an epitope on the MSG1 protein on the surface of *M. suis* (Felder et al. 2011).

Moreover, IAP is associated with coagulation dysfunction as well as hemorrhagic diathesis (Sokoli et al. 2013).

M. *suis* triggers intravasal coagulation followed by consumption coagulopathy in its host. Sokoli and co-workers proved also the affection of endothelial cells (ECs) during *M*. *suis*

infection as a possible cause of hemorrhages (Sokoli et al. 2013). The results of the study showed that the endothelial cells of the abdominal aorta are infected and destroyed during *M*. *suis* infection leading to cellular gaps within the endothelium (Sokoli et al. 2013). At these sites aggregated blood cells infected by *M*. *suis* were identified as red dots, respectively as arterial thrombi (Sokoli et al. 2013). Additionally, it was possible to visualize a network of fibrin fibers in which RBCs and leukocytes are embedded (Sokoli et al. 2013).

3.2.2.3 DIAGNOSIS AND THERAPY

Beyond the detection of acute clinical apparent infections the main challenge is the identification of asymptomatically persistent infected pigs which can act as carriers and transmitters (Hoelzle et al. 2009). Therefore, direct and indirect diagnostic methods were established to diagnose *M*. *suis* infections in pigs.

A classical direct diagnostic method is the microscopic detection of *M*. *suis* in blood smears. For this, blood slides are heated up to 37°C to avoid agglutination and stained with acridine orange (Figure 2). However, detection is only possible during maximum bacteremia when more than 80% of the RBCs contain *M*. *suis*. Comparative *M. suis* prevalence studies showed that only 35 samples out of the 164 *M. suis*-PCR positive samples were also positive by microscopy (Ritzmann et al. 2009). Therefore, microscopy is limited by its low sensitivity and specificity (Hölzle et al. 2007a).

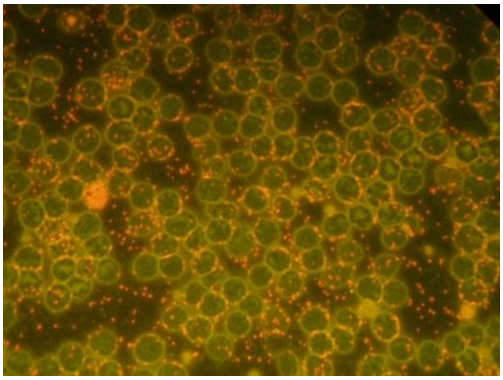

Figure 2: Microscopic picture of single *M*. *suis* cells and attached to RBCs during acute IAP in blood smears; 1000× magnification.

Today, PCR-based methods are the first choice in *M. suis* diagnosis since these approaches enable the detection of acute as well as of chronic infected pigs. The Light Cycler Rt-PCR represents a quantitative detection method (Hoelzle et al. 2007a). Due to its high degree of standardization and reproducibility as well as the low risk of contamination it is used in routine laboratory diagnostics. To achieve high sensitivity and specificity the gene sequence of the immunodominant MSG1 protein was used to generate specific primer and probes. The

test sensitivity amounts to 100% with a specificity of 97.6%. This approach is a powerful tool for studies examining the prevalence and pathogenesis of chronic and acute anemia infections in pigs (Hoelzle et al. 2007a).
Additionally, a Rt-PCR assay based on a TaqMan probe has been established (Guimaraes et al. 2011a). To obtain a highly sensitive and specific detection of *M*. *suis* the assay is based on the 16S rRNA gene encoded by *M*. *suis* strain Illinois (Guimaraes et al. 2011a).

Three different serological detection methods have been described: i) complement fixation test (Splitter 1958; Schuller et al. 1990) ii) indirect hemagglutination test (Baljer et al. 1989) iii) enzyme-linked immunosorbent-test (ELISA; Hsu et al. 1992; Hoelzle et al. 2006; Hoelzle et al. 2007d; Zhang et al. 2008).
ELISA was used for the detection of *M. suis*-specific serum antibodies. However, the major drawbacks of *M. suis* serology is the fact that during the clinically acute phase of the *M*. *suis* infection autoreactive antibodies and *M. suis*-specific serum antibodies appears simultaneously leading to a masking during the detection of *M. suis*-specific antibodies (Hoelzle et al. 2006). In addition, especially the cold agglutinins are constitutive parts in the *M*. *suis* test antigen preparations derived from infected and diseased pigs (Hoelzle et al. 2006). Therefore, the sensitivity and the specificity is limited in ELISAs using whole cell antigens from the blood of infected animals (Hoelzle et al. 2006). The depletion of immunoglobulins from the test antigen could improve the serodiagnosis by indirect ELISA (Hoelzle et al. 2006). However, further Western Blotting studies indicated that the immunodominant proteins p40, p45 and p70 can act as serological markers proving a *M. suis* infection (Hoelzle et al. 2006).
Therefore, two (p40, p70) out of the indicated three antigens were recombinantly expressed in *Escherichia coli* (Hoelzle et al. 2007d). Based on these diagnostic antigens it was possible to establish a highly sensitive and specific ELISA which is independent from animal experiments (Hoelzle et al. 2007d). Recombinant ELISA is a serological test which can be used in routine diagnostics in standard laboratories for pathogenesis studies and epidemiological researches (Hoelzle et al. 2007d).
In 2012 a novel, specific and sensitive blocking ELISA based on horseradish peroxidase (HRP)-labeled monoclonal antibodies (1A7) was established (Zhang et al. 2012).

3.2.2.4 INFECTION MODEL

Little is known about the pathophysiology of *M. suis*, due to the fact that an *in vitro* cultivation system is still missing. For the investigation of *M. suis* infections an *in vivo* model, the splenectomized *M. suis* pig model, is used. This infection model enables the experimental investigation of the acute *M. suis* infection. For this, five-week old piglets are splenectomized according to the method of Heinritzi (Heinritzi 1984a; Heinritzi 1992). The piglets are infected intramuscularly with *M. suis*-positive blood one week after splenectomy. The course of the disease and the clinical signs are comparable to natural infected animals suffering from acute anemia.

Many milestones of *M. suis* research are based on the well-established pig infection model which is used since more than 30 years. For example, the infection model was applied in several studies dealing with diverse aspects of *M. suis* infections, such as intra-uterine infections (Heinritzi 1992), the transmission by arthropods (Prullage et al. 1993), the attachment to RBCs and replication (Zachary and Basgasll 1985) and the identification of a misdirected immune response (Zachary and Smith 1985). Moreover, cold agglutinins in *M. suis*-infected pigs could be isolated (Schmidt et al. 1992) and the verification of antibodies during different time points of infection with the indirect hemagglutination test were carried out (Baljer et al. 1989). Furthermore, molecular biological studies on surface-proteins with adhesion properties could be performed without a cultivation system (Hoelzle et al. 2007b; Hoelzle et al. 2007c; Schreiner et al. 2011). Hoelzle and co-workers succeeded in the identification of anti-MSG1 and anti-HspA1 antibodies in pigs (Hoelzle et al. 2007d) and Groebel and co-workers detected the invasive behavior in RBCs (Groebel et al. 2009). Additionally, the establishment of an *in vitro* cultivating system was supported by results gained from the infection model (Schreiner et al. 2012a).

Nevertheless, at the moment there is no established pig infection model available which can be used to investigate chronic anemia in pigs. The establishment of a chronic *M. suis*-pig infection model would have some substantial benefits. Firstly, we could use the natural host resembling the natural infection. Secondly, we would have access to blood samples containing high numbers of *in vivo* grown *M. suis* at the time of bacteremia. In combination, both established infection models (acute, chronic) could allow us to investigate the pathogenesis, immunology, and clinics of acute as well as chronic IAP under standardized conditions in future. Therefore, the establishment of a second infection model is necessary.

3.3 OMICS

Omics technologies target the holistic analysis of a defined biological system at a given point of time based on the analysis of all detectable molecules of DNA, RNA, proteins or metabolites, namely genomics, transcriptomics, proteomics and metabolomics (Figure 3). Genomics deals with the complete DNA sequence of an organism and serves as the blueprint of life (Lee et al. 2005). Transcriptome analysis only deals with expressed genes based on RNA detection (Lee et al. 2005). Protein profiles (proteomics) of organisms are analyzed by gel electrophoresis coupled with liquid chromatography and tandem mass spectrometry (LC-MS/MS; Lee et al. 2005). Metabolomic analysis is based on whole sets of metabolites at a certain time point. They are usually measured by gas chromatography coupled to tandem MS (GC-MS/MS) or nuclear magnetic resonance (NMR; Lee et al. 2005).

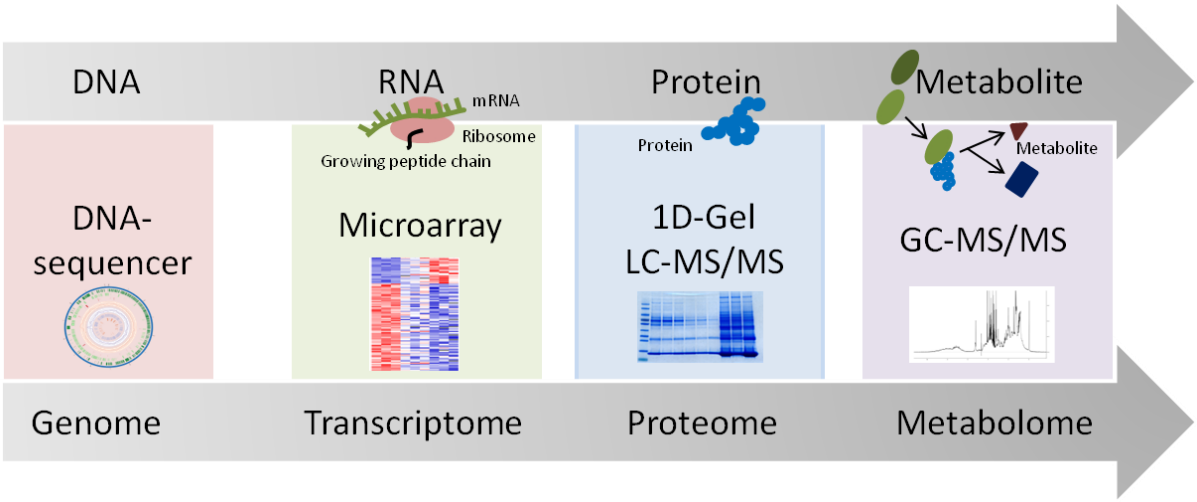

Figure 3: The field of omics-methods. Genomes are sequenced by DNA sequencer. Microarrays read mRNA-profiles to detect the transcriptome, whereby proteome reflect all expressed proteins measured combined by gel electrophoresis and LC-MS/MS analysis. In metabolome profiling GC-MS, LC-MS and NMR technologies are used.

3.3.1 PROTEOME ANALYSIS

Proteome analysis is used to gain insights into the biological mechanisms at the molecular level (Roe and Griffin 2006). They are especially performed to identify single proteins, but it is also possible to obtain information on protein modification, localization, protein-protein interactions and their functional roles (Fields 2001). The proteome is a dynamic collectivity interlocked by expressed proteins at a precise point in time in an organism, cell or tissue (Lee et al. 2005).

In proteome analysis, “gel-free” 1-dimensional electrophoresis (DE) or gel-based 2-DE can be used to separate proteins according to their biochemical properties. A lot of proteome

studies applied 2-DE for the fractionation of complex samples, but for insoluble proteins this method is of limited value (Old et al. 2005). Separated proteins from 1-DE are isolated and digested by trypsin. Subsequently, the specific spectra of peptides are measured by LC-MS/MS (Lee et al. 2005).

For the MS-based proteome analysis of complex samples, MS has progressively become the method of choice, distinguished by a Nobel Prize in chemistry in 2002 (Aebersold and Mann 2003). MS measurements scale the mass-to-charge ratio (*m*/*z*) of the ionized analyte and a detector registers the number of ions at each *m*/*z* value (Aebersold and Mann 2003). The standard types of mass analyzers are: time-of-flight (TOF), ion trap, Fourier transform ion cyclotron and quadrupole which are routinely used (Aebersold and Mann 2003).

To get more insights into *M*. *suis* pathogenesis, proteome analysis were performed. The first *M*. *suis* proteome report was published in 2009. In this study, 191 out of 794 (24.1%) encoded proteins could be detected by cross-species-identification missing a fitting protein database (Yuan et al. 2009).

The first proteome study based on specific HM genome sequences provided basic knowledge about the *M. suis* gene expression profile during an acute HM infection. In this study, 179 out of 794 encoded *M. suis* proteins (22.5%) were found to be expressed (Felder et al. 2012). The identified proteins mainly include well-defined proteins of the energy, carbohydrate and nucleotide metabolism, translation, transcription, DNA repair and some putative virulence factors (Felder et al. 2012). Only a few of the many encoded hypothetical and surface *M. suis* proteins were found to be expressed during infection by this approach (Felder et al. 2012).

4 OBJECTIVES

M. *suis* is found worldwide in the pig industry as an important cause of IAP in different age classes. An efficient diagnostic method for *M*. *suis* is the quantitative Rt-PCR. The quantitative *M*. *suis* diagnostic tool works with high sensitivity and specificity. Therefore, it can be applied during acute IAP (up to 10^6 - 10^9 *M. suis* cells/ml blood) as well as during chronical infections (up to 10^3 – 10^5 *M. suis* cells/ml blood). Chronically *M. suis* infected pigs cause significant economic losses in swine production due to growth retardation rates, reduced reproduction efficiency of sows and an increased susceptibility to secondary infections (viral, bacterial).

To get further information on the pathogenesis of the chronic persistent *M. suis* infection the corresponding pig infection model has to be established.

In addition, using experimental infection pig models (acute, chronic) investigations on possible transmission pathways of *M. suis* can be performed. Until now, only blood-associated transmission pathways have been detected for *M. suis*. However, further shedding patterns are probable.

Furthermore, bacterial preparation from the *M. suis*-pig infection model contains large numbers of *M. suis* bacteria as well as several amounts of porcine components. Based on the genome sequencing it is possible to gain insights into the gene expression profile of *M*. *suis* and *S. scrofa,* respectively. Proteomic studies help to understand the mechanism *M. suis* uses to cause disease and to establish persistence in the host. The expressed membrane-associated proteins of *M. suis* are important key players in the uptake of nutrients (transporters) from the RBCs and the interaction between the pathogen and the host. This knowledge could be helpful to establish a cultivating system for *M*. *suis*, and to get new available therapies as well as prophylaxis methods.

The aim of this work was to establish a chronic-infection pig model, to identify further transmission pathways of *M*. *suis* and to update the single existing proteome profile to gain more knowledge about the pathophysiology of *M. suis*.

The objectives are listed in detail as follows:

4.1 Establishment of a chronic IAP simulated under experimental conditions

- The characterization of the course of experimental *M*. *suis* infection in splenectomized and non-splenectomized pigs using the highly virulent and RBC-invasive strain KI_3806.
- Determine whether both, chronic and acute forms of IAP, can be simulated under experimental conditions.

Major conclusions:

- Experimental infection with the highly virulent and invasive *M*. *suis* strain KI_3806 in non-splenectomized pigs lead to chronic IAP.
- Well-established infection models are important for further pathogenesis studies in *M. suis*.
- Clinical and hematological parameters allow insights into the host immune response during acute and chronic IAP.

These topics are addressed by the following article:

Stadler J, Jannasch C, Mack SL, Dietz S, Zöls S, Ritzmann M, Hoelzle K, Hoelzle LE. Clinical and haematological characterisation of *Mycoplasma suis* infections in splenectomised and non-splenectomised pigs. Vet Microbiol. 2014 Aug 6;172(1-2):294-300. doi: 10.1016/j.vetmic.2014.05.012. Epub 2014 May 13.

Link: http://www.sciencedirect.com/science/article/pii/S037811351400248X

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4.2 Investigation of potential transmission routes of *M. suis*

- Characterization of *M. suis* shedding during experimental infection in order to get insights into potential transmission pathways.

Major conclusions:

- *M. suis* shedding is found in urine, as well as salivary, nasal and vaginal secretion but not in feces.
- Blood-independent direct transmission and indirect transmission via environmental contamination may play a role in the epidemiology of *M. suis* infections

These topics are addressed by the following article:

Dietz S, Mack SL, Hoelzle K, Becker K, Jannasch C, Stadler J, Ritzmann M, Hoelzle LE. Quantitative PCR analysis of *Mycoplasma suis* shedding patterns during experimental infection. Vet Microbiol. 2014 Aug 27;172(3-4):581-5. doi: 10.1016/j.vetmic.2014.06.019. Epub 2014 Jul 2.

Link: http://www.sciencedirect.com/science/article/pii/S0378113514003113

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4.3 Mechanisms used by *M. suis* to cause disease and to establish persistence in the host

- Comprehensive proteome analysis of *M. suis*-infected porcine blood from samples containing low and high bacterial loads.
- Establishment of an improved protocol for proteome analysis of *M. suis* derived from EDTA-blood samples.
- Identification of expressed *M. suis* transporter system during acute IAP.

Major conclusions:

- The improved protocol led to the identification of 404 *M. suis* proteins in samples containing high bacterial loads.
- Surface-localized proteins which act as connecter between host and pathogen could be predicted by software tools and identified by proteome analysis.
- Identification of membrane proteins indicate that several nutrients are taken up by *M. suis* during IAP.

These topics are addressed by the following article:

Dietz S, Lassek C, Mack SL, Ritzmann M, Stadler J, Becher D, Hoelzle K, Riedel K, Hoelzle LE. Updating the proteome of the uncultivable hemotrophic *Mycoplasma suis* in experimentally infected pigs. Proteomics. 2016 Feb;16(4):609-13. doi: 10.1002/pmic.201500238.

Link: http://onlinelibrary.wiley.com/doi/10.1002/pmic.201500238/abstract

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5 PUBLICATIONS

The scientific work especially in the leader author publications were realized by S. Dietz and supervised by L. E. Hoelzle. Conception as well as preparation of all three manuscripts was done in team work with the mentioned authors.

5.1 Clinical and haematological characterisation of *Mycoplasma suis* infections in splenectomised and non-splenectomised pigs

Veterinary Microbiology 172 (2014) 294–300

Abstract

The hemotrophic bacterium *Mycoplasma suis* provokes infectious anemia in pigs (IAP). IAP is clinically described by chronic mild or acute life-threatening hemolytic anemia. The inability to culture *M*. *suis* necessitate the experimental infection of splenectomized pigs for detailed pathogenesis studies. Aim of the present study was to establish a pig infection model remodeling the chronic course of infection. For this, the experimental course of the disease in splenectomized and non-splenectomized pigs was compared to clarify the interdependency between clinical and hematological parameters and the *M*. *suis* blood loads. Therefore, we infected two groups of pigs using the highly virulent and red blood cell invasive *M*. *suis* strain KI_3806. On day 2 post infection (p. i.), all 7 pigs of group 1 (splenectomized) were proved as PCR-positive. The maximum mean bacterial loads were reached on day 8 p. i. with concentrations of 1.6 x 10^{10} *M*. *suis*/mL. Clinical symptoms such as severe anemia as well as massive hypoglycemia could be observed, hence the pigs were euthanized. All pigs of group 2 (non- splenectomized) were PCR-positive at least on day 23 p. infection. Maximum mean bacterial loads of 1.64 x 10^{5} *M*. *suis*/mL were found on day 8 p. infection. Until day 35 p. i. all animals exhibited typically clinical symptoms, i.e. mild anemia, massive skin alterations with petechiae, hemorrhagic diathesis. Furthermore, seroconversion could be detected in blood samples.

These results demonstrate a mild form of IAP in non-splenectomized pigs infected with the highly virulent strain KI_3806 that is similar to the clinical situation found during natural infections. On contrary, infection of splenectomized pigs with KI_3806 leads to severe and life-threatening IAP. In summary, both infection models evaluated in this study can be used for further pathogenesis research of chronic and acute IAP.

5.2 Quantitative PCR analysis of *Mycoplasma suis* shedding patterns during experimental infection

Veterinary Microbiology 172 (2014) 581–585

Abstract

Mycoplasma suis belongs to the uncultivable hemotrophic mycoplasmas and induces infectious anemia in pigs. Transmission pathways as well as transmission mechanism between pigs are mostly unknown. To get deeper insights into transmission pathways of *M. suis*, we analyzed environmental samples, urine, feces as well as nasal, vaginal and saliva secretions for *M*. *suis* DNA by quantitative *LightCycler* msg1 PCR. For this, non-splenectomized pigs were experimentally infected with the highly virulent *M. suis* strain KI_3806. On day 6 post infection, *M*. *suis* DNA was detected in saliva, nasal and vaginal secretions, with a quantity of 3.4 x 10^2 to 2.7 x 10^5 *M. suis*/swab. Shedding was found in all urine samples (100%) from day 6 p. i., with a quantity of 4.7 x 10^2 to 6.3 x 10^5 *M. suis*/mL. Moreover, *M*. *suis* DNA could be detected on day 2 and 6 p. i. in dust and water samples of the pig drinking troughs. However, in all feces and air-samples *M. suis* DNA could not be detected at all. These results indicate that blood independent transmission pathways (indirect and direct) could play an important role in the epidemiology of *M. suis* infections in pig farms.

5.3 Updating the proteome of the uncultivable hemotrophic *Mycoplasma suis* in experimentally infected pigs

Proteomics 16 (2016) 609–613

Abstract

The uncultivable bacterium *Mycoplasma suis* is one member of the hemotrophic mycoplasmas which are associated with chronic and acute anemia in livestocks worldwide. Up to now, it is not possible to culture *M. suis in vitro* complicating the characterization of *M. suis* on the molecular level. Only one proteome study based on the *M. suis* KI_3806 genome has been published so far. In the present comprehensive proteome study we used three different *M. suis* protein extraction methods followed by 1D SDS-PAGE and LC-MS/MS. In total, 50.8% of the predicted *M. suis* proteins (404 out of 795) were identified. Moreover, 83.7% of the detected *M. suis* proteins could be assigned to functions. Interestingly 34.2% of the encoded *M. suis* hypothetical proteins could also be proved as expressed during acute infection. Further detailed computational analyses revealed information that some of these expressed hypothetical proteins are membrane-associated which could be involved in host-pathogen interaction or adhesion process. In addition, analyses of the expressed proteins indicate the presence of one ECF-transporter as well as a hexose-6-phosphat-transporter. Taken together, these proteome results are important steps to unravel the specific and unique life style of *M. suis* and help to establish an *in vitro* culture system in future.

6 SUMMARISING DISCUSSION

The lack of an *in vitro* cultivation system for HMs is one of the main challenges to obtain insights into their pathobiology.

Until now the establishment of an *in vitro* cultivation system for *M*. *suis* was not successful (Hoelzle 2008). Only one study succeeded to maintain *M*. *suis* in a cell-free medium (Schreiner et al. 2012a). This medium contained iron-supplements (porcine hemin, hemoglobin) since in many cases iron is the limiting factor for bacterial growth (Schreiner et al. 2012a). *M. suis* metabolism is apparently glucose-dependent, based on the fact that life-threatening hypoglycemia is one general clinical sign of IAP (Heinritzi 1990). Therefore, glucose-supplemented media were tested for growth of *M*. *suis* (Schreiner et al. 2012a). Nevertheless, the growth conditions seem to be suboptimal since the *M*. *suis* cells underwent transformation processes leading to so-called nanoforms (Schreiner et al. 2012a).

Due to this fact HM research is crucially based on the usage of experimentally infected animals for the propagation of these specialized bacteria. However, most HMs exhibit strict host specificity which means that we have to use homologous animal models.

Experimental chronic pig model

Typically, experimental *M*. *suis* infections are performed with six-week old *M*. *suis*-free piglets. One week after splenectomy (Heinritzi 1984a) piglets are intramuscularly infected using *M*. *suis*-positive blood. This model based on splenectomized piglets is very useful to induce the acute course of IAP (Hoelzle et al. 2006). In addition, animals produce high bacterial loads rising up to 10^{10} bacteria per ml blood on day 8 or 10 post infection (p. i.; Figure 4A).

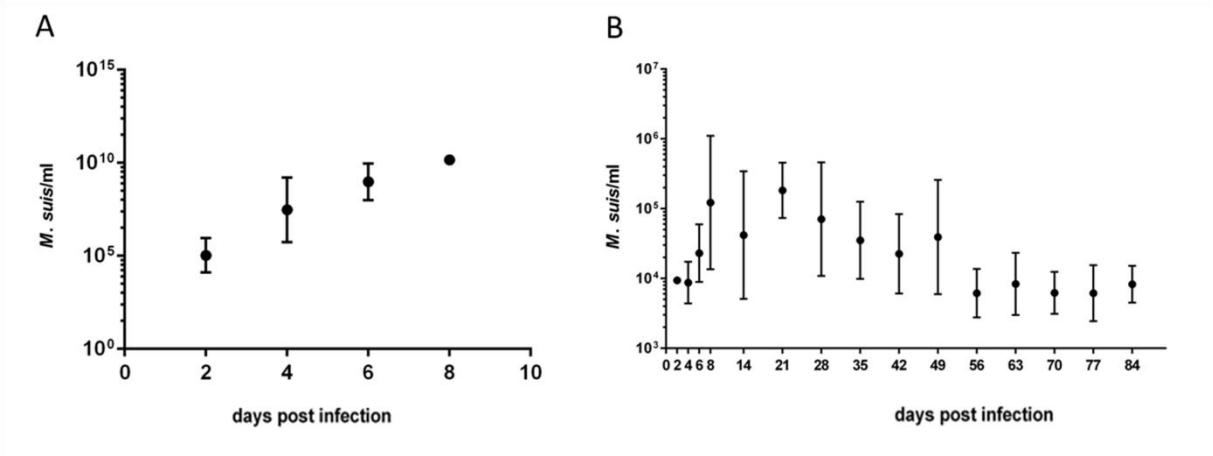

Figure 4: Bacterial loads in porcine blood samples during experimental infection with *M. suis* strain KI_3806 measured by Rt-PCR. A) Acute experimental infection model (n=7), B) chronic experimental infection model (n=7).

With regard to animal welfare, pig scoring systems are used to evaluate the clinical conditions after experimental *M. suis* infection and to define interruption criteria for the experiments (Stadler et al. 2014). Based on these scores, animals exceeding three scoring points per day are treated with glucose and tetracycline; a scoring higher than 5 leads to the euthanasia of the affected piglets.

As aforementioned, chronic *M. suis* infections in pigs are very important with the regard of epidemiology and economic losses. Therefore, the aim of this work was to establish a pig infection model which is suitable to simulate and investigate the chronic course of infection.
For this a novel model based on non-splenectomized pigs was established. Six-week old piglets were infected intramuscularly with a highly virulent and invasive *M. suis* strain (KI_3806). By doing this, the experimentally infected animals exhibited clinical symptoms typical for the chronic course of the *M. suis* infection, i.e. mild anemia and Morbus maculosus (Figure 5A, B, C).

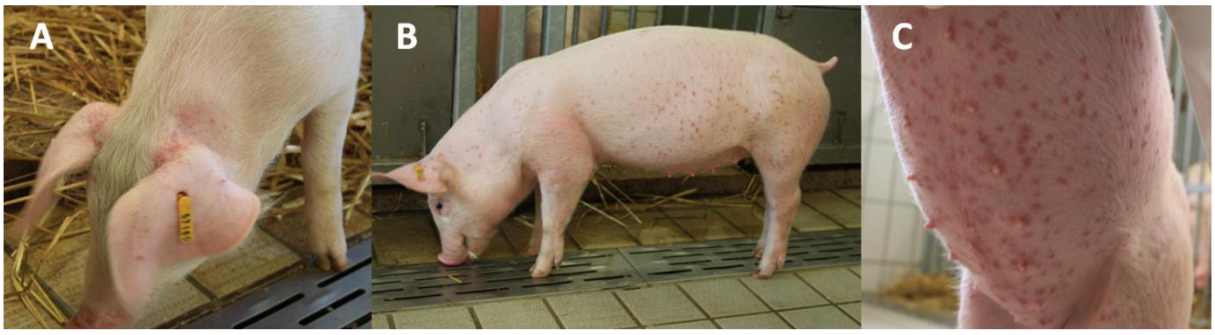

Figure 5: Clinical symptoms (Morbus maculosus) A) at the ears, B) the flanks and C) the belly of *M. suis* infected pigs

Natural chronic IAP is characterized by cyclic variations of bacterial loads, i.e. anemia attacks with high bacterial numbers in the blood are followed by low bacteremia phases (Zachary and Smith et al. 1985, Heinritzi et al. 1990a; Heinritzi et al. 1990b). Interestingly, these cyclic variations of bacterial loads in blood samples could also be observed in our chronic infection model (Figure 4B).
We found experimentally infected animals even with phases in which *M. suis* could not be detected in the blood stream (Figure 6A, B). Nevertheless, in these periodically *M. suis* blood negative animals bacterial levels increased after a few days leading even to anemia attacks. This periodically course of the bacterial blood level is a typical observation of chronic IAP and could be demonstrated also in our study (Zachary and Smith et al. 1985, Heinritzi et al. 1990a; Heinritzi et al. 1990b).

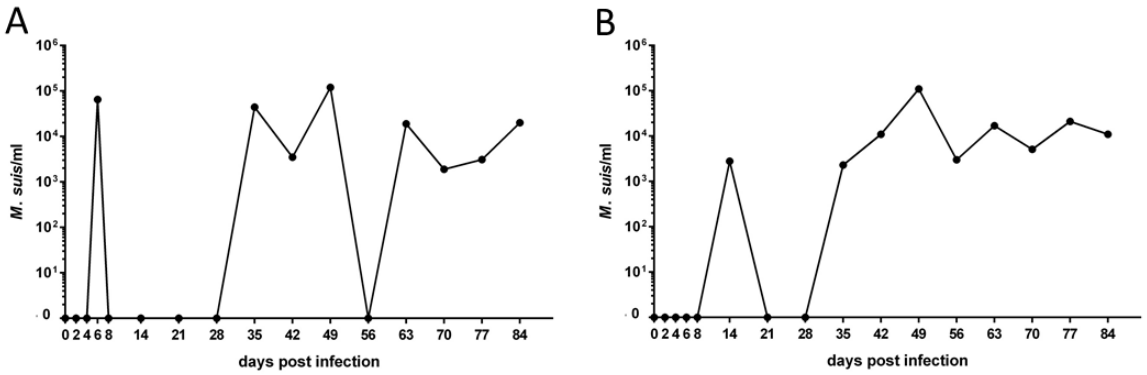

Figure 6: Bacterial loads in blood samples from two experimentally infected pigs (A, B) during the chronic course of IAP. The animals were infected by *M. suis* strain KI_3806 and bacterial loads were measured by Rt-PCR.

The localization of *M. suis* during these blood PCR-negative time points has not been clarified yet. However, we suggest some similarities to the lifestyle of *Plasmodium falciparum*. This organism also parasites the RBCs of his host, uses the bloodstream to spread throughout the host and settles in tissues (Prudêncio et al. 2006). *P. falciparum* migrates to the liver, where it can infect the hepatocytes (Prudêncio et al. 2006). For *M. suis*, the invasion into erythrocytes is already verified and the bacterial load can be measured in blood samples. Based on the periodically observed negative results for *M. suis* in blood during experimental infection, it can also be speculated that *M. suis* may be hidden in cells of the viscera during the blood negative periods. This hypothesis is supported by the fact that in immunohistochemical investigations *M. suis* could be detected in liver, mesenteric lymph nodes, heart and lungs associated with massive alterations in the tissue structure (Sokoli et al. 2013). In addition, macroscopic organ changes such as brightened or blemish livers, hyperemia, as well as single randomized beige foci up to 5 mm in diameter or single white foci could be observed in experimentally *M. suis* infected pigs (unpublished data).

The major energy source of *M. suis* seems to be glucose which is synthesized in the liver by gluconeogenesis. Additional nutrients such as iron, amino acids, sodium, calcium, magnesium and potassium are also available in the liver environment. It is possible that the invasion in liver cells represents a silent stage of infection.

In summary, the established novel chronic infection model provides some substantial benefits; first it enables the investigation of chronic infection during natural conditions (natural host) over a long period of time. Secondly, the mechanisms leading to the economically important persistent *M. suis* infections can be monitored. In combination with the acute infection model (experimental infection after splenectomy) the novel model simulating the

chronic course of infection is very worthwhile for the clarification of the *M. suis* pathobiology in future.

Transmission

One essential prerequisite in fighting against transmissible pathogens is to know all the possible epidemiological pathways that can be used by this infectious agent.

It is well known that during *M. suis* infections blood-associated pathways are of central importance. As vehicles act contaminated needles and scalpels, but also arthropods are discussed (Heinritzi 1990; Prullage et al. 1993; Messick 2004). Moreover, animals can be infected by direct blood transfer during bloody rank fights (Henderson et al. 1997; Hoelzle et al. 2010). These transmission pathways, especially the iatrogenic as well as vector based transmission pathways are efficiently reduced within the modern pig husbandries. Nevertheless, *M. suis* infections are an emerging problem in pig production. Therefore, we suggest that other pathways have to be of epidemiological significance.

In order to clarify this, the second aim of the study was to identify potential blood-independent transmission pathways also in acute and chronic *M*. *suis* infections. This was enabled by the availability of both described infection models (Heinritzi 1984b; Hoelzle et al. 2003; Stadler et al. 2014).

In samples gained from acute infected pigs, we identified and quantified *M*. *suis* DNA in nasal, vaginal and saliva swabs as well as in urine samples from day 6 p. infection. We observed that *M*. *suis* detection in secretions and urine correlated with the bacterial load in blood samples. Therefore, we suggest that blood-independent shedding of bacteria is obviously nonetheless coupled with high bacterial blood loads.

The course of chronic infection is more common in naturally infected pigs and is characterized by low bacteremia. However, employing the chronic model enabled the chronological sampling and analysis of nasal, vaginal and saliva swabs as well as urine samples for 84 days p. infection. We identified that an excretion via these secretions is isolated and rarely possible during chronic IAP in single pigs on days 6, 21, 28 and 42 p. i. (unpublished data).

For the first time our results indicate the excretion of *M. suis* via blood-independent transmission pathways including different secretions (nasal, vaginal and saliva) as well as urine during chronic IAP.

The excretion via vaginal secretions could also be responsible for *M*. *suis* transmission. A transmission via vaginal secretions could already be observed for *Streptococcus suis* during parturition (Robertson et al. 1991). Interestingly, it was shown that *M*. *suis* transmission from dam to unborn piglets *in utero* was first described a few years ago (Henderson et al. 1997).

Nevertheless, it should be clarified whether the transmission occurs via blood or by oral ingestion of vaginal secretions during parturition.

Excretion via saliva could represent an important transmission pathway of *M*. *suis*, especially in boars. Boar fattening is associated with hierarchic encounters and aggressive behavior including biting and pressing against the cages (Cronin et al. 2003). Furthermore, tail biting and ear biting constitute abnormal social behavior in pigs and could also be responsible for transmissions of *M*. *suis* through infected saliva (Brunberg et al. 2011). Moreover, we observed that after *M. suis* infection water samples from pig drinking thoughts were tested positive (Dietz et al. 2014). We hypothesize that contamination of pig drinking thoughts by *M*. *suis* excretions via saliva, could represent a potential transmission pathway especially in the group housing of pig farming.

Saliva is also used for diagnostic purposes due to its ready accessibility and ease of collection (Aps and Martens 2005). Consequently, saliva plays an important role in transmission for other pathogens. Nevertheless, our study indicates that the excretion during high bacterial loads seems to be coupled to the shedding of *M*. *suis* via secretions. This underpins the hypothesis that a bacterial load of 10^7 *M*. *suis* cells/ml blood constitutes the threshold value for excretion. Consequently, the detection of *M*. *suis* DNA in saliva and nasal secretion samples for diagnostic purposes are insufficient at this stage of infection. Therefore, blood sampling is still necessary to safely diagnose *M*. *suis* infections in pigs.

Interestingly, against former research studies (Heinritzi 1992), we could also detect *M. suis* in RBC-free urine from acute and chronically infected pigs. Therefore, RBCs-free urine can also be considered as a potential transmission pathway since pigs drink each other's urine.

Our results underpin the hypotheses that *M*. *suis* transmission during acute and chronic IAP can also occur blood-independent. These blood-independent transmission pathways via saliva, vaginal secretions and urine may take place during standard pig farming, i.e. parturition or feeding. Based on our assumptions we suggest that the epidemiological variations of *M*. *suis* was underestimated during the last decades. These results represent a milestone in the investigation of the *M. suis* epidemiology and shed light on further *M*. *suis* transmission pathways besides blood and arthropods.

Proteome analysis

The sequencing of genomes from pathogens has opened the doors for proteome analysis (Hanash 2003). The comparative investigation of the proteome of a pathogen under specified conditions (environment, course of infection etc.) can help to acquire knowledge about the differential expression of the genes within these certain situations. Therefore, proteomic analysis can be used to identify new biomarkers, and for a better understanding of the pathobiology as well as to support drug evolution (Hanash 2003). Only a few proteome

studies on cultivated mycoplasmas are published until today. These include *M*. *genitalium* strain G37, *M*. *hyopneumoniae* strain 232 and *M*. *mobile* strain 163K (Wasinger et al. 2000; Jaffe et al. 2004; Pendarvis et al. 2014). For these strains 33%, 70% and 80% of the predicted proteins could be detected, respectively (Wasinger et al. 2000; Jaffe et al. 2004; Pendarvis et al. 2014).

To deepen our knowledge about the gene expression of *M. suis* during acute infection the third goal of this work were proteomics-based investigations of *M. suis*. To this end, we performed proteome analysis of the *M*. *suis* strain KI_3806 on day 4 and day 8 p. i. (Figure 7).

A previous proteome study of *M*. *suis* from Felder and co-workers (2012) applied a *M*. *suis* enrichment prior to protein analysis. Based on this work we improved the *M*. *suis* enrichment protocol and complemented proteome analysis by two additional protein fractions to achieve higher number of protein identifications during the different phases of infection.

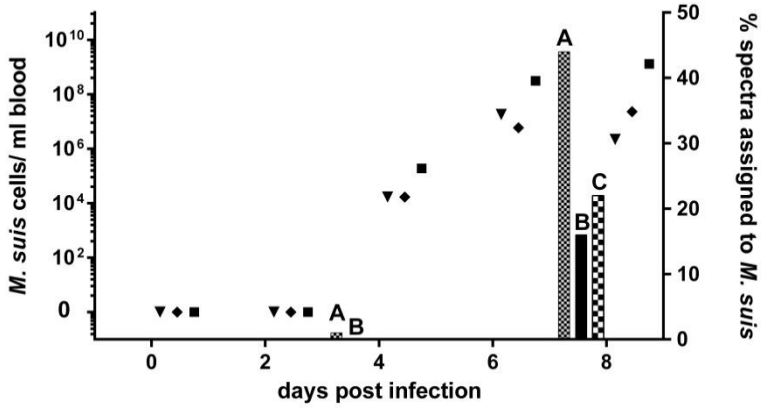

Figure 7: The diagram represents the bacterial load of porcine EDTA-blood samples on day 4 and 8 p. i. from three biological replicates measured by Rt-PCR (left x-axis). Furthermore, it depicts assigned spectra (%) detected by LC-MS/MS from three different methodological approaches targeting A) the enrichment of *M*. *suis* cells, B) the soluble and C) the insoluble fraction (right x-axis).

Using this sample preparation approach we succeed to analyze the proteome profile of *M*. *suis* during acute IAP at day 8 p. infection. By this 404 of 795 *M*. *suis* proteins could be identified which correspond to 52% of all encoded proteins. It is noteworthy that this identification ratio is in accordance with GeLC-MS/MS protein identification levels for other cultivable mycoplasmas (Wasinger et al. 2000; Jaffe et al. 2004; Pendarvis et al. 2014). In this approaches bacterial cells can be purified from pure cultures.

Unfortunately, our approach did not worked to analyze the *M*. *suis* specific expression pattern in the early phase of infection. This is especially due to the fact that in this infection period only low numbers of bacteria can be found in the blood. Therefore, the ratio between the expressed *M. suis* proteins and the expressed host proteins is in disfavor to the *M. suis* proteins.

So, only nine *M*. *suis* proteins (eight from the *M*. *suis* enriched fraction, one from the buffer soluble fraction, and zero from the buffer-insoluble fraction) could be detected. These proteins are highly abundant *M*. *suis* proteins such as DNA directed RNA polymerase subunit beta (detected in two fractions), elongation factor Tu and G, chaperone protein DnaK, glyceraldehy-3-P dehydrogenase, 2,3-bisphosphoglycerate-independent phospho-glycerate mutase, pyruvate kinase and MgpA-like protein. These results demonstrate that the modern and sophisticated proteome analysis suffers from limitations, i.e. the significant amounts of *S. scrofa* proteins overwhelm *M*. *suis* proteins and thereby their identification.

To overcome the detection limit of the system an *M*. *suis*-specific enrichment should be established in future. One possibility is the inclusion of an *M*. *suis* enrichment step into the described protocol (Dietz et al. 2015). One possible option for the enrichment could be the use of beads coated with monoclonal antibodies directed against *M*. *suis* surface antigens (e.g. pluriBeads; pluriselect, Leipzig, Germany).

Nevertheless, the pig related dataset enables the quantitative identification of host proteins also during the early phase of infection and is a reliable and worthwhile basis to get further information about the misdirected host immune response in IAP.

Comprehensive proteome results were used to unravel host pathogen interactions and host adaptation of *M*. *suis*. Our study proved that glycolysis seems to be the major energy generating pathway in *M*. *suis*. However, this leads to a life-threatening hypoglycemia and to a lactate acidosis (Heinritzi et al. 1991c; Hoelzle et al. 2014). After glycolysis, the lactate dehydrogenase catalyzes the formation of lactate out of pyruvate. Lactate is thought to be exported out of the *M*. *suis* cytoplasm into the blood which leads to a drop of the blood pH from 7.4 to 7.1, which indicates a metabolic acidosis (Heinritzi et al. 1990c).

So far, it is unclear how *M*. *suis* generates energy during the intracellular lifestyle in the RBCs. Nevertheless, we suggest that the hexose-6-phosphate-transporter is involved in the uptake of phosphorylated glucose during the intracellular lifestyle in premature as well as in mature RBCs (Figure 8). In *Listeria monocytogenes* the loss of a hexose-phosphate-transport protein results in impaired intracytosolic proliferation and attenuated virulence in mice (Chico-Calero et al. 2002). Future experiments have to clarify the importance of the hexose-6-phosphate-transporter in *M*. *suis*.

Interestingly, our PATRIC analysis revealed that the annotated cobalt ABC-transporter seems to be an energy-coupling factor (ECF)–transporter (Figure 8). The most significant difference between ABC- and ECF-transporters is that ECF-transporters do not have extra-cytoplasmic binding proteins. Instead, substrate binding is carried out by one (S component) of the two membrane localized proteins (S and T components; Song et al. 2013). Transport

is energized by two ATP-binding cassette-containing proteins (ABC proteins; Song et al. 2013). However, the associated genes of the mentioned *M*. *suis* ECF-transporter consist of one T and two ABC components and do not have adjacent candidate S-component genes. Group II ECF-transports are accompanied by different candidate S-component genes scattered randomly throughout the genome (Song et al. 2013). Some genomes encode more than 12 different S-component genes but only one or two copies of the energy-coupling ABC-T module (Song et al. 2013). The vast majority of these candidate S components probably act on new substrates. Common substrates are e.g. nickel, cobalt, biotin, methylthioadenosine, riboflavin, folate, pantothenate, thiamine, thiazole, niacin, tryptophan, pyridoxine, lipoate as well as the precursors of cobalamin, thiamine, methionine and queuosine (Song et al. 2013). We suggest that the modularity of the group II ECF-transporter also contributes to a broad substrate spectrum in *M*. *suis*, since only one S component for the uptake of folate could be identified by PATRIC annotations.

Information about the iron-uptake in mycoplasmas is still limited. Comparative genome analysis by PATRIC revealed that *Ureaplasma parvum* represents a comparable genome locus for iron uptake when compared to *M*. *suis* (unpublished data). The fact that a siderophore uptake system is encoded and expressed during infection suggests that *M*. *suis* is capable of synthesizing siderophores (Figure 8).

Information about proteins involved in nutrient uptake by *M*. *suis* from the host-environment could be one key in the establishment of an *in vitro* cultivation system. Particularly the targeted supplementation of the media with individual nutrients can play a key role in the establishment of an *in vitro M*. *suis* culture system in the future.

Based on the missing cell wall, mycoplasmas have a natural resistance to ß-lactam antibiotics. Moreover, resistance against macrolides could be enhanced by efflux-pumps which were detected during the acute phase of infection. This resistance against macrolides could be demonstrated in other Mollicutes (Figure 8; Raherison et al. 2002; Szczepanowski et al. 2004). The presence of a multi-drug resistance efflux-pump might relieve initial survival of the bacterium and facilitates the opportunity to acquire other resistance genes (Szczepanowski et al. 2004).

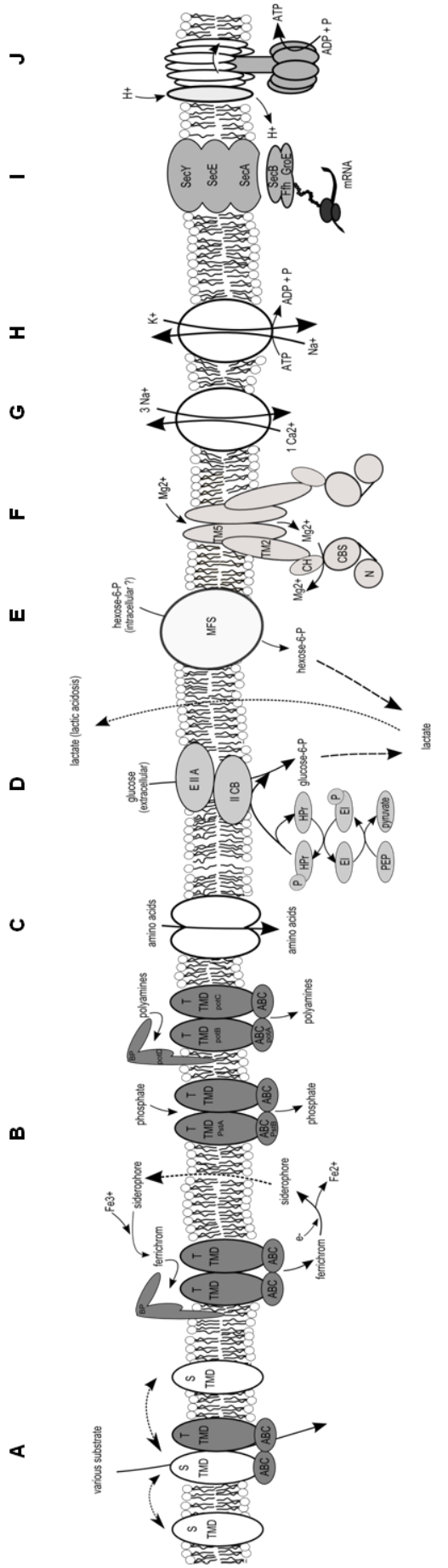

Figure 8: Overview of the different expressed and detected *M. suis* transporter systems during acute phase of infection. **A)** ECF-transporter with interchangeable S-components **B)** ABC-transporter with or without binding proteins (BP) **C)** amino acid permease **D)** phosphoenolpyruvate phosphotransferase system (PEP-PTS) **E)** hexose-phosphattransporter **F)** MgtE magnesium-transporter **G)** sodium-calcium exchanger **H)** sodium-potassium pump **I)** sec system **J)** ATP synthase.

The *M*. *suis* genome encodes a high portion of hypothetical proteins (65.9%), considerably exceeding the numbers found in other mycoplasma species e.g. 20.2% in *M*. *gallisepticum* and 22,8% in *M*. *fermentans* (Papazisi et al. 2003; Rechnitzer et al. 2011; Felder et al. 2012). In the present study 188 of the 533 *M*. *suis* KI_3806 ORFs (35.3%) encoding hypothetical proteins could be confirmed as expressed during acute infection. In contrast, the expression of only 6.0% (32/533) of these genes could be verified by the proteome approach of Felder and co-workers (2012). This is probably due to the fact, that we used both an improved *M*. *suis* enrichment protocol and a faster and more sensitive mass spectrometer (Orbitrap Elite). Based on this fact, we could prove the expression of a great proportion of these putative hypothetical proteins during the acute IAP. Therefore, we can suggest that they carry out significant functions during acute infection and the life cycle of *M. suis*. Probably, they encode unique proteins responsible for the high degree of host adaptation to the hostile environment of the porcine blood system (Felder et al. 2012). Therefore, future approaches will deal with the recombinant expression as well as the structural and functional characterization of these proteins. Hypothetical proteins which were predicted as potential membrane proteins in this study will thereby be the first choice.

Lipoproteins are considered as the most important pathogenic elements in mycoplasmas (Zuo et al. 2009). To date, it is known that mycoplasma lipoproteins play a key role in infection and are able to adjust the host immune system and are necessary during the ignition process (Zuo et al. 2009). Moreover, they are pro-inflammatory eliciting innate as well as adaptive immune responses and have a role in virulence-associated functions, like invasion, colonization, and evasion of host defense (Zuo et al. 2009; Browning et al. 2011). In HMs it was possible to predict 15 lipoproteins encoded by the genome of *M*. *haemocanis* and 17 of *M*. *haemofelis* including some hypothetical proteins (do Nascimento et al. 2012). For *M*. *parvum* three lipoproteins were predicted (do Nascimento et al. 2014). In *M. suis* we found also three lipoproteins using the LipoP 1.0 algorithm. These three lipoproteins were found to be expressed in *M*. *suis* during acute IAP (Msui06740, Msui01480 and Msui00920). Interestingly, one of these hypothetical proteins seems to be an ABC transporter permease.

M. suis is the only HM which has been investigated by proteomics until today. Moreover, to our knowledge, this study is the most successful and comprehensive proteome report. Besides the *M*. *suis* proteome the complete protein dataset (*M. suis*, *S. scrofa*) enables the quantitative identification the *M*. *suis* induced and/ or influenced gene expression of *S. scrofa* proteins during different time points of acute IAP containing low and high bacterial loads (unpublished data). Based on these datasets further evaluations could provide new insights into the pathogen-related physiology of the host during acute IAP infection.

7 CONCLUSION AND FUTURE CHALLENGES

M. *suis* is worldwide distributed in pig industry causing acute and chronic infections. The acute disease called IAP is well known since more than 80 years. However, clinically asymptomatic infected pigs constitute the major epidemiological problem and lead to high economic losses in swine production. Furthermore, the lack of a *M*. *suis in vitro* cultivation system hinders the investigation of the pathobiology of this organism.

Despite all these limitations, the work of the present PhD thesis was successful in i) establishing a pig infection model for the investigation of chronic *M. suis* infections, ii) identifying blood-independent transmission pathways of *M*. *suis*, and iii) deepening our knowledge on the gene expression of *M. suis* during acute disease by using proteomics.

The novel chronic infection model enables the analysis of *M. suis* infections in the porcine host during considerably longer investigation periods. Compared to the existing infection model, the typical cyclic course of anemia can be observed in this model. Therefore, it is possible to investigate anemia attacks containing high bacterial loads as well as clinically inapparent infection phases characterized by low bacteremia. In conclusion, both infection models are necessary and very promising for further studies on pathogenic differences between chronic and acute *M. suis* infections.

In this thesis, both infection models were used to determine potential blood-independent transmission pathways of *M*. *suis*. For this, feces, urine, air and dust as well as nasal, vaginal and saliva secretions were screened for *M*. *suis* DNA by Rt-PCR. It was possible to detect *M. suis* in secretions (nasal, vaginal and saliva) and RBC-free urine. Against this, *M. suis* was neither detected in feces nor in air samples. Results gained from these experiments indicate that *M*. *suis* transmission besides blood or arthropod-dependent pathways during chronic and acute IAP are also of epidemiological significance. Additionally, it should be queried whether *M*. *suis* transmission via oral ingestion of vaginal secretions during parturition is possible. To exclude the transmission during birth piglets from *M. suis*-infected sows and born by C-section should be investigated in future. Our results proof for the first time blood-independent *M*. *suis* transmission pathways, extend knowledge about *M*. *suis* epidemiology and will help to develop strategies to disrupt infection chains of *M. suis*.

As aforementioned information on the pathobiology of *M*. *suis* is rather limited due to different reasons. Nevertheless, novel high-throughput laboratory approaches (omics) are predestinated in such situations. To deepen our knowledge about *M. suis* gene expression during acute infections we used modern and sophisticated proteome analysis. For this, a novel enrichment method of *M*. *suis* membrane proteins was established. This method enabled an improved resolution of the protein expression profile of *M*. *suis* and thereby deeper insights into the pathogenesis of the organism. Moreover, in this work more than 50% of the encoded *M*. *suis* proteins were successfully identified as expressed during the acute

phase of infection. This identification ratio is in accordance with GeLC-MS/MS protein identification levels for other cultivable mycoplasmas.

In addition, it was possible to prove the expression of 180 so-called hypothetical proteins. Therefore, it is rather probable that these proteins are important and responsible for the unique life style of *M*. *suis*. Thus, future studies will be performed to unravel their functions, e.g. nutrient transport (as substrate binding-components in ECF-transporter system), host adaptation mechanisms or immune evasion.

Based on the new *M*. *suis* enrichment, it was possible to identify several expressed proteins involved in transport of nutrients. Our data prove that nutrients like glucose, hexose-6-phosphate, spermidine, putrescine, phosphate, amino acids, magnesium, potassium, sodium and iron are taken up from the host environment by *M*. *suis*. Moreover, information on *M*. *suis* proteins involved in transport mechanisms should also be helpful in the establishment of an *in vitro* cultivation system in future. Particularly, the supplementation of individual nutrients can play a key role in finding a suitable medium to support *M. suis* growth *in vitro*.

In conclusion, the established enrichment strategy of membrane proteins combined with enhanced proteome analysis provides excellent results during the acute phase of the *M. suis* infection associated with high bacterial loads in the blood. Against that, it was not possible to identify a comparable *M*. *suis* proteome during low bacteremia due to the high amount of pig proteins in relation to low numbers of *M*. *suis*-specific proteins. We found five proteins representing a phosphotranspherase system as well as several well-known chaperons. However, the analysis of differentially expressed proteins of *S. scrofa* during the early infection will give deeper insights into the (immune) response of the host. Besides immunology, nobody has examined the ongoing molecular processes in the host during *M*. *suis* infection. The proteome dataset acquired from the pig in this work firstly enables these studies and can help to unravel infection-relevant process in the host and provides further insights into the initiation of the misguided immune response.

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AFFIDAVIT

Pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. I hereby declare that I independently completed the doctoral thesis submitted on the topic "Microbiological and proteome analysis to gain insights into the pathogenesis of the highly adapted not-cultivated hemotrophic *Mycoplasma suis"*.

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphrasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit. I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

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