Epidemiological and clinical description of `*Candidatus* Mycoplasma haemosuis`, an emerging pathogen in pigs.

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Für meine Familie

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LIST OF ABBREVIATIONS

%	percentage
°C	centigrade / degree Celsius
Ca.	*Candidatus*
DNA	deoxyribonucleic acid
E.	*Eperythrozoon*
e.g.	for example
ELISA	enzyme-linked immunosorbent-assay
GAPDH	glyceraldehyde 3- phosphate dehydrogenase
GE	genomic equivalents
H.	*Haemobartonella*
HM	hemotrophic mycoplasma
IAP	infectious anemia in pigs
i.e.	this is
IgG	immunoglobulin G
IgM	immunoglobulin M
i.m.	intramuscular
i.v.	intravenous
kb	kilobase
LOD	lower limit of detection
M.	*Mycoplasma*
mb	megabase
MCH	mean corpuscular hemoglobin
MSG1	*M. suis* glyceraldehyde 3- phosphate dehydrogenase-like protein 1
PRRSV	porcine reproductive and respiratory syndrome virus
PCR	polymerase chain reaction
PCV	porcine circovirus
PMWS	postweaning multisystemic wasting syndrome
RBC	red blood cell
RNA	ribonucleic acid
spp.	species pluralis
s.c.	subcutaneous
µm	micrometer
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
var.	varietas

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1. **SUMMARY**

`*Candidatus* Mycoplasma haemosuis` is an emerging pathogen infecting pigs. It belongs to the group of uncultivable hemotrophic mycoplasmas. This group includes other long-known porcine representatives, i. e. *Mycoplasma parvum* und *Mycoplasma suis*. *M. suis* is the causative agent of infectious porcine anemia (IAP), a disease of great economic importance to the pig industry (HOELZLE et al. 2014). Previously, `*Ca*. M. haemosuis` was only described in China (FU et al. 2017), South Korea (SEO et al. 2019) and Thailand (THONGMEESEE et al. 2022), with no knowledge of its occurrence outside Asia or of its general clinical and economic importance in general.

The present work investigates the occurrence of the novel hemotrophic bacterium and its` clinical importance in Germany for the first time. For this, a quantitative real-time PCR was first successfully developed for the detection of `*Ca*. M. haemosuis` in pigs. The SYBR® Green-based PCR amplifies a 177-bp fragment of the `*Ca*. M. haemosuis` *gap*, which encodes the NAD-dependent glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This assay proved to be highly specific and sensitive, providing the basis for routine diagnostics and science (ADE et al. 2022a).

Using this PCR, `*Ca*. M. haemosuis` was detected in a total of seven pigs during an acute clinical disease in May 2017. In those cases, fattening pigs from a pig farm in Southern Germany showed hematological, clinical, and pathological signs similar to those found in *M. suis*-caused IAP. Clinical signs included fever, apathy, icterus, cyanoses of the ears, generalized hemorrhagic diathesis as well as a normocytic, normochromic anemia. Comprehensive differential diagnostic investigations covering *M. suis,* Classical and African Swine Fever, PRRSV, Porcine Circovirus Type 2 and 3 and *Sarcoptes scabei* var. *suis* remained all negative. Thus, `*Ca*. M. haemosuis` can be assumed to be the etiological agent of the found clinical syndrome. This represents the first detailed description of a disease induced by `*Ca*. M. haemosuis` and the first detection of this novel HM species outside of Asia. The intrinsic pathogenic potential of `*Ca*. M. haemosuis` should be further elucidated in experimental infections.

In a further study, the newly established PCR was used to comprehensively investigate the occurrence of `*Ca*. M. haemosuis` in clinically healthy animals of different age groups in Southern Germany. `*Ca*. M. haemosuis` was prevalent in 6.25% of the sows (n=208), in 4.50% of the piglets (n=622), in 17.50% of the pigs (n=200), and in 0.00% of the breeding boars (n=183). This represents the first description of the novel HM species outside of Asia in clinically healthy animals and shows an already widespread distribution in the German pig

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population in all age groups except breeding boars. Whether subclinical `Ca`. M. haemosuis` infections have the same significance as those with *M. suis* can only be assessed on the basis of the results of further work (ADE et al. 2022a).

By sampling the piglets immediately after birth and prior to the first colostrum uptake, the possibility of a vertical transmission of `Ca`. M. haemosuis` was also determined within this thesis. Since 76.92% of the `Ca`. M. haemosuis` positive sows gave birth to at least one `Ca`. M. haemosuis` positive piglet, a vertical transmission is regarded as very likely. This hypothesis is further supported by the fact that the infected piglets had high bacterial blood loads (mean blood load of $2.25 \times 10^5$ `Ca`. M. haemosuis`/mL blood) which argues against an infection during the birth process (ADE et al. 2022a).

HMs are known to be transmitted blood-dependent and thus, transmitted iatrogenic or via wounds from animal to animal. The detection of *M. suis* in blood-free excretions such as saliva, urine, nasal, and vaginal secretions from experimentally infected animals has initiated the discussion of additional, blood-independent transmission routes. Saliva (n=148) and urine samples (n=47) were also collected from the sows examined by blood sampling, semen samples (n=183) were also obtained from the examined boars and applied to `Ca`. M. haemosuis` qPCR. The pathogen was not detected in any of the saliva, urine, or semen samples. On the one hand, this demonstrates the lack of suitability of blood-free sample materials for diagnostics; on the other hand, it highlights the blood-dependent transmission pathways known to date and thus strengthens the potential to limit infections through strict hygiene measures during veterinary procedures and through the control of bloodsucking arthropods (ADE et al. 2021).

In conclusion, based on the newly established qPCR assay for the sensitive and specific detection of `Ca`. M. haemosuis`, the present work provides the first clinical and epidemiological description of the emerging hemotrophic pathogen in pigs. Further, the qPCR assay will be the basis for future studies regarding the epidemiology as well as the clinical relevance and pathogenesis of `Ca`. M. haemosuis` -infections in pigs.

2. ZUSAMMENFASSUNG

Bei `*Candidatus* Mycoplasma haemosuis` handelt es sich um ein neuartiges Pathogen des Schweines, welches zu den hämotrophen Mykoplasmen (HM), einer Gruppe von nicht-kultivierbaren Bakterien, gehört. Zur Gruppe der HM gehören weiterhin zwei schon sehr lange beim Schwein bekannte Vertreter, nämlich *Mycoplasma parvum* und *Mycoplasma suis*. *Mycoplasma suis* ist das ätiologische Agens der infektiösen Anämie der Schweine (IAP), einer Erkrankung mit großer wirtschaftlicher Bedeutung (HOELZLE et al. 2014). Der neue HM-Vertreter `*Ca*. M. haemosuis` wurde dagegen erst vor kurzem und lediglich in China (FU et al. 2017), Südkorea (SEO et al. 2019) und Thailand (THONGMEESEE et al. 2022) beschrieben. Über das Vorkommen außerhalb Asiens sowie die klinische und wirtschaftliche Bedeutung ist bisher nichts bekannt.

Im Rahmen der vorgelegten Dissertation wurde erstmals untersucht, ob die neu in Asien aufgetretene hämotrophe Bakterienspezies in Deutschland vorkommt und möglicherweise die Ursache von klinischen Symptomen sein könnte. Dazu wurde zunächst eine quantitative Real-time PCR für den Nachweis von `*Ca*. M. haemosuis` in Schweinen entwickelt. Die auf der SYBR® Green-Technologie basierende PCR amplifiziert ein 177-bp großes Fragment des `*Ca*. M. haemosuis` *gap*-Gens, welches für eine NAD-abhängige Glyceraldehyd 3-Phosphat Dehydrogenase codiert. Diese PCR erwies sich als sehr spezifisch und sensibel. Durch die Etablierung des qPCR Testsystems wurde die Grundlage für die Routinediagnostik sowie für epidemiologische und klinische Studien geschaffen (ADE et al. 2022a).

Mittels dieser PCR konnte `*Ca*. M. haemosuis` in einer akuten klinischen Bestandserkrankung in insgesamt sieben Schweinen im Mai 2017 nachgewiesen werden. Hierbei handelte es sich um Mastschweine aus einem süddeutschen Mastbetrieb, welche klinische Symptome sowie hämatologische und pathologische Veränderungen aufwiesen, die sehr eindeutig dem klinischen und pathologischen Bild einer durch *M. suis* verursachten infektiösen Anämie ähnelten. Unter anderem zeigten die betroffenen Tiere Fieber, Apathie, Ikterus, Zyanosen der Ohrränder, generalisierte Hämorrhagien der Haut sowie eine normozytäre, normochrome Anämie. Umfassende differentialdiagnostische Untersuchungen, welche *M. suis*, die Klassische und Afrikanische Schweinepest, *Sarcoptes*-Räude sowie PRRS-Virus und Porcines Circovirus 2 und 3 abdeckten, verliefen allesamt negativ. Folglich kann von `*Ca*. M. haemosuis` als ätiologisches Agens ausgegangen werden. Dies stellt die erste detaillierte Beschreibung einer durch `*Ca*. M. haemosuis` induzierten Erkrankung und den ersten Nachweis dieses Bakteriums außerhalb Asiens dar. Das tatsächlich pathogene Potenzial dieser neuen hämotrophen Mykoplasmenspezies sollte in weiterführenden Studien in experimentellen Infektionen näher aufgeklärt werden.

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In einer weiteren Fragestellung wurde die neu etablierte PCR verwendet, um das Vorkommen von `*Ca*. M. haemosuis` in klinisch gesunden Tieren verschiedener Altersgruppen und Nutzungsrichtungen in Süddeutschland zu untersuchen. Dabei war `*Ca*. M. haemosuis` in 6,25% der Sauen (n=208), in 4,50% der Ferkel (n=622), in 17,50% der Mastschweine (n=200) und in 0,00% der Zuchteber (n=183) nachweisbar. Dies stellt die erste Beschreibung der neuen hämotrophen Mykoplasmenspezies außerhalb Asiens in klinisch gesunden Tieren dar und zeigt eine bereits weit ausgedehnte Verbreitung in der süddeutschen Schweinepopulation in allen Altersgruppen ausgenommen Zuchtebern. Ob subklinische `*Ca*. M. haemosuis`-Infektionen die gleiche Bedeutung wie solche mit *M. suis* haben kann erst anhand der Ergebnisse weiterführender Arbeiten eingeschätzt werden (ADE et al. 2022a).

Durch die Beprobung der Ferkel unmittelbar nach der Geburt und vor der ersten Kolostrumaufnahme konnten Rückschlüsse über eine mögliche vertikale Übertragung des Erregers gezogen werden. Da ein hoher Anteil von 76,92% der `*Ca*. M. haemosuis` positiven Sauen mindestens ein `*Ca*. M. haemosuis` positives Ferkel geboren hatten, ist eine vertikale Übertragung als sehr wahrscheinlich einzustufen. Dafür spricht auch, dass die positiven Ferkel eine hohe Erregerlast von durchschnittlich $2,25 \times 10^5$ `*Ca*. M. haemosuis`/mL Blut aufwiesen, was gegen eine Infektion auf dem Geburtsweg spricht (ADE et al. 2022a).

Hämotrophe Mykoplasmen werden für bekanntlich blutgebunden und somit iatrogen oder über Wunden von Tier zu Tier übertragen. Der Nachweis von *M. suis* in blutfreien Ausscheidungen wie Speichel, Urin, Nasen- und Vaginalsekret von experimentell infizieren Tieren hat die Diskussion um weitere, blutunabhängige Übertragungswege eröffnet. Von den untersuchten Sauen wurden auch Speichel- (n=148) und Urinproben (n=47), von den Ebern zudem Spermaproben (n=183) gewonnen und per PCR auf `*Ca*. M. haemosuis` untersucht. In keiner der Speichel-, Urin- oder Spermaproben konnte der Erreger nachgewiesen werden. Dies zeigt einerseits, dass blutfreies Probenmaterial für eine `*Ca*. M. haemosuis`-Diagnostik nicht geeignet ist, andererseits unterstreicht es die Bedeutung der bislang bekannten blutabhängigen Übertragungswege und auch die prophylaktische Bedeutung strikter Hygienemaßnahmen bei iatrogenen Eingriffen und der Bekämpfung von blutsaugenden Arthropoden (ADE et al. 2021).

Zusammenfassend wurde in dieser Arbeit die diagnostische Grundlage für den Nachweis von `*Ca*. M. haemosuis` geschaffen. Mittels des neu etablierten qPCR Tests erfolgte die erste klinische und epidemiologische Beschreibung des Erregers in Schweinen. Darüber hinaus stellt der qPCR-Test die Grundlage für künftige Studien zur Epidemiologie sowie zur klinischen Relevanz und Pathogenese von `*Ca*. M. haemosuis` Infektionen bei Schweinen dar.

3. INTRODUCTION

3.1 Hemotrophic mycoplasmas in general

3.1.1 History and taxonomy

Bacteria that are classified into the group of hemotrophic mycoplasmas (HMs), synonymously named as hemoplasmas, were first described in the late 1920`s. In Berlin, Amsterdam and in Leuven, Schilling, Dinger as well as Bruynoghe and Vassilliadis independently described small, round organisms on the surface of red blood cells (RBCs) in murine blood smears. Those observed organisms were similar to the then known *Anaplasma* spp. or *Rickettsia* spp.. In accordance to their location on RBCs they were subsequently named *Eperythrozoon (E.) coccoides* (SCHILLING 1928; DINGER 1928; BRUYNOGHE and VASSILIADIS 1929; ELIOT and FORD 1930). During the 20th century a variety of *Eperythrozoon* species in different animals (wildlife, pets and livestock) were delineated (DOYLE 1932; KINSLEY 1932; ADLER and ELLENBOGEN 1934; NEITZ et al. 1934; SPLITTER 1950a, b; SPLITTER and WILLIAMSON 1950; HOYTE 1962; TUOMI and VON BONSDORFF 1967; EWERS 1971; MOULDER 1974; PETERS W et al. 1974; KREIER and GOTHE 1976; KREIER and RISTIC 1981).

Moreover, bacteria formerly known as *Haemobartonella* are also classified into the group of HMs. The first representatives of the genus *Haemobartonella* were also described as early as the 1920s, e.g., in a splenectomized dog (*Haemobartonella canis*). The most popular representative of *Haemobartonella* was first found in 1942 by CLARK in blood smears of anemic cats in South Africa (KIKUTH 1928; TYZZER and WEINMAN 1939; CLARK 1942). Due to the close resemblance to representatives of the genus *Eperythrozoon* the newly observed feline bacterium initially was designated as *E. felis*. However, later *E. felis* was renamed as *Haemobartonella (H.) felis* (present name: *M. haemofelis*) due to morphological characteristics. (CLARK 1942, FLINT and MC KELVIE 1955; RIKIHISA et al. 1997, WILLI et al. 2007). Following the course of time, several animal-specific species of *Haemobartonella* were described (DONATIEN and LESTOQUARD 1934; MC NAUGHT et al. 1935; FLINT and MOSS 1953; BENJAMIN and LUMB 1959; BROCKLESBY 1970; FRERICHS and HOLBROOK 1971; PETERS W et al. 1974; KREIER and GOTHE 1976).

Figure 1 (A-C) represents the original microscopic images of the publication by Schilling in 1928 as the first description of the genus *Eperythrozoon*. A blood smear can be seen, cells of *E. coccoides* (small, round particles) colonize the murine RBCs and appear to occur in various forms. On the images A and B, colonized RBCs appear much darker, and the small dots

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represent *E. coccoides* cells. Image C shows a perishing RBC colonized by a very high amount of *E. coccoides* cells (SCHILLING 1928).
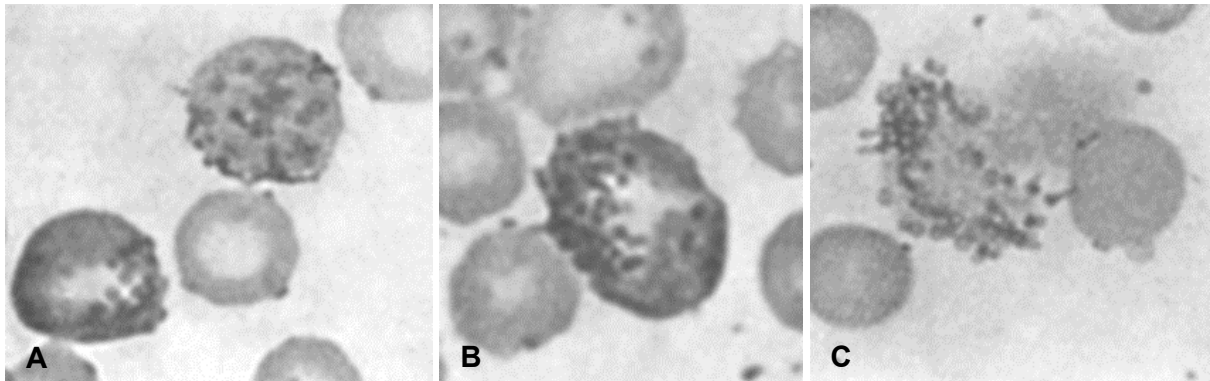
Figure 1: Original microscopic images of a murine blood smear from the first description of *E. coccoides* by SCHILLING 1928. RBCs are densely populated with cells of *E. coccoides*, which occur in different forms.

Today the taxonomic classification of HMs is not yet concluded. Due to phenotypical characteristics as well as due to 16S rDNA-based sequencing and analyses of the RNase P RNA gene (*rnpB*), the genera *Eperythrozoon* and *Haemobartonella* were reclassified from the family *Anaplasmataceae*, order *Rickettsiales* into the class *Mollicutes*, order *Mycoplasmatales*. Within this order the group of HMs are allocated in the family *Mycoplasmataceae* as *incertae cedis* in an own cluster, which clearly can be differentiated from the remaining *Mycoplasma* species (RIKIHISA et al. 1997, NEIMARK et al. 2001,2002; MESSICK et al. 2002; TASKER et al. 2003b; PETERS et al. 2008; HICKS et al. 2014; HOELZLE et al. 2014). The differentiation between the members of the HM group into the previous genera *Eperythrozoon* and *Haemobartonella* is still present as the group is divided into two subgroups/ clusters. Here, the so-called "Haemominutum group" (also "Suis group") presents the representatives of the former *Eperythrozoon* species and the so-called "Haemofelis group" the former *Haemobartonella* species, respectively (TASKER et al. 2003b, PETERS et al. 2008; HOELZLE et al. 2011; GUIMARAES et al. 2014; FU et al. 2017). Recently, Gupta et al. proposed a revision of the taxonomy with the introduction of an own genus for the HMs for which they suggested the name *Eperythozoon* again. However, this has not been implemented practically yet (GUPTA et al. 2018).

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Figure 2 shows the phylogenetically tree of genus *Mycoplasma* spp. and the cluster of HMs (HOELZLE, unpublished).
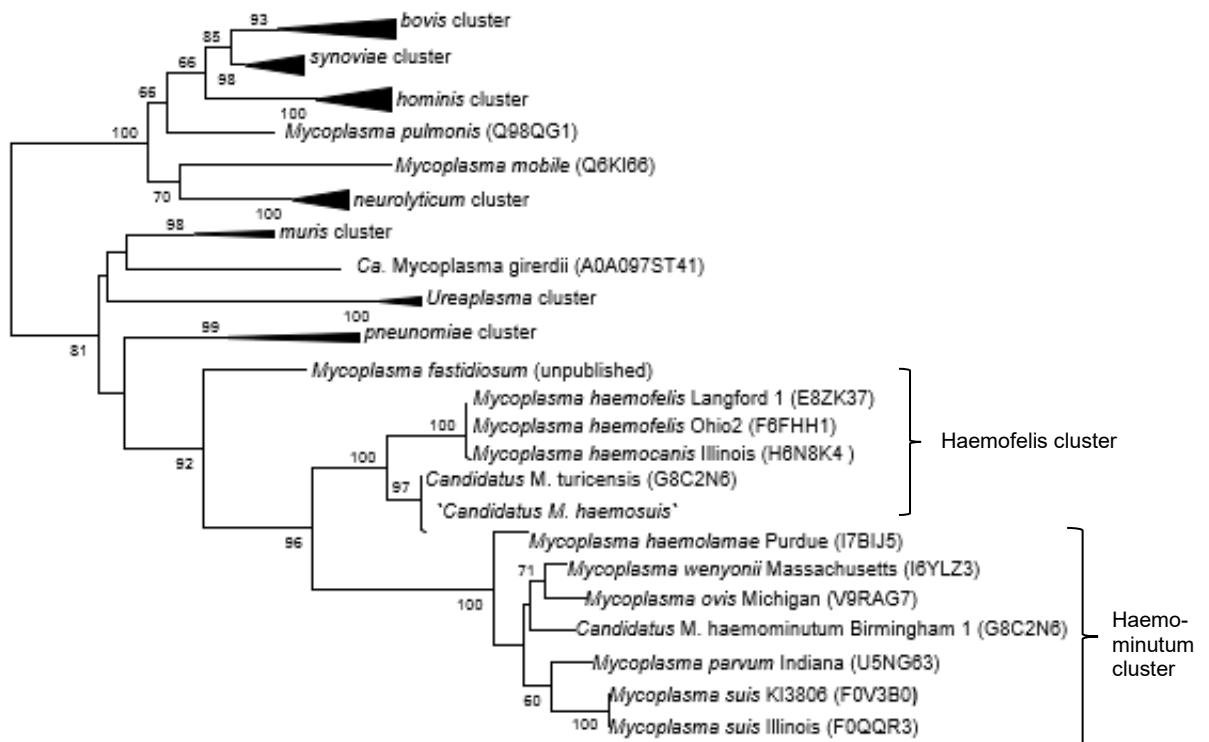
Figure 2: Phylogenetic tree showing hemotrophic mycoplasma species in their clusters as well as the distinction to other mycoplasma species (HOELZLE, unpublished).

3.1.2 Microbiological characteristics

HMs are quite small (usually >1 µm), pleomorphic bacteria which cannot be cultured *in vitro* yet. According to present knowledge, they are among the smallest existing self-replicating organisms. Unlike other bacteria but equal to other *Mycoplasma* species, they lack a cell wall. Instead, only a single membrane is present for the differentiation against the outside. Consequently, they show no sensitivity to ß-lactam antibiotics such as penicillin, as these agents act as inhibitors of the bacterial cell wall synthesis (NEIMARK et al. 2001, 2002; MESSICK 2004; HOELZLE 2008; HOELZLE et al. 2011, 2014). The bacterias` genome is double-strained, circular and very small when compared to the genomes of other bacteria. Depending on the HM species, it amounts between 564 kb and 1.2 mb (DO NASCIMENTO et al. 2013; BARKER et al. 2011a). HMs further have a very high number of hypothetical genes within their protein-coding sequences (more than 50%), with the exact percentage again varies within the species. However, it is important to mention here that not all HM species has been completely sequenced yet (BERENT and MESSICK 2003; BARKER et al. 2011a; b; 2012;

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MESSICK et al. 2011; OEHLERKING et al. 2011; GUIMARAES et al. 2011; 2014; SANTOS et al. 2011; DO NASCIMENTO et al. 2012a; b; 2013, 2014; DOS SANTOS et al. 2012; MARTÍNZEZ-OCAMPO et al. 2016; QUIROZ-CASTAÑEDA et al. 2018).

Currently, little is known about the way of life and the pathobiology of HMs owed to the absence of an *in vitro* cultivation system and additionally due to the high number of hypothetical protein coding sequences within their genomes. The best-studied species among the HM species is the porcine species *M. suis* as well as the feline representatives, i.e. *M. haemofelis*, `*Ca.* M. haemominutum` and `*Ca*. M. turicensis`.

Analyses of some previously sequenced species yielded a particular profile of their metabolic pathways, which seem to be highly conserved. Genome sequencing and proteomic studies for *M. suis* revealed the complete presence of the Embden-Meyerhoff-Parnas pathway (glycolysis) so the degradation of glucose appears to be a possibility to generate energy in the form of ATP (OEHLERKING et al. 2011; GUIMARAES et al. 2011; FELDER et al. 2012; DIETZ et al. 2016). This aspect can also be noticed in sick animals suffering from hemolytic anemia induced by HM infections. Strong hypoglycemia correlated to increasing bacterial blood loads could be observed chiefly in infected pigs (ZACHARY and SMITH 1985; HEINRITZI et al. 1990a, b; SMITH JE et al. 1990), calves (LOVE and MC EWEN 1972; LOVE et al. 1977), cows (YAN et al. 2008; GENOVA et al. 2011), sheep (SUTTON 1977), llamas (MC LAUGHLIN et al. 1990), lambs (BURKHARD and GARRY 2004), cats (HARVEY and GASKIN 1978; WILLI et al. 2006) and further in a case report of an infected opossum (MESSICK et al. 2000). Moreover, in a comparative study from 2014 regarding the genomes of *M. suis*, *M. haemofelis*, *M. haemocanis*, *M. wenyonii*, `*Ca*. M. haemolamae`, and `*Ca*. M. haemominutum`, Guimaraes et al. characterized the complete lack of the pentose phosphate pathway, the pyruvate dehydrogenase complex and within this also the lack of the citrate cycle and the metabolism of coenzyme A in each of the included HM representatives. According to the HM species, different enzymes of the nucleotide metabolism pathways are available. The possibility to use hypoxanthine in the purine metabolism is common to all of the just described HM species (GUIMARAES et al. 2014).

HMs show an unique tropism for RBCs. In infected animals they adhere on the RBC`s surface (ZACHARY and BASGALL 1985). At least one *M. suis* isolate is able to invade porcine RBCs (GROEBEL et al. 2009). Among the tropism for RBCs, endothelial cells are known to be target cells, however, this is only described for *M. suis* but not for other HM species so far. Whether or not RBCs and endothelial cells also represent the site of replication is still unclear (SOKOLI et al. 2013).

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HM species actually are considered to be specific for their related host animal species and for humans, respectively (KREIER and RISTIC 1981; PITCHER and NICHOLAS 2005, STEER et al. 2011). Nevertheless, several cases have been published suggesting this assumption probably needs to be reconsidered. This is for example the description of the ovine HM species *M. ovis* in reindeers (STOFFREGEN et al. 2006) as well as the occurrence of the bovine HM species `*Ca*. M. haemobos` in sheep and goats (SHI et al. 2019) and furthermore the one case of the porcine species *M. suis* in dogs (MASCARELLI et al. 2016). In addition, the feline species `*Ca*. M. haemominutum` could be detected in goats (GALON et al. 2019). Moreover, in the USA, in China and Brazil human co-infections with different non-human HM species (*M. haemofelis*, *M. suis*, *M. ovis* and `*Ca*. M. haematoparvum`) were observed (DOS SANTOS et al. 2008; YUAN et al. 2009; SYKES et al. 2010; MAGGI et al. 2013a, b). Chapter 3.1.3 below will review the cases of human HM infections again.

3.1.3 Zoonotic potential

Up to now, *M. haemofelis*, *M. suis*, *M. ovis* and `*Ca*. M. haematoparvum` were detected in humans as already briefly noted in chapter 3.1.2 (DOS SANTOS et al. 2008; YUAN et al. 2009; SYKES et al. 2010; MAGGI et al. 2013a, b). Recently, Descloux et al. detected the human HM species `*Ca*. M. haemohominis` in bats and discussed a possible connection to human infections (DESCLOUX et al. 2020). Other reports on human infections have been published from Croatia (PUNTARIC et al. 1986; BOSNIC et al. 2010), Brazil (DUARTE et al. 1992) and China (HUANG et al. 2012). In the Chinese study more than 14,000 HM-infected people have been identified (HUANG et al. 2012). However, the HM species had not been determined in these cases and it remains unclear if those were zoonotic infections or infections with the then unknown human HM species `*Ca*. M. haemohominis` (STEER et al. 2011; HATTORI et al. 2020). Nevertheless, as a result of all those reports a possible zoonotic potential of HMs must be taken into consideration. Clarifications require further studies including precise genomic identifications of the pathogens (MILLÁN et al. 2020).

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3.2 Porcine HM species

3.2.1 Taxonomy

Three HM species are described in pigs so far: *Mycoplasma suis*, *Mycoplasma parvum* and `*Candidatus* Mycoplasma haemosuis`. *Mycoplasma suis* and *M. parvum* have already been observed as *Eperythrozoon* species (*E. suis* and *E. parvum*) since the 1930`s. Back then, Doyle described them in blood smears of pigs. Both are closely related species and today are classified in the "Haemominutum group" within the cluster of HMs, as they are representatives of the former genus *Eperythrozoon* (DOYLE 1932; KINSLEY 1932; SPLITTER 1950a). In 2017, a third porcine HM species emerged in China (FU et al. 2017) and was further named as `*Ca*. M. haemosuis` In contrast to *M. suis* and *M. parvum*, this species is clustered in the "Haemofelis group" which includes the HM species formerly classified into the genus *Haemobartonella* (FU et al. 2017).

3.2.2 Microbiology and Pathobiology

Generally, the basic properties of hemotrophic mycoplasmas mentioned in chapter 3.1.2 (i.e. lack of cell wall, uncultivability, association to RBCs) are also valid for the porcine HM species. The state of knowledge regarding the special microbiological and pathobiological properties of the three porcine HM species varies considerably, with *M. suis* being the best investigated one.

As already outlined in chapter 3.1.2, *M. suis* is known to be not only able to adhere to the RBCs` surface, as every HM species does, but is also able to invade porcine RBCs (GROEBEL et al. 2009). Further, *M. suis* is so far the only HM species with a known tropism for endothelial cells. Both, RBCs and endothelial cells are being severely damaged by the colonization of *M. suis* (SOKOLI et al. 2013).

Figure 3 (A+B) shows porcine RBCs being colonized with a variety of *M. suis* cells after experimental infection of a splenectomized pig. The deformation of RBCs due to the attachment of *M. suis* is illustrated in image B.

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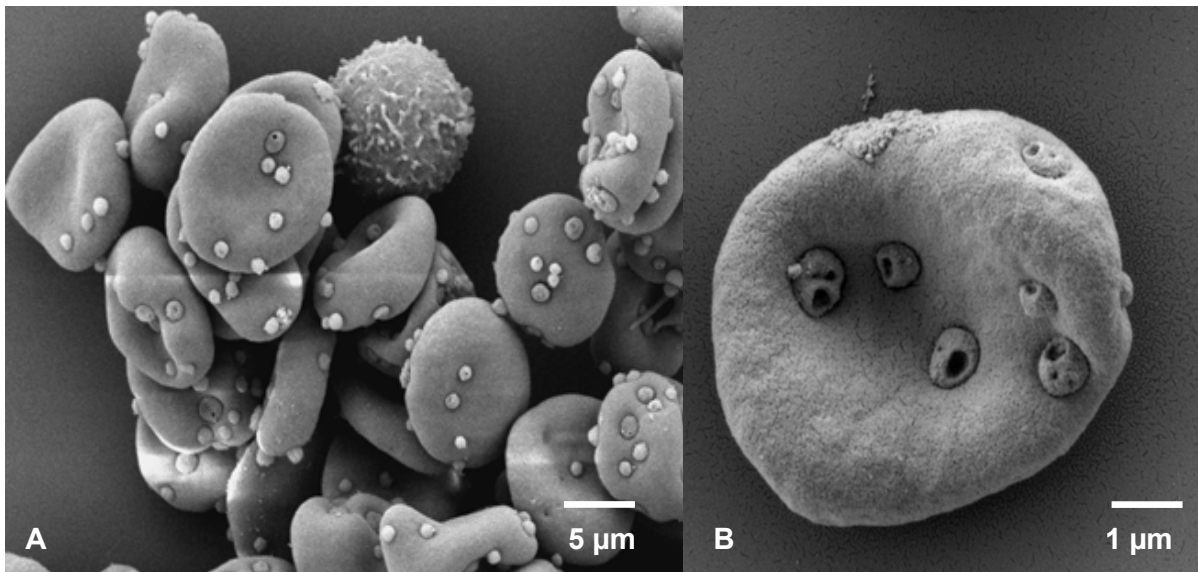
Figure 3: Electron microscopic images of porcine RBCs being colonized with *M. suis* (HOELZLE 2009, unpublished).

Compared to *M. suis*, *M. parvum* cells are smaller in diameter (*M. parvum*: 0.2–0.8 µm diameter, *M. suis*: 0.8–2.5 µm diameter; SPLITTER 1950a; 1953; POSPISCHIL and HOFFMANN 1982; LIEBICH and HEINRITZI 1992; DO NASCIMENTO 2014).

According to experimental infections in pigs, *M. parvum* is regarded as a non-pathogenic HM species. Nevertheless, *M. parvum* was found to colonize porcine RBCs. Even if *M. parvum* seems to colonize less RBCs than *M. suis* does, the number of *M. parvum* cells on colonized RBCs was similar to the number of *M. suis* cells on colonized RBCs (SPLITTER 1950a, GUIMARAES et al. 2014). On genomic level, all *M. parvum* protein-coding sequences with known functions are shared with *M. suis* and their difference in pathogenicity have not been clarified yet (GUIMARAES et al. 2014).

`*Candidatus* M. haemosuis` has not been described on a microbiological, genomic or pathobiological level yet. Only in the study of Fu et al. it is demonstrated by blood smears that this bacterium can also be found on porcine RBCs (FU et al. 2017). Figure 4 shows a Giemsa-stained blood smear of a pig infected with `*Ca*. M. haemosuis` from this publication. RBCs appear pale red, `*Ca*. M. haemosuis` cells are marked by arrows and appear as small, violet points (FU et al. 2017).

3. INTRODUCTION
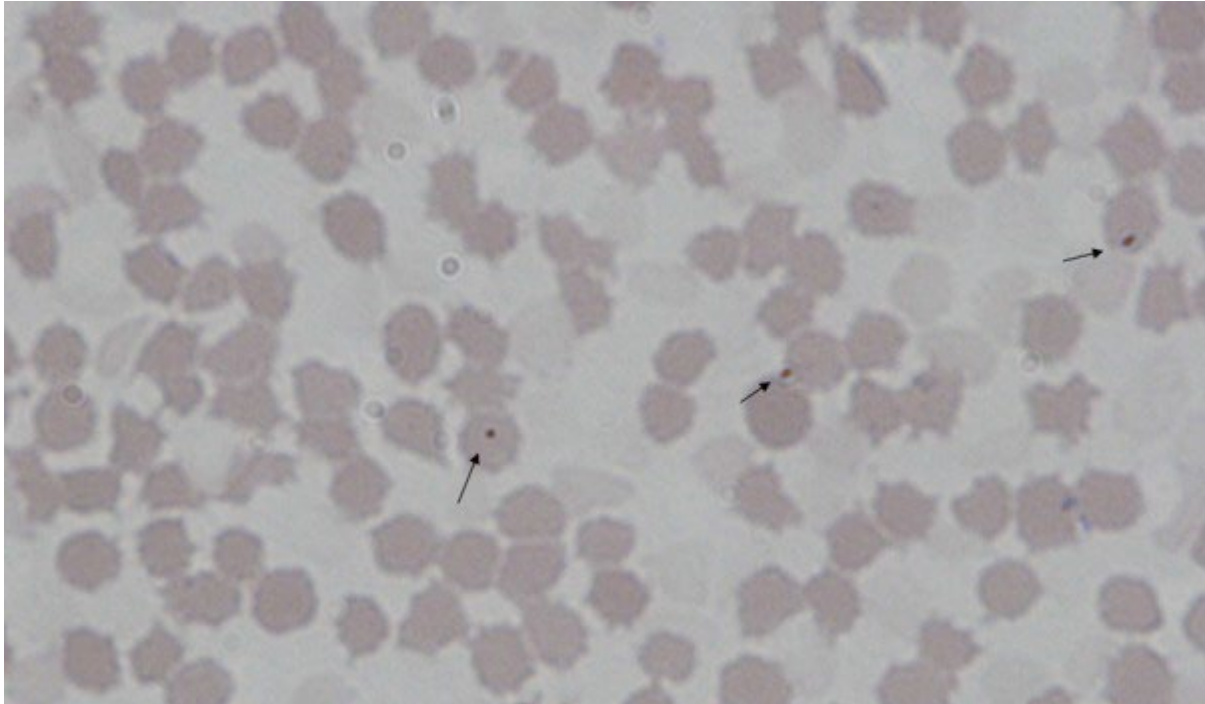
Figure 4: Giemsa-stained porcine blood smear showing RBCs infected with cells of `Ca. M. haemosuis` (marked by arrows), which appear as small, violet dots (FU et al. 2017).

Regarding the molecular level, *M. suis* is so far the only HM species for which pathogenicity factors have been demonstrated. Two proteins are known to mediate adhesion to RBCs: α-enolase and MSG1, which is a protein with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) activity. Apart of acting as adhesion and therefore as pathogenicity factors, both proteins function as enzymes in the degradation of glucose (glycolysis/ Embden-Meyerhoff-Parnas pathway; HOELZLE et al 2007a, 2014; SCHREINER et al. 2012a).

Autoimmune mechanisms seem to be substantial during the course of infections. IgG and IgM autoreactive antibodies were described in *M. suis* infected pigs. Those antibodies play a crucial role in hemolysis and eryptosis (ZACHARY and SMITH 1985; SCHMIDT et al. 1992; JÜNGLING et al. 1994; GROEBEL et al. 2009; FELDER et al. 2010; HOELZLE et al. 2014). Eryptosis caused by *M. suis* was described in detail during an experimental infection of pigs by Felder et al. (FELDER et al. 2011). Further, *M. suis* infections are thought to cause a strong modulation and suppression in their hosts` immune system (HENRY 1979; ZACHARY and SMITH 1985; DO NASCIMENTO et al. 2018, MACK 2019). According to Mack, the modulation of the immune system by *M. suis* is in fact quite significantly involved in the pathogenesis, however, participating mechanisms need to be further investigated. (MACK 2019).

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3.3 HM infections in pigs

3.3.1 Clinical signs

The disease caused by *M. suis* is known as infectious anemia in pigs (IAP; HOELZLE et al. 2014). Clinical course of IAP is either acute or chronic (HOELZLE 2008). Clinical signs of both types have been described intensively in literature and seem to depend on the animals` age, direction of use and general health as well as on the infection dose and virulence of the *M. suis* isolate (BRÖMEL and ZETTL 1985, GROEBEL et al. 2009; STADLER et al. 2014). All groups of ages can be affected (POSPISCHIL and HOFFMANN 1982; HOELZLE et al. 2011). Infected animals, however, do not exhibit the same clinical signs all the time. In addition, severity of the clinical signs varies from animal to animal (HENRY 1979; BUGNOWSKI et al. 1986; HOELZLE 2008; GROEBEL et al. 2009; HOELZLE et al. 2011).

The acute form of IAP usually affects single animals in a herd and then often occurs after stress situations such as rehousing, the implementation of zootechnical measures or, in the case of piglets, also after weaning (HEINRITZI 1989). Affected fattening pigs as well as suckling and post-weaning piglets usually react with a high-grade icteroanemia and up to 42°C high fever, which occurs in episodes and attacks. The accompanying general health is very poor, affected animals are partly apathetic and present a pale-anemic appearance. In severe cases, dyspnea continues to occur with anorexia and lethargy. Necroses are also frequent, especially on the edges and tips of the ears, but also on other parts of the body such as the tail and the distal ends of the limbs. Very often splenomegaly can also be detected (PRESTON and GREVE 1965; HOFFMANN and SAALFELD 1977; SMITH AR 1978; HENRY 1979; BUGNOWSKI et al. 1986; MADSEN 1986; HEINRITZI 1990a; GROEBEL et al. 2009; DENT et al. 2013). Infected suckling piglets additionally often show tachycardia and have an increased respiratory rate, further there are increased rates of runts and animal losses (PRESTON and GREVE 1965; HOFFMANN H 1984; BRÖMEL and ZETTL 1985; HENDERSON et al. 1997). During acute IAP, life-threatening conditions can develop and the infection can end fatally for piglets and fattening pigs (PRESTON and GREVE 1965; HENDERSON et al. 1997; WU et al. 2006). In affected sows, acute disease is described with less serious signs. Anorexia accompanied by fever can occur and appear in a milder form. More often dysgalactia as well as edema of the mammary gland and of the vulva arise (BRÖMEL and ZETTL 1985; HEINRITZI 1990a; SOBESTIANSKY et al. 1999; MESSICK 2004; STRAIT et al. 2012). Clinical signs of acute IAP resemble clinical signs of swine fever, which must be excluded by diagnostics (KORN and MUSSGAY 1968).

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Experimental infections are commonly carried out on splenectomized pigs. The absence of the spleen results in the fact that affected RBCs can no longer be excreted which leads to a faster and severe progress of the infection. Clinical signs just described for the acute form of IAP are much more gravely and can be assigned to two phases during the infection. In the first and early phase of such infections, animals are apathetic, febrile and anorectic. This correlates with a high amount of *M. suis* cells in the blood as well as with a high-grade and life-threatening hypoglycemia (SPLITTER 1950a; b; ZACHARY and SMITH 1985; HEINRITZI et al. 1990a; b; SMITH JE et al. 1990; STADLER et al. 2014; STADLER et al. 2021). In the following second and late phase, a high-grade icteroanemia develops attended by pale icterically discolored mucous membranes, dyspnea and apathy. Circulatory disturbances at the acres can lead to cyanosis (mainly on the edges of the ears). In this phase the *M. suis* blood loads and the body temperature of the infected animals are again decreasing. The latter even falls below the normal range (SPLITTER 1950a; b; ZACHARY and SMITH 1985; HEINRITZI 1990a).

Chronic *M. suis* infections often progress subclinical or are accompanied by milder clinical signs. Infected animals often show a poor general picture and reduced performance parameters compared to non-infected animals. Infected juvenile animals hardly gain weight whereas adult animals may experience moderate to severe weight loss (BRÖMEL and ZETTL 1985; HEINRITZI 1990a). Pereyra et al. describe a possible relationship between an *M. suis* infection and the "postweaning multisystemic wasting syndrome" (PMWS), which is usually attributed to infections with the porcine Circovirus type 2 (PCV 2). PMWS is marked by worries and generally reduced vitality as well as by pallor, inflammation of the eyes and of the respiratory tract and other unspecific signs (PEREYRA et al. 2006). Some studies also describe a negative effect of chronic *M. suis* infection on the reproductive performance. In affected sows, cycle irregularities as well as poorer fertility rates are described. In addition, an increased number of stillborn or weak piglets is reported, which in turn show typical signs of an *M. suis* infection (CLAXTON and KUNESH 1975; BROWNBACK 1981; ZINN et al. 1983; BRÖMEL and ZETTL 1985; SCHWEIGHARDT et al. 1986; GRESHAM et al. 1994; GWALTNEY 1995). A more current study was able to demonstrate a higher number of stillbirths in *M. suis* infected gilts but failed to reconstruct an impact of *M. suis* to other reproductive parameters (BRISSONNIER et al. 2020). Another recent study was able to detect *M. suis* in sows with reproductive disorders (stillborn fetuses, fetal mummification, abortion). *M. suis*, however, was not detected in any of the fetal material (BORDIN et al. 2021). Further, in chronic infected animals also necrosis of the ear margins could be observed as well as the occurrence of petechial subcutaneous bleedings and ecchymosis. Those hemorrhages are then described in the clinical picture called *Morbus maculosus* (HEINRITZI 1984; BRÖMEL and ZETTL 1985; BUGNOWSKI 1988; HEINRITZI 1990b). Another point in chronic *M. suis*

infections is the suppression of the hosts` immune system, and thus, a general higher susceptibility to secondary infections. In particular, a higher occurrence of bacterial intestinal and respiratory infections is described in *M. suis* infected pigs (ZACHARY and SMITH 1985; GWALTNEY 1995; DO NASCIMENTO et al. 2018). Clinical manifestation of the disease can reoccur from subclinical infected carrier animals due to immunosuppressive events as stress conditions (HOELZLE 2008).

Figure 5 presents two of the above-mentioned typical signs of *M. suis* infections. On the left picture (A) a pig with a severe cyanosis of the ear tip caused by a high-grade *M. suis* bacteremia is shown. The right picture (B) shows a healthy piglet on the left side and a *M. suis* infected pig suffering from anemia on the right side. A clear difference in skin color is noticeable.

Figure 5: A pig with high-grade *M. suis* bacteremia suffering from severe cyanosis of the ear tip (A). *M. suis* induced anemia of a piglet (right) which is expressed by strong pallor compared to a healthy pig (left) (B) (HEINRITZI et al. 1990a).

Contrary to *M. suis, M. parvum* is considered as a non-pathogenic porcine HM species as referenced in chapter 3.2.2 above. Only few studies investigating *M. parvum* are available (SPLITTER 1950a; JANSEN 1952; JENNINGS and SEAMER 1956; DO NASCIMENTO et al. 2014). In most of the cases, a measurable proliferation of *M. parvum* cells in the blood as well as the adhesion of the bacteria to RBCs was observed while the affected pigs stayed without

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any clinical signs. Only in the study of Jennings and Seamer one of seven affected animals developed a mild anemia and in the study of Seamer, affected piglets developed a more severe anemia (JENNINGS and SEAMER 1956; SEAMER 1960). Further, do Nascimento and co-workers describe a transient and slight increase in the body temperature of the affected pig (DO NASCIMENTO et al. 2014). However, the two studies mentioned first were carried out in the pre-PCR era and, as we know today, a distinction of HM species through microscopic findings is unreliable. Accordingly, it cannot be clearly said if those were studies with *M. parvum* but with the pathogenic *M. suis* instead.

The clinical relevance of `*Ca*. M. haemosuis` is merely described yet. So far, this third porcine HM species was observed in of clinically inconspicuous animals or detailed descriptions of the clinical signs are missing (FU et al. 2017; SEO et al. 2019; THONGMEESEE et al. 2022).

3.3.2 Pathological findings

Pathological and histopathological examinations are currently only available for *M. suis* infected animals but not for *M. parvum* or `*Ca*. M. haemosuis` infected pigs. In addition, observations usually belong to acute clinically diseased animals, mainly from experimental infections. What is missing, however, are pathological descriptions of subclinical or chronically infected animals.

Icteroanemia, as the primary clinical sign of *M. suis* infections, is also reflected in observed pathological changes. Dissected carcasses show strong, generalized yellow discolorations. These are not only visible on the body surface, in the fat including the subcutis, but also in organs and vascular linings (DOYLE 1932; SPLITTER 1951; HOFFMANN and SAALFELD 1977, THIEL 1983, STADLER et al. 2021). In addition, icteric discolorations are also visible in digestive tract contents (DOYLE 1932, SPLITTER 1950a, QUIN 1960). The accumulation of yellowish fluids in the abdominal and thoracic cavity as well as in the pericardium was already described by DOYLE in 1932 (DOYLE 1932). Stadler and co-workers were also able to observe this during necropsy in the abdominal cavity of an examined animal (STADLER et al. 2021). In addition, pale musculature and hyperemic tissue in lungs, kidneys and the liver of infected animals can be observed as a striking sign of anemia (STADLER et al. 2021).

Figure 6 presents the aorta of a *M. suis* infected pig. The vascular lining (tunica intima) shows a strongly yellow discoloration as a sign of icteroanemia.

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Figure 6: High-grade icteric discoloration on the tunica intima of the aorta of a *M. suis* infected pig (STADLER et al. 2021).

Further macroscopic and histopathological abnormalities are evident in numerous organs.

In the kidneys, in addition to macroscopically visible multiple lesions, interstitial nephritis but also inflammations in the renal pelvis were described (STADLER et al. 2021). Macroscopically, the liver shows unspecific degenerative signs (SPLITTER 1951). Edema of the gallbladder bed occurs frequently (STADLER et al. 2021). In histopathological examinations, both centrilobular and periportal necroses as well as low-grade interstitial hepatitis can be observed (CLAXTON and KUNESH 1975; THIEL 1983; STADLER et al. 2021). If present, the spleen of infected animals is usually hyperplastic (SPLITTER 1951; QUIN 1960; CLAXTON and KUNESH 1975), lymph nodes are edematous, markedly swollen and often macroscopically altered by blood resorption (QUIN 1960; KORN and MUSSGAY 1986; STADLER et al. 2021). Stadler et al. were also able to diagnose pulmonary edema and emphysema in infected animals (STADLER et al. 2021). Various pathological findings were observed in the brain, e.g. low-grade bleeding and edema in the leptomeninx (STADLER et al. 2021).

M. suis has a known tropism for endothelial cells (SOKOLI et al. 2013). These show massive damages during the course of a *M. suis* infection (SOKOLI et al. 2013; WENTZEL 2017). A histological image of a small intestinal venule originated from a *M. suis* infected pig is shown in Figure 7. Endothelial cells show seriously damages, which include massive cytoplasmic

swelling, progressive cellular necrosis and the detachment of the cells from their basement membrane. The short arrows mark areas of cellular denudation of the luminal surface, the long arrows mark immunoreactive *M. suis* cells (SOKOLI et al. 2013).
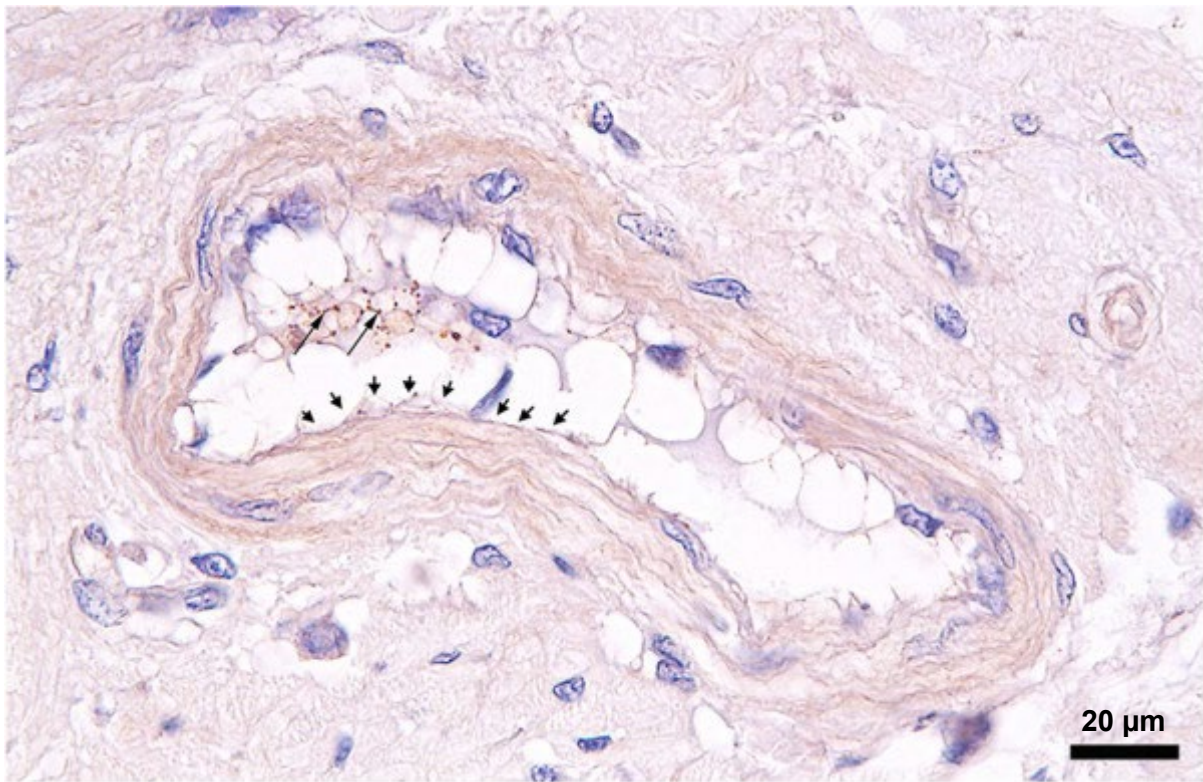
Figure 7: Histological picture of a small intestinal venule of a *M. suis* infected pig showing a severe degeneration of the endothelial cells the small arrows mark areas of cellular denudation of the luminal surface, the long arrows mark immunoreactive *M. suis* cells (SOKOLI et al. 2013).

Numerous effects on the blood and hematological system have been associated for *M. suis* infections. Regarding the hematopoietic system, the bone marrow of infected animals is hyperplastic and activated through an enhanced erythropoiesis (CLAXTON and KUNESH 1975; HOFFMANN et al. 1981; STADLER et al. 2021). Macrophages of the lymph nodes, liver and spleen are shown to sedimentations of hemosiderin. In addition, erythrophagocytosis was intensely increased (CLAXTON and KUNESH 1975; HOFFMANN et al. 1981; THIEL 1983; WENTZEL 2017). Many authors were also able to characterize generalized bleeding into various organs, such as lymph nodes, the bladder or the gastrointestinal tract (KORN and MUSSGAY 1986; PLANK and HEINRITZI 1990). Further, Stadler and co-workers were able to characterize multiple signs of blood-clotting disorders, i.e. the presence of fibrin and the detection of hyaline globules in capillaries and small pulmonary vessels (STADLER et al. 2021).

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The blood of *M. suis* infected animals itself appears aqueous and bright. Often changes in the blood count can be observed. The most common disorder, described in both, experimentally and naturally infected animals, is normocytic normochromic anemia as the typical sign of hemolytic anemia (HEINRITZI 1984; PETERANDERL 1988; RITZMANN et al. 2009; STADLER et al. 2014; STADLER et al. 2021).

3.3.2 Therapeutic and preventive measures

Clinically affected pigs are usually treated with antibiotics. Due to the missing cell wall in HMs, substances targeting the cell-wall synthesis, such as ß-lactams, are ineffective. Instead, good results were achieved using tetracyclines (HEINRITZI 1984). During the course of acute IAP in experimentally infected pigs, improvement of the clinical signs can be achieved by combination of antibiotics (tetracycline), analgesics (metamizole) and oral glucose application (STADLER et al. 2014; 2021). However, antibiotic treatment only leads to a reduction of clinical signs and bacterial loads but not to an eradication of the pathogens. Once infected, animals become long-life carriers of HMs (HOELZLE 2008).

To date, the prevention of HM infections is only based on biosecurity measures and the control of blood-sucking arthropods. Due to the lack of *in vitro* cultivation systems, whole cell vaccines are not available. As an alternative, Hoelzle K. et al. used recombinant MSG1 as well as *Escherichia coli* transformants expressing MGS1 as vaccine candidates. Immunization with both recombinant candidates induced a strong humoral and cellular immunity in vaccinated animals, however, in the following experimental infection (challenge test) the vaccination did not provide a protective immunity against *M. suis*. Vaccinated animals developed the same clinical signs of IAP compared to unvaccinated animals. Merely animals immunized with the *Escherichia coli* expressing MSG1 transformants revealed higher hemoglobin and hematocrit values than unvaccinated animals (HOELZLE K. et al. 2009).

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3.3. Epidemiology of porcine HM species

3.3.1 Occurrence, frequency, and significance

Mycoplasma suis infections are distributed worldwide and literature on the detection of the bacterium in domestic pigs is available from several countries around the globe as shown in Table 1 below.

Table 1: Reports on the detection of *Mycoplasma suis* in domestic pigs worldwide

Europe	**Austria** (SCHWEIGHARDT et al. 1986; SCHULLER et al. 1990; SCHWARZ et al. 2020); **Belgium** (DE BUSSER et al. 2008); **France** (BRISSONNIER et al. 2020; NORMAND et al. 2020); **Germany** (KORN and MUSSGAY 1968; HOFFMANN and SAALFELD 1977; MÜLLER and NEDDENRIEP 1979; HOFFMANN R et al. 1981; BRÖMEL and ZETTEL 1985; HEINRITZI 1989; BALJER et al. 1989; PLANK and HEINRITZI 1990; HOELZLE et al. 2007b; RITZMANN et al. 2009; STADLER et al. 2019; ADE et al. 2022a); **Great Britain** (HENDERSON et al. 1997); **Hungary** (KANTAS and ANDO 1987); **Italy** (CERUTTI 1939); **Portugal** (PERESTRELO-VIEIRA et al. 1997); **Serbia** (LAKO et al. 2009; POTKONJAK et al. 2009; 2013); **Switzerland** (HOELZLE et al. 2007b)
North America	**Canada** (SAVAGE and ISA 1958; WILSON et al. 1988; AYROUD et al. 1994); **USA** (DOYLE 1932; KINSLEY 1932; BIBERSTEIN et al. 1956; ADAMS et al. 1959; PRESTON and GREVE 1965; HENRY 1979; SISK et al. 1980; ZINN et al. 1983; OBERST et al. 1990; 1993; MESSICK et al. 2011; STRAIT et al. 2012; DENT et al. 2013)
Central- and South America	**Argentina** (ANZIANI et al. 1986; KLOSTER et al. 1987; PORTIANSKY et al. 2004; ACOSTA et al. 2019); **Brazil** (LOPES et al. 1982; BORETTI 1984; GUIMARAES et al. 2007; TOLEDO et al. 2016; GATTO et al. 2019; MARTINS et al. 2019; PETRI et al. 2020; BORDIN et al. 2021); **Cuba** (CRUZ et al. 1985); **Trinidad** (SMITH MW 1975)
Asia	**China** (WU et al. 2006; YUAN et al. 2009; ZHOU et al. 2009; SONG et al. 2014; FU et al. 2017; ZHONGYANG et al. 2017); **Japan** (WATANABE et al. 2011, 2012); **South Korea** (JEON 1971; RIM et al. 1984; SEO et al. 2019); **Thailand** (THONGMEESEE et al. 2022)
Africa	**Nigeria** (OKON 1976; DIPEOLU et al. 1982; 1983); **Ghana** (ASSOKU 1979); **South Africa** (HENNING 1956)

There are also publications from Germany and Brazil on the occurrence of *M. suis* in wild boars (*Sus scrofa* L.; HOELZLE K. et al. 2010; DIAS et al. 2019; FERNANDES et al. 2022) and white-lipped peccaries (*Tayassu pecari*; DIAS et al. 2019).

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Infections with *M. parvum* are only described to a limited extent. Few studies report the occurrence of this species in domestic pigs, among others in Brazil (GATTO et al. 2019; Petri et al. 2020), China (FU et al. 2017), Germany (ADE et al. 2022b), Great Britain (JENNINGS and SEAMER 1956), Japan (WATANABE et al. 2011; 2012), Kenia (BARNETT 1963), Korea (SEO et al. 2019), the Netherlands (UILENBERG et al. 1981), Nigeria (DIPEOLU et al. 1982), South Africa (JANSEN 1952; HENNING 1956), Thailand (THONGMEESEE et al. 2022) as well as in the US (SPLITTER 1950a; DO NASCIMENTO et al. 2013; 2014). Further, Fernandes et al. were able to detect *M. parvum* in wild boars in Brazil (FERNANDES et al. 2022).

Since `*Ca*. M. haemosuis` is an emerging pathogen, there are currently only published data on the occurrence of `*Ca*. M. haemosuis` in domestic pigs from Asia (China (FU et al. 2017); Korea (SEO et al. 2019); Thailand (THONGMEESEE et al. 2022)).

To better illustrate the occurrence of porcine HM-infections, those countries, in which porcine HM species were found, are marked in the world map in Figure 8 below.
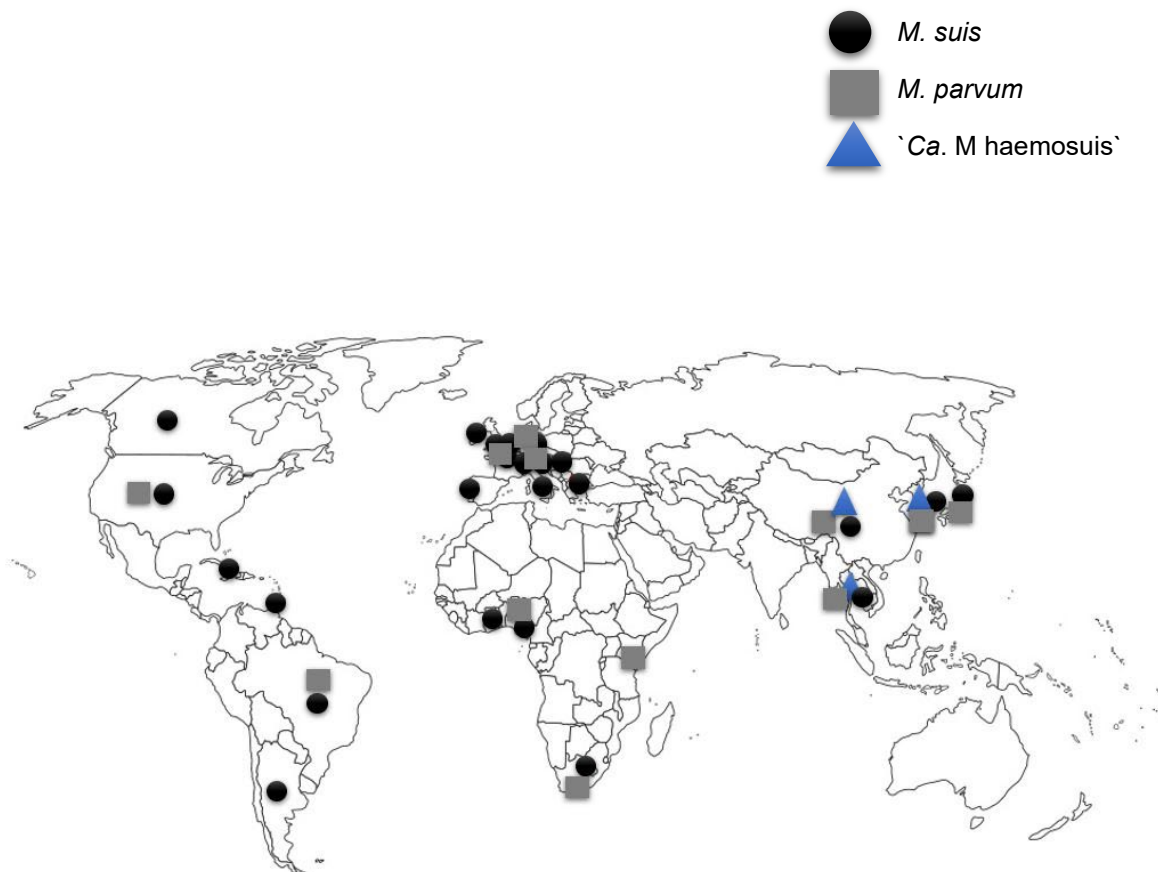
Figure 8: World map with markings for the detection of porcine HMs.

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Regarding all the mentioned descriptions of HMs in pigs, it must be mentioned that most of the cases illustrated here are not prevalence/ frequency studies but individual evidences, mostly in clinically ill animals. The number of more intensive evidence studies based on reliable, modern detection methods (PCR, serology) is unfortunately infrequently and limited to a few countries as shown in Table 2 below.

Table 2: Frequency studies of *M. suis*, *M. parvum* and `*Ca*. M. haemosuis` based on PCR or serological findings.

country	authors	age group	prevalence on single animal level		prevalence on herd level	
frequencies of *M. suis* based on PCR findings						
Brazil	GUIMARAES et al. 2007	sows	18.2%	(22/121)	100%	(4/4)
		piglets	1.64%	(1/61)	25%	(1/4)
		boars	25.0%	(1/4)	25%	(1/4)
	TOLEDO et al. 2016	slaughter pigs	76.2%	(112/147)	not available	
	MARTINS et al. 2019	sows and boars	54.7%	(35/64)	not available	
	BORDIN et al. 2021	sows	18,75%	(15/80)	40.6%	(11/27)
France	BRISSONNIER et al. 2020	sows	53,0%	(105/198)	100%	(10/10)
Germany	HOELZLE et al. 2007b	piglets	10.6%	(17/160)	not available	
	RITZMANN et al. 2009	feeder pigs	13.9%	(164/1176)	47.6%	(79/166)
Japan	WATANABE al. 2012	feeder pigs and sows	5.0%	(6/120)	9.1%	1/11
Serbia	POTKONJAK et al. 2009	not specified	100%	(40/40)	not available	
South Korea	SEO et al. 2019	all age groups	0.2%	(83/1867)	not available	
Switzerland	HOELZLE et al. 2007b	sows	19.0%	(19/100)	not available	
frequencies of *M. suis* based on serological findings						
China	SONG et al. 2014	all age groups	31.9%	(1277/4004)	95.6%	(66/69)
	ZHONGYANG et al. 2017	all age groups	33.3%	(1150/3458)	not available	
Serbia	POTKONJAK et al. 2013	feeder pigs	44.5%	(20/46)	100%	(4/4)
frequencies of *M. parvum* based on PCR findings						
Germany	ADE et al. 2022b	sows	25.0%	(15/60)	not available	
		fattening pigs	36.0%	(72/200)	not available	
		boars	4.4%	(8/183)	not available	
Japan	WATANABE et al. 2012	feeder pigs and sows	15.0%	(18/120)	54.5%	(6/11)
South Korea	SEO et al. 2019	all age groups	2.7%	(51/1867)	not available	

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frequencies of `Ca. M. haemosuis` based on PCR findings						
China	FU et al. 2017	feeder pigs and sows	50.0%	(162/324)	100%	(53/53)
South Korea	SEO et al. 2019	all age groups	0.1%	(1/1867)	not available	

The economic significance of HMs to the pig industry is controversially discussed. On the one hand, many authors consider infections to be of great economic impact (PRULLAGE 1983; HOELZLE 2007). This statement is mainly supported by the description of general poor reproductive performance in *M. suis* infected animals as well as the evidence of a higher stillbirth rate in *M. suis* infected sows (HENRY 1979; STADLER et al. 2019, BRISSONNIER et al. 2020). Moreover, Zinn et al. observed decreased birth weights in infected piglets (ZINN et al. 1983). On the other hand, there is no further detailed evidence for the scientific verification of this considered economic impact. Additionally, a recent study failed to reproduce the negative correlation between reproductive parameters and HM infections in pigs (BRISSONNIER et al. 2020). Further, the recent study by Petri et al. showed only a very weak correlation between the average daily weight gain in fattening pigs and HM infections (PETRI et al. 2020).

3.3.2 Transmission routes

To date, transmission routes are only known for *M. suis* and, with less extend, also for *M. parvum*, but not for `Ca`. M. haemosuis.

In experimental infections, *M. suis* and *M. parvum* could be transmitted via parenteral blood inoculation (STADLER et al. 2021; DO NASCIMENTO et al. 2014). Consequently, hematogenous transmission routes seem to be the most common way for animals to become infected. This might happen through iatrogenic and zootechnical measurements, through licking of wounds, during ranking fights and any other situation with direct contact to blood of infected animals (SEAMER et al. 1960; HEINRITZI 1990a; 1992; DO NASCIMENTO et al. 2014). Moreover, a transmission via blood-sucking arthropods was described by some authors. Prullage verified the transmission of *M. suis* via *Stomoxys calcitrans* (stable fly) and *Aedes aegypti* (yellow fever mosquito) (PRULLAGE 1993); Acosta et al. detected *M. suis* further in *Haematopinus suis* (pig louse) (ACOSTA et al. 2019). The transmission of *M. parvum* via *Haemoatopinus suis* was outlined in the study of Seamer (SEAMER 1960). Transmission of *M. suis* could also be described with blood-contaminated semen (HEINRITZI 1999; MAES et al. 2008).

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Oral infection using *M. suis* containing blood as an inoculum was also investigated in certain studies. Heinritzi successfully infected *M. suis* negative piglets by feeding them a high amount of *M. suis* containing blood (HEINRITZI 1992). In contrast, a recent study failed to verify this transmission route in an experimental infection model. Authors explain the differing results in various inoculation doses and *M. suis* strains used (ADE et al. 2021).

The possibility of vertical transmissions from sows to their offspring was already described for *M. suis* in a couple of older studies (BERRIER and GOUGE 1954; PRESTON and GREVE 1965; CLAXTON and KUNESH 1975). However, this small number of studies makes it very difficult to assess the likelihood and significance of this transmission pathway in natural infections. A more recent study demonstrated the occurrence of *M. suis* in piglets sampled prior to colostrum uptake, which indicates more likely a vertical transmission of *M. suis* (STADLER et al. 2019).

Despite blood dependent transmissions, very little is known about other further natural transmission and excretion pathways of HMs. Among the porcine HM species, only one study has been published to date that addresses this topic. Dietz et al. were able to demonstrate the existence of *M. suis* in urine, saliva as well as in nasal and vaginal secrete of experimentally infected pigs (DIETZ et al. 2014).

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## 3.4 Laboratory diagnostics

3.4.1. Microscopy

Historically, it was common to detect HMs microscopically via stained blood smears. For light microscopy, several Romanowsky-type stains are suitable to detect HMs (Giemsa, May-Grundwald-Giemsa, Wright, Wright-Giemsa, Pappenheimer). Here, depending on the staining type and on the pH value, HMs can be identified as pale red to reddish-purple colored dots arranged individually, in pairs or in chains and are located on the surface of RBCs or free in the plasma. The biggest problem with this methodology is that artifacts in the dyes can easily be confused with bacteria. Furthermore, the so-called Howell-Jolly bodies, which are pathological fragments of RBCs, stain like HMs. This can lead to a misinterpretation of the samples (THIEL 1983; BOBADE and NASH 1987; BERENT et al. 1998; SMALL and RISTIC 1967, HEINRITZI 1990a, b; BOUJON et al. 1991; LIEBICH and HEINRITZI 1992; TASKER and LAPPIN 2002; RITZMANN et al. 2009; HOELZLE et al. 2011). More sensitive than Romanowsky dyes and light microscopy is the staining of blood smears with acridine orange, a dye for the detection of nucleic acids, and subsequent fluorescence microscopic examination. On this occasion, HMs appear as light orange dots with a yellow-green undertone. This allows to identify also a smaller number of bacteria than light microscopy does. However, there are also some error susceptibilities here. Besides the bacterial DNA, other nucleic acids and above all the Howell-Jolly bodies might also be colored, what can easily lead to false positive results. Additionally, in blood smears of chronic and subclinical infected animals usually no organisms appear, which leads to false negative findings. This problem also applies to Romanowsky stained blood smears (BOBADE and NASH 1987; BERENT et al. 1998; SMALL and RISTIC 1967, HEINRITZI 1990a, b; BOUJON et al. 1991; LIEBICH and HEINRITZI 1992; TASKER and LAPPIN 2002; MESSICK 2004; MC AULIFFE et al. 2006; RITZMANN et al. 2009; HOELZLE et al. 2011). Compared to modern PCR methods, microscopic detections showed to be less specific and sensitive in the detection of HMs (MC AULIFFE et al. 2006; RITZMANN et al. 2009; NORMAND et al. 2020).

A Giemsa-stained porcine blood smear presenting cells of `*Ca*. M. haemosuis` is illustrated in Figure 4 in chapter 3.2.2.

An acridin orange-stained blood smear (1000 x magnification) of a pig infected with *M. suis* is shown on Figure 9. The high amount of *M. suis* cells appear as bright-orange points, RBCs are pale green (HOELZLE 2008).

3. INTRODUCTION
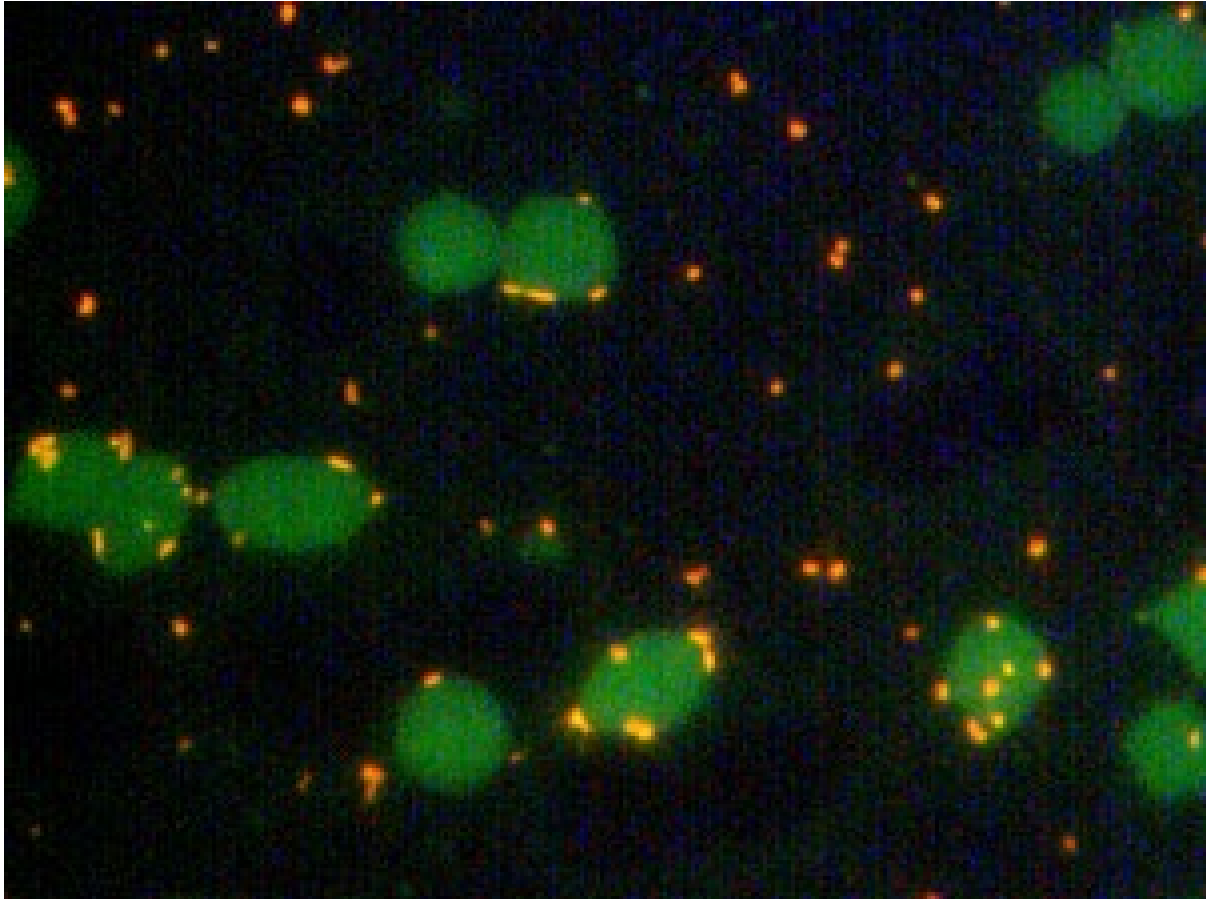
Figure 9: Acridin orange-stained porcine blood smear (1000x magnification) showing a high amount of *M. suis cells* appearing as bright orange dots while RBCs are pale green (HOELZLE et al., 2007a).

3.4.2 Culture

Today, there is no possibility to cultivate HMs *in vitro*. Cultivation attempts have been published for *M. suis*, however, it was not possible to multiply the bacteria in any of these experiments. Nonaka et al. tried to cultivate *M. suis* in petri dish cultures containing RBCs. Here, merely a short maintenance of the bacteria could be observed. Similar observations were made by Schreiner et al. trying to cultivate *M. suis* in cell-free medium enriched with glucose and iron-binding proteins. Maintenance of *M. suis* was hold for a period of 12 weeks and in addition, morphological chances of the bacteria could be observed using electron microscopy. During the cultivation, *M. suis* cells altered into irregularly shaped nanoforms arranged in microcolonies. It is assumed that a suboptimal environment could be a cause of this nanotransformation, what also means that the used media do not provide appropriate growth conditions for *M. suis* (NONAKA et al. 1996; SCHREINER et al. 2012b).

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3.4.3 Molecular methods

Since HMs cannot be cultivated *in vitro* yet and microscopic diagnostics have proven to be less specific, molecular methods play a particularly important role in the diagnosis of these pathogens. Among a variety of available methods including *in-situ* hybridization (HA et al. 2005; PETERS et al. 2011), loop-mediated isothermal amplification assay (LAMP; SONG et al. 2013) and Southern Blotting (MACIEIRA et al. 2009) numerous Polymerase-Chain-Reaction (PCR) protocols have been developed. In the meantime, conventional PCR protocols based on the amplification of 16S rDNA have been published for almost all HM species. Moreover, Hoelzle K. et al. developed a conventional PCR assay with universal primers targeting the 16S rDNA of all HM species (HOELZLE K. et al. 2011). Real-time PCRs, especially those that amplify more specific genes than 16S rDNA fragments and further have the possibility of quantification, are limited to only a few HM species, i.e. *M. suis* (HOELZLE et al. 2007b); the bovine *M. wenyonii* and `*Ca*. M. haemobos` (MELI et al. 2010; ADE et al. 2018), the canine *M. haemocanis* and `*Ca*. M. haematoparvum` (BARKER et al. 2010) and the feline *M. haemofelis*, `*Ca*. M. haemominutum` and `*Ca* M. turicensis` (TASKER et al. 2003a; WILLI et al. 2005; 2006);

3.4.4. Serology

Serological diagnostic methods are well suited for intensive epidemiological studies. In the case of HM infections, they further can make an important contribution in the diagnosis of infected animals with weak bacterial blood loads, which may be below the detection limit of the available PCRs (WOLF-JÄCKEL et al. 2010). However, the development and use of meaningful serological diagnostic systems requires the availability of suitable test antigens, what is extremely difficult for HMs due to the fact that *in vitro* cultivability has not been successful so far. That is why just a few specific serological test systems are available yet. Only for the detection of antibodies against the porcine *M. suis* (HOELZLE et al. 2006; HOELZLE K. et al. 2007; LIU et al. 2012; ZHONGYANG et al. 2017), the bovine *M. wenyonii* (ZHAO et al. 2017) and the three feline HM species (WOLF-JÄCKEL et al. 2010) specific protocols have been published based on recombinant proteins.

4. **OBJECTIVES**

Mycoplasma suis, *Mycoplasma parvum* and `*Candidatus* Mycoplasma haemosuis` are porcine hemotrophic mycoplasma species. The current knowledge concerning the microbiology, pathobiology and epidemiology of those bacteria is highly limited. Especially incomplete is the expertise on the 2017 described species `*Ca*. M. haemosuis` compared to the long-studied *M. suis* and, additionally, the understanding of the meaning, frequency and transmission of subclinical and chronic infections of all three porcine HM species. This is particularly a result of the bacterias` uncultivability and the associated lack of specific diagnostic possibilities. However, a further increase of knowledge is justified for a variety of reasons. For one thing, there seems to be a problem of economic damages due to pathogenic hemotrophic mycoplasmas in pig husbandry. This may be caused by performance degradations and increased susceptibilities for further infections as they are reported for infections with *M. suis*. Additionally, it is well known that HM infections can lead to a high demand on antibiotics as antibiotic treatments are the only available therapy and there is no effective possibility to forestall infections with preventive measures such as vaccinations. Also, a zoonotic potential of hemotrophic mycoplasmas cannot be ruled out.

In addition to baseline studies regarding microbiological and pathobiological characteristics of hemotrophic mycoplasmas, epidemiological studies based on reliable diagnostic tools provide a comprehensive insight into the frequency of naturally appearing diseases as well as into possible shedding and transmission routes. They are therefore essential to emphasize the meaning of hemotrophic mycoplasmas as well as to have a reliable basis for eradication measures.

For this reason, the first aim of this work was to develop a quantitative real-time PCR assay for the detection of `*Ca*. M. haemosuis` in samples of pigs, since no diagnostic tool was published and available to date. The novel established qPCR assay served as a specific and sensitive diagnostic tool for the quantitative detection of this HM species in pigs.

Applying this novel established PCR assay to porcine blood samples, this work secondly aimed to analyze the involvement of `*Ca*. M. haemosuis` in an acute disease event in fattening pigs showing IAP-like clinical signs. This reflects the first detailed description of clinical signs in pigs caused by `*Ca*. M. haemosuis`.

The third aim was to gain further insight into the occurrence of `*Ca*. M. haemosuis` in Germany. Therefore, blood samples from pigs concerning to different age groups (piglets, fattening pigs, sows, boars) were collected and conducted to the novel qPCR. This represents the first prevalence study of `*Ca*. M. haemosuis` in Europe as well as the first determination of

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`*Ca*. M. haemosuis` blood loads. Further, piglets were sampled before colostrum uptake to illustrate the meaning of a possible vertical transmission of `*Ca*. M. haemosuis` from mothers to their offspring under field conditions, as this aspect has not been evaluated before. In addition to blood samples, saliva and urine samples of the blood-sampled sows and semen samples of the blood-sampled boars were collected and examined with qPCR in regard to evaluate a possible blood-independent shedding and transmission of `*Ca*. M. haemosuis`.

The following sections provide a detailed overview on the objectives which are reflected by scientific publications.

4. OBJECTIVES

4.1 Establishment of a quantitative real-time PCR assay for the detection of `*Ca*. M. haemosuis` in pigs

- Establishment of a `*Ca*. M. haemosuis` specific quantitative real-time SYBR green PCR assay (StepOne™ System Applied Biosystems®) using novel primers targeting the *gap*

- **Major conclusions:**

  – The novel qPCR assay was found to be a highly specific and sensitive tool for the diagnosis of `*Ca*. M. haemosuis` in porcine samples. No cross-reactivities with other mycoplasma species and other porcine pathogens were determined.

  – The novel qPCR assay enables the quantification of `*Ca*. M. haemosuis` in clinical samples by applying the standard curve method (plasmid standard dilutions).

- This topic is present in the following article:

  Ade J, Stadler J, Ritzmann M, Zübert C, Hoelzle K, Hoelzle LE. 2022a. Occurrence of `Candidatus Mycoplasma haemosuis` in fattening pigs, sows and piglets in Germany using a novel gap-based quantitative real-time PCR assay. BMC Vet Res. 18(1): 40

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4.2 Involvement of `*Ca*. M. haemosuis` in an acute disease event with IAP-like clinical signs in fattening pigs

- The emerging hemotrophic *Mycoplasma* species `*Ca*. M. haemosuis` was detected in fattening pigs during an acute disease outbreak in Southern Germany.

- **Major conclusions:** `*Ca*. M. haemosuis` was present in the blood of seven pigs showing typical IAP like clinical and pathological signs. Extensive differential diagnostic investigations including *M. suis*, Classical and African Swine Fever and others all remained negative. Thus, it seems likely, that `*Ca*. M. haemosuis` is also able to cause infectious anemia in pigs.

- This topic is present in the following article:

  Stadler J, Ade J, Ritzmann M, Hoelzle K, Hoelzle LE. 2020. Detection of a novel haemoplasma species in fattening pigs with skin alterations, fever and anemia. Vet Rec. 187(2), 66

4. OBJECTIVES

4.3 Occurrence of `Ca. M. haemosuis` in pigs of different age groups in Germany

- The occurrence of `Ca. M. haemosuis` was investigated in pigs belonging to different age groups (piglets, fattening pigs, sows, boars). A total of 208 sows and 622 piglets out of 21 piglet producing farms (10 sows per farm and 2-3 piglets of each sow) as well as 183 boars were sampled. Further, 200 fattening pigs out of 20 farms were sampled at the time of slaughter. Obtained results were compared to the *M. suis* status, which was also determined by qPCR.

- **Major conclusions:**

  - `Ca. M. haemosuis` (and *M. suis*) were present in the blood of piglets, sows, and fattening pigs but not in the blood of boars.
  - Table 3 summarizes the observed qPCR results of all sampled animals.

    Table 3: Hemotrophic mycoplasma qPCR results of the sampled animals from different age groups on herd and single animal level

	`Ca. M. haemosuis`		*M. suis*	
piglet producing farms	14.29 %	(3/21)	76.19 %	(16/21)
farrowing sows	6.25 %	(13/208)	31.25 %	(65/208)
piglets	4.50 %	(28/622)	14.35 %	(68/474)
farms	45.00 %	(9/20)	50.00 %	(10/20)
fattening pigs	17.50 %	(35/200)	19.90 %	(38/200)
boars	00.00%	(0/183)	00.00%	(0/183)

- These topics are present in the following articles:

  - Occurrence of `Ca. M. haemosuis` (and *M. suis*) in blood samples of sows, piglets and fattening pigs:

    Ade J, Stadler J, Ritzmann M, Zübert C, Hoelzle K, Hoelzle LE. 2022a. Occurrence of `Candidatus Mycoplasma haemosuis` in fattening pigs, sows and piglets in Germany using a novel gap-based quantitative real-time PCR assay. BMC Vet Res. 18(1): 40

4. OBJECTIVES

- Occurrence of `Ca. M. haemosuis` (and *M. suis*) in blood samples of boars:

  Ade J, Ritzmann M, Wöstmann C, Eddicks M, Reese S, Hoelzle K, Hoelzle LE, Stadler J. 2021. Update on shedding and transmission routes of porcine haemotrophic mycoplasmas in naturally and experimentally infected pigs. Porcine Health Manag 7(1): 49

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4.4 Investigation on the vertical transmission of \`*Ca*. M. haemosuis\` under field conditions

- \`*Candidatus* M. haemosuis\` qPCR results of farrowing sows (n=208) and their piglets (n=622, 2 or 3 piglets of each sow) (see chapter 4.3) were used to evaluate the possibility of a vertical transmission of \`*Ca*. M. haemosuis\` under field conditions as the piglets were sampled immediately after birth and prior to the first colostrum uptake.

- **Major conclusions**: \`*Ca*. M. haemosuis\` was detected in the blood of pre-suckling sampled piglets. High bacterial blood loads in piglets argue against an infection during birth but indicate the possibility of a vertical transmission of \`*Ca*. M. haemosuis\` from sows to their piglets.

- This topic is present in the following articles:

  Ade J, Stadler J, Ritzmann M, Zübert C, Hoelzle K, Hoelzle LE. 2022a. Occurrence of \`*Candidatus* Mycoplasma haemosuis\` in fattening pigs, sows and piglets in Germany using a novel gap-based quantitative real-time PCR assay. BMC Vet Res. 18(1): 40

4. OBJECTIVES

4.5 Shedding of `Ca`. M. haemosuis` and *M. suis* in blood-free secretes (i.e. saliva, urine and semen samples) under field conditions

- Saliva (n=148) and urine (n=47) samples were obtained from sows, and semen samples (n=183) were also collected from boars with a known `Ca`. M. haemosuis` and *M. suis* status. All secretes and excretes were tested for the occurrence of `Ca`. M. haemosuis` and *M. suis* by qPCR. In addition, urine samples were tested for RBC residues using Servotest® 5 + NL stripes (Servoprax, Wesel, Germany).

- **Major conclusions:**

  - Blood independent shedding routes are not likely to play an important role under field conditions.
  - Urine samples: Both, `Ca`. M. haemosuis` and *M. suis* could not be detected in any of the 47 tested urine samples.
  - Saliva samples: All 148 saliva samples were tested negative for `Ca`. M. haemosuis` and *M. suis* by qPCR, also from animals whose blood sample showed a positive qPCR result for `Ca`. M. haemosuis` or *M. suis*, respectively.
  - Semen samples, as well as corresponding blood samples were qPCR negative for `Ca`. M. haemosuis` or *M. suis*.

- This topic is present in the following article:

  Ade J, Ritzmann M, Wöstmann C, Eddicks M, Reese S, Hoelzle K, Hoelzle LE, Stadler J. 2021. Update on shedding and transmission routes of porcine haemotrophic mycoplasmas in naturally and experimentally infected pigs. Porcine Health Manag 7(1): 49

5. PUBLICATIONS

5.1 Detection of a novel haemoplasma species in fattening pigs with skin alterations, fever and anaemia

Stadler J, Ade J, Ritzmann M, Hoelzle K, Hoelzle LE.

Detection of a novel haemoplasma species in fattening pigs with skin alterations, fever and anaemia

Julia Stadler [ID] ,[1] Julia Ade,[2] Mathias Ritzmann,[1] Katharina Hoelzle,[2] Ludwig E Hoelzle[2]

Abstract

Background In a fattening farm in Southern Germany, skin alterations (urticaria, haemorrhagic diathesis) and high fever were observed in 30% of the pigs 2 weeks after arrival. Feed intake was severely compromised in affected pigs.

Methods After detailed clinical observation, blood samples from affected pigs were collected for haematological, PCR and serological investigations. In addition, pathological investigations were performed on one pig.

Results and conclusion Analysis of blood parameters revealed a normocytic, normochromic anaemia. A novel porcine haemoplasma species was detected in blood samples of affected pigs and spleen sample of the necropsied pig by PCR. Phylogenetic analyses based on the 16S rDNA showed 99% identity to a novel porcine haemoplasma ('*Candidatus* (*Ca*.) M. haemosuis') species which has recently been described in China. Interestingly, this is the first report of '*Ca*. M. haemosuis' in pigs with clinical signs resembling those of *Mycoplasma (M) suis* and the first description of this novel haemoplasma species outside Asia. On-farm affected pigs were treated with oxytetracycline and non-steroidal anti-inflammatory drugs. Clinical signs improved after implementation of treatment and optimisation of management procedures. This case might indicate that other porcine haemoplasma species than *M suis* can induce fever and skin alterations and may have an economic impact on affected farms.

Introduction

Haemotrophic mycoplasmas (haemoplasmas) are uncultivable, small epicellular, cell wall-less, tetracycline-sensitive bacteria that attach to the surface of host erythrocytes.[1] Haemoplasmas were formerly classified in the two genera *Eperythrozoon* and *Haemobartonella* within the family *Anaplasmataceae*. Phylogenetic analyses of the 16S rDNA and RNase P RNA gene led to a reclassification into the family *Mycoplasmataceae*.[2–6]

Haemotrophic mycoplasmas are detected in a large number of vertebrate hosts.[7–16] Despite the description of host adaptation and strict host specificity for most haemoplasma species, interspecies transmission and zoonotic potential of haemoplasma infections have to be considered.[17–20] Until 2017, two porcine haemoplasma species have been described in pigs, that is, *Msuis* and *M parvum*. While *M parvum* is considered non-pathogenic, *M suis* is known as the major cause of infectious anaemia in pigs (formerly known as eperythrozoonosis).[1 21 22] Depending on the host susceptibility and the virulence of *M suis*, the disease manifestation in pigs varies from acute life-threatening anaemia with high fever, icterus, cyanosis and necrosis in the ears and massive hypoglycaemia to chronic disease associated with mild anaemia, reduced growth rate, poor reproductive performance and increased susceptibility to secondary infections of the respiratory and enteric system as well as asymptomatic infections.[1 22–24] *M suis* and *M parvum* are phylogenetically closely related, both clustering in the 'Haemominutum' group within the phylogenetic cluster of haemoplasmas.[5] This 'Haemominutum' group mainly represents the former

species *Eperythrozoon*. Recently, a novel porcine haemoplasma species was described in clinically healthy pigs in China with a prevalence of 26.5% and in Korea with a prevalence of 0.01%.[25] Phylogenetic analyses revealed that this potentially novel haemoplasma species is closely related to the feline haemoplasma '*Ca*. M. turicensis', in the 'Haemofelis' group which mainly includes the former members of the species *Haemobartonella*.[5 25] The novel species was provisionally named '*Candidatus* (*Ca*.) M. haemosuis'.[25] In the present case, skin alterations, fever and anaemia were reported in a fattening farm. '*Ca*. M. haemosuis' was diagnosed in blood samples of affected pigs and spleen sample of a necropsied pig by PCR and subsequent sequencing of the 16S rDNA amplicons.

Clinical report

Case history and clinical findings
The case described here occurred on a fattening farm with 1200 pigs located in a high pig density area in Southern Germany. The farm is single sourced from a 300-sow self-recruiting piglet producing farm in Southern Germany located at a distance of 50 km from the fattening farm. Every 4 weeks, 360 pigs with an average weight of 30 kg which were vaccinated against Porcine Circovirus Type 2 and *Mycoplasma hyopneumoniae* in the third week of life are delivered. Upon arrival at the fattening farm, pigs are vaccinated with a PRRSV-1 MLV. The fattening farm consists of four barns each containing six unequally sized pens. On the fattening farm, pigs are housed on fully slatted floor in groups of 15 (smaller pens) and 80 (bigger pens) pigs per pen, respectively. Pigs are fed with liquid feed which consists of commercial feed and fresh water from the public supplier. All-in all-out with cleaning and disinfection between groups is performed. During the fattening period, no antimicrobials are used and deworming is routinely performed on arrival at the fattening farm. The average length of the fattening period is 115 days, with an average mortality rate of 1% and average daily weight gain of 780 g.

Clinical problems in the herd started beginning of May 2017, when approximately 30% of the pigs showed reduced feed intake and high fever (>40°C) 2 weeks after arrival. The herd veterinarian initiated treatment of affected pigs with cefquinomsulfat (2 mg/kg body weight/q 24 hours) via intramuscular injection for three consecutive days and dexamethasone (0.05 mg/kg body weight/q 24 hours) for two consecutive days. However, treatment did not result in improvement of clinical signs. The Clinic for Swine was involved into the clinical problem of the farm by the herd attending veterinarian end of May 2017. Clinical signs observed during the herd visit were characterised by apathy, fever and skin alterations. Clinical examination of the skin revealed pallor, icterus, petechia, ecchymosis and cyanosis of ears, ventral abdomen and legs. In several pigs, generalised skin reactions like small round pink to purple raised wheals in common with urticaria and haemorrhagic diathesis were observed (figure 1). Clinical signs also included necrosis of the edge and tip of the ears. Further anamnesis revealed no changes of housing, water supply or the feeding procedure. During the herd visit, an elevated temperature of 26.0°C–27.0°C was evident in all fattening barns. Subsequently, in the affected age group the mortality increased to 4.8% and a prolonged fattening period (132 fattening days) and decreased average daily weight gain of 680 g were recorded.

Diagnostic methods and laboratory findings
For diagnostic work-up, blood samples (EDTA-anti-coagulated blood and serum) from 20 pigs with skin alterations and/or fever were collected during the farm visit by the University Clinic for Swine for haematological, PCR and serological investigations. Blood samples were investigated for Classical and African swine fever (PCR and ELISA), PCV-2 (PCR[26]), PCV-3 (PCR[27]), *M suis* (PCR[28]), PRRSV (PCR[29]) and *Sarcoptes scabiei* var. *suis* (ELISA). All samples were negative for Classical and African Swine Fever, PCV-2 and PCV-3, *M suis*, PRRSV and *Sarcoptes scabiei* var. *suis*. Haematological parameters, that is, red blood cell count, haemoglobin, packed cell volume (PCV), mean corpuscular volume, mean corpuscular

Figure 1 Fifteen-week-old pig (ID 82/17) with cutaneous haemorrhages on the ears (A) and on the entire body (B), housed at the University Clinic for swine for further investigations.

Table 1	Results of haematological examination and qPCR investigation			
Animal ID	RBC 10⁶/μl	PCV %	Haemoglobin mmol/l	Novel haemoplasma species GE/ml blood*
Reference values	‹5.80	‹32	‹6.6	
1	5.28	0.31	5.82	5.42E+02
2	6.95	0.36	6.64	Negative
3	5.6	0.29	5.47	5.34E+05
4	4.95	0.25	4.36	Negative
5	6.28	0.37	6.95	Negative
6	5.57	0.3	5.03	3.08E+02
7	6.95	0.37	6.89	Negative
8	6.36	0.37	6.76	Negative
9	6.68	0.35	6.89	Negative
10	5.38	0.3	5.07	Negative
11	5.77	0.31	6.5	Negative
12	6.54	0.34	6.7	Negative
13	6.26	0.33	6.76	Negative
14	5.34	0.29	4.9	3.96E+07
15	5.98	0.33	5.88	Negative
16	3.75	0.22	3.78	1.75E+03
17	2.98	0.15	2.61	2.14E+06
18	6.15	0.33	6.64	Negative
19	5.48	0.3	5	Negative
20	6.55	0.32	6.45	Negative
82/17	5.62	0.32	6.24	2.01E+04

Red blood cell (RBC) count, haemoglobin, packed cell volume (PCV) and bacterial load of qPCR-positive pigs are displayed in bold. Reference values (Kixmöller et al[47]) are listed in brackets. *Detection and quantification of 'Ca. M. haemosuis' genome equivalents (GE) using a gap-based quantitative PCR (paper in preparation).

haemoglobin (MCH) and MCH concentration were determined using the Scil Vet ABC tool (Scil Animal Care Company GmbH). Analysis of haematological parameters revealed normocytic, normochromic anaemia in 11 out of 20 blood samples (table 1). A haemoplasma-specific 16S rDNA PCR[30] was performed at the Institute of Animal Science, University of Hohenheim, Germany. Seven out of 21 blood samples were 16S rDNA PCR positive. Sequencing of the 16S rDNA amplicons revealed a 99% identity to the 16S rDNA of a novel porcine haemoplasma species (accession no. JX489601) which has so far been found in China[25] and Korea.[31] In addition, all blood samples were further investigated using a novel quantitative PCR (qPCR) specific for the novel porcine haemoplasma species ('Candidatus (Ca.) M. haemosuis'; paper in preparation). Briefly, we used primers targeting the 'Ca. M. haemosuis' gap gene (CMhsuisF 5′-TGCTTTGGCTCCTGTGGTTA-3′ and CMhsuisR 5′- GCAG CAGCACCTGTAGAAGTA-3′; accession no. MN896988). 'Ca. M. haemosuis' DNA was detected with the StepOne System (Applied Biosystems) using Fast SYBR Green Master Mix and a melting curve analysis was performed after each PCR run. The lower limit of detection was 0.45 fg of the plasmid DNA per reaction (C_T <35 cycles) corresponding to 10 genome equivalents per reaction and 2.5×10^3 bacteria per millilitre of blood. The PCR was considered highly specific as no cross-amplification was detected using DNA-derived several haemotrophic and non-haemotrophic Mycoplasma species (including

M suis, M parvum and M hyopneumoniae) as well as other porcine bacterial pathogens. We further included plasmid DNA standard dilutions containing 'Ca. M. haemosuis' gap amplicons in each run to quantify 'Ca. M. haemosuis' DNA. Table 1 includes the qPCR results. All 16S rDNA PCR-positive samples also reacted qPCR positive.

In addition, one pig (ID 82/17) with severe skin alterations and fever was selected and forwarded to the University Clinic for Swine for further diagnostic work-up. Pathological investigations were performed at the Institute of Veterinary Pathology, Ludwig-Maximilians-University Munich, Germany. Postmortem examination revealed multifocal generalised cutaneous haemorrhages and pallor of mucus membranes. Gross lesions were further characterised by icterus of the liver and vascular endothelium as well as ascites. Microscopic examination of the liver revealed a splenic sinus hyperplasia and a generalised follicular hyperplasia. In addition, multifocal lymphoplasmacytic dermatitis was microscopically found. An aerobic and anaerobic bacteriological examination from various organs (brain, spleen, liver, kidney, heart, lung, lymph nodes and intestines) was performed (Institute of Infectious Diseases and Zoonosis, Ludwig-Maximilians-University Munich, Germany) and revealed growth of commensal pathogens (lungs: Rothia nasimurium, Aerococcus viridans; intestines: Bacillus subtilis, Enterococcus faecalis). In addition, organ samples (tonsils, lungs, lymph nodes, spleen and kidneys) were investigated at the Institute of Infectious Diseases and Zoonosis, Ludwig-Maximilians-University Munich, Germany for the presence of PRRSV by PCR and PCV-2, PCV-3 by PCR and in situ hybridisation for PCV-2 and at the Institute of Animal Science, University of Hohenheim, Germany for porcine haemoplasma by PCR as previously described. All samples were negative for PRRSV, PCV-2 and PCV-3. Spleen samples of the necropsied pig were negative for M suis but positive for the novel porcine haemoplasma species.

Further steps and outcome of the case

Pigs with skin alterations and fever were each treated twice with intramuscular injection of long-acting oxytetracycline (20 mg/kg body weight, q 48 hours) and metamizol (20 mg/kg body weight q 24 hours). In addition, to reduce predisposing factors the farmer was advised to decrease the barn temperature. The clinical signs improved after implementing treatment with oxytetracycline and adaptation of barn temperature. Moreover, blood samples (n=11) were collected from sows at the piglet producing farm and investigated for M suis and 'Ca. M. haemosuis' by qPCR. Despite qPCR-negative results for both M suis and 'Ca. M. haemosuis', the farmer of the fattening farm decided to change the piglet supplier. No new cases of skin alterations and fever occurred after the fattening farm purchased piglets from a new supplier located in Northern Germany.

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Discussion

The present report describes a case of skin alterations and fever in a fattening farm in Southern Germany caused by the novel porcine haemoplasma species ('*Candidatus* (*Ca.*) M. haemosuis'). Haemotrophic mycoplasma can induce haemolytic anaemia in a wide range of mammalian species. Clinical manifestations can range from life-threatening haemolytic anaemia to mild chronic anaemia, ill thrift, infertility and immune suppression as well as clinically inapparent infections.[32–34] Until 2017, only two species have been described in pigs: *M parvum* and *M suis*. Research has mainly focused on the latter, as *M parvum* is regarded non-pathogenic.[21 35] In 2017, a novel haemoplasma species was identified in apparently clinically healthy pigs in China[25] and recently in Korea in one pig without further information on the clinical manifestation.[31] This novel haemoplasma species was most closely related to a feline haemoplasma species, '*Ca.* M. turicensis'. To the best of the author's knowledge, this is the first report on the occurrence of '*Ca.* M. haemosuis' in clinically affected pigs. In the present case report, clinical signs, haematological alterations and lesions resembling an acute *M suis* infection were found. However, *M suis* could not be confirmed by PCR investigation.

Cutaneous manifestations in terms of urticaria and haemorrhagic diathesis as observed in the present case have also been described after experimental infection with *M suis*.[32 36] Interestingly, dermatological disorders including erythematous maculopapular and vesicular rashes are frequently found in human medicine as complication of *M pneumoniae* infections.[37] During the last decades, intravasal coagulation and subsequent consumption coagulopathy was regarded as the primary cause of haemorrhagic diathesis during acute *M suis* infection.[24] However, more recent research indicates that interaction between *M suis* and endothelial cells resulting in endothelial damage, adhesion to the endothelium and vascular occlusion might be responsible for the development of haemorrhagic diathesis.[38] Further research is certainly needed to elucidate the exact pathogenesis responsible for cutaneous manifestation.

In the present case, all '*Ca.* M. haemosuis'-positive pigs showed a normocytic, normochromic anaemia, usually observed during natural and experimental *M suis* infection.[32 39 40] Interestingly, in cats severe anaemia was only detected after co-infection with *M haemofelis* and '*Ca.* M. haemominutum' and not after infection with '*Ca.* M. turicensis' or '*Ca.* M. haemominutum' alone.[41] Macroscopic examination of one pig in the present case revealed similar results as observed after infection with *M suis* (icterus, ascites, liver alterations, pale mucus membranes).[42] However, pathognomonic findings during necropsy were neither detected by Dent *et al*[42] nor in our investigations. The field case illustrates that this novel porcine haemoplasma species might pose a pathogenic potential for pigs. However,

considering the high prevalence reported by Fu *et al*[25] in apparently asymptomatic carrier pigs, it might be speculated that the clinical presentation described here was exacerbated by predisposing factors as elevated barn temperature observed during the farm visit. It is well known that non-infectious and infectious co-factors support the clinical manifestation of *M suis* infections.[1] The potential of this novel haemoplasma species to induce haemolytic anaemia, corresponding clinical signs and pathological lesions needs to be further investigated in experimental studies.

Several transmission routes for *M suis* have been described in literature including blood-dependent and blood-independent horizontal transmission routes.[1 43] A recent publication also indicates the possibility of vertical transmission under field conditions.[32] However, despite extensive research on transmission routes of *M suis* within the last decades, the exact mechanism of inter-transmission and intra-transmission routes needs to be further evaluated. To elucidate the potential source of introduction of this novel haemoplasma species in the present case, blood samples from 11 sows were tested for *M suis* and '*Ca.* M. haemosuis' by qPCR at the piglet producing farm. The sample size was based on previous investigations determining the intra-herd prevalence of *M suis* in a German pig population.[40] However, as data on the intra-herd prevalence of '*Ca.* M. haemosuis' are lacking so far, and clinical signs disappeared after the change of the piglet producing farm, the sample size might have been insufficient to detect '*Ca.* M. haemosuis'.

The diagnosis of haemoplasma infections is impaired by the lack of an in vitro cultivation system. However, different techniques are applied for the diagnosis of *M suis*.[28 39 44–46] The formerly used microscopic detection of the agent in chemically stained blood smears has been mainly replaced by more specific, sensitive, reproducible and reliable modern molecular techniques. Next to the establishment of different PCR assays, serological diagnostic methods based on whole-cell ELISA or recombinant ELISAs have been widely used for prevalence studies in recent years. For the differentiation of porcine haemoplasma species, phylogenetic analyses based on 16S rDNA sequences or species-specific PCRs are needed. As both methods are not always available for routine diagnostic, it is supposed that infections with this novel haemoplasma species are underdiagnosed. In this report, the novel haemoplasma species was identified by a haemoplasma-specific 16S rDNA PCR[30] and subsequent sequencing of the 16S rDNA amplicons. Additional diagnostic investigations using a newly established qPCR (paper in preparation) confirmed the results of the conventional PCR and sequencing. Bacterial loads in blood ranged from 3.08×10^2 to 3.96×10^7 '*Ca.* M. haemosuis' cells/ml blood and were thus within the range found for *M suis* in feeder pigs in Germany (range 1.2×10^2 to 1.1×10^{10} *M suis* cells/ml blood).[40] Due to the lack of vaccination against haemoplasma, treatment with

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oxytetracycline combined with a reduction of infectious and non-infectious predisposing factors are currently the method of choice.

Conclusion

In conclusion, the present case report resembles the first description of '*Ca*. M. haemosuis' in pigs outside Asia. The occurrence of skin alterations, fever and anaemia in '*Ca*. M. haemosuis'-positive pigs indicates that this novel haemoplasma species might induce clinical signs comparable with *M suis*. However, further studies are certainly needed to assess the prevalence of this novel porcine haemoplasma and gain more information on transmission routes as well as its impact on the European swine industry.

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5. PUBLICATIONS

5.2 Update on shedding and transmission routes of porcine haemotrophic mycoplasmas in naturally and experimentally infected pigs

Ade J, Ritzmann M, Wöstmann C, Eddicks M, Reese S, Hoelzle K, Hoelzle LE.

Update on shedding and transmission routes of porcine haemotrophic mycoplasmas in naturally and experimentally infected pigs

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Abstract

Horizontal transmission of *Mycoplasma suis* via parenteral exposure during standard practices or through bites during fightings have been identified as key epidemiological routes. However, as knowledge gaps on other potential shedding and transmission routes exist, the present study combines both laboratory experiments and field surveys to gain new insights into the epidemiology of porcine haemotrophic mycoplasmas. Splenectomised pigs were orally inoculated with a *M. suis* field strain and investigated for clinical signs related to infectious anaemia of pigs (IAP) and the presence of *M. suis* in blood, urine and saliva samples by qPCR. All blood samples were negative for *M. suis* and animals did not show obvious clinical signs of IAP throughout the entire study period. Additionally, urine, nasal and saliva samples from sows of conventional piglet producing farms and semen samples from a boar stud revealed no detection of *M. suis* and '*Candidatus* Mycoplasma haemosuis' by qPCR. Thus, the results indicate that blood-independent transmission routes might be of minor relevance under field conditions.

Keywords: Porcine haemotrophic mycoplasmas, *Mycoplasma suis*, '*Candidatus* Mycoplasma haemosuis', Oral inoculation, Shedding patterns

Background

Haemotrophic mycoplasmas (HMs) are uncultivable bacteria found on the surface of red blood cells (RBCs) of numerous domestic and wild mammals [1]. *Mycoplasma suis*, the mostly studied porcine HM species, is considered as the causative agent of infectious anaemia in pigs (IAP), causing important economic losses in pig production [2, 3]. The disease can either occur as an acute, haemolytic anaemia attended by high fever and life-threatening conditions or as a chronic or even subclinical form of disease with mild to moderate anaemia and unspecific clinical signs [2, 3]. Recently, a novel HM species currently named as '*Candidatus* (*Ca*.) Mycoplasma haemosuis' was described in subclinical diseases as well as in accordance with IAP-like signs in pigs in China, Korea and Germany [4–6].

Natural routes of porcine HM transmission remain rather unknown [7–9]. Experimental transmission by intravenous, intramuscular, subcutaneous, intraperitoneal and oral inoculation of *M. suis* containing blood has been successfully performed [7, 10]. However, oral infection experiments were conducted prior to the establishment of specific and sensitive PCR assays using microscopic methods [7]. Thus, the first objective of the study was to demonstrate the possibility of oral infection in experimentally infected pigs compared to a subcutaneous infected control group.

Recently, *M. suis* shedding was demonstrated in blood-free excretions (i.e. saliva, urine, nasal and vaginal

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secretion) [11] suggesting transmission of porcine HMs through direct contact via excretions. However, the investigated excretions originated from experimentally infected pigs with high blood loads [11]. Thus, the second aim of the study was to investigate shedding of porcine HMs (*M. suis* and '*Ca.* M. haemosuis') via blood-independent excretions (saliva, urine and semen samples) under field conditions.

Results
Clinical and pathological observations during experimental *M. suis* infection
In the group of orally infected pigs (group A), clinical signs related to IAP were absent in all seven animals until the end of the study on 90 DPI and the clinical score remained at zero points for each animal. In contrast, all subcutaneously infected piglets (group B) showed typical signs of IAP as described by Stadler et al. [12]. Three animals (ID 23, 32, 76) developed fever, apathy, anorexia and skin alterations on 7 and 8 DPI. The determination criteria (i.e. high fever, anorexia, impaired general health) were reached on 8 DPI and the three pigs were humanely euthanized. The four remaining animals showed cyanoses of the ears between13 and 15 DPI. Clinical IAP signs exacerbated in those four remaining animals and euthanasia had to be performed on 17 (ID 73), 20 (ID 74), 41 (ID 31) and 62 (ID 71) DPI, respectively. Clinical score points of group B animals (ID 23, 31, 32, 71, 73, 74, 76) are shown in Fig. 1. During necropsy, none of the seven orally infected pigs (group A) showed macroscopic alterations. Thus, no further microscopic investigation was conducted in those animals. Group B animals (subcutaneously infected) showed various macroscopic

(i.e. e. severe icterus of membranes, yellowish discoloration of skin and body fluids, pale musculature) and histopathological lesions (i.e. hyaline thrombi and globules in alveolar vessels, haemosiderin deposits in macrophages, dilatation of lymph vessels, periportal and centrilobular necrosis) as described elsewhere [12].

Detection of *M. suis* in blood, saliva and urine samples of experimentally infected pigs
Mycoplasma suis qPCR remained negative for blood, urine and saliva samples of all group A piglets throughout the entire study period. In group B, positive *M. suis* results were obtained from the blood of all animals as described elsewhere [12]. Briefly, on 4 DPI *M. suis* was first detected in the blood of three animals (ID 23, 74, 76) and on 6 DPI in all seven animals of this group. Subsequently, *M. suis* was permanently present in the blood of group B animals until the individual termination point of the study [12]. *M. suis* blood loads varied between 2.2×10^3 and 8.6×10^9 *M. suis*/mL blood.

qPCR could also detect *M. suis* in the urine samples of two animals on 8 DPI (ID 73) and on 28 and 48 DPI (ID 71), respectively. *M. suis* loads in urine samples varied between 2.40×10^4 and 5.5×10^4 *M. suis*/mL urine. In each of the three urine samples, Servotest® 5 + NL stripes showed the presence of RBC residues.

Regarding the saliva samples of group B animals, *M. suis* was evident in two animals on 8 DPI (ID 31 + ID 71), and on 15 DPI in one animal (ID 71). Bacterial loads in saliva samples varied between 2.0×10^3 and 5.26×10^3 *M. suis*/mL saliva.
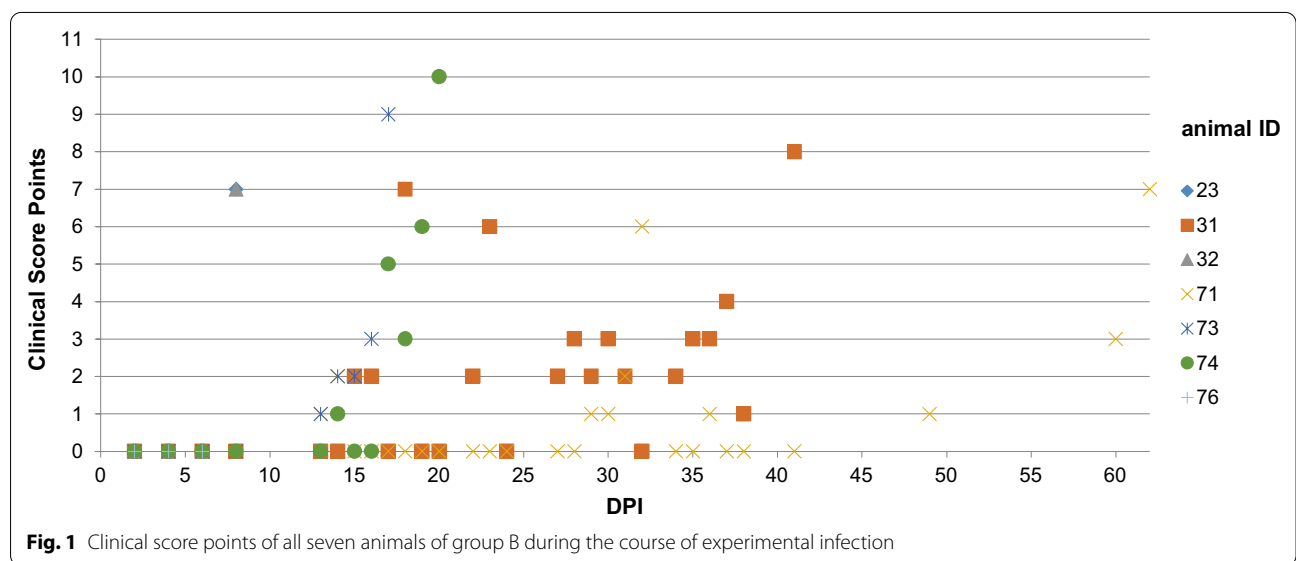
Fig. 1 Clinical score points of all seven animals of group B during the course of experimental infection

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Table 1 *Mycoplasma suis* quantification of blood, urine and saliva samples determined by qPCR during experimental infection in group B animals (subcutaneously infected animals)

Days post infection	*M. suis*/mL	Animal ID (group B animal)						
		ID 23	ID 31	ID 32	ID 71	ID 73	ID 74	ID 76
2	Blood	Negative	Negative	Negative	Negative	Negative	Negative	Negative
	Urine	Negative	Negative	Negative	Negative	Negative	Negative	Negative
	Saliva	Negative	Negative	Negative	Negative	Negative	Negative	Negative
4	Blood	2.2×10^3	Negative	Negative	Negative	Negative	2.0×10^5	6.0×10^4
	Urine	Negative	Negative	Negative	Negative	Negative	negative	Negative
	Saliva	Negative	Negative	Negative	Negative	Negative	Negative	Negative
6	Blood	4.6×10^8	1.1×10^4	4.2×10^7	3.6×10^5	1.9×10^4	6.6×10^4	1.1×10^9
	Urine	Negative	Negative	Negative	Negative	Negative	Negative	Negative
	Saliva	Negative	Negative	Negative	negative	Negative	Negative	Negative
8	Blood	3.6×10^9†	9.2×10^6	9.6×10^9†	3.0×10^9	3.0×10^7	1.8×10^7	8.6×10^9†
	Urine	Negative	Negative	Negative	Negative	5.5×10^4	Negative	Negative
	Saliva	Negative	2.0×10^3	Negative	2.84×10^3	Negative	Negative	Negative
14	Blood		1.4×10^8		1.9×10^7	3.6×10^8	2.2×10^7	
	Urine		Negative		Negative	Negative	Negative	
	Saliva		Negative		5.26×10^3	Negative	Negative	
17*	Blood		n.d		n.d	2.4×10^9†	n.d	
	Urine		n.d		n.d	Negative	n.d	
	Saliva		n.d		n.d	Negative	n.d	
20*	Blood		n.d		n.d		1.3×10^7†	
	Urine		n.d		n.d		Negative	
	Saliva		n.d		n.d		Negative	
21	Blood		1.7×10^9		2.0×10^8			
	Urine		Negative		Negative			
	Saliva		Negative		Negative			
28	Blood		5.4×10^7		4.7×10^6			
	Urine		Negative		3.5×10^4			
	Saliva		Negative		Negative			
30*	Blood		7.5×10^8		n.d			
	Urine		Negative		n.d			
	Saliva		Negative		n.d			
35	blood		4.4×10^5		7.2×10^7			
	Urine		Negative		Negative			
	Saliva		Negative		Negative			
41*	Blood		9.2×10^7†		n.d			
	Urine		Negative		n.d			
	Saliva		Negative		n.d			
42	Blood				1.8×10^7			
	Urine				Negative			
	Saliva				Negative			
48	Blood				1.4×10^9			
	Urine				2.5×10^4			
	Saliva				Negative			
49*	Blood				1.9×10^8			
	Urine				Negative			
	Saliva				Negative			

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Table 1 (continued)

Days post infection	*M. suis*/mL	Animal ID (group B animal)						
		ID 23	ID 31	ID 32	ID 71	ID 73	ID 74	ID 76
56	Blood				**1.0 × 10⁴**			
	Urine				Negative			
	Saliva				Negative			
60*	Blood				**2.9 × 10⁹**			
	Urine				Negative			
	Saliva				Negative			
62	Blood				**4.9 × 10⁷†**			
	Urine				Negative			
	Saliva				Negative			

Bold lines represent a positive detection of *M. suis*

n.d. not determined

[*] Additional sampling date due to exacerbation of clinical signs, only clinically affected animals were sampled at this study date

[†] Euthanisation

M. suis loads in blood, saliva and urine samples of group B animals as determined by qPCR are shown in Table 1.

Detection of HMs in blood, urine, saliva and semen in field samples

Mycoplasma suis was detected in the blood from 61 out of 150 sampled sows while '*Ca.* Mycoplasma haemosuis' was detected in 13 out of the 150 samples. Each of the 13 '*Ca.* M. haemosuis' positive sows were co-infected with *M. suis*. Blood loads varied from 2.04 to 3.74×10^7 *M. suis* per mL blood and from 7.16×10^3 to 4.88×10^4 '*Ca. M haemosuis*' per mL blood, respectively. All 148 corresponding saliva samples (59 samples of HM blood positive sows) revealed negative qPCR results for both HM species (all samples $p < 0.001$, power 100%; samples of bacteremic sows $p = 0.004$, power 100%).

Similarly, all 47 urine samples (16 samples of HM blood positive sows) revealed negative qPCR results for both HM species (all samples $p = 0.014$, power 99.8%; samples of blood positive sows $p = 0.371$, power < 0.1%). RBC residues were not detected in any of the urine samples.

No evidence of *M. suis* and '*Ca. M. haemosuis*' infections was detected in any of the 183 tested boars from the boar stud as determined by qPCRs.

Discussion

Up-to-date, transmission of porcine HMs is thought to mainly occur horizontally during zootechnical procedures and ranking fights. Additionally, results from Heinritzi [7] suggested transmission through oral intake of *M. suis* containing blood. However, oral infection with *M. suis* was not proven in our experimental study due to the absence of clinical signs and *M. suis* in blood samples determined by qPCR throughout the entire study period. Possible explanations for the deviating results might be the different inoculation strains or the higher inoculation dose used by Heinritzi [7] (10 mL containing 5.8×10^9–4.17×10^{10} *M. suis*/mL) compared to the present experiment (1.5 mL; 2.0×10^7 *M. suis*/mL). However, the inoculation dose chosen by Heinritzi [7] does not represent a realistic scenario for field infections as the mean bacterial blood loads found in piglets and sows in previous qPCR field studies were much lower [13, 14]. Thus, an inoculation dose in accordance with recent studies was chosen in our experiment. Additionally, in contrast to microscopic examination lacking specificity and sensitivity, our results resemble the first investigation of oral transmission routes using up-to-date real-time PCR assays. Similar results were found for the feline HM species '*Ca.* M. turicensis' [15] as oral inoculation with '*Ca.* M. turicensis' containing blood was not successful in cats.

The detection of *M. suis* in different secretes and excretes of experimentally infected pigs has raised issues on blood independent HM transmission routes [11]. In accordance with this previous study, *M. suis* could also be detected in urine and saliva samples of group B animals (subcutaneously infected) after experimental infection. Despite comparable *M. suis* loads in both studies Dietz et al. [11] found a higher number of animals and samples positive for *M. suis* in urine and saliva. Interestingly, in the previous study of Dietz et al. [11] *M. suis* was also present in urine without RBC residues whereas all *M. suis* positive urine samples in our study contained RBC

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residues. Consequently, no RBC-free secretion in urine was observed in the present study. This might be attributable to the variation of the *M. suis* strain in both studies [11], e.g., the inoculation strain used by Dietz et al. [11] displayed an additional cell tropism for endothelial cells [16].

Regarding samples of naturally infected sows, neither *M. suis* nor '*Ca*. M. haemosuis' could be identified in urine and saliva samples under field conditions despite the presence of HMs in the corresponding blood samples. This might also be explained by variation of HM strains, higher *M. suis* blood loads in experimental studies [10–12] compared to naturally infected pigs [13, 14], or a higher susceptibility for HM infections due to the splenectomised pig model used for experimental infections [17]. Furthermore, it still has to be scrutinized under experimental conditions if the PCR positive secretes and excretes of the aforementioned experimentally infected animals actually contain infectious organisms.

Despite the very successful application of oral fluid-based testing facilitates for monitoring, surveillance and detection of several pathogens relevant for the swine production [18–24] oral fluids seem not to resemble a suitable diagnostic specimen for the detection of HMs. However, limitations of our study arise from the number of investigated animals and the use of individual swabs that might provide lower detection rates compared to pen-based oral fluids. Therefore, additional studies including pen-based oral fluids with a larger number of animals are warranted to further evaluate the efficacy and sensitivity of HM detection in oral fluids.

The PCR negative blood sample results of the 183 investigated boars from 26 different multiplier farms was somehow unexpected, as previous studies revealed a high prevalence of *M. suis* in sows [25–28]. However, it might be assumed that multiplier farms have a lower risk of *M. suis* introduction due the very limited purchase of animals and strict biosecurity measures.

Semen can serve as an important route for the introduction of various pathogens into a farm [29]. Up-to-date, transmission of *M. suis* via semen is thought to occur only in case of blood contamination [29, 30]. However, those studies were performed in the pre-PCR era and shedding of the pathogen in RBC-free urine, saliva and vaginal secretions has reinforced the discussion of blood-independent transmission route. Under the condition of the present study with investigating samples from one boar stud at one sampling point we were not able to detect porcine HM species in blood and semen. Nevertheless, to exclude boars and semen as potential reservoirs for HM transmission further studies including a higher number of boar studs and boars from conventional farms are certainly needed.

Conclusion

In conclusion, our results indicate that blood independent shedding routes are unlikely to play a major epidemiological role under field conditions. In addition, the results of our experimental study did not confirm the possibility of an oral transmission for *M. suis* via infected blood. Despite several benefits over the more invasive blood sampling, individual saliva samples might not represent an appropriate sample type for the detection of *M. suis*.

Materials and methods

Experimental design

For experimental infection, a splenectomised pig model was used [10, 17]. The experimental protocol was approved by the Government Office of Upper Bavaria, Munich (authorization reference number 55.2-1-54-2532-87-12). A total of 14 piglets at the age of 28-days originating from the same *M. suis* negative farm were included in the study. The *M. suis* negative status was confirmed by qPCR as previously described [13, 31]. One week after placement, each piglet was splenectomised according to the protocol of Heinritzi [17]. For experimental studies with *M. suis* splenectomy is usually performed since the absence of the spleen reduces the incubation period, exacerbates the clinical signs of disease and enhances the replication rate of the pathogen within the host animal [10].

One week after splenectomy, piglets were randomly assigned into two groups (group A: n=7; ID 33, 34, 37, 64, 65, 55, 69; group B: n=7; ID 23, 31, 32, 71, 73, 74;) for experimental infection. The previously described *M. suis* field strain K323/13 [12] was used as inoculation strain.

Piglets of group A were inoculated orally, piglets of group B subcutaneously with *M. suis* containing blood (1.5 mL; 2.0×10^7 .*M. suis*/mL). Daily clinical observation, treatment and determination of the experiment were performed as previously described [12]. Briefly, the clinical scoring system shown in Table 2 was used for daily observation of animals. Upon acute IAP attack, which is delineated by three clinical score points, animals were treated with oxytetracycline (20 mg/kg body weight/24 h, i.m.) and glucose (35 g/L, oral). Additionally, Metamizole (30 mg/kg body weight) was administered intramuscularly if the body temperature exceeded 42 °C. The termination criteria of the experiment were defined as follows: a clinical score of >3 remaining constant over 48 h despite antibiotic treatment, sustained fever of >40 °C and impaired general health and anorexia. On reaching these criteria, the affected animal was euthanized by intravenous pentobarbital injection (45 mg/kg body weight).

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Table 2 Clinical scoring system used for daily animal observation during experimental *M. suis* infection (in accordance with Stadler et al. [12])

Score points	Ears	Skin	Body temperature	Behavior	Feed intake	Respiration
0	No alterations	No alterations	< 40 °C	No alterations	No alterations	No alterations
1	Mild cyanosis	Moderate pallor	40–42 °C	Reduced	Reduced	Mild dyspnoe
2	Moderate cyanosis and necrosis	Generalised petechiae	> 42 °C	Apathy	Anorexia	Severe dyspnoe
3	–	Icterus	–	–	–	–

EDTA-anticoagulated blood samples (puncture of *V. jugularis*), urine and saliva samples were collected every two days for the first 8 days post infection (DPI) and subsequently once a week until the end of the trial on 90 DPI. Furthermore, individual samples were taken on additional time points when clinical signs exacerbated in the affected animal.

Individual saliva collection was performed without restraining of the animals. Saliva samples were obtained as described elsewhere [32, 33]. Briefly, the pigs were allowed to chew on a cotton swab with the help of a metal rod, until the swab was thoroughly soaked with saliva (Salivette®, Sarstedt, Aktiengesellschaft and Company, Nümbrecht, Germany). After sample collection, the swab was placed in a sealed plastic vial and was centrifuged at $4000 \times g$ for 8 min. Urine samples were taken by spontaneous urination in sterile tubes. Saliva and urine samples were stored at -80 °C until further processing.

Gross-necropsy and histopathological examination was performed of all animals. as previously described [12]. In brief, tissues were fixed in paraformaldehyde, embedded in plastic and were stained for Giemsa and haematoxyline-eosin-phloxin.

Field samples

Blood, saliva and urine samples of 150 sows were available from a previous study (stored at -80 °C) [34] (authorization reference number 55.2-154-2532.2-16-13) and collected as described above. The samples originated from 15 piglet producing farms in Southern Germany. The chosen farms were preselected as being positive for *M. suis* by detection of *M. suis* in blood samples of sows by qPCR. In total, 148 saliva samples (8–10 saliva samples and corresponding blood samples per farm) from 15 *M. suis* positive farms and 47 urine samples (1–8 urine samples and corresponding blood samples per farm) from 11 M. *suis* positive farms were investigated for the presence of *M. suis* by qPCR. A minimum of 10% positive results were assumed for the statistical analysis (Binomial test and Power calculation), which was calculated with BIAS for Windows 11.01 (Epsilon-Verlag, Frankfurt; Germany).

Additionally, semen and EDTA-blood samples obtained from a German boar stud during regular on-farm health monitoring were also included in the present study. In total semen and EDTA-samples of 183 boars originating from 26 different multiplier farms were collected. The boars' age ranged from 9 to 77 months. Samples were collected within one day and stored at -80 °C until further processing. Investigations were approved by the ethical commission of the veterinary faculty of the LMU, Munich (authorization reference number: 245-17-12-2020).

Methods

DNA was extracted from EDTA-anticoagulated blood, urine, and saliva samples as described previously [11, 13]. Urine samples were further tested with Servotest® 5 + NL stripes (Servoprax, Wesel, Germany) for RBC residues. Semen samples were pooled to five and DNA was extracted by using the QIAamp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA samples were investigated for *M. suis* and '*Ca*. M. haemosuis' by qPCR as previously described [6, 13, 31]. Briefly, the following primers targeting the *msg1* gene of *M. suis* (msg1-Fw 5′-ACAACT AATGCACTAGCTCCTATC-3′ and msg1-Rv 5′-GCT CCTGTAGTTGTAGGAATAATTGA) and the gap gene of '*Ca*. M. haemosuis' (CMhsuisF 5′-TGCTTTGGC TCCTGTGGTTA-3′ and CMhsuisR 5′- GCAGCAGCA CCTGTAG AAGTA-3′) were used. The 178 bp fragment (*M. suis*) and the 177 bp fragment ('*Ca*. M. haemosuis') were each detected and quantified using the StepOne™ System (Applied Biosystems®). QPCR was carried out with Fast SYBR® Green PCR and the following cycling conditions: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s and subsequent melting curve analysis.

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Availability of data and materials
All datasets used in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The protocol for experimental infection as well as all procedures were officially approved by the Government Office of Upper Bavaria, Munich, Germany (authorization reference number 55.2-1-54-2532-87-12). The study was compliant with all relevant European guidelines and regulations for animal experiments. The study was carried out in compliance with the ARRIVE guidelines. Collection of field samples from sows were also approved approved by the Government Office of Upper Bavaria, Munich, Germany (authorization reference number 55.2-154-2532.2-16-13). Sampling of the boars was approved by the ethical commission of the veterinary faculty of the LMU, Munich (authorization reference number: 245-17-12-2020).

Consent for publication
Not applicable.

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

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Publisher's Note

5. PUBLICATIONS

5.3 Occurrence of `*Candidatus* Mycoplasma haemosuis` in fattening pigs, sows and piglets in Germany using a novel *gap*-based quantitative real-time PCR assay

Occurrence of '*Candidatus* Mycoplasma haemosuis' in fattening pigs, sows and piglets in Germany using a novel *gap*-based quantitative real-time PCR assay

Julia Ade[1], Julia Stadler[2], Mathias Ritzmann[2], Christina Zübert[1], Katharina Hoelzle[1] and Ludwig E. Hoelzle[1*]

Abstract

Background: The appearance of the novel porcine haemotrophic mycoplasma (HM) species '*Candidatus* Mycoplasma haemosuis' was reported in apparently healthy but also in clinically sick animals in China, Korea and in a case report from Germany. Outside of Asia, however, nothing further is known about the frequency of '*Ca*. M. haemosuis' in pigs to date. To investigate the distribution of this novel HM species in Germany, fattening pigs, sows and pre-suckling piglets were examined using a herein developed quantitative real-time PCR assay (qPCR). Because the piglets were sampled before the first colostrum uptake, additional information on a possible vertical transmission from dams to their offspring was obtained.

Results: Our novel qPCR assay successfully detected '*Ca*. M. haemosuis' in all blood samples from the '*Ca*. M. haemosuis'-infected pigs. No cross-reactivity was detected when DNA from non-target *Mycoplasma* spp. and other bacterial species representing 10^5 bacteria/reaction were used as a template. The lower limit of detection of the qPCR was thus 10 *gap* gene copies per reaction and 2.5×10^3 genome equivalents (GE) per mL blood.

'*Candidatus* M. haemosuis' was detected by this qPCR in blood samples from a total out of 6.25% sows (13/208), 4.50% pre-suckling piglets (28/622) and 17.50% fattening pigs (35/200). On farm level, 3 of 21 piglet producing farms (14.28%) and 9 of 20 fattening farms (45.00%) were positive for '*Ca*. M. haemosuis'. Co-infections with *M. suis* were evident in all age groups.

Conclusion: '*Candidatus* M. haemosuis' infection is present in German pig farms and the detection of the novel porcine HM species in piglets immediately after birth before colostrum intake indicates vertical transmission. The novel qPCR assay specific for '*Ca*. M. haemosuis' described herein will be a prerequisite for future studies on the prevalence, epidemiology as well as the clinical and economic impact of '*Ca*. M. haemosuis' infections.

Keywords: Haemotrophic mycoplasmas, '*Candidatus* Mycoplasma haemosuis', Quantitative PCR, Diagnosis, Vertical transmission , Pre-suckling piglets, Vertical transmission, Farrowing sows

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Background

Haemotrophic mycoplasmas (HMs) are a group of cell-wall less bacteria with a unique tropism for erythrocytes. HMs were found worldwide in a wide range of mammals, including livestock and companion animals as well as wild animals and humans [1–5]. In pigs, the two

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HM species *Mycoplasma suis* and *Mycoplasma parvum* were first described in the first half of the last century [6]. While *M. parvum* seems to be an apathogenic HM species, *M. suis* is the causative agent of infectious anaemia in pigs (IAP) [4, 7, 8]. Acute *M. suis* infections are characterised by haemolytic anaemia, high fever, icterus, hypoglycaemia, intravascular coagulopathy, and endothelial damages [4, 6, 9, 10]. Of most importance are chronic infections with subclinical or mild to moderate clinical signs including low-grade anaemia, poor reproduction performance, and reduced growth rates [1, 11]. Both, acute and chronic *M. suis* infections have been reported worldwide causing significant economic losses to the pig industry [12–17].

Recently, a third porcine HM species was discovered in Zhejiang, China by Fu and co-workers in 2017 and designated as 'Candidatus Mycoplasma haemosuis' [18]. Phylogenetic analyses of the 16S rDNA revealed that this novel HM species is most closely related to the feline HM '*Candidatus* Mycoplasma turicensis', the murine species *Mycoplasma muris* and the human HM '*Candidatus* Mycoplasma haemohominis' [18]. This novel porcine HM species was also described in obviously healthy animals in Korea [19] and, most recently, in clinically affected fattening pigs in Germany with skin alterations, fever, and anaemia [20]. This clinical case indicated the need to establish a specific and sensitive detection method to investigate the spread of the novel emerging pathogen in the pig population.

In the last two decades, molecular detection methods for the so far uncultivable HMs have proven to be the basic prerequisite to get insights into the epidemiology, species, and strain differentiation and the clinical impact of HM infections [14, 21–26].

Thus, the aims of the present study were (i) to develop a specific real-time PCR diagnostic assay (qPCR) for the detection and quantification of '*Ca*. M. haemosuis' in pigs and (ii) to apply this novel qPCR and investigate the occurrence of '*Ca*. M. haemosuis' in fattening pigs as well as in sows and piglets in Germany. Because the piglets were sampled before the first colostrum uptake, additional information on a possible vertical transmission from dams to their offspring was obtained.

Results
Development of a real-time PCR assay
We developed a real-time qPCR assay for the specific detection of '*Ca*. M. haemosuis' from the blood of pigs. The real-time qPCR is targeting the *gap* gene encoding the NAD-dependent glyceraldehyde 3-phosphate dehydrogenase of '*Ca*. M. haemosuis' (GAPDH). For the establishment of the qPCR '*Ca*. M. haemosuis' positive samples were available from a previous study [20].

In this study, pigs (n = 7) suffering from anaemia, fever, and skin alterations, were tested '*Ca*. M. haemosuis'-positive by 16S rDNA PCR, sequencing, and subsequent sequence analysis [20]. Our novel qPCR assay successfully detected '*Ca*. M. haemosuis' in all blood samples from the '*Ca*. M. haemosuis'-infected pigs. Melting curve analyses revealed a specific melting temperature of 74.676 °C ($\pm$ 0.32 °C). Moreover, we tested the specificity of the assay using bacterial strains and isolates as listed in Table 3. Positive qPCR reactions were found for '*Ca*. M. haemosuis' DNA. No cross-reactivity was detected when DNA from non-target *Mycoplasma* spp. and other bacterial species representing 10^5 bacteria/reaction were used as a template.

Sequence analyses of the qPCR amplicons from all available '*Ca*. M. haemosuis' isolates (n = 7; 20) revealed 100% identity among each other and to the *gap* gene deposited in GenBank (Accession No. KU246051). The analytical sensitivity of the qPCR assay was determined using serial dilutions of the plasmid pC_*CMhsuis* standard corresponding to 10^7 to 100 GE. The highest dilution yielding consistently positive qPCR results (Ct < 35 cycles) contained 0.45 fg pC_*CMhsuis* DNA per reaction corresponding to 10 gene copies per reaction. The lower limit of detection of the qPCR was thus 10 *gap* gene copies per reaction and 2.5 x 10^3 genome equivalents per mL blood.

Quantitative PCR data analysis revealed a linear regression curve between 45 pg and 0.45 fg of the plasmid DNA. The PCR efficiency was calculated to be 97.3%. The intra-assay and inter-assay repeatability are shown in Table 1. All different concentrations in the standard dilutions from 10^7 to 10^1 GE were consistently detected by the qPCR assay.

Table 1 Inter-assay and intra-assay repeatability of the '*Ca*. M. haemosuis' qPCR

Copy number[a]	inter-assay repeatability (15 runs) mean Ct[b] ($\pm$SD)	intra-assay repeatability (5 runs) mean Ct ($\pm$SD)
10^7	15.27 ($\pm$ 0.27)	15.31 ($\pm$ 0.15)
10^6	17.61 ($\pm$ 0.82)	17.67 ($\pm$ 0.13)
10^5	21.08 ($\pm$ 0.60)	21.33 ($\pm$ 0.10)
10^4	24.62 ($\pm$ 0.64)	24.80 ($\pm$ 0.10)
10^3	27.68 ($\pm$ 0.82)	27.69 ($\pm$ 0.09)
10^2	30.79 ($\pm$ 0.70)	30.84 ($\pm$ 0.15)
10^1	33.51 ($\pm$ 0.75)	33.52 ($\pm$ 0.14)

[a] Copy numbers (GE/reaction) of the plasmid pC_*CMhsuis* were calculated from spectrophotometrically quantified DNA as described in Methods

[b] Mean threshold cycle values and standard deviations

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Quantification of '*Ca*. M. haemosuis' in the infected pigs [20] revealed blood loads from 3.08×10^2 to 3.96×10^7 bacteria/mL blood.

The ten randomly selected qPCR positive samples also revealed positive results in haemotrophic mycoplasma-specific16S rDNA amplification and sequencing. As all obtained sequences were identical, we uploaded one sequence to the GenBank (Accession No. MZ614253).

'*Candidatus* M. haemosuis' infections in fattening pigs

To investigate the occurrence of '*Ca*. M. haemosuis' in fattening pigs, we tested a total of 200 animals from 20 fattening farms at the time of slaughter using the novel qPCR assay. '*Candidatus*. M. haemosuis' was detected in blood samples of 35 out of 200 investigated animals (17.50%) originating from 9 out of the 20 farms (45.00%). Quantification of bacterial loads in qPCR-positive pigs revealed a mean value of 1.61×10^5 '*Ca*. M. haemosuis'/mL blood (range: 5.52×10^3 to 1.55×10^6 '*Ca*. M. haemosuis'/mL). A total of 12 '*Ca*. M. haemosuis' positive fattening pigs were co-infected with *M. suis* (Table 2). Data of all animals are included in Supplementary File 1.

'*Candidatus* M. haemosuis' infections in farrowing sows and pre-suckling piglets

As shown in Table 2, a total of 13 out of 208 sows (6.25%) were positive for '*Ca*. M. haemosuis' and the number of positive sows within a herd varied between one and ten. All 13 '*Ca*. M. haemosuis' positive sows were co-infected with *M. suis*. On-farm level, '*Ca*. M. haemosuis' was

detected in three out of 21 investigated piglet-producing farms (14.29%) in at least one animal, whereas in the remaining 18 farms all animals were qPCR negative. In all farms, no clinical signs of IAP were obvious at the time of the investigation.

A total of 28 out of the 622 (4.50%) pre-suckling piglets reacted '*Ca*. M. haemosuis' qPCR positive. All '*Ca*. M. haemosuis' infected piglets originating from one farm and were born from 10 (76.92%) of the 13 qPCR positive sows. The number of positive piglets per sow varied between one and three. Four out of the 28 '*Ca*. M. haemosuis' positive piglets were co-infected with *M. suis*.

Quantification of bacterial loads in qPCR-positive sows revealed a mean value of 3.83×10^4 '*Ca*. M. haemosuis'/mL blood (range: 3.21×10^4 to 6.44×10^4 '*Ca*. M. haemosuis'/mL) and in qPCR-positive piglets a mean value of 2.25×10^5 '*Ca*. M. haemosuis'/mL blood (range: 1.13×10^4 to 2.48×10^6 '*Ca*. M. haemosuis'/mL), respectively. Data of all animals are further included in Supplementary File 1.

Discussion

In this study, we describe the establishment of a novel quantitative real-time PCR for the specific detection of the emerging porcine HM species '*Ca*. M. haemosuis' in blood samples as well as the quantitative detection of '*Ca*. M. haemosuis' in sows, piglets and fattening pigs from Germany. To our knowledge, our SYBR® green qPCR assay is the first quantitative diagnostic tool specific for '*Ca*. M. haemosuis'. The novel qPCR assay is targeting the '*Ca*. M. haemosuis' gene encoding the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. The *gap* was chosen over the 16S rRNA gene because of an identity of >97% of the 16S rRNA gene of *Ca*. M haemosuis to other hemotrophic mycoplasmas (e. g. '*Ca*. M. turicensis', '*Ca*. M. haemobos', '*Ca*. M. haemomuris', *M. haemocanis*, and *M. haemofelis*), Glyceraldehyde-3-phosphate dehydrogenase encoding genes were used as targets in -specific qPCR assays for the detection of haemotrophic and non-haemotrophic Mycoplasma species including *M. suis*, *M. wenyonii*, '*Ca*. M. haemobos', *M. genitalium* and *M. hominis* [28, 29]. All these PCR assays have proven to be reliable and robust for use in prevalence studies and routine diagnostics [13, 17, 26, 30].

So far, '*Ca*. M. haemosuis' was detected using 16S rDNA targeting primers without quantification of bacterial loads in infected pigs [18, 19]. The assay described herein allows quantification with a sensitivity of 10 genome equivalent per PCR corresponding to 2.5×10^3 bacteria per mL blood. Comparable results have been obtained from previously published qPCR assays for the detection of other HMs, e.g. *M. suis*, the canine HM species *M. haemocanis*, '*Ca*. M. haematoparvum' as well as

Table 2 Occurrence of '*Ca*. M. haemosuis' and *M. suis* in farms and on single animal level

	piglet producing farms	
	'*Ca*. M. haemosuis'[b]	*M. suis*[a]
positive farms	3/21 (14.28%)	16/21 (76.19%)
farms with co-infections		3/21 (14.28%)
positive sows	13/208 (6.25%)	65/208 (31.25%)
positive piglets	28/622 (4.50%)	68/474 (14.35%)
born from positive sows	28	50
born from negative sows	0	18
co-infected sows		13/208 (6.25%)
co-infected piglets		4/474 (0.84%)
	fattening farms	
	'*Ca*. M. haemosuis'	*M. suis*
positive farms	9/20 (45.0%)	10/20 (50.0%)
farms with co-infections		8/20 (40.0%)
positive pigs	35/200 (17.5%)	38/200 (19.9%)
co-infected pigs		12/200 (6.0%)

[a] In the study of Stadler and co-workers [17]

[b] This study

the bovine HM species *M. wenyonii* and '*Ca*. M. haemobos' [23, 26, 31]. This high analytical sensitivity enables the identification of asymptomatic chronically infected carrier animals that may serve as important epidemiological reservoirs [24, 32]. The analytical specificity of the novel '*Ca*. M. haemosuis' qPCR assay as predicted *in silico* was confirmed by the negative PCR results obtained with all tested *Mycoplasma* spp. (HMs and non-haemotrophic mycoplasmas) and other porcine pathogens.

Reports about '*Ca*. M. haemosuis' infections are scarce because the agent was recently discovered in 2017 [18]. In the study reported herein, we surveyed for the first time a European sample panel (n = 1080 pigs) for '*Ca*. M. haemosuis' infections. We confirmed that '*Ca*. M. haemosuis' is prevalent in Germany. Interestingly, we found considerable variation in the prevalence depending on the population studied. The highest detection rate with 17.5% was found in fattening pigs, followed by 6.25% in sows, and by 4.50% in piglets. In the two previous studies, the '*Ca*. M. haemosuis' prevalence was higher in China with 36% positive sows and 24.1% positive growing pigs [18] and lower in Korea with only one '*Ca*. M. haemosuis' positive animal (0.01%) out of 1867 tested pigs from 464 farms [19]. On-farm level, the higher detection rates for fattening pigs could be confirmed: only 3 out of the 21 piglet producing farms (14.29%) but 9 out of the 20 fattening farms (45.00%) were shown to be '*Ca*. M. haemosuis' positive. Various factors could be responsible for the differences in the detection rates found between piglet producing and fattening farms including the purchase of pigs from different piglet-producing farms and potential higher biosecurity levels in piglet producing farms. In the current study, all positive sows were co-infected with '*Ca*. M. haemosuis' and *M. suis*, but only 4 out of 28 piglets, and 12 out of 200 slaughter pigs were positive for both porcine HM species. The evidence of co-infections is in line with other HM studies. Co-infections with two or three HM species were also found in sows (26.7%) and growing pigs (13.9%) in China [18], in cattle [25, 26], in sheep [33], and cats [34].

So far, our knowledge regarding the pathogenicity of '*Ca*. M. haemosuis' is rather limited. In the Chinese study, the novel porcine HM species was first detected in one diseased pig showing typical clinical signs of IAP [18]. In Europe, Stadler and co-workers (2020) identified '*Ca*. M. haemosuis' first in diseased pigs showing also typical clinical signs of an *M. suis* induced IAP (i.e. anaemia, fever, skin alterations) [20]. In the present study '*Ca*. M. haemosuis' was detected in obviously healthy pigs as it was also shown for *M. suis* in recent studies [13, 17, 35]. Typically, such chronic HM infections predominate in the pig population causing significant economic loss and welfare concern due to immune dysregulation, higher susceptibility to other infectious agents, extended feeding periods or increased stillbirth rates [4, 13, 17, 35, 36]. In addition, chronic HM infections can lead to increased and metaphylactic antibiotic usage contributing to the development of antibiotic resistance [36]. Further studies focusing on individual health parameters of '*Ca*. M. haemosuis' positive pigs are certainly needed.

The '*Ca*. M. haemosuis' loads found in the present study seem to be lower than those found for *M. suis* in sows (mean blood load of 3.15×10^7 *M. suis*/mL [17];), in pre-suckling piglets (mean loads of 5.09×10^7 *M. suis*/mL blood) or in fattening pigs (mean loads of 7.62×10^7 *M. suis*/mL blood [13];, respectively. Interestingly, 76.92% of the positive sows have born at least one '*Ca*. M. haemosuis' positive piglet indicating that '*Ca*. M. haemosuis' is transmitted vertically within the pig herds. The possibility of vertical transmission of HMs has also been described for *M. suis* [17] as well as for *M. wenyonii* and '*Ca*. M. haemobos' [37, 38]. But it is worth noting when comparing the vertical transmission of *M. suis* [17] and '*Ca*. M. haemosuis' (present study) that a considerably higher percentage of 76.92% of the '*Ca*. M. haemosuis' infected sows delivered infected pre-suckling piglets whereas only 50% of the *M. suis* infected sows have born positive piglets.

Conclusion
We showed for the first time that '*Candidatus* Mycoplasma haemosuis' infection is prevalent in Germany in piglet producing farms as well as in fattening farms and coinfections with *M. suis* are common. Our data on the detection of '*Ca*. M. haemosuis' in pre-suckling piglets indicate that the pathogen is transmitted vertically. Further studies are needed to investigate the pathogenic potential, the clinical impact, prevalence, and epidemiology including transmission routes of '*Ca*. M. haemosuis' to clarify the significance of this emerging pathogen. The herein-described novel qPCR assay can be used to accurately diagnose infections with the new HM species in pigs and to perform these studies.

Methods
Sample and data collection
Blood samples (n = 7) of '*Ca*. M. haemosuis' positive fattening pigs were available from a previous study [20]. As samples were taken as diagnostic material during acute disease, no ethical approval was required according to the German Animal Welfare Law. The pigs suffering from skin alterations (urticaria, haemorrhagic diathesis), high fever and anaemia were shown to be '*Ca*. M. haemosuis' positive by 16S rDNA amplification and subsequent sequence analysis [20]. The seven animals revealed a negative *M. suis*-qPCR result.

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In addition, DNA samples extracted from the blood of farrowing sows (n = 208) and corresponding pre-suckling piglets (n = 622) from 21 piglet producing farms were available from a previous study [17]. From each farm, nine to ten farrowing sows and two or three pre-suckling piglets per sow have been sampled. DNA quality was checked using a NanoDrop™ 2000 to assure the quality of the samples. Animal sampling was performed in accordance with the German animal welfare law using a protocol officially approved by the Government (Az. 55.2–154–2532.2-16-13).

For the group of fattening pigs, EDTA-anticoagulated blood samples were taken from 200 animals (20 different farms in Germany, 10 animals per farm) at the time of slaughter. All 20 farms were located in the South of Germany (Federal States of Bavaria and Baden-Wuerttemberg). Blood collection was performed after slaughtering, thus, no ethical approval was needed for those samples according to the German Animal Welfare Law and the DIRECTIVE 2010/63/EU.

Bacterial DNA was extracted as described elsewhere [13, 17]. Briefly, blood samples were preconditioned by mixing 200 µl of EDTA-anticoagulated blood with an equal volume of lysis buffer (10 mM Tris pH 7.5, 5 mM MgCl2, 330 mM sucrose, 1% (v/v) Triton X-100). The mixure was centrifuged (8000 x g, 1 min, 20 °C) and the pellet was again washed twice with 400 µl lysis buffer. Subsequently, a total amount of 200 µl bacterial DNA was using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Steinheim, Germany). One PBS control was included into each extraction run of ten blood samples. For all samples, the *M. suis* status was determined by a quantitative PCR [17, 23] either in a previous study (sows and piglets, [17]) or in the present study (fattening pigs).

Primer design and amplicon sequencing

To develop a specific '*Ca*. M. haemosuis' qPCR two primers were designed based on the gene *gap* encoding the NADP-dependent glyceraldehyde-3-phosphate dehydrogenase of '*Ca*. M. haemosuis' (Accession No. KU246051) using the Primer3 software [39, 40]: *CMhsuis*F 5´-TGCTTTGGCTCCTGTGGTTA-3´ and *CMhsuis*R 5´- GCAGCAGCACCTGTAGAAGTA-3`. The Blast algorithm, which is provided by NCBI, was used to test the specificity of the primers *in silico*. Specificity was further investigated by sequencing (Seqlab Sequence Laboratories, Göttingen, Germany) of the resulting 177-bp *gap* fragment of '*Ca*. M. haemosuis'. Obtained sequences were compared to GenBank entries using the Blast tool provided by NCBI.

Cloning and Preparation of Standard DNA

The 177-bp qPCR fragment of '*Ca*. M. haemosuis' was cloned into the plasmid vector pCR2.1 (Invitrogen) according to the manufacturer's instructions. Plasmid DNA was purified from the *Escherichia coli* transformant (pC_*CMhsuis*) using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich). Plasmid DNA was quantified using a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific). DNA concentrations were adjusted to 45 pg/2 µL representing 1×10^7 GE according to the calculation described below.

Quantitative real-time PCR

'*Candidatus*. M. haemosuis' DNA was detected and quantified with the StepOne™ System (Applied Biosystems). The 20 µL reaction mixtures contained 10 µL of the 2x SYBR® Green PCR Master Mix (Thermo Fisher Scientific), 8 µL primer mixture (containing 0.5 µM primer each), and 2 µL template DNA. Cycling conditions consisted of 95 °C for 15 min and 40 cycles at 95 °C for 15 s and 60 °C for 1 min followed by a melting curve analysis. Plasmid pC_*CMhsuis* DNA standard dilutions (450 fg/2 µl, 45 fg/2 µl, and 4.5 fg/2 µl) representing 10^5, 10^4, and 10^3 GE per reaction were included in each qPCR run for quantification. Obtained Ct values were extrapolated into '*Ca*. M. haemosuis' GE/reaction using the Standard Curve Method of the StepOne™ Software Version 2.2 (Applied Biosystems). The '*Ca*. M. haemosuis' GE/mL blood were determined considering the factor 200 (2 µL template out of 200 µL DNA volume out of 200 µL EDTA blood (software Microsoft® Excel, 2016).

Since the diagnostics of hemotrophic mycoplasmas in Germany has to be covered by the farmers themselves, we decided to establish a SYBR Green assay, to use the range of 10^5–10^3 GE/reaction and to omit an internal control. Despite some disadvantages compared to the use of probe-based PCRs, the SYBR Green assay offers a clear economic advantage with nevertheless good diagnostic specificity [41]. This allows us to keep costs as low as possible and to offer farmers an incentive to send in diagnostic samples. *Analytical specificity and lower limit of detection of the real-time qPCR.*

The specificity of the qPCR assay was tested by using template DNA from the porcine HM species *M. suis*, *M. parvum*, other haemotrophic and non-haemotrophic *Mycoplasma* spp. and a panel of other porcine pathogens (Table 3). Bacteria were cultivated and/or DNA was isolated as described elsewhere [17, 23, 26].

For measuring the lower limit of detection (LOD) pC_*CMhsuis* plasmid DNA concentration was adjusted to 45 pg/2 µL representing 1×10^7 GE/2 µL and the LOD

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Table 3 Bacterial isolates for testing the specificity of the '*Ca.* M. haemosuis' qPCR

Bacterial species	Origin
'*Candidatus* M. haemosuis' (n = 7)	[20], University of Hohenheim[a] (DNA)
Mycoplasma suis KI82 (n = 4) *Mycoplasma parvum*	[27], University of Hohenheim[a] (DNA) University Hohenheim[a](DNA)
Mycoplasma wenyonii (n = 3)	[26], University of Hohenheim[a] (DNA)
'*Candidatus* M. haemobos' (n = 3)	[26], University of Hohenheim[a] (DNA)
Mycoplasma haemofelis	University Zurich[b] (DNA)
Mycoplasma fastidiosum	ATCC 33229
Mycoplasma hyorhinis	ATCC 17981
Mycoplasma hyosynoviae	University Bern[c] (DNA)
Mycoplasma arginini	University Bern[c] (DNA)
Mycoplasma dispar	University Bern[c] (DNA)
Mycoplasma flocculare	University Bern[c] (DNA)
Mycoplasma bovis (n = 3)	University Bern[c] (DNA)/ATCC 25523
Mycoplasma bovirhinis	ATCC 27748
Mycoplasma bovigenitalium	University Bern[c] (DNA)
Mycoplasma bovoculi	University Bern[c] (DNA)
Mycoplasma capricoum spp. capricolum	University Bern[c] (DNA)
Salmonella Typhimurium (n = 2)	University Hohenheim[a]
Staphylococcus aureus	University Hohenheim[a]
Escherichia coli (n = 2)	University Hohenheim[a]
Pasteurella multocida	CVUA Stuttgart[d]
Streptococcus suis	ATCC 43765

[a] Institute of Animal Science, University Hohenheim, Stuttgart, Germany

[b] Institute of Veterinary Bacteriology, University Zurich, Switzerland

[c] Institute of Veterinary Bacteriology, University Bern, Switzerland

[d] Chemical and Veterinary Investigations Office, Stuttgart, Germany

was measured by testing ten-fold dilutions (from 10^7 to 1 GE/reaction) in 15 runs.

The '*Ca*. M. haemosuis' genome was calculated as 0.85 fg, and the pC_*CMhsuis* plasmid corresponds to 4.5 ag per copy (genome weight = genomic length (bp) x 10^5 x 665 Da/bp x 1.67 x 10^{24} g/Da) (http://cels.uri.edu/gsc/cndna.html). Since the actual genome size of '*Ca*. M. haemosuis is unknown so far, we used a mean genome size of all so far sequenced haemotrophic mycoplasmas of 750 kb. For the plasmid, a size of 4.106 kb was used to calculate the concentrations in plasmid copies per microliter corresponding to genome equivalents (GE) of '*Ca*. M. haemosuis'.

Four replicates of the plasmid dilutions (10^7 to 10^1 GE/reaction) were tested in the same run to assess the intra-assay repeatability. The inter-assay repeatability was determined by running duplicates of the same plasmid dilutions in five different runs on different days carried out by two persons.

Conventional PCR

For confirmation of the new developed qPCR, we tested a total out of ten randomly selected qPCR positive results by haemotrophic mycoplasma-specific16S rDNA PCR as described elsewhere [42]. The ten amplicons were sequenced (Seqlab Sequence Laboratories, Göttingen, Germany). Obtained sequences were compared to GenBank entries using the Blast tool provided by NCBI.

Abbreviations
Ca.: *Candidatus*; IAP: Infectious anaemia in pigs; HM: Haemotrophic mycoplasma; *M.*: *Mycoplasma*; qPCR: Quantitative polymerase chain reaction.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12917-022-03147-1.

> **Additional file 1.**

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Animal sampling of sows and piglets was performed in accordance with the German animal welfare law using a protocol officially approved by the government (Regierung von Oberbayern) (Az. 55.2–154–2532.2-16-13). Sampling of fattening pigs was performed after the slaughtering process which does not require any ethical approval according to the German Animal Welfare Law. `*Candidatus* M. haemosuis` positive blood samples (availiable from a previous study [20] were taken during diagnostic procedures and therefore are not subject to approval requirements.

Consent for publication
Not applicable.

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6. SUMMARIZING DISCUSSION

Compared to long known *Mycoplasma suis*, `Candidatus` Mycoplasma haemosuis` is a recent and merely described porcine hemotrophic mycoplasma species. Apart from individual descriptions in China (FU et al. 2017), South Korea (SEO et al. 2019) and Thailand (THONGMEESEE et al. 2022), this emerging species has not yet been further described. In this context, the clinical significance as well as epidemiological principles had not been clarified yet. As with other representatives of the HM group, the lack of an *in vitro* cultivation system is the greatest challenge when working with `*Ca*. M. haemosuis`. Specific molecular biological detection methods are therefore an indispensable basis for clinical diagnostics and epidemiological questions. Thus, the first aim of this work was to establish a specific real-time PCR assay for the quantitative detection of `*Ca*. M. haemosuis` in pigs. The novel `*Ca*. M. haemosuis` PCR assay uses the `*Ca*. M. haemosuis` *gap* as a target gene. *Gap* is encoding the NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. *Gap* was chosen prior to the 16S rRNA gene, which is a popular target in PCR diagnostics of infectious agents. However, in the case of HMs, 16S rRNA genes are quite similar in between the individual HM species and in the case of `*Ca*. M. haemosuis` the 16S rRNA gene shows an identity of >97% to related HM species (i.e. `*Ca*. M. turicensis`, `*Ca*. M. haemobos`, `*Ca*. M. haemomuris`, *M. haemocanis*, and *M. haemofelis*). *Gap* was further favored since it has been successful in diagnosing other HM species as *M. suis* (HOELZLE et al. 2007b), the feline *M. haemofelis*, `*Ca*. M. haemominutum` and `*Ca* M. turicensis` (TASKER et al. 2003a; WILLI et al. 2005; 2006), the canine *M. haemocanis* and `*Ca*. M. haematoparvum` (BARKER et al. 2010), the bovine *M. wenyonii* and `*Ca*. M. haemobos` (ADE et al. 2018) and other non-hemotrophic mycoplasma species as *M. hominis* (BACZYNSKA et al. 2004) and *M. genitalium* (SVENSTRUP et al. 2005). Additionally, it can be suspected that also the glycolytic *gap* is highly conserved in HM species, since the degradation of glucose seems to be essential in the otherwise metabolically restricted HMs in their unique blood environment (OEHLERKING et al. 2011; GUIMARAES et al. 2011; FELDER et al. 2012; DIETZ et al. 2016). Specificity of the primers was tested *in silico* by Blast algorithm provided by NCBI. The test specificity of this *gap* based SYBR® green qPCR assay was evaluated by using DNA of other hemotrophic and non-hemotrophic mycoplasma species, including the porcine ones, as a template. To verify the successful amplification of `*Ca*. M. haemosuis`, seven samples of `*Ca*. M. haemosuis` DNA were available (STADLER et al. 2020). `*Ca*. M. haemosuis` status of those DNA samples was confirmed by 16S rRNA gene amplification and sequencing. Since all test runs with `*Ca*. M. haemosuis` DNA showed positive qPCR results and all runs with other DNA templates others than `*Ca*. M. haemosuis` showed negative results, a high specificity can be attributed to the established *gap*-based qPCR. For the determination of the analytical sensitivity and the

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quantitation of `Ca. M. haemosuis` within the qPCR runs, the 177 bp *gap* fragment of `Ca. M. haemosuis` was cloned into the plasmid vector pCR2.1 (Invitrogen) and plasmid DNA was used for the calculation of `Ca. M. haemosuis` genomic equivalents (GE). By means of this approach, other hemotrophic mycoplasmas as *M. suis*, *M. wenyonii* and `Ca. M. haemobos` could already be quantified (HOELZLE et al. 2007b; ADE et al. 2018). Due to the uncultivability of HMs, this is so far the only possibility to determine the amount of this bacterial group in clinical samples. Ten-fold dilutions of the plasmid representing 10^7 to 1 GE/reaction were tested in different reaction runs and a lower limit of detection (LOD) of 10 *gap* gene copies per reaction and 2.5×10^3 GE per mL blood was determined. This is in line with comparable qPCR assays published for other HMs (HOELZLE et al. 2007b; BARKER et al. 2010; ADE et al. 2018; STADLER et al. 2019).

Within these results, the newly developed *gap*-based qPCR represents the first published valid, specific and sensitive diagnostic tool for the detection of `Ca. M. haemosuis` in pigs. This diagnostic tool served now as the first and most crucial basis for further investigations performed within this thesis. Thereby, the second aim and investigation of the present thesis was to evaluate the clinical manifestations of infections with `Ca. M. haemosuis` in pigs.

While *Mycoplasma suis* is able to cause the disease known as infectious anemia in pigs (IAP), no detailed description of the clinical signs in `Ca. M. haemosuis` infected pigs are available from the then known evidences of this novel porcine HM species in Asia (FU et al. 2017; SEO et al. 2019; THONGMEESEE et al. 2022). In the case described in this study, a fattening farm in Southern Germany was affected by an acute disease event in May 2017. The farm housed 1200 fattening pigs and purchased piglets from a single, self-recruiting piglet producing farm which was also located in Southern Germany. Pigs were routinely vaccinated against *Mycoplasma hyopneumoniae*, Porcine Circovirus Type 2 and PRRSV. Disease event started in 30% of the pigs about two weeks after housing. Clinical signs included high fever, poor general behavior, pallor of the skin, icterus, cyanoses of the ears and further also generalized hemorrhagic skin alterations consisting of petechiae and ecchymoses. Unspecific signs as fever and apathy but above all the concise signs i.e. icteroanemia, cyanoses are typical signs for the acute form of infectious anemia (IAP) normally induced by *M. suis* (PRESTON and GREVE 1965; HOFFMANN and SAALFELD 1977; SMITH AR 1978; HENRY 1979; BUGNOWSKI et al. 1986; MADSEN 1986; HEINRITZI 1990a; GROEBEL et al. 2009). This form of disease is usually found in younger age groups (HEINRITZI 1989). Besides, hemorrhagic diathesis of the skin is also found in *M. suis* infected animals, although this tends to affect older animals in the course of the chronic form of IAP. In IAP, this condition is usually named *Morbus maculosus* (HEINRITZI 1984; BRÖMEL and ZETTL 1985; BUGNOWSKI 1988). Analyses of the obtained hematological parameters, that are red blood cell count,

hemoglobin, packed cell volume mean corpuscular volume, mean corpuscular hemoglobin (MCH) and MCH concentration, revealed normochromic, normocytic anemia in eleven out of 20 collected blood samples. Interestingly, this form of anemia is commonly observed during *M. suis*-induced IAP (HEINRITZI 1990b; RITZMANN et al. 2009; STADLER et al. 2014; 2021).

Cross-necropsy of one of the affected animals revealed a high-grade icterus of the liver and the vascular endothelium. Further, ascites was evident. Additionally, microscopy showed a sinus hyperplasia of the liver and the spleen and generalized splenic follicular hyperplasia. Concerning the skin, generalized hemorrhagic disorders were quite prominent and microscopically multifocal lymphoplasmacytic dermatitis was present. Interestingly, the major observed pathological findings are also evident in *M. suis* infected pigs. This especially covers the high-grade icteric findings as well as ascites (SPLITTER 1950a, QUIN 1960; HOFFMANN and SAALFELD 1977, THIEL 1983; DENT et al. 2013; STADLER et al. 2021). Despite the extensive similarities in clinical, hematological and pathological signs to *M. suis*-induced IAP, *M. suis* could not be detected in the samples at any time of the disease event using long-established specific *M. suis* qPCR assays (HOELZLE et al. 2007b; STADLER et al. 2019). Comprehensive differential diagnostics for other possible diseases including Classical and African Swine Fever Virus (PCR and ELISA), Porcine Circovirus 2 and Porcine Circovirus-3 (PCRs), Porcine Reproductive and Respiratory Disease Virus (PCR) and *Sarcoptes scabei* var. *suis* (ELISA) all revealed negative results. In contrast, seven of 21 blood samples from affected pigs revealed positive results in a HM-specific 16S rDNA PCR (HOELZLE et al. 2011). Alignments of the subsequently performed sequence analyses of those PCR amplicons showed 99% identity to the 16S rDNA of `*Ca*. M. haemosuis` described in China in 2017 (FU et al. 2017; accession No. JX489601). Further, those seven samples also showed positive results in the novel established qPCR targeting the *gap* gene of `*Ca*. M. haemosuis` (ADE et al. 2022a). Based on the `*Ca*. M. haemosuis` positive PCR results and the exclusion of the aforementioned differential diagnoses, affected pigs were each treated twice with oxytetracycline and metamizole. Those therapeutic procedures are usually applied to pigs affected by *M. suis*-induced IAP, where they improve clinical disease outcome und also result in a reduction of *M. suis* blood loads (STADLER et al. 2014; 2021). Likewise, in the herein described case, antibiotic and analgetic treatment improved clinical signs of the observed IAP-like disease in affected fatting pigs.

Pathomorphological and clinical signs, the course of disease known from *M. suis*-induced IAP, the negative results for other probable differential diagnoses including *M. suis,* as well as the positive PCR results for `*Ca*. M. haemosuis` in both, 16S rDNA and *gap*-based PCR assays, indicate that this acute disease event in fattening pigs was caused by `*Ca*. M. haemosuis`.

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Experimental studies with splenectomized pig models as also used for studies on *M. suis* and *M. parvum* are needed to confirm the pathological potential of `*Ca*. M. haemosuis` in pigs.

Apart from the use in the diagnosis of clinical disease events, the novel established qPCR assay was also used for the first part of an epidemiological survey as knowledge on the frequency of `*Ca*. M. haemosuis` on different farms is essential. As previously described, data on the occurrence of this novel porcine HM species are so far only available from Asia (i.e. China and Korea) (FU et al. 2017; SEO et al. 2019). Thus, the third aim of this work was to investigate the occurrence of `*Ca*. M. haemosuis` in different aged pigs from Germany without obvious signs of a HM infection. For this, blood samples of piglets (n=622) and sows (n=208) from a total out of 21 piglet producing farms as well as fattening pigs (n=200) from a total out of 20 farms were collected applied to the novel `*Ca*. M. haemosuis` specific qPCR described above (ADE et al. 2022a). Additionally, 183 boars from a boar stud were sampled. In comparison, the *M. suis* status of all samples was assessed by *M. suis* specific qPCR (HOELZLE et al. 2007b; STADLER et al. 2019). `*Ca*. M. haemosuis` was evident in obvious healthy pigs as determined by qPCR. Subclinical infections in pigs have also extensively been described for *M. suis* (RITZMANN et al. 2009; STADLER et al. 2019; BRISSONNIER et al. 2020). For *M. suis*, such infections are predominant and of major interest. They can lead to immune dysregulation, higher susceptibility to other pathogens, and therefore result in various performance breakdowns (e.g. extended feeding periods, increased stillbirth rates) and in total to significant economic losses to the pig industry (RITZMANN et al. 2009; STADLER et al. 2014; 2019; BRISSONNIER et al. 2020; HOELZLE K. et al. 2020). Prevalences of `*Ca*. M. haemosuis` differed between the different age groups. Regarding the piglet producing farms, `*Ca*. M. haemosuis` was found in three out of the 21 farms. On single animal level, 13 out of the 208 sampled sows (6.25%) were qPCR positive for `*Ca*. M. haemosuis`, whereas 28 out of the 622 sampled pigs (4.50%) obtained qPCR positive results. In fattening pigs, `*Ca*. M. haemosuis` was evident in 35 of 200 fattening pigs (17.50%) in nine out of the 20 fattening farms. Contrary, `*Ca*. M. haemosuis` was not found in any of the 183 sampled boars. For the differences in the detection rates between fattening and piglet producing farms, several factors could be responsible. This includes differences in biosecurity standards or the differences in the purchase rates of animals from different farms. Since `*Ca*. M. haemosuis` was found in sows and thus in elderly animals, it was quite surprising that each of the 183 sampled boars revealed negative qPCR results. However, these animals also were free of *M. suis*. HM negative results of boars could result from a possible lower risk of infections in boar studs due to strict hygiene measures and a limited purchase of animals. When compared to the previous Asian studies, `*Ca*. M haemosuis` prevalence in this study was lower than in China with 36% positive sows and 24.1% positive growing pigs but higher than in Korea with

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only 0.01% `Ca`. M haemosuis` as `Ca`. M haemosuis` was only found in one out of 1876 sampled pigs. Compared to the *M. suis* results in the same farms and animals with 65 of 208 (31.25%) *M. suis* positive sows, 48 of 474 (14.35%) *M. suis* positive piglets and 38 of 200 (19.90%) *M. suis* positive fattening pigs, frequency of `Ca`. M. haemosuis` was little lower than the frequency of *M. suis*. Each of the 13 `Ca`. M haemosuis` infected sows, four out of the 28 `Ca`. M haemosuis` infected piglets and twelve of the 35 `Ca`. M haemosuis` fattening pigs were simultaneously infected with *M. suis*. The Chinese research group (FU et al. 2017) also described co-infections among pigs. Moreover, concurrent infections with more than one HM species were also described for other animals. For example, cattle were co-infected with *M. wenyonii* and `Ca`. M haemobos` (NISHIZAWA et al. 2010; TAGAWA et al. 2013; ADE et al. 2018; NIETHAMMER et al. 2018), sheep were simultaneously infected with *M. ovis* and `Ca`. M. haemovis` (TAGAWA et al. 2012), and cats were infected with both, `Ca`. M. haemominutum` and `Ca`. M. turicensis` (WILLI et al. 2006). Mean `Ca`. M. haemosuis` blood loads of fattening pigs found in this study (1.61 x 10$^5$ `Ca`. M. haemosuis`/mL blood) were lower than those found in fattening pigs affected by `Ca`. M. haemosuis`-induced IAP (6.04 x 10$^6$ `Ca`. M. haemosuis`/mL blood) (STADLER et al. 2020). Higher bacterial blood loads are also observed in *M. suis* infections in acute disease than in subclinical and chronic infections (RITZMANN et al. 2009; STADLER et al. 2014; 2021). Further, `Ca`. M. haemosuis` blood loads of all animals in this study seem to be lower than *M. suis* loads with mean loads of 3.83 x 10$^4$ `Ca`. M. haemosuis`/mL and 3.15 x 10^7 *M. suis*/mL in sows, 2.25 x 10^5 `Ca`. M. haemosuis`/mL and 5.09 x 10$^7$ *M. suis*/mL in piglets and 1.61 x 10$^5$ `Ca`. M. haemosuis`/mL and 7.62 x 10^7 *M. suis*/mL in fattening pigs, respectively. However, `Ca`. M. haemosuis` blood loads found in this study were in line with *M. suis* blood loads found by Ritzmann and co-workers (RITZMANN et al. 2009). In conclusion, this section of the thesis demonstrates the occurrence of `Ca`. M. haemosuis` in apparently clinically healthy animals of different age groups as well as the presence of co-infections with *M. suis*. This represents the first evidence of `Ca`. M. haemosuis` in clinically health pigs outside of Asia. It still hast to be scrutinized whether subclinical infections with `Ca`. M. haemosuis` have the same impact to pigs as subclinical *M. suis* infections.

In the course of epidemiological investigations, it is essential not only to collect prevalence data but also to gain an insight into the infection dynamics in terms of naturally occurring transmission routes. According to current knowledge, blood-dependent routes are considered as the major transmission way for HMs. This covers iatrogenic procedures, transmission via ranking fights within an animal group and also blood-sucking arthropods (SEAMER et al. 1960; HEINRITZI 1990a; 1992; PRULLAGE 1993; DO NASCIMENTO et al. 2014). Since these transmission pathways were described for *M. suis*, *M. parvum* as well as for numerous other

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HMs, especially for the feline HM species, they can also be expected for `Ca. M. haemosuis`. In addition, vertical transmissions from sows to piglets are recently discussed *M. suis* (STADLER et al. 2019). To investigate this option for `Ca. M. haemosuis` infections in the field, samples of the above-mentioned piglets (n=22) were collected immediately after birth and prior to colostrum uptake to investigate the possibility of a vertical transmission under field conditions. Evaluation of qPCR results showed that 76.92% of the 13 `Ca. M. haemosuis` positive sows have born at least one `Ca. M. haemosuis` positive piglet indicating the vertical spread of this HM species within the herd. High mean `Ca. M. haemosuis` blood loads in piglets (2.25 x 10^5 `Ca. M. haemosuis`/mL blood) underline this suspicion and contradict a possible infection during labor. The possibility of a vertical transmission was also observed for *M. suis* in the same animals (STADLER et al. 2019) and in other studies (BERRIER and GOUGE 1954; HENDERSON et al. 1997) as well as in other animal species and HMs, e.g. in cattle for *M. wenyonii* and `Ca. M. haemobos` (FUJIHARA et al. 2011; HORNOK et al. 2011; SASAOKA et al. 2015; GIROTTO-SOARES et al. 2016), in Llamas for `Ca. M. haemolamae` (FISHER and ZINKL et al. 1996; ALMY et al. 2006; TORNQUIST et al. 2011) as well as in humans as reported from China (YANG et al. 2000).

Up-to-date, only a few studies detected HMs in other excretions than in blood. For the porcine HM group, the evidence of *M. suis* in urine, saliva as well as in nasal and vaginal secrete of experimentally infected pigs has raised interests in blood-independent shedding routes (DIETZ et al. 2014). Whether and how blood-independent excretion also occurs in naturally infected animals in the field has not yet been investigated but is of great interest to potentially identify other sample material than blood for HM diagnostics. Thus, a further aim of this work to outline the appearance of `Ca. M. haemosuis` in blood-free secretes of pigs under field condition. For this purpose, saliva (n=148) and urine samples (n=47) of the formerly described blood-sampled sows as well as semen samples from each of the 183 blood sampled boars were applied to `Ca. M. haemosuis`-specific qPCR (ADE et al. 2022a). The *M. suis* status of the same samples was also determined by terms of *M. suis*-specific qPCR (HOELZLE et al. 2007b; STADLER et al. 2019). The total sample panel of saliva, urine and semen samples remained qPCR negative for `Ca. M. haemosuis`. This is in line with the obtained *M. suis* results in the same specimens. Of the urine samples, 16 corresponding blood samples were HM positive and of the saliva samples, 59 corresponding blood samples were HM positive. The deviating results to the occurrence of *M. suis* in blood-free excretes (i.e. urine and saliva samples) during experimental infections could be explained by different bacterial strains or higher bacterial blood loads observed in experimental studies than in naturally and subclinical infected pigs. The missing detection of *M. suis* in semen samples in this study is contrary to the study of Maes and co-workers, who were able to demonstrate the occurrence of *M. suis* in semen

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samples. However, in their case, *M. suis* positive semen samples enclosed RBC residues and thus are not proving a blood-independent existence of *M. suis*. On the one hand, results suggest that blood-free sampling material are not suitable for HM diagnostics in pigs as they are for several other pathogens (HENAO-DIAZ et al. 2020). Additionally, results show a minor to non-existent possibility of blood-free HM transmission within herds thus underlining the already known routes of blood-dependent routes of infection. This also means that strict hygienic measures during veterinary procedures as well as the control of blood-sucking arthropods can contribute significantly to the interruption of infection chains. To sum up, this part of the thesis dealing with transmission pathways of `*Ca*. M. haemosuis` in the field revealed a high probability of a vertical transmission from sows to their piglets but was unable to provide evidence for blood-independent transmission of this pathogen.

7. CONCLUSION

This thesis is the first to describe and investigate the novel emerging porcine hemotrophic pathogen `*Ca*. M. haemosuis` in Europe. For this, the thesis comprises five objectives that are reflected by three scientific publications.

First of all, the novel established quantitative PCR assay has been proven to be suitable for daily routine diagnostics, but also enabled the subsequent investigation aspects of the present thesis to be carried out including the occurrence, distribution, and epidemiology of `*Ca*. M. haemosuis` in Germany. Overall, the findings from this research suggest that `*Ca*. M. haemosuis` can cause acute disease in pigs. `*Candidatus*. M. haemosuis` infections were diagnosed during an acute disease event similar to *M. suis*-induced IAP. After excluding other possible causative pathogens including *M. suis*, clinical signs were finally attributed to a `*Ca*. M. haemosuis` infection. Thus, it seems quite probable that `*Ca*. M. haemosuis` is also able to cause IAP in pigs. However, to approve this, further investigations, especially experimental infections, are needed. Further epidemiological investigations within this thesis revealed a wide distribution of `*Ca*. M. haemosuis` in German pigs of different age groups as well as the high probability of a vertical transmission of `*Ca*. M. haemosuis` from sows to their piglets.

All issues, i.e. the novel established quantitative real-time PCR as well as the clinical and epidemiological descriptions provide an important basis for further investigations with `*Ca*. M. haemosuis` in the future.

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CURRICULUM VITAE

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