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Evaluation of the efficacy of two different tulathromycin treatments in weaned piglets infected intratracheally with *Haemophilus parasuis* serovar 5

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Contents

Abbreviations

ADG	Average daily gain
AGPT	Agar gel precipitation test
BALF	Bronchoalveolar lavage fluid
BW	Body weight
CD	Colostrum-deprived
CDCD	Caesarean derived-colostrum-deprived
CFU	Colony forming units
CNS	Central nervous system
DIC	Disseminated intravascular coagulation
ELISA	Enzyme linked immunosorbent assays
ERIC-PCR	Enterobacterial repetitive intergenic consensus polymerase chain reaction
HE	Haematoxlylin and eosin
HEPA	High efficiency particulate arresting filter
HPS	*Haemophilus parasuis*
IHC	Immunohistochemistry
IHA	Indirect haemagglutination
IM	Intramuscular
ISH	In situ hybridisation
KDa	Kilodalton
MIC	Minimum inhibitory concentration
NAD	Nicotine adenine dinucleotide
NADH	Nicotine adenine dinucleotide plus hydrogen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV-2	Porcine circovirus type 2
PMWS	Post weaning multisystemic wasting syndrome
PPLO	Pleuropneumonia like organism
PRRSV	Porcine reproductive and respiratory syndrome virus
rep-PCR	Repetitive element-based polymerase chain reaction
RFLP	Restriction fragment length polymorphism

SDS-PAGE	Sodium dodecyl sulfate polyacrimde gel eletrophorese
SEW	Segregated early weaning
SPF	Specific pathogen free
SRDC	Swine respiratory disease complex

1 Introduction

Once viewed as an infrequent disease of young pigs, Glässer's disease has emerged as a major pathogen affecting naïve swine herds. *Haemophilus parasuis* (HPS), the etiological agent of Glasser's disease, causes characteristic fibrinopurulent polyserositis, arthritis and meningitis in swine (BLANCO et al., 2008). Present day production practices such as segregated early weaning, mixing swine from different herds, high-herd health status, and the isolated swine populations have increased the prevalence and severity of the disease (OLVERA et al., 2006b; RAPP-GABRIELSON et al., 1997; SOLANO-AGUILAR et al., 1999). The production losses incurred by the disease are immense and are reflected in nursery mortality, decreased weight gain, and lower meat value at slaughter (OLIVEIRA and PIJOAN, 2004).

The pathogenic outcome of an infection depends on the immune status of an animal (NIELSEN and DANIELSEN, 1975). Various authors have shown that a swine herd may be infected simultaneously with different serovars of HPS, which may occur solely or in combination within a pig (RAPP-GABRIELSON and GABRIELSON, 1992; SMART et al., 1989). Furthermore, the prevalent serovar within a pig and subsequently within a herd can change over time (KIRKWOOD et al., 2001). Current trends recognize serovar 5 as one of the most virulent and most prevalent serovars in swine herds capable of eliciting sudden death and systemic infection in swine. Due to the considerable genotypic diversity of HPS strains (DE LEY et al., 1990; DEWHIRST et al., 1992; KIELSTEIN and LEIRER, 1990; RAPP-GABRIELSON et al., 2006), vaccination strategies are not always effective.

Alternative methods of control are essential and the prudent use of antimicrobials is considered to be an important component in the control of the septicemic spread of a HPS infection within a pig (DONE, 1999; MACINNES and DESROSIERS, 1999; RAPP-GABRIELSON et al., 2006). Antimicrobials have the advantage of not requiring specific knowledge about maternal antibody status nor of the dominant HPS serovar present in an infected herd (MCORIST and BOWLES, 2009). Experimental models of HPS infection are necessary to identify the clinical effectiveness of antimicrobial agents. However, the reproduction of systemic infection in pigs is difficult since HPS is a commensal of the upper respiratory tract of healthy pigs and most swine have a certain degree of protective immunity. So far HPS challenge

models have been restricted to using specific-pathogen-free (SPF) or caesarian-derived-colostrum deprived (CDCD) piglets (BAK and RIISING, 2002; MACINNES, 2003). Although clinical and pathological lesions of HPS can be successfully reproduced in such pigs due to their immune status, study success depends heavily on the use of antibiotics and gnotobiotic conditions; all entailing a very high cost (BLANCO et al., 2004).

Tulathromycin, a triamilide, is an approved antimicrobial in many countries and has demonstrated efficacy in reducing the incidence and severity of some respiratory disease caused by certain pathogens in swine including HPS (HOOVER, 2009; NANJIANI et al., 2005). Its duration of efficacy against HPS beyond 2-3 days after metaphylactic treatment, however, has not been fully demonstrated. The objective of this study was to evaluate the efficacy of two different tulathromycin treatments in commercially weaned piglets challenged intratracheally with HPS serovar 5.

2 Literature review

2.1 *Haemophilus parasuis*

2.1.1 Etiology

Haemophilus parasuis (HPS) is the causative agent of Glässer's disease. This disease is characterized by serofibrinous to fibrinopurulent serositis and polyarthritis in swine (NICOLET, 1968; RAPP-GABRIELSON et al., 2006). Pneumonia, sudden death, high morbidity and mortality in naïve swine herds has also been reported (MINIATS et al., 1986). In 1910, GLÄSSER (1910) first recognized the association between the presence of the bacteria with the occurrence of fibrinous serositis and polyarthritis in swine, and isolation of this organism was done by SCHERMER and EHRLICH in 1922 (LITTLE, 1970). Later in 1943, HJÄRRE and WRAMBY first identified the bacterium under the name *Haemophilus suis*. The first *Haemophilus* species, however, was identified in 1892 by Richard Pfeiffer. *Haemophilus suis* requires both X (iron porphyrin), and V (nicotinamide adenine dinucleotide = NAD) factors for growth (NEDBALCOVA et al., 2006). The name *Haemophilus parasuis* was suggested by BIBERSTEIN AND WHITE (1969) since they could demonstrate that the etiological agent of Glässer's disease only required the V growth factor hence the "para" before "suis". Factor V is present in heated blood and is also synthesized by some bacteria e.g. *Staphylococcus* spp. (RAPP-GABRIELSON *et al.*, 2006).

HPS, a gram-negative bacterium, belongs to the family *Pasteurellacae* although its exact position within the family has not been fully determined due to its considerable genotypic diversity (DE LEY et al., 1990; DEWHIRST et al., 1992; KIELSTEIN and LEIRER, 1990; RAPP-GABRIELSON et al., 2006). The bacterium is less than 1 µm in size, non-motile and capable of variable lengths and forms (NEDBALCOVA et al., 2006; RAPP-GABRIELSON et al., 2006). Many HPS strains appear to possess a polysaccharide capsule but capsule expression possibly depends on the culture medium (MOROZUMI and NICOLET, 1986a; RAPP-GABRIELSON et al., 1992). Furthermore, the noncapsulated strains have specific rod-like to filamentous morphology in contrast to the cocobacillary form of capsulated strains (MOROZUMI and NICOLET, 1986b). HPS forms colonies on chocolate agar which are usually

round with a slight conical surface, nonhemolytic, translucent and odorless (RAPP-GABRIELSON et al., 2006).

Since other members of the family *Pasteurellacae* are nonhemolytic and either NAD and or V factor dependent, biochemical differentiation is necessary to identify HPS. HPS can be differentiated from other members of the *Pasteurellacae* based on its ability to illicit a negative urease and oxidase reaction including a positive catalase reaction. HPS also has the ability to ferment such sugars as glucose, galactose, fructose, mannose, maltose, sucrose, and reduce nitrates. In addition to these biochemical properties, HPS does not produce indol (KIELSTEIN et al., 2001).

BAKOS et al. (1952) described the first HPS serovars based on observations made after a precipitation test to examine the existence of HPS serovars. These serovars were assigned the letters A through D. Thirty four years later, MOROZUMI and NICOLET (1986a) described an additional 7 serovars after serotyping isolates from Switzerland and Japan, and designated the numbers 1 through 7. KIELSTEIN et al. (1991) identified 6 further serovars (Jena 6- Jena 12), and later in 1992 an additional 5 (ND1-ND5) were identified (RAPP-GABRIELSON and GABRIELSON, 1992). A standardized serovar classification was first defined by KIELSTEIN and RAPP-GABRIELSON in 1992. They suggested a novel classification based on the results of an immunodiffusion test with heat-stable rabbit antigen. According to this now internationally recognized nomenclature, the previous strains 1-7 retained their original classification but all other strains were designated numbers until a total of 15 serovars were established.

In addition to these serovars, there exist numerous nontypeable isolates. After examining 103 HPS Danish field isolates, ANGEN et al. (2004) reported that 15 % of the isolates were nontypeable. OLVERA et al. (2007a) also reported similar figures after investigations of a Spanish farm. In Germany, the percentage of nontypeable isolates is approximately 26 % (KIELSTEIN and RAPP-GABRIELSON, 1992), and in China 12 % (CAI et al., 2005). It has been postulated that the existence of so many nontypeable isolates may be due to the insufficient type-specific antigen expression, the existence of further serovars or the highly genetic diversity within serovars (RAPP-GABRIELSON et al., 2006).

The high genetic variance among HPS serovars is the focus of many epidemiological studies worldwide (BLACKALL et al., 1997; DE LA PUENTE REDONDO et al., 2003; NEDBALCOVA et al., 2006; RAPP-GABRIELSON and GABRIELSON, 1992; RUIZ et

al., 2001; SMART et al., 1988). Based on molecular techniques such as Enterobacterial Repetitive Intergenic Consensus polymerse chain reaction (ERIC-PCR) testing, wide strain variation within serovar groups was identified (OLIVEIRA et al., 2003a; RAFIEE et al., 2000; RUIZ et al., 2001). A total of 12 different strains were detected for serovar 4 (OLIVEIRA et al., 2003b). One strain was identified for serovar 5, 2 strains for serovars 1, 3 and 7, 3 strains for serovars 12 and 14 and 4 strains for serovar 2.

2.1.2 Epidemiology

2.1.2.1 HPS prevalence

Since the introduction of herds with high health status, increased spread of the disease has been reported (DE LA FUENTE et al., 2007). RITZMANN and HEINRITZI (2005) noted that transport, mixing swine from different herds, introduction of naïve animals into infected herds, feed changes, and poor stall climate are supporting factors in the development of an HPS infection in a herd. Furthermore, because of the lower prevalence of infected animals and heterogeneity of HPS strain, specific-pathogen-free (SPF) and segregated-early-weaning (SEW) herds are more affected than commercial one-site farms (PIJOAN and OLIVEIRA, 2002).

MACINNES et al. (2008) and MINIATS et al. (1986) performed comprehensive studies in Ontario and southwestern Ontario swine herds. Both studies found conclusive evidence of widespread HPS in swine herds. A previous study by SMART et al. in 1989 in Ontario SPF herds showed that more than 70 % of the herds were positive for HPS. After examining over 400 isolates from southeast China, LI et al. (2009) found a wide prevalence of HPS. HPS prevalence has even been documented in feral pigs. OLVERA et al. (2007b) described the presence of HPS in wild boars in north-eastern Spain. A prevalence of 18 % was reported in wild boars in Slovenia (VENGUST et al., 2006). REINER et al. (2007) determined that HPS was present in 58.6 % of tonsil and lung samples from wild boars in Germany. In some German states, this prevalence was nearly 100 % making wild boars a possible HPS reservoir.

2.1.2.2 Serovar prevalence

In addition to HPS prevalence studies, several studies were carried out worldwide to determine the prevalence of the individual HPS serovars. Generally, serovars 4 and 5 are the most prevalent serovars worldwide (ANGEN et al., 2004). In Germany, serovars 4 and 5 appear to occur most frequently followed by serovars 2, 1, 7, 12, 13, and 14 (KIELSTEIN and RAPP-GABRIELSON, 1992; KIELSTEIN et al., 2001). After examining Danish isolates, ANGEN et al. (2004) found that serovar 5 was most prevalent followed by serovar 4 and serovar 13. The remaining serovars 1, 2, 3, 6, 7, 9, 12, 14, and 15 were present in small numbers. In contrast, serovar 10 was the most dominant serovar reported in UK followed by serovars 4, 5 and 7 (MORRIS et al., 2006). In North America, TADJINE et al. (2004) determined that serovars 4, 5, 13 and 7 were the most prevalent serotypes after examining 300 isolates. Serotyping of isolates from Australia, China, and Japan described similar trends (RAPP-GABRIELSON et al., 2006).

2.1.2.3 HPS in swine

HPS is a swine specific pathogen and can be isolated from the nasal cavities, the tonsils, the trachea, and the lungs of healthy pigs (KIRKWOOD et al., 2001; MOLLER and KILIAN, 1990; OLIVEIRA and PIJOAN, 2004; OLVERA et al., 2006a; RAPP-GABRIELSON et al., 2006). Some serovars can be isolated from either the respiratory tract or systemic sites (OLIVEIRA et al., 2003a; OLIVEIRA et al., 2003b; OLVERA et al., 2006a; RUIZ et al., 2001; SMART et al., 1988). Serovars 1, 2, 4, 5, 12, 13, 14 including some nontypeable isolates are frequently isolated from systemic sites such as body cavities whereas serotype 3 and other nontypeable isolates are found more frequently in the upper respiratory tract (OLIVEIRA et al., 2003b). ANGEN et al. (2004) observed the higher prevalence of serovar 4 in respiratory cases and the presence of nontypeable isolates in systemic infection. RAPP-GABRIELSON (1993) suggested the possibility of a subpopulation of HPS strains existing in the respiratory tract which cause systemic infection.

The HPS infection is enzootic and direct animal contact is necessary for transmission (AMANO et al., 1996). Sows are generally viewed as the reservoir for HPS in infected herds (HAEDKE, 2008). Occurrence of the disease in such herds are infrequent and clinical signs are often less severe. The introduction of new virulent strains can occur in swine of all age groups and prevalent serovars can persist for up to 1 year in

infected herds (OLIVEIRA et al., 2003b; OLIVEIRA et al., 2004b; RAPP-GABRIELSON et al., 2006). Despite the high heterogeneity among HPS strains, only 2 to 3 prevalent strains are normally involved in mortality within a swine herd (OLIVEIRA et al., 2003b; OLIVEIRA et al., 2004b; RAFIEE and BLACKALL, 2000; RUIZ et al., 2001). Experimental studies demonstrated morbidity between 50-70 %, in some cases even 90 % was reported. Lethality was nearly 10 % (VOS, 2004; WIEGAND et al., 1997).

Piglets are usually infected from their mother within a few days after birth. PIJOAN and OLIVEIRA (2003) were able to isolate HPS from the upper respiratory tract of newborn piglets as early as 5 days of age. Sows shed both pathogenic and non-patogenic strains during suckling (NEDBALCOVA et al., 2006). Therefore colonization by virulent and avirulent HPS serovars in a litter can increase with increasing time spent with the sow (KIRKWOOD et al., 2001; OLIVEIRA and PIJOAN, 2004), and litters from older sows have a higher colonization level than younger sows (TURNI and BLACKALL, 2007). However, relatively few piglets are infected before weaning since HPS shedding in sows is low and the prevalence of HPS among piglets is relatively low (HAEDKE, 2008).

Practical experiences show that the ability to induce HPS infections depends on the titer of maternal antibodies of the young pig (JAYAPPA et al, 1998). Antibodies associated with protective immunity against HPS appear to be serovar specific, and different strains of HPS offer little cross-protection to pigs infected with other strains (MCORIST and BOWLES, 2009; OLIVEIRA and PIJOAN, 2004). HPS maternal antibodies normally decrease after the fifth or sixth week of life but the immune competence in weaned piglets occurs much later after weaning. This explains the typical occurrence of the disease at the age of five-six weeks after stressful experiences such as weaning (OLIVEIRA and PIJOAN, 2002; SOLANO-AGUILAR et al., 1999). Development of the disease is triggered when a piglet becomes infected with HPS before weaning. An immune response occurs in the infected piglet and this animal becomes a subclinical carrier of HPS, assuming that sufficient protective colostral antibodies were consumed. However, in situations where insufficient colostral antibodies are consumed, then that pig may develop clinical signs of Glässer's disease. Other piglets which are not infected, but are still under the protection of maternal antibodies up to the fifth or sixth week of life, are highly susceptible for infection after this period (NEDBALCOVA et al., 2006). Furthermore, if

piglets are mixed after weaning from different farm sources that have a different flora of HPS, then piglets will lack protective maternal antibody and develop symptoms of Glässer's disease (MCORIST and BOWLES, 2009).

2.1.2.4 HPS co-infections with other pathogens

Various studies report about different co-infections between HPS and other swine pathogens (OLIVEIRA et al., 2001b). KIM et al. (2002) examined Korean pigs farms for the presence of porcine circovirus type 2 (PCV-2) and found that HPS occurred in 32 % of the examined cases that were also positive for PCV-2. KUKUSHIN and BAIBIKOV (2007) found in 10 from 12 farms a coinfection with HPS, PRRSV and PCV-2. However, PALZER et al. (2007b) did not find a significant association between PCV-2 and HPS after examining collective serosal swabs from infected pigs. However, the same author found a significant association between PRRSV and HPS. TIMINA et al. (2005) detected in 55.5 % of Russian pigs suffering respiratory symptoms a co-infection of HPS and PCV-2. Mean nasal colonization by HPS were higher in pigs that were infected with *Bordetella bronchiseptica* prior to intranasal inoculation with HPS serovar 4 (BROCKMEIER, 2004).

Some field observations suggest that HPS plays a significant role in the swine respiratory disease complex either as a predisposing agent or a primary agent (BROCKMEIER, 2004; KIELSTEIN et al., 1994; MULLER et al., 2003; SOLANO-AGUILAR et al., 1999). Trends show that HPS isolation has increased with the increased prevalence of *Mycoplasma pneumonia* including viral respiratory agents such as porcine reproductive and respiratory syndrome virus (PRRSV), swine influenza virus, and porcine corona virus (RAPP-GABRIELSON et al., 2006). KOBAYSHI et al. (1996) frequently isolated HPS from the lungs of 51.2 % of piglets that were infected with PRRSV which displayed clinical symptoms of the disease. Higher clinical scores were recorded in animals that were dually infected with PRRSV and HPS than in a monoinfection of both pathogens (PALZER et al., 2007b). A significant association between the HPS and *Mycoplasma hyorhinis* was demonstrated after identification of HPS genome from collective serosal swabs (PALZER et al., 2006b). After PCR analysis, STRUTZBERG-MINDER et al. (2008) detected a significant association between the identification of HPS and *Mycoplasma hyorhinis* in animals that had a serositis. Further studies are necessary to investigate the mechanism responsible for such dual infections.

2.1.3 Experimental infection

Several experimental infections have been developed using not only swine but also other species to study the virulence mechanism, pathogenesis of HPS and immune response towards HPS. In addition to these investigations, further experiemental models were tested to evaluate efficacy of antimicrobial treatments and vaccines. Such investigations usually require high doses of challenge inoculum to produce characteristic HPS lesions (MACINNES, 2003). BLANCO et al. (2004) found a positive relationship between the size of inocula and infection observed in sow reared and colostrum-deprived piglets.

Pigs used predominantly in HPS experiments are often SPF, colostrum-deprived (CD), or caesarean derived-colostrum-deprived (CDCD) pigs (BLANCO et al., 2004). The main advantage of using such animals compared with commercial pigs is the lack of prior colonization by HPS and interference due to maternal antibodies (RAPP-GABRIELSON et al., 1997; VAHLE et al., 1997). BLANCO et al. (2004) assessed disease development in sow reared and CD piglets after they were inoculated intratracheally with 10^6 to 10^9 CFU HPS serovar 5. Better disease development was observed in the CD pigs which developed typical symptoms and lesions of Glässer's disease than the sow reared pigs. OLIVEIRA et al. (2003b) confirmed the use of naturally farrowed, colostrum-deprived piglets as an alternative model to study HPS infections. The advantage of this model is the easy availability of animals even from herds which are HPS positive. However, the success of this model depends heavily on strict hygiene regimes and the use of antibiotics. Although the survival rate of these pigs has reached 80 %, high prechallenge death losses still occur (OLIVEIRA et al., 2003b).

Based on the current literature, intratracheal inoculation is the most frequent technique used in experimental studies. DE LA FUENTE et al. (2008a) challenged male 13-week old CD piglets with 10^9 CFU of HPS serovars 2, 4, and 5 to evaluate different vaccine formulations. Glässer's disease was observed in pigs which did not receive any vaccines. In a study to assess the antimicrobial efficacy of soluble florfenicol, IGLESIAS et al. (2002) experimentally inoculated weaned piglets with 10^8 CFU of a HPS solution. Two challenged-unmedicated animals were found dead shortly after infection and an overall lower weight gain was seen in the surviving challenged-unmedicated animals. To study the distribution and localization of PRRSV and HPS, SEGALES et al. (1999) infected 13 to 16-day old conventional pigs by the

intractracheal route with a reference strain of HPS serovar 5. Based on immunohistochemical and bacterial isolation results and clinical symptoms, pigs were infected with HPS. To investigate the protective role of maternal antibodies against HPS infection, SOLANO et al. (1999) infected piglets from vaccinated gilts, vaccinated and nonvaccinated piglets approximately 3 and 4 weeks of age with 10^6 CFU of HPS serovar 5. Pigs originating from gilts which were not vaccinated displayed severe signs of HPS infection and macroscopic lesions of polyserositis and pneumonia were also observed.

Another infection route which also reflects the natural path of infection in pigs is the introduction of HPS via the nasal cavities. BROCKMEIER (2004) inoculated 3 to 4 week old piglets intranasally with 10^5, 10^6 and 10^7 CFU of HPS serovar 4 and determined that prior infection with *Bordetella bronchiseptica* increases nasal colonization by HPS. To demonstrate the relationships between bacteria and pathological lesions using the immunoperoxidase technique, AMANO et al. (1996) inoculated 7 to 13-week old SPF pigs intranasally with 10^6 to 10^{10} CFU of strains from serovar 1 and 5. Eleven from thirteen pigs which died between days 1 and 6 had septicemic lesions consistent with Glässer's disease. VAHLE et al. (1995) could reproduce clinical signs and lesions of polyserositis and polyarthritis after intranasal infection of 5 week old CDCD with 10^9 CFU of a virulent HPS field strain. One study reported inoculating 23 to 26 day old piglets with 1 x 10^5 CFU of HPS serovar 5 via a nebulizer to test the efficacy of tilmicosin (MACINNES et al., 2003). Lower mean final body weights, average daily weight gains and total feed intakes including higher clinical scores, gross lesions, culture and mortality were seen in pigs which were not administered tilmicosin.

The infection of pigs via the intraperitoneal route is based on previous studies performed on mice and guinea pigs. SIDROV et al. (1977) found that inoculation of guinea pigs resulted in disease similar to Glässer's disease. After this discovery, MOROZUMI et al. (1981) inoculated both mice and guinea pigs with varying doses of HPS serovar 5 to examine the pathogenicity. Mice which were inoculated intraperitoneally with 10^9 CFU died but those infected with 10^7 to 10^8 CFU remained unaffected. Guinea pigs were inoculated by nasal, intranasal, intrapulmonary, abdominal, intramuscular, subcutaneous, oral, and conjunctival routes with 10^8 to 10^{10} CFU. Lesions were seen after intramuscular or intrapulmonary infection in the guinea pigs. Noticeable was that the infection of mice required high numbers of cell

to elicit disease in comparison to guinea pigs. In a similar study, RAPP-GABRIELSON et al. (1992) inoculated guinea pigs intratracheally and intraperitoneally to compare the virulence of HPS serovars 1 through 7. Serovars 1 and 5 were identified as the most pathogenic causing morbidity or death at higher doses. Later, BAK and RISSING (2002) infected commercial pigs of varying ages by the intraperitoneal route with different strains of serovar 5 using 10^7 and 10^8 CFU to test vaccine efficacy. Most of the control animals that were infected at 8 weeks of age became severely ill whereas those infected at 15, 17, 24 weeks of age developed varying degrees of clinical disease. FRAYSSINET et al. (2004) successfully infected 4 to 5 week old piglets intraperitoneally with 1 x 10^8 CFU of HPS to test antimicrobial efficacy of amoxicillin via drinking water. According to OLIVEIRA and PIJOAN, (2004) development of clinical symptoms and lesions of disease in conventional pigs after intraperitoneal infection are dose-dependent. Pigs inoculated with high doses of HPS such as 10^8 to 10^9 CFU developed fibrinous polyserositis, arthritis, and meningitis whereas pigs with lower doses did not develop lesions.

A rather seldomly used method of infection is the intramuscular route. Clinical signs and postmortem lesions of Glässer's disease were seen when CDCD pigs were infected using this route to evaluate the duration of immunity in vaccinated pigs (SICK and HAYES, 2002).

Other experimental studies focused on developing new culture media or dilution techniques to produce more effective inoculum for experimental infection. O' REILLY and NIVEN (1986) investigated tryptone-yeast extract broth as a culture medium. This broth supports HPS growth and produces exponential and early stationary phase cultures of optimum viability. Such cultures may be suited for the preparation of challenge inoculum if consideration is taken by the selection of suspension fluid and storage conditions. MOROZUMI and HIRAMUNE (1982) successfully demonstrated that HPS survival in saline and phosphate-buffered-saline (PBS) is temperature dependent. Other culture attempts were successful if the growth medium contained defibrinated sheep blood, horse or bovine blood instead of the commonly used whole sheep blood (AMANO et al., 1994; ANGEN et al., 2007; KILIAN, 1976; PIJOAN et al., 1983).

2.1.4 Pathogenesis and virulence

2.1.4.1 Pathogenesis

The pathogenesis and virulence factors of HPS are not fully defined. Therefore factors predisposing colonization and septicemic spread need to be confirmed. RITZMANN and HEINRITZI (2005) reported a clear affinity of HPS to serosal surfaces. Replication at serosal surfaces produces typical fibrinosuppurative polyserositis, polyarthritis and meningitis (AMANO et al., 1994; VAHLE et al., 1995). After experimental infection, VAHLE et al. (1995) isolated HPS in the nasal cavities and trachea 12 hours after infection, in blood 36 hours later, and afterwards from systemic tissues up until 108 hours post infection. Later studies by immunohistochemistry and transmission electron microscope confirm the early colonization of the nasal cavity particularly the middle and caudal cavities and the trachea (VAHLE et al., 1997).

Septicemic spread was observed after lesions indicative of petechiae or ecchymoses in the liver, kidney, meninges were detected (AMANO et al., 1994). For HPS to cause meningitis, the blood-brain barrier, composed of brain microvascular endothelial cells would have to be overcome. Studies show that HPS is able to adhere and invade brain microvascular endothelial cells (BOUCHET et al., 2008). Moreover, HPS induces apoptosis of these cells. Comparisons of the levels of adhesion and invasion by several HPS field strains showed that isolates of serovars 4 and 5 had a higher invasion capacity than isolates belonging to other serovars (VANIER et al., 2006).

2.1.4.2 Virulence factors

HPS serovars are known to have significant differences in their virulence (OLVERA et al., 2006a; RAPP-GABRIELSON et al., 2006). Furthermore, strains belonging to the same serovars demonstrate varying degrees of virulence (AMANO et al., 1994;1996; NIELSEN, 1993; OLIVEIRA and PIJOAN, 2004; RAPP-GABRIELSON and GABRIELSON, 1992; ROSNER et al., 1991).

Assignment of microorganism to a specific serovar group is generally viewed as an indicator of virulence (NEDBALCOVA et al., 2006). The virulence of certain serovars was demonstrated in numerous experimental challenges and epidemiological data. KIELSTEIN and RAPP-GABRIELSON (1992) demonstrated that among the 15 known serovars, serovars 1, 5, 10, 12, 13, and 14 were highly virulent with the ability

to cause death or morbidity within four days after intraperitoneal infection in SPF pigs. In the same study, severe polyserositis was observed after an infection with serovars 2, 4, and 15 and these serovars were therefore characterized as intermediately virulent. Serovar 8 produced mild clinical symptoms and was classified as less virulent. The remaining serovars (3, 6, 7, 9 and 11) did not produce any clinical symptoms and were viewed as avirulent. Further studies by the same author support this suggested classification. Similar results were obtained after studies were performed in guinea pigs to examined the virulence of serovars 1 through 7 (AMANO et al., 1994). After examining the virulence of serotypes 1, 2, 3, and 7, ROSENDAL et al. (1985) identified serotypes 1 and 2 as most virulent and 7 and 3 less virulent.

After inoculating pigs with either serovars 1 and 5, BAK and RIISING (2002) observed sudden death and polyserositis of varying severity. The pathogenicity of serovar 5 was further demonstrated in a study by TAKAHASHI et al. (2001). After intratracheal infections, pigs developed clinical signs of the disease and more than 50 % of the pigs died before study termination. OLIVEIRA et al. (2004a) were able to induce severe clinical signs associated with HPS after infection pigs with serovar 5. Similar results were reported by several authors (BLANCO et al., 2004; DE LA FUENTE et al., 2008b; DE LA FUENTE et al., 2009a).

Recently, the lipooligosaccharide of HPS was shown to play a minor role in adhesion to new born tracheal cells (BOUCHET et al., 2009). Lipooligosaccharide is a major component of the outer membrane of Gram-negative bacteria and is released with bacterial lysis. Most biological activities of lipooligosaccharide can be attributed to mediators which macrophages have produced through stimulation by lipooligosaccharide (AMANO et al., 1997; KHAIR et al., 1996). Lipooligosaccharide is capable of eliciting apoptosis in endothelial and epithelial cells as well as the release of proinflammatory mediators and is involved in bacterial adhesion to host cells (SYLTE et al., 2001). AMANO et al. (1997) linked the presence of lipooligosaccharide in the blood of diseased pigs to endotoxin shock and disseminated intravascular coagulation (DIC).

LICHTENSTEIGER and VIMR (2003) proposed neuraminidase as a possible HPS virulence factor. Similar to the neuraminidase from other *Pasteurella* spp, HPS neuraminidase is cell associated and it plays a possible role as nutrient supplier. Neuraminidase activity demonstrates the ability of a pathogen to colonize or invade host cells. It could be demonstrated that HPS colonies displayed greater growth after

in vivo passage than colonies without this enzyme when silica acid was added to the growth medium (LICHTENSTEIGER and VIMR, 1997).

Another possible virulence factor is the ability to build a biofilm by some serovars. ZHOU et al. (2006) postulated that bacteria which are capable of producing a biofilm can evade the immune system of the host cell, therefore increasing the chances of persistent and chronic infections. In addition to evading the immune system, bacteria with a biofilm have a higher tolerance towards antimicrobial agents. JIN et al. (2006) tested the biofilm forming ability of various strains from 13 HPS serovars (with the exception of serovars 3 and 8) including nontypeable isolates. Only serovars 2, 9, 12, 13 and 15 were unable to form biofilms. Also, it was shown that biofilm formation was retained when biofilm-forming serovars were injected into the nasal cavities of pigs. In contrast, this ability was lost in bacteria that colonized the lung and brain. These observations were later confirmed in a further study by the same author (JIN et al., 2008).

Some authors suggested the potential association between presence of a capsule and HPS virulence using experimental infections. The capsular material is thought to be an acidic polysaccharide although earlier studies by WILLIAMSON and ZAMENHOF (1964) reported a structure of repeating units of polymerized α-galactosyl-α-N-acetylglucosaminide. MOROZUMI and NICOLET (1986a) showed the presence of a capsule in 12 of 32 investigated HPS strains. Generally, serovars 1, 2 and 3 possess a capsule and serovars 4 and 5 are noncapsulated. Capsules were observed most often in isolates that originated from the nasal cavities. In another study, investigators found that noncapsulated strains were more virulent that the capsulated ones (KOBISCH et al., 1980). ROSNER et al. (1991) studied various HPS serovars and strains from different farms in Germany and found that noncapsulated strains and isolates of serovar 5 dominated in pigs with Glässer's disease.

Still, other authors attribute the virulence among certain strains to the presence of certain outer membrane proteins (MINIATS et al., 1991a; RUIZ et al., 2001). The outer membrane proteins of HPS therefore may contribute to immunity and virulence. After sodium dodecyl sulfate polyacrimde gel eletrophorese (SDS-Page) analysis, results showed that strains recovered from systemic sites had more homogeneous outer membrane proteins than those isolated from respiratory sites (RUIZ et al., 2001). The outer membrane proteins were identified as having two different profiles; biotype 1 and biotype II (MOROZUMI and NICOLET, 1986a; NICOLET et al., 1980).

Biotype I, with a molecular weight from 23-40 and 68 KDa, was found more often in the nasal cavity of healthy pigs. In contrast, biotype II which is characterized by a molecular weight of 37 KDa, was found in swine herds with Glässer's disease. MINIATS et al. (1991a) demonstrated that pigs challenged with various strains of HPS survived the challenge if they were vaccinated with outer membrane proteins antigens prior to challenge.

CERDA-CUELLAR and ARAGON (2008) proposed serum-resistance (normal and immune rabbit serum) as a possible virulent mechanism for HPS. Strains isolated from the nasal mucosa of healthy animals were sensitive to the bactericidal effect of the serum. In contrast, systemic strains were mainly resistant.

MUNCH et al. (1992) showed that HPS was able to form fimbriae-like structures after in vivo passage. Bacteria fimbriae are nonflagellar proteinaceous surface appendages which are often necessary for adherence to host cells (BEACHEY, 1982; STULL et al., 1984). However, the role of these structures as a virulence factor has yet to be clarified.

Several molecular techniques have been employed to identify virulence-associated genes in bacteria. These genes are presumably active (up-regulated) in response to restrictive host environment. SACK and BALTES (2009) identified five potential virulence associated genes and the hemolysin operon (hhdBA) was found to be most present in investigated isolates. METCALF and MACINNES (2007) detected eight virulent genes of serovar 5 that were up-regulated in response to iron depletion. Iron is indispensable for the growth and survival of microbial pathogens in the course of infection. HILL et al. (2003) also conducted a search for virulence associated genes in strain 1185 serovar 5 HPS. Three genes appeared to be more upregulated than others and a total of seven gene fragments were identified.

Based on the multitude of possible virulence factors, further studies are clearly necessary to confirm and detect virulence factors of HPS.

2.1.5 Clinical signs

HPS infection usually occurs in young pigs 4 to 6 weeks after weaning (RAPP-GABRIELSON et al., 2006). In some swine herds, clinical signs are evident one week after weaning. Such early symptoms are possibly due to insufficient colostrum intake (OLIVEIRA and PIJOAN, 2002; ZIMMERMANN and MÜLLER, 2004). The severity of clinical signs depend not only on the virulence of the strain present in the herd but

also on the immune status and the current stage of infection (OLIVEIRA and PIJOAN, 2002). The incubation period is between five to seven days (BAEHLER et al., 1974). Incubations times of 24 hours were also reported after experimental infection (DE LA FUENTE et al., 2008a; DE LA FUENTE et al., 2009; NEIL et al., 1969; OLIVEIRA et al., 2003a; VAHLE et al., 1995). In naïve herds or in swine either infected with highly virulent HPS strain or weakened by a preexisting disease, outbreaks of Glässer's disease can occur relatively shortly after exposure to HPS (RAPP-GABRIELSON et al., 2006).

Following an acute infection, clinical signs are dominated by pyrexia (41.2 °C), apathy, anorexia, kyphosis, pain (squealing), cyanosis, and lateral recumbency (RAPP-GABRIELSON et al., 2006; RITZMANN and HEINRITZI, 2005). Abortion in gilts and chronic lameness in boars following an acute infection have also been reported (RAPP-GABRIELSON et al., 2006). Sudden death is seen most often in young piglets after septicemia spread of the infection (HILL et al., 1996; PIJOAN and OLIVEIRA, 2002; RAPP-GABRIELSON et al., 2006). Auscultation of lungs frequently results in pleural friction rub if a pleuritis is present. Further respiratory symptoms often observed include coughing and dyspnoea. Palpation of the hock, the tarsal and carpal joints reveal increased synovial fluid (RAPP-GABRIELSON et al., 2006; RITZMANN and HEINRITZI, 2005). Following such changes, lameness can be observed to varying degrees of severity. If the meninges become infected, neurologic deficits such as ataxia, tremor, spasms, nystagmus, and paddling have been reported (KIELSTEIN, 1985). In one rare case, panniculitis of the ears, myositis and fascitis of the neck musculature including edema of the head region was observed in SPF sows (HOEFLING, 1991;1994). Chronic infection is manifested by rough hair, body weight loss and poor growth (RAPP-GABRIELSON *e*t al., 2006). In endemically infected herds where mostly older animals are affected, clinical symptoms are less evident than in naïve herds and are mostly restricted to symptoms of respiratory distress (OLIVEIRA and PIJOAN, 2002).

Blood examination of pigs experimentally inoculated with serovar 5 detected DIC, severe leucopenia, hypoglycemia, prolonged prothrombin and activated partial thromboplastin time, and increased fibrinogen-fibrin degradation (AMANO et al., 1997). After inoculation of SPF pigs with HPS serovar 5, endotoxin could be detected in the plasma of all the inoculated pigs from 16 hours post-infection until death, and its concentration rose dramatically just before death of the animal. All pigs died 28 to

42 hours after challenge (AMANO et al., 1997). A severe leucopenia was seen 24 hours after challenge until death. HEINRITZI (2006) noted that the blood leucocytes level decreases 1 to 2 days after an infection only to then dramatically rise afterwards.

2.1.6 Gross and microscopic lesions

2.1.6.1 Gross lesions

The severity of macroscopic lesions displayed often depends on the duration of the infection (SCHULZ, 1991). Macroscopic lesions by an HPS infection generally consists of serofibrinous or fibrino-purulent exudates on the pleura, pericardium, peritoneum or joint surface (hock, carpal and tarsal joints) all occurring in various combinations. (BLANCO et al., 2004; BLANCO et al., 2008; NEDBALCOVA et al., 2006; SOLANO-AGUILAR et al., 1999). By chronic infections, adherences in pleura, pericardium, and peritoneum are frequently present including hydrothorax, hydropericardium and hydroperitoneum (BLANCO et al., 2004). In some cases severe fibrino-purulent adhesions of the gastrointestinal tract with fibrin deposits on the surface of all abdominal organs can be observed (TURNI and BLACKALL, 2007). Organs generally appear hyperemic with petechiae or ecchymoses evident in the cardiac coronary sulcus, the liver, the kidneys, and the meninges (AMANO et al., 1994). Following septicemic spread, enlarged lymph nodes especially the mesenteric and mediastinal lymph nodes can be seen (DE LA FUENTE et al., 2008a). Thrombosis of the lungs, lymph nodes, kidneys, liver, and brain occurs infrequently. Lesions mainly in the cranial lung lobes are evident following an exudative or bronchopurulentpneumonia after HPS infection (AMANO et al., 1997; DE LA FUENTE et al., 2008a). OLIVEIRA and PIJOAN (2002) reported finding antero-ventral consolidation of the lungs with purulent exudates in the bronchi and bronchioli. Infrequent cases of purulent rhinitis were also reported by VAHLE et al. (1995). If a septicemia accompanies an HPS infection, subcutaneous and pulmonary edema including the occurrence of sudden death without the typical inflammatory lesions on the serosal surfaces may also be observed (RILEY et al., 1977).

2.1.6.2 Microscopic lesions

Histological findings in serofibrinous to fibrino-purulent inflammations usually consist of infiltrates of fibrin, neutrophils, lymphocytes and to a lesser extent macrophages (NEDBALCOVA et al., 2006; VAHLE et al., 1995). Fibrinous microthrombi were observed in organs that had petechiae or ecchymoses and in the capillaries of the pulmonary alveolar walls of some pigs (AMANO et al., 1994). This author also found fibrinopurulent meinigitis in several pigs. A mild to moderate splenitis with neutrophils attached to the capsule was noted by SOLANO-AGUILAR et al. (1999) after intratracheal infection in pigs with serovars 4 and 5. In a similar study, spleen lesions were characterized by perifollicular hemorrhage, perifollicular necrosis and fibrinious serositis (NOGUEIRA et al., 2000). AMANO et al. (1994) observed a depletion of periarteriolar lymphoid tissue and follicles in the spleen with hemorrhages in the marginal zone of white and red pulp of some pigs infected intranasally with serovars 1, 4 and 5. After experimental infection, fibrinous thrombi in the renal glomeruli and necrosis of renal tubules, with calcification and non-purulent interstitial nephritis were observed (DE LA FUENTE et al., 2008b; SOLANO-AGUILAR et al., 1999). The parenchyma of enlarged lymph nodes contains increased numbers of mononuclear or polymorphonuclear cell infiltrates and germinal centers are activated (NOGUEIRA et al., 2000; OLIVEIRA and PIJOAN, 2004). Lung lesions are characterized by infiltrates of neutrophils and macrophages (NEIL et al., 1969). If an exudative pneumonia is present, alveolar edema and edematous or serofibrinous exudates within the alveolar lumina and septa can also be seen. In addition, small areas of lung collapse as a result of bronchiolar plugging by fibrin and leucocytes can be found.

2.1.7 Differential diagnosis

Clinical signs and lesions produced by an HPS infection are often quite similar to symptoms produced by other viral and bacterial pathogens. Already in 1969, NEIL et al. (1969) discussed the similarity of the polyserositis and arthritis produced by *Mycoplasma hyorhinis* with that of HPS. Similar to Glässer's disease, particularly piglets between three to ten weeks old are prone to a *Mycoplasma hyorhinis* infection (RAPP-GABRIELSON et al., 2006; RITZMANN and HEINRITZI, 2005). The polyarthritis caused by *Mycoplasma hyosynoviae* and purulent arthritis due to streptococcal infections or *Arcanobacter pyogenes* are further possible differentials. If

a septicemic infection is suspected, bacterial involvement by *Streptococcus suis*, *Erysipelothrix rhusiopathiae*, *Actinobacillus suis*, *Salmonella cholerasuis var. kunzdorf* and *Escherichia coli* need to be considered as alternative differential diganoses (RAPP-GABRIELSON et al., 2006). If symptoms of central nervous impairment are displayed, edema disease and meningitis caused by *Streptococcus suis* are possible differential diagnoses. Prominent features of edema disease are the subcutaneous edema of the eyelids, nose and lips; swollen joints are not typical for this disease (RAPP-GABRIELSON et al., 2006). Streptococcal infections are also characterized by swollen joints and symptoms of respiratory distress (HEINRITZI, 2006). By respiratory symptoms or gross lesions, identification of other known viral and bacterial pathogens involved in the swine respiratory disease complex is necessary before a conclusive diagnosis can be made (RAPP-GABRIELSON et al., 2006).

2.2 Diagnosis

A preliminary diagnosis of Glässer's disease is based on the clinical signs, the presence of gross lesions such as polyserositis and polyarthritis, and histopathological investigations. A definitive diagnosis of any infection requires the isolation of the causal agent. In the case of HPS, isolation and identification is not always conclusive due to the fastidious nature of the microorganism and its commensal status in the upper respiratory tract of healthy pigs.

2.2.1 Selection of animals and sampling sites

An essential factor in the diagnosis of HPS is the use of appropriate animals and tissues for sampling. Successful isolation was observed when animals with clinical signs of HPS infection are used (OLIVEIRA, 2004). Furthermore, to optimize isolation, animals in the early stages of infection which have not been treated with antimicrobials for at least 1 week should be used (OLIVEIRA and PIJOAN, 2002). According to OLIVEIRA (2004), isolation from chronically affected pigs is usually unsuccessful. Since HPS is an epiphyte of the upper respiratory tract of healthy pigs, non-respiratory sites are preferred for sampling and isolation (MOLLER and KILIAN, 1990; VAHLE et al., 1997). Ideally are sites such as the brain (meninges), the pericardium, the pleura, the peritoneum, and the joints (OLIVERIA; 2004). SEGALES et al. (1998) were able to recover HPS from the peritoneal and articular fluid. Various

authors were also able to isolate HPS in the pleural fluid, peritoneal fibrin and pericardial fluid (AMANO et al., 1997; NEIL et al., 1969; TURNI and BLACKALL, 2007). The same authors found that the lung, the heart, the heart blood, affected joints and brain to be the best sites for sampling when an acute infection is present. However, other authors listed the isolation from blood during acute infection as infrequent (OLIVEIRA et al., 2003b; OLIVEIRA and PIJOAN, 2004).

2.2.2 Sampling procedures

Samples for HPS isolation should be collected aseptically whenever possible. Collection can be done using sterile swabs placed in transport systems containing Stuart or Amies media. The Amies system maintains viability of HPS better than Stuart system (DEL RIO et al., 2003). Even complete organs are suitable for transport. However, swabs have been successfully used for sampling diseased animals for diagnosis of HPS particularly by PCR (ANGEN et al., 2007). Furthermore, swabs allow easier handling and transportation compared with tissue samples. Immunohistochemical studies indicate that free HPS cells are concentrated in fibrinous exudates, therefore , swabs from organs with fibrinous exudates on the surface are preferred (SEGALES et al., 1997). Fluids from joints, peritoneum, pericardium, thoracic cavity are also useful and can be obtained using sterile syringes (OLIVEIRA, 2004).

MOZUMI and HIRAMUNE (1982) found that HPS is temperature sensitive. The authors established that HPS organisms are undetectable in physiological saline at 42 °C within 1 hour, at 37 °C within 2 hours, and at 25 °C within 8 hours. Because different HPS strains may be isolated from different body sites in the same pig, tissues samples should be submitted in separate bags. The use of formalin is not necessary for tissue samples (OLIVEIRA, 2004). The survival rate of HPS can be extended if samples are stored at 4 °C until submission to a diagnostic laboratory (MOROZUMI and HIRAMUNE, 1982; OLIVEIRA et al., 2004b).

2.2.3 Bacterial isolation

Clinical symptoms of Glässer's disease are diverse. Therefore, bacterial isolation of HPS is indispensible. Since HPS is V factor dependent, NAD is supplied when the blood agar is heated or on NAD supplemented pleuropneumonia like organism (PPLO) agar. Since *Staphylococcus aureus* produces NAD, a nurse strain can also

be streaked onto a blood agar plate thus facilitating satellite growth. When a liquid culture is required such as for biochemical tests, HPS can be cultured in liquid pleuropneumonia like organism agar broth supplemented with NAD (OLVERA et al., 2007c). HPS is a very fastidious microorganism and growth depends often not only on the suitable agar but also the correct atmosphere. CO_2 supplemented atmosphere has been shown to support HPS growth (BLANCO et al., 2008; DE LA FUENTE et al., 2009b). Incubation time varies between 24-48 hours (SEGALES et al., 1997). Due to the fact that other bacteria such as *Actinobacillus indolicus*, *Actinobacillus porcinius* and *Actinobacillus minor* are also NAD dependent and are found in the upper respiratory tract of swine, culture in media supplemented with antibiotics such as lincomycin and bacitracin are sometimes necessary (PIJOAN et al., 1983). Nevertheless, confirmation of HPS by biochemical differentiation is necessary to differentiate these bacterial species. Furthermore, if samples were obtained from the respiratory tract, successful bacterial culture does not confirm a systemic HPS infection. Particularly samples from the brain (meninges), the joints, and the serosal membranes are suitable for bacterial culture (NEDBALCOVA et al., 2006). In addition to these sites, swabs from fibrinous exudates, peritoneal fluid, synovial or cerebrospinal fluid including samples from the liver and the spleen are also suitable (OLIVEIRA, 2007; RITZMANN and HEINRITZI, 2005). HPS may also be isolated from lungs with severe pneumonia (OLIVEIRA, 2007). MOORKAMP et al. (2008) proved that isolation from bronchoalveloar lavage fluid (BALF) is comparable to detection from lung tissue. However, PALZER et al. (2005) noted that isolation from BALF does not necessarily confirm a clinical infection since HPS is a commensal in the respiratory tract of healthy pigs. Furthermore, the same author did not find a significant relationship between the cultural isolation from BALF with clinical symptoms and gross lesions of fibrinous serositis (PALZER et al., 2006b).

2.2.4 Molecular biological methods

2.2.4.1 PCR

The development of a specific PCR for HPS in 2001 was a major advance in the diagnosis of HPS infections particularly since HPS is such a fastidious bacterium (OLIVEIRA et al., 2001a). The PCR could detect a minimum of 10^2 bacteria and 0.69 pg of DNA. The gene sequence for the PCR is approximately 821 base pairs from the 16S small subunit ribosomal RNA of HPS. The high sensitivity of this PCR

was demonstrated due to its detection of HPS in tissue or swab samples from subclinic to dead animals including pure culture (colony PCR). The PCR test could also detect HPS from samples of acute lesions with negative bacterial isolation results. It is a far more sensitive method than bacterial culturing for diagnosis of HPS systemic infections (OLIVEIRA et al., 2006). Most PCR positive results are obtained after sampling fibrinous exudates in the pleura, pericardium, peritoneum, spleen, liver, joints, and meninges. Results from histopathological examination show a higher success rate when sample material from animals in an acute infection are used (OLIVEIRA, 2007). However the diagnostic value of the PCR method is limited to sample material which does not originate from the upper respiratory tract due to the presence of HPS in this region in healthy pigs. Positive PCR results from the upper respiratory tract including the lungs are only valid if HPS was not detected prior to testing (OLIVEIRA, 2007). Further limitations of the PCR include a positive non-specific reaction with *Actinobacillus indolicus*. This bacterium is also a commensal of the upper respiratory tract of pigs. Due these limitations, the PCR is suitable for diagnosing HPS isolates recovered from systemic tissues.

JUNG et al. (2004) developed a nested PCR to improve the sensitivity of the technique. Moreover, this PCR is applicable for use by formalin-fixed, paraffin-embedded tissues. Sensitivity was increased to 3 CFU due to the amplification of a 313 base pair sequence. The specificity is however the same as the conventional PCR since the primers of the nested PCR are identical to the same gene sequence of *Actinobacillus indolicus*.

An improved version of the original PCR by OLIVERA et al. (2001) was recently developed by ANGEN et al. (2007). The 16S r DNA sequence used in this PCR originated from the 15 known serovars of HPS. In comparison to the PCR by OLIVEIRA et al. (2001) which also tested positive for *Actinobacillus indolicus*, this PCR is 100 % species specific for HPS. The sensitivity of the PCR is slightly lower when applied on clinical samples from diseased pigs and pure HPS cultures. Also, the chances of false positive results of samples from systemic sites are also lower in the PCR test by OLIVEIRA et al. Both PCR methods demonstrated similar sensitivity when tested on purified DNA. Since both PCRs have similar sensitivity but differing specificity, their use depends on the purpose of the diagnostic (ANGEN et al., 2007).

2.2.4.2 In situ hybridisation

An in situ hybridisation (ISH) for HPS was developed by JUNG and CHAE (2004). In situ hybridisation allows the direct association of HPS with tissues lesions similar to immunohistochemistry (IHC) without the problems associated with antibody availability. Also, this method allows the detection of HPS in formalin-fixed, paraffin-embedded tissues (JUNG and CHAE, 2004). Since a high similarity between the probe used for this method and fragments in the genes of *Actinobacillus indolicus*, and *Actinobacillus porcinus* exists, a potential cross-reaction with these bacteria can occur. Further investigations are necessary to evaluate the use of in situ hybridisation as a routine diagnostic method.

2.2.5 Further diagnostic methods

2.2.5.1 Serotyping

After bacterial culture and biochemical differentiation, HPS may be further characterized by serotyping. Serotyping of HPS can be performed using either indirect haemagglutination (IHA) or the agar gel precipitation test (AGPT) (DEL RIO et al., 2003a; TADJINE et al., 2004). Both techniques are based on the reaction between serovar-specific polyclonal antiserum and heat-stable antigens extracted from the bacterial cultures (OLIVEIRA, 2007). In contrast to the agar gel precipitation test method, the indirect haemagglutination is specific and sensitive for HPS with fewer cross-reactions being reported. Also a higher percentage of nontypeable isolates (15-40 %) was seen for agar gel precipitation test compared with the indirect haemagglutination test (<10 %) (OLIVEIRA and PIJOAN, 2004; TADJINE et al., 2004). Although interpretation of agar gel precipitation test and indirect haemagglutination results is uncomplicated, the analysis of the results is subjective. Some field isolates do not produce sufficient antigen in vitro for serotyping. Moreover, cross-reactions between serovars are possible. Nontypeable isolates can even interfere with serotype assignment since they can produce cross-reactions. Also, nontypeable may constitute serovars for which antisera do not yet exist (OLIVEIRA, 2007).

An alternative method which is not only used for serotyping but also as a routine diagnostic method is the enzyme-linked immunoassays (ELISA). SOLANO-AGUILAR et al. (1999) developed a HPS ELISA using formalin-inactivated whole cells as a

coating antigen to study maternal antibodies and humoral response of piglets after vaccination. ELISA is routinely used because it is more sensitive than other assays and it is compatible with high volume testing. ELISA is a good method to detect antibodies if antigens used in the test match the serovars infecting the population (OLIVEIRA, 2005).

2.2.5.2 Genotyping

Similar to serotyping, genotyping methods are used to characterize HPS isolates. Genotyping is performed by sequencing either whole bacterial genomes or a single gene (OLVERA et al., 2007c). Currently, there exist four methods of genotyping HPS isolates. The first technique is the repetitive element-based-PCR pattern (rep-PCR) typing and consists of typing sequences which are repetitive within the HPS genome. Identified sequences are strain specific. The second technique is a modified version of the rep-PCR, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). These DNA sequences (ERIC elements) are distributed throughout the bacterial genome and are highly conserved among different bacterial species. Different strains have different distribution of these sequences in their genome which can be used to identify groups of related strains. ERIC-PCR is presently widely used to identify sources of strain introduction into a herd, to detect strains involved in mortality, and for the selection of strains to be used in autogenous vaccines (OLIVEIRA, 2007). It is particularly suitable for epidemiological studies since it is fast, inexpensive and interpretation of results is simple in comparison to other genotyping methods. Recently, DE LA FUENTE (2007) developed a restriction fragment length polymorphism (RFLP) PCR to genotype HPS strains. RFLP-PCR is based on the amplification of a single gene. The main advantage of this technique in comparison to serotyping and ERIC-PCR is that it can be used directly on clinical samples without the need for bacterial isolation (OLVERA et al., 2007c). A further genotyping method, mutlilocus sequence typing, was developed by OLVERA et al. (2006a) to overcome the resolution and implementation problems of the other genotyping methods.

2.2.5.3 Immunohisotchemistry

Immunohistochemistry (IHC) has been used to detect HPS antigen in formalin-fixed, paraffin embedded tissues (AMANO et al., 1994; SEGALES et al., 1997; VAHLE et al., 1997). Immunohistochemistry has the advantage of providing direct visual

demonstration of bacterical antigens in affected tissues which facilitates the association between the presence of antigens and histopathological changes. NOGUEIRA et al. (2000) found that lung and spleen showing lesions are the most suitable organs for detection of HPS antigen. In contrast, the same author found that mediastinal lymph nodes unsuitable for immunohistochemistry. Immunohistochemistry has not been used extensively for diagnostic purposes, since the availability of monoclonal antibodies to HPS is very limited and polyclonal antibodies have shown cross-reactivity with other bacteria such as *Actinobacillus pleuropneumoniae* (OLVERA et al., 2007c; SEGALES et al., 1997).

2.3 Therapy

2.3.1 Antimicrobial use in food animal production

The use of antimicrobials is an integrate part of the food animal production system. In the EU, an estimated 6750 tonne of antibiotics were used for animal health purposes (SCHWARZ and CHASLUS-DANCLA, 2001). Tetracyclines amounted to 66 % of this therapeutic use while macrolides and penicillins amounted to 12 % and 9 % respectively. The other antimicrobial groups together comprised 12 % (BOATMAN, 1998; SCHWARZ and CHASLUS-DANCLA, 2001). In food production, 60 % of all antibiotics are used mainly for swine.

Antibiotics used in veterinary medicine are similar or structurally related to antibacterials used in human medicine and repeated and indiscriminate usage poses the risk of developing antibiotic resistance or inducing cross-resistance pertaining to human medicine (UNGEMACH et al., 2006). However, since antibiotics of the same class, such as tetracyclines, macrolides and beta-lactams have been used for decades in both humans and animals, resistance to these antibiotics has been selected for and transferred in both groups of hosts (SCHWARZ et al., 2001). Based on previous studies, antimicrobial use in animals and resistance resulting from such use generally does not account for resistance problems in human medicine (SCHWARZ et al., 2001).

2.3.2 Antimicrobial treatment for HPS

Antibiotics are used to prevent septicemic spread of the microorganism and reduce the severity of clinical symptoms. High doses are often need during an acute

outbreak of Glässer's disease regardless of the active ingredient or method of administration (DESROSIERS, 1986). This is to ensure that antimicrobials actually penetrate into affected areas such as the joints and cerebrospinal fluids (NEDBALCOVA et al., 2006).

Penicillins and tetracyclines are one of the most widely used antibiotics to combat HPS (SAN MILLAN et al., 2007). Cephalosporin, the combination product trimetoprim and sulfonamide, ampicillin, fluroquinolon, gentamycin, spectinomycin, and tiamulin are also effective (HEINRITZI, 2006; RAPP-GABRIELSON et al., 2006). In addition, *in vivo* experiments with tilmicosin showed good efficacy against HPS (DEROSA et al., 2000; MACINNES *et al.*, 2003; PARADIS et al., 2002). Improvements in all clinical and growth measures were seen in challenged pigs which received tilmicosin. In field trials, a single intramuscular dose of ceftiofur was effective for the treatment of naturally occurring bacterial swine respiratory disease including HPS (MEEUWSE et al., 2002). Mortality rates were significantly lower and clinical cure rates higher in pigs that receive ceftiofur than placebo-treated pigs. FRAYSSINET et al. (2004) also observed significantly lower mortalities and clinical signs in pigs which receive amoxicillin after infection with 10^8 CFU of HPS. In another study, IGLESIAS et al. (2002) infected weaned piglets with 10^8 CFU of HPS to assess the efficacy of florfenicol against HPS. The amount and severity of clinical disease manifestations were lower in pigs treated with florfenicol compared to pigs from the control group.

2.3.3 Antimicrobial susceptibility patterns for HPS

Antibiotic use against septicemic spread of HPS can increase the potential selection of bacteria resistant to the antibiotic administered. In some countries, HPS is almost susceptible to all tested antimicrobial agents, whereas in other countries high resistance exists (AARESTRUP et al., 2008).

In one German study, less than 10 % of all HPS strains were resistant towards tetracycline, oxytetracycline, enrofloxacine, and canamycin. 5 % of the investigated strains were resistance for sulfonamide (VON ALTROCK, 1998). In a Danish study, the susceptibility of HPS towards ampicilin, ceftiofur, ciprofloxacin, erythromycin, florfenicol, penicillin, spectinomycin, tetracylince, tiamulin, and tilmicosin was investigated. Almost all of the antimicrobial agents were sensititve for HPS with the exception of isolated resistance towards trimethoprim-sulfamethoxazol (AARESTRUP et al., 2004). DE LA FUENTE et al. (2007) tested the antimicrobial

susceptibility of 30 British and Spanish HPS isolates towards 19 currently available antimicrobial agents. British strains were mainly resistant towards ampicillin, gentamicin, spectinomycin, tetracycline and trimethoprim-sulfonamide. All other antimicrobials showed favorable susceptibility patterns. In contrast, the Spanish isolates were only sensitive to florfenicol. A high resistance was seen towards the remaining antibiotics. Correspondingly, SAN MILIAN et al. (2007) found that 14 % of Spanish HPS isolates were resistance towards penicillin. PEJSAK et al. (2005) tested 40 HPS strains collected from different Polish regions to determine their antimicrobial sensitivity. More than 95 % of the isolates were sensitive to amoxicillin, amoxicillin with clavulanic acid, ampicillin, doxycycline, cefquinome, enrofloxacine, norfloxacine and lincosamide. Contrastingly, 85 % were moderately resistant towards penicillin, tetracycline, oxytetracycline, and tiamulin. Relatively high resistance patterns were seen towards sulfonamides and trimetoprim. In China, high levels of resistance amount isolates from thirteen serovars (1-5, 7-10, and 12-15) were seen for enrofloxacine (70.9 %) and trimetoprim-sulfamethoxazol (44.5 %). 23.6 % of the isolates were resistant to more than 3 antimicrobial agents (ZHOU et al., 2009). In contrast, Korean HPS isolates are highly resistant to tylosin, lincosamide and tilmicosin (KOH et al., 2006).

2.3.4 Tulathromycin

Tulathromycin, the active ingredient of Draxxin® Injectable Solution (Pfizer GmbH, Berlin), is approved in the European Union as a single dose application for the treatment and metaphylaxis of swine respiratory diseases (SRD) associated with *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Mycoplasma hyopneumoniae* and HPS (EVANS, 2005). NANJIANI et al. (2005) and HOOVER (2009) found that tulathromycin was safe and effective in the treatment of natural outbreaks of SRDC. Recently, tulathromycin was demonstrated to significantly reduce the positive identification of HPS in BALF after a five day treatment (PALZER et al., 2007c). Various authors found that tulathromycin provides protection even up to 9 days against death and severe morbidity caused by *Actinobacillus pleuropneumoniae* in pigs (HART et al., 2006; SCHEIDT, 2007; WAAG et al., 2008). MCKELVIE et al. (2005) and STANFORD (2008) demonstrated the efficacy of tulathromycin in a swine respiratory disase challenge model using a U.K and US.A. *Mycoplasma hypneumoniae* field isolates respectively.

The unique structural features of this antibiotic make it the first member of a new macrolide class which have been termed triamilides (LETAVIC et al., 2002). Tulathromycin is structurally related to erythromycin which is monobasic and to azithromycin which is dibasic. Tulathromycin consists of a regioisomeric, equilibrated mixture of a 13-member ring azalide (10 %) and a 15-membered ring azalide (90 %) in aqueous media, both with three basic amine groups (Figure 1). The two isomers differ through a lactone bond formation at the C11 and C13 regions (EVANS, 2005).

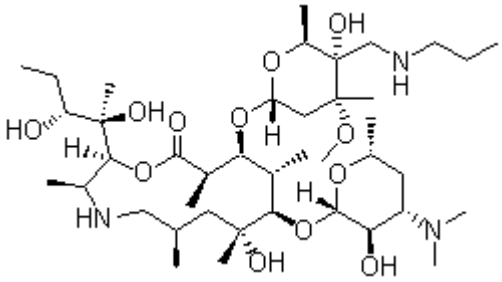

Figure 1: Chemical structure of tulathromycin

The molecular formula for tulathromycin is $C_{41}H_{79}N_3O_{12}$; its molecular weight is 806.23, and the chemical name is (2R, 3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S, 14R)-13-[[2,6-dideoxy-3-C-methly-3-O-methly-4-c[(propylamino)methy]-α-Lribo-hexopyransoyl]oxy]-2-ethyl-3, 4, 10-trihydroxy-3, 5, 8, 10, 12, 14-hexamethyl-11-[[3, 4, 6-trideoxy-3-(diamethylamino)-β-Dxylohexopyranosyl]oxy]-1-oxa-6-azacyclo-pentadecan-15-one (EVANS, 2005). In its raw form, tulathromycin is a white to off-white crystalline powder that is readily soluble in water at pH 8.0. The injectable solution is photostable and can be stored at room temperature for 36 months.

2.3.4.1 Pharmacokinetic properties of tulathromycin

The efficacy of any antimicrobial is determined in part by its pharmacokinetic properties. In swine, tulathromycin is registered for a single intramuscular use at the recommended dose of 2.5 mg/kg body weight for swine of all ages. Approximately 25 minutes after intramuscular application, the mean maximum plasma concentration in pigs weighing 21.0 kg and 31.3 kg was 0.616 µg//ml. One hour after administration, the plasma concentration was 0.58 µg/ml. Generally, lung concentrations are 24.9 to 181 times higher than plasma concentration (EVANS, 2005). The mean concentration in the lung tissue is 2.840 µg/g twelve hours post injection. The maximum plasma concentration In the lungs one hour after application is 3470 ng/ml

(BENCHAOUI et al., 2004). The slow decline in lung drug concentrations is reflected in a lung elimination half-life of 6 days (BENCHAOUI et al., 2004; NOWAKOWSKI et al., 2004). The bioavailability of tulathromycin in swine is approximately 88 % (EVANS, 2005). Tulathromycin is metabolized slowly and the majority of drug is excreted unchanged in faeces and urine. The pre-slaughter withdrawal time for tulathromycin in swine is 33 days.

2.3.4.2 Pharmacodynamic properties of tulathromycin

Although most macrolides are generally considered bacteriostatic, tulathromycin exhibits mixed bacteriostatic and bacteriocidal activity. In comparison to other macrolides, tulathromycin binds to the 50S subunit of bacterial ribosomes and inhibits protein synthesis which leads to inhibition of cell division and cell death (EVANS, 2005). The structure of tulathromycin is thought to render physico-chemical properties that enhance the displacement of magnesium ions that stabilize the bacterial outer lipopolysaccharide layer, thereby disrupting its structural integrity. This disruption facilitates all molecular forms of tulathromycin to pass through the outer layer and subsequently the peptidoglycan cell wall. An additional feature which affects intrinsic antibacterial potency relates to the observed poor affinity of tulathromycin for efflux pumps in bacterial membranes. With a low affinity to the efflux pumps, tulathromycin can accumulate in bacteria, allowing up to four times the normal minimum inhibitory concentration (MIC) thus enhancing antibacterial efficacy (TRAEDER and GROTHUES, 2004). In addition to accumulating in bacteria, tulathromycin accumulates in blood polymorphonuclear leukocytes or neutrophils and alveolar macrophages and is slowly released from these cells (SIEGEL et al., 2004).
This potential to accumulate in bacteria is the main factor inhibiting ribosomal based resistance as seen in other macrolides e.g. tilmicosin. Few studies exist about the antimicrobial resistance of relevant veterinary bacteria towards tulathromycin. NANJIANI et al. (2005) evaluated the susceptibility of various HPS isolates originating from five European countries. The average minimum inhibitory concentration value required of all HPS pathogens was 4 µg/ml.

2.3.5 Prophylaxis and immunity

Strict hygiene practices and effective management are recommended methods for a successful HPS prophylaxis (NEDBALCOVA et al., 2006; RITZMANN and

HEINRITZI, 2005). Also, other pathogens affecting the swine herd should be eliminated and mixing of weaned piglets of varying ages should be minimized. Furthermore, mixing herds from different locations should also be reduced. Any new introduction of pigs to a preexisting herd should be accompanied by sufficiently long isolation and acclimation periods to allow the development of protective immunity from either vaccination or natural exposure (RAPP-GABRIELSON et al., 2006).

Since HPS is an early colonizer, practices such as segregated early weaning are often ineffective. Elimination of the pathogen is best achieved when coupled with parenteral and oral administration of high doses of antibiotics (OLIVEIRA and PIJOAN, 2004). However, elimination of HPS is not always suitable since the introduction of naïve pigs into infected herds may lead to disease with severe economic losses (RAPP-GABRIELSON et al., 2006).

Several authors emphasize the importance of maternal and natural immunity for controlling the disease process particularly because of the septicemic nature of the disease (BLANCO et al., 2008; NIELSEN and DANIELSEN, 1975). Recently, the use of controlled exposure of young pigs to low doses of live virulent HPS strains was assessed as an alternative method to control nursery losses due to HPS (OLIVEIRA et al., 2001b; OLIVEIRA et al., 2004b). This method is based on the hypothesis that early colonization of piglets with the prevalent HPS strain in the presence of maternal immunity reduces the risk of systemic infection after weaning. Exposed pigs showed a reduction in mortality but this method is not suitable for sow herds currently infected with active PRRSV (OLIVEIRA et al., 2001b; OLIVEIRA et al., 2004a).

Outbreaks of Glässer's disease can be controlled by means of commercial vaccine (BAK and RIISING, 2002; SOLANO-AGUILAR et al., 1999) or autogenous bacterins (KIRKWOOD et al., 2001; SMART et al., 1993). They are either chemically inactivated or attenuated vaccines with either serotypes 4 or 5, a strain possessing a "key outer membrane protein", or they are of unstated composition (MACINNES and DESROSIERS, 1999; MARTIN DE LA FUENTE et al., 2009). In Germany, the current HPS vaccines consists of an inactivated serovar 5 formulation for 5 week old piglets and a combination vaccine containing inactivated HPS serovars 4, 5, and *Mycoplasma hyopneumoniae* for pigs 1 week or older (HEINRITZI, 2006). According to the manufacturers, gilts should be vaccinated in two week intervals before introduction into the herd and similarly, sows six to eight weeks and two to three weeks *ante partum*. Piglets can be vaccinated twice either in the first or fifth week of

life with a two week interval. Simultaneous vaccination of sows and their piglets is not recommended (OLIVEIRA, 2005).

Timing of vaccination is very important, and the most effective time to vaccinate depends on the occurrence of clinical signs in the herd, management, and the presence of protective maternal antibodies (OLIVEIRA and PIJOAN, 2002; RITZMANN and HEINRITZI, 2005). If problems are experienced after weaning, vaccination of sows is recommended to boost maternal immunity. But if pigs show clinical signs of disease at a later time during the growing-finishing phase, vaccination of the piglet is more suitable (OLIVEIRA, 2005).

Both types of vaccines usually protect against challenge with a homologous serovar (HOFFMANN and BILKEI, 2002; MCORIST and BOWLES, 2009; MINIATS et al., 1991b; PALZER et al., 2007a; SMART and MINIATS, 1989). But variable results have been reported concerning the lack of cross-protection between different strains and serovars of HPS (BAK and RIISING, 2002; RAPP-GABRIELSON et al., 1997).

Most farms have a stable flora of endemic HPS among their pigs, but this flora usually varies from farm to farm in its range of specific strains of HPS due to the heterogeneity of strains (BLACKALL et al., 1996; KIELSTEIN and RAPP-GABRIELSON, 1992; OLIVEIRA et al., 2003b). Therefore, as swine farm systems enlarge, the possibility of a single strain commercial or autogenous bacterins covering all strains of etiological significance on such a farm becomes more difficult (NEDBALCOVA et al., 2006; OLIVEIRA and PIJOAN, 2004). Thus, control of the disease should not be limited to vaccine programs but also changes in management and hygiene practices at all stages of swine production.

3 Material and Methods

3.1 Study aim

The aim of this study was to assess the metaphylactic efficacy of tulathromycin (Draxxin®, Pfizer, GmbH, Berlin) administered seven and four days before experimental infection in weaned piglets that were subsequently challenged intratracheally with HPS.

3.2 Study animals

Thirty-six castrated-male-crossbred piglets (Landrace x Large White x Pietrain), three weeks of age, were obtained from a commercial farrowing farm in Lower Austria. Piglets were randomly selected from a total 18 litters. The sow herd had no previous history of porcine reproductive and respiratory syndrome virus (PRRSV) or HPS. Serum samples of 18 sow were taken from the 18 litters from which the study animals originated and samples were submitted to Clinic for Swine Department for Farm Animals and Veterinary Public Health, Veterinary University Vienna, Austria) for testing by HPS ELISA (INGEZIMA® Haemophilus 11.HPS.K 1, Ingenasa, Madrid, Spain). This was done to facilitate the selection of serologically negative piglets for the study. As part of the routine piglet processing after birth, piglets were ear tagged, the canine teeth were shortened and an iron supplement was administered. In addition, piglets received Cobactan® 2.5 % (Intervet, Unterschleissheim Germany) for three days, and they were vaccinated against *Mycoplasama hyopneumoniae* (Hyoresp®, Merial, Paris, France) and PCV2 (Porcillis® PCV, Intervet, Boxmeer, The Netherlands) in their 1st and 3rd week and 2nd week of life respectively.

The piglets were transported from the farm to the study facility directly after weaning. Upon arrival, the piglets were unloaded into the main room of the facility (R2, Figure 3). Piglets were housed in their respective rooms after assignment to their treatment groups and allowed a 17 day acclimatization period before the first antibiotic treatment (Figure 2). A nursery diet (Garant, Poechlarn, Austria) was fed ad libitum in addition to water throughout the entire study.

Animal care and euthanasia during this study were approved and conducted in accordance to the current Animal Experiments Act and in accordance with the Good

Scientific Praxis guidelines of the Ethics Commimission of the Veterinary University Vienna, Austria. This study is registered by the Austrian Federal Ministry for Science and Research, Department for Genetics and Animal Experiments/Testing under the file number GZ 68.205/0030-II/10b/2009.

3.3 Study design

The study was designed to evaluate the efficacy of tulathromycin by treating weaned piglets according to two different treatment protocols. Piglets received either a tulathromycin treatment seven or four days before an infection with a virulent HPS serovar. The study was conducted on approximately 32 consecutive days from the beginning of July to mid August (Figure 2).

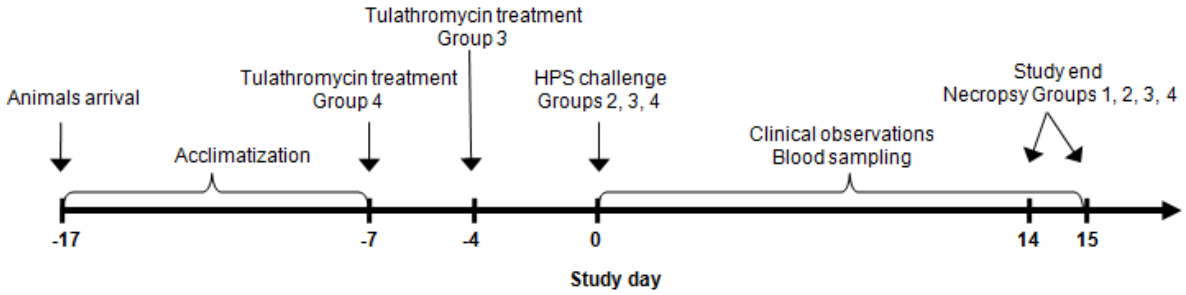

Figure 2: Experimental timeline of the study indicating arrival, treatment, challenge and necropsy

Animals arrived at the Biosafety Level 3 Facility, Clinic for Swine, Veterinary University Vienna Austria 17 days prior to the challenge day (Day -17), and all animals were immediately weighed with an electronic scale that was accurate to 0.01 kg (PS 60 SST, Bosche, Damme). The weights of each animal were recorded in an Excel sheet in descending order starting with the heaviest weight (Microsoft Excel; Microsoft Corporation). Based on this information, piglets were assigned to four different treatment groups (Table 1) so that a uniform weight was represented in each group: control (Group 1), nontreated-challenged (Group 2), tulathromycin 4 day pretreated-challenged (Group 3) and tulathromycin 7 day pretreated-challenged (Group 4). Four unique sets of ear tags with different numbers and colors were allocated to the four different treatment groups and the piglets were ear tagged accordingly.

Table 1: Treatment groups in the study assessing the efficacy of tulathromycin against HPS

Group		No. of pigs	Treatment administered	Challenge (Day 0)
1	Control	9	none	Phosphate-buffered saline (PBS)
2	Nontreated-challenged	9	none	5 x 10^8 CFU HPS
3	Tulathromycin 4 day pretreated-challenged	9	2.5 mg/kg BW tulathromycin 4 days prior to challenge	5 x 10^8 CFU HPS
4	Tulathromycin 7 day pretreated-challenged	9	2.5 mg/kg BW tulathromycin 7 days prior to challenge	5 x 10^8 CFU HPS

Pigs from Groups 4 and 3 received an intramuscular injection with tulathromycin on Days -7 and -4 respectively. On the challenge day, Day 0, pigs from Groups 2 through 4 were inoculated with HPS serovar 5. To maintain blinding, personnel who administered treatments and gave the challenge dose were not involved with animal removals, recording clinical observations, or pathological assessments. Pigs from the control Group 1 received a phosphate buffered solution (PBS) instead. Clinical examination of all pigs occurred once daily on Days -17, -7, -4, -1 and every day after challenge until the termination of the study (Days 14 and 15). All animals were weighed on Days -17, 0 at the end of the study (Days 14 and 15) or at removal. Serum samples were collected from all animals on Days -17, -7,-1, 1, 3, 5, 7, 8, 9, 11, 14, 15 and at removal for testing blood work and by APP ELISA.

Animals that survived to study termination (Days 14 and 15) or were euthanized due to humane reasons were subjected to a necropsy. In addition to gross pathology, and microbiological confirmation by culture of all serosal surfaces, histopathological examinations of selected organs and tissues were also performed. Furthermore, serosal swabs, joint capsule, synovial and cerebrospinal fluid were investigated by PCR.

3.3.1 Housing

The study was conducted in the Biosafety Level 3 Facility (Figure 3) of the Veterinary University Vienna Austria, Clinic for Swine. The facility was divided into two main rooms which were completely separated from each other through a 2 m^2 aisle. Each main room was further subdivided into two main pen areas used for animal housing.

Pens were completely separated from each other by a standard concrete wall. An area designated for necropsy examination was located outside the pen areas.

For simplification purposes, the main room to the left of the aisle entrance was designated Room 1 (R1) and Room 2 (R2) was the room directly opposite R1. Pens in R1 were approximately 20 m^2 and 15 m^2 in size. The layout of the second main room (R2) was identical to the first main room. Each pen had a solid concrete floor, two nipple waterers and two feeding troughs. Also, each pen had its own slurry/manure pit, located directly under the pens, which was automatically emptied on a regular basis after undergoing a thermal and biological disinfection. A constant temperature range from 23-28 °C was maintained in both main rooms and pens.

In this experiment, one of the pens in R1 was used to house pigs from Group 1. The remaining three Groups (2, 3 and 4) were housed in the R2 whereby Group 3 was housed in the larger pen and Group 4 in the smaller pen. Five pigs from Group 2 were housed together with Group 3 and four were placed in the smaller pen with Group 4.

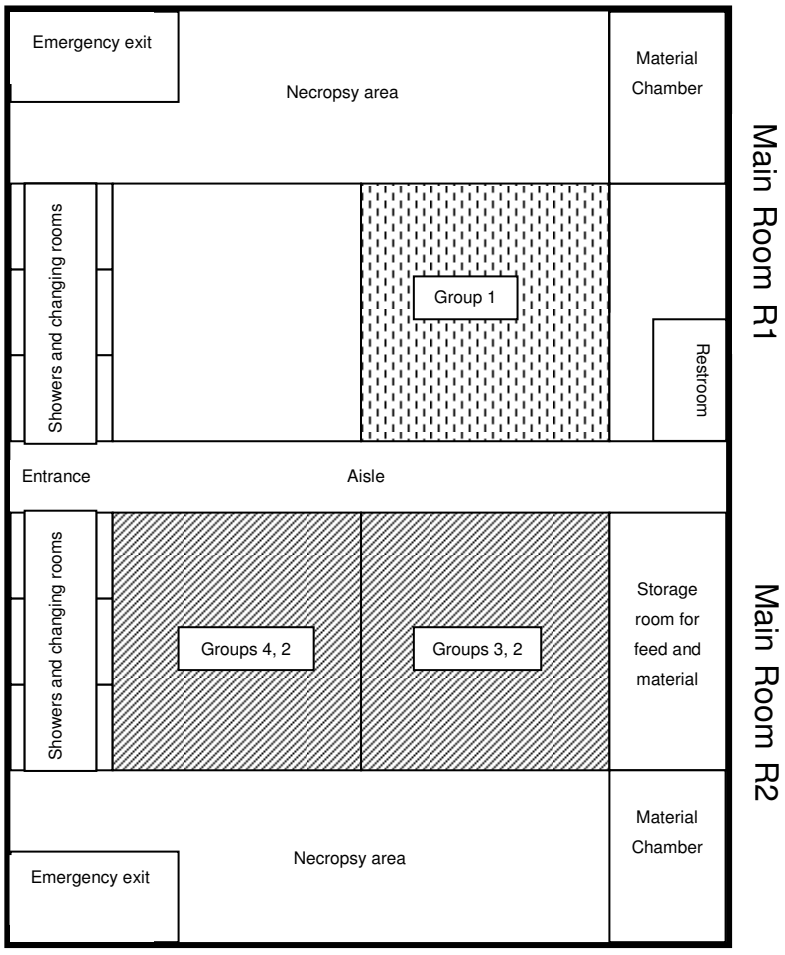

Figure 3: Schematic diagram of the housing facility (Biosafety Level 3) for the pigs during the challenge

3.3.2 Biosecurity

Strict delivery protocols were in place when the animals arrived and the truck driver did not enter the facility or assist in offloading the animals into the facility. Personnel entering both of main rooms, pens and material chambers had to go through a double-door zone with sealed door penetrations. Also, all persons were required to shower and change clothes before and after animal contact.

Adjacent to each main room was a separate chamber for feed and material delivery. All material used during the study such as clothing, boots, syringes, and feed were brought into the rooms via this chamber. Care was taken to ensure that all materials met strict hygienic conditions. Materials were stored in a storage room (Figure 3) that was only accessible by personnel who were involved with the study and who did not have any animal contact prior to entering this room. Each main room had its own designated material and feed in order to prevent cross-contamination.

Both main rooms and pens were mechanically ventilated with air flowing in and out of the facility after passing through high efficiency particulate air filters (HEPA). Ventilation systems for both main rooms and pens were operated and managed independently of each other. Water for the facility came from the local municipal water supply. The water supply for each main room and pen were sourced from separate pipe systems to ensure that no cross-contamination occurred. Waste water and slurry/manure pit sewage from each main room and pen was handled separately from each other.

3.3.3 Administration of tulathromycin

Piglets from Groups 3 and 4 received the label dosage of 2.5 mg/ kg BW tulathromycin on Days -4 and -7 respectively. Animals were manually restrained and administered the tulathromycin as a single intramuscular injection in the neck. The injection was performed using a sterile 21-gauge needle (Braun, Melsungen) and a sterile disposable 2 ml syringe (Heiland, Gallin).

3.3.4 Preparation of challenge inoculum

The challenge strain used in this study was a HPS serovar 5 isolate. Preparation and antimicrobial sensitivity testing of the challenge strain was done by the Institute for Bacteriology, Mycology and Hygiene, Veterinary University Vienna, Austria. The strain was streaked onto a chocolate agar that consisted of 10 % sheep blood agar

(Fiebrig Animalblood Products, Taunus) supplemented with 0.008 % nicotinamide adenine dinucleotide (NADH) (Sigma-Aldrich). The agar plate was incubated for 3 to 4 days at 37 °C in an atmosphere of 7 % carbon dioxide. Colonies were harvested in phosphate-buffered saline (PBS) and aliquots from this medium were frozen at -80 °C in sterile 2 ml tubes (Eppendorf, Hamburg). The amount of CFUs in one aliquote was determined using serial dilution and plating. One day prior to challenge, Day -1, one aliquot was selected, thawed at 37 °C and the suspension was adjusted until the colony count was approximately 1x 10^8 CFU per ml. On the challenge day, a suspension of 5 ml (5x 10^8 CFU) was filled in sterile 20 ml syringes (Heiland, Gallin) for intratracheal inoculation.

3.3.5 Administration of inoculum

On Day 0,5 ml of the HPS serovar 5 was administered intratracheally to all pigs from treatment Groups 2 through 4. Pigs from treatment Group 1 received 5 ml PBS instead. To eliminate bias, personnel who administered the challenged dose were not involved in recording clinical observations. Prior to inoculation, the animals were sedated using a combination of 1.2 mg/kg BW azaperon (Stresnil®, Janssen GmbH, Neuss Germany) and 10 mg/kg BW ketamin (Narketan 10 %®, Vétoquinol Paris France). Animals were manually restrained and administered the solution intramuscular in the neck with a sterile 25-gauge needle (BD Microlance® 3, New Jersey; USA). After the animals lost consciousness, they were positioned in sternal-abdominal recumbency and the upper jaw was fixed with a sterile gauze. A sterile laryngoscope (Heine Optotechnik, Herrsching) was inserted into the mouth, the larynx exposed and the epiglottis lid was fixed with the end of the laryngoscope. An open end suction catheter (Medinorm, Speisen-Elversberg) was inserted between the vocal cords and into the trachea for a distance of 9 cm. The 5 ml inoculum was applied directly through the catheter and 10 ml of air was applied afterwards to ensure complete emptying of the catheter tube. After application, the tube and laryngoscope were quickly removed. The animals were placed in an area designated as the infirmary until they regained consciousness. An assisting veterinarian attended to the animals until they regained consciousness. Once the animals fully regained consciousness as determined by the assisting veterinarian, they were promptly returned to their pens.

3.3.6 Euthansia

Animals were euthanized in order to facilitate necropsy and histopathological examinations. Euthanasia was performed on Days 14 and 15 or when the animals were removed due to humane reasons. Animals were sedated using a combination of 2 mg/kg BW azaperon (Stresnil®, Janssen GmbH, Neuss) and 15-30 mg kg BW ketamin (Narketan 10 %®, Vétoquinol Paris France). Pigs were manually restrained in dorsal recumbency and the solution was administered intramuscularly in the neck with a 25-gauge sterile needle. Afterwards, animals were euthanised with T61® (20 mg/kg BW embutramid, 5000 mg/kg BW mebenzoniumjodid, 500 mg/kg BW tetracainhydrochlorid) (Intervet, Boxmeer, The Netherlands) via intracardiac injection. All animals were weighed following euthanasia.

3.4 Clinical examinations

After challenge, pigs were clinically examined at least once daily until study termination. Prior to challenge, pigs were clinically examined upon arrival (Day -17), at each tulathromycin administration (Days -7 and -4) and on Day -1. 16 parameters (Table 1) were selected for clinical examination such as body condition, hair coat, behavior and gait. Parameters were evaluated and assigned a qualitative score with a numeric grading scale from 0 through 3. Pigs displaying no clinical symptoms received a score of 0. Rectal body temperatures (Vet-Life, Microlife Ag, Switzerland), changes in the joint fluid of the tarsal and carpal joints, conjunctiva, and sclera of all pigs were measured once daily on Days -17, -7, -4, -1 and every second day thereafter up to Day 11. On Days 12 and 13, the following parameters were not assessed: rectal temperature, sclera, conjunctiva, carpal and tarsal joints. A score of 2 was assigned by the parameter CNS (central nervous system) when signs such as head tilt, opisthotonus, ataxia, lying on side or paddling were displayed. Also, the parameters backline, abdomen, coughing and teeth grinding received a double score if clinical signs were not normal. If a joint or limb area was completely swollen during palpation to assess joint fluid in both the tarsal and carpal joint, a score of 3 was assigned for that tarsal or carpal joint. A total score for clinical signs was calculated each day for each pig based on the sum of the scores for all 16 parameters. Animals with a clinical score < 4 were classified as clinically healthy, pigs with a score of 4-5 were mildly ill, 5-6 were moderately ill and a score above 6 indicates severe illness.

For humane reasons, pigs were euthanized if they exhibited severe central impairment (lying on the side, paddling), immobility or if they were found moribund.

Table 2: Scoring system for clinical signs of pigs challenge or not challenged intratracheally with HPS

Parameter	Score			
	0	1	2	3
Body condition	very good	good	moderate	cachectic
Snout color	pink	pallor	hyperaemia	cyanosis
Gait	moves freely	slight reluctance to move or rise	difficulty in rising or moving, ataxie	recumbent, unable to rise
Dyspnoea	absent	slight dyspnea	moderate dyspnoe	labored abdominal breathing, open mouth breathing
Tarsal joint filling	no swelling	slight swelling	moderate swelling	severe swelling
Carpal joint filling	no swelling	slight swelling	moderate swelling	severe swelling
Conjunctiva appearance	pink	slight hypermia	moderate hypermia	severe hypermia
Behaviour	attentive	depression, apathy	stupor	coma, moribund
Sclera apperance	white, scleral capillaries fine	scleral capillaries blurry	scleral capillaries excessively filled	
Hair coat	short, smooth, shinny	rough long		
Backline	straight		kypohotic	
Abdominal appearance	flat		swollen	
Lameness	absent		present	
Coughing	absent		present	
Pain (teeth grinding)	absent		present	
CNS sypmtoms	absent		present	

3.5 Necropsy

Pigs euthanized for humane reasons prior to Days 14 and 15 and all remaining pigs that were euthanized at study termination (Days 14 and 15) were subjected to a routine necropsy examination. Particular attention was made to the pleural, pericardial, and peritoneal cavities and surfaces, the basal meninges, the lungs, and the carpal and tarsal joints when examining each pig. To eliminate bias, the pathologist performing the necropsies was blinded to the treatment groups.

When an inflammation in any one of the body cavities (pleuritis, pericarditis, peritonitis) and its serosal membrane was observed, the presence and of lesions were recorded (Table 3). A qualitative score ranging from 0 to 3 was assigned for the presence and the extension of each individual inflammation and a total score from these two parameters was calculated to assign a single score for each of the three inflammations in each pig. A total inflammation score for each pig was summarized from the final score of each of the three inflammations. Animals with a total inflammation score of 0 had no inflammations. Pigs with scores of 1or 2 had a mild inflammation, 3 and 4 (moderate inflammation) and 5, 6 (severe inflammation).

Table 3: Assessment table for inflammation of the pleural, pericardial and peritoneal cavities

Parameter	Pleuritis	Pericarditis	Peritonitis
Presence of inflammation			
0= none			
1= fibrin strands			
3= severe adhesion, inseparable			
Extension of inflammation			
0= 0%			
1= <30%			
2= >30%			
3= diffuse			
Total score			

Assessment of the lung was done according to a modified method as stated in CHRISTENSEN et al. (1999) (Table 4). To assign a lung score, the proportion of the area of consolidated lung tissue each of the seven lung lobes was assessed and recorded in percent. For each lung lobe, this percent of affected lung tissue was multiplied by the estimated lung capacity of that lobe (10 % each for right and left cranial, right and left middle and accessory lobes; 25 % each for the right and left caudal lobes) and the results for the seven lobes were added to obtain the total percent of lung affected. Each of the seven lung lobes were inspected visually and palpated to determine the amount of consolidation or other lesions in each of the lobes including the appearance of each lobe (Table 4).

The remaining organs and the brain were assessed and assigned a qualitative score of either 0 (unchanged) or 1 (changed). A score of 0 was assigned if the brain was homogenous light pink with the surface of the gyri clearly defined and not shrunken or edematous. If a score of 1 was assigned, a detail description of the alteration was made.

Table 4: Lung lesion score sheet for necropsy examination

Parameter	Left cranial (10% of total lung)	Left middle (10% of total lung)	Left caudal (25% of total lung)	Accessory (10% of total lung)	Right cranial (10% of total lung)	Right middle (10% of total lung)	Right caudal (25% of total lung)
% affected							
Consistency							
0= soft elastic							
1= firm							
Appearance							
0= homogenous pink							
1= dark red, mottled parenchyma							

3.6 Sample collection and Diagnostic

3.6.1 Blood sampling

Blood samples were collected from all animals on Days -17, -7 and -1 through 15 in regular two day intervals. Samples were also taken before an animal was euthanized if this occurred on a day when blood was not collected. Animals were manually restrained in dorsal recumbency and blood was obtained by puncturing the right jugular vein using either a sterile 18- or 19-gauge needle (Sterican®, Braun, Melsingen) depending on the animal's weight. Blood was collected in 7.5 and 10 ml serum vials (Primavette® V Serum, Kabe Labortechnik, Sarstedt). Blood samples were centrifuged at 3000g for 10 minutes at 20 °C. Afterwards, the sera were collected in sterile 2.0 ml safe-lock tubes (Eppendorf, Hamburg) and stored at - 20 °C. Samples were submitted to the Central Diagnostic Unit of the Veterinary University Vienna Austria for blood work. In addition, blood samples were analysed for the presence of *Actinobacillus pleuropneumoniae* by the Institute for Bacteriology, Mycology and Hygiene, Veterinary University Vienna Austria.

3.6.2 Synovial and cerebrospinal fluid collection

Following euthanasia, synovial and cerebralspinal fluid were obtained from all animals for identification of HPS by PCR. Joint fluid was collected from both tarsal joints and any other swollen joints. Prior to punctuation, designated areas were shaved, washed with antiseptic soap (Jodosept®, Vetoquinol, Paris France) and disinfected with 70 % alcohol. The joint was punctured with a sterile 19-gauge needle and the joint fluid was collected in a sterile 2 ml syringe. Cerebrospinal fluid was obtained either from the occipital or lumbosacral region using a sterile 14-gauge needle (Bovivet®, Kruuse, Langeskov Denmark) attached to a sterile 2 ml syringe.
The appearance of the synovia and the cerebrospinal fluid from each pig were evaluated and assigned a qualitative score from 0 to 4 depending on the severity of changes observed whereby 0 represents a physiological clear synovial or cerebrospinal fluid; 1 = clear, light yellow; 2 = cloudy, light yellow; 3 = cloudy, amber 4 =cloudy, flocculent, light yellow or amber (Table 5). After assessment, the synovial or the cerebrospinal fluid were pipetted in sterile safe-lock tubes and stored at -80 °C before being submitted to the Institute for Bacteriology, Mycology and Hygiene, Veterinary University Vienna Austria for PCR diagnostic.

Table 5: Scoring system for the assessment of the synovial and cerebrospinal fluid

Score
0 = normal
1 = clear, light yellow
2 = cloudy, light yellow
3 = cloudy, amber
4 = cloudy,flocculent, light yellow or amber

3.6.3 Collection and processing of serosal swabs

All the serosal surfaces of each pig with the exception of the joints were swabbed directly after the animal was opened for necropsy. Cotton swabs (Heinz Herenz, Hamburg) were used to take collective samples from the peritoneum, pleura, pericardium, and the meninges whereby the meninges were sampled with two

separate swabs. Following collection, swabs were stored at -80 °C before being submitted to the Institute for Bacteriology, Mycology and Hygiene, Veterinary University, Vienna Austria for testing of HPS by PCR. Samples were also tested by PCR for the presence of *Mycoplasma hyorhinis*.

3.6.4 Bacteriological culture

For bacterial isolation, sterile cotton swabs in Aimes transport medium without charcoal (Medi Swab®, Heinz Herenz, Hamburg) were used to take samples from 4 sites for bacterial culture. Swabs were taken aseptically from the peritoneum, pleura, pleural cavity and pericardium directly after the pig was opened for necropsy. Samples were submitted to the Institute for Bacteriology, Mycology and Hygiene, Veterinary University Vienna Austria and streaked within 30 minutes of collection onto the same chocolate agar used for preparing the challenge inoculum. The cultures were incubated at 37 °C for 2-3 days in an atmosphere containing 7 % carbon dioxide. Colonies from this agar plate were selected with a sterile loop and inoculated onto a second agar plate, identical to the first culture, to facilitate maximum growth of HPS colonies. This subculture was incubated for another 3-4 days and the presence and appearances of HPS colonies were assessed. A sample was positive when at least one typical HPS colony was observed.

3.6.5 Histopathological examination

Postmortem samples of the lung, heart, liver, kidney, spleen, duodenum, colon, inguinal lymph node, left tarsal joint capsule and brain were collected in 7 % formalin. These samples were submitted to the Institute for Pathology and Forensic Veterinary Medicine, Veterinary University Vienna, Austria for histopathological examination. Samples were fixed in formalin for 24 hours before being placed in commercial embedding cassettes and then processed for an additional 24 hours in the Tissue-Tek VIP® machine (Sakura Finetek, Netherlands). The samples were subsequently embedded in paraffin wax (Tissue-Tek TEC®, Sakura Finetek, Netherlands). Paraffin blocks were sectioned at 1-2 µm and stained with haematoxlylin and eosin (HE). Evaluation of these slides was performed under a light microscope by a pathologist blinded to the treatment group. A qualitative score was assigned for the outcomes from the examination. If an inflammation was not present, a score of 0 was assigned.

Scores ranged from 1 to 3 depending on the severity of lesions with 1 (mild), 2 (moderate) and 3 (severe).

3.6.6 Polymerase chain reaction (PCR) analysis for HPS

Samples (joint capsule, joint and cerebrospinal fluid, sera and serosal swabs) for PCR diagnostic were submitted to the Institute for Bacteriology, Mycology and Hygiene, Vetmeduni Vienna Austria. DNA was extracted from the samples using the GenElute® Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) according to the manufacture's user guide. Approximately 250 µl undiluted DNA was obtained after extraction and 5 µl of DNA was used for the PCR analysis. Reagents for the PCR were obtained from the RedTaq® ReadyMix PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich Biotechnology). Identification of HPS was done according to the PCR method described by ANGEN et al. (2007) and primers for the PCR were those mentioned by Angen et al. The reaction was run on a Eppendorf Matercycler Personal® (Eppendorf, Hamburg) machine with the following steps: 1 cycle at 94 °C for 3 minutes, 35 cycles of denaturation at 94 °C for 1minute, annealing at 56 °C for 45 seconds, and extension at 72 °C for 1 minute. The PCR product was separated on a 1.5 % agarose gel (Roti®garose, Carl Roth Co KG, Karlsruhe). Following this step, the PCR product was visualized using ethidium bromide and UV fluorescence. Results from the PCR test were assigned a qualitative score whereby a positive sample was assigned the score 1 and a negative sample a score of 0.

3.7 Statistics

The statistical analysis was performed using the statistic program SPSS 17.0 for Windows XP. Descriptive statistics were done using either the SPSS 17.0 or Microsoft Office Excel 2007. An analysis of the significance was done by comparing Groups 1 and 2, Group 2 with 3 and 4, and finally Group 3 with Group 4. The 1 % level of significance using the Bonferroni correlation to eliminate error was used to assess statistical differences for all tests with the exception of the following parameters: association of detection frequencies of HPS and the results from the clinical examinations, gross and histopathological lesions. A 5 % level of significance was used for these parameters instead. With the exception of mortalitiy rates, all group comparisons were analyzed using the Mann-Whitney U test. The Kaplan-Meier

test was used to calculate overall mortalities between the individual groups. The comparison of the HPS detection methods was performed using a Kappa test.

Each animal was considered a statistical unit. Animal days were calculated for each pig and consisted of the number of days each pig contributed to the study (from Day -17 to euthanasia). Average daily weight gain was defined as body weight gain (from Day -17 to Day 0 and Day 0 to Days 14/15) divided by animal-days. To analyze clinical scores, a total clinical score was created that consisted of the sum of each individual clinical score for each pig. The median was then calculated from this score to create a group score for the individual clinical symptom. Similarly, the average was determined for rectal temperatures and calculated for each study group.

4 Results

4.1 Screening, animal numbers and data collection

Screening for HPS antibodies was performed by HPS ELISA using serum from 18 sows to facilitate the selection of piglets without HPS antibodies. According to the manufacture, a ratio value > 0.9 is positive, 0.6-0.9 suspect and <0.6 negative. Sixteen samples were negative (0.27 ± 0.10) and 2 samples were suspect (0.73; 0.66) after serological analysis. Thus piglets from sixteen litters were used for the study. To rule out possible concurrent infection, collective serosal swabs were obtained from these piglets during the study and tested for *Mycoplasma hyorhinis* by PCR. All swab samples were negative for *Mycoplasma hyorhinis*. Furthermore, serums from these 36 piglets were also negative for *Actinobacillus pleuropneumoniae* after ELISA testing. To ensure that the HPS strain used in this study was sensitive for tulathromycin, an antimicrobial susceptibility test was performed. Test results showed that HPS was susceptible to tulathromycin and the minimum inhibitory concentration value (MIC) was 0.5 mg/l.

Tissue samples collected from all 36 animals after necropsy were histologically evaluated either at removal (death or euthanasia) or at study termination. The lungs of 1 pig from Groups 2, 3 and 2 pigs from Group 4 were not examined during necropsy. Data from these animals were excluded from all analysis. A total of 36 swabs from the serosal surfaces were submitted for bacterial culture and HPS PCR. Brain swabs from all 36 pigs were also submitted for HPS PCR. Cerebrospinal fluids from 33 animals were collected for PCR analysis. The cerebrospinal fluids of 4 animals from Group 1 and one pig from Groups 2, 3 and 4 were not macroscopically assessed. Data pertaining to the results from the macroscopic assessment of the liquor from these animals including the PCR results were excluded from statistical analysis where necessary. Joint capsules and synovial fluids from the left tarsal joint were used for HPS PCR analysis. Synovial fluid was not obtained from one pig of each tulathromycin group. Furthermore, joint capsules of two pigs from Group 2 were also not obtained. Data pertaining to the synovial fluid and the joint capsule from these animals were excluded from all statistical analysis.

4.2 Clinical results

4.2.1 Mortalities

A total of four pigs from each challenged group (44.4% of study animals) were removed (death and euthanasia) within 10 days post infection. With the exception of two pigs found dead in their pens, all of the animals which were removed were found in lateral recumbency and unable to rise. The distribution of the pig deaths per study group is shown in Figure 4. None of the control animals were euthanized or died before the end of the study on Day 15. Four pigs from Groups 2 and 3 were euthanized between Days 2 and 4. One pig from Group 4 was euthanized during this time. The remaining 3 pigs from Group 4 were euthanized on Days 6 and 10, with one pig found dead on Day 6. There was no significant difference in the time of death among the three challenged groups (P=0.15).

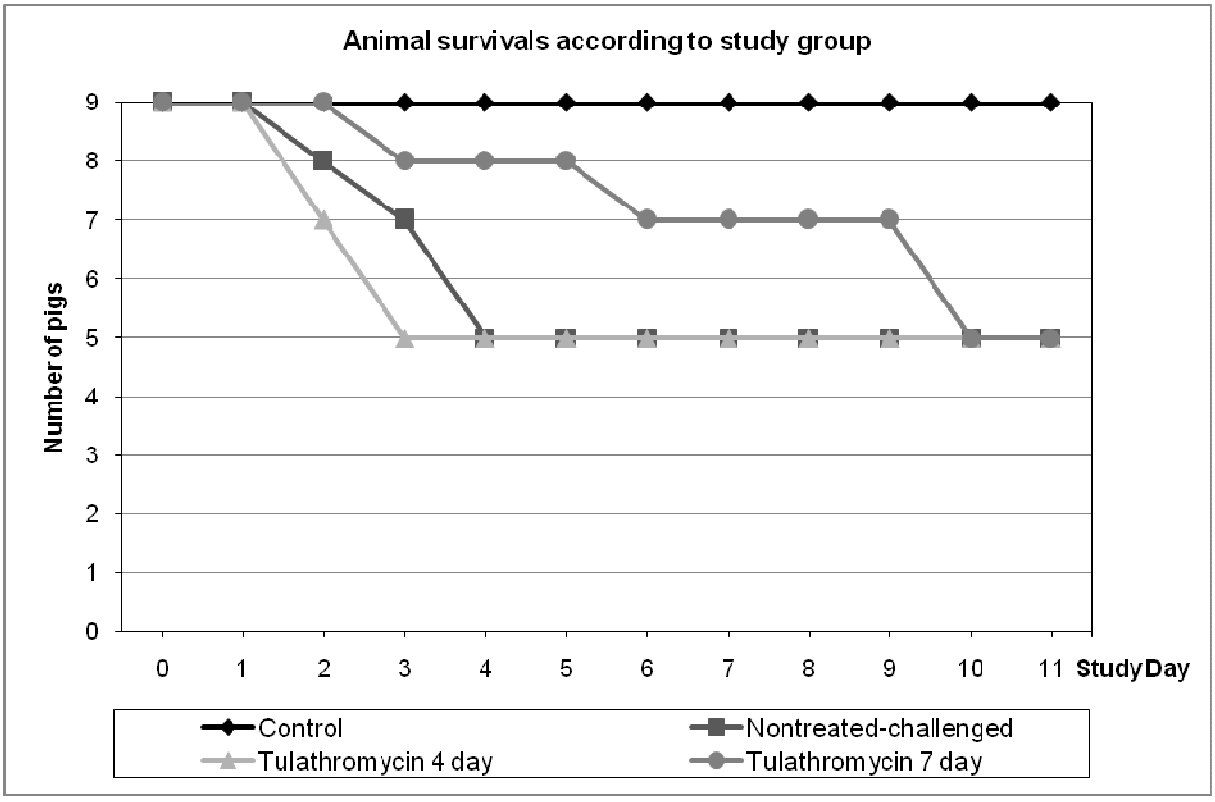

Figure 4: Distribution of pig survivals by study group before study termination

4.2.2 Clinical examinations

Examinations for sixteen clinical parameters were performed at two day intervals from study day -17 to study termination on Days 14/15. The median total daily clinical scores of all four study groups over the course of the study are displayed in Figure 5. Pigs with a total daily score up to 4 were classified as clinically healthy and a score of 4-5 indicated moderate illness. Severe illness was present if the total daily clinical score recorded was from 5 to 8. Based on clinical scores throughout the entire study, pigs from the control group did not display any signs of clinical illness, and the highest clinical score of 4 was recorded on Day -4 for this group. Clinical scores indicating severe illness were also seen on Day -4 for pigs from the tulathromycin 7 day group. Following this day, the total daily clinical score for this group decreased below 4 until study day 5. Clinical signs of mild to moderate sickness were seen on Days 5, 9, 11 for these pigs. Prior to challenge, scores under 4 were recorded for pigs from Groups 2 and 3. After challenge on Day 0, scores indicating severe sickness were seen on several days for pigs from the challenged groups. On Day 3, pigs from the nontreated-challenged group had a clinical score indicating severe illness. Following this peak, the total daily clinical score decreased to 4 until study termination on Days14/15. At study termination, scores indicating mild to moderate sickness were displayed for pigs from Group 2. The clinical scores of pigs from the Group 3 group were below 4 until study day 9 after challenged. On Day 9, scores indicating mild illness were observed and then the total score decreased to 4 until study termination on Days 14/15. After comparing the clinical scores of all study groups, significant differences for the total daily clinical score on any study day among the study groups could not be established ($P>0.01$).

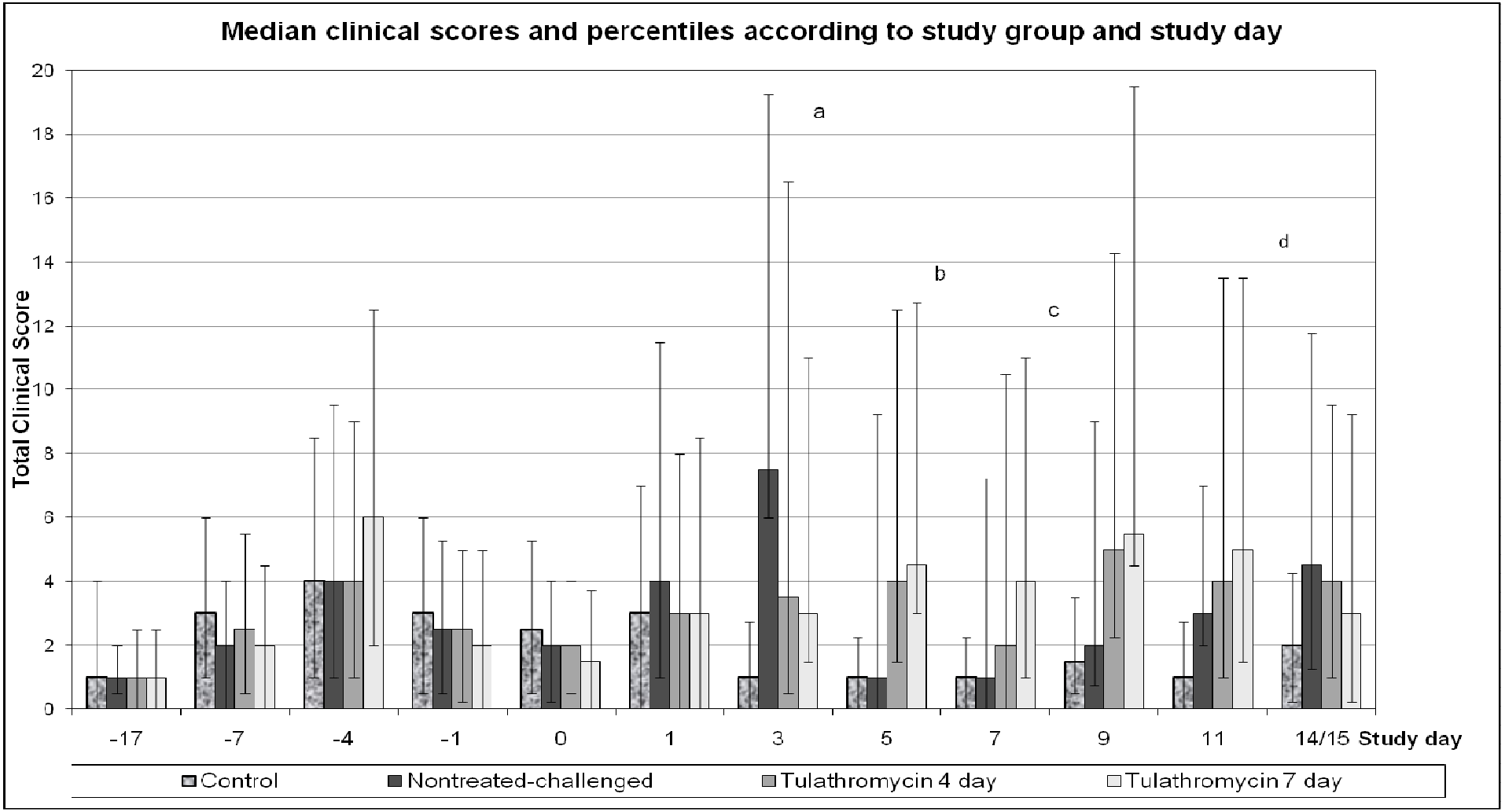

Figure 5: Median clinical scores and percentiles according to the study group

a: 2 pigs from Group 2, 4 pigs from Group 3 and 1 pig from Group 4 were removed from the study; **b**: 2 pigs from Group 2 were removed; **c**:1 pig from Group 4 was removed; **d**: 2 pigs from Group 4 were removed

4.2.3 Average daily gain (ADG)

Animals from all study groups were weighed on Days -17, 0, 14/15 or when a pig died prior to these times. The ADGs including their standard deviations of all pigs which survived to study termination (Days 14/15) are shown in Figure 6. A significant difference was not seen for the ADGs of pigs during Days -17 and 0. The average weight gain of pigs from Group 1 (633.7 g/day) was significantly higher than pigs from the challenged groups between Days 0 to 14/15 (P=0.01). Among the challenged groups, pigs from Group 2, 3 and 4 had an ADG of 371.3, 478.0 and 287.3 g/day during the study days 0 and 14/15 respectively. A significant difference was not seen among the ADGs of the challenged pigs during Days 0 and 14/15 (P>0.01).

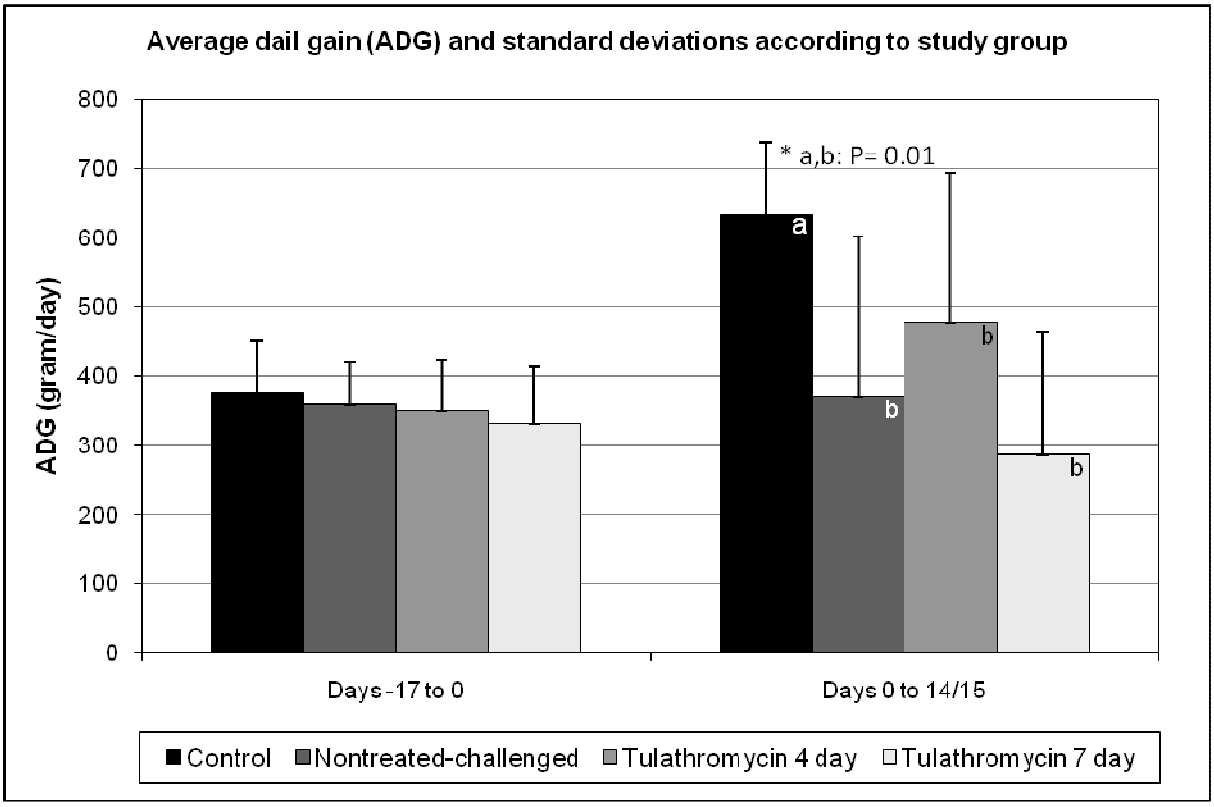

Figure 6: Average daily gain (g/day) and standard deviations according to study group

4.2.4 Rectal temperatures

The average rectal temperatures for all study groups during the entire study are depicted in Figure 7. Rectal temperatures were measured on twelve days over the course of the study. At arrival, temperatures of all pigs were between 39.0 °C - 39.3 °C. The rectal temperatures of all study groups on Day -7 were between 39.5-40.5 °C and remained within this range until Day -1. On Day -1, temperatures of pigs from all groups decreased to a range of 39.5 °C -40.1 °C. After challenge on Day 0, temperatures rose to 40.2 °C - 41.1 °C in all study groups with the exception of the control group. Pigs from the control group had an average temperature of 39.7 °C on this day. On Day 3, the temperatures decreased in all groups including temperatures of pigs from the control group. With the exception of pigs from Group 4, the rectal temperatures of pigs from all groups rose to 40.3 °C on Day 5. The temperatures of pigs from the challenged groups continually increased up to Day 7. The rectal temperatures of pigs from the control group rose to 39.6 °C on Day 7 and remained within this range until Day 11. At study termination, the average rectal temperature of pigs from the control group was 39.5 °C. Rectal temperatures of pigs from the nontreated-challenged group decreased to 39.5 °C on Day 9 and increased 1 °C for the remaining two days. On Day 7, the rectal temperature of pigs from Groups 4 steadily decreased until study end on Days 14/15. The rectal temperature of pigs from Group 3 rose to 40.0 °C on Day 9, decreased on Day 11 and rose once again on Day 14/15. The highest temperature from pigs of all study groups before challenge was seen on Days -7 and -4. The highest temperatures of pigs from all study groups after challenge were seen on Day 1. On Day 7, the second highest temperatures for Groups 2 and 4 were observed. A significant difference in the rectal temperatures among all challenged groups was not observed ($P>0.01$). Also, significant difference between the control group and the challenged groups was only was not observed ($P>0.01$).

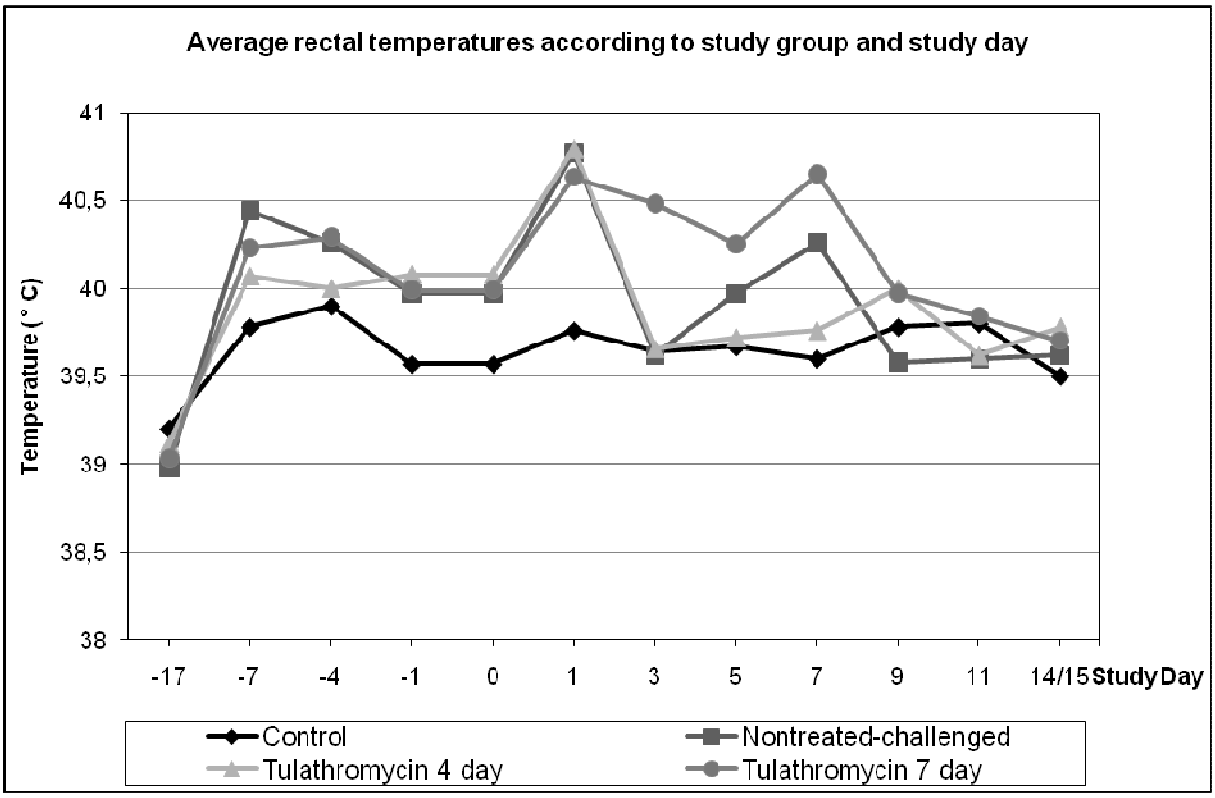

Figure 7: Average rectal temperatures according to study group and study day

4.2.5 Blood examination (Leukocyte population)

A total of 180 blood samples were collected from pigs of all four study groups on 5 days during the study (Figure 8). The total number of leukocytes of the Group 1 pigs at the first blood collection on Day -1 was 17.4 g/l. A continual decrease in the total number of leukocytes was observed for the remaining blood collection days until Days 14/15. The leukocyte population of pigs from the control group was approximately 13.5 g/l at study termination. A significant difference in the average leukocyte cell population of Group 1 pigs for all 5 days was not observed. A significant difference between the leukocytes population of the control group and the challenged groups was not observed (P=0.012).

The average leukocyte population of pigs from all challenged groups underwent a nearly identical change over the course of the study (Figure 8). A total leukocyte population between 19-22.6 g/l was recorded on Day -1. Afterwards, the leukocytes population increased to a range of 25.3 - 27.1 g/l on Day 1. Following this increase, the leukocyte population of all challenged pigs decreased to a range of 16.41 - 23.3 g/l up to Day 3. Afterwards, the leukocyte population of all challenged pigs increased to a range of 24.5- 35.6 g/l until Day 7. A steady decrease in the leukocyte population was observed until study termination on Days 14/15. A comparison of the

leukocyte population between the challenged groups demonstrated no statistical significance over the course of the entire study (P>0.01).

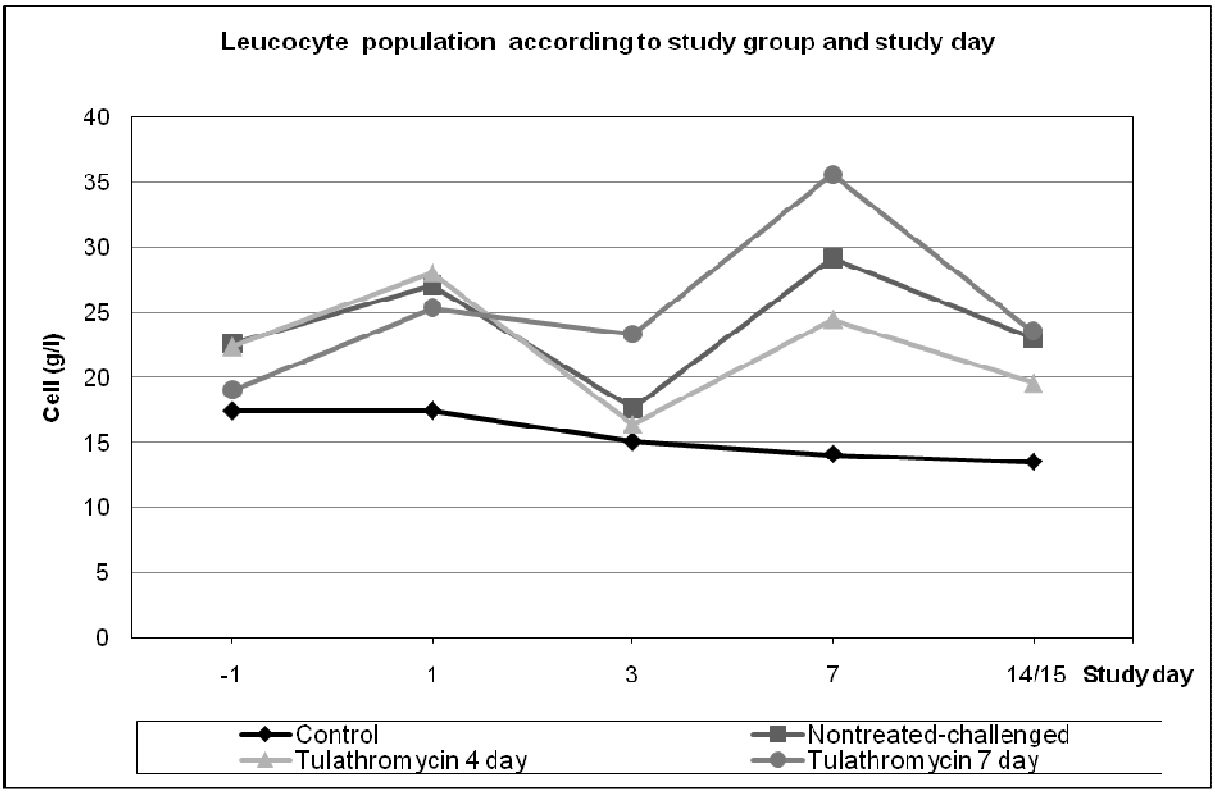

Figure 8: Average leukocyte numbers (g/l) according to study group and study day

4.3 Gross lesions

4.3.1 Lung assessment

The average overall percentage of total lung lesions for all pigs without taking time of death into consideration is shown in Figure 9. Lung assessment was performed on 32 lungs from pigs of all four study groups. Lung lesions were not observed in the lungs of pigs from the control group and this difference was significant in comparison to the challenged groups (P<0.001). On average, fifty percent of the lung lobes of pigs from Group 2 displayed macroscopic lesions. Likewise, 47 % and 37.8 % of the lung lobes of pigs from Groups 3 and 4 were covered with lung lesions. A significant difference among the three challenged groups was not observed (P>0.01). Lesions in all pigs were characterized by a dark red mottled appearance and the consistency of the lung was firm elastic (Figure 10). Lesions were diffusely spread throughout the entire lung parenchyma of all lung lobes.

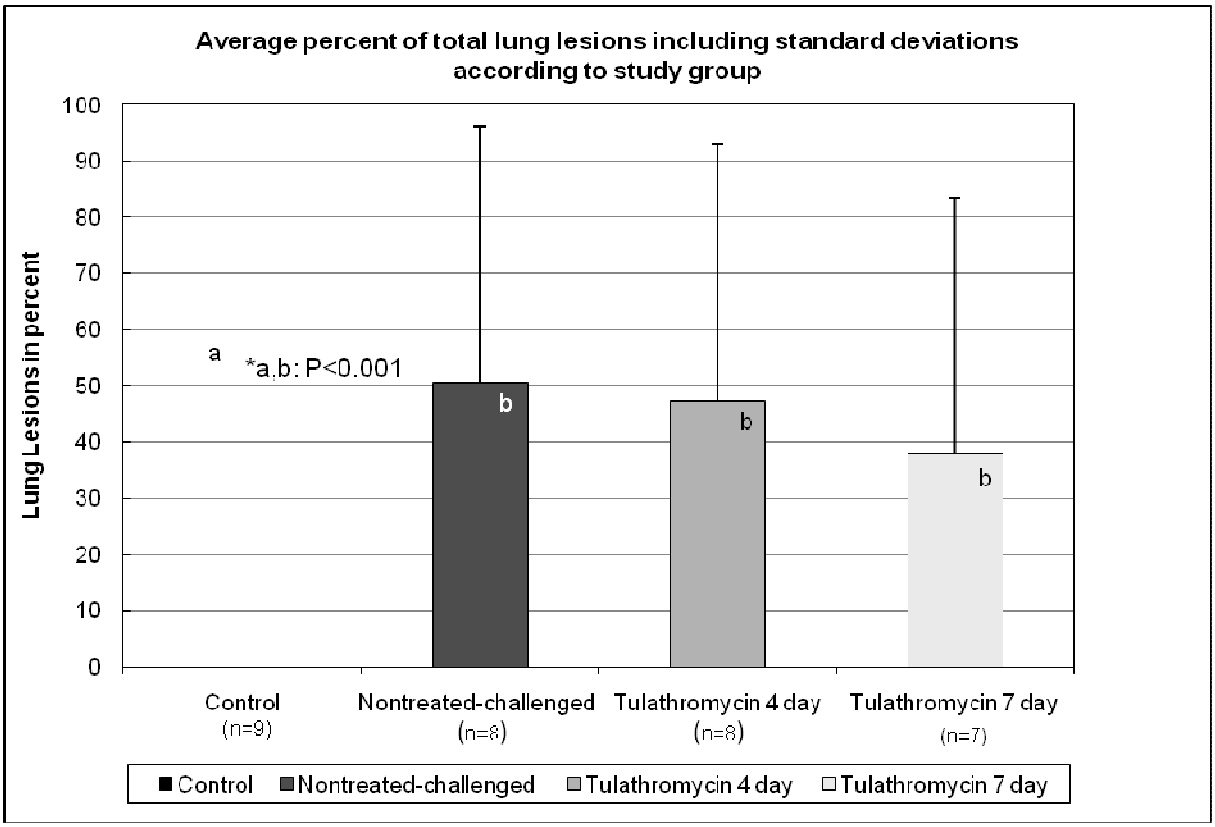

Figure 9: Average total lung lesions and standard deviations according to study group

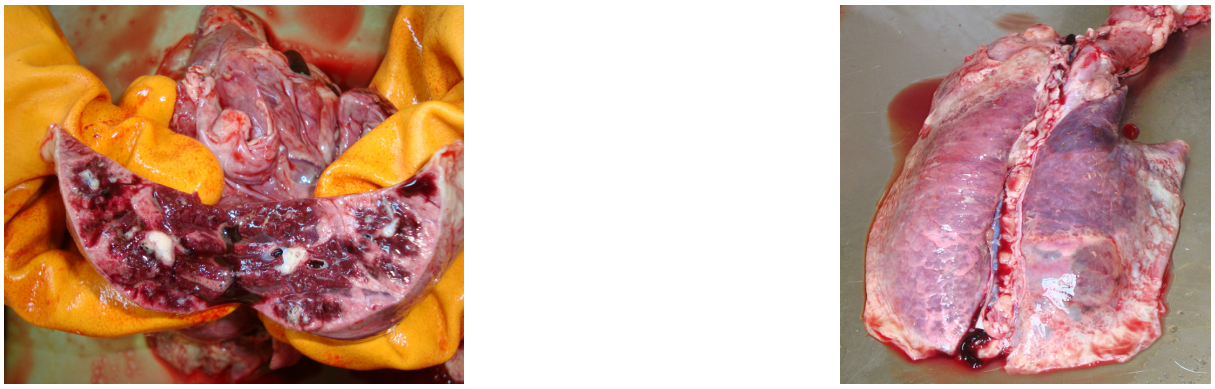

Figure 10: Severe lung lesions characterized by a mottled, dark red appearance of lung parenchyma

4.3.2 Synovial fluid assessment

Synovial fluids were collected from a total of 34 pigs at removal (death or euthanasia) or at study termination from all four study groups. The assessment of the synovial fluid from each study group is illustrated in Figure 11. Mild and severe changes in the synovial fluid of 3 Group 1 pigs were observed. The synovial fluids from the remaining 6 pigs were macroscopically unchanged. A significant difference between the macroscopic assessment of the synovial fluids of the control and challenged animals was not observed ($P>0.01$). All synovial fluids collected from pigs of Group 2 were macroscopically changed. Seven pigs from this group had mild synovial changes and two pigs had moderate to severe changes. The synovial fluids of one pig from Group 3 and three pigs from Group 4 were unchanged. Four pigs from the Group 3 had mild synovial changes and the remaining 3 pigs had severely changed synovial fluids. Three pigs from the Group 4 day group had mildly changed synovial fluid and the remaining two pigs had moderate to severely changed synovial fluid. The differences in synovial fluid assessment among the challenged groups were not statistically significant ($P>0.01$).

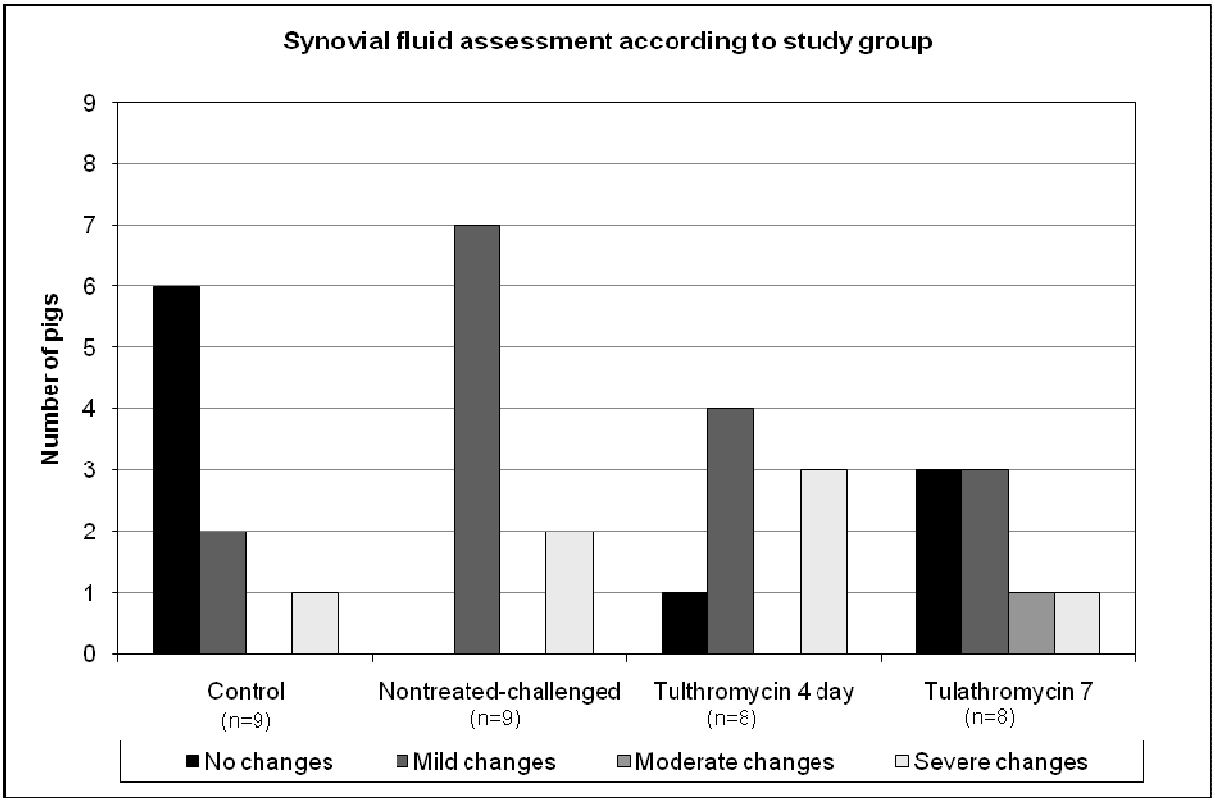

Figure 11: Synovial fluid assessment according to study group

4.3.3 Cerebrospinal fluid assessment

A total of 29 cerebrospinal fluids were obtained from pigs of all four study groups. The assessment of the cerebrospinal fluid for each study group is displayed in Figure 12. The cerebrospinal fluids of 3 pigs from each study group were unchanged. The remaining 2 pigs of the control group had moderately changed cerebrospinal fluids. The cerebrospinal fluids of 5 pigs from Group 2 were severely changed. Five pigs from Groups 3 and 4 had mild or severely changed cerebrospinal fluid. The differences in cerebrospinal fluid assessment among all study groups were not statistically significant ($P>0.01$).

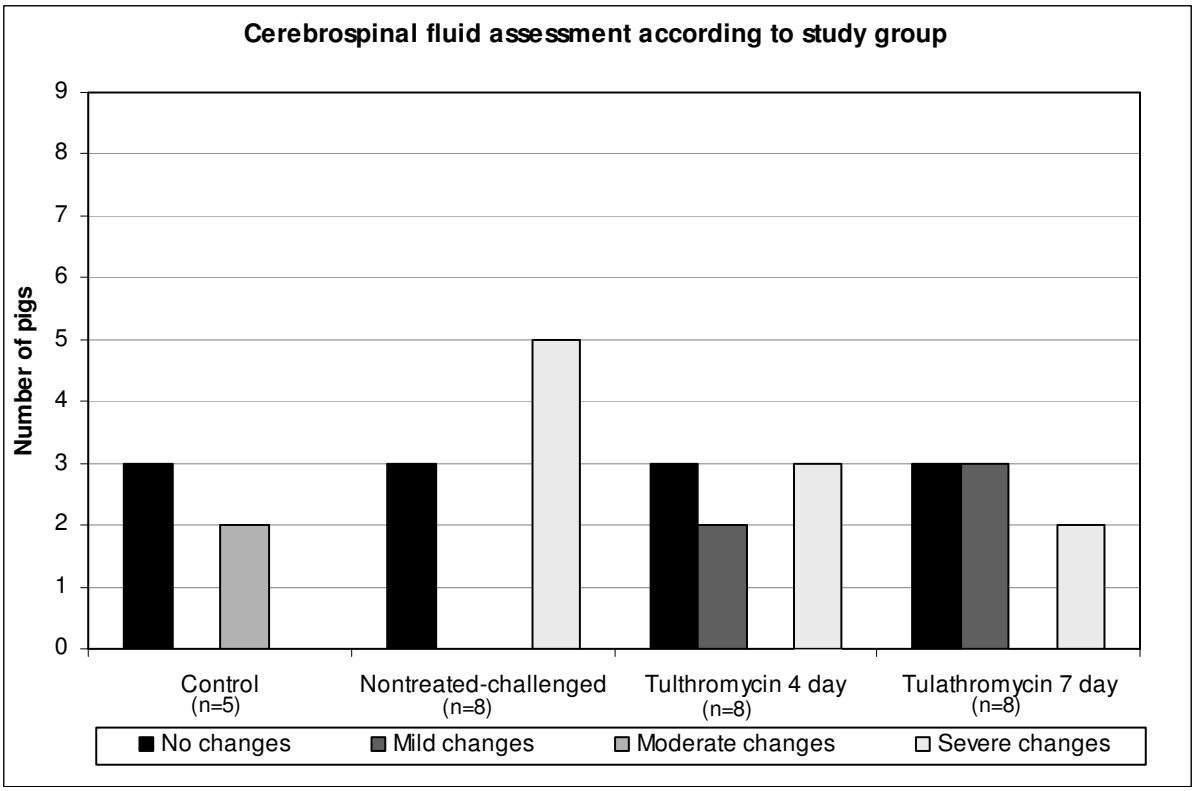

Figure 12: Cerebrospinal fluid assessment of pigs according to study group

4.3.4 Brain assessment

The brains of all 36 pigs were assessed at either removal (sudden death, euthanasia) or study end and macroscopic changes were not observed in the brains of the pigs from the control group (Figure 13). This difference in brain assessment was significant between the control pig and pigs from the challenged group ($P<0.001$). The brains of all pigs from Group 3 were macroscopically altered. The brains of 6 pigs from Group 2 and the brains of 7 pigs from Group 4 were macroscopically changed. The brains of 3 pigs from the Group 2 and 2 pigs from the Group 4 were macroscopically unchanged. Macroscopic changes in the brain included hyperemia, edema and pallor (Figure 14). A significant difference in the brain assessment of pigs from all three challenged groups was not observed ($P>0.01$).

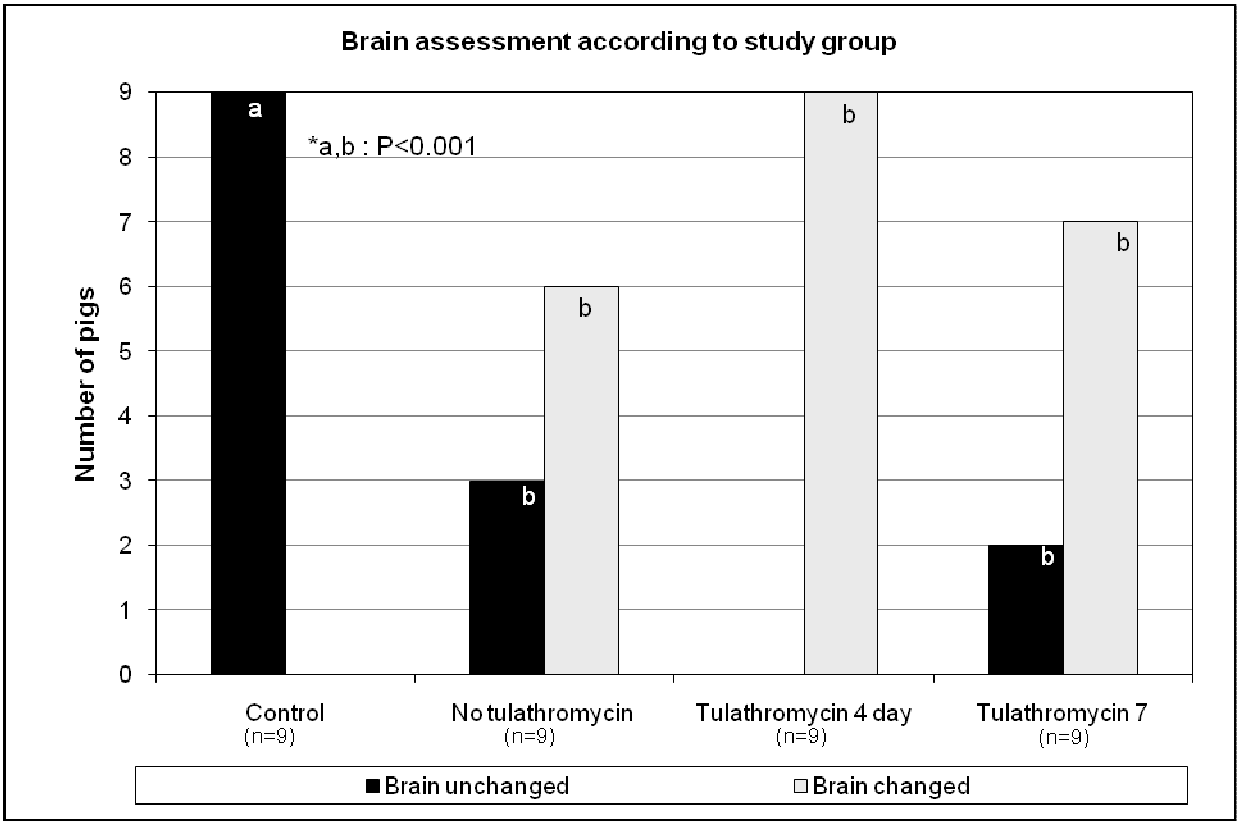

Figure 13: Brain assessment according to study group

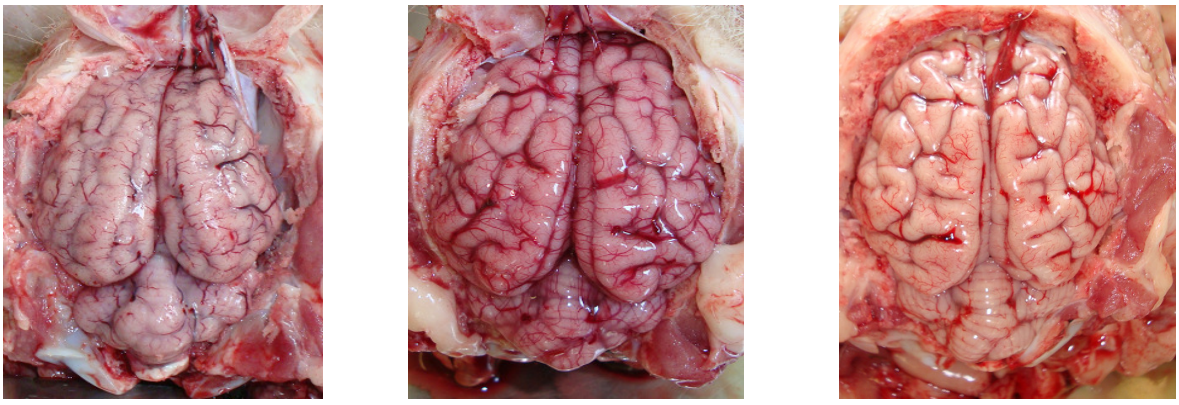

Figure 14: Characteristic pathological changes observed after brain assessment (from left to right): brain with pallor, edematous and hyperemic brain, brain without pathological changes

4.3.5 Assessment of the serosal membranes and cavities

At necropsy, all grades of inflammation on all serosal surfaces and cavities as described in Section 3.5 were seen (Figure 19). Furthermore, hydropericardium, hydrothorax, hydroperitoneum including adherences in pleura, pericardium and peritoneum were also observed. With the exception of two pigs from Group 1, all inflammations were seen in the challenged pigs. These difference between the control group and the challenged groups was significant ($P<0.01$). Pigs from Group 3 had the most cases of inflammations followed by pigs from Groups 4 and 2, and significant difference among the challenged groups was not observed ($P>0.01$) (Figure 15).

As shown in Figure 16, pleuritis was not observed in any of the pigs from the control group, and in four pigs from the nontreated-challenged group. The remaining 5 pigs of Group 2 had a severe pleuritis. Pleuritis was also not identified in two pigs from Group 3 and five pigs from Group 4. Seven pigs from Group 3 had either a mild or severe pleuritis and four pigs from Group 4 had a severe pleuritis. A significant difference among all study animals for this inflammation type was not observed ($P>0.01$).

Results from the assessment of the pericardium are illustrated in Figure 17. Pericarditis was not observed in pigs from the control group. Pigs from all challenged groups had either no pericarditis or a severe pericarditis. Severe pericarditis was seen in 2, 5 and 6 pigs from Groups 2, 3 and 4 respectively. A significant difference among all study groups for this inflammation type was not found ($P>0.01$). The results from the peritonitis assessment of all four study groups are depicted in Figure 18. Two pigs from the control group had a mild peritonitis and the remaining 7 pigs did not have any lesions of peritonitis. A significant difference between the control pigs and pigs from the challenged group was observed ($P<0.01$). Mild to moderate cases of peritonitis were found in 6 pigs from Group 2. Three pigs from this group and two pigs from Group 4 did not have lesions indicative of peritonitis. Six pigs from the Group 3 had a mild peritonitis and 3 pigs had a severe peritonitis. A significant difference among the challenged groups for the occurrence of the individual inflammation type was not established ($P>0.01$).

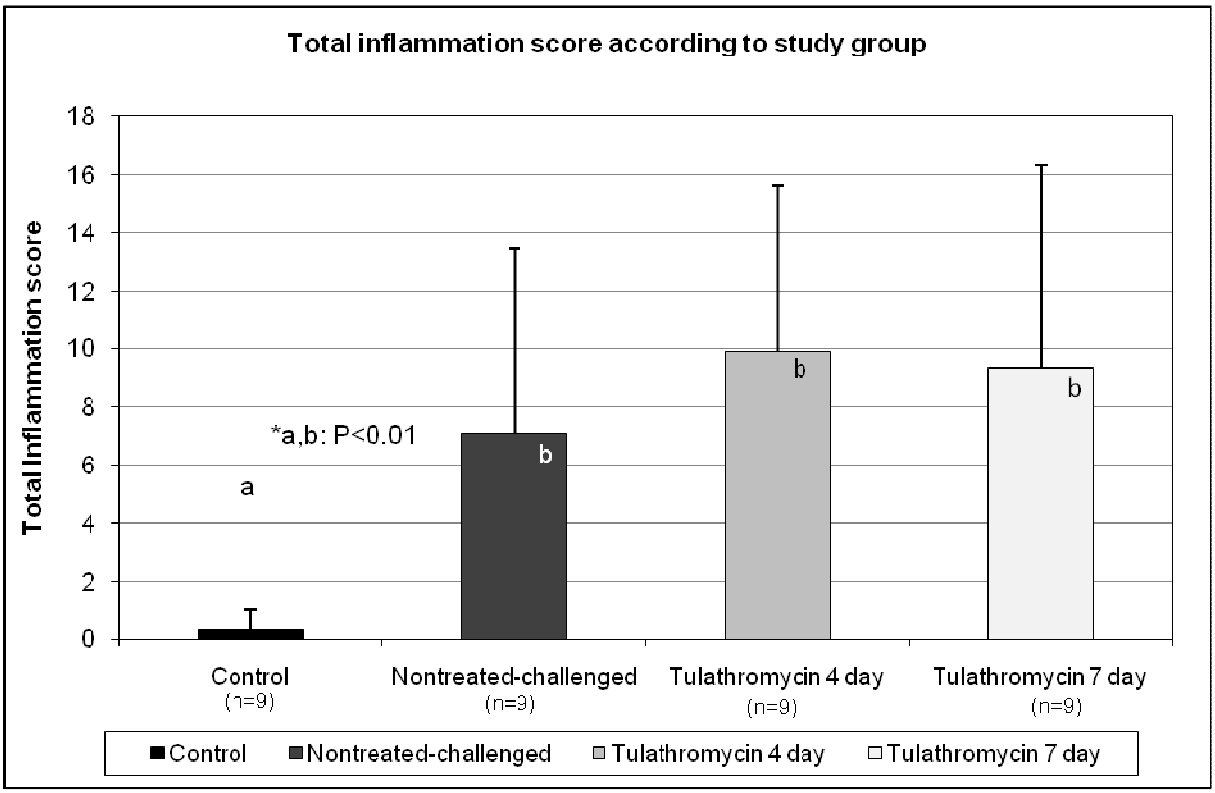

Figure 15: Total inflammation score according to study group

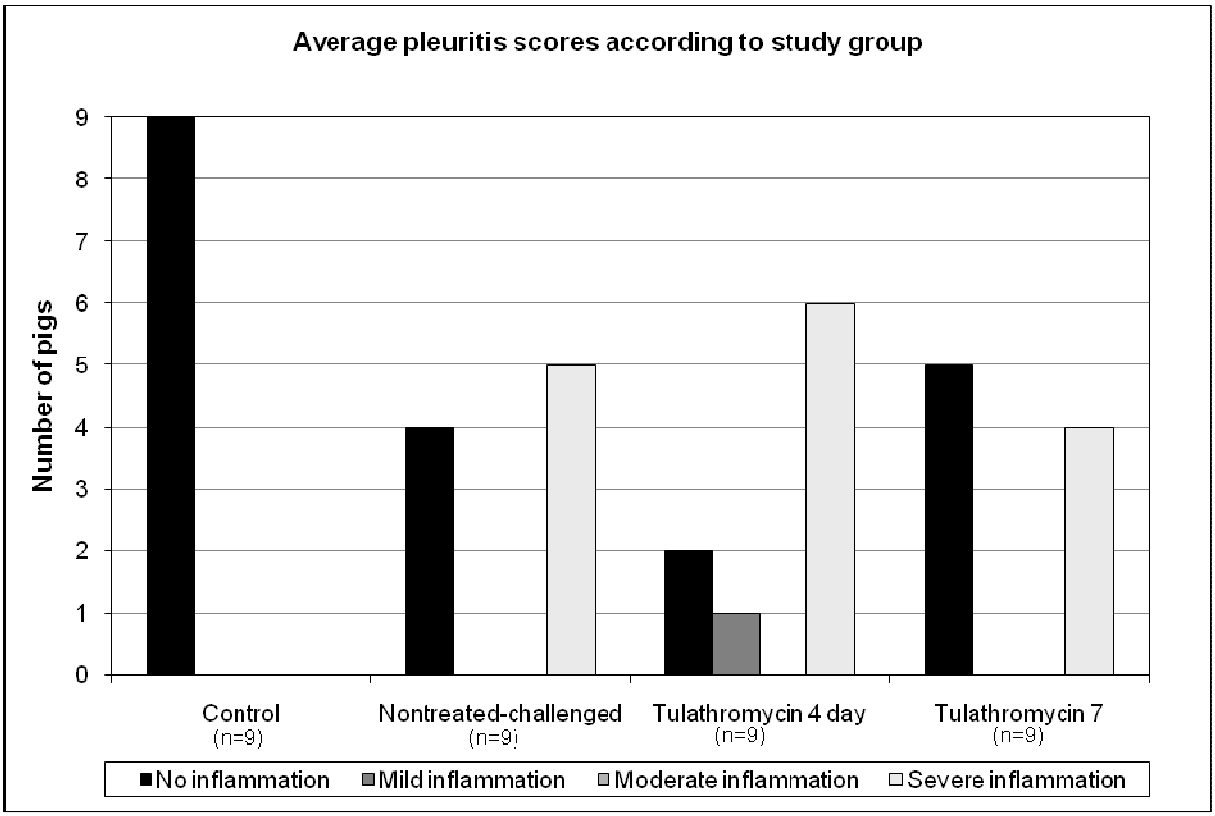

Figure 16: Average pleuritis scores according to study group

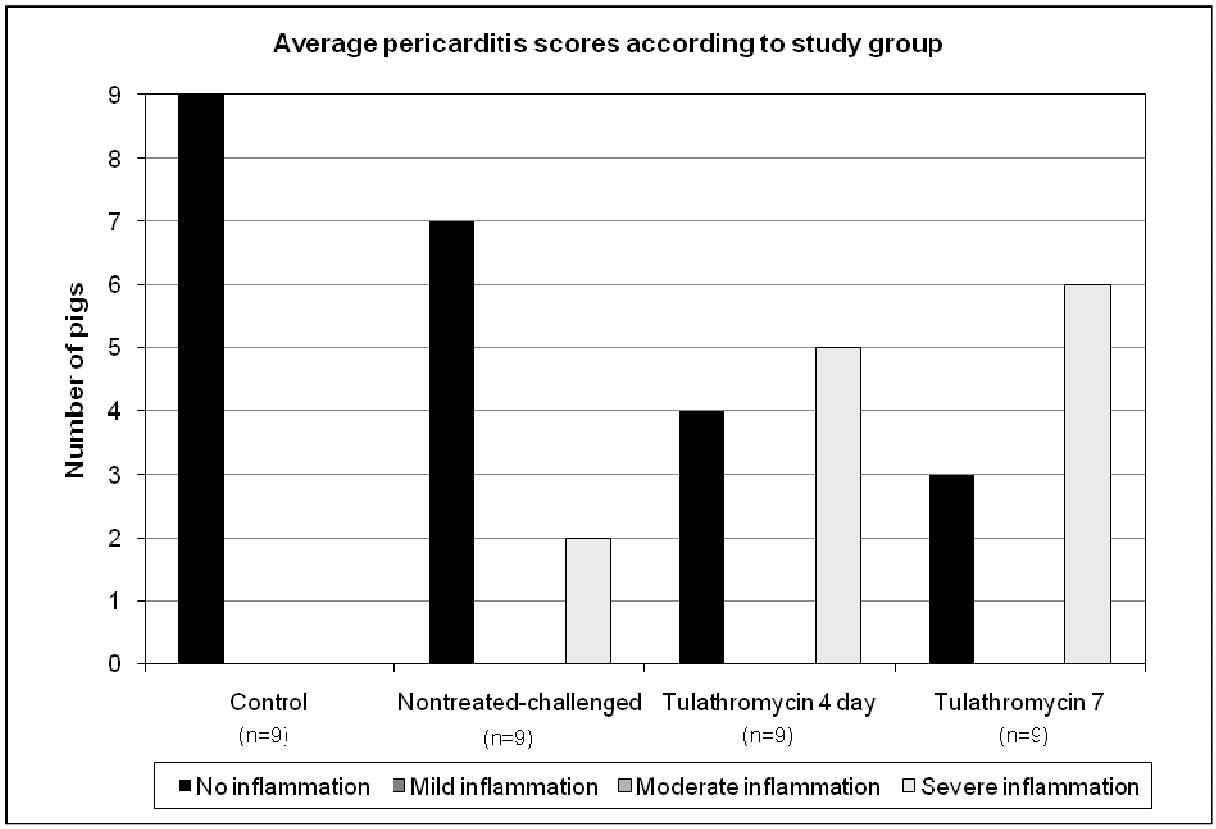

Figure 17: Average pericarditis scores according to study group

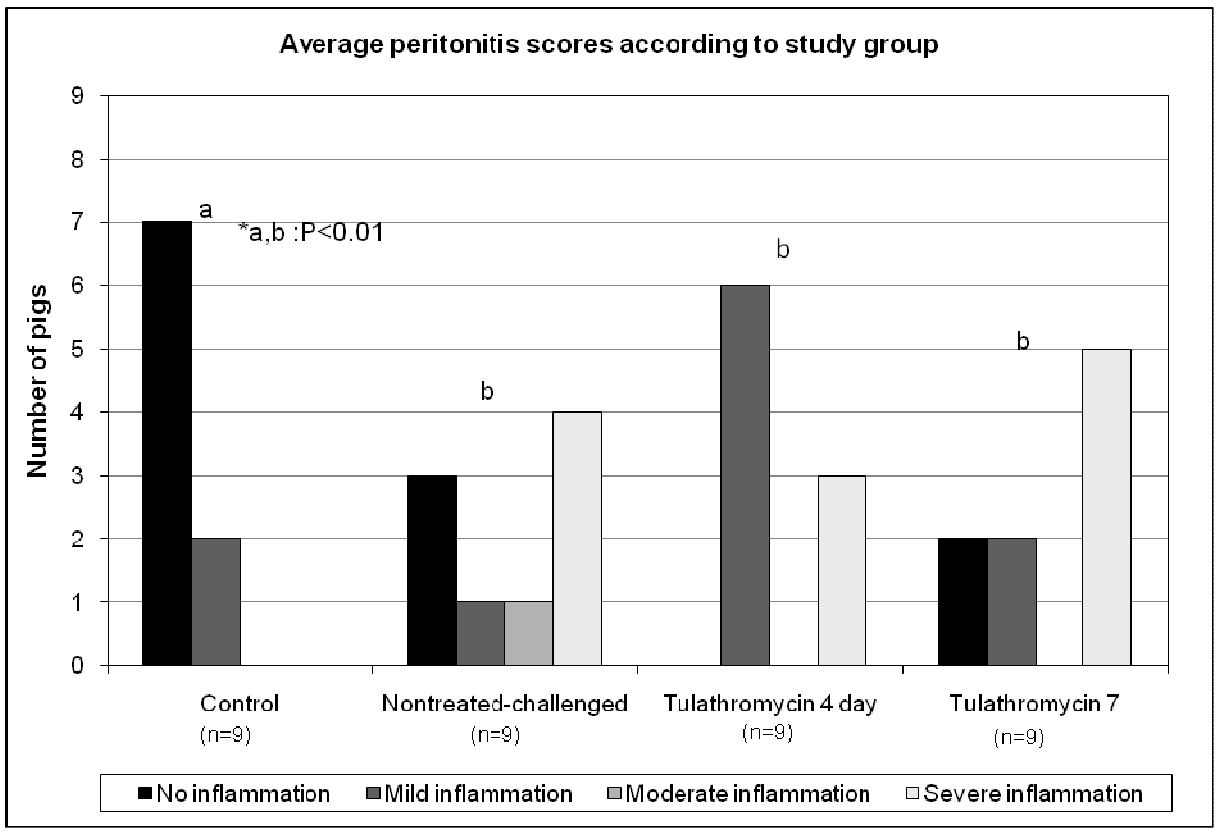

Figure 18: Average peritonitis score according to study group

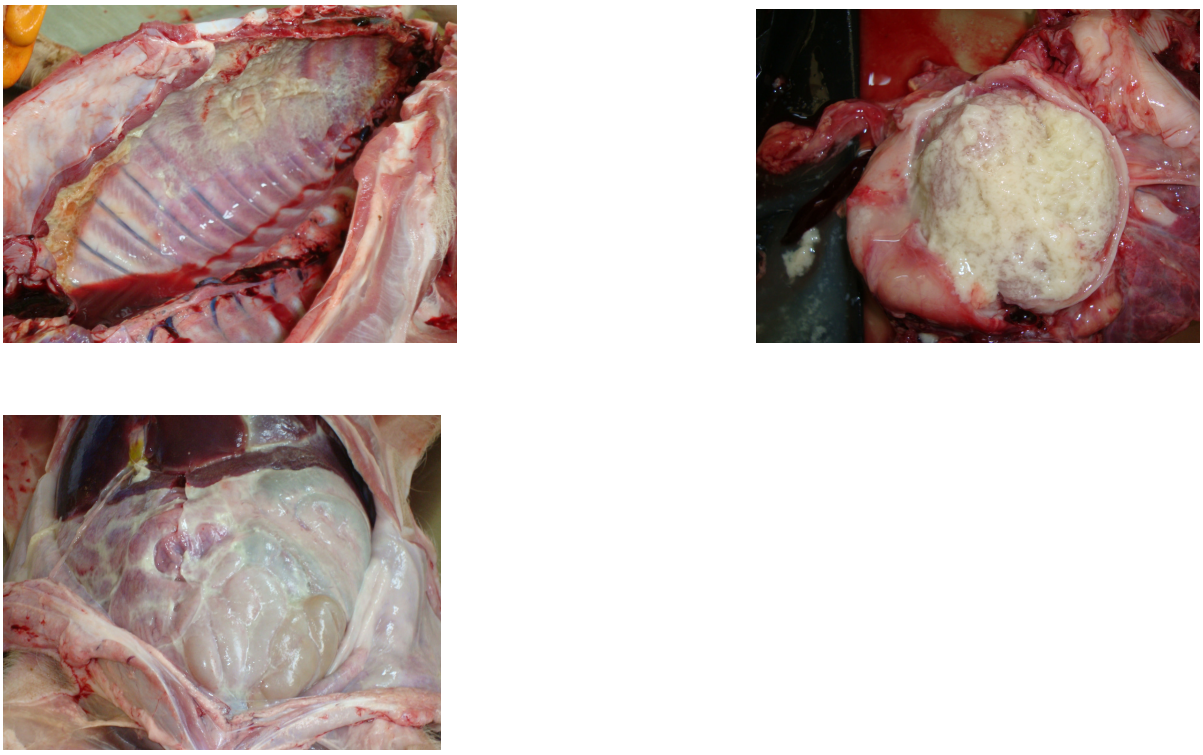

Figure 19: Severe case of pleuritis, pericarditis and peritonitis observed in challenged pigs at necropsy (clockwise from left to right)

4.4 Histopathological lesions

Histopathological examination of all tissue samples mentioned in section 3.6.5 identified synovitis, pyelitis and meningitis in samples from the challenged pigs. Histopathological lesions were not found in any of the tissues samples from pigs of the control group ($P<0.001$). With the exception of 1 pig from Group 3, these findings were identified in the challenged pigs which died prior to study termination (Days 14/15). All other animals including pigs from the control group which died on Days 14/15 displayed no lesions indicative of an inflammation. The occurrence of the individual inflammations is displayed in Table 6. A total of 3 pigs from Group 2 and 4 pigs Groups 3 and 4 had at least one of the three inflammation types after histological assessment. The most frequent inflammation type found was pyelitis, which occurred either alone or together with meningitis or synovitis. Similarly, meningitis was only identified when a pyelitis or synovitis was present. A significant difference for the occurrence of these inflammations among the challenged pigs was not observed ($P>0.01$).

Table 6: Histopathological examination results according to study group (n= number of animals)

	Pyelitis	**Synovitis**	**Meningitis and Pyelitis**	**Meningitis and Synovitis**	**Meningitis, Pyelitis and Synovitis**	
Control	0	0	0	0	0	n=0
Nontreated-challenged	2	0	0	0	1	n=3
Tulathromycin 4 day	0	1*	1	0	2	n=4
Tulathromycin 7 day	0	0	1	1	2	n=4
	n=2	n=1	n=2	n=1	n=5	

*Pig euthanized at study termination (Days 14/15) in comparison to all other pigs

4.5 PCR detection of HPS genome

The results of the PCR detection of all 5 sample materials mentioned in section 3.6.6 are depicted in Table 7. All samples from the control group were negative for HPS genome. A total of 129 sample materials were collected from all three challenged groups and examined by PCR. HPS genome was detected in at least one of the five sample materials of 18 pigs from all three challenged groups. HPS genome was most frequently detected in sample materials from pigs of Group 3 followed by pigs from Groups 2 and 4. HPS genome was most frequently detected in the collective serosal swabs and the cerebrospinal fluid (Table 7). The sensitivity of these two sample material types for HPS genome was 44 % and 50 % respectively. HPS genome was only detected in the synovial fluids from animals that died at study termination. HPS genome was detected in the brains swabs and the collective serosal swabs from animals which died prior to study termination. A significant difference for HPS detection in the individual sample material was not observed ($P>0.05$).

Table 7: PCR results from all five sample materials according to study group, presented as number of positive samples

	Joint capsule	Synovial fluid	Cerebrospinal fluid	Brain swab	Collective serosal swab	
Control	0	0	0	0	0	n=0
Nontreated-challenged	2	1	4	0	4	n=11
Tulathromycin 4 day	1	2	3	3	4	n=13
Tulathromycin 7 day	2	1	3	1	4	n=11
	n=5	n=4	n=10	n=4	n=12	n=35

4.6 Bacterial isolation of HPS

Results of the bacterial examinations of the 36 collective serosal swabs are presented in Figure 20. All samples from the control group were negative for HPS after culture. Positive samples were obtained from the challenged pigs that died prior to study termination (Days 14/15). HPS was isolated in four swabs from pigs of Groups 2 and 3, and in two collective serosal swabs from pigs of Group 4. A significant difference for the frequency of HPS detection among the challenged groups was not observed (P>0.01).

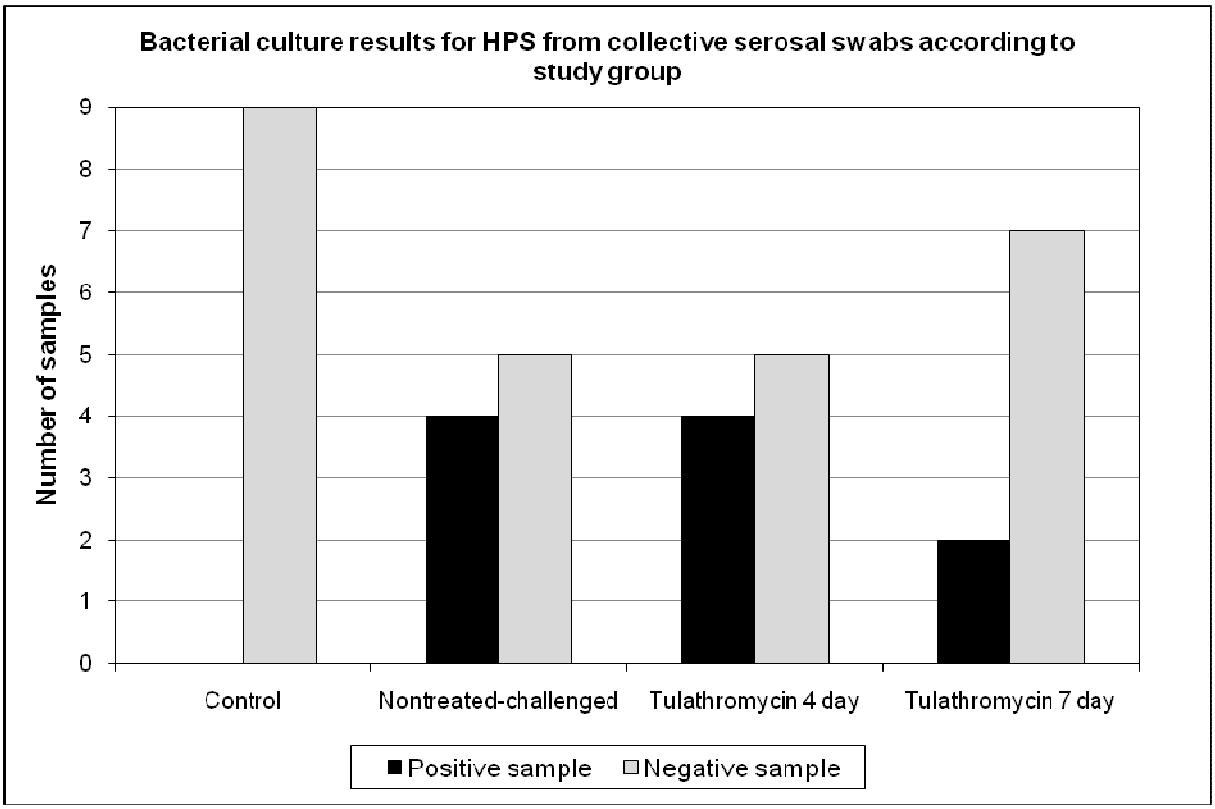

Figure 20: Bacterial culture results of the collective serosal swabs according to study group

4.7 Association between HPS detection in collective serosal swabs by PCR and bacterial culture

Collective serosal swabs from all 36 animals were tested by both PCR and bacterial culture for the presence of HPS genome and HPS respectively. A comparison of the HPS detection by PCR and bacterial culture in collective serosal swabs from the 27 challenged pigs is displayed in Table 8. HPS genome was detected in 12 swabs by PCR and in 10 swabs by bacterial culture. HPS was detected by both PCR and bacterial culture in 10 swab samples. After statistical analysis, HPS was detected equally well by both PCR and bacterial culture (Ƙ=0.81).

Table 8: Frequency of HPS detection by PCR and bacterial examination in collective serosal swabs from challenged pigs

	HPS PCR pos	**HPS PCR neg**	
Bacterial culture pos	10	0	n=10
Bacterial culture neg	2	15	n=17
	n= 12	n=15	

4.8 Associations between HPS detection in samples from challenged pigs and clinical results

4.8.1 Association between HPS detection by PCR and bacterial culture and the median total daily clinical score

Animals with a median total daily clinical score greater than 4 were classified as clinically sick and animals with a clinical score lower than four were clinically healthy. After challenge, 13 infected pigs showed clinical signs and symptoms indicative of mild to severe sickness. HPS genome was detected by PCR in sample materials from 9 clinically sick animals and in three sample materials from clinically healthy animals (Table 9). After bacterial culture of the collective serosal swabs, HPS was detected in swabs from 8 clinically sick animals (Table 10). HPS was significantly more frequently detected by PCR ($P=0.02$) and bacterial culture ($P<0.001$) in animals which had a median total daily clinical score greater than 4 in comparison to pigs with a clinical score lower than 4.

Table 9: Frequency of HPS genome detection by PCR in samples from challenged pigs and the median total daily clinical score

	HPS PCR pos pigs	HPS PCR neg pigs	
Clinical score> 4	9	4	n=13
Clinical score<4	3	11	n=14
	n=12	n=15	

Table 10: Frequency of HPS detection by bacterial culture from collective serosal swabs of challenged pigs and the median total daily clinical score

	HPS pos pigs- bacterial culture	HPS neg pigs-bacterial culture	
Clinical score> 4	8	5	n=13
Clinical score<4	2	12	n=14
	n=10	n=17	

4.8.2 Association between HPS detection by PCR and bacterial culture and time of death

A total of 12 animals from all three challenged groups were removed (sudden death or euthanized) over the course of 10 days after infection. Fifteen challenged pigs were euthanized at study termination on Days 14/15. HPS genome was detected by PCR in at least one of the five sample materials collected from the 12 pigs which died or were euthanized before study end and in samples from six pigs which died at study termination (Table 11). HPS was detected by bacterial culture in collective serosal swabs from 10 pigs and all samples were from pigs which died prior to study termination (Table 12). HPS was detected significantly more frequently by PCR ($P<0.001$) and bacterial culture ($P<0.001$) in samples from animals that died prior to study end in comparison to samples from animals which died at study termination.

Table 11: Frequency of HPS genome detection by PCR in samples from challenged pigs which died prior to and a study termination

	HPS PCR pos pigs	HPS PCR neg pigs	
Pigs dead before study end	12	0	n=12
Pigs dead at study end	6	9	n=15
	n= 18	n=9	

Table 12: Frequency of HPS detection by bacterial culture in samples from challenged pigs which died prior to and a study termination

	HPS pos pigs- bacterial culture	HPS neg pigs- bacterial culture	
Pigs dead before study end	10	2	n=12
Pigs dead at study end	0	15	n=15
	n= 10	n=17	

4.9 Association between HPS detection in samples from challenged pigs and gross lesions

4.9.1 Association between HPS detection in collective serosal swabs and the total inflammation score

If an animal had a total inflammation score greater than 6, a polyserositis was assumed to present. From 27 challenged pigs, polyserositis was seen in a total of 17 pigs from all three challenged groups. Polyserositis was not observed in pigs from the control group. HPS genome was detected by PCR in the collective serosal swabs from 11 pigs which had a polyserositis and in 1 sample from a pig which did not have a polyserositis (Table 13). After the bacterial culture, HPS was identified in the collective serosal swabs from 10 animals which had a polyserositis (Table 14). HPS was detected more frequently by PCR (P=0.01) and bacterial culture (P<0.001) in collective serosal swabs from animals with a polyserositis than in samples from animals without a polyserositis.

Table 13: Frequency of HPS genome detection by PCR in collective serosal swabs from challenged pigs and the occurrence of polyserositis

	PCR HPS pos	PCR HPS neg	
Polyserositis present	11	6	n=17
Polyserositis absent	1	9	n=10
	n=12	n=15	

Table 14: Frequency of HPS identification by bacterial examination in collective serosal swabs from challenged pigs and the occurrence of polyserositis

	Bacterial culture HPS pos	Bacterial culture HPS neg	
Polyserositis present	10	7	n=17
Polyserositis absent	0	10	n=10
	n=10	n=17	

4.9.2 Association between HPS detection in collective serosal swabs and pleuritis, pericarditis, and peritonitis

With the exception of two pigs from the control group, all three inflammation types were observed in pigs which were infected with HPS. As shown in Figure 21, HPS genome was detected by PCR in the collective serosal swabs of 11, 7 and 9 pigs that had a pleuritis, pericarditis and peritonitis respectively. HPS genome was also detected in a swab sample from 1 animal that did not have lesions of pleuritis and in samples from 5 animals which did not have either a pericarditis or peritonitis. After bacterial culture, HPS was isolated in the swabs from 10, 6 and 8 pigs which had pleuritis, pericarditis and peritonitis respectively (Figure 22). HPS was also found in swabs from 4 pigs that did not have a pericarditis and 2 pigs which did not have peritonitis. HPS genome was significantly more frequently detected by PCR in samples from animals that had a pleuritis ($P<0.001$) and peritonitis ($P=0.03$) than animals without these two inflammation types. Similarly, HPS was isolated more frequently by bacterial culture in samples from animals that had a pleuritis ($P<0.001$) and peritonitis ($P<0.001$). An association between the positive identification of HPS genome in the collective serosal swab by either PCR or bacterial culture and the occurrence of pericarditis was not observed ($P>0.05$).

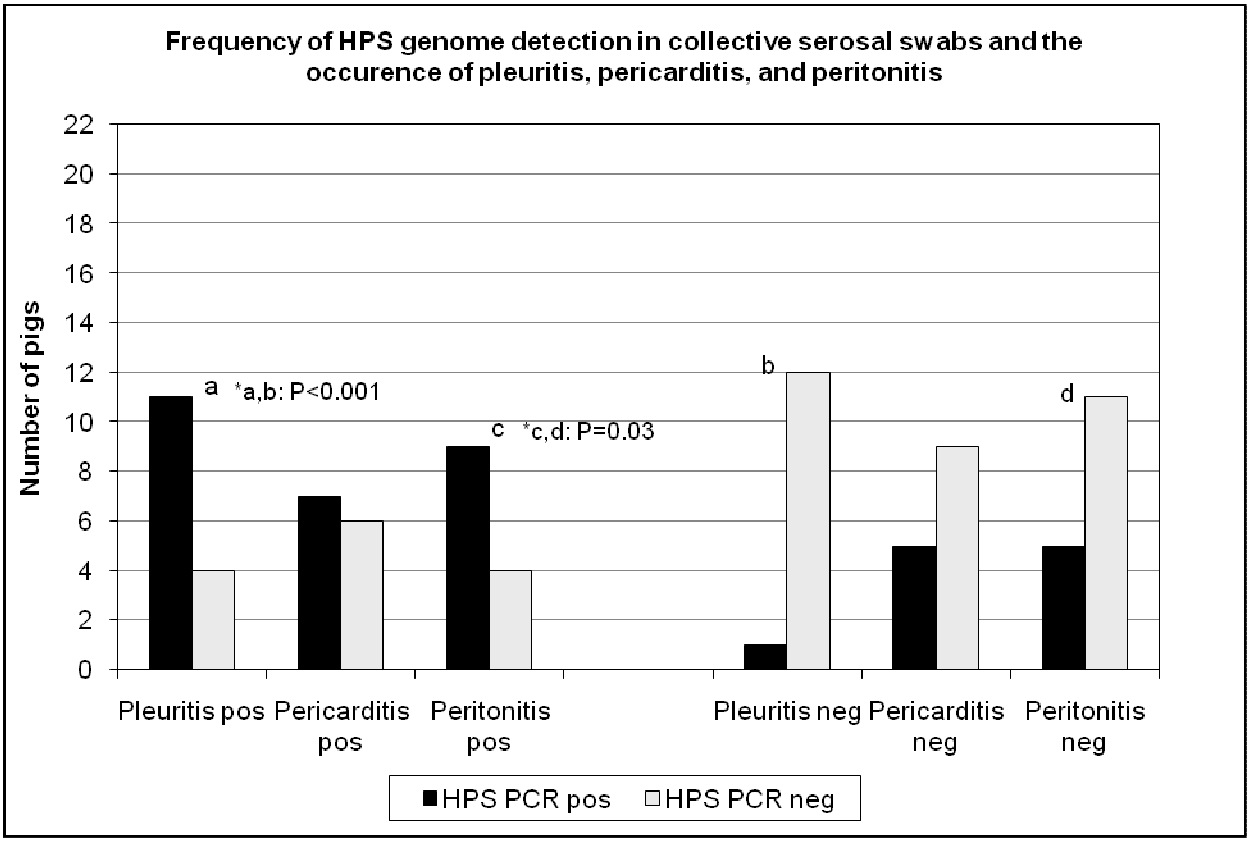

Figure 21: Frequency of HPS genome isolation by PCR in collective serosal swabs from challenged pigs and the occurrence of pleuritis, pericarditis and peritonitis

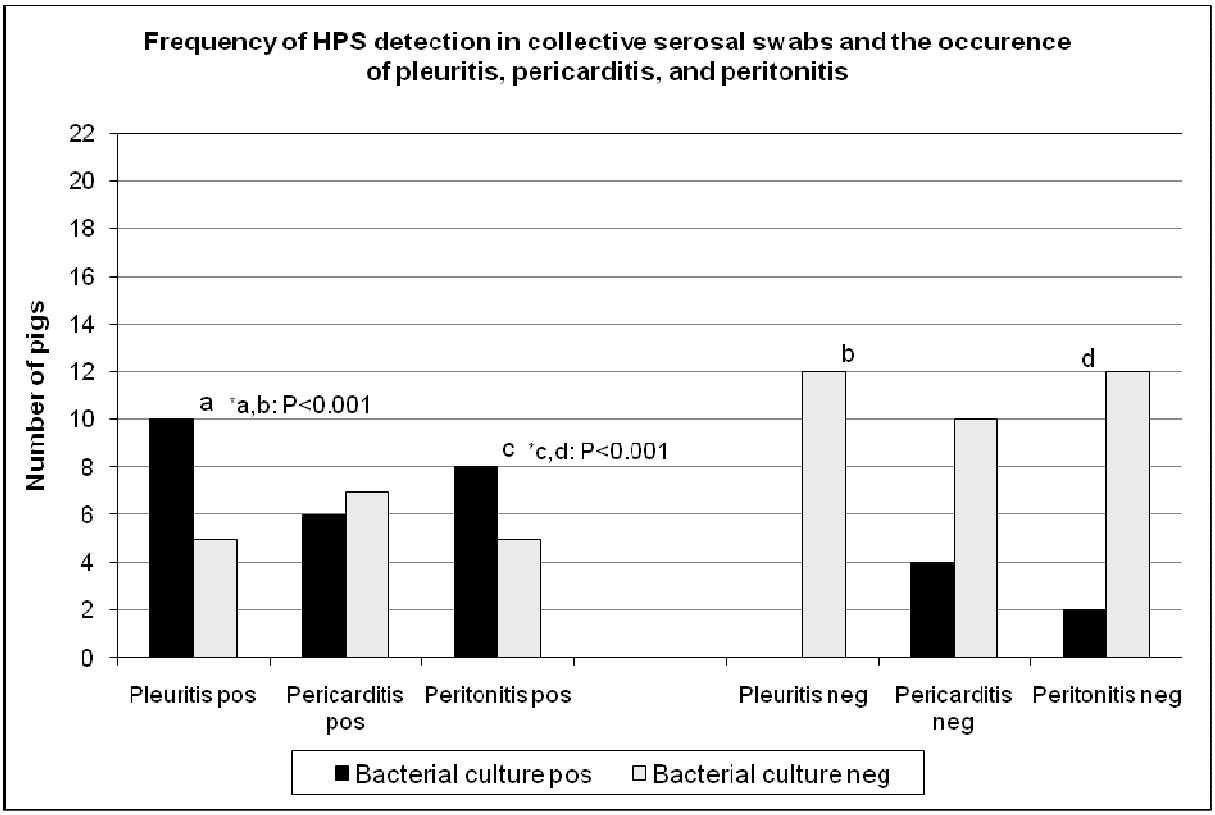

Figure 22: Frequency of HPS detection by bacterial culture in collective serosal swabs from challenged pigs and the occurrence of pleuritis, pericarditis, and peritonitis

4.9.3 Association between HPS genome detection in cerebrospinal fluids, brain swabs and the assessment of the brains and cerebrospinal fluids

The brains of 22 pigs from all challenged groups were macroscopically changed. Cerebrospinal fluids from 24 challenged pigs were macroscopically examined and mild to severe changes in samples from 15 challenged pigs were observed. HPS genome was detected by PCR in the brain swabs of 4 pigs whose cerebrospinal fluid and brains were macroscopically altered (Figure 23). Also, HPS genome was detected in the brain swabs from 4 pigs whose cerebrospinal fluids were macroscopically changed (Figure 24). HPS genome was detected in the cerebrospinal fluids from 6 pigs whose brains were macroscopically changed. Also, HPS genome was detected in the cerebrospinal fluids from 4 pigs whose brains were macroscopically unchanged (Figure 25). In addition, HPS genome was detected in the cerebrospinal fluids of 7 pigs whose cerebrospinal fluids were macroscopically altered and in 2 pigs whose cerebrospinal fluids were macroscopically unchanged (Figure 26). A significant association was not found between the frequency of HPS genome detection in brain swabs and cerebrospinal fluids and the macroscopic assessment of the brain and cerebrospinal fluid ($P>0.05$).

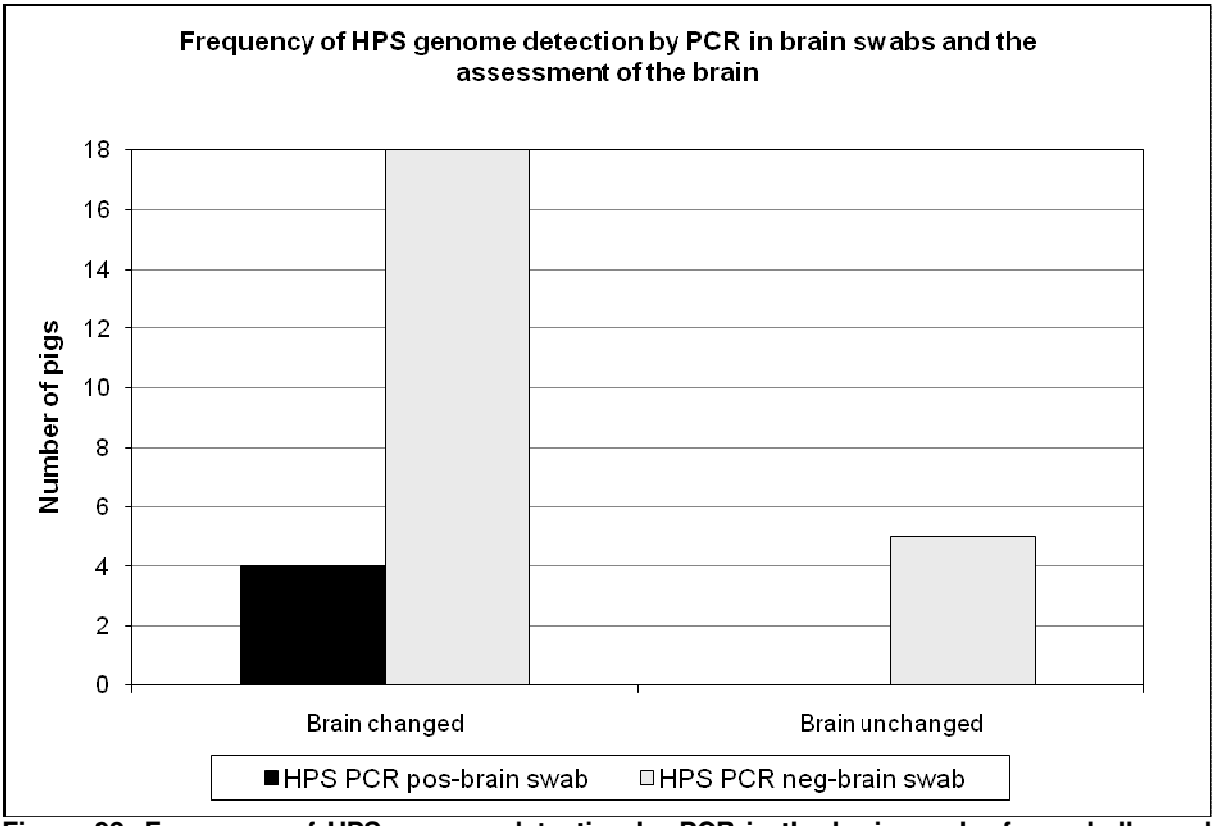

Figure 23: Frequency of HPS genome detection by PCR in the brain swabs from challenged pigs and the assessment of the brain

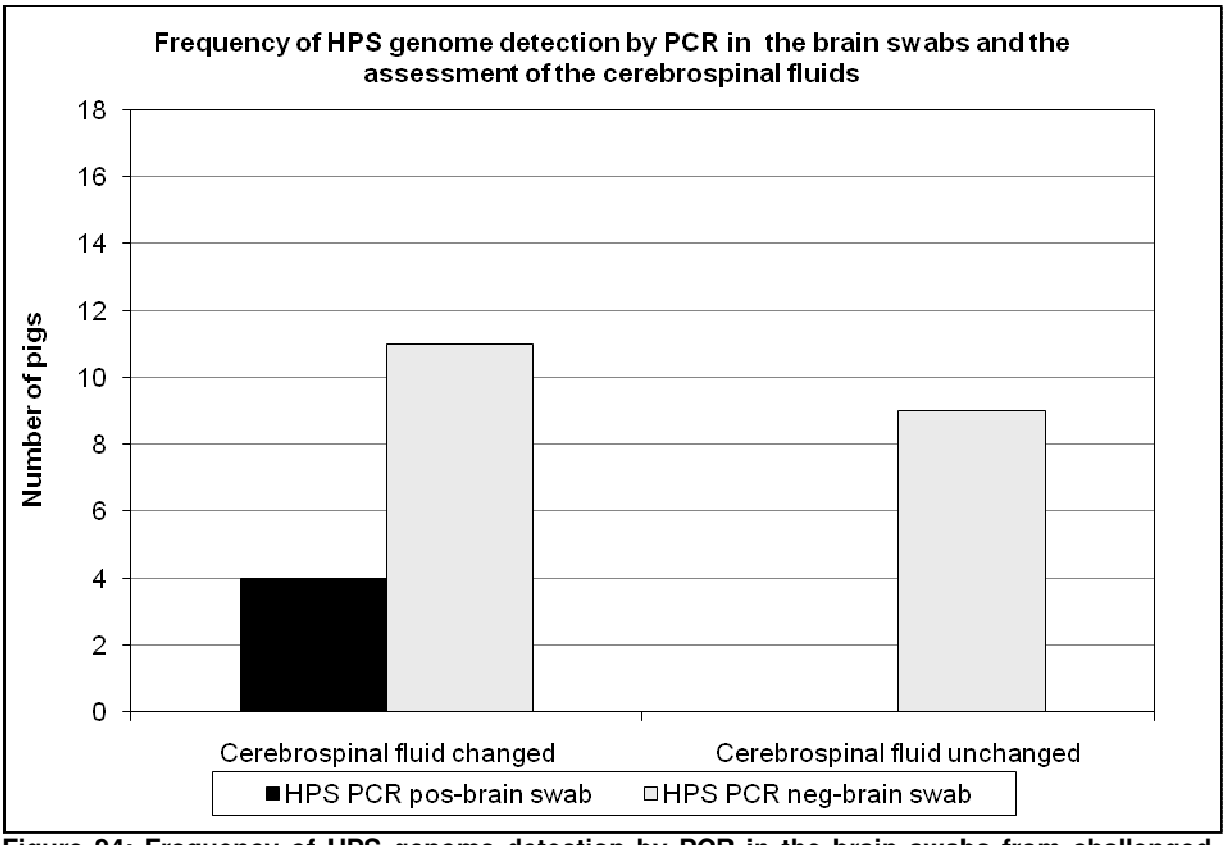

Figure 24: Frequency of HPS genome detection by PCR in the brain swabs from challenged pigs and the assessment of the cerebrospinal fluids

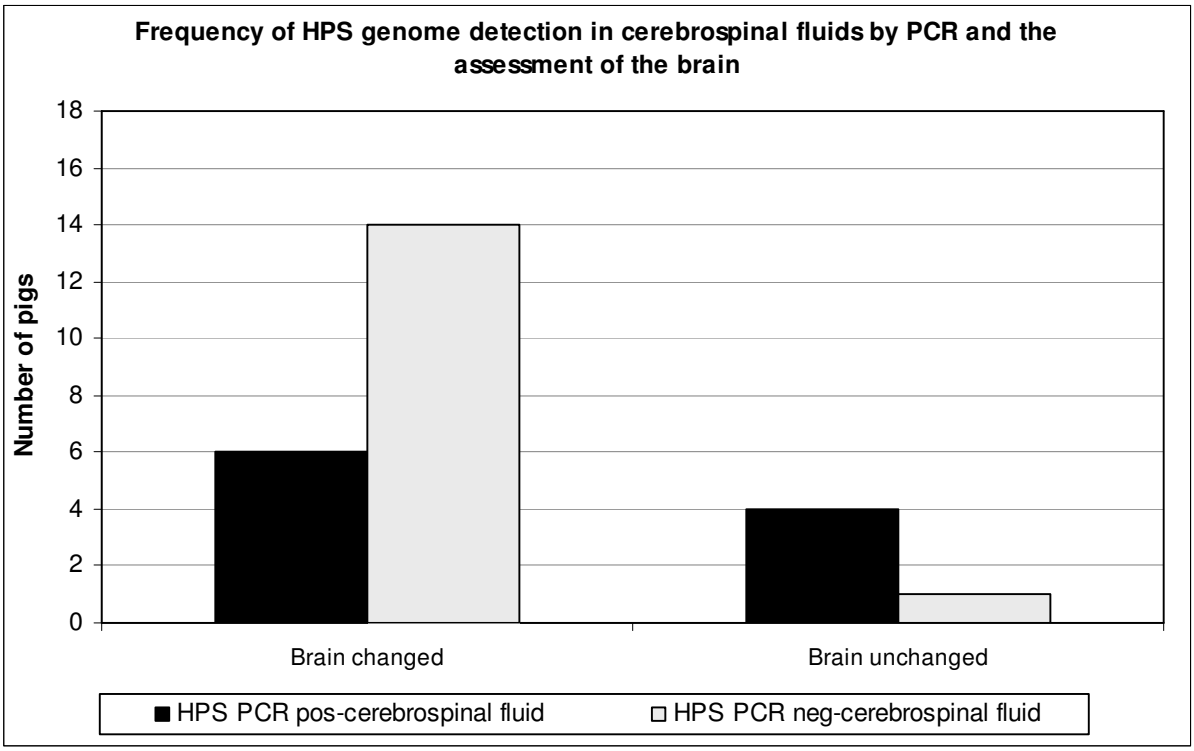

Figure 25: Frequency of HPS genome detection by PCR in cerebrospinal fluids from challenged pigs and the assessment of the brain

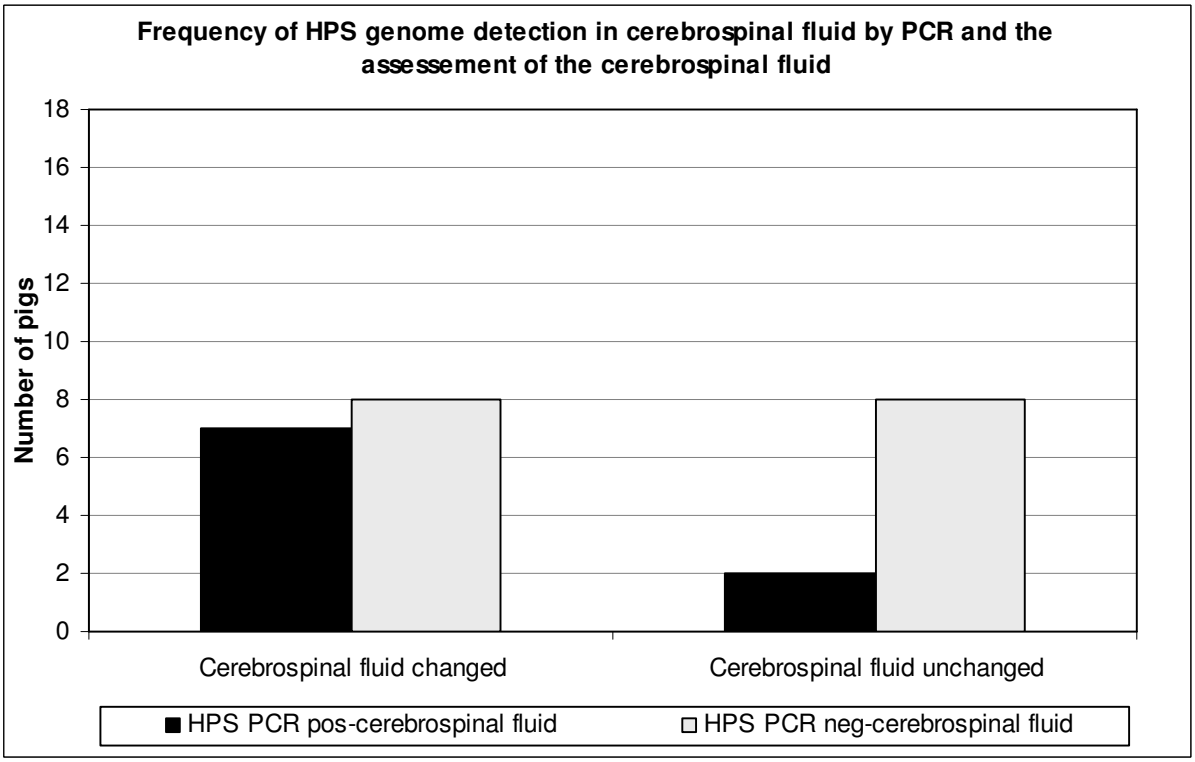

Figure 26: Frequency of HPS genome detection by PCR in cerebrospinal fluids from challenged pigs and the assessment of the cerebrospinal fluids

4.9.4 Association between HPS genome detection in synovial fluids, joint capsules and the synovial fluid assessment

The synovial fluids of 25 challenged pigs were macroscopically examined and mild to severe changes were seen in samples from 21 pigs. A total of 25 joint capsules and synovial fluid samples were obtained from all 27 challenged animals for HPS PCR testing. HPS genome was detected in the synovial fluids of 3 pigs whose synovial fluids were macroscopically altered (Table 15). In addition, HPS genome was detected in the synovial fluid from 1 pig whose synovial fluid was macroscopically unchanged. Also, HPS genome was found in the joint capsules of 5 pigs whose synovial fluids were macroscopically altered (Table 16). A corresponding identification of HPS genome in both joint capsule and synovial fluid was not observed. A significant association was not found between the frequency of HPS genome detection in the joint capsule and synovial fluid and the macroscopic assessment of the synovial fluid ($P>0.05$).

Table 15: Frequency of HPS genome detection by PCR in synovial fluids from challenged pigs and the assessment of the synovial fluid

	HPS PCR pos-synovial fluid	HPS PCR neg-synovial fluid	
Synovial fluid changed	3	18	n=21
Synovial fluid unchanged	1	3	n=4
	n=4	n=21	

Table 16: Frequency of HPS genome detection by PCR in joint capsules from challenged pigs and the assessment of the synovial fluid

	HPS PCR pos-joint capsule	HPS PCR neg-joint capsule	
Synovial fluid changed	5	14	n=19
Synovial fluid unchanged	0	4	n=4
	n=5	n=18	

4.10 Association between HPS detection in samples from challenged pigs and histopathological lesions

4.10.1 Association between HPS genome detection in synovial fluids and joint capsules and synovitis

After microscopic examination, synovitis was observed in tissue samples from 7 challenged pigs. A total of 25 joint capsules and synovial fluid samples were obtained from the 27 challenged animals for HPS PCR diagnostic. HPS genome was detected in the joint capsules of 2 pigs with a synovitis and also in the capsules of 3 pigs without synovitis (Table 17). Additionally, HPS genome was detected in the synovial fluids of 4 pigs without a synovitis (Table 18). A corresponding detection of HPS genome in both the synovial fluid and the joint capsule was not observed. A significant association was not seen between the frequency of HPS genome detection in the joint capsule and the synovial fluid and the occurrence of synovitis ($P>0.05$).

Table 17: Frequency of HPS genome detection by PCR in the joint capsules from challenged pigs and the occurrence of synovitis

	HPS PCR pos-joint capsule	HPS PCR neg-joint capsule	
Synovitis present	2	5	n=7
Synovitis absent	3	15	n=18
	n=5	n=20	

Table 18: Frequency of HPS genome detection by PCR in the synovial fluids from challenged pigs and the occurrence of synovitis

	HPS PCR pos-synovial fluid	HPS PCR neg-synovial fluid	
Synovitis present	0	7	n=7
Synovitis absent	4	14	n=18
	n=4	n=21	

4.10.2 Association between HPS genome detection in cerebrospinal fluids and brain swabs and meningitis

After histopathological examination of tissues from the 27 challenged pigs, mild to severe meningitis was observed in 7 pigs from all three challenged groups. A total of 27 brain swabs and 25 cerebrospinal fluid samples were collected from pigs of all challenged groups for PCR testing. HPS genome was detected in the cerebrospinal fluid of 6 pigs with meningitis and in samples from 4 pigs which did not have meningitis (Table 19). Furthermore, HPS genome was detected in the brain swabs of 4 pigs which had meningitis (Table 21). HPS was significantly more frequently detected in the cerebrospinal fluids ($P<0.001$) and the brain swabs ($P<0.001$) of animals with meningitis than in animals without meningitis.

Table 19: Frequency of HPS genome detection in cerebrospinal fluids from challenged pigs and the occurrence of meningitis

	HPS PCR pos- cerebrospinal fluid	HPS PCR neg- cerebrospinal fluid	
Meningitis present	6	0	n=6
Meningitis absent	4	15	n=19
	n=10	n=15	

Table 20: Frequency of HPS genome detection in brain swabs from challenged pigs and the occurrence of meningitis

	HPS PCR pos- brain swab	HPS PCR neg- brain swab	
Meningitis present	4	3	n=7
Meningitis absent	0	20	n=20
	n=4	n=23	

4.10.3 Association between HPS detection and pyelitis

Pyelitis was identified in tissue samples of 10 pigs from all three challenged groups. A total of 129 sample materials and 27 serosal swabs were collected for HPS PCR and bacterial examination respectively. HPS genome was detected by PCR in at least one of the five sample materials from the 10 pigs which had a pyelitis (Table 21). Additionally, HPS genome was also found in samples from 2 pigs which did not have a pyelitis after histopathological examination. Following bacterial culture, HPS was isolated in 8 collective serosal swabs from pigs with pyelitis and also in 2 samples from pigs without pyelitis (Table 22). A corresponding identification of HPS by both PCR and bacterial culture was seen in samples from 8 pigs with pyelitis. HPS was significantly more frequently detected by PCR ($P<0.001$) and bacterial culture ($P<0.001$) in challenged pigs which had a pyelitis than in samples from animals without a pyelitis.

Table 21: Frequency of HPS genome detection by PCR in samples from challenged pigs and the occurrence of pyelitis

	HPS PCR pos	HPS PCR neg	
Pyelitis present	10	0	n=10
Pyelitis absent	2	15	n=17
	n=12	n=15	

Table 22: Frequency of HPS detection bacterial culture in collective serosal swabs from challenged pigs and the occurrence of pyelitis

	HPS PCR pos- bacterial culture	HPS PCR neg- bacterial culture	
Pyelitis present	8	2	n=10
Pyelitis absent	2	15	n=17
	n=10	n=17	

5 Discussion

5.1 Clinical results

5.1.1 Mortalities

The HPS challenge model used in this study produced peracute, severe clinical signs which resulted in the removal (death or euthanasia) of four pigs from each challenged group within ten days post infection. The high number of deaths corresponds with the findings of previous studies about the highly pathogenic nature of HPS serovar 5. According to RAPP-GABRIELSON et al. (2006), serovar 5 is highly virulent capable of eliciting death in pigs. Similarly, other authors reported the deaths of several pigs shortly after experimental infection with HPS serovar 5 (AMANO et al., 1997; BAK and RIISING, 2002; DE LA FUENTE et al., 2008b). Serovar 5 was selected for this study since it is one of the most prevalent serovars affecting swine producers in Germany and therefore suitable for testing the efficacy of tulathromycin (KIELSTEIN et al., 2001; RAPP-GABRIELSON et al., 2006).

The timing of these the deaths, however, differed numerically but not statistically between the individual study groups. Four pigs from the nontreated-challenged and tulathromycin 4 day groups died within four days after challenge. In contrast, only one pig from the tulathromycin 7 day group died during this time. The remaining 3 pigs from this group died between days 6 and 10. HOOVER (2009) investigated the efficacy of tulathromycin in weaned pigs over a 56-day period and found that mortality rates were significantly lower in tulathromycin-treated pigs than pigs which did not receive tulathromycin. Likewise, NUTSCH et al. (2005) reported significantly lower mortality rates in feeder pigs with swine respiratory disease including HPS which received tulathromycin. In these and other tulathromycin studies, the therapeutic efficacy of tulathromycin was assessed based on either reports from field studies or based on experimental infection where tulathromycin was always administered directly after infection with HPS (EVANS, 2005; NANJIANI et al., 2005). This is the first challenge study which investigates the efficacy of tulathromycin in pigs which were infected after treatment.

It could be inferred from these study results that an antimicrobial effect of tulathromycin was present which may have prolonged the lethal effects of the serovar

5 in both tulathromycin groups. Currently, 2.5 mg/kg BW is the only registered dose for tulathromycin application in pigs. This dose was selected for the study since it corresponds to the present dose that swine producers are currently using, and is the dose proven to be effective in extensive laboratory and field trials against a variety of pathogens including HPS. Another reason for the apparent absence of a significant antimicrobial effect may be that the HPS strain used in this study was resistant towards tulathromycin. However, the results of the MIC test showed that the HPS serovar 5 strain used in this study was susceptible towards tulathromcyin (MIC 500 µg/l). NANJIANI et al. (2005) determined that the MIC of various HPS strains of swine farms from four European countries was 4 µg/ml. The differences in challenge strain MIC compared to the pan-European survey values may be due to the fact that either the HPS strains or serovars used in this study were not identical to those tested by NANJIANI et al. The application of the relatively high inoculum dose intratracheally may have overcome any significant antimicrobial effect. According to AMANO et al. (1997), an organism inoculated intratracheally circumvents the mucosal immunity of the upper respiratory tract and can cause systemic infection more easily. Thus an antimicrobial effect may have existed but due to the high dose and intratracheal application, a significant antimicrobial effect was not observed. Furthermore, the administration time of tulathromycin may have been unsuitable for this challenge model. After intramuscular application, the plasma concentration of tulathromycin is 0.58 µg/ml. In contrast, the lung concentrations are up to 181 times higher than the plasma concentration (EVANS, 2005). The lung-half life time of tulathromycin is 6 days. Therefore, an application of tulathromycin seven and four days prior to infection means that only half of the active ingredient is available to protect the pig against septicemic spread. Further studies are necessary to confirm the results observed in this study.

5.1.2 Clinical examinations

In this study, in addition to high mortalities, all characteristic clinical signs indicative of Glässer's disease were observed within 48 hours after challenge. Generally, there are a number of pathogens which can elicit clinical signs similar to HPS infection such as *Mycoplasma hyorhinis*. Screening of all study animals prior to and during the study showed that all animals were negative for HPS, *Mycoplasma hyorhinis* and *Actinobacillus pleuropneumoniae*. Based on the clinical scores throughout the entire

study, pigs from the control group did not display signs of clinical illness and the highest clinical score of 4 was recorded on Day -4 for this group. On this particular day, a median clinical score of 6 indicating severe sickness were seen in pigs from the tulathromycin 4 group. As demonstrated in the following days leading up to the challenge day (Day 0), clinical scores indicating that all 36 pigs were clinically healthy were recorded. Since an infection was not present in any of the pigs before the scheduled infection on Day 0 and screening results indicated that all pigs were clinically healthy until challenge Day 0, this peak may have been due to higher scoring in parameters such as sclera or conjunctiva appearance for a few pigs which subsequently led to an overall high total daily clinical score for that day. Furthermore, a clinical score of four was recorded for this group partly because this was an infection of commercial pigs and not an infection of gnotobiotic animals. Previous HPS experimental studies predominantly used either SPF animals or gnotobiotic pigs (AMANO et al., 1997; BLANCO et al., 2008; OLIVEIRA et al., 2003a). Following challenge on Day 0, animals from the challenged groups displayed severe clinical symptoms from all 16 parameters mentioned in the material and methods section. Although significant differences among the three challenged groups were not found, differences in the severity and occurrence of the sickness were evident among the challenged groups. Pigs from the nontreated-challenged group were the first to showed signs of mild to severe sickness in comparison to pigs from the tulathromycin groups. High scores were recorded up to study day 5 for this group. Meanwhile scores indicating mild to moderate illness were seen in both tulathromycin groups from study day 5 to study day 11. Within the first four days after challenge, 4 pigs from the nontreated-challenged group displayed symptoms of severe illness which led to the death or removal of these pigs. The score from these pigs influenced the overall total daily clinical score for that group hence the peaks until all four pigs and their clinical data were removed from the study after Day 4. During this time, the same number of deaths was reported only from pigs of the tulathromycin 4 day group. Only one pig died from the tulathromycin 7 day group during this time. Therefore, these pigs with clinical scores indicating sickness, survived longer and subsequently influenced the overall total daily clinical score of their respective groups until these pigs along with their data were also excluded from the study on Day 10. Hence the longer display of high clinical scores as seen in Figure 5 until Day 10 for both tulathromycin groups.

OLIVEIRA and PIJOAN (2002) found that the severity of clinical signs depended not only on the virulence of the strain but also on the immune status of the pig and the current stage of infection. In this study, a relatively high infection dose (5 x10^8 CFU) was used to infect the pigs. According to OLIVEIRA et al. (2003a), the ideal dose used for reproduction of HPS infections depends on the characteristics of the aims of the study. Depending on the dose, either a colonization of the pig is achieved or the manifestation of clinical signs of disease. In order to test the efficacy of tulathromycin, the manifestation of clinical signs in the pigs were required for this study. The clinical signs manifested in the pigs without taking treatment into consideration correspond to the findings of other experimental studies. OLIVEIRA et al. (2003a) found that animals inoculated with 10^8 to 10^9 CFU developed systemic infections compared with animals inoculated with a lower dose such as 10^6 to 10^7 CFU. After infecting SPF piglets with 10^6 to 10^9 HPS serovar 5, BLANCO et al. (2008) found that piglets displayed symptoms of severe illness such as dyspnoea, cyanosis, lameness and central nervous impairment. Similarly, SEGALES et al. (1999) and MACINNES et al. (2003) observed severe clinical symptoms such as lethargy, recumbency and even sudden death in infected pigs shortly after infection with 10^7 CFU HPS serovar 5.
Notwithstanding this apparent success of the infection model, a significant difference between the nontreated-challenged pigs and the pigs receiving tulathromycin was not observed. In pigs treated with therapeutically with tulathromycin, the overall cure rate was significantly higher in comparison to cure rates of pigs which did not receive tulathromycin (NUTSCH et al., 2005). NANJIANI et al. (2005) found that the severity of clinical symptoms were lower in the tulathromycin–treated animals than animals without tulathromycin. Similarly, clinical symptoms were significantly lower in tulathromycin-treated pigs over a 56-day study period than pig which did not receive tulathromycin (HOOVER, 2009). As mentioned previously, these studies tested tulathromycin under field conditions or in experimental studies whereby tulathromycin administration occurred directly after the infection. Factors such as too high inoculum dose or the untimely application of tulathromycin may have hindered tulathromycin from preventing the septicemia spread and thus the severe clinical symptoms seen in both tulathromycin treated groups.

5.1.3 Average daily gain (ADG)

The average daily weight gains of pigs from all groups before challenge (Days-17 to 0) were comparable and a significant difference among the groups was not observed. In contrast, the average daily weight gains of pigs from all groups between Days 0 to 14/15 varied widely. The average daily gains of pigs from the control group were higher than the ADGs of pigs from the challenged groups between Days 0 to 14/15 but a significant difference could not be established. The low weight gains seen in all challenged pigs in comparison to the control pigs corresponds to the total daily clinical score for these animals observed over the course of the study. Mild to severe illness were seen in all challenged pigs, and therefore a subsequent decrease in body weight or slow weight gain is expected in animals that are sick.

Only pigs from the tulathromycin 4 day group had an ADG comparable to the ADGs of the control group. Similarly, the third highest average daily gain between Days 0 to 14/15 was recorded by pigs from the nontreated-challenged. Pigs from the tulathromycin 7 day group had the lowest ADGs from all challenge groups during this period. On European farms infected with pathogens of the swine respiratory disease complex such as HPS, NANJIANI et al. (2005) found that pigs administered different doses of tulathromycin did not show any significantly improved ADG in comparison to pigs which did not receive tulathromycin. Conversely, HOOVER (2009) investigated the efficacy of tulathromycin against swine respiratory disease including HPS and found that tulathromycin treated animals gained significantly more weight than pigs which did not receive tulathromycin. In contrast to this present study, both studies tested the efficacy of tulathromycin in pigs which were not only sick with an unverifiable amount of HPS but also infected with other swine pathogens. Since pigs from the tulathromycin 4 day group had the best ADG's from all challenged pigs, an antimicrobial effect may have been present. However, an explanation as to why pigs from the tulathromycin 7 day group had worse ADGs in comparison to the ADGs of the nontreated-challenged pigs remains to be determined. Further investigations are necessary to confirm these results.

5.1.4 Rectal temperatures

The rectal temperatures of pigs from all study groups were relatively high for the entire study period and a significant difference among the four study groups was not seen either prior to or after challenge. Nevertheless, peak temperatures were seen

one day before challenge on Day -7, and on Days 1 and 7 directly after challenge. According to STRAW et al. (2008), the rectal temperatures of healthy pigs between 9-45 kg ranges between 39.3 °C and 39.0 °C respectively. It is most likely that the septicemic spread of HPS in the pigs resulted in fever which was responsible for the peak temperatures seen on Days 1 and 6 in challenged pigs. SOLANO et al. (1997) reported pronounced but not significant rectal temperatures differences among challenged and control pigs one day after infection with HPS. Similarly, DE LA FUENTE et al. (2009) and VAHLE et al. (1995) observed a mild transient rise in rectal temperature between 40.0 °C and 40.5 °C directly after challenge with HPS. Screening results of all pigs prior to and after challenge indicated that all animals were healthy until infection with HPS on challenge day 0; therefore, some other factor must have influenced the rectal temperature measurements of the pigs before HPS infection. Furthermore, extreme variations in the temperatures of all pigs over the course of the study were observed. The extremely high and fluctuating temperatures observed throughout this study were most likely due to the manual restraint of the pigs and not solely the result of the HPS challenge. In order to measure the rectal temperature and assess the synovial fluid in each joint, every animal was manually restrained by at least one assisting veterinarian in this study. While under restraint, the pigs struggled and made several attempts to escape. Various authors demonstrated that pigs are very susceptible to stress and manual handling in pigs is associated with an extreme stress reaction which leads to elevated cortisol levels and rectal temperatures (MADEJ et al., 1996; ROOZEN et al., 1995).

5.2 Blood examination (Leukocyte population)

The course of the average leukocyte population of the four study groups differed throughout the study. Since the control pigs were not infected with HPS, an increase in the leukocyte population was not expected. In contrast, the leukocyte population of the challenged animals was notably higher than the leukocyte population of the control animals. According to FRIENDSHIP et al. (1984), the reference value for the leukocyte count in weaned piglets is 8.7 - 37.9 x 10^9 cells/L. Elevated leukocyte populations were seen one day directly after challenge. This corresponds to the short incubation time of 24 hours reported by DE LA FUENTE et al. (2008b) after experimental infection with HPS serovar 5 in weaned piglets. After this rise in leukocyte numbers, the number of leukocytes decreased on Day 3, then gradually

rose up to Day 7 and finally decreased until study termination. HEINRITZI (2006) described a similar course of the leukocyte population after HPS infection. However, AMANO et al. (1997) observed a severe leucopenia 24 hours after challenge until death.

Contrary to the expectations of this study, a significant difference between the leukocyte population of the tulathromycin groups and the untreated-challenge group was not observed. Only pigs from the tulathromycin 4 day group had lower leukocyte populations in comparison to pigs from the nontreated-challenged group. Pigs from the tulathromycin 7 day group had the highest leukocyte populations during the entire study. For reasons previously discussed, apparently the tulathromycin administration was unable to significantly prevent or lessen the septicemic spread of the disease in the tulathromycin groups which led to the dramatic increase in the leukocyte population.

5.3 Gross lesions

5.3.1 Lung lesions

Lung lesions indicative of pneumonia were seen in the lungs of pigs from all three challenged groups. As expected, no lung lesions were observed in the lungs of pigs from the control group. According to SOLANO-AGUILAR et al. (1999) and RITZMANN and HEINRITZI (2005), gross lesions of Glässer's disease are characterized by a catarrhal-purulent bronchopneumonia. OLIVEIRA and PIJOAN (2002) found that pneumonia is characterize by antero-ventral consolidation with purulent exudate in the bronchi and bronchioli. Moreover, DE LA FUENTE (2008a) found lesions of exudative pneumonia with alveolar edema and hyperemia in the cranial lung lobes of HPS experimentally infected weaned piglets. In this study, lesions in all pigs were diffusely spread throughout the entire lung parenchyma of all lung lobes. A consolidation of an individual lung lobe was not observed. The lack of a purulent component in the lung lesions most likely indicates an early infection stage in the pigs.

Although the lungs of pigs from the nontreated-challenged group showed the most lung lesions, a significant difference among the three challenged groups was not observed. Based on the pharmacokinetic properties of tulathromycin, fewer lung lesions were expected in the pigs that received tulathromycin in comparison to the

nontreated-challenged pigs. According to BENCHAOUI et al. (2004) the high distribution and slow elimination following a single dose of tulathromycin are desirable pharmacokinetic properties for an antibiotic indicated for the treatment of respiratory disease in swine. In a study evaluating the therapeutic activity of tulathromycin against pathogens of the swine respiratory disease complex on farms in Europe, NANJIANI et al. (2005) found that pigs treated with tulathromycin showed better clinical improvements in comparison to pigs without tulathromycin. The same author noted that the improvement lasted at least 10 days after treatment. Similarly, NUTSCH et al. (2005) and HOOVER (2009) found that a single intramuscular dose of tulathromycin was effective in the treatment of swine respiratory disease. Furthermore, in studies evaluating the efficacy of tulathromycin for the treatment of pneumonia following experimental infection of swine with *Mycoplasma hyopneumoniae*, STANFORD (2008) and MCKELVIE et al. (2005) found that tulathromycin was effective in the treatment of pneumonia.

After intramuscular injection, tulathromycin has a high bioavailability (88 %) and achieves concentrations in the lungs which are significantly higher than plasma concentrations (EVANS, 2005). Moreover, tulathromycin has a lung half-life of 6 days (BENCHAOUI et al., 2004; NOWAKOWSKI et al., 2004). This long half-life in lung tissues means that the drug concentrations are at therapeutic levels for several days, increasing the exposure time of pathogens such as HPS to tulathromycin and thus possibly optimizing the antibacterial activity. Therefore, in the case of the pigs which received tulathromycin seven and four days prior to HPS infection, the maximum active ingredient concentration possible to prevent septicemic spread was not present. It is also possible that the intratracheal administration of the relatively high challenge inoculums dose was sufficient to overcome any antimicrobial effect of tulathromycin in the lung thus resulting in lung lesions in pigs from both treatment groups. Another explanation for the study results may be due to the infection route used. According to AMANO et al. (1997), an organism inoculated intratracheally circumvents the mucosal immunity of the upper respiratory tract, and can cause systemic infection more easily. Also, if the intratracheal application is performed incorrectly, the tracheal catheter can enter the lung lobe and significantly damage lung tissue while doing so. Furthermore, various authors noted that pathogens of the oropharyngeal cavity such as *Streptococcus suis and Escherichia coli* could enter the lungs if the tracheal catheter comes in contact with the mucous membrane or tonsils

(GANTER et al., 1993; HENNIG-PAUKA et al., 2007; KIPPER, 1990). These additional pathogens could compound the pathogen level which the tulathromycin would have to work against.

5.3.2 Synovial fluid assessment

Pathological changes in the synovial fluids were seen in all pigs from each study group including the control pigs. However, the synovial fluids of pigs from the control group were less pathologically changed in comparison to the fluids from the challenged pigs. Since the control pigs were not infected with HPS, it is assumed that a noninfectious source such as blunt trauma was responsible for the synovial changes observed in this group. Severe changes in appearance and/or content implies a fibrinous to fibrinopurulent inflammatory process in the joint. The synovial fluid changes seen in the challenged pigs without taking treatment into consideration are consistent with findings from other HPS experimental studies. After infection of both colostrum-deprived and sow-reared piglets with HPS serovar 5, more than half of the animals had distended joint capsules (BLANCO et al., 2004). DE LA FUENTE (2008a) found that the joints were distended by variable amounts of fibrinopurulent exudates. Similarly, SOLANO-AGUILAR et al. (1998) noted that some of the infected pigs had swollen joints due to fibrinous exudate. Comparable results were also reported by VAHLE et al. (1995). This author found that the carpal joints were covered by variable amounts of fibrinopurulent exudate which often extended into adjacent periarticular soft tissues and fascial planes.

Although a significant difference among the groups was not found, the synovial fluids obtained from the nontreated-challenged pigs were all pathologically altered in contrast to fluids from pigs of both tulathromycin groups. Furthermore, synovial fluid from pigs of the tulathromycin 7 day group had the least amount of pathological changes. A literature survey did not yield any information about pharmacokinetic properties of tulathromycin in the joint. Due to the fact that mild to severe changes in the synovial fluids were seen in tulathromycin treated pigs, a significant antimicrobial effect of tulathromycin is possible. It is possible that tulathromycin may have been present in the joint but in too low concentrations to elicit a significant antimicrobial effect to prevent synovial fluid changes. Tulathromycin has the pharmacodynamic property of accumulating in blood polymorphonuclear leukocytes or neutrophils (SIEGEL et al., 2004). Thus pigs which receive tulathromycin earlier, in this case the

tulathromycin 7 day pigs, would subsequently have high plasma concentrations of tulathromycin. Further investigations are necessary to confirm these observations.

5.3.3 Cerebrospinal fluid assessment

The cerebrospinal fluid was assessed based on the same criteria used for the synovial fluid assessment. Pathological changes in the cerebrospinal fluids were seen in samples from all four study groups. However, the severity of the changes differed significantly among the groups. Mild pathological changes in the cerebrospinal fluids from two control pigs were seen. These changes are probably due to a noninfectious origin since screening results showed that the pigs were clinically healthy and not infected with HPS. Moreover, the corresponding brain assessments demonstrated that the brains were pathologically unchanged. In contrast to the results from the control pigs, moderate to severe pathological changes in the cerebrospinal fluids were seen in 5 pigs from each challenge group. A detailed description of cerebrospinal fluid assessment by an HPS infection in the literature is very seldom. Based on previous studies, characteristic gross pathological lesions by HPS infection include all stages of meningitis (BLANCO et al., 2004; DE LA FUENTE et al., 2008a; MACINNES and DESROSIERS, 1999). It can be assumed that meningitis is accompanied by corresponding changes in the cerebrospinal fluid.
Although pathological changes were seen in the cerebrospinal fluids from 5 pigs from all challenged groups, pigs from the nontreated-challenged group had the most cases of pathologically changed cerebrospinal fluid. This improvement in the fluids of the tulathromycin group may be attributed to tulathromycin. However, there exist no current literature about the pharmacokinetic properties of tulathromycin and its influence on the central nervous system. Further investigations are necessary to confirm the results seen in this study.

5.3.4 Brain assessment

Pathological changes in the brains were only observed in the challenged pigs. Since the control pigs were not challenged and the screening results indicated that these pigs were healthy, pathological changes were not expected. Changes seen consisted primarily of hyperemia, edema and pallor. DE LA FUENTE et al. (2008b) found that the brains of HPS infected weaned piglets were hyperemic with petechiae or echymoses. Similarly observations were also made by AMANO et al. (1994) after the

inoculation of pigs with HPS serovars 1, 4, and 5. Although petechiae or ecchymoses were not seen in this study, the macroscopic observations after the brain assessment are consistent with the results from previous experimental HPS studies.

In contrast to pigs from the nontreated-challenged and tulathromycin 7 day groups, the brains of pigs from the tulathromycin 4 day group were all pathologically changed. Only 6 pigs from the nontreated-challenged group and 7 pigs from the tulathromycin 7 day group had pathological brain changes. Due to the fact that even the tulathromycin pigs had brain lesions, a significant antimicrobial effect of the tulathromycin treatment cannot be established. As mentioned in the previous section, the pharmacokinetic properties of tulathromycin and its effect on the central nervous system have yet to be determined. Further studies are necessary to confirm the observations made in this study.

5.3.5 Assessment of serosal membranes and cavities

According to RITZMANN and HEINRITZI (2005), HPS has a clear affinity to serosal surfaces, and bacteremia follows replication at serosal surfaces which subsequently produces the characteristic polyserositis observed in pigs (AMANO et al., 1994; VAHLE et al., 1995). After challenge, polyserositis was observed in several pigs from all the challenged groups, and pigs from the tulathromycin 4 day group had the most cases of inflammations followed by pigs from the tulathromycin 7 day and nontreated-challenged groups. Moreover, adherences in pleura, pericardium, and peritoneum including hydrothorax, hydropericardium and hydroperitoneum were also seen. In previous studies by BLANCO et al. (2004) and BAK and RIISING (2002) severe polyserositis and adherences in the serosal cavities after experimental infection with HPS were observed. Similarly, SOLANO-AGUILAR et al. (1999) found that severe serositis was consistently observed in most HPS infected pigs.

All three inflammation types were seen in pigs from all study groups. However, the severity of the individual inflammations pleuritis, pericarditis and peritonitis differed among the groups. Pleuritis was only observed in pigs from the challenged groups. The most cases of severe pleuritis were seen in pigs from the tulathromycin 4 day followed by pigs from the nontreated-challenged and the tulathromycin 7 day groups. In contrast to pigs from the nontreated-challenged group, the most cases of severe pericarditis were seen pigs from both tulathromycin groups. In contrast to the pleuritis and pericarditis, peritonitis was observed in pigs from all study groups. Two pigs from

the control group had a mild peritonitis, and all the pigs from the tulathromycin 4 day group had some degree of peritonitis in contrast to pigs from the nontreated-challenged and tulathromycin 7 day groups. However, statistical analysis demonstrated that the control pigs had significantly fewer cases of peritonitis in comparison to challenged pigs. Since the control pigs were not infected with HPS and HPS was not detected in any samples from these animals at any time during the study, it is assumed that the peritonitis was caused by another pathogen for example *Escherichia coli*. *Escherichia coli* is not only a commensal of the digestive tract of pigs but also responsible for manifestations such peritonitis (MEYER et al., 1971).
Since pleuritis, pericarditis and peritonitis were observed in both the nontreated-challenged pigs and the tulathromycin pigs, a significant antimicrobial effect of tulathromycin was not established. Detailed studies about the efficacy of tulathromycin and its effectiveness at lessening gross lesions such as pleuritis, pericarditis and peritonitis are currently unavailable. This is the first description about the association of such lesions and the efficacy of tulathromycin. Further examinations are necessary to confirm the results observed in this study.

5.4 Histopathological lesions

After histopathological examination, synovitis, pyelitis and meningitis were observed predominantly in tissue samples from challenged pigs which died prior to study termination. Pyelitis was the most frequent inflammation type found and it occurred either alone or together with a meningitis or synovitis. Although both meningitis and synovitis were observed, meningitis always occurred together with a pyelitis, synovitis or both. In an experimental study to identify lesions and colonization locations of HPS, VAHLE et al. (1995) found fibrinopurulent synovitis in challenged pigs. Numerous studies confirm the findings of meningitis in pigs infected with HPS (AMANO et al., 1994; BLANCO et al., 2004; MACINNES and DESROSIERS, 1999; PALZER et al., 2006a; SOLANO-AGUILAR et al., 1999). Only a few studies described finding kidney lesions to varying degrees. DE LA FUENTE et al. (2008a) described a few cases of necrosis of the renal tubules including calcification and non-purulent interstitial nephritis in HPS infected pigs. Likewise, SOLANO et al. (1999) described the finding of fibrin deposits in the renal glomeruli of one pig experimentally infected with HPS serovar 5. In a field infection of weaned piglets in Australia, PEET et al. (1983) found kidney lesions in four pigs which were characterized by glomerular

thrombosis. Although the pigs in these studies demonstrated kidney lesions, pyelitis was never observed. This is the first study which describes a pyelitis in an HPS infected pig and further studies are necessary to confirm these observations.
Despite tulathromycin application, a significant difference in the occurrence of the individual inflammations between the nontreated-challenged group and pigs from the tulathromycin groups was not observed. As mentioned previously in section 5.3.5, detailed study results about the efficacy of tulathromycin and its antimicrobial efficacy at preventing gross or histopathological lesions do not exist. This is the first description about the association of such lesions and tulathromycin efficacy and further studies are necessary to confirm the finding of these studies.

5.5 Detection of HPS by PCR and bacterial culture

HPS could be detected by PCR and bacterial culture in sample materials from at least 18 challenged pigs. HPS genome was detected in at least one of five sample materials from pigs of all three challenged groups, and HPS was reisolated in 10 collective serosal swabs of pigs from all three challenged groups. As expected, HPS was not detected by PCR or culture in samples from the control pigs. Although a significant difference among the sample materials for HPS genome was not established in this study, HPS genome was most frequently detected in the collective serosal swabs and cerebrospinal fluids. This detection frequency corresponds to findings by PALZER et al. (2006c). ANGEN et al. (2007) noted that swabs were successfully used for sampling diseased animals for HPS diagnosis particularly by PCR. Furthermore, swabs allow easier handling and transportation compared to tissue samples. BLANCO et al. (2008) reported that the best chances for HPS detection by either PCR or bacterial culture are achieved when a combination of samples from the meninges, peritoneum and pleura were used. Likewise, OLIVEIRA et al. (2004) noted that ideal sites for isolation are the brain (meninges) pericardium, pleura, peritoneum, and joints. All of these recommended sampling sites were sampled when the five sample materials were used in this study.
Although HPS was detected in samples from nontreated-challenged pigs and tulathromycin pigs, a significant difference of HPS detection among the challenged groups was not observed. These results demonstrate that HPS detection is possible despite previous antibiotic treatment. Similar results were observed by HAEDKE

(2008) for the detection of HPS genome by PCR in collective serosal swabs from pigs treated with antibiotics.

After PCR detection, HPS was detected in the synovial samples from animals which died at study termination. Notably, HPS was not detected in the corresponding joint capsules. Similarly, HPS was detected predominantly in joint capsules from animals which died prior to study termination while the corresponding synovial fluids were negative for HPS genome. Therefore, at no point in this study, a corresponding identification of HPS genome in both synovial fluid and joint capsules was observed. Differences in the detection frequency of HPS may be due to different amount of the bacterium present in the synovial fluid and joint capsules. OLIVEIRA et al. (2001a) reported that the PCR required a minimum of 100 CFU ml^{-1} for detection. Another explanation may be that the bacterium is first localized in the joint capsule during the early stages of infection and in later stages of infection only in the synovial fluid. This is assumed since HPS was only detected in synovial fluids from animal which died at study termination. It is possible that different results may have been obtained if sample material from different joints including the right tarsal joint were used since only sample material from the left tarsal joint was used for PCR diagnostic. Further examinations are necessary to understand the mechanism of HPS colonization in the joint.

5.6 Comparison of HPS detection by PCR and bacterial culture in collective serosal swabs from challenged pigs

HPS genome was detected by PCR in twelve collective serosal swabs from challenged pigs. In contrast, HPS was only detected after bacterial culture in collective serosal swabs from 10 challenged pigs which died prior to study termination. A corresponding identification of HPS by both PCR and bacterial samples was seen for 10 samples. Although a significant difference between the successful detection of HPS was not established among both detection methods, HPS was more frequently detected by PCR testing than bacterial culture. Similarly, OLIVEIRA et al. (2006) found that PCR is a far more sensitive method for HPS detection in comparison to bacterial culture. According to RAPP-GABRIELSON et al. (2006) PCR is useful for diagnosis when bacterial isolation is negative. Conversely, TURNI and BLACKALL (2007) found that the PCR did not perform as well as culture due to the low amount of HPS present in samples. Likewise, BLANCO et al. (2008)

found that HPS isolation was more successful after using bacterial culture than PCR. However, this author noted that PCR is still an appropriate tool for detection of HPS since bacterial isolation is limited due to the fastidious nature of the bacterium. Furthermore the same author reported that bacterial isolation is not always possible when samples are isolated form dead pigs due to the possible contamination by other bacteria.

5.7 Associations between HPS detection, the clinical results and pathological lesions

5.7.1 Association between HPS detection and clinical results

After HPS challenge on Day 0, mild to severe clinical signs associated with Glässer's disease were seen in all challenged animals. Moreover, 12 pigs from all three challenge groups died within ten days post challenge as a result of their severe clinical symptoms. HPS genome was detected in sample materials from 9 animals which were clinically sick and in all sample materials from animals which died prior to study termination. HPS genome was also detected in a total of 6 sample materials from pigs which died at study termination and in sample materials from 3 animals which were clinically healthy. It is possible that given more time, those pigs could have developed more obvious lesions, or perhaps the immune system of the pig was able to control bacterial growth and prevent the lesions from developing in those tissues. Similarly, HPS was isolated by bacterial culture in sample materials from 8 animals which were clinically sick and in 10 animals which died prior to study termination. Based on these results, a significant association between HPS detection by PCR or bacterial culture and sample materials which came from animals with clinical signs and from animals that died prior to study termination was established. This finding is consistent with the results from HAEDKE (2008) and PALZER et al. (2006b). OLIVEIRA (2004) observed successful HPS detection when animals with clinical signs of HPS infection are used. The same author also noted that isolation from chronically affected pigs is usually unsuccessful. TURNI and BLACKALL (2007) found more frequent HPS detection in sample materials from animals which were euthanized in comparison to animals which only showed mild clinical signs. In one of the first studies on HPS detection, HPS was readily recovered from pigs in the acute stages of experimental infection, but was isolated only sporadically from pigs with lameness at later time (NEIL et al., 1969).

As mentioned previously in section 5.5, HPS synovial fluids were only positive in samples from animals that died at study termination. Notably, HPS genome was not identified in the corresponding joint capsules. The joint capsules and cerebrospinal fluids were the only sample materials which successfully identified HPS genome in samples from animals that died both prior to and at study termination. However, HPS genome could not be detected in the corresponding synovial fluids of these animals. An explanation for these observations could be that the bacterium is first localized in the joint capsule at the early stages of infection and in later stages of infection only in the synovial fluid. Notably, only samples from the left tarsal joint were used for HPS diagnostic. Further examination is necessary to see if a similar detection pattern exists for samples from other joints.

5.7.2 Association between HPS detection and pathological lesions

Gross and histological lesions characteristic of Glässer's disease were seen in several pigs from all three challenged groups. Polyserositis was found in 17 pigs from three challenged groups. Findings of inflammations of the serosal membranes with fibrinous to fibrinopurulent exudates is a characteristic feature of an acute HPS infection (OLIVEIRA and PIJOAN, 2002). In this study, HPS was detected significantly more frequently by PCR and bacterial culture in collective serosal swabs from animals with a polyserositis than animals without a polyserositis. These results are consistent with the findings of HAEDKE (2008) who also found a significant association between the detection of HPS in collective serosal swabs and polyserositis. In one case, HPS was detected although a polyserositis was not present. According to PALZER et al. (2006c), the detection of HPS genome in collective serosal swabs from animals without pathological lesions are possibly due to the fact that an early stage of infection is present.

By the differentiation between inflammations of the pleura, pericardium and peritoneum, significant associations were not observed in all cases. HPS was detected significantly more frequently in samples from animals with a pleuritis and peritonitis. Similar observations were made by HAEDKE (2008) who found a significant association between the detection of HPS genome and pleuritis and pericarditis. HPS could be detected in samples from pigs with and without pericarditis but a significant association between HPS detection and pericarditis was not established. In this case, as previously mentioned, the possibility of HPS detection

without pathological lesions due to an early infection stage needs to be taken into consideration (PALZER et al., 2006c).

Although HPS genome was found in the brain, synovial and cerebrospinal fluids of animals with and without meningitis and synovitis, a significant association between HPS detection and these gross and histopathological lesions was not observed in this study. HADEKE (2008) also did not find a significant association between the detection of HPS genome and changes in the joint. In contrast, PALZER et al. (2006c) found an association between the detection of HPS genome from collective serosal swabs and pathologically changed joints. As mentioned previously, the fact that sample materials for the HPS genome detection were only obtained from the left tarsal joint, it is possible that differing results may have been observed if samples from other joints were also used for diagnostic in this study.

SOLANO et al. (1997) and VAHLE et al. (1995) found that cerebrospinal fluid was an excellent material for isolation of the bacteria when animals showed central nervous signs. It is possible that the bacterium was present in the respective tissue and fluids but it was not in sufficient amounts to be detected by this HPS PCR. The dynamics of how the bacterium interacts with joint tissues and meninges remains to be determined (BLANCO et al., 2008).

One notable discovery after histopathological examination was the pyelitis occurrence in 10 challenged pigs. HPS genome was detected significantly in sample materials from 10 of these pigs and in collective serosal swabs of 8 pigs with pyelitis. HPS was detected by both PCR and bacterial culture in samples from two pigs without a pyelitis. As previously mentioned, the bacteria was probably present in the tissue and given more time, maybe corresponding histopathological lesions would have appeared. Histopathological examinations of pigs from some previous studies indicated kidney and pelvis lesions after HPS infection (DE LA FUENTE et al., 2008a; PEET et al., 1983; SOLANO et al., 1997). Pyelitis has never been mentioned in these studies or any other publications as a lesion of HPS infection. Furthermore, a correlation between the occurrence of pyelitis or kidney and pelvis lesions and the detection of HPS has never been determined. This study found that HPS was more frequently detected in samples from animals with a pyelitis than animals without a pyelitis. These results are being reported for the first time and further studies are necessary to confirm these results.

5.8 Conclusion

The aim of this study was to evaluate the efficacy the two different tulathromycin treatments administered as a single dose prior to an intratracheally challenge with HPS serovar 5 in 36 weaned piglets. The efficacy the two tulathromycin treatments was evaluated based on parameters such as mortalities, the results from the clinical examinations and pathological lesions.

The HPS challenge model produced peracute, severe clinical signs which resulted in the death or euthanasia of four pigs from all three challenged groups within ten days post infection. A significant difference among the challenged groups was not observed. Likewise, the average daily weight gains (ADG) and rectal temperatures of tulathromycin-treated animals did not differ significantly from the nontreated-challenged pigs. Furthermore, a significant difference among the leukocyte population of the pigs was only observed between the control pigs and the challenged pigs. Typical gross and histopatholgical lesions of Glässer's disease such as pleuritis, pericarditis, peritonitis, meningitis, and synovitis were observed after necropsy. But a significant difference among the challenged groups was not seen. In addition to these lesions, pyelitis was identified in seven challenged pigs. This is the first study which reports the findings of these lesions in HPS infected pigs and further studies are necessary to confirm these observations.

The fact that clinical signs, gross and histopathological lesions indicative of Glässer's disease were observed after intratracheal challenge with HPS serovar 5, confirms the success of this infection model. However, according to the efficacy evaluation parameters of this study, the administration of tulathromycin 4 and 7 days prior to an infection with HPS serovar 5 did not appear to have a significant antimicrobial effect against the infection dose used in this study. This may have been due to the challenge model setup. For example, the challenge inoculum dose may have been too high or the inoculation route used in the study was inappropriate. Intratracheal administration has the advantage of overcoming the mucosal defense thus facilitating systemic infection more easily. Other routes such as intranasal infection should be taken into consideration since it represents the most natural route of HPS infection. Further studies examining the efficacy of tulathromycin taking these changes into consideration are necessary.

In addition to clinical and pathological evaluation, associations between the frequency of HPS detection by PCR and bacterial culture and the results from the clinical and pathological parameters were assessed. HPS was significantly detected by PCR and bacterial culture in samples from animals with clinical symptoms and animals which died acutely prior to study termination. Furthermore, a significant association between the detection of HPS and sample materials from animals with pleuritis, peritonitis, meningitis and pyelitis was established. Bacterial isolation of HPS was only successful in collective serosal swabs from animals which died prior to study termination. HPS genome was detected by PCR in all five sample materials but the most successful detection was seen after the testing of the collective serosal swabs and cerebrospinal fluids. Notably, HPS genome was only detected in synovial fluids from animals which died at study termination, while the corresponding joint capsules were negative for HPS genome. Similarly, HPS was detected predominantly in joint capsules from animals which died prior to study termination but the corresponding synovial fluids were negative for HPS genome. Further investigations are necessary to confirm the HPS detection results observed by the synovial fluids and joint capsules since PCR testing was only performed on sample materials from the left tarsal joint. Moreover, studies are required to examine the relationship between gross lesions such as pyelitis and the detection of HPS. Notwithstanding these facts, the detection results demonstrate that the PCR and bacterial examination of various sample materials is a suitable diagnostic tool to detect all stages of HPS infection, even in animals treated with antibiotics.

6 Summary

Once viewed as an infrequent disease of young pigs, Glässer's disease has emerged as a major pathogen affecting naïve swine herds (BLANCO et al., 2008). This disease is characterized by polyserositis, arthritis and meningitis in swine (RITZMANN and HEINRITZI, 2005). Due to the genetic diversity of *Haemophilus parasuis* (HPS), the etiological agent of Glässer's disease, vaccine regimes are not always effective and prudent use of antimicrobials is considered an important component in controlling the disease. The aim of this study was to evaluate the efficacy of two different tulathromycin treatments (Draxxin®, Pfizer, GmbH, Berlin) administered as a single dose prior to an intratracheally infection with HPS serovar 5 in weaned piglets. The efficacy of the two tulathromycin treatments was evaluated based on mortalities and the results from the clinical examinations and pathological lesions.

Thirty-six piglets, 3 weeks of age, were obtained from a commercial nursery site with no previous history of porcine reproductive and respiratory syndrome virus (PRRSV), *Mycoplasma hyopneumoniae* or HPS. Antibodies for *Actinobacillus pleuropneumoniae* were also not detected in blood samples from the study pigs. After arrival at the biosafety level 3 facility of the Clinic for Swine, Veterinary University Vienna Austria on study day -17, piglets were housed in separate units and assigned to four study groups: control, nontreated-challenged, tulathromycin 4 day pretreated-challenged, and tulathromycin 7 day pretreated-challenged. All piglets were allowed a 10-day acclimatization period before piglets of the tulathromycin-7-day group received the registered dose of 2.5 mg/kg BW tulathromycin intramuscularly on study day -7. Three days later (Day -4) piglets from the tulathromycin-4-day group were also administered 2.5 mg/kg BW tulathromycin per intramuscular injection. With the exception of pigs from the control group, all pigs were administered 5 ml of 5 x 10^8 CFU HPS serovar 5 intratracheally on study day 0. Pigs from the control group received phosphate-bufferedsaline (PBS) instead. Clinical examinations of all pigs occurred at least once daily until study termination on Days 14 and 15. Furthermore, necropsy and histopathological examination was performed at study termination or prior to study termination if a pig died or was euthanized due to severe clinical symptoms. In addition to these examinations, synovial fluid, cerebrospinal fluid, joint capsules and brain swabs (meninges) were

collected from all animals for PCR. Collective serosal swabs from all 36 piglets were also tested for HPS by PCR and bacterial culture.

After challenge, severe clinical signs indicative of Glässer's disease were seen in pigs from all three challenged groups, and 44 % of the challenged pig (4 pigss from each challenged group) died within ten days post infection. Significant differences between the nontreated-challenged group and pigs which were administered tulathromycin for these observations were not established. Rectal temperatures and average daily weight gains (ADGs) of tulathromycin-treated animals did not differ significantly from those of the nontreated-challenged pigs. Gross and histopathological lesions of Glässer's disease were seen in pigs from all three challenged groups but a significant difference among these groups was not found. Furthermore, pyelitis was observed in seven challenged pigs. This is the first study which reports the findings of these lesions in HPS infected pigs and further studies are required to confirm these findings. HPS was significantly more frequently detected by PCR and bacterial culture in sample materials from animals which had clinical symptoms and animals which died prior to study termination in comparison to animals which died at study termination and which were clinically healthy. Furthermore, HPS was significantly more frequently detected by PCR and bacterial culture in sample materials from animals with gross lesions such as pleuritis, pericarditis, peritonitis and pyelitis than swabs from animals without such lesions.

In conclusion, based on the results of the clinical examinations, gross and histopathological lesions, the administration of tulathromycin 4 and 7 days prior to the experimental HPS infection did not appear to have a significant antimicrobial effect against the infection dose and serovar used in this study. Reasons for this finding are most likely due to the challenge model used in this study. Factors such as unsuitable application times may have contributed to these findings. Furthermore, the challenge inoculum dose may have been too high or the inoculation route used in the study was inappropriate. Further studies examining the efficacy of tulathromycin taking these changes into consideration are necessary. The results from this study confirm the PCR testing and bacterial culture of various sample materials as suitable diagnostic tools for all stages of HPS infection, even in animals treated with antibiotics prior to testing.

7 Zusammenfassung

Beurteilung der Wirksamkeit von zwei unterschiedlichen Tulathromycin-Behandlungen bei intratracheal mit *Haemophilus parasuis* Serovar 5 infizierten Absetzferkeln

Während die Glässer'sche Krankheit früher als eine sporadische Erkrankung bei jungen Schweinen galt, hat sie sich inzwischen zu einer bedeutenden Erkrankung in naiven Schweineherden entwickelt (BLANCO et al., 2008). Wegen der genetischen Vielfalt von *Haemophilus parasuis* (HPS), dem Erreger der Glässer'schen Krankheit, sind Impfprogramme nicht immer effektiv, und eine gezielte Antibiotikatherapie ist häufig erforderlich, um die Krankheitssymptome zu kontrollieren. Ziel dieser Studie war es, die Wirksamkeit von zwei unterschiedlichen, einmaligen Behandlungen mit Tulathromycin (Draxxin®, Pfizer, USA) vor einer intratrachealen Infektion mit HPS von Absetzferkeln zu evaluieren. Die Wirksamkeit wurde anhand der Mortalitätsraten, der Ergebnisse der klinischen Untersuchungen sowie der pathomorphologischen und pathohistologischen Untersuchungen evaluiert.

Die 36 Ferkel für diese Studie stammten aus einem Ferkelproduktionsbetrieb, der frei ist von Porcinem Respiratorischen und Reproduktiven Syndrom-Virus (PRRSV), *Mycoplasma hyopneumoniae* und HPS. Die Tiere waren zum Zeitpunkt des Einschlusses drei Wochen alt und wurden in vier Gruppen eingeteilt: eine Kontrollgruppe, eine unbehandelt-infizierte Gruppe, eine Gruppe, die am vierten Tag vor der Infektion mit Tulathromycin behandelt wurde (4-Tage-Tulathromycin-infiziert), sowie eine Gruppe, die am siebten Tag vor der Infektion mit Tulathromycin behandelt wurde (7-Tage-Tulathromycin-infiziert). Schweine der 7-Tage-Tulathomycin-Gruppe und der 4-Tage-Tulathromycin-Gruppe bekamen am Studientag -7 bzw. -4 die zugelassene Dosis von 2,5 mg/kg Körpergewicht Tulathromycin intramuskulär appliziert. Am Studientag 0 wurde allen Schweinen, mit Ausnahme der Tiere der Kontrollgruppe, 5 ml einer Suspension aus HPS Serovar 5 (5×10^8 CFU) intratracheal appliziert. Die Tiere wurden während des gesamten Studienverlaufes mindestens einmal täglich klinisch untersucht. Eine pathologische Untersuchung wurde am letzten Studientag oder bei Tieren mit schwerwiegenden klinischen Symptomen am Tag der Tötung bzw. des Verendens durchgeführt. Im Rahmen der Sektion wurden von allen Tieren Synovia, Liquor, Gelenkkapsel und

Gehirntupferproben für eine Untersuchung mittels PCR entnommen. Weiters wurden Serosensammeltupfer von allen 36 Studientieren mittels PCR und Kulturversuch auf HPS untersucht.

Charakteristische Symptome der Glässer'schen Krankheit konnten nach der Infektion bei Tieren aller drei infizierten Gruppen beobachtet werden. Vier Tiere aus jeder infizierten Gruppe verendeten innerhalb von zehn Tagen nach der Infektion. Es konnten keine signifikanten Unterschiede zwischen der unbehandelt-infizierten Gruppe und den Tulathromycin-behandelten Gruppen festgestellt werden. Es konnten sowohl in der pathomorphologischen als auch in der pathohistologischen Untersuchung Veränderungen beobachtet werden, die für die Glässer'sche Krankheit charakteristisch sind. Es gab jedoch keine signifikanten Unterschiede zwischen diesen Gruppen. Darüber hinaus wurde bei sieben der infizierten Tiere eine Pyelitis diagnostiziert. Da in dieser Studie erstmalig solche Veränderungen bei mit HPS infizierten Schweinen beschrieben wurden, sind weitere Untersuchungen nötig, um dies zu bestätigen. Es konnte signifikant häufiger HPS mittels PCR und bakteriologischer Untersuchung bei Tieren nachgewiesen werden, die klinische Symptome zeigten oder vor Studienende verstarben. Des Weiteren konnte HPS signifikant häufiger in Probenmaterialien von Tieren nachgewiesen werden, die pathologische Veränderungen wie Pleuritis, Peritonitis und Pyelitis zeigten.

Aufgrund der Ergebnisse der klinischen Untersuchung, sowie der pathomorphologischen und pathohistologischen Veränderungen scheint die Applikation von Tulathromycin vier bzw. sieben Tage vor der experimentelle Infektion mit HPS keine ausreichende antibakterielle Wirksamkeit bei der verwendeten Infektionsdosis und dem verwendeten Isolat gezeigt zu haben. Dies ist wahrscheinlich im Versuchsaufbau dieser Studie begründet, die verwendete Infektionsdosis war entweder zu hoch oder der verwendete intratracheale Infektionsweg ungeeignet. Andere Faktoren, wie zum Beispiel die ungeeigneten Applikationszeitpunk von Tulathromycin könnten ebenso zu diesen Resultaten führen. Um dies zu verifizieren/falsifizieren sind Folgestudien, die die Wirksamkeit von Tulathromycin weiter untersuchen, notwendig.

Die Ergebnisse dieser Studie bestätigen, dass die Untersuchung von verschiedenen Probenmaterialien mittels PCR und Kulturversuch sowohl bei mit Antibiotika vorbehandelten als auch bei unbehandelten Tieren geeignet ist, um eine HPS Infektionen nachzuweisen.

8 List of Figures

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10 References

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