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Andreas Beineke, Katharina Bennecke, Christina Neis, Charlotte Schröder, Karl-Heinz Waldmann, et al.. Comparative evaluation of virulence and pathology of serotypes 2 and 9 in experimentally infected growers. *Veterinary Microbiology*, 2008, 128 (3-4), pp.423. <10.1016/j.vetmic.2007.10.028>. <hal-00532349>

HAL Id: hal-00532349

<https://hal.science/hal-00532349v1>

Submitted on 4 Nov 2010

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Accepted Manuscript

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PII: S0378-1135(07)00531-7
DOI: doi:10.1016/j.vetmic.2007.10.028
Reference: VETMIC 3867

To appear in: *VETMIC*

Received date: 16-8-2007
Revised date: 25-10-2007
Accepted date: 30-10-2007

Please cite this article as: Beineke, A., Bennecke, K., Neis, C., Schröder, C., Waldmann, K.-H., Baumgärtner, W., Valentin-Weigand, P., Baums, C.G., Comparative evaluation of virulence and pathology of *Streptococcus suis* serotypes 2 and 9 in experimentally infected growers, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.10.028

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Comparative evaluation of virulence and pathology of *Streptococcus suis* serotypes 2 and 9 in experimentally infected growers

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Abstract

Streptococcus (S.) suis is an invasive porcine pathogen causing meningitis, septicemia, arthritis and other diseases. Studies on pathogenesis as well as vaccine trials have focused on serotype 2 strains, which are worldwide the most prevalent among invasive isolates. However, in Europe serotype 9 strains also contribute substantially to *S. suis*-associated invasive diseases of piglets. The objective of this study was to determine the virulence of an MRP⁺ SLY⁺ serotype 9 *S. suis* strain in comparison to an MRP⁺ EF⁺ SLY⁺ serotype 2 strain. Experimental intranasal and intravenous infections of 7-8 week old SPF piglets were investigated with regard to clinic and pathology. In contrast to the virulent serotype 2 strain, the serotype 9 strain did not cause disease with clinical manifestations after intranasal administration. However, histological screenings of these animals revealed pathological lesions, such as mild focal suppurative meningitis. Clinical manifestations related to meningitis, arthritis and serositis could be induced by intravenous application of this serotype 9 strain. Bacteriological culture, immunohistochemistry of the brain and nested PCR of cerebrospinal fluid confirmed association with the *S. suis* challenge strains in all cases with clinical manifestations. Interestingly, expression of MRP within meningitis lesions was demonstrated for both pathotypes via immunohistochemistry. In conclusion, this study demonstrates that MRP⁺ SLY⁺ serotype 9 strains are less virulent for growers than MRP⁺ EF⁺ SLY⁺ serotype 2 strains. Thus, intravenous application of this serotype 9 strain is required to evaluate heterologous protection in the course of vaccine development based on serotype 2 strains in the future.

Keywords: *Streptococcus suis*, meningitis, MRP, immunohistochemistry

1. Introduction

Streptococcus (S.) suis is worldwide an important porcine pathogen, causing different diseases such as meningitis, septicemia, pneumonia, polyarthritis and polyserositis. These diseases occur mainly in suckling and weaning piglets. Invasion of the

cerebrospinal fluid (CSF) compartment, the joint spaces and serosal cavities by *S. suis* is predominantly associated with fibrinosuppurative inflammations of the respective tissues (Williams and Blakemore, 1990; Madsen et al., 2002). *S. suis* might also cause meningitis and other diseases in humans (Arends and Zanen, 1988; Gottschalk et al., 2007).

S. suis isolates from diseased animals express a capsule, which is, at least in serotype 2 strains, protective against phagocytosis (Smith et al., 1999). Other factors such as the hemolysin suilysin (SLY), a fibrinogen and fibronectin-binding protein and a serum opacity factor have also been shown to contribute to the pathogenesis of *S. suis* (Allen et al., 2001; de Greeff et al., 2002; Baums et al., 2006).

Epidemiological studies revealed a high diversity of serotypes among *S. suis* isolates. In Europe serotypes 1, 2, 7 and 9 are far more common among invasive isolates than any of the other 29 known serotypes (Wisselink et al., 2000; Silva et al., 2006). Pathogenesis studies focused on serotype 2 strains, because they are worldwide the most prevalent strains in pigs, and the vast majority of isolates from humans belong to serotype 2. Experimental infection models have only been described for serotype 1, 2 and 7, but not for serotype 9 strains. These experimental infections revealed, in addition to epidemiological data, that the 136 kDa muramidase-released protein (MRP) and the 110 kDa extracellular factor (EF) are reliable virulence markers for serotype 2 isolates (Vecht et al., 1992; Wisselink et al., 2000). The majority of invasive serotype 9 isolates from Central Europe express a larger variant of MRP, termed MRP*, which shares high homology with the 136 kDa MRP protein of serotype 2 strains (Wisselink et al., 2000; Silva et al., 2006). The objective of this study was to establish and characterize an experimental infection model with an MRP* SLY+ serotype 9 reference strain in pigs.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. suis strain 10 is an MRP+ EF+ SLY+ serotype 2 strain which has been shown to be highly virulent in experimental infections of piglets (de Greeff et al., 2002; Baums et al.,

2006). A3286/94 is a serotype 9 *S. suis* strain, which was originally isolated from a pig with meningitis. This strain expresses a large variant of MRP (MRP*) and carries the suilysin gene *sly* (Silva et al., 2006). Multilocus sequence typing of A3286/94 revealed that it belongs to sequence type 99, which is part of the clonal complex 87 (Rehm et al., 2007). *S. suis* and *Escherichia coli* strains were cultured as described (Baums et al., 2006).

2.2. Cloning of *mrp*

For expression of recombinant His-tagged MRP (rMRP) the plasmid pQE_{mrp} was constructed as follows. The 3520 bp PCR amplification product generated with Pfu polymerase (Promega, Mannheim, Germany) and the primer pair mrppostssBamHI (CTGAGGATCCTGTTGCTTCATCAGAACC) and mrppraeanchorPstI (TTACCTGCAGCGGTTTTACCTGCTTG) was cut with *Bam*HI and *Pst*I (New England Biolabs, Frankfurt, Germany) and cloned into pQE31 (Qiagen, Hilden, Germany). Purified plasmid DNA was verified by restriction analysis. Routine DNA manipulations were performed as described (Sambrook et al., 1989).

2.3. rMRP expression, purification and generation of polyclonal antisera in rabbits

IPTG-induced expression of rMRP and subsequent purification by Ni²⁺-affinity chromatography under native conditions were carried out as described for rOFS (Baums et al., 2006). Western blot analysis with monoclonal anti-MRP antibodies was performed for verification (Silva et al., 2006). Polyclonal antiserum against purified rMRP was raised in a New Zealand White rabbit (Charles River Laboratories, Sulzfeld, Germany) through three consecutive immunizations with 0,1 mg of purified protein and Freund's incomplete adjuvant. In addition, antiserum against formalin inactivated *S. suis* serotype 2 was raised respectively.

2.5. Animal experiments

Sixteen German Landrace piglets (#1-16) from a herd known to be free of *sly+* *mrp+* *epf+* *cps2+* and *sly+* *mrp** *cps9+* strains were infected experimentally and cared for in accordance with the principles outlined in the *European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes* [European Treaty Series, no. 123: <http://conventions.coe.int/treaty/EN/Menuprincipal.htm>; permit no. 509.6-42502-04/829]. The piglets were 7 to 8 weeks old on the day of transport to the experimental facility. They were randomly divided into the different groups outlined in Table 1. Intranasal infections of piglets #1-12 were performed as described (Baums et al., 2006). To apply *S. suis* intravenously four piglets (#13-16) were anaesthetized the same way and *S. suis* A3286/94 resuspended in PBS was injected into the peripheral ear vein. The health status of the animals was monitored as described (Baums et al., 2006). All surviving piglets were sacrificed 20 days post infection. Differences between groups were assessed with the Fisher's exact test.

2.6. Haematologic analysis

Blood samples were collected before experimental infection (0 dpi), on day 3, 5, 7 and 11 post infection and prior to euthanasia. White blood cells were counted on haemocytometer chamber. Leucocytes were differentiated on classical Wright stained blood smears.

2.7. Histopathological screening and immunohistochemistry

The histological screenings were carried out and scored with blinded experiments as described elsewhere (Baums et al., 2006). In addition, formalin-fixed and paraffin-embedded brain tissue was evaluated for the presence of streptococcal antigen by immunohistochemistry using the avidin-biotin complex (ABC) method. Primary antibodies consist of rabbit anti-*S. suis* and anti-MRP polyclonal antibodies (concentrations: 1:1600 and 1:1000). Binding of secondary goat anti rabbit antibodies

and formation of the ABC was visualized by a chromogen reaction using 3,3'-diaminobenzidine-tetrachloride (Beineke et al., 2001; Vector Laboratories, Burlingame, USA). Positive controls consisted of formalin-fixed and paraffin-embedded pellets of cultured *S. suis*. For negative controls primary antibodies were replaced by rabbit preimmune serum.

2.9. Bacteriological screening

All tissues screened histologically were also investigated bacteriologically through culture and PCR-based detection of putative isolates of the challenge strain as described (Baums et al., 2006).

3. Results

3.1 Clinical and haematological findings

In this study an intranasal infection model of piglets was used to compare two different *S. suis* pathotypes. Five of the six growers (#7-12) infected with the serotype 2 reference strain 10 developed severe clinical symptoms with fever and leucocytosis as outlined in Table 1. Two of these growers (#7 and #9) showed nervous dysfunctions, in particular tremor, opisthotonus and ataxia. In contrast, clinical signs were not observed in the 6 piglets (#1-6) infected intranasally with the serotype 9 reference strain A3286/94 during the 20 days after infection. The body temperature was below 40°C at all measurements (Table 1). However, in all serotype 9 infected piglets a temporary increase of blood neutrophils and lymphocytes was observed within 11 days after infection (Fig. 1).

Three of the four growers infected intravenously with A3286/94 developed severe signs of acute arthritis within the first 24 h post infection. The fourth piglet (#13) showed very high fever (42.7°C), anorexia, kyphosis, ataxia and tremor. All four growers infected intravenously were euthanized for reasons of animal welfare within the first 24 hours post infection. In conclusion, the serotype 9 strain A3286/94 caused severe diseases

with high mortality in growers after intravenous application, but not in an intranasal infection model in contrast to the highly virulent serotype 2 strain 10.

3.2 Histopathology

Severe or moderate fibrinosuppurative lesions were found by histopathological examination in a number of different tissues of animals intranasally infected with strain 10 as outlined in Table 2. Among all animals, inflammation of the brain was most advanced in strain 10 infected piglets #7 and #9 which showed in association with neural dysfunctions moderate to severe fibrinosuppurative meningitis (Fig. 2A; Table 3). Piglets infected intranasally with A3286/94 demonstrated mostly mild and focal lesions of serosal surfaces, meninges and choroid plexuses (Table 2). The lower severity of fibrinosuppurative lesions registered in the serotype 9 i. n. infected group resulted in a lower pathological score of 2.5 in comparison to the serotype 2 infected group which received 3.2 (Table 2). In both intranasally infected groups infiltrations were not always dominated by neutrophils, but in a substantial proportion of animals also by mononuclear cells as outlined for the brain in Table 3 and illustrated in Fig. 2B.

A high pathological score of 4.0 was also found in the group infected intravenously with A3286/94. Three of these four growers (#14-16) showed moderate to severe neutrophilic infiltrations of at least one tarsal joint. In addition, all four piglets demonstrated neutrophilic accumulation of the splenic red pulp and pneumonia of different severity as outlined in Table 2.

3.3. Detection of the challenge strains

In general, the challenge strain was isolated from a number of affected tissues in the group infected with serotype 2 and also in the group infected intravenously with serotype 9. However, the serotype 9 challenge strain was not isolated from the piglets infected intranasally, except for the tonsil of one animal (# 3). All culture positive samples except for the tonsils were associated with fibrinosuppurative inflammations. Specifically, the CSF of the animals with severe meningitis (#7 and #9) were positive for *sly+* *mrp+* *epf+*

cps2+ *S. suis*. The challenge strain (*sly+ mrp* cps9*) was also isolated from the CSF of piglet #13. All other CSF samples were culture negative. The joint fluids were positive for the challenge strain only in the three piglets with severe arthritis (synovialitis) infected i. v. with serotype 9 (#14-16). Furthermore, this challenge strain was also detected in the spleen, liver and pleural swab of piglet #13 as well as in the peritoneal swab of piglet #14.

In this study, immunohistochemistry was performed with an antiserum against whole bacteria of *S. suis* serotype 2, which was known to cross react with serotype 9. In addition to the two piglets with severe serotype 2-associated meningitis (#7 and #9), immunoreactivity was also observed in meningeal lesions of piglets #1 and #4 infected intranasally with serotype 9. Immunolabeling of extracellular coccoid bacteria was present in all four animals, whereas extensive immunolabeling within macrophages and neutrophils was observed in piglets #4, #7 and #9 (Fig. 2C). Additionally, a polyclonal serum against rMRP, which was found to recognize also MRP* of serotype 9, was used for immunohistochemistry. In animals #4, #7 and #9 immunolabeling with the anti-rMRP serum was very similar to the results with the serum against whole bacteria and was present in different lesions of the brain, such as fibrinosuppurative meningitis and granulomatous encephalitis (Table 3, Fig. 2D). In addition extensive immunolabeling with the anti-MRP serum was also observed within macrophages and neutrophils in piglet #13 (Table 3). In conclusion, expression of MRP within lesions of the brain was demonstrated by immunohistochemistry for serotypes 2 and 9.

4. Discussion

In our opinion it is important to challenge piglets with different *S. suis* serotypes in vaccination trials in order to evaluate heterologous protection. As MRP* serotype 9 strains are responsible for a substantial fraction of invasive *S. suis* diseases of piglets in Europe (Wisselink et al., 2000; Silva et al., 2006), we included this pathotype in our studies. Here, we describe for the first time systematically the outcome of experimental infections with a well characterized MRP* serotype 9 strain (A3286/94), which was originally isolated from a piglet with meningitis. Intravenous application of 10^8 CFU of

241 this strain resulted in disease with clinical manifestation and should allow evaluation of
242 protection against serotype 9 strains in future vaccination trials.

243 Comparison of this serotype 9 strain with the MRP+ EF+ serotype 2 reference strain 10
244 in an intranasal infection model of 7 to 8 week old SPF piglets demonstrated that
245 A3286/94 is less virulent than strain 10. It appears unlikely that maternal antibodies
246 might have interfered with the comparison of these pathotypes, as sera taken from all
247 animals prior to experimental infection were negative in an α MRP-ELISA. Furthermore,
248 these animals had comparable low titers in ELISAs measuring antibodies against all
249 lysozyme-released proteins of either strain 10 or A3286/94 (results not shown). Low
250 virulence of MRP* serotype 9 strains in intranasal infections is in agreement with
251 preliminary results of Vecht et al. (1996).

252 *S. suis* is generally regarded as a causative agent of fibrinous inflammations and
253 neutrophilic infiltrations (Williams and Blakemore, 1990; Madsen et al., 2002). In
254 accordance these alterations were observed in different tissues of piglets either infected
255 with the serotype 2 or the serotype 9 strain. However, numerous mild infiltrations
256 observed in the experimentally infected piglets of this study were not dominated by
257 neutrophils but by mononuclear cells. These alterations were very frequent in the
258 meninges and the choroid plexuses of intranasally infected piglets. A number of different
259 findings suggest that at least the lymphoplasmacytic infiltrations of the brain were
260 caused by the experimental *S. suis* infection. First, the meninges and the choroid
261 plexuses are well known target tissues of *S. suis*. Mononuclear infiltrations at these sites
262 have not been registered in other piglets from this herd used in other studies (data not
263 shown). Secondly, *S. suis* was detected by immunohistochemistry in two of these
264 animals. Finally, the majority of these growers did not only show an increase of blood
265 neutrophils within 11 dpi, but also a mild increase of blood lymphocytes. Thus, in older
266 piglets infection with *S. suis* might also cause lymphoplasmacytic infiltrations due to the
267 activation of memory cells. The association of *S. suis* with lymphoplasmacytic
268 infiltrations in the meninges is in agreement with findings by Sanford (1987) who
269 describes subacute meningoencephalitis or meningoencephalomyelitis associated with
270 mostly mononuclear cell infiltrations in 53 piglets.

This study revealed differences of virulence between an MRP⁺ serotype 9 and an MRP⁺ EF⁺ serotype 2 strain. With regard to pathogenesis it is remarkable that expression of MRP was demonstrated for both pathotypes within meningeal lesions via immunohistochemistry, though Smith et al. (1996) showed that an isogenic *mrp* mutant of the same MRP⁺ EF⁺ serotype 2 strain was not attenuated in virulence and was frequently isolated from the central nervous system of infected piglets. However, the etiology of the differences in virulence between the two *S. suis*-pathotypes remains to be elucidated in future studies.

Acknowledgements

We thank Hilde Smith for providing strain 10 and the monoclonal antibodies against MRP as well as Julia Schirrmeier for excellent technical support. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG), Bonn, Germany (SFB587).

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345

346

TABLE 1. Virulence of *S. suis* strain 10 (*mrp+* *epf+* *sly+* *cps2*) and A3286/94 (*mrp** *sly+* *cps9*) in experimentally infected piglets^a

number infected piglets	experimental infection		<i>S. suis</i>			severe clinical symptoms ^b	max. body temperature (°C)			max. WBC (10 ⁹ /l) ^c		
	strain	appliance	CFU	morbidity	mortality		≤40	40 – 40.5	≥40.5	≤ 22	22 – 30	≥ 30
6	10	i. n. ^d	10 ⁹	5/6	4/6	4/6	1/6	3/6	2/6	1/6	4/6	1/6
6	A3286/94	i. n. ^d	10 ⁹	0/6 ^e	0/6 ^e	0/6 ^e	6/6	0/6 ^e	0/6 ^e	3/6	3/6	0/6
4	A3286/94	i. v. ^d	10 ⁸	4/4	4/4	4/4	1/4	0/4	3/4	2/4	2/4	0/4

^a German landrace piglets from a herd free of *sly+* *epf+* *mrp+* *cps2* and *cps9* *S. suis* strains

^b in particular severe depression, persistent anorexia, neural disorder and acute severe lameness

^c White blood cell (WBC) counts were performed on day 3, 5, 7 and 11 post infection. All piglets had WBCs below 20 pre-infection.

^d i. n. = intranasal; i. v. = intravenous

^e significantly different from strain 10 infected group.

TABLE 2. Scoring of fibrinosuppurative lesions of piglets infected with *S. suis* strain 10 (*mrp+* *epf+* *sly+* *cps2*) and A3286/94 (*mrp** *sly+* *cps9*)

Number of infected piglets	strain	experimental infection	S. suis CFU	brain			serosae			joint			spleen and liver			lung			ω ^e	
				meningitis, chorioiditis, ventriculitis	pleuritis or peritonitis	synovialitis	Splentitis ^a or hepatitis	pneumonia												
5 ^b	3 ^c	1 ^d	4 ^b	2 ^c	1 ^d	4 ^b	2 ^c	1 ^d	4 ^b	2 ^c	1 ^d	4 ^b	2 ^c	1 ^d						
6	10	i. n.	10 ⁹	2/6	0/6	0/6	1/6	0/6	2/6	0/6	1/6	1/6	2/6 ^e	1/6	1/6	1/6	1/6	1/6	2/6	3.2
6	A3286/ 94	i. n.	10 ⁹	1/6	1/6	0/6	0/6	4/6	0/6	0/6	1/6	1/6	0/6	1/6	2/6	0/6	2/6	1/6		2.5
4	A3286/ 94	i. v.	10 ⁸	0/4	1/4	0/4	1/4	0/4	1/4	3/4	0/4	0/4	1/4	2/4	1/4	1/4	0/4	3/4		4.0

359

360 ^a neutrophilic accumulation of the splenic red pulp

361 ^b Scoring of 4 and 5 indicates moderate to severe diffuse or multifocal fibrinosuppurative inflammations.

362 ^c Scoring of 2 and 3 indicates mild focal fibrinosuppurative inflammation.

363 ^d Individual single perivascular neutrophils received a score of 1.

364 ^e $\omega = \sum \text{score}_{\text{max}} / n_{\text{animals}}$ (Baums et al., 2006).

TABLE 3. Histopathological findings in the brain of piglets infected with *S. suis* strain 10 (*mrp+* *epf+* *sly+* *cps2*) and A3286/94 (*mrp** *sly+* *cps9*)

piglet # ^b	<i>S. suis</i> challenge strain	appli- cation	necropsy dpi	Histopathology ^a					Immunohistochemistry			
				fibrinosuppurative		lymphoplasmacytic		granulo- matous	anti- <i>S. suis</i>		anti-MRP	
				menin- gitis	choroiditis	menin- gitis	choroiditis	ence- phalitis	extra- cell.	intra- cell.	extra- cell.	intra- cell.
7	10	i. n.	3	+++	-	-	-	-	+	++	+	++
8	10	i. n.	20	-	-	+	+	-	-	-	-	-
9	10	i. n.	6	++++	++++	-	-	-	++	+++	+	+++
10	10	i. n.	5	-	-	+	++	-	-	-	-	-
11	10	i. n.	3	-	-	+	+	-	-	-	-	-
1	A3286/94	i. n.	20	-	-	+	+	-	+	-	-	-
2	A3286/94	i. n.	20	-	-	+	-	-	-	-	-	-
3	A3286/94	i. n.	20	-	-	-	++	-	-	-	-	-
4	A3286/94	i. n.	20	++	-	+	-	++	+	+	-	+
5	A3286/94	i. n.	20	++	+++ ^c	++ ^c	-	-	-	-	-	-
6	A3286/94	i. n.	20	-	-	+	-	++	-	-	-	-
13	A3286/94	i. v.	1	++	++	-	-	-	-	-	-	++

367

368 ^a Lesions were assigned + to ++++ depending on the severity (+ individual perivascular immune cells, ++ mild,
369 +++ moderate and ++++ severe).

370 ^b Piglet #12 infected with strain 10 intranasally (i. n.) and piglets #14-16 infected with A3286/94 intravenously (i. v.) did
371 neither show lesions nor immunolabeling in the brain.

372 ^c Severe fibrinous ventriculitis and mild lymphoplasmacytic encephalitis was also present in piglet #5.

373

374

375 **Figure legends**

376

377 Fig. 1. Neutrophil (A) and lymphocyte (B) concentrations in the jugular vein after experimental infection of growers with
378 serotype 9 strain A3286/94. Piglet #5 was not sampled 11 dpi. The number of each piglet (#) is indicated in the box.

379

380 Fig. 2. Histological findings (A, B) and immunohistochemistry (C, D) of the brain of growers infected intranasally with *S.*
381 *suis*. (A) Severe diffuse suppurative meningitis in piglet #9 infected with serotype 2 strain 10; bar = 100 μ m, HE. (B) Focal
382 lymphocytic infiltration (arrow) of the choroid plexus (CP) in strain A3286/94 infected piglet #3; bar = 25 μ m, HE. (C)
383 Immunolabeling in macrophages and neutrophils of serotype 2 strain 10 infected piglet #9 with an antiserum raised
384 against *S. suis* serotype 2, bar = 10 μ m. (D) Intrahistiocytic MRP-specific immunoreactivity associated with granulomatous
385 encephalitis in piglet #4 infected intranasally with *S. suis* serotype 9 strain A3286/94, bar = 25 μ m.

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