Production of Virulence-Related Proteins by Canadian Strains of *Streptococcus suis* Capsular Type 2

Marcelo Gottschalk, Annie Lebrun, Henk Wisselink, J. Daniel Dubreuil, Hilde Smith, and Uri Vecht

ABSTRACT

The production of muramidase-released protein (MRP), extracellular protein factor (EF) and hemolysin (suilysin) by 101 Canadian field strains of *Streptococcus suis* capsular type 2 is described. Most strains (72%) isolated from diseased pigs were MRP−EF− and only 1 strain was MRP+EF+. This strain was also the only 1 to produce the hemolysin. Thirteen strains (15%) were MRP+ EF− and only 3 strains were MRP* EF−. All the strains isolated from clinically healthy pigs as well as a bovine and 2 human isolates had a MRP-EF- phenotype. In addition, 7 strains (8%) had a MRP_S phenotype, which had so far been described for *S. suis* capsular type 1. In conclusion, most Canadian field isolates of *S. suis* capsular type 2 tested in this study do not produce the virulence-related proteins described so far for this bacterial pathogen.

RÉSUMÉ

La production de la protéine relâchée par la muramidase (MRP), du facteur protéique extracellulaire (EF) et de l'hémolysisne (suilysine) par 101 isolats de champs canadiens de *Streptococcus suis* sérotype 2 est rapportée. La plupart des isolats (72 %) provenant de porcs malades étaient MRP− EF− et seulement un isolat était MRP+ EF+. Ce dernier isolat était également le seul à produire l'hémolysine. Treize isolats (15 %) se sont avérés MRP+ EF− et seulement trois isolats présentaient un profil MRP * EF−. Tous les isolats provenant de porcs cliniquement sains ainsi qu'un isolat provenant d'un bovin et deux isolats provenant de cas humains présentaient un phénotype MRP− EF−. De plus, sept isolats (8 %) présentaient un phénotype MRP_S; ce phénotype avait été décrit chez *S. suis* sérotype 1. En conclusion, la plupart des isolats canadiens de *S. suis* testés dans cette étude ne produisent pas les protéines décrites comme reliées à la virulence pour cette espèce bactérienne.

(Traduit par docteur Serge Messier)

Streptococcus suis is one of the most important bacterial swine pathogens worldwide and it is associated with cases of meningitis, arthritis, septicemia and sudden death (1). There are 35 capsular types, with capsular type 2 being the most frequently isolated from diseased animals and humans (2,3,4,5).

The study of virulence factors of *S. suis* has increased in recent years. It is now accepted that highly virulent and completely avirulent *S. suis* capsular type 2 strains do exist (6,7,8). Different bacterial structures or products, such as the capsule, fimbriae, extracellular and membrane-associated proteins, and hemolysin have been thought to be virulence factors (7,8,9,10). In The Netherlands, 2 proteins were detectable only in virulent strains of *S. suis* capsular type 2 (7,10,11). The 1st protein, which is known as muramidase-released protein (MRP), is a 136 kDa cell-wall-associated protein which can also be found in the culture supernatant. The 2nd one, called extracellular protein factor (EF), is a 110 kDa extracellular protein. At 1st, 3 different phenotypes were recognized: MRP+EF+, MRP+EF− and MRP−EF−. Most isolates from diseased pigs were MRP+EF+, whereas most human isolates and isolates from clinically healthy pigs were MRP+EF− and MRP−EF− respectively (11). An association of these phenotypes with strains isolated from diseased pigs was also reported in other countries (12,13,14). Two double antibody sandwich enzyme-linked immunoassays (ELISA) using specific monoclonal antibodies (MAbs) against MRP and EF proteins were used to phenotype field strains (10). However, when culture supernatants of MRP+EF− strains were tested by immunoblotting using EF specific MAbs, antigenically related proteins of variable size and with a higher molecular mass, designed EF*, were recognized in all MRP+EF− strains tested (11). Hence, the phenotype of these strains was designed MRP+EF*. Only MRP+EF+ strains were shown to be highly pathogenic after experimental inoculations of newborn germfree pigs; MRP+EF* and MRP−EF− strains appeared to be weakly and non-pathogenic, respectively (8). More recently, 2 new phenotypes, MRP−EF+ and MRP*EF−, have been reported (6,12,15). The MRP* is a MRP-related protein, also detected in immunoblots by a MRP-specific MAb, which has a molecular weight slightly higher than conventional 136 kDa MRP (12). Finally, some strains of *S. suis* capsular type 1 produce a MRP protein with a considerably lower molecular weight (< 136 kDa), which has been called MRP_s (15).

Groupe de Recherche sur les Maladies Infectieuses du Porc (GREMIP), Département de pathologie et microbiologie, Faculté de médecine vétérinaire, Université de Montréal, C.P. 5000, Saint-Hyacinthe (Québec) J2S 7C6, (Gottschalk, Lebrun, Dubreuil); DLO Institute for Animal Sciences and Health, P.O. Box 65, 8200, Lelystad, The Netherlands (Wisselink, Smith, Vecht).

This work was supported by Grant OGP0154280, from the Natural Sciences and Engineering Research Council of Canada and by Grant 97-NC-1037 from the Fonds pour la Formation de Chercheurs et l'Aide à la recherche.

Received January 31, 1997.

TABLE I. Distribution of MRP and EF phenotypes in 98 Canadian strains of *Streptococcus suis* capsular type 2 isolated from diseased (85 strains) or clinically healthy (13 strains) pigs

	No (%) of strains isolated from			
	Diseased pigs			
Phenotype	Systemic[a] infection	Lungs[b]	Total	Clinically healthy pigs
MRP+ EF+	1 (2)[c]	0 (0)	1 (1)	0 (0)
MRP+ EF−	9 (16)	4 (14)	13 (15)	0 (0)
MRP* EF−	0 (0)	3 (11)	3 (4)	0 (0)
MRP*EF−	3 (5)	4 (14)	7 (8)	0 (0)
MRP− EF−	44 (77)	17 (61)	61 (72)	13 (100)

[a] Strains isolated from septicemia, meningitis, endocarditis and/or arthritis
[b] Strains isolated from cases of pneumonia
[c] This strain also produces the hemolysin

Recently, an extracellular protein with hemolytic properties, called suilysin, has been described (16,17,18). This protein belongs to the family of toxins known as antigenically related cholesterol-binding cytolytic toxins, since it is sensitive to oxidizing agents, activated by reducing agents, inhibited by low concentrations of cholesterol and forms transmembrane pores by a multi-hit mechanism of action (17). A recent report indicates that most *S. suis* capsular type 2 field strains from 4 different European countries produce this hemolysin (19). Since very few data for the production of MRP and EF (6,12) or no data for the production of hemolysin by *S. suis* North American strains were available, the objective of this work was to study the production of these virulence-related proteins by Canadian field isolates of *S. suis* capsular type 2.

A total of 98 field isolates of *S. suis* capsular type 2 from pigs originating from different farms were studied. Of these strains, 85 were from diseased pigs (28 from cases of pneumonia and 57 from cases of systemic disease) and 13 were from tonsils of clinically healthy animals (from herds where no clinical cases of *S. suis* capsular type 2 were diagnosed). In addition, 1 strain isolated from a case of bovine abortion and 2 strains of human origin isolated from a case of endocarditis and from a case of meningitis, were also studied. Most of these strains were provided by R. Higgins, clinical bacteriology laboratory, Faculty of Veterinary Medicine, University of Montreal, St-Hyacinthe, Quebec. Additional strains were obtained from Quebec and other Canadian provincial laboratories. *S. suis* was identified and serotyped using a standard procedure (20). Control strains for the production of hemolysin (S735, reference strain) as well as strains with the phenotype MRP+EF+ (D-282), MRP+EF* (S735) or MRP−EF− (T15) were used. Selected strains from different European countries were also tested as controls.

Once identified and serotyped, each field strain was cultured overnight in 5 mL of Todd-Hewitt broth (Difco Laboratories, Detroit, Michigan, USA) and, after centrifugation at 3000 g for 20 min, the supernatant was concentrated 10 times by evaporation (Speed-Vac Concentrator, SVC 200H, Savant) and stored at −20°C until tested by Western blotting with murine MAbs and rabbit monospecific polyclonal antibodies (PAbs). The supernatant of some strains were also tested by the double sandwich ELISA previously reported (10). Monoclonal antibodies against the MRP and EF (MRP_3 and EF_3 respectively) and PAb against MRP used in this study were those used in other studies (10). Polyclonal antibodies against EF were produced by inoculation of rabbits with polyacrylamide gel bands of the 110 kDa protein from an European EF+ strain. Briefly, the concentrated supernatant was separated on a SDS-polyacrylamide gel and stained with Coomassie blue. The 110 kDa band was excised from the gel, mixed with Freund's incomplete adjuvant and injected to rabbits as previously described (21). Sensitivity and specificity of this PAb was similar to that of antibodies used in an earlier study (10).

Western blotting was performed as previously described (6). Briefly, concentrated supernatant was mixed with an equal volume of solubilization buffer and separated in 5% SDS-polyacrylamide vertical slab gels. Following electrophoresis, the supernatant material was transferred from the slab gel to nitrocellulose membranes in methanol-Tris-glycine buffer. After blocking unreacted sites with casein (2%, w/v), the membranes were incubated with the Mabs or Pabs. Each strain was tested for the 4 different antibodies on separate membranes. After additional washing with Tris-saline, peroxidase-labeled immunoglobulin G fraction (IgG) of goat anti-rabbit IgGs or goat anti-murine IgG and IgM (Jackson Immunoresearch Laboratories Inc. West Grove, Pennsylvania, USA) at appropriate dilution was added. Reaction was visualized following incubation of the nitrocellulose membrane with 0.06% 4-chloro-1-naphthol (Sigma Chemicals, St-Louis, Missouri, USA) in cold methanol mixed with H_2O_2 in Tris-HCl. Apparent molecular masses were calculated by comparison with standards of known molecular mass (Bio-Rad Laboratories Ltd, Mississauga, Ontario). For every membrane, and depending on the antibody used, supernatants from MRP+, MRP−, EF+, EF− or EF* control strains were included. Western blotting of each single strain was carried out in 2 independent laboratories (The Netherlands and Canada) using coded strains (blind testing). The results were identical in both laboratories.

For the hemolysin test, supernatant of overnight cultures grown in Todd-Hewitt broth were tested with 1.4% of horse red blood cells as previously described (17). Following incubation for 1 h at 37°C, the cells were sedimented by centrifugation (16 000 g for 10 min) and the supernatants were transferred to polystyrene microplates (Dynatech). Optical densities were read at 450 nm on a spectrophotometer (Molecular Devices, Menlo Park), following corrections for controls.

Since it was reported that monospecific PAbs (especially the MRP-specific PAb) are slightly more sensitive than the MAbs (14), we have used both monospecific PAbs and MAbs to study the Canadian strains. In our hands, the phenotypes obtained with the Mabs and the PAbs were identical for all isolates. These results differed from those of Quessy et al (6), who observed some strains with a

110 kDa protein that reacted with a PAb but not with the same MAb used in this study. Since the strain used by these authors to produce the PAb (strain 1591) was confirmed to be EF-negative in the present study, it is most likely that the 110 kDa protein described by Quessy et al (6) was not related to the EF. Finally, some faint high-molecular-weight bands (> 150 kDa) in culture supernatants of certain strains were detected only with the EF-specific PAbs. These proteins are probably not related to the EF since only certain rabbit sera reacted with these strains and no reaction could be obtained with the MAbs. Similar results were obtained in the past with some strains in The Netherlands (11).

Western blot results obtained with the Canadian *S. suis* strains are summarized in Table I. An example of results obtained with some strains is also shown in Figure 1. Since it has been suggested that *S. suis* is not a primary cause of pneumonia and strains isolated from lungs may be less virulent (8), strains used in this study and isolated from lungs in cases of pneumonia were studied separately from those isolated from animals suffering from systemic infections, such as meningitis, septicemia and arthritis. Nevertheless, most results were similar for both groups of strains. Most strains (72%) isolated from diseased pigs were MRP−EF−. In fact, only 1 strain (isolated from a case of meningitis) was classified as MRP+EF+. A total of 13 strains (15%) were MRP+ and EF− and three strains produced the MRP*EF− profile. All the strains isolated from clinically healthy animals were MRP−EF− (Table I). In addition, 7 strains (8%) had a MRP_s EF− phenotype which has only been described for *S. suis* capsular type 1 so far (15). The 2 human and the bovine strains also had an MRP−EF− phenotype. Finally, none of the Canadian strains had the MRP+ EF* or MRP−EF+ phenotypes previously described (6,11,12). Results obtained by the double-sandwich ELISAs were in accordance with those obtained by Western blots (results not-shown), confirming previous reports that these tests are reliable diagnostic tools for MRP and EF detection (10). All the European strains used as controls showed the expected MRP+EF+ or MRP+EF* phenotype (results not shown).

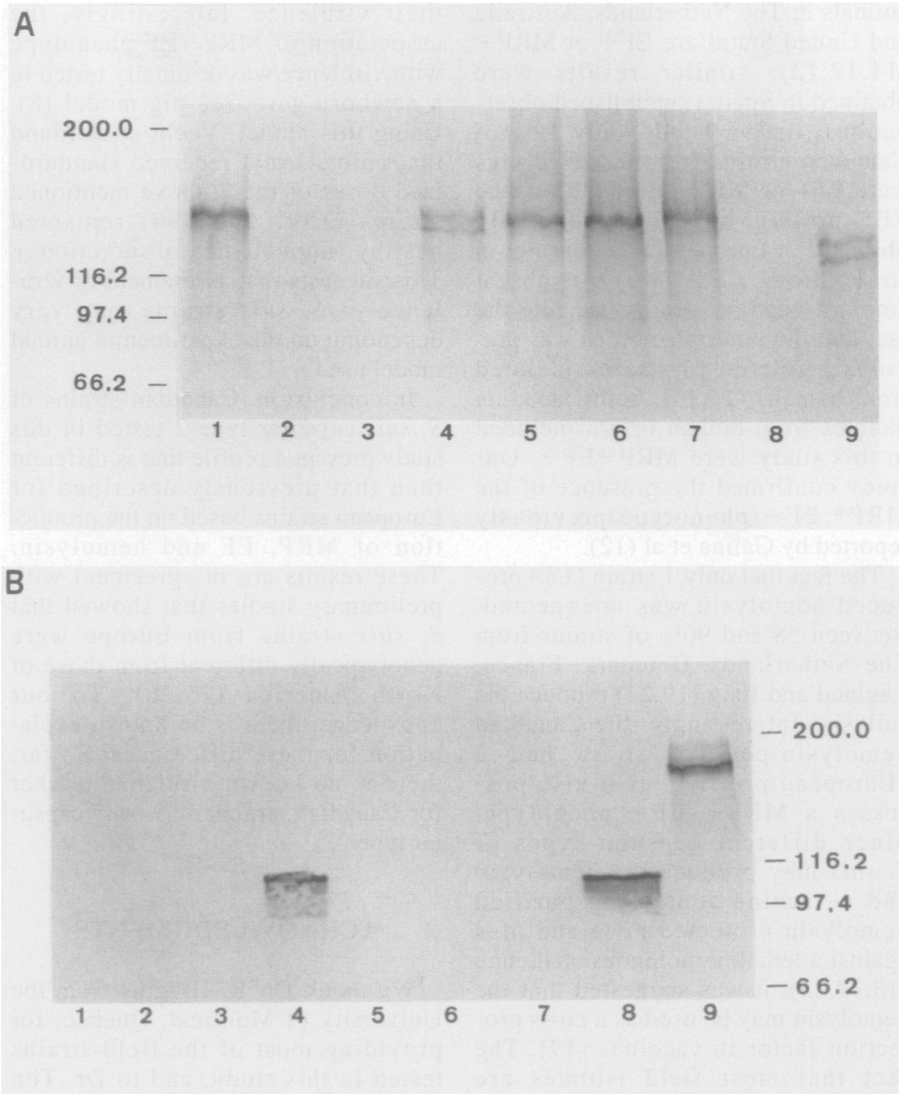

Figure 1. (A) Western blot of the cell culture supernatants of selected *S. suis* type 2 strains probed with a monoclonal antibody directed to the muramidase-released protein (MRP). Lane 1: control European strain D-282 (MRP+); Lane 2: control strain T15 (EF−); Lanes 3 to 9: Canadian field strains from diseased animals. Lane 3: 89-5694 (MRP−); Lane 4: 89-4984 (MRP*); Lane 5: 95-8242 (MRP+); Lane 6: strain 95-920 (MRP+); Lane 7: 95-7693 (MRP*); Lane 8: 89-88-377 (MRP−); Lane 9: 95-1249-7063 (MRP_s). Numbers on the left indicate molecular weight standards in kDa.

(B) Western blot of cell culture supernatants of selected *S. suis* type 2 strains probed with a monoclonal antibody directed to the extracellular protein factor (EF). Lane 1 to 6: Canadian field strains from diseased animals. Lane 1: strain 89-4984 (EF−); Lane 2: 88-5303 A (EF−); Lane 3: 95-7159 (EF−); lane 4: 95-8242 (EF+); Lane 5: 89-88-633 (EF−); Lane 6: 89-4297 (EF−); Lane 7: control strain T15 (EF−); Lane 8: control European strain D-282 (EF+); Lane 9: control European strain S735 (EF*). Numbers on the right indicate molecular weight standards in kDa.

Only 1 of the Canadian strains tested produced suilysin. Interestingly, this strain was the only MRP+EF+ strain found in this study. Since the technique used for testing the hemolytic properties of our strains (17) differs slightly from that described by Jacobs et al (19), hemolysin-negative strains were retested using both techniques in parallel with identical results. All selected European strains used as controls were positive in both hemolysin tests.

The results obtained in this study indicate that virulence-related protein patterns of Canadian strains isolated from diseased pigs differ from those isolated in some other countries. For example, it was reported that more than 50% of strains from diseased

animals in The Netherlands, Australia and United States are EF+ or MRP+ (11,12,13); similar results were obtained in Spain (unpublished observations). In our hands, only 19% of Canadian strains from diseased pigs were EF+ or MRP+. In addition, the EF* protein which is frequently observed in Europe (11,22) but not in strains from U.S.A. (12) was absent among Canadian strains. Despite the fact that the latter phenotype was previously related to strains isolated from humans (11,13), both Canadian isolates from human origin included in this study were MRP−EF−. Our study confirmed the presence of the MRP* EF− phenotype previously reported by Galina et al (12).

The fact that only 1 strain (1%) produced hemolysin was unexpected. Between 58 and 90% of strains from The Netherlands, Denmark, France, England and Italy (19,22) produce the suilysin. Interestingly, the Canadian hemolysin-positive strain had a "European profile," as it also possesses a MRP+ EF+ phenotype. Since different capsular types of *S. suis* may produce the hemolysin and a vaccine containing purified hemolysin protected mice and pigs against a lethal homologous challenge (18,19,23), it was suggested that the hemolysin may be used as a cross protection factor in vaccines (19). The fact that most field isolates are hemolysin negative probably precludes its use, at least, in Canada.

It has been recently shown that MRP−EF− isogenic mutant strains are still as virulent for pigs as the parent strain (24). The authors mentioned that these proteins do not contribute to the pathogenicity of the *S. suis* type 2 infections, but the synthesis of these proteins is coincidentally associated with pathogenicity. However, in our hands, at least 2 Canadian MRP−EF− strains tested in this study (1591 and 999) were shown to cause meningitis, pericarditis and arthritis in conventional pigs after intravenous inoculation (6, unpublished observations). In addition, the fact that these 2 strains, as well as other strains used in this study, were isolated in pure culture from different tissues from systemically diseased pigs (or only from meninges and brain in cases of meningitis) in endemically affected herds, is an indication of their virulence. Interestingly, the association of MRP−EF phenotype with virulence was originally tested in a newborn germfree pig model (8). Using this model, Vecht et al found that animals that received standardized doses of the 2 above mentioned strains (1591 and 999) remained healthy (unpublished observations). This suggests that assessment of virulence of *S. suis* strains may vary depending on the experimental animal model used.

In conclusion, Canadian strains of *S. suis* capsular type 2 tested in this study present a profile that is different than that previously described for European strains based on the production of MRP, EF and hemolysin. These results are in agreement with preliminary studies that showed that *S. suis* strains from Europe were genotypically different from those of North America (25,26). To our knowledge, there is no known explanation for these differences. So far, there is no known virulence marker for Canadian strains of *S. suis* capsular type 2.

ACKNOWLEDGMENTS

We thank Dr. R. Higgins from the University of Montreal, Quebec, for providing most of the field strains tested in this study, and to Dr. Ton Jacobs, from Intervet International B.V., The Netherlands, for providing the detailed protocol for the detection of hemolysin. We are also in debt to Mariela Segura and Sonia Lacouture for their expert technical assistance.

REFERENCES

1. **ALEXANDER TJL.** *Streptococcus suis*: An update. Pig Vet Soc Proc 1992; 21: 50–60.
2. **ARENDS J, ZANEN H.** Meningitis caused by *Streptococcus suis* in humans. Rev Infect Dis 1988; 10: 131–137.
3. **HIGGINS R, GOTTSCHALK M, BEAUDOIN M, RAWLUK S.** Distribution of *Streptococcus suis* capsular types in Quebec and western Canada. Can Vet J 1992; 33: 27–30.
4. **HIGGINS R, GOTTSCHALK M, LEBRUN M, HENRICHSEN J.** Description of six new capsular types of *Streptococcus suis*. J Vet Diagn Invest 1995; 7: 405–406.
5. **TROTTIER S, HIGGINS R, BROCHU G, GOTTSCHALK M.** A case of human endocarditis due to *Streptococcus suis* in North America. Rev Inf Dis 1991; 13: 1251–1252.
6. **QUESSY S, DUBREUIL JD, CAYA M, HIGGINS R.** Discrimination of virulent and avirulent *Streptococcus suis* capsular type 2 isolates from different geographical origins. Infect Immun 1995; 63: 1975–1979.
7. **VECHT U, ARENDS JP, VAN DER MOLEN EJ, VAN LEENGOED LAG.** Differences in virulence between two strains of *Streptococcus suis* type II after experimentally induced infection of newborn germ-free pigs. Am J Vet Res 1989; 50: 1037–1043.
8. **VECHT U, WISSELINK HJ, VAN DICK JE, SMITH H.E.** Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depends on phenotype. Infect Immun 1992; 60: 550–556.
9. **GOTTSCHALK M, HIGGINS R, JACQUES M, DUBREUIL JD.** Production and characterization of two *Streptococcus suis* capsular type 2 mutants. Vet Microbiol 1992; 30: 59–71.
10. **VECHT U, WISSELINK HJ, ANAKOTTA J, SMITH HE.** Discrimination between virulent and non-virulent *Streptococcus suis* type 2 strains by enzyme-linked immunosorbent assay. Vet Microbiol 1993; 34: 71–82.
11. **VECHT U, WISSELINK HJ, JELLEMA ML, SMITH HE.** Identification of two proteins associated with virulence of *Streptococcus suis* type 2. Infect Immun 1991; 59: 3156–3162.
12. **GALINA L, VECHT U, WISSELINK HJ, PIJOAN C.** Prevalence of various phenotypes of *Streptococcus suis* isolated from swine in the U.S.A. based on the presence of muraminidase-released protein and extracellular factor. Can J Vet Res 1996; 60: 72–74.
13. **MWANIKI CG, ROBERTSON D, TROTT DJ, ATYEO RF, LEE BJ, HAMKSON DJ.** Clonal analysis and virulence of Australian isolates of *Streptococcus suis* type 2. Epidemiol Infect 1994; 113: 321–334.
14. **SALASIA SIO, LÄMMLER C.** Detection of virulence-associated proteins of *Streptococcus suis* by cultivation of the bacteria on nitrocellulose membranes. Med Sci Res 1994; 22: 347–348.
15. **VECHT U, WISSELINK HJ, REEK FH, STOCKHOFE-ZURWIEDEN N, SMITH HE.** Diagnosis of several capsular serotypes of *Streptococcus suis* by phenotype and PCR and the relation with virulence for pigs. In Proceed 14th Internat Pig Vet Soc Cong, Bologna, Italy. 1996; p. 298.
16. **FEDER I, CHENGAPPA MM, FENWICK B, RIDER M, STAATS J.** Partial characterization of *Streptococcus suis* type 2 hemolysin. J Clin Microbiol 1994; 32: 1256–1260.
17. **GOTTSCHALK M, LACOUTURE S, DUBREUIL JD.** Characterization of *Streptococcus suis* capsular type 2 haemolysin. Microbiol 1995; 141: 189–195.
18. **JACOBS AAC, LOEFFEN L, VAN DEN BERG J, STORM P.** Identification of a

thiol-activated hemolysin (suilysin) of *Streptococcus suis*. Infect Immun 1994; 62: 1742–1748.

19. **JACOBS AAC, VAN DEN BERG J, BAARS JC, NIELSEN B, JOHANNSEN LW.** Production of suilysin, the thiol-activated haemolysin of *Streptococcus suis*, by field isolates from diseased pigs. Vet Rec 1995; 16: 295–296.
20. **HIGGINS R, GOTTSCHALK M.** An update on *Streptococcus suis* identification. J Vet Diagn Invest 1990; 2: 249–252.
21. **HOLT ME, ENRIGHT MR, ALEXANDER TJL.** Protective effects of sera raised against different fractions of *Streptococcus suis* type 2. J Comp Pathol 1990; 103: 85–94.
22. **SALA V, ANTONINI M, VISCHI O, ANSUINI A, GUADAGNINI P, CONEDERA G, FABBI M, PERINNI S.** Distribution of capsular types and hemolysin production of *Streptococcus suis* isolates in Northen Italy. In Proc 14th Internat Pig Vet Soc Cong, Bologna, Italy. p. 307.
23. **STAATS JJ, OKWUMABUA , FUNK P and CHENGAPPA MM.** 1995. Expression of cloned *Streptococcus suis* type 2 hemolysin gene in *Bacillus subtilis* and protection by the gene product in mice against lethal *S. suis* type 2 challenge. Proc Conf Res Work in Anim Dis 1996; 76: 14.
24. **SMITH HE, VECHT U, WISSELINK HJ, STOCKHOFE-ZURWIEDEN N.** *Streptococcus suis* mutants in which the genes encoding muramidase-released proteins (MRP) and extracellular protein factor (EF) are inactivated are virulent for pigs. In Proc 14th Internat Pig Vet Soc Cong, Bologna, Italy. 1996; 302.
25. **CANTIN M, HAREL J, HIGGINS R, GOTTSCHALK M.** Antimicrobial resistance patterns and plasmid profiles of *Streptococcus suis* isolates. J Vet Diagn Invest 1992; 4: 170–174.
26. **BEAUDOIN M, HAREL J, HIGGINS R, GOTTSCHALK M, FRENETTE M, MACINNES J.** Molecular analysis of isolates of *Streptococcus suis* capsular type 2 by restriction-endonuclease-digested DNA separated on SDS-PAGE and by hybridization with an rDNA probe. J Gen Microbiol 1992; 138: 2639–2645.