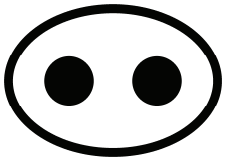
Population dynamics of swine influenza virus in finishing pigs



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Population dynamics of swine influenza virus in finishing pigs

Populatie dynamica van varkensinfluenzavirus in vleesvarkens

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Leven en laten leven…

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Chapter 1

General introduction

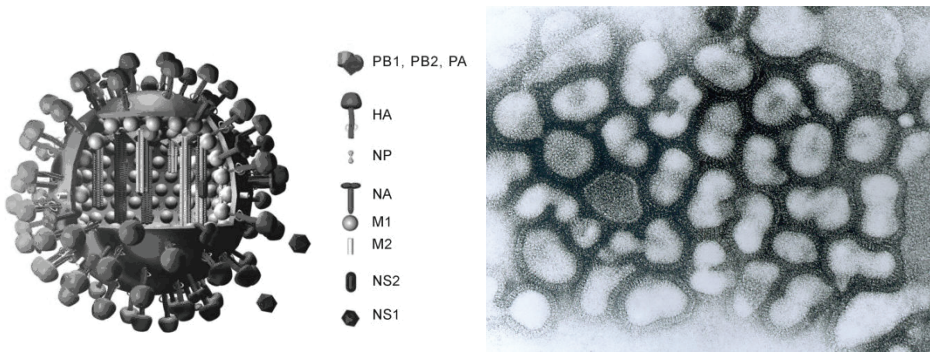
General introduction

Influenza viruses

Influenza viruses are part of the family of Orthomyxoviridae, which includes the genera of influenza A, influenza B and influenza C viruses. They are ssRNA viruses. Virus particles are enveloped and approximately 80-120nm in diameter. The genome is segmented, with 8 RNA fragments (7 for influenza C). Two glycoproteins are present on the surface of the virus. The haemagglutinin (HA) is made up of 2 subunits, HA1 and HA2. HA mediates the attachment of the virus to the cellular receptor. Neuraminidase (NA) molecules are present in lesser quantities in the envelope. NA supports an efficient release of virus from cells. Influenza A virus subtypes are characterised by their types of HA and NA. There are currently 16 different HA types, designated H1 to H16, and 9 different NA types, designated N1-N9 (Fouchier et al., 2005; Alexander, 2007).

History of influenza viruses in swine in Europe

Influenza virus infections in swine were first noticed in the US in 1918, during the human pandemic of the Spanish flu (Shope, 1931; Dowdle and Hattwick, 1977; Schultz et al., 1991). Both were caused by an influenza virus subtype H1N1. In the following decades, some outbreaks of influenza, also caused by influenza virus subtype H1N1,



Left: Three-dimensional view of influenza virus (source: Wikipedia, released into the public domain by the author). Right: Transmission electron micrograph of influenza A virus (Source: Wikipedia. This image is a work of the Centers for Disease Control and Prevention, part of the United States Department of Health and Human Services. As a work of the U.S. federal government, the image is in the public domain.).

were reported in swine in Europe (Blakemore and Gledhill, 1941; Nardelli et al., 1978). The H2N2 strain, which caused the 1957 Asian flu pandemic in humans, has, however, never been detected in swine.

It was not until the late 1960's that influenza virus infections in swine became widespread in Europe as well. The H3N2 subtype, which was introduced in the European pig population at that time, was related to the human H3N2 strains that caused the 1968 Hong Kong flu pandemic in humans (Bibrack, 1972; Harkness et al., 1972; Popovici et al., 1972). This strain did not cause severe clinical symptoms in swine (Harkness et al., 1972).

In 1979 an influenza virus subtype H1N1 of avian origin was introduced into the European swine population (Ottis et al., 1981; Pensaert et al., 1981; Vanderputte et al., 1981; Witte et al., 1981; Masurel et al., 1983; Scholtissek et al., 1983; Hinshaw et al., 1984; Burki et al., 1985). This introduction occurred independently of the re-emergence of influenza virus subtype H1N1 in the human population in 1977 (Russian flu). H1N1 strains isolated from humans in 1977 were closely related to the human influenza strains from before 1957 (Scholtissek et al., 1978; Kendal et al., 1979; Kozlov et al., 1981). The 1979 introduction in the swine population was accompanied by widespread and severe acute respiratory disease in pig herds. Furthermore, reproductive failure in sows was noticed, possibly due to the high fever that accompanies the influenza virus infection in fully susceptible hosts. After the initial epidemic, the virus became endemic in the European pig population.

A reassortment between this newly introduced H1N1 strain and the already endemic H3N2 somewhere in the mid-eighties resulted in a novel H3N2 strain, with all the internal genes of the H1N1 and the heamagglutinin and neuraminidase of the H3N2 strain (Castrucci et al., 1993). This novel H3N2 strain was apparently more fit than the old one and became the only H3N2 subtype to circulate amongst pigs in Europe. Moreover, this new H3N2 strain seemed to be more virulent than the old one and respiratory disease related to this strain became common.

In Europe, an influenza virus of the H1N2 subtype was first found in France in 1987 (Gourreau et al., 1994), but no subsequent isolates of this particular strain were ever reported. The real place of origin of the influenza virus subtype H1N2 currently circulating in Europe seems to be the UK, where it was first found in 1994 (Brown et al., 1995a). This UK H1N2 strain is the result of multiple reassortments and contains the haemagglutinin (H1) gene from a human H1N1 strain, the neuraminidase (N2) gene from human-like swine H3N2 strains and the remaining genes from avian-like swine H1N1 strains (Brown et al., 1998). Subsequently, this subtype became endemic in the UK pig population. In the late 1990's and at the beginning of the current century, an H1N2 subtype was also reported from Belgium (Van Reeth et al., 2000), Italy and France (Marozin et al., 2002), Germany (Schrader and Suss, 2003), and Spain (Maldonado et al., 2006). Many, if not all of these strains are related to the original H1N2 strain from the UK (Marozin et al., 2002).

Since the late 1990's, commonly circulating influenza virus strains in swine in Europe have therefore been of the H1N1, H3N2 and H1N2 subtypes. High seroprevalences have generally been found among sows and finishing pigs, but the

extent to which influenza virus infections had been responsible for respiratory disease in swine remained unknown. Other subtypes were occasionally detected in swine, within and outside Europe, but have so far not become endemic (Brown et al., 1994; Karasin et al., 2000; Peiris et al., 2001; Tsai and Pan, 2003; Karasin et al., 2004; Xu et al., 2004; Ma et al., 2006; Shin et al., 2006; Ma et al., 2007).

Respiratory disease due to influenza virus infections in swine

Many influenza virus infections in swine run a subclinical course. If clinical symptoms do occur, there can be a wide range in their severity. In its most typical and severe form, influenza is an acute respiratory disease, characterised by high fever (40.5-41.5°C), apathy, anorexia and laboured breathing (Bachmann, 1989; Easterday and Van Reeth, 1999). In the initial stage, coughing is not a very common symptom, but may be apparent during the later stages of the disease. Other symptoms, seen less frequently, are conjunctivitis, nasal discharge and sneezing. In a typical influenza outbreak, morbidity is high, but with a very low mortality and rapid recovery after five to seven days. Complications such as bacterial infections can however increase the severity, as well as prolong the duration of the disease. Pneumonia, with chronic respiratory disease, mainly characterised by coughing and suboptimal growth performances may be the result of these secondary bacterial infections.

Uncomplicated swine influenza virus infections result in superficial lesions in the lungs, mainly the bronchi and bronchioles. Epithelium cells become infected and ultimately necrotic. Airways can be filled with blood-tinged, fibrinous exudate. Macroscopically this can be seen as gross lesions, mostly of the apical and cardiac lobes of the lungs. Affected lung parts are dark red and are in sharp contrast to normal lung tissue. Recovery of epithelium can already be seen after a few days (Guarda and Sidoli, 1983; Bachmann, 1989; Brown et al., 1993; Easterday and Van Reeth, 1999).

During an outbreak, economic damages are said to be caused by severe growth retardation, increased food conversion ratios, use of medication, and mortality, although the latter is limited in uncomplicated cases. However, quantitative data on economic damages in the field are lacking.

Population dynamics of influenza virus in swine herds

Influenza virus infections in swine are very common. In Europe, seroprevalences for the three most common swine influenza strains, H1N1, H3N2 and H1N2, range from 20-80% in finishing pigs at the end of the finishing period and in sows (Masurel et al., 1983; Haesebrouck and Pensaert, 1986; Yus et al., 1989; Elbers et al., 1990; Teuffert et al., 1991; Elbers et al., 1992; Groschup et al., 1993; Ewald et al., 1994; Brown et al., 1995b; Maes et al., 1999; Van Reeth et al., 2000; Maldonado et al., 2006). However, little is known about the population dynamics of influenza virus within herds, or between herds. Year-round continuous circulation within a herd was shown by Nakamura et al. (1972) but the mechanism behind this was not clear. Carrier pigs were suggested as a reservoir. Persistent infections in individual animals, including persistence of virus in several internal organs, have been reported (Mensik and Valicek, 1969; Blaskovic et

al., 1970). Later studies were however unable to reproduce these results (Wallace and Elm, 1979; Brown et al., 1993), nor have there been any more recent reports of the existence of carriers. Furthermore, in experimental infections with a wide range of influenza virus strains, virus excretion is usually limited to 5-9 days (Easterday, 1971; Kundin and Easterday, 1972; Brown et al., 1993; Brown et al., 1994; Kida et al., 1994; Lee et al., 1995; Heinen et al., 2000; Heinen et al., 2001a; Heinen et al., 2001b). The early reports on carriers of influenza virus, and especially their role in the transmission of virus, may therefore be questioned. An alternative explanation put forward suggests that virus persistence in a herd is mainly maintained by continual passage in young susceptible pigs (Brown, 2000), but data to support this theory is lacking. Instead of persisting in a herd, influenza may also be introduced repeatedly through the purchase of infected pigs (Van Reeth and Pensaert, 1994) or through other transmission routes, which may be part of continuous virus circulation on a regional level or within a production chain.

Increased knowledge on these dynamics could have direct consequences for intervention strategies in the swine population, whether these be vaccination or zoosanitary measures. Furthermore, this knowledge will help in our understanding of how influenza viruses evolve.

Scope of the thesis

This research started in 1995. At that time the extent to which influenza virus infections were responsible for acute respiratory disease in swine was not yet known. Respiratory disease, however, was, and still is, a major problem in swine herds, especially in finishing pigs. Most of the infectious agents that are known to cause respiratory disease are endemic in the swine population, although often causing subclinical infections. Detecting the presence of these infectious agents doesn't therefore necessarily prove that they caused the symptoms of respiratory disease or were even involved, making it sometimes hard to diagnose the cause of respiratory disease correctly.

Chapter two describes a study that was carried out to find out what infectious agents are associated with acute respiratory disease in finishing pigs in the Netherlands. It was found that influenza virus infections were responsible for more than half of the outbreaks of acute respiratory disease which was the reason for the subsequent studies in this thesis.

Chapters three and four are devoted to a study of the population dynamics of influenza virus in weaned piglets and finishing pigs. Chapter three addresses the question to which extent influenza virus infections already occur in weaned piglets, which may act as a reservoir for continued virus circulation in breeding herds. Furthermore, the decay of maternal antibodies was studied, as this may affect piglets' susceptibility to an infection and virus transmission within a population of piglets with (different levels of) maternal antibodies. In chapter four, influenza virus infections in finishing pigs from farrow-to-finish and specialised finishing herds are compared. Longitudinal studies were performed to find out whether differences exist in the

population dynamics of influenza virus in both herd types, as such differences may affect the timing of intervention measures.

Chapter five describes an investigation of whether air filtration and hygienic measures are able to reduce the incidence of influenza virus infections. Studying the role of repeated introduction versus virus persistence in subpopulations of pigs also enabled more insight to be gained in the population dynamics of influenza virus in endemically infected herds.

Chapter six describes a study of the influence of maternal antibodies. This study investigated whether maternal antibodies affect the severity of clinical symptoms and the development of both humoral and cellular immunity against a homologous influenza virus strain.

The thesis will conclude with a general discussion.

References

Alexander, D.J., 2007, An overview of the epidemiology of avian influenza. Vaccine 25, 5637-5644.

Bachmann, P.A., 1989, Swine influenza virus, In: Pensaert, M.B. (Ed.) Virus infections of porcines. Elsevier Science Publishers, Amsterdam, pp. 193-207.

Bibrack, B., 1972, [Comparative serological studies on the occurrence of porcine influenza and influenza A2-Hongkong in pigs in Bavaria]. Zentralbl.Veterinarmed.[B] 19, 397-405.

Blakemore, F., Gledhill, A.W., 1941, Some observations on an outbreak of swine influenza in England. Veterinary Record 53, 227-230.

Blaskovic, D., Jamrichová, O., Rathová, V., Kaplan, M.M., Kocisková, D., 1970, Experimental infection of weanling pigs with A/swine influenza virus. 2. The shedding of virus by infected animals. Bull.World Health Organ. 42, 767-770.

Brown, I.H., 2000, The epidemiology and evolution of influenza viruses in pigs. Vet.Microbiol. 74, 29-46.

Brown, I.H., Alexander, D.J., Chakraverty, P., Harris, P.A., Manvell, R.J., 1994, Isolation of an influenza A virus of unusual subtype (H1N7) from pigs in England, and the subsequent experimental transmission from pig to pig. Vet.Microbiol. 39, 125-134.

Brown, I.H., Chakraverty, P., Harris, P.A., Alexander, D.J., 1995a, Disease outbreaks in pigs in Great Britain due to an influenza A virus of H1N2 subtype. Vet.Rec. 136, 328-329.

Brown, I.H., Done, S.H., Spencer, Y.I., Cooley, W.A., Harris, P.A., Alexander, D.J., 1993, Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. Vet.Rec. 132, 598-602.

Brown, I.H., Harris, P.A., Alexander, D.J., 1995b, Serological studies of influenza viruses in pigs in Great Britain 1991- 2. Epidemiol.Infect. 114, 511-520.

Brown, I.H., Harris, P.A., McCauley, J.W., Alexander, D.J., 1998, Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J.Gen.Virol. 79 (Pt 12), 2947-2955.

Burki, F., Werner-Tutschku, V., Nowotny, N., Werner-Tutschku, G., 1985, Swine influenza A/H1N1 on breeding premises of Austria. Wiener Tierarztliche Monatsschrift 72, 173-178.

Castrucci, M.R., Donatelli, I., Sidoli, L., Barigazzi, G., Kawaoka, Y., Webster, R.G., 1993, Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology 193, 503-506.

Dowdle, W.R., Hattwick, M.A., 1977, Swine influenza virus infections in humans. J.Infect.Dis. 136 Suppl, S386-S389.

Easterday, B.C., 1971, Influenza virus infection of the suckling pig. Acta Veterinaria Brno 40 Suppl 2, 33-42.

Easterday, B.C., Van Reeth, K., 1999, Swine Influenza, In: Straw, B.E., D'Allaire, S., Mengeling, W.L., Taylor, D.J. (Eds.) Diseases of Swine. Iowa State University Press, Ames, pp. 277-290.

Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1990, Sero-epidemiological screening of pig sera collected at the slaughterhouse to detect herds infected with Aujeszky's disease virus, porcine influenza virus and Actinobacillus (Haemophilus) pleuropneumoniae in the framework of an integrated quality control (IQC) system. Vet.Q. 12, 221-230.

Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1992, Variation in seropositivity for some respiratory disease agents in finishing pigs: epidemiological studies on some health parameters and farm and management conditions in the herds. Vet.Q. 14, 8-13.
Ewald, C., Heer, A., Havenith, U., 1994, Factors Associated with Influenza-A Virus-Infections in Finishing Pigs. Berliner und Munchener Tierarztliche Wochenschrift 107, 256-262.
Fouchier, R.A., Munster, V., Wallensten, A., Bestebroer, T.M., Herfst, S., Smith, D., Rimmelzwaan, G.F., Olsen, B., Osterhaus, A.D., 2005, Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79, 2814-2822.
Gourreau, J.M., Kaiser, C., Valette, M., Douglas, A.R., Labie, J., Aymard, M., 1994, Isolation of two H1N2 influenza viruses from swine in France. Arch.Virol. 135, 365-382.
Groschup, M.H., Brun, A., Haas, B., 1993, Serological studies on the potential synergism of porcine reproductive and respiratory syndrome virus and influenza-, corona- and paramyxoviruses in the induction of respiratory symptoms in swine. Zentralbl.Veterinarmed.[B] 40, 681-689.
Guarda, F., Sidoli, L., 1983, Swine influenza: gross and histological respiratory tract lesions. Deutsche Tierarztliche Wochenschrift 90, 356-358.
Haesebrouck, F., Pensaert, M.B., 1986, Prevalence of H1N1- and H3N2-influenza A viruses in fatteners [swine] in Belgium (1984). Vlaams Diergeneeskundig Tijdschrift 55, 12-16.
Harkness, J.W., Schild, G.C., Lamont, P.H., Brand, C.M., 1972, Studies on relationships between human and porcine influenza. 1. Serological evidence of infection in swine in Great Britain with an influenza A virus antigenically like human Hong Kong/68 virus. Bulletin of the World Health Organization 46, 709-719.
Heinen, P.P., Boer-Luijtze, E.A., Bianchi, A.T., 2001a, Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. J.Gen.Virol. 82, 2697-2707.
Heinen, P.P., Van Nieuwstadt, A.P., Boer-Luijtze, E.A., Bianchi, A.T., 2001b, Analysis of the quality of protection induced by a porcine influenza A vaccine to challenge with an H3N2 virus. Vet.Immunol.Immunopathol. 82, 39-56.
Heinen, P.P., Van Nieuwstadt, A.P., Pol, J.M.A., De Boer-Luijtze, E.A., Van Oirschot, J.T., Bianchi, A.T.J., 2000, Systemic and mucosal isotype-specific antibody responses in pigs to experimental influenza virus infection. Viral Immunol. 13, 237-247.
Hinshaw, V.S., Alexander, D.J., Aymard, M., Bachmann, P.A., Easterday, B.C., Hannoun, C., Kida, H., Lipkind, M., MacKenzie, J.S., Nerome, K., Schild, G.C., Scholtissek, C., 1984, Antigenic comparisons of swine-influenza-like H1N1 isolates from pigs, birds and humans: an international collaborative study. Bulletin of the World Health Organization 62, 871-878.
Karasin, A.I., Brown, I.H., Carman, S., Olsen, C.W., 2000, Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J.Virol. 74, 9322-9327.
Karasin, A.I., West, K., Carman, S., Olsen, C.W., 2004, Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. J Clin Microbiol 42, 4349-4354.
Kendal, A.P., Joseph, J.M., Kobayashi, G., Nelson, D., Reyes, C.R., Ross, M.R., Sarandria, J.L., White, R., Woodall, D.F., Noble, G.R., Dowdle, W.R., 1979, Laboratory-based surveillance of influenza virus in the United States during the winter of 1977-1978. I. Periods of prevalence of H1N1 and H3N2 influenza A strains, their relative rates of isolation in different age groups, and detection of antigenic variants. Am J Epidemiol 110, 449-461.
Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K.F., Kawaoka, Y., Webster, R.G., 1994, Potential for transmission of avian influenza viruses to pigs. Journal of General Virology 75, 2183-2188.
Kozlov, J.V., Gorbulev, V.G., Kurmanova, A.G., Bayev, A.A., Shilov, A.A., Zhdanov, V.M., 1981, On the origin of the H1N1 (A/USSR/90/77) influenza virus. J Gen Virol 56, 437-440.
Kundin, W.D., Easterday, B.C., 1972, Hong Kong influenza infection in swine: experimental and field observations. Bulletin of the World Health Organization 47, 489-491.
Lee, B.W., Bey, R.F., Baarsch, M.J., Larson, M.E., 1995, Class specific antibody response to influenza A H1N1 infection in swine. Vet.Microbiol. 43, 241-250.
Ma, W., Gramer, M., Rossow, K., Yoon, K.J., 2006, Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States. J Virol 80, 5092-5096.
Ma, W., Vincent, A.L., Gramer, M.R., Brockwell, C.B., Lager, K.M., Janke, B.H., Gauger, P.C., Patnayak, D.P., Webby, R.J., Richt, J.A., 2007, Identification of H2N3 influenza A viruses from swine in the United States. Proc Natl Acad Sci U S A 104, 20949-20954.

Maes, D., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., Vrijens, B., De Kruif, A., 1999, Risk indicators for the seroprevalence of Mycoplasma hyopneumoniae, porcine influenza viruses and Aujeszky's disease virus in slaughter pigs from fattening pig herds. Zentralbl.Veterinarmed.[B] 46, 341-352.

Maldonado, J., Van Reeth, K., Riera, P., Sitja, M., Saubi, N., Espuna, E., Artigas, C., 2006, Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet J 172, 377-381.

Marozin, S., Gregory, V., Cameron, K., Bennett, M., Valette, M., Aymard, M., Foni, E., Barigazzi, G., Lin, Y., Hay, A., 2002, Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. J.Gen.Virol. 83, 735-745.

Masurel, N., De Boer, G.F., Anker, W.J., Huffels, A.D., 1983, Prevalence of influenza viruses A-H1N1 and A-H3N2 in swine in the Netherlands. Comp.Immunol.Microbiol.Infect.Dis. 6, 141-149.

Mensik, J., Valicek, L., 1969, Pathogenesis of experimental influenza in sucking piglets. 2. Persistence of virus in the lungs and in organs outside the respiratory tract. Zentralbl.Veterinarmed.[B] 16, 808-817.

Nakamura, R.M., Easterday, B.C., Pawlisch, R., Walker, G.L., 1972, Swine influenza: epizootiological and serological studies. Bulletin of the World Health Organization 47, 481-487.

Nardelli, L., Pascucci, S., Gualandi, G.L., Loda, P., 1978, Outbreaks of classical swine influenza in Italy in 1976. Zentralblatt fur Veterinarmedizin Reihe B 25, 853-857.

Ottis, K., Bollwahn, W., Bachmann, P.A., Heinritzi, K., 1981, Outbreak of swine Influenza in the Federal Republic of Germany: clinical symptoms, virus isolation and identification. Tierarztliche Umschau 36, 608-612.

Peiris, J.S., Guan, Y., Markwell, D., Ghose, P., Webster, R.G., Shortridge, K.F., 2001, Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J.Virol. 75, 9679-9686.

Pensaert, M., Ottis, K., Vandeputte, J., Kaplan, M.M., Bachmann, P.A., 1981, Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. Bulletin of the World Health Organization 59, 75-78.

Popovici, V., Hiastru, F., Draghici, D., Zilisteanu, E., Matepiuc, M., Cretescu, L., Niculescu, I., 1972, Infection of pigs with an influenza virus related to the A2-Hong Kong-1- 68 strain. Acta Virol. 16, 363.

Scholtissek, C., Burger, H., Bachmann, P.A., Hannoun, C., 1983, Genetic relatedness of hemagglutinins of the H1 subtype of influenza A viruses isolated from swine and birds. Virology 129, 521-523.

Scholtissek, C., von Hoyningen, V., Rott, R., 1978, Genetic relatedness between the new 1977 epidemic strains (H1N1) of influenza and human influenza strains isolated between 1947 and 1957 (H1N1). Virology 89, 613-617.

Schrader, C., Suss, J., 2003, Genetic characterization of a porcine H1N2 influenza virus strain isolated in Germany. Intervirology 46, 66-70.

Schultz, U., Fitch, W.M., Ludwig, S., Mandler, J., Scholtissek, C., 1991, Evolution of pig influenza viruses. Virology 183, 61-73.

Shin, J.Y., Song, M.S., Lee, E.H., Lee, Y.M., Kim, S.Y., Kim, H.K., Choi, J.K., Kim, C.J., Webby, R.J., Choi, Y.K., 2006, Isolation and characterization of novel H3N1 swine influenza viruses from pigs with respiratory diseases in Korea. J Clin Microbiol 44, 3923-3927.

Shope, R.E., 1931, Swine influenza. III. Filtration experiments and aetiology. J Exp Med 54, 373-385.

Teuffert, J., Sinnecker, R., Karge, E., 1991, Serological survey of HI antibodies against porcine and human type A influenza viruses among domestic and wild pigs in East Germany.; [Seroepidemiologische Untersuchungen mit dem HAHT zum Vorkommen porciner und humaner Infl-A-Viren bei Haus- und Wildschweinen in der ehemaligen DDR]. Monatsh.Veterinarmed. 46, 171-174.

Tsai, C.P., Pan, M.J., 2003, New H1N2 and H3N1 influenza viruses in Taiwanese pig herds. Vet Rec 153, 408.

Van Reeth, K., Brown, I.H., Pensaert, M., 2000, Isolations of H1N2 influenza A virus from pigs in Belgium. Veterinary Record 146, 588-589.

Van Reeth, K., Pensaert, M., 1994, Prevalence of infections with enzootic respiratory and enteric viruses in feeder pigs entering fattening herds. Veterinary Record 135, 594-597.

Vanderputte, J., Pensaert, M.B., Andries, K., 1981, Swine Influenza in Belgium: Virus Isolation and Experimental Infection. Vlaams Diergeneeskundig Tijdschrift 50, 291-300.

Wallace, G.D., Elm, J.L., Jr., 1979, Transplacental transmission and neonatal infection with swine influenza virus (Hsw1N1) in swine. Am.J.Vet.Res. 40, 1169-1172.
Witte, K.H., Nienhoff, N., Ernst, H., Schmidt, U., Prager, D., 1981, The first appearance of swine influenza in pig herds in the Federal Republic of Germany. Tierarztliche Umschau 36, 591-606.
Xu, C., Fan, W., Wei, R., Zhao, H., 2004, Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. Microbes Infect 6, 919-925.
Yus, E., Laviada, M.D., Moreno, L., Castro, J.M., Escribano, J.M., Simarro, I., 1989, The prevalence of antibodies to influenza virus and respiratory coronavirus in fattening pigs in Spain. Zentralbl.Veterinarmed.[B] 36, 551-556.

Chapter 2

A survey of infectious agents involved in acute respiratory disease in finishing pigs

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A survey of infectious agents involved in acute respiratory disease in finishing pigs

Abstract

Outbreaks of respiratory disease constitute a major health problem in pig finishing herds. The aetiology of these outbreaks often remains unclear. In this study, sixteen outbreaks of respiratory disease with acute clinical signs in finishing pigs were investigated thoroughly to determine which infectious agents were involved in these outbreaks. From each herd with an acute outbreak four diseased and two clinically healthy pigs were pathologically examined and examined for the presence of viruses, bacteria and mycoplasmas. Furthermore, paired blood samples from ten groupmates of the diseased pigs were tested for antibodies against commonly known causal agents of respiratory disease. A clear diagnosis was possible in twelve of the sixteen outbreaks. Seven were due to an infection with influenza virus and five were due to an infection with *Actinobacillus pleuropneumoniae*. A combination of influenza virus and *A. pleuropneumoniae* may have caused one other outbreak, whereas in the other three outbreaks no clear cause could be established. The important role of influenza virus in these outbreaks was unexpected and deserves further research, especially with respect to prevention of disease caused by this virus.

Introduction

Respiratory diseases are considered to be the most important health disorders in finishing pigs. They are responsible for over half of all antibiotic treatments in these pigs (Elbers et al., 1990a). In addition to the costs of these treatments, they are also responsible for losses from mortality, decreased growth rates, reduced feed conversion efficiency and reduced carcass quality (Straw et al., 1990; Paisley et al., 1993).

Viruses, bacteria or mycoplasmas are usually the primary cause of respiratory disease in pigs, although for many of these agents the exact role in the often complex pathogenesis is not fully understood. Environmental factors, farm management and defence mechanisms of the pig may also play an important role in the onset of respiratory disease or the severity of the clinical signs of disease (Christensen and Mousing, 1992).

The two most important primary bacterial agents involved in respiratory disease are considered to be *Mycoplasma hyopneumoniae*, which causes enzootic pneumonia (Ross, 1992), and *Actinobacillus pleuropneumoniae*, which causes pleuropneumonia (Sebunya and Saunders, 1983). The most important secondary agent, involved in respiratory disease only after lung defence mechanisms have been impaired, is considered to be *Pasteurella multocida* (Ciprián et al., 1988; Pijoan, 1992). The most important viruses in respiratory disease are swine influenza virus, Aujeszky's disease

virus, Porcine Reproductive and Respiratory Syndrome (PRRS) virus and Porcine Respiratory Corona virus (PRCV), although especially for the latter two the exact role in respiratory disease is unclear (Laude et al., 1993; Wensvoort, 1993; Done and Paton, 1995).

Dual infections, in which two infectious agents cause respiratory disease after infecting the host at the same time or subsequently with a short interval, have gained more attention during the last decade. Dual infections, involving several viruses, bacteria and mycoplasmas, have been studied under experimental conditions with inconclusive results (Fuentes and Pijoan, 1987; Iglesias et al., 1992; Lanza et al., 1992; Amass et al., 1994; Kay et al., 1994; Van Reeth and Pensaert, 1994; Van Reeth and Pensaert, 1996; Van Reeth et al., 1996a,b).

Although some field studies were published on etiological agents of respiratory disease, these studies were usually limited to certain pathogens. Also, some of these studies were carried out in finishing pigs at the slaughterhouse, where there is no clear relation to clinical problems. Studies to associate bacterial agents with respiratory disease were, amongst others, published by Awad-Masalmeh et al. (1990), Castryck et al. (1990), Falk et al. (1991), Falk and Lium (1991) and Høie et al. (1991), whereas serological investigations were carried out by Callebaut et al. (1986), Elbers et al. (1992), Nowotny et al. (1994) and Maes et al. (1996). Results of a more elaborate study in five herds, combining serological investigation, isolation of bacteria and immunofluorescence microscopy were published recently by Runge et al. (1996). To our knowledge, studies with extensive investigations into infectious causes of acute respiratory disease in a substantial number of herds, during the acute phase and considering all possible etiological agents, have not been published recently.

The main purpose of this study was to identify infectious agents involved in acute respiratory disease in finishing pigs, preferably during the first half of the finishing period. We also searched for clues of dual infections and the importance of dual infections in the field.

Materials & methods

Selection of herds.

Forty finishing or farrow-to-finish herds in the southern Netherlands were selected out of approximately 200 herds with a known history of recurrent respiratory problems in the finishing pigs. Criteria for selection were: recurring respiratory problems with acute symptoms, preferably during the first half of the finishing period, frequent use of curative medication and restricted use of preventive medication. Further criteria were a herd size of more than 500 finishing pigs, compartments of 60-100 pigs, and the practice of an all-in-all-out system.

Sampling.

Farmers were requested to notify the Animal Health Service as soon as possible, and before starting medication, if at least one of the following clinical symptoms was observed in a compartment of finishing pigs without a history of respiratory disease:

decrease of feed intake with 10% or more, coughing or laboured breathing. Farms were usually visited the same day, but always within 24 hours. An outbreak was included for further investigation when clinical symptoms of respiratory disease were present, with several pigs having a fever (>40°C, temperatures were only taken from pigs with clear clinical signs) and no antibiotics had been used for at least one week. From December 1, 1995 until April 15, 1996 sixteen outbreaks where reported that met these criteria. Only one outbreak per herd was investigated.

From each outbreak, four diseased pigs from one compartment and two clinically healthy pigs of approximately the same age, but from another compartment, were admitted for pathological examination and isolation of viruses, bacteria and mycoplasmas. Diseased and healthy pigs were kept separately during transport.

In the compartment where the outbreak occurred, blood samples were taken from ten groupmates of the four selected pigs, all with acute symptoms of respiratory disease (acute sera). The same pigs were bled again four weeks later (convalescent sera) and, if they stayed at least another three weeks on the farm, at the end of the finishing period (designated end sera). Furthermore, blood samples were taken from ten randomly chosen pigs in one or two other compartments at the end of the finishing period (random end sera).

Pathology.

All six pigs were anaesthetized and exanguinated at the research facilities of ID-DLO in Lelystad. A post-mortem examination was carried out with special attention to the lungs. When present, fluid from the thoracic cavity was collected in a sterile syringe and preserved in heparin. The percentage of macroscopically affected lung tissue was estimated and the types of lung lesions were recorded. The right cranial lobe was flushed with 30ml of sterile Eagle's Minimal Essential Medium (EMEM, Flow Laboratories, Irvine, England) and the right caudal lobe was flushed with 30 ml of sterile physiological saline. The recovered lung lavage fluids were examined for the presence of bacteria and mycoplasmas. Tissue specimens were taken from the right cranial lobe, the right caudal lobe and the caudal part of the left cranial lobe for histological, bacteriological and virological examination. Additional specimens were taken in cases where pneumonic lesions were not present at these sites. For histological examination samples of these specimens were fixed in 4% formalin and tissue slides were stained with hematoxylin-eosin stain. Tonsils were collected for virus isolation.

Isolation of viruses.

In each outbreak approximately 20-25 pieces of lung tissue and tonsil were used for virus isolation. Tissue was homogenised by grinding it with sand in EMEM with 0,5% lactalbumin hydrolysate and antibiotics in a mortar. Tissue homogenates were clarified at 1500g for 10 minutes and passed through a 200 nm filter. The filtrate was inoculated on monolayers of secondary pig thyroid cells and on primary pig lung macrophages. Monolayers were incubated in a CO2-incubator at 36°C for one to two weeks and examined for cythopathic effects every day. Viruses isolated on pig thyroid cells were tested in a haemagglutination test. Haemagglutinating viruses were tested in a

haemagglutination inhibition test with specific antisera against influenza virus A, subtype H1N1 (A/Swine/Netherlands/80) and H3N2 (A/Port Chalmers/1/73). Viruses isolated on pig lung macrophages were tested in an immuno-peroxidase test with a specific conjugate against PRRS virus.

Isolation of bacteria.

Lung tissue specimens were decontaminated for 6-8 seconds in boiling water, cut aseptically into small pieces and minced in 5 ml Brain Heart Infusion broth (Difcco Laboratories, Detroit, US) by use of a Stomacher 80 lab blender. Lung lavage fluids were centrifuged for 10 minutes at 1000g and supernatants were discarded.

Thorax fluids, supernatants of minced lung tissues, and the pellet of lung lavages were inoculated with a loop onto sheep blood agar and sheep blood agar supplemented with 0.1% NAD. Plates were incubated at 37°C under aerobic, anaerobic, and micro-aerophilic conditions for at least 48 to 72 hours. Plates were read at least at two occasions. Bacterial growth was further identified according to Cowan and Steels (1993) manual for the identification of medical bacteria. Known pathogens were always recorded. Other bacteria were only identified when isolated in pure culture.

Isolation of mycoplasmas.

Lung tissue specimens were minced as described above, except that the Brain Heart Infusion broth contained 40 I.U. penicillin and 0.08% thallium acetate.

Thorax fluid, supernatants of minced lung tissues, and the lung lavage fluids were immediately inoculated onto modified Edward agar and Friis agar (Friis 1975) and were incubated at 37°C under micro-aerophilic conditions. In addition, specimens were serially diluted tenfold to 10-5 in NHS25 broth and to 10-2 in NHS25 with a rabbit antiserum against *M. hyorhinis*. The broth media were incubated at 37°C in rolling drums in which the tubes rotated once per minute. Media were inspected every other day for three weeks. Acidified cultures were transferred twice in tenfold dilutions and then stored at -70°C until further use.

The isolated mycoplasmas were identified using an indirect immuno-fluorescence test (Rosendal and Black, 1972) and two PCR assays (Stemke et al., 1994; Mattsson et al., 1995), which enable discrimination between *M. hyorhinis*, *M. hyopneumoniae* and *M. flocculare*.

Serological examination.

All sera collected in compartments with a reported outbreak (acute sera, convalescent sera and designated end sera), and all random end sera were tested for antibodies against the following pathogens:

- Aujeszky's disease virus, using a gE-ELISA (van Oirschot et al., 1988).
- PRRS virus, using an immuno-peroxidase monolayer assay (IPMA) as described by Wensvoort et al. (1991). Random end sera were tested in a 1:200 dilution. Paired sera and designated end sera were tested in serial twofold dilutions to determine antibody titres.

- Influenza virus, using a haemagglutination inhibition (HAI) test (Kendal et al., 1982) for antibodies against H1 and H3. All sera were tested in serial twofold dilutions, starting at 1:9. Titres ≥18 were considered positive. Influenza virus strains A/swine/Neth/Best/96 and A/swine/Neth/St Oedenrode/96 were used to test for H1 and H3 antibodies respectively. Both strains were isolated in this study.
- *A. pleuropneumoniae*, using a complement fixation (CF) test (Nicolet et al., 1971) with a mixture of a serotype 2 and a serotype 9 strain as antigen. Sera were tested in a 1:40 dilution. No further differentiation was made between serotypes 2 and 9.
- *M. hyopneumoniae*, using a commercially available ELISA which specifically detects an epitope of *M. hyopneumoniae* without cross-reacting with *M. flocculare* or *M. hyorhinis* (DAKO *M. hyopneumoniae* ELISA Kit, DAKO, Glostrup, Denmark).

Results

Clinical symptoms.

Clinical symptoms in the first 15 outbreaks were very similar: feed intake was decreased, pigs were depressed (recumbent, slow, 'sick'), and respiratory signs were present (coughing, laboured breathing). Clear symptoms were usually seen in 10-30% of the pigs at the time of the first visit. Most pigs with clinical symptoms also had fever (40-42°C). Although similar, clinical symptoms in herd 16 were less severe and fewer pigs were affected than in the other 15 herds.

In herds 1 and 3-15, clinical outbreaks occurred in pigs of 14 to 20 weeks old, covering the second month of the finishing period. In herd 2, the pigs were 22 weeks old and in herd 16, the pigs were only 11 weeks old.

Pathological examination.

Macroscopic lung lesions were found in 61 of the 64 clinically diseased pigs. Percentages of affected lung ranged from 5 to 50%. Most lesions were multilobular and catarrhal, sometimes with purulent exudate. In herds 3, 9, 10 and 11 focal or multifocal haemorrhagic (pleuro)pneumonia was predominant. In herds 8 and 16 (multi)lobular as well as (multi)focal lesions were present.

In most pigs acute lesions were predominant. However, next to acute lesions, subacute or chronic lesions were also present in the pigs from herds 2, 5, 9, 10, 11, 12, 14 and 16. Notable were also the lesions in the liver, due to migrating Ascaris suum larvae, in all pigs from herd 12.

Macroscopic lung lesions were also found in 16 of the 32 clinically healthy pigs in herds 2, 3, 5, 8, 9, 11, 12, 15 and 16. Ten pigs had pneumonia and six pigs had a chronic pleuritis. Lesions of these 'healthy' pigs were always less severe than those of the diseased pigs from the same herd. Percentage of affected lung rarely exceeded 5%.

Isolation of viruses, bacteria and mycoplasmas.

Influenza virus, *A. pleuropneumoniae*, *S. suis* and *P. multocida* were frequently isolated and more often from diseased pigs and clinically healthy pigs with lung lesions

than from healthy pigs without lung lesions (Table 1). The isolation of *P. multocida* was usually associated with the presence of chronic or subacute lesions. *M. hyorhinis*, PRRS virus, *H. parasuis* and *B. bronchiseptica* were also frequently isolated, but from both diseased as well as healthy pigs, with or without lung lesions. *M. hyopneumoniae* was not frequently isolated and from both diseased and clinically healthy pigs (Table 1).

Influenza virus was isolated from pigs from nine herds (Table 2). Subtype H1 was predominant, but H3 was also isolated from two herds. In seven of the herds (5, 6, 7, 12, 13, 14 and 15) the virus was isolated from the lungs of three or four diseased pigs, while histological examination revealed a high correlation with the presence of endobronch(iol)itis (Table 2). From some of the pigs in herds 9 and 16, influenza virus was isolated from tonsils only. *A. pleuropneumoniae* was isolated from pigs from seven herds (Table 3). All isolates were serotype 2. In five of the herds (3, 8, 9, 10 and 11) bacteria were isolated from the lungs of three or four diseased pigs. Pathological and

Table 1: Isolated agents in diseased and clinically healthy pigs in sixteen acute outbreaks of respiratory disease

Agent	Diseased (n=64)		Clinically healthy			
			With lung lesions (n=16)		Without lung lesions (n=16)	
	n	(%)	n	(%)	n	%
Viruses						
Influenza virus	32	(50)	5	(31)	3	(19)
PRRS virus	18	(28)	3	(19)	6	(38)
Bacteria						
App serotype 2	21	(33)	4	(25)	-	(-)
P. multocida	12	(19)	1	(6)	-	(-)
H. parasuis	16	(25)	3	(19)	2	(13)
B. bronchiseptica	14	(22)	4	(25)	2	(13)
S. suis	16	(25)	1	(6)	-	(-)
A. pyogenes	1	(2)	-	(-)	-	(-)
Mycoplasmas						
M. hyopneumoniae	6	(9)	2	(13)	2	(13)
M. hyorhinis	47	(73)	12	(75)	10	(63)

histological examination revealed a high correlation with haemorrhagic and, especially, necrotising pneumonia (Table 3). In herd 7, *A. pleuropneumoniae* was isolated from one pig only and no other indications were found for an infection. In herd 16, *A. pleuropneumoniae* was isolated from two pigs. Only one of them had pathological and histological signs of haemorrhage and necrosis.

In four herds influenza virus or *A. pleuropneumoniae* was not the single most important isolated agent. In table 4 the main pathological findings and isolated agents are summarised for each individual pig in these four herds. PRRS virus was isolated from the lungs of three or four diseased pigs from herds 1 and 4. However, it was also

Table 2: Influenza-virus (H1 and H3) isolations and pathological findings in four diseased and two clinically healthy pigs from each of sixteen acute outbreaks of respiratory disease

Outbreak	Diseased pigs				Healthy pigs	
	1	2	3	4	5	6
1	I[a]	I	I	I		
2		I	I	I	E[b]	I
3	I	I			I	
4	I	I	IE	I	I	
5	IE	IE	E	IE	IE	I
6	IE	I	I	I	I	
7	I	IE		E		
8						
9	E		I			
10			I	I	I	
11		I				I
12	E	IE	E			
13	E	IE	IE	IE		
14		IE	E	E	I	I
15	IE	E	IE	E	IE	IE
16		IE	I	IE	I	IE

[a]) I = Interstitial pneumonia (microscopic), [b]) E = Endobronch(iol)itis (microscopic), Shaded = Influenza-virus isolated from lungs and/or tonsils (H3 in herds 7 and 12, all others H1. Virus was isolated only from the tonsils of pigs 1 and 3 from herd 9 and pigs 4 and 5 from herd 16)

isolated from both clinically healthy pigs from herd 4. Several bacteria were isolated, but none of them consistently. In herd 16, influenza virus as well as *A. pleuropneumoniae* were isolated from some of the pigs from typical lung lesions for either of these agents. Both infections seemed to exist next to each other, with pigs infected with either one or both.

Serological examination of paired sera.

Most groupmates of the four diseased pigs in herds 5, 6, 9, 13, 14 and 15 seroconverted against influenza virus subtype H1, whereas groupmates in herds 7 and 12 seroconverted against influenza virus subtype H3 (Table 5). This correlated perfectly

Table 3: *A. pleuropneumoniae* serotype 2 isolations and pathological findings in four diseased and two clinically healthy pigs from each of sixteen acute outbreaks of respiratory disease

Outbreak	Diseased pigs				Healthy pigs	
	1	2	3	4	5	6
1						
2				H[a]		
3	HN[b]	HN	H	HN		
4						
5						H
6	H					
7						
8	HN	HN	H			
9	HN	N		HN	N	
10	HN	HN	HN	HN		
11	N	HN	HN			
12						
13	H		H	H		
14	H			H		
15						
16	HN					H

a) H = Haemorrhage (macro- or microscopic), b) N = Necrosis (macro- or microscopic) Shaded = *A. pleuropneumoniae* isolated from lungs and/or lung lavage fluids

with the isolation of influenza virus subtypes H1 and H3 from the diseased pigs of the respective herds. In herd 16 however, the groupmates did not seroconvert against

Table 4: Main pathological findings and isolated agents from all six pigs from 4 herds (1, 2, 4 and 16) with unclear diagnosis. Diseased pigs are numbered 1-4, clinically healthy pigs 5 and 6.

#	Pig	Main pathological findings		Viruses[1]		Bacteria[2]					Myc[3]	
		Description[4]	% affected lung (macrosc)	Infl	Prrs	Ap	Pm	Hp	Bb	Ss	hp	hr
1	1	broncho-interstitial pneumonia (hist)	0	-[5]	±	-	-	-	+	-	-	+
	2	catarrhal pneumonia, pleuritis	<10	-	+	-	-	+	+	-	-	+
	3	broncho-interstitial pneumonia (hist)	0	-	+	-	-	+	+	-	-	+
	4	catarrhal pneumonia	30	-	+	-	-	-	+	-	-	+
	5	peribronch /perivasc infiltrates (hist)	0	-	-	-	-	-	-	-	-	-
	6	peribronchiolar infiltrates (hist)	0	-	-	-	-	-	-	-	-	+
2	1	catarrhal purulent pneumonia	50	-	-	-	-	-	-	-	-	+
	2	catarrhal purulent pneumonia	50	-	-	-	-	-	-	+	-	+
	3	catarrhal purulent pneumonia	40	-	-	-	+	-	-	+	-	+
	4	catarrhal purulent pneumonia	40	-	-	-	+	-	-	+	-	-
	5	pleuritis	5	-	-	+	-	-	-	-	+	+
	6	catarrhal purulent pneumonia	5	-	-	-	-	-	-	-	-	-
4	1	catarrhal purulent pneumonia	30	-	+	-	-	+	-	+	-	+
	2	catarrhal purulent pneumonia	15	-	+	-	-	-	+	-	-	+
	3	catarrhal pneumonia	10	-	+	-	-	+	+	+	-	+
	4	broncho-interstitial pneumonia (hist)	0	-	+	-	-	-	-	-	-	-
	5	broncho-interstitial pneumonia (hist)	0	-	+	-	-	-	-	-	+	+
	6	-	0	-	+	-	-	-	-	-	-	+
16	1	necrotizing pleuropneumonia	15	-	-	+	-	-	-	-	-	+
	2	catarrhal purulent interst pneumonia	25	+	-	-	-	-	-	-	-	+
	3	catarrhal purulent pneumonia	20	-	-	-	+	-	-	-	-	+
	4	interstitial pneumonia, pleuritis	10	±	-	+	-	-	-	-	-	+
	5	interstitial pneumonia (hist)	0	±	-	-	-	-	-	-	-	+
	6	catarrhal pneumonia	5	+	-	-	-	-	-	-	-	+

[1]) Infl=Influenzavirus, [2]) Ap=*A. pleuropneumoniae*, Pm=*P. multocida*, Hp=*H. parasuis*, Bb=*B. bronchiseptica*, Ss=*S. suis*, [3]) Myc=mycoplasma, hp=*M. hyopneumoniae*, hr=*M. hyorhinis*, [4]) Macroscopic lesions, unless followed by (hist)=histological findings. [5]) +=isolated from lungs, ±=isolated from tonsils only, -=not isolated

influenza virus, even though the virus, subtype H1, was isolated from two of the diseased pigs. Antibodies against subtype H1 were already present in the acute sera.

At least four of the ten groupmates of the four diseased pigs in herds 3, 8, 9, 10, 11, and 16, seroconverted against *A. pleuropneumoniae* (Table 5). At least nine of these groupmates were seropositive for *A. pleuropneumoniae* at the end of the finishing period (data not shown). *A. pleuropneumoniae* serotype 2 was isolated from the lungs of the diseased pigs from all these herds. In herds 1 and 13, at least five out of ten tested groupmates seroconverted, although *A. pleuropneumoniae* was not isolated from the four diseased pigs.

Table 5: Serological results of paired sera of pigs in sixteen acute outbreaks of respiratory disease (total number of blood samples taken each time = 10)

Outbreak	Number of acute, resp. convalescent sera that tested positive					
	Infl[a].H1	Infl.H3	Auj[b].gE	PRRS	App[c]	Mhyo[d]
1	9-7	10-8	1-0	10-10	0-5	0-1
2	10-10	10-10	0-0	10-10	0-0	4-9
3	9-10	1-2	0-0	10-10	0-8	3-4
4	0-1	6-6	0-0	10-10	0-0	2-5
5	0-10	6-5	0-0	10-10	0-0	6-5
6	0-10	3-4	0-0	1-10	1-0	0-0
7	4-3	1-10	0-0	9-10	0-0	0-0
8	7-6	0-0	0-0	10-10	0-7	4-6
9	0-9	10-10	0-0	9-10	4-9	2-2
10	7-9	5-4	0-0	9-9	0-4	0-1
11	9-9	0-0	0-0	10-10	2-9	4-2
12	1-4	0-10	0-0	10-10	0-0	6-7
13	1-10	0-0	0-0	9-9[e]	0-8	0-0
14	0-10	10-10	0-0	10-10	6-6	7-1
15	0-10	0-0	0-0	10-10	0-0	5-10
16	9-10	4-1	1-0	7-10	0-4	0-0

[a]) Influenza, [b]) Aujeszky's disease, [c]) *A. pleuropneumoniae*, [d]) *M. hyopneumoniae*, [e]) Although nine acute sera were already positive, a four fold rise in titre was observed in 6 pigs, Shaded = seroconversion (negative-positive or four fold rise of titre) in ≥50% of tested pigs

Serological examination of random end sera and designated end sera.

Pigs in most compartments became seropositive for one or both subtypes of influenza virus, PRRS virus, and *M. hyopneumoniae*. In only half of the compartments pigs became seropositive for *A. pleuropneumoniae*, whereas none became seropositive for Aujeszky's disease virus (Table 6).

Some of the designated end sera were positive for influenza virus (herds 4 and 11) or *M. hyopneumoniae* (herds 6 and 16), whereas the convalescent sera were still negative for these agents. Apparently these infections occurred after the investigated outbreaks.

Overall findings and diagnosis

Influenza virus was the most important infectious agent involved in acute respiratory disease. In herds 5, 6, 7, 12, 13, 14 and 15, we concluded that no other detectable agent but influenza virus was responsible for the outbreak of respiratory disease (Table 7). Influenza virus was isolated from three or four of the diseased pigs, endobronch(iol)itis was frequently observed, and nine or ten of the groupmates seroconverted against the isolated subtype. Except for herd 5, few secondary bacteria were isolated.

In herds 9 and 16, influenza virus was also isolated from several pigs, but sometimes only from the tonsils. Even though endobronch(iol)itis was found in some pigs and the groupmates in herd 9 seroconverted for the isolated subtype, we concluded that influenza virus was not the sole or most important cause of these outbreaks.

Table 6: Serological results of all designated and random end sera in sixteen herds with a clinical outbreak (Compartment is called positive if at least two individual pigs were seropositive, doubtful results and results of vaccinated pigs omitted)

	Compartments			Individual pigs		
	pos[a]	n[b]	**%**	pos	n	**%**
Influenza H1	38	40	**95**	327	400	**82**
Influenza H3	33	40	**83**	222	400	**56**
Aujeszky-gE	0	40	**0**	0	400	**0**
PRRS	40	40	**100**	400	400	**100**
App 2/9	20	39	**51**	170	365	**47**
M. hyopneumoniae	31	43	**72**	166	303	**55**

[a]) pos = number of positive compartments respectively individual pigs, [b]) n = number of compartments respectively individual pigs tested

A. pleuropneumoniae was the second most important agent involved in acute respiratory disease. In herds 3, 8, 10 and 11, *A. pleuropneumoniae* serotype 2 was isolated from three or four of the diseased pigs, pigs had haemorrhagic and necrotic lung lesions, and groupmates seroconverted against *A. pleuropneumoniae*. In herd 9, a concurrent infection with influenza virus occurred, but based on isolation and pathological findings (including histology) we concluded that *A. pleuropneumoniae* was the most important agent causing the outbreak (Table 7). In herds 1 and 13, the groupmates of the diseased pigs seroconverted, but *A. pleuropneumoniae* was not isolated, nor were typical lung lesions observed. The *A. pleuropneumoniae* infection

Table 7: History of infections in sixteen compartments with a clinical outbreak, based on serology, isolations of infectious agents and pathological findings

#	Before clinical outbreak	**Cause of clinical outbreak**	Concurrent infection	After clinical outbreak	Secondary bacteria
1	PRRS/Influenza	**Sec. infections?**[a]		App	++[b]
2	Mhyo[c]/PRRS/Infl.[d]	**Sec. infections?**			++
3	Mhyo/PRRS/Infl.	**App[e] type 2**			-
4	PRRS/Influenza	**Sec. infections?**	Mhyo	Influenza H1	++
5	Mhyo/PRRS	**Influenza H1**			+++
6		**Influenza H1**	PRRS	Mhyo	+
7	PRRS	**Influenza H3**			+
8	Mhyo/PRRS/Infl.	**App type 2**			+
9	PRRS	**App type 2**	Influenza H1		++
10	PRRS/Influenza	**App type 2**			++
11	Mhyo/PRRS/Infl.	**App type 2**		Influenza H3	++
12	Mhyo/PRRS/Ascaris	**Influenza H3**			+
13	PRRS	**Influenza H1**		App	+
14	Mhyo/PRRS/App	**Influenza H1**			+
15	Mhyo/PRRS	**Influenza H1**			+
16	PRRS/Infuenza?	**Infl. H1/App 2**		Mhyo	+

[a]) Infection with secondary bacteria, [b]) Number of isolates (*P. multocida*, *H. parasuis*, *B. bronchiseptica*, *S. suis*) in four diseased pigs: - = none, + = few (1-3), ++ = moderate (4-6), +++ = many (≥7), [c]) *M. hyopneumoniae*, [d]) Influenza, [e]) *A. pleuropneumoniae*

must have occurred shortly after the investigated outbreak.

The outbreak in herd 16 seemed to be caused by a combination of influenza and *A. pleuropneumoniae*. The causes for the outbreaks in herds 1, 2 and 4 remained unclear (Table 7).

Discussion

Influenza virus and *A. pleuropneumoniae* are the most important infectious agents involved in acute respiratory disease in finishing pigs. Twelve of the sixteen investigated outbreaks in herds with recurrent acute respiratory disease could be attributed to an infection with either one of these agents. Concurrent infections with other infectious agents did occur, but none of these agents was clearly correlated with the presence of influenza virus or *A. pleuropneumoniae*. Thus, there were no obvious clues that certain specific dual infections may be important. However, due to the fact that only sixteen outbreaks were investigated, and several combinations of dual infections may be possible, a role for dual infections can also not be excluded. The aetiology of four outbreaks remained uncertain and several infectious agents were involved in these outbreaks.

Influenza virus was responsible for seven of the sixteen outbreaks of respiratory disease. Evidence that influenza virus was the cause of the outbreak was based the presence of endobronch(iol)itis, which is very typical for an influenza infection (Easterday and Hinshaw, 1992), virus isolation and serology of paired sera. Furthermore, influenza virus was also involved in two other outbreaks.

Even though many clinical outbreaks can be explained by an infection with influenza virus, infections with influenza virus do not always lead to an outbreak with clinical signs. Pigs in all sixteen compartments with an outbreak went through an infection with one or both subtypes of influenza virus. Whereas some of these infections caused the investigated outbreaks, most of these infections occurred before the clinical outbreak, thus at a time when no clinical signs of respiratory disease were observed by the farmer. This suggests that infections in young pigs often remain subclinical. Also, random end sera showed a high percentage of compartments where an infection with one or both subtypes had occurred. This included many compartments where, according to the farmer, no clinical signs of respiratory disease were noticed. This is in agreement with results of earlier studies, where high seroprevalences were found in finishing pigs (Elbers et al., 1990b, 1992; Van Reeth and Pensaert, 1994; Maes et al., 1996), even in herds without a history of respiratory disease.

A. pleuropneumoniae was responsible for five outbreaks. Pathological findings of haemorrhage and necrosis are typical for an *A. pleuropneumoniae* infection, and isolation and serology of paired sera confirmed the infection. In some outbreaks only four or five out of ten groupmates seroconverted within three weeks. This is probably due to the relatively low sensitivity of the CF test (Fenwick and Henry, 1994, Sørensen et al., 1996) or a slower production of antibodies. At the end of the finishing period at least nine out of ten pigs had seroconverted, indicating that almost all pigs had become infected.

In one other outbreak *A. pleuropneumoniae* was concluded to be part of the cause, together with influenza virus. In two more outbreaks, an *A. pleuropneumoniae* infection occurred probably within days after the outbreak under investigation. In these two herds, clinical signs due to the infection with *A. pleuropneumoniae* may have occurred, but were probably indistinguishable from the clinical signs of the initial outbreak. Infections with *A. pleuropneumoniae* usually seem to lead to an outbreak with clinical signs and rarely remaining subclinical.

A. pleuropneumoniae infections were often preceded by infections with PRRS virus and/or influenza virus. Since most pigs go through these infections, a possible role as precursors for an *A. pleuropneumoniae* infection remains unclear. An earlier study revealed that virus infections previous to an infection with *A. pleuropneumoniae* may however increase the severity of clinical disease (Sakano et al., 1993).

Secondary infections with other bacteria were slightly more prominent than with influenza infections. This may be due to the fact that *A. pleuropneumoniae* infections result in more profound lesions, providing secondary bacteria with a better opportunity for infecting the lungs.

Although PRRS virus was isolated frequently, sometimes even from all four diseased pigs, there was no clear evidence that it caused respiratory disease. Because the virus can be isolated up to at least 8 weeks after the infection (Meredith, 1995), isolation of the virus does not have to indicate a recent infection. In fact, antibodies were present in convalescent sera of all sixteen outbreaks, but in at least fourteen of these outbreaks infections were not recent. In these outbreaks antibodies against PRRS virus were already present in the acute sera and no further increase of antibody titres was seen in convalescent sera. In the IPMA, antibody titres reach a maximum at 20 to 30 days after infection (Wensvoort et al., 1992) or even as late as 5 to 6 weeks after infection (Meredith, 1995), so we concluded that these infections must have taken place at least three to four weeks before the onset of the clinical outbreak. A possible role of PRRS virus in dual infection has been demonstrated by Van Reeth et al. (1996a,b). However, in one of those experiments it was suggested that possible impairment of lung defence mechanisms is overcome within two weeks (Van Reeth et al., 1996b). Therefore, we concluded that it is not likely that PRRS virus has played an important role in the influenza or *A. pleuropneumoniae* infections that occurred later in time. However, it remains unclear what the effect of persistent virus may be, once the lungs are damaged by other infectious agents.

M. hyopneumoniae was isolated only sporadically. Serological findings also indicated that *M. hyopneumoniae* infections were not a major cause of acute respiratory disease. This was not unexpected, since *M. hyopneumoniae* is usually associated with chronic respiratory disease (Ross, 1992, Kobisch et al., 1993). Because antibodies against *M. hyopneumoniae* were already present in many acute sera, we concluded that infections with *M. hyopneumoniae* often started well before the onset of the acute outbreak. Possibly, the pigs that were subclinically infected with *M. hyopneumoniae* were more susceptible for the infectious agents that caused the acute outbreaks.

The pathogenicity of *M. hyorhinis* is controversial. Although some strains are able to produce pneumonia in gnotobiotic pigs (Gois and Kuksa, 1974), *M. hyorhinis* usually

causes polyserositis and arthritis in young pigs (Ross, 1992). Even though Hensel et al. (1994) only sporadically isolated *M. hyorhinis* from the lung lavage fluids of healthy pigs, we found no difference between lungs with and without lesions. We concluded that *M. hyorhinis* is not of particular interest in acute respiratory disease.

S. suis and *P. multocida* were both isolated from pigs with lung lesions only, whereas *H. parasuis* and *B. bronchiseptica* were isolated from pigs without lung lesions as well. The role of all these bacteria in the outbreaks we investigated remains uncertain. Under conventional circumstances, these infections are considered to be secondary after lung defence mechanisms have been impaired.

Several Streptococcus spp., including *S. suis* have been isolated from moderate to high percentages of lungs from healthy pigs (Hensel et al., 1994), as well as from pigs with lung lesions (Castryck et al., 1990, Falk et al., 1991). It has been established that some serotypes of *S. suis* may enhance clinical disease after an infection with a primary agent, like Aujeszky's disease virus, as was found by Iglesias et al. (1992) for *S. suis* type 2.

P. multocida can cause severe pneumonia after lung defence mechanisms have been impaired (Fuentes et al., 1987; Ciprián et al., 1988; Amass et al., 1994). The bacterium was not isolated from lungs without lesions, concurring with Hensel et al. (1984). *P. multocida* was in our study highly correlated with subacute or chronic lesions, like it was in earlier studies (Falk et al., 1991; Høie et al., 1991; Runge et al., 1996). This may also explain our relatively low number of isolations, since more than half of the pigs only showed acute lesions at pathological examination.

It is possible that secondary bacteria have influenced the severity of the clinical symptoms, while they may also have played a role in the clinical outbreaks in which no clear cause could be established. In these outbreaks an infection with an unknown primary agent (possibly PRRS virus or *M. hyopneumoniae*) may have been subclinical, with clinical disease occurring only after lung lesions due to secondary infections developed.

References

Amass, S.F., Clark, L.K., Van Alstine, W.G., Bowersock, T.L., Murphy, D.A., Knox, K.E., Albregts, S.R., 1994, Interaction of Mycoplasma hyopneumoniae and Pasteurella multocida infections in swine. J.Am.Vet.Med.Assoc. 204, 102-107.

Awad-Masalmeh, M., Kofer, J., Schuh, M., 1990, On the occurrence of chronic respiratory disease at swine herds in Austria, bacteriological findings, efficacy of autogenous vaccines. Proceedings of the International Pig Veterinary Society Congress, Lausanne 11, 107.

Callebaut, P., Pensaert, M.B., Miry, C., Haesebrouck, F., Vergote, J., 1986, Prevalence of influenza-, Aujeszky-, transmissible gastroenteritis- and porcine epizootic diarrhea virus infections in feeder pigs. Proceedings of the International Pig Veterinary Society Congress, Barcelona 9, 216.

Castryck, F., Devriese, L.A., Hommez, J., Cassimon, P., Miry, C., 1990, Bacterial agents associated with respiratory disease in young feeder pigs. Proceedings of the International Pig Veterinary Society Congress, Lausanne 11, 112.

Christensen, G., Mousing, J., 1992, Respiratory system, In: Leman, A.D., Straw, B.E., Glock, R.D., Mengeling, W.L., D'Allaire, S., Taylor, D.J. (Eds.) Diseases of Swine. Iowa State University Press, Ames, pp. 138-162.

Ciprian, A., Pijoan, C., Cruz, T., Camacho, J., Tortora, J., Colmenares, G., Lopez-Revilla, R., De La Garza, M., 1988, Mycoplasma hyopneumoniae increases the susceptibility of pigs to experimental Pasteurella multocida pneumonia. Can.J.Vet.Res. 52, 434-438.

Cowan, S.T., Steel, K.J., 1993, Manual for the identification of medical bacteria, 3 Edition. Cambridge University Press, Cambridge, 1-320 pp.
Done, S.H., Paton, D.J., 1995, Porcine reproductive and respiratory syndrome: clinical disease, pathology and immunosuppression. Vet.Rec. 136, 32-35.
Easterday, B.C., Hinshaw, V.S., 1992, Swine Influenza, In: Leman, A.D., Straw, B.E., Glock, R.D., Mengeling, W.L., D'Allaire, S., Taylor, D.J. (Eds.) Diseases of Swine. Iowa State University Press, Ames, pp. 349-357.
Elbers, A.R.W., Cromwijk, W.A.J., Hunneman, W.A., Tielen, M.J.M., 1990a, Log book recording on pig finishing farms in an Integrated Quality Control (IQC) project. I. Use of drugs and vaccines [Logboekregistratie op vleesvarkensbedrijven in het Integrale Keten Beheersing (IKB) project. I. Gebruik van medicijnen en vaccins]. Tijdschr.Diergeneeskd. 115, 249-261.
Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1990b, Sero-epidemiological screening of pig sera collected at the slaughterhouse to detect herds infected with Aujeszky's disease virus, porcine influenza virus and Actinobacillus (Haemophilus) pleuropneumoniae in the framework of an integrated quality control (IQC) system. Vet.Q. 12, 221-230.
Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1992, Variation in seropositivity for some respiratory disease agents in finishing pigs: epidemiological studies on some health parameters and farm and management conditions in the herds. Vet.Q. 14, 8-13.
Falk, K., Hoie, S., Lium, B.M., 1991, An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds .2. Enzootic pneumonia of pigs - microbiological findings and their relationship to pathomorphology. Acta Veterinaria Scandinavica 32, 67-77.
Falk, K., Lium, B.M., 1991, An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds .3. Serological findings and their relationship to pathomorphological and microbiological findings. Acta Veterinaria Scandinavica 32, 79-88.
Fenwick, B., Henry, S., 1994, Porcine pleuropneumonia. J.Am.Vet.Med.Assoc. 204, 1334-1340.
Friis, N.F., 1975, Some recommendations concerning primary isolation of Mycoplasma suipneumoniae and Mycoplasma flocculare a survey. Nord.Vet.Med. 27, 337-339.
Fuentes, M.C., Pijoan, C., 1987, Pneumonia in pigs induced by intranasal challenge exposure with pseudorabies virus and Pasteurella multocida. Am.J.Vet.Res. 48, 1446-1448.
Gois, M., Kuksa, F., 1974, Intranasal infection of gnotobiotic piglets with Mycoplasma hyorhinis: differences in virulence of the strains and influence of age on the development of infection. Zentralblatt fur Veterinarmedizin Reihe B 21, 352-361.
Hensel, A., Ganter, M., Kipper, S., Krehon, S., Wittenbrink, M.M., Petzoldt, K., 1994, Prevalence of aerobic bacteria in bronchoalveolar lavage fluids from healthy pigs. American Journal of Veterinary Research 55, 1697-1702.
Hoie, S., Falk, K., Lium, B.M., 1991, An abattoir survey of pneumonia and pleuritis in slaughter weight swine from 9 selected herds. IV. Bacteriological findings in chronic pneumonic lesions. Acta Vet.Scand. 32, 395-402.
Iglesias, J.G., Trujano, M., Xu, J., 1992, Inoculation of pigs with Streptococcus suis type 2 alone or in combination with pseudorabies virus. American Journal of Veterinary Research 53, 364-367.
Kay, R.M., Done, S.H., Paton, D.J., 1994, Effect of sequential porcine reproductive and respiratory syndrome and swine influenza on the growth and performance of finishing pigs. Vet.Rec. 135, 199-204.
Kendal, A.P., Skehel, J.J., Pereira, M.S., 1982, Concepts and procedures for laboratory-based influenza surveillance. US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.
Kobisch, M., Blanchard, B., Lepotier, M.F., 1993, Mycoplasma hyopneumoniae infection in pigs - duration of the disease and resistance to reinfection. Veterinary Research 24, 67-77.
Lanza, I., Brown, I.H., Paton, D.J., 1992, Pathogenicity of concurrent infection of pigs with porcine respiratory coronavirus and swine influenza virus. Res.Vet.Sci. 53, 309-314.
Laude, H., Van Reeth, K., Pensaert, M., 1993, Porcine respiratory coronavirus: molecular features and virus-host interactions. Vet.Res. 24, 125-150.
Maes, D., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., De Kruif, A., 1996, Epidemiological investigations on respiratory diseases in pigs in Belgium. Proceedings of the International Pig Veterinary Society Congress, Bologna 14, 405.
Mattsson, J.G., Bergstrom, K., Wallgren, P., Johansson, K.E., 1995, Detection of Mycoplasma hyopneumoniae in nose swabs from pigs by in-vitro amplification of the 16S ribosomal RNA gene. Journal of Clinical Microbiology 33, 893-897.

Meredith, M.J., 1995, Porcine reproductive and respiratory syndrome (PRRS). Germany, Ingelheim/Rhein, 81pp

Nicolet, J., De Meuron, P.A., Bachmann, P., 1971, Haemophilus infection in pigs. 4. The use of the complement fixation reaction in the diagnosis of Haemophilus parahaemolyticus infections. Schweizer Archiv fur Tierheilkunde 113, 191-200.

Nowotny, N., Mostl, K., Maderbacher, R., Odorfer, G., Schuh, M., 1994, Serological studies in Austrian fattening pigs with respiratory disorders. Acta Vet.Hung. 42, 377-379.

Paisley, L.G., Vraa-Andersen, L., Dybkjær, L., Møller, K., Christensen, G., Mousing, J., Agger, J.F., 1993, An epidemiologic and economic study of respiratory diseases in two conventional Danish swine herds .1. Prevalence of respiratory lesions at slaughter and their effects on growth. Acta Veterinaria Scandinavica 34, 319-329.

Pijoan, C., 1992, Pneumonic pasteurellosis, In: Leman, A.D., Straw, B.E., Glock, R.D., Mengeling, W.L., D'Allaire, S., Taylor, D.J. (Eds.) Diseases of Swine. Iowa State University Press, Ames, pp. 552-559.

Rosendal, S., Black, F.T., 1972, Direct and indirect immunofluorescence of unfixed and fixed Mycoplasma colonies. Acta Pathologica et Microbiologica Scandinavica 80B, 615-622.

Ross, R.F., 1992, Mycoplasmal diseases, In: Leman, A.D., Straw, B.E., Glock, R.D., Mengeling, W.L., D'Allaire, S., Taylor, D.J. (Eds.) Diseases of Swine. Iowa State University Press, Ames, pp. 537-551.

Runge, M., Ganter, M., Delbeck, F., Hartwick, W., Ruffer, A., Franz, B., Amtsberg, G., 1996, Etiological diagnosis of pneumonia in fattening pigs: investigation of bronchoalveolar lavage (BAL) by cultural methods and immunofluorescence microscopy, as well as serological findings. Berliner und Munchener Tierarztliche Wochenschrift 109, 101-107.

Sakano, T., Shibata, I., Samegai, Y., Taneda, A., Okada, M., Irisawa, T., Sato, S., 1993, Experimental pneumonia of pigs infected with Aujeszky's disease virus and Actinobacillus pleuropneumoniae. J.Vet.Med.Sci. 55, 575-579.

Sebunya, T.N., Saunders, J.R., 1983, Haemophilus pleuropneumoniae infection in swine: a review. J.Am.Vet.Med.Assoc. 182, 1331-1337.

Sorensen, V., Barfod, K., Nielsen, J.P., Feld, N.F., Christensen, J., 1996, Evaluation of a polyclonal blocking ELISA and a complement fixation assay detecting antibodies to Actinobacillus pleuropneumoniae serotype 2 in pigs. Proceedings of the International Pig Veterinary Society Congress, Bologna 14, 191.

Stemke, G.W., Phan, R., Young, T.F., Ross, R.F., 1994, Differentiation of Mycoplasma hyopneumoniae, M flocculare, and M hyorhinis on the basis of amplification of a 16S rRNA gene sequence. Am.J.Vet.Res. 55, 81-84.

Straw, B.E., Shin, S.J., Yeager, A.E., 1990, Effect of pneumonia on growth rate and feed efficiency of minimal disease pigs exposed to Actinobacillus pleuropneumoniae and Mycoplasma hyopneumoniae. Preventive Veterinary Medicine 9, 287-294.

Van Oirschot, J.T., Houwers, D.J., Rziha, H.J., Moonen, P.J.L.M., 1988, Development of an ELISA for detection of antibodies to glycoprotein I of Aujeszky's disease virus: a method for the serological differentiation between infected and vaccinated pigs. Journal of Virological Methods 22, 191-206.

Van Reeth, K., Nauwynck, H., Pensaert, M., 1996a, Dual infections of feeder pigs with porcine reproductive and respiratory syndrome virus followed by porcine respiratory coronavirus or swine influenza virus: a clinical and virological study. Vet.Microbiol. 48, 325-335.

Van Reeth, K., Pensaert, M., 1996, A clinical and virological study in pigs infected with Aujeszky's disease virus shortly after infection with porcine respiratory coronavirus. Proceedings of the International Pig Veterinary Society Congress, Bologna 14, 137.

Van Reeth, K., Pensaert, M.B., 1994, Porcine respiratory coronavirus mediated interference against influenza virus replication in the respiratory tract of feeder pigs. American Journal of Veterinary Research 55, 1275-1281.

Van Reeth, K., Van Hoof, D., Willems, L., Pensaert, M., 1996b, A clinical study of dual infections with porcine reproductive respiratory syndrome virus and swine influenza virus administered with different time intervals. Proceedings of the International Pig Veterinary Society Congress, Bologna 14, 59.

Wensvoort, G., 1993, Lelystad virus and the porcine epidemic abortion and respiratory syndrome. Vet.Res. 24, 117-124.

Wensvoort, G., De Kluijver, E.P., Luijtze, E.A., Den Besten, A., Harris, L., Collins, J.E., Christianson, W.T., Chladek, D., 1992, Antigenic comparison of Lelystad virus and swine infertility and respiratory syndrome (SIRS) virus. Journal of Veterinary Diagnostic Investigation 4, 134-138.
Wensvoort, G., Terpstra, C., Pol, J.M., Ter Laak, E.A., Bloemraad, M., De Kluijver, E.P., Kragten, C., Van Buiten, L., Den Besten, A., Wagenaar, F., et al., 1991, Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet.Q. 13, 121-130.

Chapter 3

Estimating the incidence of influenza-virus infections in Dutch weaning piglets using blood samples from a cross-sectional study

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Estimating the incidence of influenza-virus infections in Dutch weaning piglets using blood samples from a cross-sectional study

Abstract

A cross-sectional study was carried out on 32 Dutch breeding herds to estimate the incidence of influenza-virus infections in piglets before the start of the finishing period, at the age of approximately 10 weeks. Longitudinal studies on two herds (8 and 10 litters, respectively) were done to obtain an average decay function for maternal antibodies. Each participating farm in the cross-sectional study was visited twice within 5 months; each time, blood samples were taken randomly from one compartment (a separate room with separate air flow) of 4-5-week-old piglets and one compartment of 8-9-week-old piglets. These blood samples (a total of 2598; 16-23 per compartment, depending on its size) were tested in a haemagglutination inhibition test for antibodies against influenza-virus subtypes H1 and H3. Samples from 8-9-week-old piglets from the first sampling period (n=660) were also tested in an IgM ELISA. For each individual herd and each influenza-virus subtype separately, the decay function derived from the longitudinal studies was used to calculate an expected seroprevalence in 8-9-week-old piglets, which was then compared to the observed seroprevalence. Depending on subtype and sampling period, between 10 and 15 of the 32 herds were suspected of virus circulation during the weaning period because the observed seroprevalence was significantly higher than the expected seroprevalence ($P<0.05$). In the first sampling period the IgM ELISA confirmed six of these outbreaks. However, due to the small window of detection of the IgM ELISA (compared to the length of the weaning period), it will always underestimate the number of infections. Infections in the first half of the weaning period will no longer be detectable because IgM antibodies have already disappeared. In individual pigs, an incidence of 16-17% was estimated for each subtype over a 4-week period between the age of 4-5 and 8-9 weeks. For each influenza subtype, 80% of the piglets will enter the finishing facilities without antibodies or with decaying maternal antibodies. These piglets may be susceptible to an infection with influenza virus.

Introduction

Respiratory diseases in finishing pigs are the most important health disorders in commercial swine herds. They are responsible for over half of all antibiotic treatments in these pigs (Elbers et al., 1990a). In addition, respiratory diseases can cause major

losses due to growth retardation, reduced feed-conversion efficiency, increased mortality, and reduced carcass quality (Straw et al., 1990; Paisley et al., 1993).

A few years ago, a field survey was carried out to determine the infectious agents involved in acute respiratory disease (mainly occurring between the age of 15 and 20 weeks) in Dutch finishing pigs (Loeffen et al., 1999). Sixteen outbreaks in 16 different herds were investigated; in nine of these outbreaks, influenza virus was involved: virus was isolated from diseased pigs, microscopic lesions typical for influenza were found in affected lungs and seroconversion of 90-100% of the pigs in the compartment in question were detected. This survey established that influenza virus is important in respiratory diseases.

Several studies over the past 15 years also show that at the end of the finishing period, up to 95% of the finishing pigs have antibodies against one or both major subtypes of influenza (H1N1 and H3N2) (Haesebrouck and Pensaert, 1986; Elbers et al., 1990b; Groschup et al., 1993; Loeffen et al., 1999; Maes et al., 1999). Because often no clinical signs are noticed by the farmer, many infections with influenza virus apparently remain subclinical.

In the above-mentioned survey on 16 outbreaks, in eight of the nine outbreaks involving influenza virus, no antibodies against the involved influenza subtype were present in the acute stage (and in most cases neither against the other subtype). The ninth outbreak occurred in 10-11-week-old piglets where antibodies against the involved influenza subtype already were present. In contrast, in the seven outbreaks not involving influenza virus, antibodies against one or even both influenza subtypes already were present during the acute stage of disease (indicating an infection earlier in life that was subclinical because it was not noticed by the farmer).

The main objective of this study was to determine whether infections with influenza virus occur in Dutch piglets before the start of the finishing period (usually at 10 weeks of age). Furthermore, an overall estimate was made of the incidence of influenza infections during the weaning period. For this purpose, a serum bank was used from a cross-sectional study on 32 breeding herds. Sera from 4-5 and 8-9-week-old piglets were tested for antibodies against influenza-virus subtypes H1 and H3. The presence of maternal antibodies, which can persist up to the age of 2-4 months (Große Beilage et al., 1996), was expected to be a disturbing factor. Two longitudinal studies were therefore carried out to determine the rate of decline of maternal antibodies in field sera. The decay functions derived from these studies were used to interpret the serological results from the crosssectional study, more or less comparable to the way this was done before for PRRS (Nodelijk et al., 1997). An IgM ELISA (Heinen et al., 2000) was also used on a subset of the samples as a way to differentiate between maternal antibodies and antibodies in the early stage of an infection.

Materials & methods

Selection of herds

A serum bank from a previous cross-sectional study on porcine reproductive and respiratory syndrome virus (PRRSV) (Nodelijk et al., 1997) was used. Selection of

participating herds in that study was described there. In short, 100 farms were randomly selected out of 5712 registered breeding farms in the Netherlands with >100 sows. The first 32 farms responding positively to a written request for participation in the PRRSV study were included. Although this might introduce some bias into the selection, we believe it is very low, all the more because influenza was not mentioned at the time farmers were asked to participate in the study.

Longitudinal studies

Two separate longitudinal studies were carried out to determine the rate of decline of maternal antibodies directed against influenza virus in field sera. In the first study blood samples were collected from eight sows, within 1 week after farrowing, from a herd also participating in the cross-sectional study (herd 19). Five piglets from each of the eight litters were randomly selected and tagged with an ear mark. These piglets were sampled at weekly intervals after birth, until the age of 10 weeks. In a second study blood samples were collected from ten sows, 3 or 4 days after farrowing, from a breeding herd not participating in the cross-sectional study. Four piglets from each of the 10 litters were randomly selected and tagged with an ear mark. They were sampled at the age of 3 or 4 days and 1, 2, 4, 6, 8 and 10 weeks.

Cross-sectional study

A cross-sectional study was carried out to determine whether influenza-virus infections occurred in piglets before the start of the finishing period (usually at the age of 10 weeks). From the serum bank (Nodelijk et al., 1997), blood samples were available from two visits to each farm: one in October-November 1995 and the other in March 1996. During each visit, one compartment (separate room with separate air flow) with pigs of 4-5 weeks old and one compartment with pigs of 8-9 weeks old was sampled randomly. For that all piglets in a compartment were numbered individually and piglets were drawn from that pool, independent of the pens they were in. The sample size for each compartment was calculated so that with an estimated seroprevalence of 60% and a confidence level of 95%, the margin of error was no more than 20%. This resulted in sample sizes of 16-23 pigs per compartment, using the formula for simple random sampling (Thrusfield, 1995). A total of 2598 blood samples were collected, each blood sample originating from a different piglet. For the first sampling, 635 samples were taken from 4-5-week-old piglets and 660 samples from 8-9-week-old piglets. For the second sampling, 662 samples were taken from 4-5-week-old piglets and 641 samples from 8-9-week-old piglets.

Haemagglutination inhibition test

All sera were tested in a haemagglutination inhibition (HAI) test (Kendal et al., 1982) for antibodies against H1 and H3. Sera were tested in serial 2-fold dilutions, starting at 1:9. Titres <9 were set to 4.5, after which all titres were log-transformed using the following formula:

$$y = \log_2 \frac{1}{9}(titre)$$

Influenza-virus strains A/swine/Neth/Best/96 (H1N1) and A/swine/Neth/St Oedenrode/96 (H3N2) were used to test for H1 and H3 antibodies, respectively. Both strains were isolated in the Netherlands at the time the study was carried out (Loeffen et al., 1999). For estimates of the prevalence, titres ≥18 (≥1 after log-transformation) were considered positive, titres ≤9 (≤0 after log-transformation) were considered negative.

IgM ELISA

All 660 sera of the 8-9-week-old piglets of the first sampling in October-November 1995 were tested in an IgM ELISA. This ELISA measures influenza nucleoprotein-specific IgM antibodies and was described by Heinen et al. (2000). This IgM ELISA detected antibodies approximately from day 7 to day 17 after infection. In short, ELISA plates (Costar EIA/RIA, Cat. no. 3590, Costar, USA) were: (i) coated overnight at 4°C with 200 ng of a mouse antibody against porcine IgM per well in 50 mM sodium bicarbonate buffer, pH 9.6; (ii) blocked with 0.3% non-fat milk powder in PBS by shaking on a microplate shaker (Vari-shaker, Dynotech, UK) for 2 h at 37°C; (iii) incubated with 2-fold serial dilutions of serum samples; (iv) incubated with a 1:200 dilution of antigen preparation (whole virus in 1% NP40); (v) incubated with HRPO conjugated MAb (ATCC no.: HB 65, H16-L10-4R5) against the NP of influenza virus; (vi) incubated at room temperature with chromogen/substrate solution (0.1 mg/ml tetramethylbenzidin (TMB), 0.006% H_2O_2 in 0.1 M sodium acetate buffer). Coloration was stopped after 30 min with 0.5 M H_2SO_4. All dilutions of reagents were made in ELISA buffer (0.5 M NaCl, 0.05% (w/v) Tween 80 in PBS, pH 7.4) to which 0.3% non-fat milk powder was added, and plates were shaken for 1 h at 37°C in each step. In each step, 100 ml of reagent was added per well and between each step plates were washed five times (10 times before adding chromogen/substrate) with 0.05% (w/v) Tween 80 in tap water. The absorbance at 450 nm was read with an ELISA reader (Spectra Reader, SLT Labinstruments, Austria). Antibody titres were expressed as the reciprocal of the sample dilution still giving optical-density (OD) values of 1.0 (three times the background value).

Statistical analysis longitudinal studies

Log-transformed titres of the 18 sows in the first week after farrowing and their respective 1-week-old piglets (averaged for each litter) were compared using the Pearson correlation coefficient. After log-transformation of the titres, a linear model was fitted for each litter separately to study the decline of maternal antibodies for both H1 and H3:

$$y(t) = a + b \cdot t + \varepsilon$$

where $y(t)$ is the average log-transformed titre of the piglets in each litter at the age of weeks. The parameters a and b are constants representing the beginning level and the rate of decline of the maternal antibodies, respectively. Residuals (ε) were assumed to be normally distributed, with expectation zero and variance σ_ε^2. For each of the 18 litters a, b and σ_ε^2 were estimated. When at least two piglets in a litter reached a titre of <9 (set to 4.5, which is -1 after log-transformation), further serological results of that litter were not included for regression anymore. This was done because titres <9 are not quantified any further and would thus eventually form a horizontal line in time. The averages of a and b were calculated and used to obtain an average decay function for both longitudinal studies separately. Variances of a and b were also calculated separately for both herds and together with σ_ε^2 used to determine the variance of $y(t)$ at the age of t weeks:

$$\sigma_{y(t)}^2 = \sigma_a^2 + (t \cdot \sigma_b)^2 + \sigma_\varepsilon^2$$

The expected proportion of piglets still seropositive for maternal antibodies at the age of t weeks then could be calculated as the probability that a random variable with distribution N($\overline{a} + \overline{b} \cdot t$, $\sigma_{y(t)}^2$) was >0.5. The value of 0.5 (the centre between 0 (negative) and 1 (positive)) was chosen because titres were not measured on a continuous scale and the normal distribution is continuous. An average b and σ_ε^2 for both herds together was calculated, corrected for clustering within the herds.

Statistical analysis cross-sectional study

To interpret the serological results of the 8-9-week-old piglets, a modified decay function was used:

$$y(8) = y(4) + b \cdot 4 + \varepsilon$$

where $y(4)$ is the average log-transformed titre at the age of 4-5 weeks as the beginning level (with $s_{y(4)}^2$ being the calculated variance of that beginning level).

Several assumptions had to be made:

- Titres at the age of 4-5 weeks are due to maternal antibodies only.
- The average titre in the 4-5-week-old group constitutes a reasonable average for the situation in each individual herd and therefore is the best predictor for the average titre in the 8-9-week-old group in the same herd and sampled at the same day.
- The average rate of decline and the variance of the residuals is identical for all herds and thus comparable to the one found in the longitudinal studies.

Titres are in fact log-normally distributed even though they were not tested on a continuous scale and titres <9 were not quantified.

The expected prevalence of piglets at 8-9 weeks was calculated for each compartment separately as the probability that a random variable with distribution N($y(4)+\overline{b}\cdot 4$, $\sigma^2_{y(8)}$) was larger than 0.5. The observed prevalence was determined and a 95% confidence interval was calculated (Thrusfield, 1995).

If the lower limit of the 95% confidence interval of the observed seroprevalence was higher than the expected seroprevalence, the observed seroprevalence was significantly higher than the expected seroprevalence and an infection was likely to have occurred. This is essentially the same method as described for PRRS (Nodelijk et al., 1997). However, we incorporated a group of 4-5-week-old piglets from each individual herd to serve as the starting point in the regression formula to lessen the effect of the large variation between herds.

While the steps above were used to determine for each individual herd whether an outbreak has occurred or not, an overall estimate of the incidence also was calculated. For this, the expected seroprevalence at 8-9 weeks was calculated for all 32 herds together using the same method. For each of the herds, the average log-transformed titre ($y(4)$) and variance ($s^2_{y(4)}$) at the age of 4-5 weeks were calculated. The averages of these results were used in the modified decay function described above. Observed seroprevalences at the age of 8-9 weeks were calculated as the average seroprevalence of the 32 herds. By subtracting the expected prevalence from the observed prevalence at the age of 8-9 weeks, the incidence of influenza-virus infections could be estimated as the percentage of piglets responding to an infection between the age of 4-5 and 8-9 weeks.

The within-farm seroprevalence of 4-5-week-old and 8-9-week-old pigs was compared for the first and second sampling with a paired t -test. Serological results of 4-5-week-old piglets were used to compute the Shapiro-Wilk statistic to determine whether they were normally distributed.

All titres were log-transformed and analysed by a linear mixed model to analyse differences between farms and between pens on farms. The analysis was done separately for the two samplings and the two age groups. ‘Animal’ was taken as the experimental unit and ‘farm’ and ‘pen’ as random terms. Components of variance were estimated for each term and a restricted maximum-likelihood (REML) test was used to determine whether these components were significantly different from zero. All calculations were carried out using the MIXED procedure in SAS 6.12 (SAS Institute, 1996).

Results

First longitudinal study

In the first longitudinal study, all eight sows were positive for antibodies against influenza H1 and H3 (log-transformed titres ranged from 3 to 9 and from 3 to 7, respectively). All piglets were seropositive during the first week, and 28% were still seropositive at the age of 10 weeks for each subtype.

Antibody titres of the sows and their piglets at the age of 1 week were positively correlated for influenza subtype H1 ($r = 0.84$; $P < 0.01$). For influenza subtype H3 the correlation coefficient was not significantly different from zero ($r = 0.54$; $P = 0.17$). Antibody titres for all litters showed a steady decline until the age of 10 weeks (Figs. 1 and 2).

The R^2's for the linear model based on the log-transformed titres (for the eight individual litters) ranged from 0.93 to 0.99 for H1 and from 0.78 to 0.98 for H3. The parameter a (representing the beginning level) was on average 4.6 (S.D. 1.5) for H1 and 5.6 (S.D. 1.1) for H3. The parameter b (the rate of decline), was on average -0.65 (S.D. 0.17) for H1 and -0.66 (S.D. 0.09) for H3. The average variances of the residuals (σ_ε^2) were 0.09 for H1 and 0.35 for H3.

For subtype H1 the expected proportions of seropositive piglets due to maternal

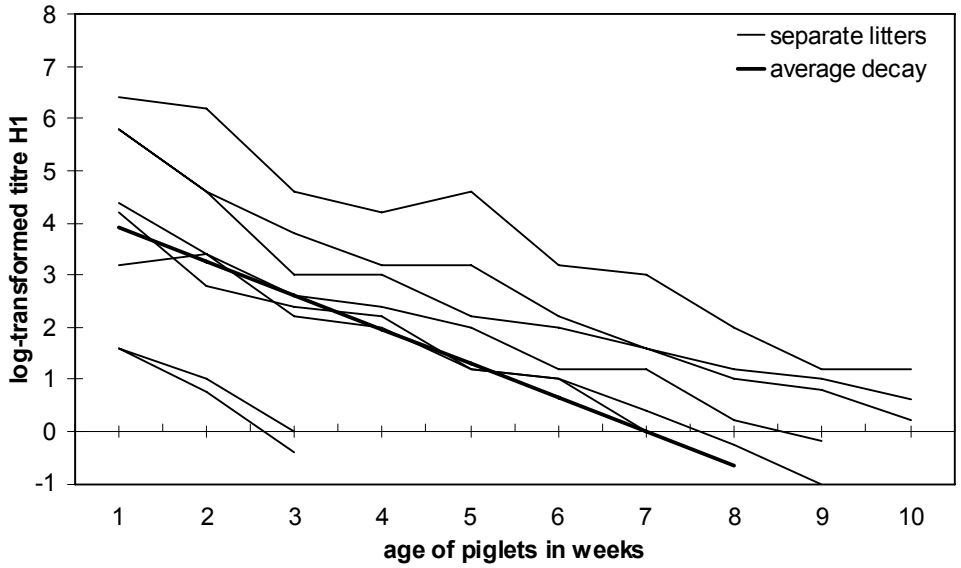

Figure 1: Decline of maternal antibodies against influenza subtype H1 in 8 litters from 1 Dutch breeding herd between birth and 10 weeks of age, for each litter separately and the average for all litters together.

antibodies were 0.80 at 4-5 weeks and 0.29 at 8-9 weeks. The observed proportions in the longitudinal study were 0.75 and 0.44, respectively. For subtype H3 the expected proportion of seropositive piglets due to maternal antibodies were 0.97 at 4-5 weeks and 0.45 at 8-9 weeks. The observed proportions were 0.97 and 0.41, respectively.

Second longitudinal study

In the second longitudinal study, all 10 sows were positive for antibodies against influenza H1 and H3 (log-transformed titres ranged from 1 to 5 and from 1 to 6, respectively). All piglets were seropositive during the first week, and 42 and 82% were still seropositive at the age of 10 weeks for subtypes H1 and H3, respectively.

Antibody titres of the sows and their piglets at the age of 1 week were positively correlated for influenza subtypes H1 ($r = 0.80$; $P < 0.01$) and H3 ($r = 0.86$; $P < 0.01$).

The R^{2}'s for the linear model based on the log-transformed titres (for the ten individual litters) ranged from 0.94 to 0.98 for H1 and from 0.85 to 0.99 for H3. The parameter a was on average 4.3 (S.D. 1.4) for H1 and 5.7 (S.D. 1.4) for H3; b was on average -0.51 (S.D. 0.06) for H1 and -0.50 (S.D. 0.04) for H3. The average variances of the residuals (σ_{ε}^{2}) were 0.11 for H1 and 0.17 for H3.

For subtype H1 the expected proportions of seropositive piglets due to maternal antibodies were 0.89 at 4-5 weeks and 0.43 at 8-9 weeks. The observed proportions in the longitudinal study were 0.82 and 0.60, respectively. For subtype H3 the expected

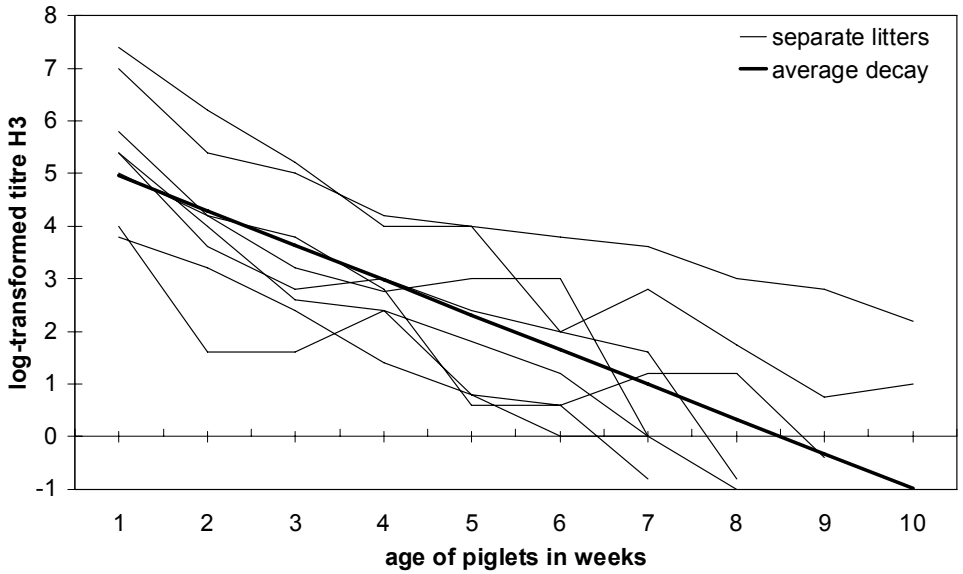

Figure 2: Decline of maternal antibodies against influenza subtype H3 in 8 litters from 1 Dutch breeding herd between birth and 10 weeks of age, for each litter separately and the average for all litters together.

proportion of seropositive piglets due to maternal antibodies were 0.99 at 4-5 weeks and 0.79 at 8-9 weeks. The observed proportions were 0.89 and 0.87, respectively.

Combined longitudinal studies

Based on the course of the antibody titres of individual piglets (Figs. 1 and 2) and the agreement between expected and observed proportions of seropositive piglets, we concluded that all titres in both studies were due to maternal antibodies and that no infection with either subtype occurred in the piglets of the 18 litters.

Combining both longitudinal studies, an average rate of decline of -0.58 for both subtypes was calculated with S.D. of 0.12 and 0.07 for subtypes H1 and H3, respectively. The combined variances of the residuals (σ_{ε}^{2}) were 0.10 for H1 and 0.25 for H3. These were the constants used to interpret the serological results of the cross-sectional study. The calculated half-lives from these rates of decline were 12 days for antibodies against both H1 and H3.

Cross-sectional study

Between 10 and 15 herds showed a higher seroprevalence at the age of 8-9 weeks than was expected, depending on influenza-virus subtype and sampling period (Table 1). We therefore concluded that these are the herds in which influenza virus probably circulated.

Observed and expected seroprevalences were also calculated for all herds together (Table 2). From that, the estimated incidences between the age of 4-5 and 8-9 weeks were calculated as 16% (range 14-18%) for H1 and 17% (range 11-24%) for H3. For

Table 1: Comparison of observed and expected seroprevalences in weaned piglets aged 8 to 9 from 32 Dutch breeding farms.

	Number of herds		
Influenza subtype and sampling dates	Observed > Expected	Observed = Expected	Observed < Expected
H1N1			
October - November 1995	11	16	5
March 1996	10	20	2
H3N2			
October - November 1995	11	15	6
March 1996	15	14	3

Table 2: Observed and expected seroprevalences in weaned piglets aged 8 to 9 weeks and estimated incidence between the ages of 4 to 5 and 8 to 9 weeks (32 herds, the Netherlands).

Influenza subtype and sampling dates	Observed seroprevalence (%)	Expected seroprevalence (%)	Incidence (%)
H1N1			
October - November 1995	36	18	18
March 1996	27	13	14
H3N2			
October - November 1995	57	46	11
March 1996	57	33	24

influenza subtype H1, we predict that 68% will enter the finishing period (age 9-10 weeks) without antibodies, 16% with detectable levels of only maternal antibodies and 16% with antibodies due to an infection. For influenza subtype H3, these percentages were 43, 40, and 17%, respectively.

The average seroprevalences and titres at the age of 4-5 weeks were significantly ($P<0.01$) higher than that at the age of 8-9 weeks for both subtypes and during both samplings (Table 3). The Shapiro-Wilk statistic for titres at the age of 4-5 weeks ranged from 0.96 to 0.97 for both sampling periods and both influenza-virus subtypes. For both samplings and both subtypes, variances of random terms (farm and pen) were significantly ($P<0.01$) different from zero (Table 3). This means that serological results of pigs within a farm and within a particular pen resemble each other more than they would have if samples had been distributed randomly over the whole population of pigs.

Furthermore there was also a significant positive correlation between the average titres in October-November 1995 and March 1996 in the 4-5-week-old groups of each herd (0.69 and 0.62 for H1 and H3, respectively, $P < 0.0001$).

IgM ELISA

In 17 of the 32 herds, antibodies against influenza virus were found in the IgM ELISA (Fig. 3). In eight of these herds only one positive piglet was found and in three other herds three or four positive piglets. In only four of these herds circulation of influenza virus was suspected, based on the statistical approach on the blood samples from the cross-sectional study. In the remaining six herds, 5-21 piglets were positive in the IgM ELISA. In all of these herds circulation of influenza virus was already suspected, based on the statistical approach on the blood samples from the cross-

Table 3: Average seroprevalences and titres and components of variance based on a linear mixed model analysis of antibody titres against both influenza subtypes in piglets in 32 Dutch breeding herds.

	Sero-prevalence		Log-transformed titre		σ^2_{farm}		σ^2_{pen}		σ^2_{animal}	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
H1N1										
Oct-Nov 1995										
4-5 weeks old	0.64	0.05	1.38	0.21	1.20	0.38	1.04	0.18	1.46	0.11
8-9 weeks old	0.37	0.04	0.22	0.16	0.72	0.21	0.48	0.09	0.80	0.06
March 1996										
4-5 weeks old	0.63	0.05	1.19	0.21	1.20	0.36	0.93	0.15	1.07	0.08
8-9 weeks old	0.27	0.03	-0.05	0.12	0.35	0.12	0.40	0.08	0.76	0.06
H3N2										
Oct-Nov 1995										
4-5 weeks old	0.78	0.04	2.62	0.29	2.19	0.68	1.26	0.26	2.49	0.18
8-9 weeks old	0.57	0.05	1.27	0.29	2.42	0.67	0.80	0.15	1.60	0.11
March 1996										
4-5 weeks old	0.77	0.05	2.08	0.24	1.69	0.51	1.40	0.21	1.37	0.10
8-9 weeks old	0.57	0.06	1.05	0.25	1.90	0.53	0.40	0.12	1.47	0.11

sectional study. Overall only 14% of all tested piglets had IgM antibodies against influenza virus.

Discussion

On a herd level, both the statistical approach and testing with an IgM ELISA suggest that virus circulation among weaned piglets occurs in one-third to half of the Dutch breeding herds. However, both methods also suggest that during the period covered, in many cases only a small percentage of piglets developed antibodies between the ages of 4-5 and 8-9 weeks. Due to the presence of maternal antibodies, spread of these infections may be limited at that time.

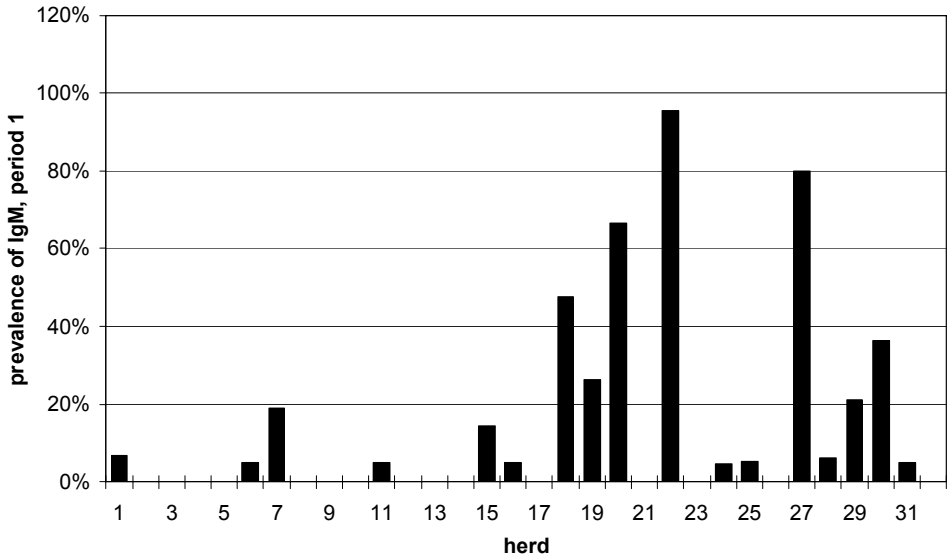

Figure 3: Percentage of 8- to 9-week-old piglets with IgM antibodies against influenza virus (not subtype specific) in 32 Dutch breeding herds, sampled in October - November 1995.

On the individual-pig level, infections with influenza virus, resulting in an antibody response in weaned piglets, are not common in Dutch breeding herds. For each subtype, on average only about 16-17% of all piglets of 8-9 weeks old have antibodies due to an infection. The remaining piglets are either seronegative or still have detectable levels of maternal antibodies.

Within each compartment, animals were sampled using a simple random sampling strategy. However, animals within a pen resembled each other more than animals between pens. In 4-5-week-old pigs this is mainly due to the practise of keeping pigs from the same litter together as much as possible after they are weaned. These piglets highly correlate with each other as far as antibody titres are concerned. On the other hand, there are also farmers who relocate all weaned piglets to different pens, usually putting piglets of approximately the same body weight together in one pen. In these cases variance between pens is likely to be lower than that of individual pigs. These management strategies are practised in approximately 60 and 40% of the herds, respectively (authors own observation, unpublished). The same explanation is true for the 8-9-week-old piglets. However, in addition to the litter effect, there may also be a major effect of field infection, lowering the variance between individual piglets, especially within one pen. Stratified random sampling instead of simple random sampling would have increased the precision of the estimate of the seroprevalence because only the variability within each stratum (pen) would contribute to the variance.

In situations like this, stratified random sampling would thus be preferable over simple random sampling.

It is not likely that antibodies at the age of 4-5 weeks are the result of an infection. At this age, the expected proportion of piglets with detectable levels of maternal antibodies was still 80 and 97% for influenza H1 and H3, respectively. Infections under the protection of these antibodies are not likely to occur or possibly do not result in a rise of antibody titres. The results of some experimental infections in the early seventies seem to indicate so (Blaskovic et al., 1970; Easterday, 1971, 1972).

It is safe to assume that the average titre in the 4-5-week-old group is the best predictor for the average titre in the 8-9-week-old group of the same herd and sampled at the same time. Both age groups are born from a similar, more or less random sample of sows from the same sow herd. Thus, on average the mothers of both age groups will have the same immune status. Because maternal antibody titres are a reflection of the immune status of the sow, it is to be expected that on average, the titres in both age groups were comparable at the time they were 4-5 weeks old. The positive correlation between the average titres in October-November 1995 and March 1996 in the 4-5-week-old groups of each herd also shows that even over a longer period of time there is a limited change in the status of the sow population of that herd. However, it is our opinion that especially the variance of this status causes a certain inaccuracy to the statistical approach that limits it value for small sample sizes, like individual herds. This is also supported by the fact that in some herds the true seroprevalence was lower than the expected seroprevalence, which can be explained by this variance in the sow population.

The half-lives of maternal antibodies for both H1 and H3 were 12 days. From data presented in literature almost identical half-lives for maternal antibodies against influenza virus could be derived (Young and Underdahl, 1949; Easterday, 1971; Bikour et al., 1994). It was also suggested in previous research that dilution caused by growth of the piglet explains most of the decline of the antibody titres (Curtis and Bourne, 1973).

An IgM ELISA is very useful to detect recent infections when antibodies are already present (either maternal or due to a previous infection). A major disadvantage, however is the small window of detection of this test. Also, the IgM ELISAwas not able to distinguish between different subtypes of influenza. With a calculated incidence of 17% over a period of approximately 30 days for each subtype, 31% of all piglets will develop IgM antibodies against either or both subtypes (if there is no correlation between subtypes). With a detection window of 10-12 days, one would expect 10-12% seropositive piglets of 8-9 weeks old. This is very close to the 14% we found, supporting our conclusions once more.

Although there were some differences between the results of the statistical approach and the results of the IgM ELISA, the overall conclusions were the same: not many infections leading to an antibody response occur in piglets up to 9 weeks old. Part of these infections may occur as major outbreaks in a few herds. Others occur as only one or a few infections per herd. Whether these are just a minor outbreaks under the

protection of maternal antibodies or the beginning of a major outbreak could not be established.

Conclusion

We conclude that the statistical approach is useful in estimating the prevalence and incidence of certain infections in the presence of maternal antibodies, especially if an IgM ELISA is not available or not discriminatory between subtypes of an infectious agent. For individual herds both methods have disadvantages. The statistical approach relies heavily on the predictability of the presence of maternal antibodies and will therefore result in randomerrors. Any inaccuracy in this may result in either a false positive or a false negative result for an individual herd. The IgM ELISA as an alternative will certainly lead to systematic errors (underestimation of infections due to the small window of detection). For a group of herds on the other hand, the method seem to be able to get a fast and fairly reliable estimate of the number of herds in which an outbreaks has occurred or an overall prevalence and incidence of the infection. For influenza it was concluded that the majority of the infections in finishing piglets will take place during the finishing period, when the pigs are at least 10 weeks old.

References

Bikour, M. H., Cornaglia, E., Elazhary, Y., 1994. Comparative study of the immunostimulatory properties of different adjuvants administered with an inactivated influenza virus vaccine and evaluation of passive immunity in pigs. Immunol. Infect. Dis., 4: 166-172.

Blaskovic, D., Rathová, V., Kocisková, D., Jamrichová, O. , Sádecký, E., Kaplan, M. M., 1970. Experimental infection of weanling pigs with A/swine influenza virus. 3. Immunity in piglets farrowed by antibody-bearing dams experimentally infected a year earlier. Bull. World Health Organ., 42: 771-777.

Curtis, J., Bourne, F. J., 1973. Half-lives of immunoglobulins IgG, IgA and IgM in the serum of new-born pigs. Immunology, 24: 147-155.

Easterday, B. C., 1971. Influenza virus infection of the suckling pig. Acta Vet. Brno, 40 Suppl 2: 33-42.

Easterday, B. C., 1972. Immunologic considerations in swine influenza. J. Am. Vet. Med. Assoc., 160: 645-648.

Elbers, A. R. W., Cromwijk, W. A. J., Hunneman, W. A., Tielen, M. J. M., 1990a. Log book recording on pig finishing farms in an Integrated Quality Control (IQC) project. I. Use of drugs and vaccines [Logboekregistratie op vleesvarkensbedrijven in het Integrale Keten Beheersing (IKB) project. I. Gebruik van medicijnen en vaccins]. Tijdschr. Diergeneeskd., 115: 249-261.

Elbers, A. R. W., Tielen, M. J. M. , Cromwijk, W. A. J., Hunneman, W. A., 1990b. Sero-epidemiological screening of pig sera collected at the slaughterhouse to detect herds infected with Aujeszky's disease virus, porcine influenza virus and Actinobacillus (Haemophilus) pleuropneumoniae in the framework of an integrated quality control (IQC) system. Vet. Q., 12: 221-230.

Groschup, M. H., Brun, A., Haas, B., 1993. Serological studies on the potential synergism of porcine reproductive and respiratory syndrome virus and influenza-, corona- and paramyxoviruses in the induction of respiratory symptoms in swine. Zentralbl. Veterinarmed. [B], 40: 681-689.

Große Beilage, E., Schenk, L., Süss, J., Schrader, C., 1996. The influence of maternal antibodies on the frequency, severity, and serology of the infection with influenza A virus. Proc. IPVS Congr. Bologna, 14: 130.

Haesebrouck, F., Pensaert, M. B., 1986. Prevalence of H1N1- and H3N2-influenza A viruses in fatteners [swine] in Belgium (1984). Vlaams Diergen. Tijdschr., 55: 12-16.

Heinen, P. P., Van Nieuwstadt, A. P., Pol, J. M. A., De Boer-Luijtze, E. A., Van Oirschot, J. T., Bianchi, A. T. J., 2000. Systemic and mucosal isotype-specific antibody responses in pigs to experimental influenza virus infection. Viral Immunol., 13: 237-247.

Kendal, A. P., Skehel, J. J., Pereira, M. S., 1982. Concepts and procedures for laboratory-based influenza surveillance. US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.

Loeffen, W. L. A., Kamp, E. M., Stockhofe-Zurwieden, N., Van Nieuwstadt, A. P. K. M. I., Bongers, J. H., Hunneman, W. A., Elbers, A. R. W., Baars, J., Nell, T., Van Zijderveld, F. G., 1999. Survey of infectious agents involved in acute respiratory disease in finishing pigs. Vet. Rec., 145: 123-129.

Maes, D., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., Vrijens, B., De Kruif, A., 1999. Risk indicators for the seroprevalence of Mycoplasma hyopneumoniae, porcine influenza viruses and Aujeszky's disease virus in slaughter pigs from fattening pig herds. Zentralbl. Veterinarmed. [B], 46: 341-352.

Nodelijk, G., Van Leengoed, L. A. M. G., Schoevers, E. J., Kroese, A. H., De Jong, M. C. M., Wensvoort, G., Verheijden, J. H. M., 1997. Seroprevalence of porcine reproductive and respiratory syndrome virus in Dutch weaning pigs. Vet. Microbiol., 56: 21-32.

Paisley, L. G., Vraa-Andersen, L., Dybkjær, L., Møller, K., Christensen, G., Mousing, J., Agger, J. F., 1993. An epidemiologic and economic study of respiratory diseases in two conventional Danish swine herds .1. Prevalence of respiratory lesions at slaughter and their effects on growth. Acta Vet. Scand., 34: 319-329.

SAS Institute Inc., 1996. SAS/STAT software: changes and enhancements through release 6.11. SAS Institute Inc., Cary, North Carolina. 1104 pp.

Straw, B. E., Shin, S. J., Yeager, A. E., 1990. Effect of pneumonia on growth rate and feed efficiency of minimal disease pigs exposed to Actinobacillus pleuropneumoniae and Mycoplasma hyopneumoniae. Prev. Vet. Med., 9: 287-294.

Thrusfield, M., 1995. Veterinary epidemiology, second edition. Blackwell Science Ltd, Cambridge. 479 pp.

Young, G. A., Underdahl, N. A., 1949. Swine influenza as a possible factor in suckling pig mortalities. II. Colostral transfer of hemagglutinin inhibitors for swine influenza virus from dam to offspring. Cornell Vet., 39: 120-128.

Chapter 4

Population dynamics of swine influenza virus in farrow-to-finish and specialised finishing herds

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Population dynamics of swine influenza virus in farrow-to-finish and specialised finishing herds

Abstract

Influenza virus infections with subtypes H1N1, H3N2 and H1N2 in domestic pigs in Europe are very common. Data on possible differences of population dynamics in finishing pigs in farrow-to-finish herds and in specialised finishing herds are, however, scarce. The presence of sows and weaned piglets on the same premises may, however, affect the exposure of finishing pigs to influenza viruses. In a longitudinal study on 14 farrow-to-finish herds and 15 finishing herds, groups of pigs were followed by repeatedly testing the same animals for antibodies against all three influenza virus subtypes (H1N1, H3N2 and H1N2). At the end of the finishing period, the seroprevalences in farrow-to-finish and specialised finishing herds were 44.3% and 62.0% respectively for H1N1, 6.6% and 19.3% respectively for H3N2, and 57.2% and 25.6% respectively for H1N2. For all three subtypes, the incidence of influenza virus infections was highest at the beginning of the finishing period in farrow-to-finish herds, while the incidence of influenza virus infections was highest at the end of the finishing period in finishing herds. Respiratory disease, probably related to the influenza infections, was observed in five of these herds only, but also occurred at the beginning of the finishing period in farrow-to-finish herds and at the end of the finishing period in finishing herds. The observed differences of population dynamics of influenza virus may affect choice and timing of intervention measures.

Introduction

Influenza virus infections in swine are very common. In several studies in European countries, seroprevalences for the traditional swine influenza strains H1N1 and H3N2 were found to be in the range of 20-80% in finishing pigs at the end of the finishing period and in sows (Masurel et al., 1983; Haesebrouck and Pensaert, 1986; Yus et al., 1989; Elbers et al., 1990; Teuffert et al., 1991; Elbers et al., 1992; Groschup et al., 1993; Ewald et al., 1994; Brown et al., 1995b; Maes et al., 1999; Maldonado et al., 2006). A more recent subtype, H1N2, seems to originate from the UK where it was found for the first time in 1994 (Brown et al., 1995a). H1N2 was subsequently reported from Belgium (Van Reeth et al., 2000), Italy and France (Marozin et al., 2002), Germany (Schrader and Suss, 2003), and Spain (Maldonado et al., 2006). Many, if not all of these strains are related to the original H1N2 strain from the UK (Marozin et al., 2002). Seroprevalence studies in Belgium (Van Reeth et al., 2000) and Spain (Maldonado et al., 2006) resulted in high seroprevalences of approximately 70% and 50% respectively for H1N2, but both studies were carried out in sows.

Studies in finishing pigs so far give overall estimates of the seroprevalence at the end of the finishing period. However, it is not clear at what moment during the finishing period these infections take place, nor do they differentiate between finishing pigs in

FIGURE 1: Location of the three most pig-dens regions in the Netherlands and the location of the herds participating in the longitudinal study (farrow-to-finish herds (▼) and finishing herds (▲)).

specialised finishing herds and finishing pigs in farrow-to-finish herds. The presence of sows and piglets on the same premises as the finishing pigs may, however, affect the population dynamics of swine influenza in these herd types, and thus also affect the choice and timing of intervention measures, like vaccination or zoosanitary measures.

Longitudinal studies were therefore carried out to be able to compare the seroprevalences and incidences of swine influenza virus infections at different ages in finishing pigs in both herd types. This allowed us also to determine whether there are differences in the population dynamics of swine influenza virus in finishing pigs in farrow-to-finish herds versus finishing pigs in specialised finishing herds.

Materials & methods

Selection of herds

In the Netherlands, there are three regions with a high pig density (figure 1). In these regions the average pig density is more than 600 pigs per square kilometre. In contrast, the average pig density in the remaining part of the Netherlands is below 100 pigs per square kilometre. From the three pig dense regions all herds with >400 finishing pigs were selected for possible participation in the study. This selection included 27% of the Dutch swine herds, housing 69% of the Dutch finishing pig population.

In each category (farrow-to-finish and finishing herds) 64 herds were randomly selected. A written request for participation in a longitudinal serological survey was sent to these farmers. Vaccination against swine influenza was not allowed. The first 15 in each category returning a positive response were included in the study. One farrow-to-finish herd that applied for the survey withdrew shortly before the survey actually started and could not be replaced on such short notice. Thus, 29 herds finally participated.

Sampling

All farms were visited three (finishing herds) or four (farrow-to-finish herds) times during the months of January to May. In each herd one compartment was followed in a longitudinal study and blood samples were collected during each visit.

In finishing herds one compartment where the piglets were 12 weeks old was sampled during the first visit. On average, these piglets arrived 2-3 weeks before on the farm. Pigs were tagged individually during the first sampling to allow for resampling of the same pigs during subsequent visits. The second blood samples were taken four weeks later (age of 16 weeks) and a final sample was taken within one week before the first pigs from that compartment were delivered to the slaughter house (on average at the age of 22 weeks). In eleven of the finishing herds the investigated pigs originated from only one breeding herd, in three finishing herds the pigs originated two breeding herds and in one finishing herd the pigs originated from three breeding herds. During the finishing period no other pigs were added to that compartment.

In farrow-to-finish herds one compartment where the piglets were 8 weeks old was sampled during the first visit. Pigs were tagged individually during the first sampling to

allow for resampling of the same pigs during subsequent visits. The second blood samples were taken four weeks later (age of 12 weeks), the third another four weeks later (age of 16 weeks) and the final sample was taken within one week before the first pigs from that compartment were delivered to the slaughter house (on average at the age of 22 weeks). Tagged piglets were kept together in the same compartment until slaughter, but while being transferred to the finishing facilities, piglets from two compartments were sometimes mixed. However, during the finishing period no other pigs were added to that compartment anymore.

The sample size for each compartment was calculated so that with an estimated seroprevalence of 50% and a confidence of 0.95 the margin of error was less than 20%. This resulted in sample sizes of 16-24 pigs per compartment using the formula for simple random sampling (Thrusfield, 1995). Within a compartment an equal number of pigs were sampled per pen, as far as the total number of samples allowed for this. Within each pen, pigs were selected at random (haphazardly).

All farmers were asked to record all clinical signs and medications in the compartment under study.

Serological examination

All sera were tested in a hemagglutination inhibition (HI) test (Kendal et al., 1982) for antibodies against influenza virus strains A/swine/Neth/Best/96 (H1N1), A/swine/Neth/St Oedenrode/96 (H3N2) and A/swine/Gent/7625/99 (H1N2). A/swine/Neth/Best/96 (H1N1) and A/swine/Neth/St Oedenrode/96 were isolated in the Netherlands and are representative for influenza strains circulating in the Netherlands (Loeffen et al., 1999). A/swine/Gent/7625/99 (H1N2) is a Belgian strain, representative for H1N2 strains circulating among pigs in Belgium, which in turn is expected to be representative for H1N2 strains all over Western Europe (Marozin et al., 2002). Although H1N1 and H1N2 strains both possess a hemagglutinin H1, these are of different origin (Brown et al., 1998) and little or no cross reactivity was found in serological tests after experimental infections (Van Reeth et al., 2000; Van Reeth et al., 2004; Van Reeth et al., 2006).

Sera were tested in serial twofold dilutions, starting at 1:9. Titres ≥18 were considered positive. A fourfold rise in titres in consecutive samples was considered evidence of an influenza virus infection somewhere during that period (taking into account approximately one week for titres to develop after an influenza virus infection). To calculate a four-fold rise in titre, the decay of maternal antibodies was also taken into consideration (Loeffen et al., 2003). Titres of the second sera were therefore not compared to the titres of the first sera, but to extrapolated titres of the first sera, estimating what they would have been at the time of the second sampling if they were the result of maternal antibodies.

Statistical analysis

Statistical analyses for the differences between farrow-to-finish herds (FFH) and specialised finishing herds (FH) were performed per strain en per time interval. The incidence, cumulative incidence and prevalence data were expressed as fractions per

Table 1: Seroprevalences for swine influenza subtypes H1N1, H3N2, and H1N2, in farrow-to-finish herds and finishing herds. In each herd the same pigs were sampled at 4 (farrow-to-finish herds) or 3 (finishing herds) different ages. Any significant (P<0.05) differences between farrow-to-finish and finishing herds (pair wise comparisons for the same influenza virus subtype over the same period) are indicated with a shaded background. Any trends towards significance (P<0.1) are indicated by a square.

Type	Age (weeks)	Number of herds	H1N1			H3N2			H1N2		
			Number of samples	Prevalence	95%CI	Number of samples	Prevalence	95%CI	Number of samples	Prevalence	95%CI
Farrow-to-finish	8	14	290	21.7	11.1-32.3	290	21.7	12.9-30.6	290	53.8	38.4-69.2
	12	14	287	20.9	5.9-35.9	287	15.7	2.4-29.0	287	48.8	28.9-68.7
	16	14	286	44.4	22.3-66.5	286	8.7	0.0-20.1	286	60.8	42.7-79.0
	22	14	287	44.3	23.1-65.4	287	6.6	0.0-15.7	283	57.2	38.2-76.3
Finishing	12	15	322	22.0	8.1-36.0	322	35.7	19.7-51.8	322	18.6	6.6-30.6
	16	15	321	22.1	8.7-35.6	321	20.2	7.0-33.5	320	10.3	0.0-21.1
	22	15	321	62.0	44.6-79.4	321	19.3	3.7-34.9	320	25.6	7.6-43.7

farm. Farms were considered as experimental units, because of dependence between animals within a farm. The test statistic (t) for the null hypothesis of no difference between the herd types was:

$$t = a - b$$

wherein a and b are the fractions averaged over FFH and FH farms respectively. The null-distribution was obtained by random permutation of the labels FFH and FH over the farms.

Results

Seroprevalence

For H1N2, at the end of the finishing period the seroprevalences in farrow-to-finish and specialised finishing herds were 57.2% and 25.6% respectively (table 1). The seroprevalence was in all age groups higher in farrow-to-finish herds than in specialised finishing herds (P=0.01, P<0.01 and P=0.03 for 12, 16 and 22 weeks of age respectively). For H1N1 and H3N2, at the end of the finishing period the seroprevalences in farrow-to-finish and specialised finishing herds were 44.3% and 62.0% respectively for H1N1 and 6.6% and 19.3% respectively for H3N2. The differences of the seroprevalences in farrow-to-finish herds and finishing herds were not statistically significant.

Table 2: Incidences for swine influenza subtypes H1N1, H3N2, and H1N2, in farrow-to-finish herds and finishing herds. In each herd the same pigs were sampled at 4 (farrow-to-finish herds) or 3 (finishing herds) different ages. Incidences were calculated based on the number of seroconversions per period. Any significant (P<0.05) differences between farrow-to-finish and finishing herds (pair wise comparisons for the same influenza virus subtype over the same period; in farrow-to-finish herds the periods of 0-8 and 8-12 weeks are added to each other to be able to compare them to the period of 0-12 weeks in finishing pigs) are indicated with a shaded background. Any trends towards significance (P<0.1) are indicated by a square.

Type	Period (weeks of age)	Number of herds	H1N1			H3N2			H1N2		
			Number of samples	Incidence (%)	95%CI	Number of samples	Incidence (%)	95%CI	Number of samples	Incidence (%)	95%CI
Farrow-to-finish	0-8	14	290	-	-	290	-	-	290	8.6	0.0-19.0
	8-12	14	287	15.7	2.7-28.6	287	10.1	0.0-23.3	287	24.4	8.5-40.3
	12-16	14	286	31.1	8.1-54.1	286	1.4	0.0-2.9	286	25.9	7.9-43.9
	16-22	14	285	2.5	0.0-7.1	285	1.1	0.0-2.6	281	1.4	0.3-2.6
Finishing	0-12	15	320	6.9	0.0-15.7	320	4.1	0.0-8.5	320	5.0	0.0-14.5
	12-16	15	320	13.8	4.8-22.7	320	10.3	0.9-19.7	319	2.8	0.0-5.9
	16-22	15	308	41.2	22.1-60.4	308	8.8	0.0-21.1	307	18.6	1.4-35.7

Incidence

For H1N1 the differences between both herd types are statistically significant between 16 and 22 weeks of age (P<0.01) and for H1N2 between 0 and 12 (P=0.05) and 12 and 16 (P=0.01) weeks of age (table 2). The cumulative incidences are shown in table 3. They are essentially comparable to the seroprevalence, however, corrected for antibodies due to maternal immunity or antibodies from an infection that declined to a level below the detection limit of the test.

Clinical symptoms of respiratory disease

Twenty-four farmers (from 11 farrow-to-finish herds and 13 finishing herds) kept a record of clinical signs and medications. In eleven of them, an episode of respiratory disease was recorded: five in farrow-to-finish herds and six in finishing herds. The severity of the symptoms was variable, ranging from only coughing for a few days up to a combination of coughing, laboured breathing and decrease of feed intake. Five of these episodes coincided with an incidence of 80-95% of the pigs for at least one of the influenza subtypes in the same period. Two of these were in farrow-to-finish herds and both occurred between the age of 8 and 12 weeks. The other three occurred in finishing herds, all of them between the age of 16 and 22 weeks.

Table 3: Cumulative incidences for swine influenza subtypes H1N1, H3N2, and H1N2, in farrow-to-finish herds and finishing herds. In each herd the same pigs were sampled at 4 (farrow-to-finish herds) or 3 (finishing herds) different ages. Incidences were calculated based on the number of seroconversions per period. Any significant (P<0.05) differences between farrow-to-finish and finishing herds (pair wise comparisons for the same influenza virus subtype over the same period) are indicated with a shaded background.

Type	Period (weeks of age)	Number of herds	H1N1			H3N2			H1N2		
			Number of samples	Incidence (%)	95%CI	Number of samples	Incidence (%)	95%CI	Number of samples	Incidence (%)	95%CI
Farrow-to-finish	0-8	14	290	-	-	290	-	-	290	8.6	0.00-19.0
	0-12	14	287	15.7	2.7-28.6	287	10.1	0.0-23.3	287	33.1	12.7-53.4
	0-16	14	286	46.9	24.2-69.5	286	11.5	0.0-24.6	286	59.1	40.0-78.2
	0-22	14	285	49.5	26.6-72.3	285	12.6	0.0-25.8	281	60.5	41.4-79.6
Finishing	0-12	15	320	6.9	0.0-15.7	320	4.1	0.0-8.5	320	5.0	0.0-14.5
	0-16	15	320	20.6	8.6-32.6	320	14.4	3.3-25.4	319	7.8	0.0-17.5
	0-22	15	308	62.7	45.4-79.9	308	23.4	7.2-39.5	307	26.7	8.6-44.9

Discussion

In this study we found that the timing of influenza infections in finishing pigs is quite different in finishing pigs from farrow-to-finish herds compared to finishing pigs from specialised finishing herds. In farrow-to-finish herds the incidence of influenza virus infections was highest at the beginning of the finishing period, while in finishing herds the incidence of influenza virus infections was highest at the end of the finishing period. This pattern was observed for all three influenza virus subtypes. It can be speculated that the exposure of finishing pigs to influenza virus is different in both herd types.

Sows and especially weaned piglets may be a reservoir for continuous circulation of influenza viruses (Loeffen et al., 2003). Indirect exposure of finishing pigs to influenza virus from sows or weaned piglets will be more easily achieved in farrow-to-finish herds than in specialised finishing herds. Multiple virus introductions into a compartment of finishing pigs in a farrow-to-finish herd could thus result in a high incidence at the beginning of the finishing period. Large outbreaks are apparently rare at that time, possibly due to the sum of decreasing numbers of piglets with maternal antibodies and increasing numbers of piglets with antibodies due to an infection. Introductions later during the finishing period may then die out quickly due to the already high seroprevalence. A compartment of pigs in a finishing herd on the other hand may be subject to only a few virus introductions. On average this may result in populations with more susceptible animals in the second half of the finishing period, with any introduction at that time resulting in a large outbreak within that compartment.

The presence of finishing pigs in a herd might also affect influenza virus infections in sows and weaned piglets. Especially with subtype H1N2, at least 8.6% percent of the weaned piglets seroconverted before they were 8 weeks old. This could also explain the higher seroprevalence at the age of 12 weeks in farrow-to-finish herds, compared to finishing herds. It may be that in the breeding herds that supplied the piglets to the specialised finishing herds, virus circulation in weaned piglets was lower than in weaned piglets on farrow-to-finish herds. This would suggest that the presence of finishing pigs may result in an additional exposure of weaned piglets to the virus and thus may affect virus circulation in weaned piglets. It's also possible that the relatively recent introduction of the H1N2 subtype in the population somehow causes this effect. This would, however, indicate that farrow-to-finish herds and specialised breeding herds are at a different risk for virus introduction.

Even though serological differentiation between H1N1 and H1N2 is possible in experimental sera (Van Reeth et al., 2004; Van Reeth et al., 2006), nothing is known about differentiation in field sera. In the field multiple infections can be expected, and variations occur in haemagglutinin, even within one subtype (De Jong et al., 2001). Seroprevalence and incidence of H1N1 and H1N2 might as a result be overestimated. However, if the serological results of these two subtypes are compared at the age of 22 weeks, the agreement beyond chance is very low (Kappa value 0.078). Under the assumption that both subtypes occur independently, this suggests a very low level of cross-reactivity.

The seroprevalence of H1N1 is comparable to the levels that were found in finishing pigs in 1980 (44%) (Masurel et al., 1983) and 1987 (62%) (Elbers et al., 1990) in the Netherlands. The seroprevalence found for H3N2 is, however, much lower than the ones found in those same studies (68% in 1980; 33% in 1987). All other seroprevalence studies carried out in Europe in the past two decades also showed much higher seroprevalences (Haesebrouck and Pensaert, 1986; Yus et al., 1989; Teuffert et al., 1991; Groschup et al., 1993; Ewald et al., 1994). The reason for the low seroprevalence therefore remains unknown. The seroprevalence of H1N2 is not comparable to published results from other countries, because they were carried out in sows (Van Reeth et al., 2000; Maldonado et al., 2006).

While the population dynamics of influenza virus is different between farrow-to-finish and finishing herds, it would be equally important to determine whether the occurrence of clinical symptoms, and subsequent economic losses, are also different between both herd types. Unfortunately, also with respect to this, no information can be found in existing literature. Some studies that tried to determine the importance of influenza virus infections as a cause for respiratory disease, found that approximately 50% of the respiratory outbreaks were caused by influenza virus infections (Loeffen et al., 1999; Barigazzi and Donatelli, 2003; Choi et al., 2003). There was however no differentiation in herd type, or time of infection. In this study, eleven episodes of respiratory disease were noticed, almost equally distributed over both herd types. Only five of them coincided with a seroconversion against one of the influenza subtypes of at least 80% of the pigs in the same period, also equally distributed over both herd types. These five episodes are the ones most likely related to the influenza virus infections, which is also

consistent with the 50% of respiratory disease caused by influenza virus infections (Loeffen et al., 1999). Although this is not enough to allow for any statistical analysis, it is noticeable that the two episodes of respiratory disease in farrow-to-finish herds occurred between the age of 8 and 12 weeks, while the three episodes in finishing herds occurred between the age of 16 and 22 weeks. Finishing pigs in both herd types may be equally subject to respiratory disease, but at a different moment during the finishing period.

Differences in time of influenza virus infections in both herd types may ask for a different approach in preventive measures, whether these are zoosanitary measures or vaccination. If infections, and subsequent economic losses, in specialised finishing herds occur mainly at the end of the finishing period, it would be possible to wait with vaccination, and with possibly a single vaccination being sufficient, until maternal antibodies disappear. Interference of vaccination with maternal antibodies (Wesley and Lager, 2006) would thus not be a real problem and full and equal protection may be reached. In farrow-to-finish herds on the other hand, there seems to be no optimum with respect to the time of vaccination. Before all maternal antibodies disappear in a population, most of the influenza virus infections, and possibly also the economic losses due to the infection, already occur. Vaccination at a young age, and probably multiple vaccinations to circumvent the negative effect of maternal antibodies, may be indicated. While these conclusions and recommendations may apply to swine herds in general, variations in individual herds may occur. It is therefore advisable that, where possible, results from investigations in an individual herd are also taken into account before putting together a strategy of vaccination or any other kind of intervention.

References

Barigazzi, G., Donatelli, I., 2003, Swine influenza in Italy. Vet Res Commun 27 Suppl 1, 93-99.

Brown, I.H., Chakraverty, P., Harris, P.A., Alexander, D.J., 1995a, Disease outbreaks in pigs in Great Britain due to an influenza A virus of H1N2 subtype. Vet.Rec. 136, 328-329.

Brown, I.H., Harris, P.A., Alexander, D.J., 1995b, Serological studies of influenza viruses in pigs in Great Britain 1991- 2. Epidemiol.Infect. 114, 511-520.

Brown, I.H., Harris, P.A., McCauley, J.W., Alexander, D.J., 1998, Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J.Gen.Virol. 79 (Pt 12), 2947-2955.

Choi, Y.K., Goyal, S.M., Joo, H.S., 2003, Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. Can Vet J 44, 735-737.

De Jong, J.C., Heinen, P.P., Loeffen, W.L., Van Nieuwstadt, A.P., Claas, E.C., Bestebroer, T.M., Bijlsma, K., Verweij, C., Osterhaus, A.D., Rimmelzwaan, G.F., Fouchier, R.A., Kimman, T.G., 2001, Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy. Vaccine 19, 4452-4464.

Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1990, Sero-epidemiological screening of pig sera collected at the slaughterhouse to detect herds infected with Aujeszky's disease virus, porcine influenza virus and Actinobacillus (Haemophilus) pleuropneumoniae in the framework of an integrated quality control (IQC) system. Vet.Q. 12, 221-230.

Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1992, Variation in seropositivity for some respiratory disease agents in finishing pigs: epidemiological studies on some health parameters and farm and management conditions in the herds. Vet.Q. 14, 8-13.

Ewald, C., Heer, A., Havenith, U., 1994, Factors Associated with Influenza-A Virus-Infections in Finishing Pigs. Berliner und Munchener Tierarztliche Wochenschrift 107, 256-262.

Groschup, M.H., Brun, A., Haas, B., 1993, Serological studies on the potential synergism of porcine reproductive and respiratory syndrome virus and influenza-, corona- and paramyxoviruses in the induction of respiratory symptoms in swine. Zentralbl.Veterinarmed.[B] 40, 681-689.

Haesebrouck, F., Pensaert, M.B., 1986, Prevalence of H1N1- and H3N2-influenza A viruses in fatteners [swine] in Belgium (1984). Vlaams Diergeneeskundig Tijdschrift 55, 12-16.

Kendal, A.P., Skehel, J.J., Pereira, M.S., 1982, Concepts and procedures for laboratory-based influenza surveillance. US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.

Loeffen, W.L.A., Kamp, E.M., Stockhofe-Zurwieden, N., Van Nieuwstadt, A.P.K.M.I., Bongers, J.H., Hunneman, W.A., Elbers, A.R.W., Baars, J., Nell, T., Van Zijderveld, F.G., 1999, Survey of infectious agents involved in acute respiratory disease in finishing pigs. Vet.Rec. 145, 123-129.

Loeffen, W.L.A., Nodelijk, G., Heinen, P.P., Van Leengoed, L.A., Hunneman, W.A., Verheijden, J.H., 2003, Estimating the incidence of influenza-virus infections in Dutch weaned piglets using blood samples from a cross-sectional study. Vet.Microbiol. 91, 295-308.

Maes, D., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., Vrijens, B., De Kruif, A., 1999, Risk indicators for the seroprevalence of Mycoplasma hyopneumoniae, porcine influenza viruses and Aujeszky's disease virus in slaughter pigs from fattening pig herds. Zentralbl.Veterinarmed.[B] 46, 341-352.

Maldonado, J., Van Reeth, K., Riera, P., Sitja, M., Saubi, N., Espuna, E., Artigas, C., 2006, Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet J 172, 377-381.

Marozin, S., Gregory, V., Cameron, K., Bennett, M., Valette, M., Aymard, M., Foni, E., Barigazzi, G., Lin, Y., Hay, A., 2002, Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. J.Gen.Virol. 83, 735-745.

Masurel, N., De Boer, G.F., Anker, W.J., Huffels, A.D., 1983, Prevalence of influenza viruses A-H1N1 and A-H3N2 in swine in the Netherlands. Comp.Immunol.Microbiol.Infect.Dis. 6, 141-149.

Schrader, C., Suss, J., 2003, Genetic characterization of a porcine H1N2 influenza virus strain isolated in Germany. Intervirology 46, 66-70.

Teuffert, J., Sinnecker, R., Karge, E., 1991, Serological survey of HI antibodies against porcine and human type A influenza viruses among domestic and wild pigs in East Germany.; [Seroepidemiologische Untersuchungen mit dem HAHT zum Vorkommen porciner und humaner Infl-A-Viren bei Haus- und Wildschweinen in der ehemaligen DDR]. Monatsh.Veterinarmed. 46, 171-174.

Thrusfield, M., 1995, Veterinary epidemiology, second edition, 2 Edition. Blackwell Science Ltd, Cambridge, 1-479 pp.

Van Reeth, K., Brown, I., Essen, S., Pensaert, M., 2004, Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. Virus Res 103, 115-124.

Van Reeth, K., Brown, I.H., Pensaert, M., 2000, Isolations of H1N2 influenza A virus from pigs in Belgium. Veterinary Record 146, 588-589.

Van Reeth, K., Labarque, G., Pensaert, M., 2006, Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. Viral Immunol 19, 373-382.

Wesley, R.D., Lager, K.M., 2006, Overcoming maternal antibody interference by vaccination with human adenovirus 5 recombinant viruses expressing the hemagglutinin and the nucleoprotein of swine influenza virus. Vet Microbiol 118, 67-75.

Yus, E., Laviada, M.D., Moreno, L., Castro, J.M., Escribano, J.M., Simarro, I., 1989, The prevalence of antibodies to influenza virus and respiratory coronavirus in fattening pigs in Spain. Zentralbl.Veterinarmed.[B] 36, 551-556.

Chapter 5

Reduced incidence of influenza virus infections in finishing pigs by air filtration and hygienic measures

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Reduced incidence of influenza virus infections in finishing pigs by air filtration and hygienic measures

Abstract

Influenza virus infections are responsible for more than half of the outbreaks of acute respiratory disease. The objective of this study was to investigate whether the incidence of influenza virus infections can be reduced by zoosanitary intervention measures, even if piglets are born in an endemically infected environment. The zoosanitary intervention measures investigated here relied on air filtration and hygienic measures and were implemented in one building (the Air Pathogen Free (APF) house) on the premises of a large pig farm that was endemically infected with influenza virus. During a period of two years, serological investigations were carried out in pigs in the APF house, either entering the APF house at the age of three weeks (APF3) or at the age of nine weeks (APF9). Groups that were housed under conventional circumstances on the same farm at the same time were included as controls. A significantly lower incidence of influenza virus infections was found in pigs in the APF house (H1N1: 17.0 and 15.8%, H3N2: 25.3 and 34.4%, for APF3 and APF9 respectively) compared to the conventionally housed pigs (H1N1: 63.0%, H3N2: 64.9%). The differences became evident during the finishing period. The results show that our zoosanitary measures were able to significantly reduce the incidence of influenza virus infections in finishing pigs.

Introduction

High prevalences of antibodies against influenza virus in finishing pigs at the end of the finishing period are common (Elbers et al., 1990; Groschup et al., 1993; Blocks et al., 1994; Ewald et al., 1994; Maes et al., 2000). While many infections with influenza virus remain subclinical, infections can also result in acute respiratory disease. Influenza virus infections are in fact responsible for more than half of the outbreaks of acute respiratory disease in the Netherlands (Loeffen et al., 1999), but are also an important cause of respiratory disease in other countries (Barigazzi and Donatelli, 2003; Choi et al., 2003). In case of respiratory disease, antibiotics are often used, either as a curative measure, or as a preventive measure against secondary bacterial infections.

In experimental vaccination-challenge experiments it is concluded that economic losses due to influenza virus infections may be reduced by vaccination (Haesebrouck et al., 1987; Van Reeth et al., 2001; Van Reeth et al., 2003). Field data to support these conclusions are however not available. Although vaccinating pigs against influenza may

not always prevent infection, it is able to prevent clinical symptoms and related economic losses of growth retardation and veterinary treatments (Heinen et al., 2001b).

Economic losses may also be reduced by reducing the incidence of influenza virus infections. This may be achieved by zoosanitary measures that reduce the number of introductions of the virus into an epidemiological unit. An advantage of zoosanitary measures is that they can be effective against many different infectious agents, in contrast to vaccination, which is only effective for one specific infectious agent. Furthermore, zoosanitary measures are preferred over the use of antibiotics, also from the point of view of public health.

The aim of this study was to examine the possibility to reduce the incidence of influenza virus infections in finishing pigs that are born in an endemically infected environment. For this purpose an 'Air Pathogen Free' (APF) house was build in which air was filtered before entering the compartments where the pigs were housed. Furthermore, hygiene protocols applied for pigs, people and materials before entering the building.

Materials & methods

Herd

The herd in which this study took place was a conventional herd, certified free from Aujeszky's disease, DNT producing Pasteurella multocida and mange. Serological

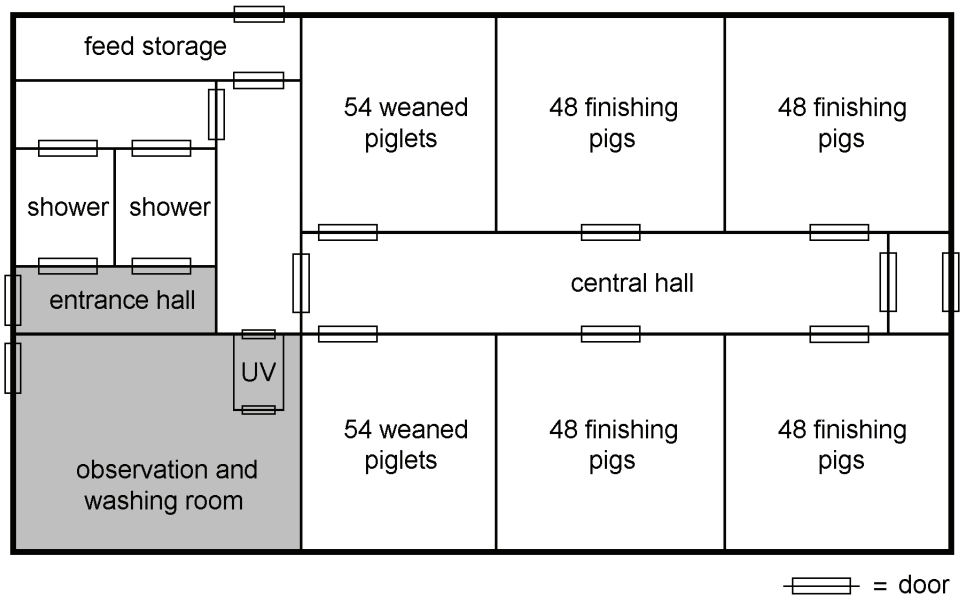

Figure 1: Plan of APF house (white area = positive air pressure, grey area = atmospheric air pressure)

investigations in the past had, however, shown that influenza virus infections were very common, although influenza as a clinical disease was rare. The herd consisted of approximately 350 sows with piglets up to 9 weeks of age and 1200 finishing pigs.

APF house

On the premises of this farm an 'air pathogen free' (APF) pig house was build (figure 1). The APF house consisted of 6 separate compartments, accessible from a central hall. Two of these compartments were rearing compartments, housing 54 weaned piglets each, in six pens of nine piglets. Piglets were reared there from three weeks of age up to nine weeks of age. The other four compartments were finishing compartments, housing 48 finishing pigs each, in six pens of eight pigs. Pigs were finished there from nine weeks of age up to live slaughter weight of approximately 110-115 kilograms (23 to 26 weeks of age). The two main differences compared to a regular pig house were the filtering of the air entering the house and the hygienic measures before people and materials could enter the building. The aim of this APF-house was to keep infectious agents from being introduced after the pigs enter.

Air filtration in APF house

Before entering the building in a central air canal, the air was filtered by a serial system of filters (table 1). The first two filters were primary filters to collect coarse dust (EU2 and EU4 respectively). The third filter was a secondary filter to collect and retain small particle dust (EU7). From the central air canal, the air went into each of the six compartments. Before entering each compartment, the air was filtered through a HEPA filter with an efficiency of 99.97-99.99% (EU12). The air pressure in the compartments was 40-80 Pascal above atmospheric pressure to allow only filtrated air in each compartment. The air shaft at the exit of each compartment was equipped with a fly screen to prevent insects from entering.

Hygienic measures in APF house

Additional hygienic measures were taken with respect to people, animals and materials entering the APF house.

People entered the building through a hygiene lock, where they had to take a shower and change completely into new clothing. This clothing was used only in the

Tabel 1: Specifications of the filters. Efficacy in weight percentage of collected particles

Class	Type	Location	Life expectancy	Efficacy		
				Coarse particles	Fine particles	Aerosols (<0.3µm)
EU2	Coarse	Central air canal	2 months	75%	-	-
EU4	Coarse	Central air canal	4 months	95%	35%	-
EU7	Fine	Central air canal	6 months	100%	85%	70%
EU12	HEPA	Compartment	10 years	100%	100%	99.97%

APF house and washed after each use.

Animals entered the building through a separate door, immediately into the central hall. From there the animals were distributed over the compartments. Afterwards, the central hall was cleaned and disinfected.

Materials entering the APF house were clean, had not been in contact with the conventional pigs, and were treated in a UV closet for 10 to 15 minutes before being used in the APF house.

Feed was delivered through a separate door and stored in the feed storage room. The feed storage room was disinfected each time new feed was delivered.

Conventional housing

Farrowing units housed 6-12 sows each and suckling piglets were raised there under conventional circumstances until they were three weeks old. Three identical compartments, for 90 piglets each, were available to house the weaning piglets. Two identical compartments, for 40 finishing pigs each, were available to house the finishing pigs.

Treatment groups

The study included the following three treatment groups:

- APF3: Piglets born and suckled in conventional housing until three weeks of age, then reared and finished in the APF house from the age of three weeks until slaughter.
- APF9: Piglets born, suckled and reared in conventional housing up to the age of nine weeks, and finished in the APF house from the age of nine weeks until slaughter.
- CONV: Piglets born, suckled, reared and finished in conventional housing until slaughter.

Study design

The following design was used (figure 2): every week 15 to 20 sows, usually housed in 2 compartments, would give birth to approximately 160-220 piglets within a period of two to three days. These piglets were suckled together until three weeks of age. A random block design was used at the time of weaning to assign piglets to the three treatment groups. Each block consisted of three pigs that closely resembled each other based on genetics, sex, weight and age, so usually they were from the same litter. From each block one random pig would be allocated to the APF3 group, one to the APF9 group and one to the CONV group. This assured the least possible variation between the three groups. Over a period of two years, this was repeated ten times (round 1 to 10). In each round, 54 piglets were reared within the APF-house from three to nine weeks of age (APF3), while 90 were reared together in a conventional compartment (APF9+CONV). From the age of nine weeks, the start of the finishing period, 48 of those 90 conventionally reared piglets were finished within the APF-house (APF9), while 40 piglets were finished in a conventional compartment (CONV). Any remaining piglets during selections at 3 or 9 weeks of age were removed from the

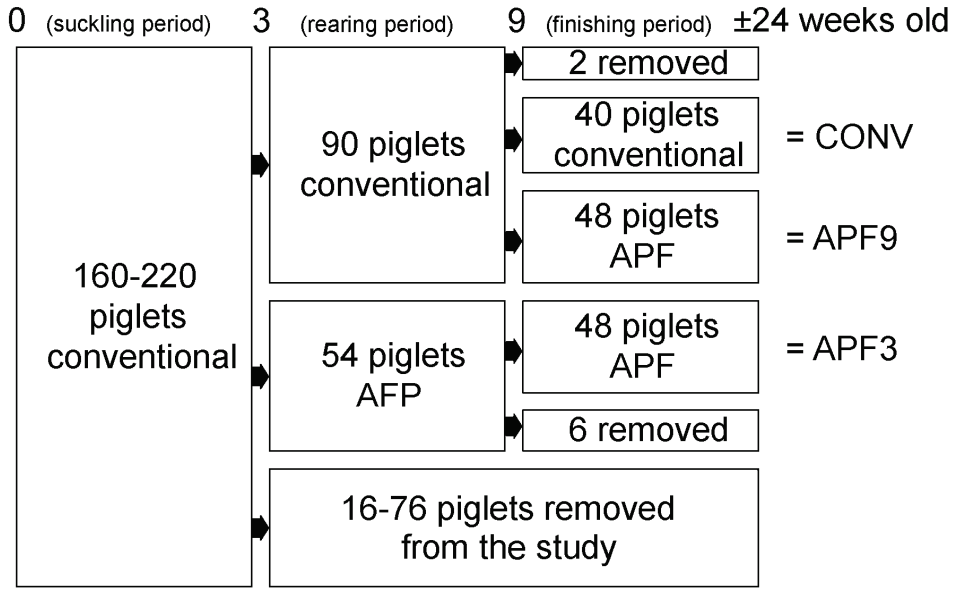

Figure 2: Experimental set-up of each round. Starting with approximately 180 piglets from 18 sows, piglets were weaned at the age of three weeks, and divided in one conventional group and one APF group (APF3). The conventionally reared group was divided iagain at the age of nine weeks (APF9 and CONV). At the age of approximately 24 weeks, all pigs were slaughtered.

study. If clinical symptoms were noticed, or medicines were used, this was recorded on a form for each group on a daily basis.

Sampling

Within each group, 20% of the pigs, which was ten, ten, and eight for the APF3, APF9, and CONV group respectively, were tagged and repeatedly sampled. Immediately after weaning, ten pigs were to that end randomly selected and tagged in the APF3 group. From the APF9 group the ten piglets belonging to the same blocks of three were tagged, and from the CONV group eight out of the ten piglets belonging to the same blocks of three. Blood samples from these pigs were taken at the age of 5, 9, 11, 17 and 23 weeks.

Serological examination

All blood samples were tested for antibodies against the two major influenza virus subtypes occurring in the Netherlands at the time of the study (H1N1 and H3N2). A hemagglutination inhibition (HAI) test (Kendal et al., 1982) was used to test for antibodies against both influenza subtypes. Influenza virus strains

A/swine/Neth/Best/96 (H1N1) and A/swine/Neth/St Oedenrode/96 (H3N2) were used to test for H1 and H3 antibodies respectively. Both strains were isolated in the Netherlands, during the 1995/1996 season (Loeffen et al., 1999).

All sera were tested in serial twofold dilutions, starting at 1:9. Titres ≥18 were considered positive. A fourfold rise of the titre was considered a seroconversion. For each pig it was estimated in which time frame the infection must have taken place, based on the period during which seroconversion was observed. Decay of maternal antibodies was taken into consideration to determine whether a seroconversion had taken place (Loeffen et al., 2003b). It was assumed that each pig could become infected with each strain only once (no evidence of multiple influenza virus infections was seen). For clarity, mainly to filter out seropositivity due to maternal antibodies, the cumulative incidences until the age of sampling were used instead of the prevalence at the age of sampling.

Taking an average delay of one week into account before antibodies can be detected after infection (Heinen et al., 2000; Kim et al., 2006), seroconversions at the time of sampling at 9, 11, 17 and 23 weeks of age actually referred to infections occurring in the time frames of 4-8, 8-10, 10-16 and 16-22 weeks of age.

Statistical analyses

Analyses were performed per strain en per time interval on the incidence and cumulative incidence expressed as fractions per treatment group. Each group was considered as an experimental unit, because of dependence between animals within the same group. A randomization test was performed, as an overall significance test for comparing the three treatments (APF3, APF9 and CONV), followed by pair wise comparisons between the treatments.

The observed fractions (either incidence or cumulative incidence) were calculated as $f_{ij} = y_{ij} / n_{ij}$ for the i-th treatment (i = 1, 2, 3) at the j-th round (j = 1, 2, …, 10), where y_{ij} is the observed frequency and n_{ij} the number of animals sampled.

The test statistic for the null hypothesis of no difference between the treatments was:

$$t = \sum_i (f_i - \bar{f})^2$$

where f_i (i = 1, 2, 3) and $\bar{f}$ denote the fractions per treatment and the overall fraction:

$$f_i = \frac{\sum_j y_{ij}}{\sum_j n_{ij}} = \frac{\sum_j n_{ij} f_{ij}}{\sum_j n_{ij}} \text{ (i = 1, 2, 3) and } \bar{f} = \frac{\sum_{i,j} y_{ij}}{\sum_i \sum_j n_{ij}} = \sum_i \left(\frac{\sum_j n_{ij}}{\sum_i \sum_j n_{ij}} \right) f_i$$

The null-distribution was obtained by random permutation of the treatment labels APF3, APF9 and CONV over the three treatment groups within each round. Pair wise comparisons, e.g. between treatments APF3 and APF9, were based on the squared differences between the treatment fractions, e.g. $t_{12} = (f_1 - f_2)^2$, again randomly permutating labels to obtain the null-distribution.

Results

Clinical symptoms

During the whole study, respiratory symptoms were seen only once, in the APF3 group of round 3, at the age of approximately 9 weeks. The clinical symptoms, recorded only as "respiratory disease", were noticed the same day the piglets were transferred from the rearing compartment to the finishing compartment. Several pigs were treated individually with tetracycline and they recovered within a week.

Tabel 2: Incidence of influenza H1N1 in 10 rounds of rearing pigs under three different conditions (number of seroconversions per period, out of n pigs tested longitudinally)

	APF3					APF9					CONV				
Round	**n**	4-8	8-10	10-16	16-22	**n**	4-8	8-10	10-16	16-22	**n**	4-8	8-10	10-16	16-22
1	**9**	-	-	-	-	**9**	-	-	-	-	**8**	-	-	-	7
2	**8**	-	-	-	-	**9**	-	-	-	-	**6**	-	-	-	5
3	**10**	1	5	4	-	**10**	2	2	1	-	**7**	-	2	3	1
4	**10**	1	1	-	1	**10**	2	-	-	-	**5**	-	-	3	1
5	**10**	1	-	-	2	**10**	1	-	1	2	**8**	4	2	1	-
6	**10**	-	-	-	-	**10**	2	1	-	1	**8**	2	-	1	-
7	**10**	-	-	-	-	**9**	-	-	-	-	**8**	-	-	-	-
8	**10**	-	-	-	-	**9**	-	-	-	-	**8**	-	-	-	-
9	**9**	-	-	-	-	**10**	-	-	-	-	**8**	-	-	5	2
10	**8**	-	-	-	-	**9**	-	-	-	-	**7**	-	-	6	1
Total	**94**	3	6	4	3	**95**	7	3	2	3	**73**	6	4	19	17
Inc./week (%)[1]		0.8^a	3.2^a	0.7^a	0.5^a		1.8^a	1.6^a	0.4^a	0.5^a		2.1^a	2.7^a	4.3^b	3.9^b
Cum. Inc. (%)[2]		3.2^a	9.6^a	13.8^a	17.0^a		7.4^a	10.5^a	12.6^a	15.8^a		8.2^a	13.7^a	39.7^b	63.0^b

[1]) Incidence per week = number of seroconversions / number of pigs tested/length of period between samplings in weeks; [2]) Cumulative incidence = total number of pigs seroconverted so far / number of pigs tested. Significant differences between results for each treatment group, over the same period, are indicated with different letters in superscript.

H1N1

Influenza virus infections were detected in 8 out of 10 CONV groups (Table 2). In 5 of these groups the first seroconversions were seen during the finishing period and most of the pigs (>70% of those tested) seemed to become infected within a short period of time. In only 3 and 4 of the APF3 and APF9 groups respectively, influenza virus infections were detected. In all cases the first influenza virus infections were already detected between the age of 4 and 8 weeks and the cumulative incidence was increasing gradually during the whole rearing and finishing period in these groups.

The overall cumulative incidence in the CONV group was significantly higher than that in the APF3 and APF9 groups at 16 weeks of age ($P=0.01$) and at 22 weeks of age ($P<0.01$). There were no significant differences between the APF3 and APF9 groups.

Serological evidence of a large outbreak in any of the APF-groups, defined as a high number of influenza virus infections (>70% of those tested) within a short period, was found only in round 3 of the APF3 group. Five out of 10 sampled pigs became infected between 8 and 10 weeks of age, and another 4 between 10 and 16 weeks of age. The time frame for these serological findings matched with the clinical symptoms of respiratory disease that were seen in the same group at the age of 9 weeks.

H3N2

Influenza virus infections were detected in all 10 out of 10 CONV groups (table 3). In 6 of these groups the first seroconversions were seen during the finishing period and in 3 of these cases most of the pigs (>70% of those tested) became infected within a short period of time. In only 5, respectively 6 of the APF3 and APF9 groups influenza virus infections were detected. In all cases the first influenza virus infections were already detected between the age of 4 and 8 weeks (APF3) or between the age of 4 and 10 weeks (APF9).

The overall cumulative incidence in the CONV group was significantly higher than that in the APF3 group ($P=0.02$) at 16 weeks of age, although not different from that in the APF9 group. At 22 weeks of age, the overall cumulative incidence in the CONV group was significantly higher than that in the APF3 and APF9 groups ($P<0.01$). There were no significant differences between the APF3 and APF9 groups.

Discussion

Measures with regard to air filtration and increased hygiene, as they were implemented in the APF house, are able to reduce the incidence of influenza virus infections in finishing pigs. Whether this will also reduce clinical symptoms and economic losses related to the influenza virus infections could not be evaluated, because only once during the two year study, respiratory disease in one of the groups was noticed. However, it can be expected that reducing the incidence of influenza virus infections will also reduce the occurrence of acute respiratory disease. This study also allowed for gaining more insight in the population dynamics of influenza virus within a herd.

During the rearing period, up to the age of 9 weeks, the incidence of influenza virus infections was comparable in all groups, inside and outside the APF house. The main

Tabel 3: Incidence of influenza H3N2 in 10 rounds of rearing pigs under three different conditions (number of seroconversions per period, out of n pigs tested longitudinally)

	APF3					APF9					CONV				
Round	**n**	4-8	8-10	10-16	16-22	**n**	4-8	8-10	10-16	16-22	**n**	4-8	8-10	10-16	16-22
1	**9**	4	-	2	3	**9**	5	-	1	1	**8**	2	-	3	1
2	**8**	4	-	-	4	**9**	6	3	-	-	**6**	3	1	-	2
3	**10**	-	-	-	-	**10**	6	1	-	-	**8**	6	-	-	1
4	**10**	-	-	-	-	**10**	1	-	-	2	**5**	-	-	-	5
5	**10**	1	-	-	-	**10**	-	-	-	-	**8**	-	-	7	-
6	**10**	1	-	-	-	**10**	-	-	-	-	**8**	-	-	3	-
7	**10**	-	-	-	-	**10**	-	-	-	-	**8**	-	-	-	1
8	**10**	-	-	-	-	**9**	-	-	-	-	**8**	-	-	1	1
9	**9**	5	-	-	-	**10**	-	5	-	1	**8**	-	5	-	-
10	**9**	-	-	-	-	**9**	1	-	-	-	**7**	-	-	5	-
Total	**95**	15	-	2	7	**96**	19	9	1	4	**74**	11	6	19	11
Inc./week (%)[1]		3.9[a]	0[a]	0.4[a]	1.2[a]		4.9[a]	4.7[a]	0.2[a]	0.7[a]		3.7[a]	4.1[a]	4.3[b]	2.5[a]
Cum. Inc. (%)[2]		15.8[a]	15.8[a]	17.9[a]	25.3[a]		19.8[a]	29.2[a]	30.2[ab]	34.4[a]		14.9[a]	23.0[a]	48.6[b]	63.5[b]

[1]) Incidence per week = number of seroconversions / number of pigs tested/length of period between samplings in weeks; [2]) Cumulative incidence = total number of pigs seroconverted so far / number of pigs tested. Significant differences between results for each treatment group, over the same period, are indicated with different letters in superscript.

risk for entry of pathogens that still remains in such a house is introducing them with the pigs themselves. Further introductions into the APF house through other routes should be rare, as the results in the finishing pigs also suggest. These ongoing infections during the rearing period are therefore most likely the result of successive infections in susceptible hosts becoming available all the time due to declining levels of maternal antibodies, although this doesn't necessarily mean that virus will remain present in the group until slaughter.

In theory, virus could also persist in individual animals. This has been reported (Mensik and Valicek, 1969; Blaskovic et al., 1970), however, later studies were unable to reproduce these results (Wallace and Elm, 1979; Brown et al., 1993), nor have there been any more recent reports of the existence of carriers. Furthermore, in experimental infections, with a wide range of influenza virus strains, duration of virus excretion was always limited, usually to some 5 to 9 days (Easterday, 1971; Kundin and Easterday, 1972; Brown et al., 1993; Brown et al., 1994; Kida et al., 1994; Lee et al., 1995; Heinen et al., 2000; Heinen et al., 2001a; Heinen et al., 2001b). The early reports on carriers may therefore be questioned and it seems reasonable to assume that virus persistence in individual animals is very unlikely.

In the finishing pigs (>9 weeks of age), influenza virus infections in the APF house also seemed to be associated with virus introduced with the animals instead of later virus introductions through other routes. After all, no infections, and therefore no virus introductions, were found in groups that were apparently virus free from the moment they entered the APF house. The virus seems to be able to keep circulating in the group for a while, after being introduced with the pigs. For that, only a few animals need to become infected at the same time and sufficient numbers of susceptible pigs need to be available each time before infectious animals stop shedding the virus. Such a situation might arise in pig populations with maternal antibodies. Maternal antibodies may last up to an age of at least 16 weeks but this is highly variable (Loeffen et al., 2003b). New susceptible piglets will therefore become available continuously and influenza virus infections may thus go on at a low level until well beyond the first half of the finishing period. This finding is also in accordance with the results of a previous cross-sectional study in weaned piglets, where also very few piglets seemed to seroconvert at any given time (Loeffen et al., 2003b).

Sometimes gaps occurred in the serological profiles, where no influenza virus infections were seen between two periods with influenza virus infections. Although we can not preclude that in some of these cases influenza virus was newly introduced, the most likely explanation is that influenza virus infections were ongoing at a low level and were sometimes missed because only 20% of the pigs in a group was sampled.

Reducing the incidence of influenza virus infections will eventually lead to fully susceptible groups of pigs. Infections in fully susceptible finishing pigs may result in severe growth retardation. However, without complications, these pigs seem to be able to recover very quickly and even compensate for their lost growth (Loeffen et al., 2003a). Overall, reducing the incidence of influenza virus infection, mainly by reducing the number of introductions, seems therefore favourable to a situation of unlimited influenza virus infections during the whole rearing and finishing period, especially if complications, like chronic respiratory disease by secondary infectious agents, are likely to occur. During this project, only one outbreak of respiratory disease was noticed that may have been due to an infection with influenza virus subtype H1N1. Therefore, no conclusion can be drawn on how the implemented measures affect the occurrence of respiratory disease, or related economic losses.

Because most of the reduction of the incidence of the influenza virus infections seems to be in the finishing period, it may be enough to implement the investigated measures only on houses for finishing pigs. Even so, implementing these measures in a pig house and keeping it operational requires not only a certain investment, but also increases operational costs. It was calculated that for this specific case the total costs could be compensated for if for instance the overall growth performance and feed conversion ratio improved by approximately 10% (or similar gains by reduced mortality and/or veterinary costs). For farms with high economic losses due to recurrent influenza virus infections, zoosanitary measures like this may be considered as a good alternative to for instance vaccination.

Several measures were taken at the same time, but may not all contribute as much towards reducing the incidence of influenza virus infections. Further research could

focus on the role of these single measures. That way it would be possible to better evaluate for which measures the costs would be proportionate to the benefits.

The advantage of zoosanitary measures compared to vaccination is that introductions and thus the incidence of infections with multiple infectious agents can be reduced at the same time. Investment into measures like these could then be even more beneficial. They could for instance also be used to preserve SPF herds in regions with a high pig density. Further investigations should, however, be carried out to find out to what extent the measures applied here would be efficient for other (respiratory) diseases as well.

References

Barigazzi, G., Donatelli, I., 2003, Swine influenza in Italy. Vet Res Commun 27 Suppl 1, 93-99.

Blaskovic, D., Jamrichová, O., Rathová, V., Kaplan, M.M., Kocisková, D., 1970, Experimental infection of weanling pigs with A/swine influenza virus. 2. The shedding of virus by infected animals. Bull.World Health Organ. 42, 767-770.

Blocks, G.H.M., Vernooy, J.C.M., Verheijden, J.H.M., 1994, Integrated Quality-Control Project - Relationships Between Pathological Findings Detected at the Slaughterhouse and Information Gathered in a Veterinary Health Scheme at Pig Farms. Veterinary Quarterly 16, 123-127.

Brown, I.H., Alexander, D.J., Chakraverty, P., Harris, P.A., Manvell, R.J., 1994, Isolation of an influenza A virus of unusual subtype (H1N7) from pigs in England, and the subsequent experimental transmission from pig to pig. Vet.Microbiol. 39, 125-134.

Brown, I.H., Done, S.H., Spencer, Y.I., Cooley, W.A., Harris, P.A., Alexander, D.J., 1993, Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. Vet.Rec. 132, 598-602.

Choi, Y.K., Goyal, S.M., Joo, H.S., 2003, Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. Can Vet J 44, 735-737.

Easterday, B.C., 1971, Influenza virus infection of the suckling pig. Acta Veterinaria Brno 40 Suppl 2, 33-42.

Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1990, Sero-epidemiological screening of pig sera collected at the slaughterhouse to detect herds infected with Aujeszky's disease virus, porcine influenza virus and Actinobacillus (Haemophilus) pleuropneumoniae in the framework of an integrated quality control (IQC) system. Vet.Q. 12, 221-230.

Ewald, C., Heer, A., Havenith, U., 1994, Factors Associated with Influenza-A Virus-Infections in Finishing Pigs. Berliner und Munchener Tierarztliche Wochenschrift 107, 256-262.

Groschup, M.H., Brun, A., Haas, B., 1993, Serological studies on the potential synergism of porcine reproductive and respiratory syndrome virus and influenza-, corona- and paramyxoviruses in the induction of respiratory symptoms in swine. Zentralbl.Veterinarmed.[B] 40, 681-689.

Haesebrouck, F., Pensaert, M., Wyffels, R., 1987, Vaccination of swine against H3N2-influenza field isolates using the human Philippines-strain. Vet.Q. 9, 9-14.

Heinen, P.P., Boer-Luijtze, E.A., Bianchi, A.T., 2001a, Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. J.Gen.Virol. 82, 2697-2707.

Heinen, P.P., Van Nieuwstadt, A.P., Boer-Luijtze, E.A., Bianchi, A.T., 2001b, Analysis of the quality of protection induced by a porcine influenza A vaccine to challenge with an H3N2 virus. Vet.Immunol.Immunopathol. 82, 39-56.

Heinen, P.P., Van Nieuwstadt, A.P., Pol, J.M.A., De Boer-Luijtze, E.A., Van Oirschot, J.T., Bianchi, A.T.J., 2000, Systemic and mucosal isotype-specific antibody responses in pigs to experimental influenza virus infection. Viral Immunol. 13, 237-247.

Kendal, A.P., Skehel, J.J., Pereira, M.S., 1982, Concepts and procedures for laboratory-based influenza surveillance. US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta.

Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K.F., Kawaoka, Y., Webster, R.G., 1994, Potential for transmission of avian influenza viruses to pigs. Journal of General Virology 75, 2183-2188.

Kim, W.I., Wu, W.H., Janke, B., Yoon, K.J., 2006, Characterization of the humoral immune response of experimentally infected and vaccinated pigs to swine influenza viral proteins. Arch Virol 151, 23-36.
Kundin, W.D., Easterday, B.C., 1972, Hong Kong influenza infection in swine: experimental and field observations. Bulletin of the World Health Organization 47, 489-491.
Lee, B.W., Bey, R.F., Baarsch, M.J., Larson, M.E., 1995, Class specific antibody response to influenza A H1N1 infection in swine. Vet.Microbiol. 43, 241-250.
Loeffen, W.L., Heinen, P.P., Bianchi, A.T., Hunneman, W.A., Verheijden, J.H., 2003a, Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. Vet Immunol Immunopathol 92, 23-35.
Loeffen, W.L.A., Kamp, E.M., Stockhofe-Zurwieden, N., Van Nieuwstadt, A.P.K.M.I., Bongers, J.H., Hunneman, W.A., Elbers, A.R.W., Baars, J., Nell, T., Van Zijderveld, F.G., 1999, Survey of infectious agents involved in acute respiratory disease in finishing pigs. Vet.Rec. 145, 123-129.
Loeffen, W.L.A., Nodelijk, G., Heinen, P.P., Van Leengoed, L.A., Hunneman, W.A., Verheijden, J.H., 2003b, Estimating the incidence of influenza-virus infections in Dutch weaned piglets using blood samples from a cross-sectional study. Vet.Microbiol. 91, 295-308.
Maes, D., Deluyker, H., Verdonck, M., Castryck, F., Miry, C., Vrijens, B., De Kruif, A., 2000, Herd factors associated with the seroprevalences of four major respiratory pathogens in slaughter pigs from farrow-to-finish pig herds. Vet Res 31, 313-327.
Mensik, J., Valicek, L., 1969, Pathogenesis of experimental influenza in sucking piglets. 2. Persistence of virus in the lungs and in organs outside the respiratory tract. Zentralbl.Veterinarmed.[B] 16, 808-817.
Van Reeth, K., Labarque, G., De Clercq, S., Pensaert, M., 2001, Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection. Vaccine 19, 4479-4486.
Van Reeth, K., Van Gucht, S., Pensaert, M., 2003, Investigations of the efficacy of European H1N1- and H3N2-based swine influenza vaccines against the novel H1N2 subtype. Vet Rec 153, 9-13.
Wallace, G.D., Elm, J.L., Jr., 1979, Transplacental transmission and neonatal infection with swine influenza virus (Hsw1N1) in swine. Am.J.Vet.Res. 40, 1169-1172.

Chapter 6

Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus

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Abstract

The aim of this study was to determine the role of maternally derived antibodies (MDA) against an influenza H1N1 virus in the clinical protection of piglets and especially their effect on the development of the active immunity after an infection with a homologous influenza H1N1 virus. Twenty piglets with MDA and 10 piglets without MDA were housed together and inoculated twice with influenza H1N1 virus, at 7 and 15 weeks of age. Nine piglets without MDA were added to these groups at 12 weeks of age to be inoculated at 15 weeks of age only. Clinical signs, body temperature, growth performance, virus excretion, antibody responses, and influenza-specific T-cell response were monitored. It was shown that MDA protect piglets against the clinical consequences of a primary influenza infection, but that this protection is not complete. A short but significant rise in body temperature was observed and growth seemed to be inhibited due to the infection. Piglets with MDA shed virus for a longer period after an infection than piglets without MDA. Piglets with and without MDA were protected against the clinical consequences of a secondary infection. However, both after primary and secondary infection significant differences in immune responses were observed that indicated that pigs with MDA developed a weaker immunity than pigs without MDA. Furthermore, overall growth performances from weaning to slaughter show a trend in favour of pigs without maternal antibodies, compared to pigs with maternal antibodies, mainly caused by a significant better performance in the second half of the finishing period. The results of this study provide us insight in the role of MDA in clinical protection and their influence on active immunity after an influenza virus infection of pigs. Furthermore, it leads us to the discussion about the profitability of massive sow herd vaccinations in an attempt to increase MDA levels in piglets, taking into account the overall performance of these piglets and the possible effects on antigenic drift.

Introduction

In 1995-1996 a field survey was carried out in swine in The Netherlands to examine which infectious agents were involved in acute respiratory disease in finishing pigs (Loeffen et al., 1999). Influenza virus turned out to be the most important agent involved. It was isolated from 9 out of 16 outbreaks of acute respiratory disease. In seven of these outbreaks, among finishing pigs 15-20 weeks old, no other infectious

agents were consistently isolated from all investigated pigs, indicating that influenza virus was the primary agent related to the clinical signs. The important role of influenza virus in acute respiratory disease was confirmed in a follow-up study of another 37 outbreaks (Loeffen, unpublished data).

In outbreaks not related to influenza but occurring at a comparable age, haemagglutinating inhibiting (HI) antibodies against one subtype of influenza virus or even against both H1 and H3 were often already present. These antibodies must have been the result of infections earlier in life. The absence of clinical signs related to these infections suggests that many influenza infections remain subclinical but do result in immunity at least for the rest of the finishing period. This is also in agreement with studies in which high seroprevalences were observed in finishing pigs, even in herds without a history of respiratory disease (Elbers et al., 1990; Elbers et al., 1992; Van Reeth and Pensaert, 1994; Maes et al., 1999, 2000). Protection by maternally derived antibodies (MDA) during early infections may have accounted for the absence of clinical signs.

Studies from the early 70s show that MDA protect up to a certain level against clinical disease after infection (Blaskovic et al., 1970) although other studies find no protection (Mensik et al., 1971a, 1971b) or a level of protection depending on the level of MDA (Renshaw, 1975). Besides differences in levels of MDA, the age at the moment of infection or the influenza strain used may also have influenced the results of these studies. There is a certain consensus about the fact that influenza infections in the presence of MDA do not cause any rise in HI antibody titres (Blaskovic et al., 1970; Mensik and Pokorny, 1971; Mensik et al., 1971b; Renshaw, 1975).

The aim of this study was to determine the role of MDA in the clinical protection of piglets and especially their effect on the development of active immunity against a homologous influenza H1N1 virus. For this, virus excretion, HI antibody titres, IgM, IgA, IgG titres against influenza nucleoprotein, and influenza-specific T-cell responses after both the primary and secondary inoculation were analysed in pigs with and without MDA.

Materials & methods

Housing

The experiment took place in a small, conventional pig stable. The stable consisted of two identical compartments (A and B) that were completely separated from each other, including a separate air flow. Each compartment consisted of five separate pens that could house either one sow with piglets or up to nine finishing pigs. Pens were separated with fully closed dividing boards and no direct contact between pigs from adjacent pens was possible. In another part of the building, accessible only through another outside door and separate hygiene lock, a small compartment (C) with only one pen and different climate control and light regime was available to temporarily separate 10 piglets from the other piglets in compartments A and B.

Feeding

All piglets had free and unlimited access to feed and water. Two consecutive feeds were used: a diet feed for weaned piglets that was given between the age of 2 and 10 weeks (initially as a supplement to the mother's milk) and a general feed for finishing pigs, that was given from the age of 10 weeks until slaughter. Both feeds were regular and commercially available pelleted feed-mixtures without any additional supplements. The transition of the first feed to the second took 2 weeks, in which gradually a higher percentage of the second feed was given.

Experimental pigs

For the experiment 40 piglets were needed: 20 with maternal antibodies against the same H1N1 influenza strain they would be inoculated with, and 20 without maternal antibodies. To obtain these piglets, eight pregnant sows were bought from a conventional sow herd in The Netherlands. This herd had been closed for over 8 years, without any import of animals and was situated in a region with a low pig density. Serological testing of the herd showed that no influenza infections had occurred in the last 2.5 years because all sows under that age were seronegative for both subtypes present in The Netherlands (H1N1 and H3N2). All eight sows were tested again individually, both in a haemagglutination inhibition (HI) assay as well as in an ELISA for influenza nucleoprotein-specific IgG, just before they were transported to the experimental facilities.

Five of the eight sows were transported to the experimental facilities 7 weeks before farrowing and housed in compartment B (Fig. 1). They were allowed to acclimatise for 1 week and were then inoculated with an influenza virus, subtype H1N1. The remaining three sows were transported to the experimental facilities 10 days before farrowing and were housed in compartment A.

All sows farrowed within a period of 2 days. Piglets received an injection with iron and penicillin on day 3. Boars were not castrated. At 4 weeks of age, piglets were weaned by removing the sows. Blood samples from all sows and piglets in both compartments were collected at the same day and tested for antibodies against influenza subtypes H1N1 and H3N2. At 6 weeks of age all test results were available and 40 piglets were assigned to the experimental groups. Piglets showing signs of disease were excluded from the experiment. All piglets not assigned to any of the groups were removed from the stable.

Twenty piglets with the highest levels of maternal antibodies from compartment B were selected and designated group MDA+7 (MDA positive, first inoculation at 7 weeks of age). Ten piglets were randomly selected from compartment A and were designated group MDA-7 (MDA negative, first inoculation at 7 weeks of age). Ten other piglets were randomly selected from the remaining piglets in compartment A and were designated group MDA-15 (MDA negative, first inoculation at 15 weeks of age).

Piglets from groups MDA+7 and MDA-7 were randomly (although stratified by sex as much as possible) assigned to the 10 available pens in compartments A and B, resulting in one piglet of group MDA-7 and two piglets of group MDA+7 in each pen. By allocating pigs this way, effects of pen or compartment are for the most part eliminated

in comparisons between the experimental groups. Piglets from group MDA-15 were relocated to compartment C, where they were kept separated from groups MDA-7 and MDA+7 for the next 6 weeks. No contact with these piglets in compartment C was allowed for any person, unless at least 12 h had passed since their last contact with other pigs (including those in compartments A and B), and they had showered and changed to clean clothing. Separate clothing, boots, feed storage and other supplies were used for these piglets. These measures were carried out to prevent these piglets from any infection with an influenza virus, including spread of the inoculation strain to compartment C. The same hygienic measures were applied for compartments A and B although previous contact with pigs in compartment C was allowed.

Groups MDA+7 and MDA-7 were inoculated with an influenza virus, subtype H1N1, for the first time (day 0) when they were 7 weeks of age. Piglets from group MDA-15, housed in compartment C, were tested for antibodies against both influenza subtypes when they were 11 weeks of age. They all tested seronegative and at 12 weeks of age they were regrouped with groups MDA+7 and MDA-7 by randomly assigning one piglet

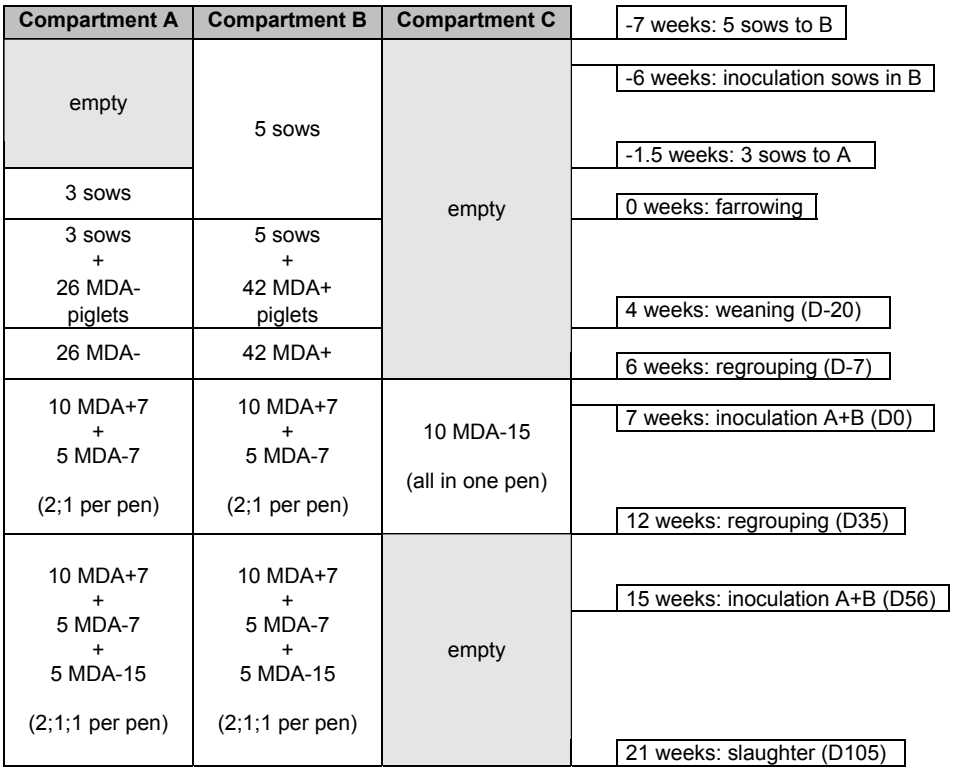

Figure 1. Schematic and chronological outline of the experiment, showing main events with regard to farrowing (weeks) and 1st inoculation (D-20 until D105)

to each pen in compartments A and B. Because one of the piglets in compartment C had suddenly died, one pen contained only three piglets, while the other all contained four piglets. Cause of death was unrelated to the experiment (Mulberry heart disease).

All pigs were inoculated with an influenza virus, subtype H1N1, when they were 15 weeks of age (day 56), either for the first time (group MDA-15) or for the second time (groups MDA+7 and MDA-7).

Virus and experimental inoculation

The influenza strain A/swine/Neth/Best/96 (H1N1) was isolated form pneumonic lung tissue of a pig from an outbreak of influenza during a recent field survey (Loeffen et al., 1999). The virus was isolated on secondary porcine thyroid cells and passaged three times in these cells, after which it was passaged twice on Madin Darby canine kidney (MDCK) cells. A virus stock containing 10^8 $TCID_{50}$/ml was produced as described before (Heinen et al., 2000) and stored at -70°C. This same batch was used for inoculation of the sows, the piglets at 7 weeks of age (day 0) and the pigs at 15 weeks of age (day 56). All pigs were inoculated into the nostrils with an aerosol produced by nebulization of 1.5 ml of the culture, using an airbrush device (Badger, No.: 100LG, Franklin Park, IL, USA).

Clinical observations and sampling

Clinical observations and measurements of rectal body temperatures were carried out from day 1-8 and from day 55-67. On the day of the inoculation and the following 4 days, pigs were observed twice a day, on the other days only once. Clinical scoring was carried out by one and the same veterinarian during the whole experiment. This veterinarian did not know to which group each pig belonged at the time he was scoring them. Pigs were always observed and scored at rest, before body temperatures were measured. The clinical signs that were scored were: overall activity, breathing frequency, abdominal breathing, and coughing (Table 1). Scores for each observation were accumulated to obtain a clinical score for each moment, ranging from 0 to 7. Pigs were weighed at 4 weeks of age (day 20, at the time of weaning), and again at day 0, 7, 21, 56, 63, 77 and 105.

Pharyngeal swabs for virus isolation were taken on day 1, 2, 4, 6, 8, and 10, as well as on day 57, 58, 60, 62 and 64. Multi layered gauze dressings in a pair of tweezers were used to scrape the dorsal pharyngeal wall, behind the soft palate. Tools used for sampling were disinfected, rinsed with water and dried between sampling each pig. Swabs were stored in 2 ml Eagle's minimal essential medium (EMEM) (Flow Laboratories) at -70°C until analysis.

Nasal swabs for detection of IgA antibodies and blood samples for detection of IgM, IgG and HI antibodies were taken on day -20 (HI assay only), 3, 4, 7, 14, 21, 28, 53, 60, 63, 70, 77, 84, and 105. Nasal swabs were stored in 1.5 ml EMEM at -70°C and serum samples at -20°C until all samples were analysed at the same time. Heparinised blood samples for the T-cell proliferation test were taken on day -3, 7, 14, 53, 60, 63, 70, and 84. These samples were transported to the laboratory for immediate analysis.

Table 1: Scores for clinical signs in pigs, experimentally infected with an influenza H1N1 virus. For a total clinical score all scores per topic are accumulated.

Topic	Scores	Description
Activity	0	Active, alert
	1	Diminished activity, not fully alert, gets up after little urge, but tends to lie down again after a moment
	2	Apathy, unwilling to get up, immediately lying down after getting up under heavy pressure
Breathing frequency	0	Normal
	1	Slightly elevated
	2	Clearly elevated
Abdominal breathing	0	Normal
	1	Slight abdominal breathing
	2	Distinct abdominal breathing, jerking
Coughing	0	Absent
	1	Present, at rest or during exertion

Virus isolation

Oropharyngeal swab samples were centrifuged at 1000 g for 10 min. Ten-fold serial dilutions of the supernatant were prepared in cell culture infection medium (McCoy's medium without serum, supplemented with 5 mg/ml trypsin). Dilutions were inoculated on MDCK cells in microtitre plates, which were incubated at 37°C and examined for a cytopathic effect after 4 days. Of the samples that were negative in the microtitre assay, 1 ml was tested for the presence of virus by inoculating a monolayer in 25 ml tissue culture flasks. Virus titres were calculated by the method of Spearman-Kärber.

Haemagglutination inhibition (HI) assay

The HI assay detects antibodies against the haemagglutinin (HA) and was performed essentially as described previously (Kendal et al., 1982), using 0.5% chicken erythrocytes for haemagglutination and strains A/swine/Neth/Best/96 (H1N1) and A/swine/Neth/St Oedenrode/96 (H3N2) to test for antibodies against H1 and H3,

respectively. All sera were tested in serial twofold dilutions, starting at 1:9. Titres ≥18 were considered positive.

Nucleoprotein (NP)-specific and isotype-specific ELISAs

The ELISAs to measure influenza NP-specific IgM, IgG, and IgA antibodies in pigs were described recently (Heinen et al., 2000). An antibody capture assay (ACA) format of the ELISA was used to detect IgM and IgA. In short, ELISA plates (Costar EIA/RIA, Cat. no. 3590, Costar, USA) were coated with monoclonal antibodies (MAbs) specific for porcine IgM (28.4.1) or IgA (27.9.1b). Subsequently, they were incubated with three-fold serial dilutions of serum samples or nasal swab samples, antigen (complete virus disrupted in 1% NP-40 in phosphate-buffered saline (PBS)), a horseradish peroxidase (HRPO) conjugated MAb (ATCC No.: HB 65, H16-L10-4R5) against the NP of influenza virus, and a chromogen/substrate solution.

An indirect double antibody sandwich (IDAS) format of the ELISA was used for the detection of specific IgG. Plates were coated with the MAb against the NP. Subsequently, they were incubated with antigen, 3-fold serial dilutions of serum samples, a MAb against IgG (23.49.1) conjugated to HRPO, and chromogen/substrate solution.

The absorbance at 450 nm was read with an ELISA reader (Spectra Reader, SLT labinstruments, Salzburg, Austria), and antibody titres were expressed as the reciprocal of the sample dilution still giving an optical density (OD) value of 1.0. In each ELISA, positive control samples obtained from a previous influenza infection experiment were included to enable correction for plate differences. However, differences in titres of control samples on different plates were never more than one dilution step. Therefore, no correction was needed for any of the titres measured.

T-cell proliferation assay

The T-cell proliferation assay to measure influenza-specific T-cell responses of pigs was developed essentially as described for pseudorabies virus (Kimman et al., 1995). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinised blood samples by centrifugation onto Lymphoprep (Nycomed Pharma A., Oslo, Norway), and were washed twice with PBS. The isolated PBMC were seeded in 96-well flat-bottom plates (M29, Greiner, The Netherlands) at a density of $5*10^5$ cells per well in 100 µl medium (RPMI 1640 containing 10% porcine serum (a pool from 2 euthanised SPF pigs that did not have antibodies to influenza)), 2 mM L-glutamine, 50 µM ß-mercaptoethanol, 200 U/ml penicillin, 200 µg/ml streptomycin, and 100 U/ml mycostatin). To the PBMC, 100 µl medium containing 400 haemagglutinating units and $5*10^5$ $TCID_{50}$ of influenza A/swine/Neth/Best/96, a control sample prepared from non-infected cells (mock control) or 5 µg/ml of Con A (vitality control) were added in quadruplicate. After 4 days incubation at 37°C in a 5% CO_2 atmosphere, the cultures were pulsed with 0.4 µCi [3H]-thymidine (Amersham, The Netherlands). After 4 h of incubation, cells were harvested and the incorporated radioactivity was measured in a Betaplate scintillation counter (Wallac, EG&G Instruments, The Netherlands). Proliferation was expressed as the number of counts (mean of quadruplicate) of

influenza-stimulated PBMC minus the number of counts of the mock control-stimulated PBMC (delta counts).

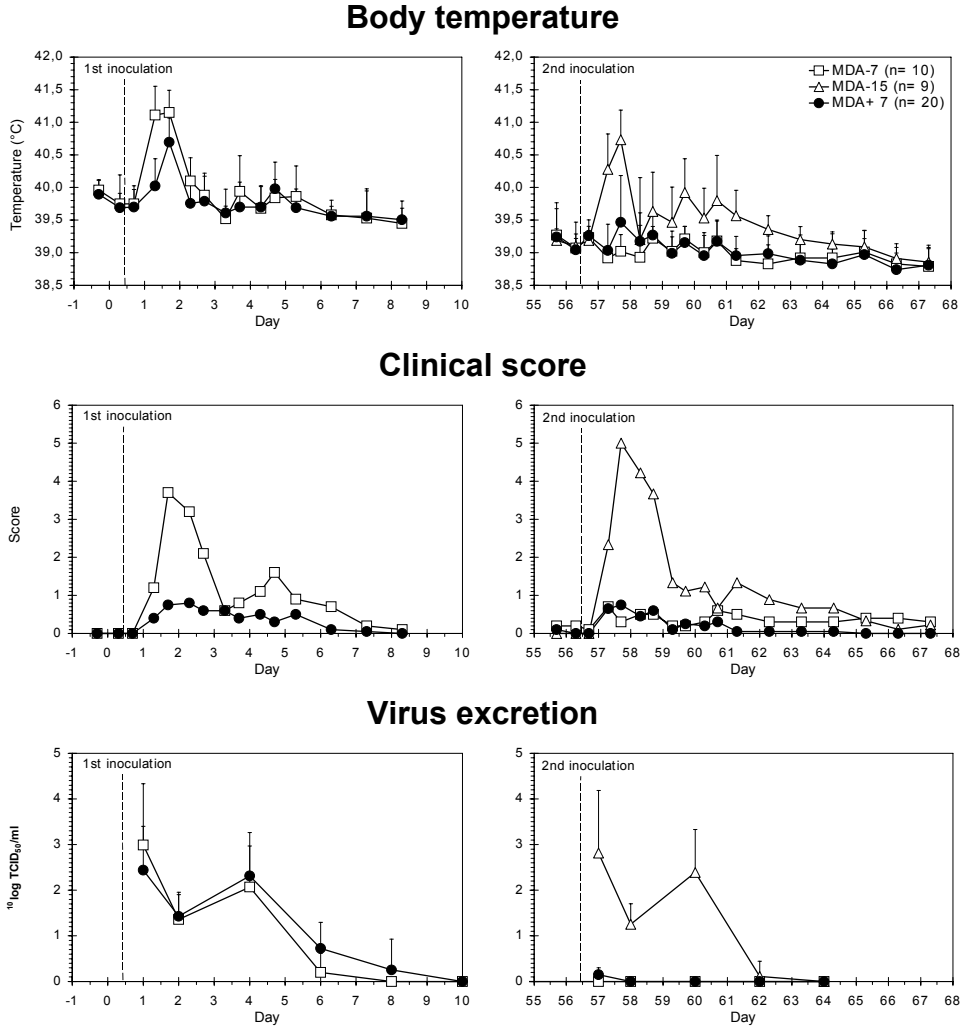

Figure 2. Mean clinical scores, body temperatures, and virus titres in pharyngeal swabs, after inoculation of pigs at the age of 7 and 15 weeks with an influenza H1N1 virus. Pigs were either MDA positive (MDA+7) or MDA negative (MDA-7 and MDA-15). At the age of 15 weeks, group MDA-15 was inoculated for the first time, while the other two groups were inoculated for the second time with the same influenza virus strain. For body temperature and virus excretion, error bars represent standard deviations.

Statistics

Comparisons of growth rates, body temperatures, virus isolation, IgM, IgA, IgG, HI, and T-cell proliferation in all three experimental groups were evaluated in a analysis of variance with pen as a block variable, allowing for comparison of treatment within blocks. Comparisons of clinical scores were evaluated by a non-parametric analysis on ranks (Wilcoxon rank-sum test). All comparisons were made by comparing two groups at a time.

Results

Clinical signs

The first clinical signs were noticed within 24 h after inoculation and the clinical score peaked at approximately 30 h after the inoculation (Fig. 2). Only minor or no signs were noticed in groups MDA+7 after both inoculations and MDA-7 after the secondary inoculation at 15 weeks of age. The course of the clinical signs was similar after primary inoculation at 7 or 15 weeks of age in groups MDA-7 and MDA-15, respectively. However, the peak score in group MDA-15 seemed a little higher, indicating slightly more severe clinical signs. In general, coughing was a rare symptom, noticed in a few pigs and for a few days only.

Body temperature curves showed a course similar to that of the clinical score. In groups MDA-7 and MDA-15 after primary inoculation at 7 and 15 weeks of age, respectively, a rise of body temperature was noticed within 24 h after the inoculation and peaked at approximately 30 h after the inoculation (Fig. 2). A second peak, much lower but lasting longer, was observed, most notably in group MDA-15, after the inoculation at 15 weeks. Piglets from group MDA+7 showed a very short rise in body temperature approximately 30 h after the inoculation at 7 weeks of age (Fig. 2). On the other hand, most pigs with antibodies due to a previous inoculation showed no increase of body temperature at all after the secondary inoculation at 15 weeks of age. Note that before the second inoculation, body temperatures are lower than before the first inoculation, which is a physiological age effect.

In the first week after the primary inoculation at 7 weeks of age, growth rates in the MDA-7 group were significantly lower than in the MDA+7 group (Table 2). In the second and third week however, there was some compensation in the MDA-7 group and overall, growth performance from day 0-21 was not significantly different between both groups. Much higher growth rates between day 7 and 21 suggested that the first inoculation also reduced the growth rate in group MDA+7 in the first week after inoculation.

In the first week after the inoculation at 15 weeks of age, growth rates in the primary inoculated group MDA-15, were severely reduced, compared to the secondary inoculated groups MDA+7 and MDA-7 (Table 2). Again, in the second and third week after inoculation, growth rates were partly compensated. Both groups MDA+7 and MDA-7 showed a minor reduction in growth during the first week after secondary inoculation, compared to growth rates right before and after that week.

Right before slaughter, at day 105 of the experiment, pigs from group MDA-7 were significantly heavier than pigs from group MDA+7 (Table 3). In the overall growth performance from weaning until slaughter (day -20-105) there was a trend in favour of group MDA-7 (P=0.07), mainly caused by a significantly higher growth rate between day 56 and 105, after the second inoculation (P=0.02). Group MDA-15, although included in Table 3, was not compared to the other two groups because these pigs were kept under different circumstances in compartment C between the age of 6 and 12 weeks, which may have had an impact on their overall growth performance.

Virus isolation

After the primary inoculation at 7 weeks of age, their was no significant difference between groups MDA+7 and MDA-7 in virus isolation from pharyngeal swabs during the first 4 days (Fig. 2). However, piglets with MDA excreted virus longer than pigs without MDA. On day 6 post-inoculation virus could be isolated from only 2 out of 10 piglets from group MDA-7, against 13 out of 20 piglets from group MDA+7. After the secondary inoculation at 15 weeks of age, low amounts of virus were isolated from only 3 pigs

Table 2. Growth performances in pigs, experimentally infected with an influenza H1N1 virus, before and after inoculations at 7 (D0) and 15 (D56) weeks of age (group MDA 15 only inoculated at the age of 15 weeks). Different letters in a columns are significant differences between values (P<0.05).

Group	n	Growth performance after inoculation at 7 weeks of age							
		D-20/0		D0/7		D7/21		D0/21	
		g/day	SD	g/day	SD	g/day	SD	g/day	SD
MDA+7	20	378[a]	66	513[a]	103	784[a]	95	694[a]	66
MDA-7	10	379[a]	64	397[b]	182	807[a]	129	670[a]	92

Group	n	Growth performance after inoculation at 15 weeks of age									
		D38/56		D21/56		D56/63		D63/77		D56/77	
		g/day	SD	g/day	SD	g/ day	SD	g/day	SD	g/day	SD
MDA+7	20	-	-	818[a]	93	764[a]	160	903[a]	164	856[ab]	148
MDA-7	10	-	-	873[a]	92	825[a]	128	1087[b]	150	975[a]	134
MDA-15	9	912	110	-	-	41[b]	422	1158[b]	255	786[b]	110

n=number of pigs in each group, SD=standard deviation.

from group MDA+7 at day 1 post-inoculation, and no virus was isolated from pigs in group MDA-7. Virus isolation from group MDA-15 was similar to group MDA-7 after the primary inoculation at 7 weeks of age (Fig. 2).

Antibody response

IgM antibody titres were highest at 7 days after inoculation and persisted longer in pigs inoculated at a later age (MDA-7 versus MDA-15, both after primary inoculation) (Fig. 3). IgM antibody titres were almost completely suppressed after inoculation in the presence of either MDA or actively produced antibodies.

The IgG response was initially also inhibited in the presence of MDA (MDA+7 after primary inoculation), but in time, titres in groups MDA+7 and MDA-7 after the primary inoculation became comparable. The secondary inoculation, however, resulted in a

Table 3. Live body weights (at weaning, before second inoculation and before slaughter) and growth performances (weaning to second inoculation, second inoculation to slaughter and overall) in pigs, experimentally infected with an influenza H1N1 virus, once (group MDA-15, at 15 weeks of age) or twice (groups MDA+7 and MDA-7, at 7 and 15 weeks of age). Different letters in a columns are significant differences between values (*P*<0.05). Only groups MDA+7 and MDA-7 were compared for significant differences (see text for explanation).

Group	n	Live body weight					
		Day -20		Day 56		Day 105	
		kg	SD	kg	SD	kg	SD
MDA+7	20	8.7[a]	0.30	59.5[a]	1.1	103.0[a]	2.1
MDA-7	10	9.9[b]	0.74	62.1[a]	1.4	112.2[b]	2.9
MDA-15	9	9.8	0.58	65.7	1.8	109.9	2.6

Group	n	Growth performance					
		D-20/56		D56/105		D-20/105	
		g/day	SD	g/day	SD	g/day	SD
MDA+7	20	668[a]	62	887[a]	130	754[a]	76
MDA-7	10	687[a]	63	1022[b]	123	818[a]	81
MDA-15	9	735	61	901	135	800	66

n=number of pigs in each group, SD=standard deviation

booster effect in group MDA+7, but not in group MDA-7. The IgG response in group MDA-15 was comparable to that in group MDA-7 after the primary inoculation at 7 weeks of age (Fig. 3).

The IgA response was also suppressed after inoculation in the presence of MDA. However, when the same pigs (MDA+7) were inoculated again, the IgA response was faster than in naive pigs (MDA-15) (Fig. 3), although the antibodies were detectable for a shorter period of time.

The formation of HI antibodies was almost completely suppressed after inoculation in the presence of MDA. However, secondary inoculation at 15 weeks of age in that group (MDA+7) resulted in antibody titres significantly higher than in the other two groups (Fig. 3).

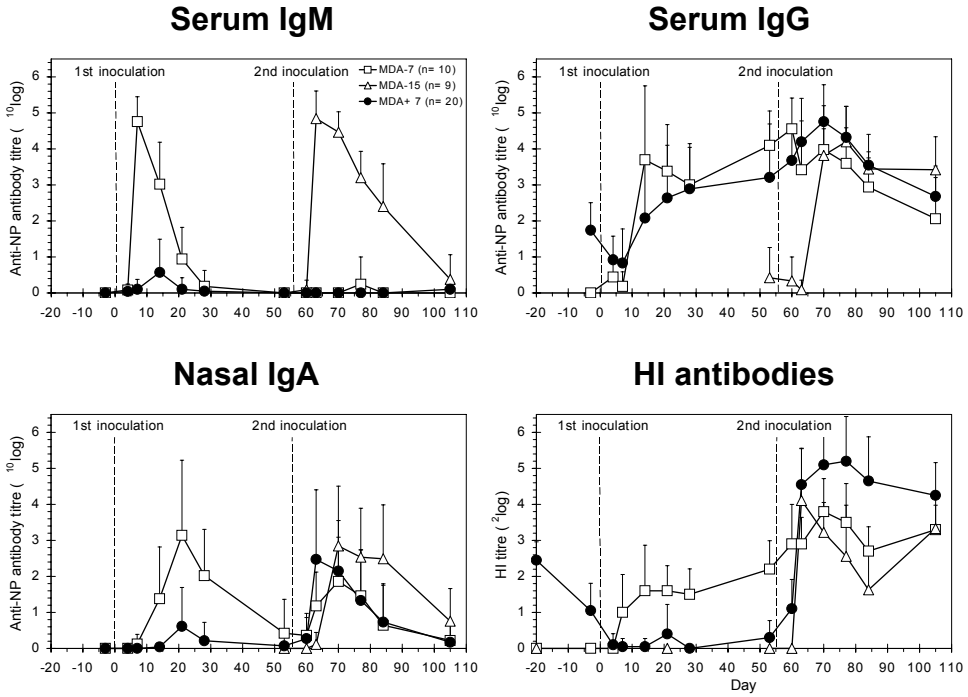

Figure 3. Mean influenza nucleoprotein-specific serum IgM, serum IgG, and nasal IgA, and mean serum HI antibody titres after inoculation of pigs at 7 and 15 weeks old with an influenza H1N1 virus. Groups MDA+7 and MDA-7 were MDA positive and MDA negative respectively and were inoculated with the same influenza virus strain at 7 and 15 weeks of age. Group MDA-15 was MDA negative and was inoculated at 15 weeks of age only. Error bars represent standard deviations.

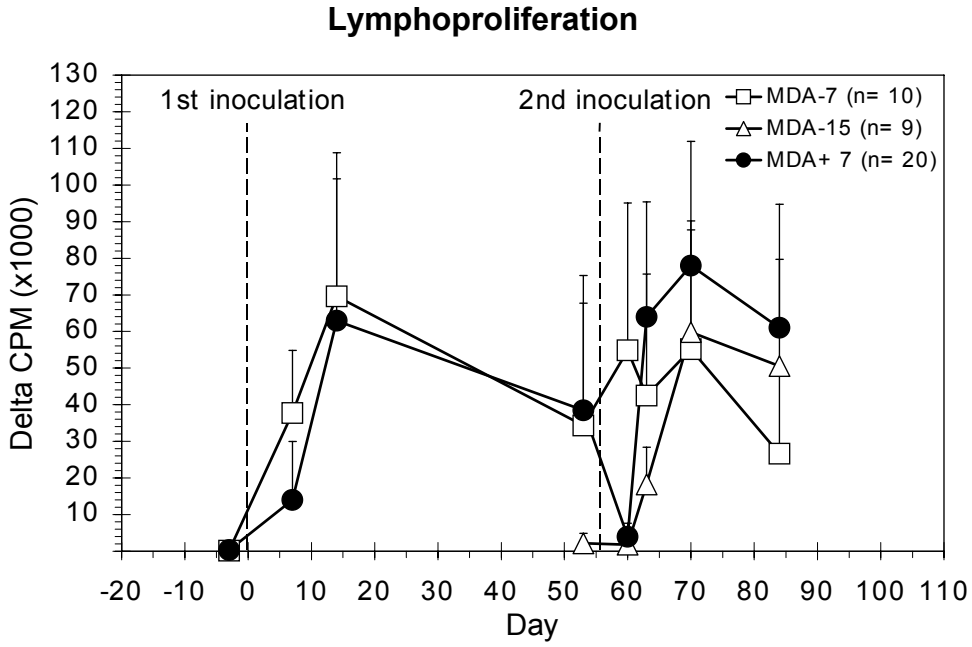

Figure 4. Mean influenza-specific lymphoproliferation response of PBMC after inoculation of pigs at 7 and 15 weeks old with an influenza H1N1 virus. Groups MDA+7 and MDA-7 were MDA positive and MDA negative respectively and were inoculated with the same influenza virus strain at 7 and 15 weeks of age. Group MDA-15 was MDA negative and was inoculated at 15 weeks of age only. Error bars represent standard deviations.

T-cell response

After the inoculation at 7 weeks of age, an influenza-specific T-cell response of PBMC was measured in groups MDA+7 and MDA-7. Kinetics of the responses were similar, although the early response at day 7 was significantly higher in group MDA-7 than in group MDA+7 (Fig. 4).

After the inoculation at 15 weeks of age, kinetics of the responses were quite different. In group MDA+7 there was a sharp drop 4 days after the inoculations, before it went up to be the highest response of all groups. In group MDA-7 the response remained more consistent, and the response in group MDA-15 was similar to that of group MDA-7 after the primary inoculation at 7 weeks of age.

Discussion

Maternally derived antibodies (MDA) against an influenza H1N1 virus protect piglets against the clinical effects of an influenza infection with the same virus strain. However,

protection is not complete, at least not at the levels of maternal antibodies present at challenge in our study. In piglets partially protected by MDA a small peak of fever was noticed approximately 30 h after the infection. Also, growth rates during the first week after the infection, compared to the 2 weeks following, indicate a minor growth retardation due to the infection. Moreover, the virus excretion was not significantly reduced by the presence of MDA, but lasted even longer than in piglets without MDA.

MDA mainly consist of IgG antibodies, which predominantly protect the lungs against virus replication and invasion. Local antibodies of the IgA isotype on the other hand are important in the inhibition of virus replication in the upper airways (Ada and Jones, 1986; Renegar and Small, 1991; Underdown and Mestecky, 1994; Tomoda et al., 1995). Observations in the present study confirm the inhibition or at least delay of the active antibody response to primary infection (including IgA, but also IgM, IgG, and HI antibody response) in the presence of MDA. Moreover, MDA not only inhibited the antibody responses, but also affected the proliferative T-cell response after primary infection. Although the response was ultimately comparable to that of piglets without MDA, the response was initially inhibited in the presence of MDA, resulting in a significantly lower response at 7 days after primary infection. The inhibition of the immune response and the inability of the MDA to counter virus replication in the upper airways may explain why the virus is excreted for a longer period in piglets with MDA.

Prolonged virus excretion, as well as an initial reduction of the lymphoproliferation response in piglets with MDA was found in pseudorabies virus infections as well (Bouma et al., 1998). In addition, Renshaw (1975) described several experiments, which suggested that influenza virus excretion from piglets with low levels of MDA lasted at least as long as from MDA-negative piglets. In some of these experiments, higher levels of MDA, however, resulted in a much shorter period of excreting influenza virus. Because these experiments were performed using piglets from different ages and different inoculation protocols, interpretations have to be made with caution, but the level of MDA may be an important factor determining the duration of virus excretion. Unfortunately, we were not able to confirm this, as all MDA positive piglets in our experiment had only low levels of MDA.

The implications of the extended virus excretion in MDA positive piglets are hard to assess. Vaccinating sows, thereby increasing levels and duration of MDA, may not affect virus circulation among piglets. Therefore, the only effect would be a clinical protection of influenza virus infections in young piglets, also resulting in a less than optimal active immunity, as discussed further on. However, transmission of virus is not only affected by virus excretion, but by susceptibility of the host as well. Transmission experiments with pseudorabies virus in MDA positive piglets showed that, possibly depending on the levels of MDA, MDA can significantly inhibit transmission of the virus (Bouma et al., 1997), even though under certain circumstances excretion of virus may be extended compared to piglets without MDA.

Antigenic drift of influenza viruses in pigs is less pronounced then in humans, although limited antigenic drift has been reported to occur for influenza subtypes H1N1 (De Jong et al., 1999) and H3N2 (De Jong et al., 2001). This limited antigenic drift is likely to be caused by immune pressure, either in sows who live long enough to be

infected more than once with the same subtype, or in young piglets in the presence of MDA. Excretion, and, therefore, replication of virus seems not to be inhibited in the presence of MDA. It is, however, conceivable that the extensive replication in the presence of antibodies results in antigenic drift. Transmission experiments wherein influenza virus is passaged several times in MDA-positive piglets and antigenetically compared to the original strain would show the effect of MDA on virus transmission and in how far they can cause antigenic drift. This information would be valuable to assess the effect of massive sow vaccination in order to increase levels and duration of MDA in piglets.

The presence of MDA also inhibited the development of immunity against a secondary influenza infection. The differences in immune responses after the second inoculation at the age of 15 weeks were even more pronounced than after primary inoculation. The stronger HI, IgG, IgA, and ultimately also lymphoproliferation responses in the MDA-positive pigs after the second inoculation, compared to the responses in the MDA-negative pigs, indicate that the immunity of MDA-positive pigs prior to secondary inoculation was weaker. In addition, in the MDA-positive pigs a sharp drop in the proliferative T-cell response was observed 4 days after the secondary inoculation, while the response in MDA-negative pigs remained more or less the same in time. This drop may have resulted from an initial massive relocation of T-cells to the lungs, dramatically lowering the presence of influenza-specific T-cells in the peripheral blood. This relocation could compensate for lower protective antibody levels in the MDA-positive pigs, rather than for a lower level of cell-mediated immunity, because previous studies indicated that MDA do not affect the establishment of cell-mediated immunity (Pertmer et al., 2000; Radu et al., 2001). The possible influx of lymphocytes into the lung was described by Heinen et al. (2001a), who found an increase of lymphocytes in the lungs, especially in piglets with a low immunity. In a second study, a vaccination/challenge experiment, they also found the same dramatic drop of influenza-specific T-cells in the peripheral blood after challenge. In this case the protection of the vaccine was sub-optimal compared to the protection after a previous infection. A relocation of influenza-specific T-cells from the blood to the respiratory tract was suggested here as well (Heinen et al., 2001b).

There was a clear difference between the antibody response to the NP measured in the IgG ELISA and the HI antibody response. The IgG response to the NP in MDA-positive piglets was comparable to the response in MDA-negative piglets, after a primary inoculation. However, the HI antibody response was much lower in MDA-positive piglets. This is consistent with the findings of Pertmer et al. (2000) who demonstrated that the IgG response to the HA, but not the IgG response to the NP was inhibited by MDA. It was suggested that the immune response against HA, embedded in the virus membrane and thus fully accessible to antibodies, would be blocked by maternal antibodies because they neutralise the HA before it is presented to the immune system. On the other hand maternal antibodies would not block the response to the NP because they have little access to NP, which is either shielded by the viral lipid membrane or inside the infected host cell. However, it is also possible that the strong immunogenicity is an intrinsic characteristic of the NP. Because antibodies to the

NP do not inhibit viral replication, the NP may thus act as a decoy to misdirect the antibody response.

Besides a difference in clinical signs and immune response caused by the presence of MDA, some differences also seemed to be caused by age. Fully susceptible piglets at the age of 7 weeks showed less growth retardation and less severe clinical signs than pigs at the age of 15 weeks. However, because these inoculations were not carried out at the same time, these conclusions can not statistically be accounted for.

In conclusion it can be stated that maternal antibodies do protect piglets against most of the clinical consequences after an influenza virus infection and that this infection also leads to an active immunity, protecting the pig probably at least until the end of the finishing period. This may explain why so many infections in the field are subclinical and pass unnoticed for most farmers. Both clinical protection and development of active immunity, however, are sub-optimal in MDA-positive pigs as several parameters showed. Furthermore, it may be that the overall growth performance in MDA-positive pigs is lower than in MDA-negative pigs in cases where there is repeated contact with influenza virus. In our experiment this difference was mainly caused by a highly significant difference in growth performances in the period from the second inoculation until slaughter (allthough a sow effect, additional to treatment effect, in these differences can not completely be ruled out because of the experimental design). Thus, it remains to be seen whether massive vaccination of sow herds will be profitable in terms of performance of piglets and finishing pigs, to say nothing of the possible effects on antigenic drift and its attendant necessity to update influenza vaccines regularly (De Jong et al., 1999; De Jong et al., 2001). Depending on the rate of transmission of influenza virus among MDA positive piglets, this effect on genetic drift may or may not be a problem, but should certainly be investigated in detail.

References

Ada,G.L., Jones,P.D., 1986. The immune response to influenza infection. Curr. Top. Microbiol. Immunol. 128, 1-54.

Blaskovic,D., Rathová,V., Kocisková,D., Jamrichová,O., Sádecký,E., Kaplan,M.M., 1970. Experimental infection of weanling pigs with A/swine influenza virus. 3. Immunity in piglets farrowed by antibody-bearing dams experimentally infected a year earlier. Bull. World Health Organ. 42, 771-777.

Bouma,A., De Jong,M.C., Kimman,T.G., 1998. The influence of maternal immunity on the development of the in vitro lymphocyte proliferation response against pseudorabies virus in pigs. Res. Vet. Sci. 64 (2), 167-171.

Bouma,A., De Jong,M.C.M., Kimman,T.G., 1997. The influence of maternal immunity on the transmission of pseudorabies virus and on the effectiveness of vaccination. Vaccine 15 (3), 287-294.

De Jong,J.C., Heinen,P.P., Loeffen,W.L., Van Nieuwstadt,A.P., Claas,E.C., Bestebroer,T.M., Bijlsma,K., Verweij,C., Osterhaus,A.D., Rimmelzwaan,G.F., Fouchier,R.A., Kimman,T.G., 2001. Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy. Vaccine 19 (31), 4452-4464.

De Jong,J.C., Van Nieuwstadt,A.P., Kimman,T.G., Loeffen,W.L., Bestebroer,T.M., Bijlsma,K., Verweij,C., Osterhaus,A.D., Claas,E.C., 1999. Antigenic drift in swine influenza H3 haemagglutinins with implications for vaccination policy. Vaccine 17 (11-12), 1321-1328.

Elbers,A.R.W., Tielen,M.J.M., Cromwijk,W.A.J., Hunneman,W.A., 1990. Sero-epidemiological screening of pig sera collected at the slaughterhouse to detect herds infected with Aujeszky's disease virus, porcine influenza virus and Actinobacillus (Haemophilus) pleuropneumoniae in the framework of an integrated quality control (IQC) system. Vet. Q. 12 (4), 221-230.

Elbers,A.R.W., Tielen,M.J.M., Cromwijk,W.A.J., Hunneman,W.A., 1992. Variation in seropositivity for some respiratory disease agents in finishing pigs: epidemiological studies on some health parameters and farm and management conditions in the herds. Vet. Q. 14 (1), 8-13.

Heinen,P.P., Boer-Luijtze,E.A., Bianchi,A.T., 2001a. Respiratory and systemic humoral and cellular immune responses of pigs to a heterosubtypic influenza A virus infection. J. Gen. Virol. 82 (11), 2697-2707.

Heinen,P.P., Van Nieuwstadt,A.P., Boer-Luijtze,E.A., Bianchi,A.T., 2001b. Analysis of the quality of protection induced by a porcine influenza A vaccine to challenge with an H3N2 virus. Vet. Immunol. Immunopathol. 82 (1-2), 39-56.

Heinen,P.P., Van Nieuwstadt,A.P., Pol,J.M.A., De Boer-Luijtze,E.A., Van Oirschot,J.T., Bianchi,A.T.J., 2000. Systemic and mucosal isotype-specific antibody responses in pigs to experimental influenza virus infection. Viral Immunol. 13 (2), 237-247.

Kendal,A.P., Skehel,J.J., Pereira,M.S., 1982. Concepts and procedures for laboratory-based influenza surveillance. US Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta

Kimman,T.G., De Bruin,T.M., Voermans,J.J., Peeters,B.P., Bianchi,A.T., 1995. Development and antigen specificity of the lymphoproliferation responses of pigs to pseudorabies virus: dichotomy between secondary B- and T-cell responses. Immunology 86 (3), 372-378.

Loeffen,W.L.A., Kamp,E.M., Stockhofe-Zurwieden,N., Van Nieuwstadt,A.P.K.M.I., Bongers,J.H., Hunneman,W.A., Elbers,A.R.W., Baars,J., Nell,T., Van Zijderveld,F.G., 1999. Survey of infectious agents involved in acute respiratory disease in finishing pigs. Vet. Rec. 145 (5), 123-129.

Maes,D., Deluyker,H., Verdonck,M., Castryck,F., Miry,C., Vrijens,B., De Kruif,A., 1999. Risk indicators for the seroprevalence of Mycoplasma hyopneumoniae, porcine influenza viruses and Aujeszky's disease virus in slaughter pigs from fattening pig herds. Zentralbl. Veterinarmed. [B] 46 (5), 341-352.

Maes,D., Deluyker,H., Verdonck,M., Castryck,F., Miry,C., Vrijens,B., De Kruif,A., 2000. Herd factors associated with the seroprevalences of four major respiratory pathogens in slaughter pigs from farrow-to-finish pig herds. Vet Res 31 (3), 313-327 .

Mensik,J., Pokorny,J., 1971. Development of antibody response to swine influenza virus in pigs. I. The influence of experimental infection of pregnant sows on serum antibody production by their progeny during postnatal development. Zentralbl. Veterinarmed. [B] 18 (3), 177-189.

Mensik,J., Pospisil,Z., Franz,J., Dreslerova,Z., 1971b. Local effect of passively acquired colostral antibody on the development of experimental swine influenza infection in suckling pigs. Zentralbl. Veterinarmed. [B] 18, 804-818.

Mensik,J., Valicek,L., Pospisil,Z., 1971a. Pathogenesis of swine influenza infection produced experimentally in suckling piglets III. Multiplication of virus in the respiratory tract of suckling piglets in the presence of colostrum-derived specific antibody in their blood stream. Zentralbl. Veterinarmed. [B] 18, 665-678.

Pertmer,T.M., Oran,A.E., Moser,J.M., Madorin,C.A., Robinson,H.L., 2000. DNA vaccines for influenza virus: differential effects of maternal antibody on immune responses to hemagglutinin and nucleoprotein. J Virol 74 (17), 7787-7793.

Radu,D.L., Antohi,S., Bot,A., Miller,A., Mirarchi,A., Bona,C., 2001. Effect of maternal antibodies on influenza virus-specific immune response elicited by inactivated virus and naked DNA. Scand J Immunol 53 (5), 475 -482.

Renegar,K.B., Small,P.A., Jr., 1991. Passive transfer of local immunity to influenza virus infection by IgA antibody. J. Immunol. 146 (6), 1972-1978.

Renshaw,H.W., 1975. Influence of antibody-mediated immune suppression on clinical, viral, and immune responses to swine influenza infection. Am. J. Vet. Res. 36 (1), 5-13.

Tomoda,T., Morita,H., Kurashige,T., Maassab,H.F., 1995. Prevention of influenza by the intranasal administration of cold- recombinant, live-attenuated influenza virus vaccine: importance of interferon-gamma production and local IgA response. Vaccine 13 (2), 185 -190.

Underdown,B.J., Mestecky,J., 1994. Mucosal immunoglobulins. In: Ogra,P.L., Strober,W., Mestecky,J., McGhee,J.R., Lamm,M.E. and Bienenstock,J. (Editors), Handbook of mucosal immunology. Academic Press, San Diego. pp. 79-85 .

Van Reeth,K., Pensaert,M., 1994. Prevalence of infections with enzootic respiratory and enteric viruses in feeder pigs entering fattening herds. Vet. Rec. 135, 594-597.

Chapter 7

General discussion

General discussion

In this chapter, the role of influenza virus infections in swine will be discussed in the aetiology of acute respiratory disease. In addition, the findings with respect to the population dynamics of influenza virus will be discussed and the potential of new influenza strains to become endemic in the swine population. This will be concluded with the possible implications of the findings for intervention strategies, such as vaccination or the implementation of zoosanitary measures.

Influenza virus infections in swine

Influenza virus as a cause of respiratory disease

First, sixteen outbreaks of acute respiratory disease were investigated in swine herds in the Netherlands (chapter 2). These outbreaks were meticulously investigated, using virus isolation, isolation of bacteria and mycoplasmas, serology, macroscopic pathology and histopathology. Eight out of these sixteen outbreaks could be attributed to an infection with influenza virus, either subtype H1N1 or H3N2. In a nineth outbreak, an influenza infection occurred as well, although pathological findings indicated that an *A. pleuropneumoniae* infection was the dominant infection. Concurrent infections with other infectious agents occasionally occurred in these outbreaks, but none of these agents was unambiguously correlated with the presence of influenza virus or could be held responsible for clinical symptoms or pathological findings.

This was the first indication that influenza virus infections are responsible for a large portion of the acute respiratory disease outbreaks in swine. However, the number of investigated outbreaks was fairly small. Following the study described in chapter 2, an additional 37 outbreaks of acute respiratory disease were therefore investigated during a three-year period, from 1996 to 1999. The results (not published) were similar to those found in the first study, with 21 out of 37 (57%) investigated outbreaks of acute respiratory disease attributed to an infection with influenza virus subtypes H1N1 or H3N2. Other, more recent, publications also confirm that influenza virus is an important cause of respiratory disease (Barigazzi and Donatelli, 2003; Choi et al., 2003).

Influenza virus subtype H1N2

In 1999 a third subtype of swine influenza virus was found to be endemic in the Netherlands as well (chapter 4). This is an H1N2 strain that is now also endemic in the UK, Belgium, France, Italy, Germany and Spain (Brown et al., 1995; Brown et al., 1998; Van Reeth et al., 2000; Marozin et al., 2002; Schrader and Suss, 2003; Maldonado et al., 2006). Serological screenings at the end of the finishing period showed seroprevalences of 26% and 57% for specialised finishing herds and finishing pigs in farrow-to-finish herds respectively.

In none of the outbreaks from chapter 2, or in the additional investigations between 1996 and 1999, acute respiratory disease could be attributed to influenza virus subtype H1N2. Neuraminidase from all H1-isolates from the first study in chapter 2 was typed and found to be N1 (De Jong et al., 2001). However, during the investigations of the additional 37 herds, the neuraminidase of the isolated influenza viruses was not determined. On the other hand, if H1N2 had been involved, positive virus isolations with a lack of seroconversions against H1N1 would have been found, given the lack of cross-reactivity between antibodies against H1N1 and H1N2 (Van Reeth et al., 2000; Van Reeth et al., 2004; Van Reeth et al., 2006). Because no such observation was made in any of the respiratory outbreaks, the involvement of any H1N2 strain can be ruled out with a high degree of certainty. Probably the H1N2 strain was introduced somewhere between 1996 and 1999 in the Netherlands but not widespread rare during most of this period in which the outbreaks of acute respiratory disease were investigated.

Other influenza virus subtypes in swine

Pigs are considered to be susceptible to influenza viruses of both avian and mammalian origin. This is due to the presence of both types of receptors in the upper airways to which these viruses can bind. Pigs have therefore always been considered possible ‘mixing vessels’, in which concurrent infections with different subtypes of influenza virus may result in new viruses through reassortment. The currently circulating H3N2 and H1N2 subtypes are examples of such a reassortment (Castrucci et al., 1993; Castrucci et al., 1994; Brown et al., 1998). These new viruses could pose a threat to the pig population itself, but also to the human population if a virulent and transmissible new influenza virus were to evolve against which immunity is absent in the human population. Infections with several subtypes of influenza virus have been described in pigs, both in the field and under experimental conditions (Brown et al., 1994; Kida et al., 1994; Karasin et al., 2000; Peiris et al., 2001; Tsai and Pan, 2003; Karasin et al., 2004; Xu et al., 2004; Choi et al., 2005; Ma et al., 2006; Shin et al., 2006; Ma et al., 2007). In 2003, during an outbreak of avian influenza H7N7 in poultry in the Netherlands, H7N7 infections in pigs were detected in several mixed herds (having both poultry and pigs). No clinical symptoms were observed in the pigs. Evidence from the field and transmission experiments suggests that the infections were the result of exposure to infected poultry, with no transmission amongst the pigs themselves (Loeffen et al., 2004). Ongoing exposure to viruses like this may, however, support subsequent adaptation to the new host. Surveillance in pigs, both virological and serological, is therefore indicated, not only to monitor currently prevalent strains, but also to find newly introduced subtypes that may ultimately become a threat for either the pig population itself, or for the human population. New subtypes will, however, always be hard to detect, unless they result in clinical symptoms or can be targeted specifically because of known occurrence in other species in the neighbourhood.

Population dynamics of influenza virus in swine herds

Seroprevalence

In the study described in chapter 4, seroprevalences were measured for Dutch finishing pigs at the end of the finishing period, in regions of the Netherlands with a high pig density. Seroprevalences of approximately 44% and 62% for H1N1, 7% and 19% for H3N2 and 57% and 26% for H1N2 were found for farrow-to-finish and specialised finishing herds respectively. Furthermore, this was the first report of H1N2 seroprevalence in the Netherlands and it showed that this subtype was already widespread and had become endemic in the Dutch pig population in 1999. The seroprevalence of H1N1 was comparable to the levels that were found in finishing pigs in 1980 (44%) (Masurel et al., 1983) and 1987 (62%) (Elbers et al., 1990) in the Netherlands. The seroprevalence found for H3N2 in chapter 4 was, however, much lower than the ones found in those studies (68% in 1980; 33% in 1987). All other seroprevalence studies carried out in Europe in the past two decades also showed much higher seroprevalences (Haesebrouck and Pensaert, 1986b; Yus et al., 1989; Teuffert et al., 1991; Groschup et al., 1993; Ewald et al., 1994). The reason for the low seroprevalence therefore remains unknown.

Population dynamics in weaned piglets

Virus circulation in weaned piglets is very common (chapter 3), although mostly only a limited number of piglets in a group become infected during the weaning period. Thus, at the time these piglets enter the finishing facilities, a high percentage may either be susceptible or soon become susceptible, due to declining levels of maternal antibodies. The half-life of the maternal antibodies is more or less constant (chapter 3). The initial amount of maternal antibodies in a piglet will therefore determine when maternal antibodies ultimately disappear and piglets become susceptible to an influenza virus infection. This, in turn, mainly depends on the immune status of the sow, which can be highly variable, even within a herd. Maternal antibodies may therefore be detectable for only a few weeks in some piglets, but up to an age of 16 weeks in other piglets (Kim et al., 2006). Even within a group of piglets of the same age, susceptible piglets, in low numbers, will thus be available at any given time. As a consequence, ongoing but limited transmission may occur in such a group of piglets. Weaned piglets could under those circumstances serve as a reservoir for influenza virus in a herd.

Chapter 6 shows that infections in the presence of maternal antibodies may result in a lesser immunity than infections in fully susceptible pigs. In these experiments, piglets that were infected in the presence of maternal antibodies showed hardly any clinical symptoms at that time and the infection also led to an active immunity. However, compared to fully immune pigs (previously infected without any maternal antibodies being present), a stronger HI, IgG, IgA, and ultimately also lymphoproliferation response was seen after a second inoculation. Excretion of influenza virus on the other hand was not reduced in piglets with maternal antibodies. Whether this also means that transmission is not reduced under these circumstances is not known. Neither is it clear whether piglets with maternal antibodies could thus also play a role in the population

dynamics of influenza virus. Studies of Aujeszky's disease showed that transmission in the presence of maternal antibodies was reduced, even though virus excretion was not, resulting in a reproduction ratio significantly below 1 (Bouma et al., 1997).

Population dynamics of influenza virus in finishing pigs

The occurrence of influenza virus infections in finishing pigs was quite different in farrow-to finish herds when compared to finishing herds (chapter 4). For all subtypes, influenza virus infections in farrow-to-finish herds occurred mainly at a young age, up to and including the first half of the finishing period. In finishing herds on the other hand, the influenza virus infections mainly occurred in the second half of the finishing period. One possible explanation may be that the exposure of finishing pigs to influenza viruses is different in both herd types. Because of the presence of either sows or weaned piglets as a possible reservoir on the same premises, the virus may be introduced more often through indirect (other than animal-animal) contacts, and on average at a younger age, in a compartment of finishing pigs. This would result in the highest incidence during the early finishing period. In specialised finishing herds on the other hand, the virus may be introduced less frequently in a compartment of finishing pigs, because indirect contact with sows and weaning piglets is far more limited. Introductions may on average occur at a later age and furthermore result in large outbreaks, as a relatively high proportion of the animals will be susceptible by then.

The exact nature of circulation of influenza viruses within herds, and also transmission between herds, could be further investigated with new and more modern tools. Sequence analysis has been used for molecular epidemiology and to establish direct links between infected herds in cases of disease outbreaks. For example, this was recently carried out for avian influenza subtype H5N1 to prove that the presence of this virus in two Nigerian poultry farms was the result of three independent introductions (Ducatez et al., 2006). Whether tools like this turn out to be useful for swine influenza as well, especially to elucidate population dynamics within a single herd, remains to be seen. This would require a sufficiently high mutation rate within a single farm. This will in turn depend on selection pressure and total time frame of the investigation. Furthermore it will depend on the size of the genome part to be sequenced. Estimates of mutation rates from single farms are rare and do not exist yet for swine influenza. For avian influenza in poultry though, the mutation rate within a single farm was high enough to enable molecular epidemiological analyses (Ducatez et al., 2007). In future studies it would therefore also be desirable to collect multiple virus isolates from single swine herds over a period of time, to determine the mutation rates.

The basic techniques for sequencing (Sanger et al., 1977) lead to single consensus sequences only, which may not be enough to draw unambiguous conclusions on timelines of and relations between virus mutations, which form the basis for molecular epidemiological analyses. Techniques such as deep sequencing, allowing for determination of hundreds of sequences and mutations in a population of viruses would result in much more information to determine how viruses are spreading from one place to the other (Shendure et al., 2005; Velicer et al., 2006; Hall, 2007). If classical sequencing techniques for swine influenza are not sufficient, these kinds of techniques

may offer some further prospects to investigate population dynamics of influenza virus in the future.

Implications for intervention

The difference found between finishing pigs in finishing herds and farrow-to-finish herds may have implications for the choice of intervention measures, whether this is intervention through vaccination, or intervention through zoosanitary measures.

Vaccination as an intervention tool

To reduce the economic losses caused by outbreaks of respiratory disease due to influenza virus infections, vaccination is a viable option. Vaccines containing H1N1 and H3N2 antigen are commercially available for use in pigs and have proven to be efficacious under experimental conditions (Haesebrouck and Pensaert, 1986a; Bikour et al., 1996; Heinen et al., 2001; Van Reeth et al., 2001). In general, most farmers and veterinarians are, however, reluctant to use the vaccine, due to the costs and scepticism, justified or not, regarding its efficacy.

Frequent outbreaks of acute respiratory disease in certain herds and the fact that more than 50% of these outbreaks are caused by influenza virus infections (chapter 2) should allow for realistic field trials on the efficacy of vaccination. A follow-up of multiple groups of finishing pigs per herd, both vaccinated and non-vaccinated, should allow for a comparison, both in terms of clinical protection and economic losses. Investigating all outbreaks of respiratory disease in these herds, especially confirming or invalidating the role of influenza virus would also be essential to evaluate the efficacy of the vaccine.

Antigenic drift and shift

One of the reasons why the efficacy of influenza vaccines is often questioned, is the ongoing changes of influenza viruses as a result of antigenic drift and shift (Scholtissek, 1995; Brown, 2000). In contrast to human vaccines, where the virus strains in the vaccine are updated every few years on average, vaccines for use in pigs often contain strains that are several decades old.

Influenza virus strains from subtype H1N1 and H3N2 that were isolated during the first field study (chapter 2) and from subsequently investigated outbreaks were tested for antigenic variation and compared to vaccine strains (De Jong et al., 1999; De Jong et al., 2001). It was shown that cross-reactivity between antibodies against influenza vaccines and recent field strains may be limited and it was suggested that vaccine strains may need to be updated as is regularly done with human influenza vaccines. Nevertheless, it was shown in animal experiments that vaccines containing relatively old influenza virus strains are still able to protect the pigs against clinical symptoms, following a challenge infection with a recent influenza virus strain that under in vitro conditions does not cross-react with the vaccine strain (Heinen et al., 2001; Van Reeth et al., 2001).

The introduction of the H1N2 subtype, which subsequently became endemic, may have resulted in an additional problem. While it also possesses a haemagglutinin of the H1 subtype, cross-reactivity with the current H1N1 subtypes is almost nonexistent. This

has been shown under in-vitro conditions (Van Reeth et al., 2000; Van Reeth et al., 2004; Van Reeth et al., 2006), but was also shown by correlating field samples that contain antibodies against either, or both subtypes (chapter 5). Furthermore, previous infections with either H1N1 or H3N2 did not provide adequate protection against a challenge infection with H1N2 (Van Reeth et al., 2003a). Neither did vaccination with a commercially available vaccine containing H1N1 and H3N2 (Van Reeth et al., 2003b). On the other hand, previous infections with an H1N1 and an H3N2 field strain were able to protect adequately against an H1N2 challenge (Van Reeth et al., 2003a), as did a vaccine supplemented with an H1N2 component (Van Reeth et al., 2003b).

Maternal antibodies

Another reason to question the efficacy of influenza vaccines may be the interference with maternal antibodies (Kitikoon et al., 2006). Under field conditions, pigs will be vaccinated in the presence of variable amounts of maternal antibodies, and vaccination in the presence of maternal antibodies yields a less than optimal immunity and protection against field infections (Kitikoon et al., 2006). Interference of maternal antibodies may thus pose a problem in case of vaccinations in the field and it may be difficult to find the right vaccination moment, and ascertain whether at that time one vaccination will be enough or a double vaccination will be needed. For that, a relationship between maternal antibody titre and the immune response after vaccination should be investigated to determine at what levels of maternal antibodies vaccination is feasible. Due to the variation of maternal antibody titres within a population, it may, however, still be difficult to find the right moment of vaccination.

Perspective of vaccination

In conclusion, while it is clear that vaccination can work under experimental conditions, there are some serious issues that need to be considered before vaccines can confidently be used in the field. For optimal protection it may be necessary to update virus strains in the vaccine on a regular basis. This could be done in the same way as it is done for influenza vaccines for humans. Ongoing surveillance, focusing on antigenic drift and shift, but also on the introduction of new subtypes, is one of the cornerstones of adapting vaccines. This would, however, require at least a representative number of field isolates from a certain population.

Influenza vaccines, even in human medicine, are still very conservative and conventional. Virus is grown on eggs and inactivated to subsequently serve as a vaccine. Modern developments, especially in the field of molecular biology, provide tools for a different approach. Reverse genetics allows for the production of live vaccines that are claimed to be safe, efficacious and that can easily be manipulated and updated by exchanging genes or implementing mutations (Rimmelzwaan and Osterhaus, 2001; Neumann et al., 2003; Subbarao and Katz, 2004; Bardiya and Bae, 2005; Marsh and Tannock, 2005; Palese, 2006). These modern vaccines could deal with both the need for updating vaccines on a regular basis, and improve efficaciousness in piglets with maternal antibodies. To allow for surveillance in vaccinated populations, it is even possible to introduce the DIVA principle (Differentiate

Vaccinated from Infected Animals). In terms of evaluating vaccine efficacy, not only with respect to clinical protection, but also transmission, DIVA vaccines are extremely valuable. For swine influenza there does not seem to be a good enough reason yet to use DIVA vaccines, however.

Zoosanitary measures as an intervention tool

Vaccines, while potentially highly efficacious, have the disadvantage that they are very specific for one infectious agent, or even one subtype of an infectious agent. In case of multiple disease problems, different vaccines will thus be necessary. One of the immediate consequences of this is the increased costs of the preventive measures. Zoosanitary measures may also be used to either limit the risk of infection, or increase the resistance of an animal and thus protect (partially) against the consequences in terms of severity of clinical symptoms. Management options and increased hygiene, limiting the number of contacts between different age groups, as well as improved climate control, are often applied tools in this context. The big advantage of zoosanitary measures compared to vaccination is that multiple infectious agents can be addressed at the same time with the same or similar measures.

In chapter 6, measures with respect to air filtration and increased hygiene were evaluated. The measures that were implemented in the Air Pathogen Free (APF) house significantly reduced the number of influenza virus infections during the finishing period. Whether these measures will also reduce clinical symptoms related to the influenza virus infections could not be evaluated. Respiratory disease was only noticed once in one of the groups during the two year study.

When implementing these kinds of measures, the investment and the increased operational costs will always have to be taken into account and balanced against the possible gains. For the APF house it was calculated that the costs for all the measures taken could for example be compensated for if the overall growth performance and feed conversion ratio improved by approximately 10%. Although there is no reliable data available on economic losses due to influenza virus infections, this seems a rather unrealistic target for only one infectious agent. In cases where recurrent respiratory disease, especially if caused by multiple infectious agents, causes high economic losses, zoosanitary measures could, however, be a good alternative to measures such as vaccination. Furthermore, in regions with a high pig density, these kinds of measures may also be used to preserve Specific Pathogen Free (SPF) herds and therefore have a much broader applicability. The feasibility of implementing these kinds of measures would increase if they could be used to reduce other diseases as well as just influenza. It remains to be seen, however, whether such measures are also effective in reducing other infections.

During this study, several measures were taken at the same time: air filtration and a variety of hygienic measures. It is unknown which of the measures taken contribute the most towards reducing influenza virus infections. Further research could be focussed on the role of such single measures and their cost-benefits.

Concluding remarks

- The importance of influenza virus infections as a cause of acute respiratory disease was clearly established during this project. More than 50% of the outbreaks of acute respiratory disease are caused by influenza virus infections.
- Virus persistence may occur within herds through ongoing infections in weaning piglets, which become susceptible on an ongoing basis when maternal antibodies decline. It is likely that weaning piglets are also subject to virus introductions from outside. The source could be either finishing pigs on the same premises, participating in a larger within-herd cycle of infections, or occasionally from outside the herd.
- Finishing pigs in farrow-to-finish herds seem to be subject to repeated virus introductions, most likely from the reservoir within the weaning piglets, resulting in multiple small outbreaks already at the start of the finishing period. Less frequent virus introductions in specialized finishing herds may result in a large outbreak within a compartment, on average more towards the end of the finishing period.
- Infections in the presence of low levels of maternal antibodies are often subclinical, but also evoke a lesser immunity than when animals get infected while fully susceptible. Infections in fully susceptible finishing pigs may cause severe growth retardation, but increased growth afterwards may fully compensate for any loss of growth.
- Zoosanitary measures can be implemented to reduce the number of influenza virus infections in finishing pigs and therefore most likely also the magnitude of acute respiratory disease. These measures are unlikely to be cost-beneficial when they are only applied for influenza virus infections, but the application of such measures in a broader context may offer some prospects.

References

Bardiya, N., Bae, J.H., 2005, Influenza vaccines: recent advances in production technologies. Appl Microbiol Biotechnol 67, 299-305.

Barigazzi, G., Donatelli, I., 2003, Swine influenza in Italy. Vet Res Commun 27 Suppl 1, 93-99.

Bikour, M.H., Cornaglia, E., Elazhary, Y., 1996, Evaluation of a protective immunity induced by an inactivated influenza H3N2 vaccine after an intratracheal challenge of pigs. Can.J.Vet.Res. 60, 312-314.

Bouma, A., De Jong, M.C.M., Kimman, T.G., 1997, The influence of maternal immunity on the transmission of pseudorabies virus and on the effectiveness of vaccination. Vaccine 15, 287-294.

Brown, I.H., 2000, The epidemiology and evolution of influenza viruses in pigs. Vet.Microbiol. 74, 29-46.

Brown, I.H., Alexander, D.J., Chakraverty, P., Harris, P.A., Manvell, R.J., 1994, Isolation of an influenza A virus of unusual subtype (H1N7) from pigs in England, and the subsequent experimental transmission from pig to pig. Vet.Microbiol. 39, 125-134.

Brown, I.H., Chakraverty, P., Harris, P.A., Alexander, D.J., 1995, Disease outbreaks in pigs in Great Britain due to an influenza A virus of H1N2 subtype. Vet.Rec. 136, 328-329.

Brown, I.H., Harris, P.A., McCauley, J.W., Alexander, D.J., 1998, Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in the emergence of an H1N2 virus of novel genotype. J.Gen.Virol. 79 (Pt 12), 2947-2955.

Castrucci, M.R., Campitelli, L., Ruggieri, A., Barigazzi, G., Sidoli, L., Daniels, R., Oxford, J.S., Donatelli, I., 1994, Antigenic and sequence analysis of H3 influenza virus haemagglutinins from pigs in Italy. Journal of General Virology 75, 371-379.

Castrucci, M.R., Donatelli, I., Sidoli, L., Barigazzi, G., Kawaoka, Y., Webster, R.G., 1993, Genetic reassortment between avian and human influenza A viruses in Italian pigs. Virology 193, 503-506.

Choi, Y.K., Goyal, S.M., Joo, H.S., 2003, Retrospective analysis of etiologic agents associated with respiratory diseases in pigs. Can Vet J 44, 735-737.
Choi, Y.K., Nguyen, T.D., Ozaki, H., Webby, R.J., Puthavathana, P., Buranathal, C., Chaisingh, A., Auewarakul, P., Hanh, N.T., Ma, S.K., Hui, P.Y., Guan, Y., Peiris, J.S., Webster, R.G., 2005, Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 79, 10821-10825.
De Jong, J.C., Heinen, P.P., Loeffen, W.L., Van Nieuwstadt, A.P., Claas, E.C., Bestebroer, T.M., Bijlsma, K., Verweij, C., Osterhaus, A.D., Rimmelzwaan, G.F., Fouchier, R.A., Kimman, T.G., 2001, Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy. Vaccine 19, 4452-4464.
De Jong, J.C., Van Nieuwstadt, A.P., Kimman, T.G., Loeffen, W.L., Bestebroer, T.M., Bijlsma, K., Verweij, C., Osterhaus, A.D., Claas, E.C., 1999, Antigenic drift in swine influenza H3 haemagglutinins with implications for vaccination policy. Vaccine 17, 1321-1328.
Ducatez, M.F., Olinger, C.M., Owoade, A.A., De Landtsheer, S., Ammerlaan, W., Niesters, H.G., Osterhaus, A.D., Fouchier, R.A., Muller, C.P., 2006, Avian flu: multiple introductions of H5N1 in Nigeria. Nature 442, 37.
Ducatez, M.F., Olinger, C.M., Owoade, A.A., Tarnagda, Z., Tahita, M.C., Sow, A., De Landtsheer, S., Ammerlaan, W., Ouedraogo, J.B., Osterhaus, A.D., Fouchier, R.A., Muller, C.P., 2007, Molecular and antigenic evolution and geographical spread of H5N1 highly pathogenic avian influenza viruses in western Africa. J Gen Virol 88, 2297-2306.
Elbers, A.R.W., Tielen, M.J.M., Cromwijk, W.A.J., Hunneman, W.A., 1990, Sero-epidemiological screening of pig sera collected at the slaughterhouse to detect herds infected with Aujeszky's disease virus, porcine influenza virus and Actinobacillus (Haemophilus) pleuropneumoniae in the framework of an integrated quality control (IQC) system. Vet.Q. 12, 221-230.
Ewald, C., Heer, A., Havenith, U., 1994, Factors Associated with Influenza-A Virus-Infections in Finishing Pigs. Berliner und Munchener Tierarztliche Wochenschrift 107, 256-262.
Groschup, M.H., Brun, A., Haas, B., 1993, Serological studies on the potential synergism of porcine reproductive and respiratory syndrome virus and influenza-, corona- and paramyxoviruses in the induction of respiratory symptoms in swine. Zentralbl.Veterinarmed.[B] 40, 681-689.
Haesebrouck, F., Pensaert, M.B., 1986a, Effect of intratracheal challenge of fattening pigs previously immunised with an inactivated influenza H1N1 vaccine. Vet.Microbiol. 11, 239-249.
Haesebrouck, F., Pensaert, M.B., 1986b, Prevalence of H1N1- and H3N2-influenza A viruses in fatteners [swine] in Belgium (1984). Vlaams Diergeneeskundig Tijdschrift 55, 12-16.
Hall, N., 2007, Advanced sequencing technologies and their wider impact in microbiology. J Exp Biol 210, 1518-1525.
Heinen, P.P., Van Nieuwstadt, A.P., Boer-Luijtze, E.A., Bianchi, A.T., 2001, Analysis of the quality of protection induced by a porcine influenza A vaccine to challenge with an H3N2 virus. Vet.Immunol.Immunopathol. 82, 39-56.
Karasin, A.I., Brown, I.H., Carman, S., Olsen, C.W., 2000, Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J.Virol. 74, 9322-9327.
Karasin, A.I., West, K., Carman, S., Olsen, C.W., 2004, Characterization of avian H3N3 and H1N1 influenza A viruses isolated from pigs in Canada. J Clin Microbiol 42, 4349-4354.
Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K.F., Kawaoka, Y., Webster, R.G., 1994, Potential for transmission of avian influenza viruses to pigs. Journal of General Virology 75, 2183-2188.
Kim, W.I., Wu, W.H., Janke, B., Yoon, K.J., 2006, Characterization of the humoral immune response of experimentally infected and vaccinated pigs to swine influenza viral proteins. Arch Virol 151, 23-36.
Kitikoon, P., Nilubol, D., Erickson, B.J., Janke, B.H., Hoover, T.C., Sornsen, S.A., Thacker, E.L., 2006, The immune response and maternal antibody interference to a heterologous H1N1 swine influenza virus infection following vaccination. Vet Immunol Immunopathol 112, 117-128.
Loeffen, W., De Boer-Luijtze, E.A., Koch, G., 2004, Transmission of a highly pathogenic avian influenza virus to swine in the Netherlands. Proceedings of the in-between congress of the International Society for Animal Hygiene, St. Malo, France, 329-330.
Ma, W., Gramer, M., Rossow, K., Yoon, K.J., 2006, Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States. J Virol 80, 5092-5096.

Ma, W., Vincent, A.L., Gramer, M.R., Brockwell, C.B., Lager, K.M., Janke, B.H., Gauger, P.C., Patnayak, D.P., Webby, R.J., Richt, J.A., 2007, Identification of H2N3 influenza A viruses from swine in the United States. Proc Natl Acad Sci U S A 104, 20949-20954.
Maldonado, J., Van Reeth, K., Riera, P., Sitja, M., Saubi, N., Espuna, E., Artigas, C., 2006, Evidence of the concurrent circulation of H1N2, H1N1 and H3N2 influenza A viruses in densely populated pig areas in Spain. Vet J 172, 377-381.
Marozin, S., Gregory, V., Cameron, K., Bennett, M., Valette, M., Aymard, M., Foni, E., Barigazzi, G., Lin, Y., Hay, A., 2002, Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. J.Gen.Virol. 83, 735-745.
Marsh, G.A., Tannock, G.A., 2005, The role of reverse genetics in the development of vaccines against respiratory viruses. Expert Opin Biol Ther 5, 369-380.
Masurel, N., De Boer, G.F., Anker, W.J., Huffels, A.D., 1983, Prevalence of influenza viruses A-H1N1 and A-H3N2 in swine in the Netherlands. Comp.Immunol.Microbiol.Infect.Dis. 6, 141-149.
Neumann, G., Hatta, M., Kawaoka, Y., 2003, Reverse genetics for the control of avian influenza. Avian Dis 47, 882-887.
Palese, P., 2006, Making better influenza virus vaccines? Emerg Infect Dis 12, 61-65.
Peiris, J.S., Guan, Y., Markwell, D., Ghose, P., Webster, R.G., Shortridge, K.F., 2001, Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southeastern China: potential for genetic reassortment? J.Virol. 75, 9679-9686.
Rimmelzwaan, G.F., Osterhaus, A.D., 2001, Influenza vaccines: new developments. Curr Opin Pharmacol 1, 491-496.
Sanger, F., Nicklen, S., Coulson, A.R., 1977, DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74, 5463-5467.
Scholtissek, C., 1995, Molecular evolution of influenza viruses. Virus Genes 11, 209-215.
Schrader, C., Suss, J., 2003, Genetic characterization of a porcine H1N2 influenza virus strain isolated in Germany. Intervirology 46, 66-70.
Shendure, J., Porreca, G.J., Reppas, N.B., Lin, X., McCutcheon, J.P., Rosenbaum, A.M., Wang, M.D., Zhang, K., Mitra, R.D., Church, G.M., 2005, Accurate multiplex polony sequencing of an evolved bacterial genome. Science 309, 1728-1732.
Shin, J.Y., Song, M.S., Lee, E.H., Lee, Y.M., Kim, S.Y., Kim, H.K., Choi, J.K., Kim, C.J., Webby, R.J., Choi, Y.K., 2006, Isolation and characterization of novel H3N1 swine influenza viruses from pigs with respiratory diseases in Korea. J Clin Microbiol 44, 3923-3927.
Subbarao, K., Katz, J.M., 2004, Influenza vaccines generated by reverse genetics. Curr Top Microbiol Immunol 283, 313-342.
Teuffert, J., Sinnecker, R., Karge, E., 1991, Serological survey of HI antibodies against porcine and human type A influenza viruses among domestic and wild pigs in East Germany.; [Seroepidemiologische Untersuchungen mit dem HAHT zum Vorkommen porciner und humaner Infl-A-Viren bei Haus- und Wildschweinen in der ehemaligen DDR]. Monatsh.Veterinarmed. 46, 171-174.
Tsai, C.P., Pan, M.J., 2003, New H1N2 and H3N1 influenza viruses in Taiwanese pig herds. Vet Rec 153, 408.
Van Reeth, K., Brown, I., Essen, S., Pensaert, M., 2004, Genetic relationships, serological cross-reaction and cross-protection between H1N2 and other influenza A virus subtypes endemic in European pigs. Virus Res 103, 115-124.
Van Reeth, K., Brown, I.H., Pensaert, M., 2000, Isolations of H1N2 influenza A virus from pigs in Belgium. Veterinary Record 146, 588-589.
Van Reeth, K., Gregory, V., Hay, A., Pensaert, M., 2003a, Protection against a European H1N2 swine influenza virus in pigs previously infected with H1N1 and/or H3N2 subtypes. Vaccine 21, 1375-1381.
Van Reeth, K., Labarque, G., De Clercq, S., Pensaert, M., 2001, Efficacy of vaccination of pigs with different H1N1 swine influenza viruses using a recent challenge strain and different parameters of protection. Vaccine 19, 4479-4486.
Van Reeth, K., Labarque, G., Pensaert, M., 2006, Serological profiles after consecutive experimental infections of pigs with European H1N1, H3N2, and H1N2 swine influenza viruses. Viral Immunol 19, 373-382.
Van Reeth, K., Van Gucht, S., Pensaert, M., 2003b, Investigations of the efficacy of European H1N1- and H3N2-based swine influenza vaccines against the novel H1N2 subtype. Vet Rec 153, 9-13.

Velicer, G.J., Raddatz, G., Keller, H., Deiss, S., Lanz, C., Dinkelacker, I., Schuster, S.C., 2006, Comprehensive mutation identification in an evolved bacterial cooperator and its cheating ancestor. Proc Natl Acad Sci U S A 103, 8107-8112.

Xu, C., Fan, W., Wei, R., Zhao, H., 2004, Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. Microbes Infect 6, 919-925.

Yus, E., Laviada, M.D., Moreno, L., Castro, J.M., Escribano, J.M., Simarro, I., 1989, The prevalence of antibodies to influenza virus and respiratory coronavirus in fattening pigs in Spain. Zentralbl.Veterinarmed.[B] 36, 551-556.

Samenvatting

Influenzavirussen maken deel uit van de familie van Orthomyxoviridae, die bestaat uit de genera van influenza A, influenza B en influenza C virussen. Het zijn allen enkelstrengs RNA virussen, met een genoom dat bij de influenza-A-virussen is opgebouwd uit 8 RNA fragmenten. Op het oppervlak bevinden zich twee glycoproteïnen: het haemagglutinine en het neuraminidase. Van het haemagglutinine zijn 16 varianten bekend (H1-H16) en van het neuraminidase 9 (N1-N9). Influenza subtypen worden genoemd naar de combinatie van de beide glycoproteïnen, bv. H2N3.

Influenza bij varkens werd voor het eerst waargenomen in 1918 in de Verenigde Staten, tijdens de humane pandemie van de Spaanse griep. In Europa werd influenza bij varkens zelden waargenomen, tot eind jaren 60 een influenzavirus van het subtype H3N2 werd geïntroduceerd. Aanvankelijk veroorzaakte dit virus weinig ziekteverschijnselen. In 1979 maakte in Europa een influenzavirus subtype H1N1 de overstap van watervogels naar varkens. Dit virus verspreidde zich snel door de Europese varkensstapel en veroorzaakte veel respiratoire problemen. Een reassortment tussen deze H1N1 en de reeds circulerende H3N2 leidde tot een nieuwe variant van het H3N2 subtype. Alle interne genen werden overgenomen van het H1N1 subtype en vanaf dit moment leidde ook H3N2 infecties tot meer klinische problemen. Tenslotte begon zich in de jaren 90 een derde influenzavirus door Europa te verspreiden, van het subtype H1N2. Alle drie subtypes komen inmiddels wijdverspreid in veel Europese landen voor.

In een eerste studie werd mid-jaren 90 vastgesteld dat influenzavirusinfecties de belangrijkste oorzaak zijn van acute respiratoire problemen bij vleesvarkens. Zestien uitbraken van acute respiratoire problemen werden daarvoor uitgebreid en zorgvuldig onderzocht. Pathologie (macroscopisch en microscopisch), virusisolaties, bacteriologisch onderzoek, isolatie van mycoplasmata en serologie maakten deel uit van dit onderzoek. Acht van deze uitbraken werden veroorzaakt door een infectie met het influenzavirus. In een negende uitbraak speelde influenzavirus eveneens een rol, maar uit pathologisch onderzoek werd geconcludeerd dat *A. pleuropneumoniae* primair verantwoordelijk was voor de geconstateerde ziekteverschijnselen. Hoewel infecties met andere virussen of bacteriën optraden kort voor of tegelijkertijd met de influenza-infectie, konden deze niet in relatie gebracht worden met de klinische verschijnselen of pathologische afwijkingen. Van specifieke dubbelinfecties was dus geen sprake.

In een vervolgstudie werd het belang van influenza bij acute respiratoire problemen bevestigd. In de periode van 1996 tot 1999 werden nog 37 uitbraken van acute respiratoire problemen onderzocht. De resultaten waren vergelijkbaar met de eerste studie: 21 van de 37 onderzochte uitbraken (57%) werden veroorzaakt door een infectie met influenzavirus.

Alle uitbraken in deze periode werden op basis van virusisolatie en serologie toegeschreven aan infecties met het subtype H1N1 of H3N2. In 1999 werd echter

vastgesteld dat ook het subtype H1N2 inmiddels wijdverspreid in Nederland voorkwam. Bij vleesvarkens op gesloten bedrijven (waar zowel zeugen als vleesvarkens aanwezig zijn) werd aan het einde van de mestperiode een prevalentie van 57% gevonden, tegen 26% bij vleesvarkens op gespecialiseerde vleesvarkensbedrijven.

Viruscirculatie bij gespeende biggen is niet ongebruikelijk. Toch raakt, waarschijnlijk onder invloed van bescherming van maternale antistoffen, maar een deel van de gespeende biggen geïnfecteerd. Een groot gedeelte van de biggen gaat dus de mestperiode in zonder antistoffen tegen influenza, of met een restant maternale antistoffen. Zij zijn, of worden na verloop van tijd, volledig gevoelig voor een infectie met influenzavirus. Hoe lang maternale antistoffen aanwezig blijven, wordt hoofdzakelijk bepaald door de hoeveelheid antistoffen die via de biest wordt opgenomen, en is dus in belangrijke mate afhankelijk van de titerhoogte bij de zeug. De halfwaardetijd is vervolgens redelijk constant en is ongeveer 12 dagen. Bij hoge begintiters zijn maternale antistoffen daarbij tot maximaal zo'n 16 weken leeftijd aantoonbaar.

Maternale antistoffen hebben hun effect op de ontwikkeling van een immunologische respons na een infectie. Hoewel infecties in aanwezigheid van maternale antistoffen nauwelijks tot enige klinische verschijnselen leiden, is de opgebouwde immuniteit minder goed dan na een infectie bij volledig gevoelige dieren. Los van de aanwezigheid van maternale antistoffen, leidden infecties bij vleesvarken (15 weken oud) tot veel ernstiger verschijnselen dan infecties bij biggen (7 weken oud). Er lijkt dus sprake te zijn van een leeftijdsgebonden effect dat los staat van de aanwezigheid van specifieke antistoffen.

Bij vleesvarkens op gesloten bedrijven en gespecialiseerde vleesvarkensbedrijven werden verschillende infectiepatronen gezien. Op gesloten bedrijven kwamen infecties vooral voor in het eerste deel van de mestperiode. Mogelijk is dit het gevolg van de voortdurende blootstelling van virus dat bij de gespeende biggen circuleert. Dit veronderstelt dat de gespeende biggen een reservoir vormen op een bedrijf, waar langdurig, of zelfs permanent, influenzavirus kan blijven circuleren.

Bij vleesvarkens op gespecialiseerde vleesvarkensbedrijven komen infecties daarentegen vooral voor in het tweede deel van de mestperiode. Op deze bedrijven zijn de contacten met gespeende biggen veel beperkter, waardoor de kans op introductie van het influenzavirus in een afdeling met varkens ook kleiner is. Een introductie op oudere leeftijd, in een volledig gevoelige populatie, zorgt echter wel voor omstandigheden waarbij het virus zich snel kan verspreiden en er alsnog een hoge prevalentie bereikt wordt in een afdeling.

Om de gevolgen van een influenza-infectie te beperken, is het mogelijk om te vaccineren. Onder experimentele omstandigheden zijn vaccins in staat om klinische verschijnselen geheel of vrijwel geheel te voorkomen. Zelfs als door antigene drift het challengevirus sterk afwijkt van het vaccinvirus (van hetzelfde subtype), wordt een goede bescherming tegen de challenge waargenomen. Van vaccinatie onder

praktijkomstandigheden zijn echter geen betrouwbare gegevens beschikbaar. Effectiviteit in het veld kan mede afhankelijk zijn van de aanwezigheid van maternale antistoffen in relatie tot tijdstip van vaccinatie, antigene drift van influenzavirussen in de tijd en de aanwezigheid van een meer of minder grote variatie in gelijktijdig circulerende virusstammen. Wat in de laatste jaren bovendien een rol speelt is de circulatie van het H1N2 subtype, wat nog niet in vaccins is opgenomen.

Zoosanitaire maatregelen vormen mogelijk een alternatief voor vaccinatie. Deze maatregelen trachten het contact tussen virus en varken te verminderen, waardoor infecties, en dus ook ziekteverschijnselen, minder vaak voorkomen. In een speciaal hiervoor ingerichte stal (APF-stal) werd binnenkomende lucht gefilterd over absoluutfilters en werden hygiënische maatregelen genomen om virusinsleep via personen, materialen en voer te reduceren. In deze stal werden bij de vleesvarkens significant minder influenza infecties waargenomen dan bij vleesvarkens die elders op hetzelfde bedrijf werden gehuisvest. Het maakte daarbij niet veel uit of de varkens vanaf een leeftijd van 3 weken of 9 weken in de APF-stal werden gehuisvest. Een voordeel van dergelijk zoosanitaire maatregelen is dat zij niet alleen tegen influenza werkzaam zijn, maar potentieel ook tegen een groot aantal andere ziektekiemen.

Overall konden de volgende conclusies worden getrokken uit het onderzoek in dit proefschrift:

- Infecties met influenzavirus vormen de belangrijkste oorzaak van acute respiratoire problemen bij vleesvarkens in Nederland.
- Gespeende biggen vormen een potentieel reservoir van influenzavirus op een bedrijf. Door het voortdurend beschikbaar komen van nieuwe gevoelige dieren (o.a. door aflopende maternale immuniteit van steeds weer nieuw geboren biggen) vindt het virus steeds nieuwe gastheren, zodat de infectie niet snel doodloopt.
- Vleesvarkens op gesloten bedrijven lijken onderhevig aan een voortdurende blootstelling aan influenzavirus vanuit de gespeende biggen, waardoor infecties vooral in de eerste helft van de mestperiode optreden.
- Infecties in aanwezigheid van maternale antistoffen verlopen veelal subklinisch. De immuniteit die daarbij wordt opgebouwd is echter minder dan bij dieren die een infectie doormaken terwijl zij volledig gevoelig zijn. Infecties bij oudere vleesvarkens kunnen een aanzienlijke groeivertraging geven, maar voor deze groeivertraging kan tijdens de herstelperiode volledig gecompenseerd worden.
- Zoosanitaire maatregelen kunnen het aantal influenza-infecties reduceren. Alleen voor influenza zijn deze maatregelen waarschijnlijk niet kosteneffectief, maar in een bredere context bieden zij mogelijk wel perspectief.

Dankwoord

Hoewel alleen mijn naam voor op dit boekje staat, moge het duidelijk zijn dat dit het werk van velen is. En dan zijn er nog al die mensen die op een andere manier hebben bijgedragen aan het uiteindelijke verschijnen van dit boekje. Er zijn dus veel mensen die ik wil bedanken, en als ik alle namen ga noemen ga ik er zeker een paar vergeten. Bovendien wordt het boekje dan twee keer zo dik en dat kost weer een extra boom.

Ik ga dus alleen mijn ouders speciaal noemen, omdat zij dit al 44 jaar mogelijk maken en er als enigen vanaf het begin bijgeweest zijn. Verder, iedereen bedankt!

Curriculum vitae

Willie Loeffen werd geboren op 24 juli 1964, te Oss. In 1981 behaalde hij zijn HAVO-diploma en een jaar later zijn VWO-diploma. In 1982 begon hij zijn studie Diergeneeskunde in Utrecht, die in februari 1991 werd afgerond met de uitreiking van zijn diploma. In september 1991 ging hij aan het werk bij de Gezondheidsdienst voor Dieren in Boxtel. In een periode van iets meer dan 10 jaar werkte hij daar op de varkensafdeling in het veldonderzoek aan de ziekte van Aujeszky, acute fase eiwitten bij slachtvarkens, respiratoire aandoeningen bij vleesvarkens en varkensinfluenza. Deze laatste twee onderwerpen vormen de basis voor zijn proefschrift. In December 2001 maakte hij de overstap naar het toenmalige divisie WOT van het ID-Lelystad als projectleider klassieke varkenspest. Een maand later werd de divisie verzelfstandigd tot CIDC-Lelystad en weer 6 jaar later veranderde die naam door een fusie in Centraal Veterinair Instituut van Wageningen UR.

List of publications

Weesendorp, E., Stegeman, A., Loeffen, W.L., 2008, Dynamics of virus excretion via different routes in pigs experimentally infected with classical swine fever virus strains of high, moderate or low virulence, Veterinary Microbiology (accepted)

Weesendorp, E., Stegeman, A., Loeffen, W.L., 2008, Survival of classical swine fever virus at various temperatures in faeces and urine derived from experimentally infected pigs, Veterinary Microbiology (accepted)

Weesendorp, E., Landman, W.J., Stegeman, A., Loeffen, W.L., 2008, Detection and quantification of classical swine fever virus in air samples originating from infected pigs and experimentally produced aerosols. Veterinary Microbiology 127, 50-62.

Dortmans, J.C., Loeffen, W.L., Weerdmeester, K., van der Poel, W.H., de Bruin, M.G., 2008, Efficacy of intradermally administrated E2 subunit vaccines in reducing horizontal transmission of classical swine fever virus. Vaccine 26, 1235-1242.

Elbers, A.R., Gorgievski-Duijvesteijn, M.J., van der Velden, P.G., Loeffen, W.L., 2007, [Nonspecific clinical signs in pigs and use of exclusion diagnosis for classical swine fever: a survey among pig farmers and veterinary practitioners]. Tijdschrift voor Diergeneeskunde 132, 340-345.

Blome, S., Meindl-Bohmer, A., Loeffen, W., Thuer, B., Moennig, V., 2006, Assessment of classical swine fever diagnostics and vaccine performance. Scientific and Technical Review (Rev Sci Tech) 25, 1025-1038.

Wieringa-Jelsma, T., Quak, S., Loeffen, W.L., 2006, Limited BVDV transmission and full protection against CSFV transmission in pigs experimentally infected with BVDV type 1b. Veterinary Microbiology 118, 26-36.

Geenen, P., Van Der Gaag, L., Loeffen, W., Elbers, A., 2004, On the robustness of feature selection with absent and non-observed features. Lecture Notes in Computer Science (including subseries Lecture Notes in Artificial Intelligence and Lecture Notes in Bioinformatics) 3337, 148-159.

Loeffen, W.L., Heinen, P.P., Bianchi, A.T., Hunneman, W.A., Verheijden, J.H., 2003, Effect of maternally derived antibodies on the clinical signs and immune response in pigs after primary and secondary infection with an influenza H1N1 virus. Veterinary Immunology and Immunopathology 92, 23-35.

Loeffen, W.L.A., Nodelijk, G., Heinen, P.P., Van Leengoed, L.A., Hunneman, W.A., Verheijden, J.H., 2003, Estimating the incidence of influenza-virus infections in Dutch weaned piglets using blood samples from a cross-sectional study. Veterinary Microbiology 91, 295-308.

De Jong, J.C., Heinen, P.P., Loeffen, W.L., Van Nieuwstadt, A.P., Claas, E.C., Bestebroer, T.M., Bijlsma, K., Verweij, C., Osterhaus, A.D., Rimmelzwaan, G.F., Fouchier, R.A., Kimman, T.G., 2001, Antigenic and molecular heterogeneity in recent swine influenza A(H1N1) virus isolates with possible implications for vaccination policy. Vaccine 19, 4452-4464.

De Jong, J.C., Van Nieuwstadt, A.P., Kimman, T.G., Loeffen, W.L., Bestebroer, T.M., Bijlsma, K., Verweij, C., Osterhaus, A.D., Claas, E.C., 1999, Antigenic drift in swine influenza H3 haemagglutinins with implications for vaccination policy. Vaccine 17, 1321-1328.

Loeffen, W.L.A., Kamp, E.M., Stockhofe-Zurwieden, N., Van Nieuwstadt, A.P.K.M.I., Bongers, J.H., Hunneman, W.A., Elbers, A.R.W., Baars, J., Nell, T., Van Zijderveld, F.G., 1999, Survey of infectious agents involved in acute respiratory disease in finishing pigs. Veterinary Record 145, 123-129.

Van Nes, A., Stegeman, J.A., De Jong, M.C., Loeffen, W.L., Kimman, T.G., Verheijden, J.H., 1996, No major outbreaks of pseudorabies virus in well-immunized sow herds. Vaccine 14, 1042-1044.

Van Nes, A., Stegeman, J.A., De Jong, M.C., Loeffen, W.L., Kimman, T.G., Verheijden, J.H., 1997, No massive spread of pseudorabies virus in vaccinated sow herds. Veterinary Microbiology 55, 147-151.

Stegeman, A., Elbers, A.R.W., Loeffen, W., De Jong, M.C.M., Tielen, M.J.M., 1996, Rate of successful pseudorabies virus introductions in swine breeding herds in the southern Netherlands that participated in an area-wide vaccination programme. Preventive Veterinary Medicine 27, 29-41.