THE DETECTION OF RESPIRATORY DISEASES IN SWINE HERDS
BY MEANS OF ANTIBODY ASSAY ON COLOSTRUM FROM SOWS

BY

KATRI LEVONEN

Department of Food- and Environmental Hygiene
Faculty of Veterinary Medicine
University of Helsinki

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Hämeentie 57, 00550 Helsinki

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Summary 1
List of original articles 5
Introduction 6
1. Review of the literature 9
1.1 Diagnostics within disease-control programs in pig farming 9
1.2 Enzootic pneumonia caused by Mycoplasma hyopneumoniae 13
1.2.1 The agent M. hyopneumoniae 13
1.2.2 Enzootic pneumonia disease 13
1.2.3 Epidemiology of M. hyopneumoniae 14
1.2.4 Diagnosis of M. hyopneumoniae 16
1.3 Catarrhal pneumonia and pleuritis caused by Actinobacillus pleuropneumoniae 18
1.3.1 A. pleuropneumoniae 18
1.3.2 Disease caused by A. pleuropneumoniae 19
1.3.3 Epidemiology of A. pleuropneumoniae 21
1.3.4 Diagnosis of A. pleuropneumoniae 22
1.4 Progressive atrophic rhinitis (PAR) caused by toxigenic P. multocida 24
1.4.1 Toxigenic P. multocida 24
1.4.2 The disease progressive atrophic rhinitis (PAR) 26
1.4.3 Epidemiology of toxigenic P. multocida 27
1.4.4 Diagnosis of PAR 28
2. Aims 30
3. Materials and methods 31
3.1 Collection of colostrum and serum samples 31
3.2 Bacterial strains for A. pleuropneumoniae antigen and for immunisation of rabbits 34
3.3 Control serums in A. pleuropneumoniae ELISA 34
3.4 Performing the M. hyopneumoniae ELISA 35
3.5 Immunisation of rabbits to obtain A. pleuropneumoniae antisera (III, IV) 37
3.6 Preparation of A. pleuropneumoniae antigen 38
3.7 Coating of microwell plates with A. pleuropneumoniae antigen 38
3.8 A. pleuropneumoniae ELISA assay (III) 39
3.9 The ELISA assay for 12 A. pleuropneumoniae serotypes (IV) 40
3.10 Testing cross reactions in A. pleuropneumoniae ELISA (III, IV) 41
3.11 Repeatability of the test in A. pleuropneumoniae ELISA (III) 41
3.12 ELISA for P. multocida toxin antibodies (V) 42
4. Results 43
4.1 The *M. hyopneumoniae* ELISA test
4.2 Cross-reactions with *M. hyorrhinis*
4.3 Repeatability of the Chekit®Hyoptest
4.4 Comparison of the Chekit®Hyoptest and DAKO *M. hyopneumoniae* ELISA
4.5 Control serums in the *A. pleuropneumoniae* ELISA
4.6 Repeatability of the *A. pleuropneumoniae* ELISA
4.7 Cross-reactions in *A. pleuropneumoniae* ELISA
4.8 Positive samples in anti-PMT ELISA
4.9 Specificity and sensitivity of the anti-PMT ELISA
4.10 *M. hyopneumoniae* survey
4.11 *A. pleuropneumoniae* serotype 12 survey
4.12 *A. pleuropneumoniae* 12 serotypes
4.13 Anti-PMT survey

5. Discussion
5.1 *M. hyopneumoniae* infections in disease-control herds
5.2 Specificity and sensitivity of *M. hyopneumoniae* ELISA
5.3 An exceptional herd in the *M. hyopneumoniae* survey
5.4 The *A. pleuropneumoniae* serotype 2 survey
5.5 *A. pleuropneumoniae* 12 serotypes survey
5.6 The *A. pleuropneumoniae* ELISA
5.7 Progressive atrophic rhinitis (PAR) survey
5.8 Specificity and sensitivity of the blocking ELISA test
5.9 Colostrum sample

6. Conclusions
7. References
Acknowledgements
Summary

The control of infectious diseases in pig farming is of great importance from the point of view of animal welfare and economic profitability. Pneumonias have been shown to cause the greatest economic losses due to disease in pig houses. Prevention of infections is the most humane way of fighting against disease. Through the prevention of infections the use of antibiotics and thus antibiotic residues can also be avoided.

*Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae* and toxigenic *Pasteurella multocida* are the most common and most detrimental respiratory pathogens of swine, and they all may have periods of subclinical existence in swine herds. Antibody assays of serum and colostrum have great potential for the diagnosis of subclinical infections. In this study the ELISAs for *M. hyopneumoniae* antibodies were evaluated, an ELISA for *A. pleuropneumoniae* antibodies (12 serotypes) was developed and the ELISA for *P. multocida* toxin was modified to detect antibodies against the toxin. The prevalence of *M. hyopneumoniae*, *A. pleuropneumoniae* serotype 2 and *P. multocida* toxin antibodies in disease-control breeding herds was determined (193 herds, mean number of sows 30), as was the seroprevalence of 12 different serotypes of *A. pleuropneumoniae* in Finnish slaughter sows (n=700).

The serological ELISA tests for *M. hyopneumoniae* antibodies were found to be specific and sensitive. The specificity of the Chekit Hyoptest was 98.0%, and that of the DAKO *M.
hyopneumoniae ELISA, 99.3% in this study. In a M. hyopneumoniae conducted on disease-control herds, six herds out of 185 were found to be infected. These herds were detected by serological means at an earlier stage than by traditional diagnostic methods, i.e. by observing clinical signs and pathological findings at slaughterhouses.

An ELISA for each of the 12 A. pleuropneumoniae serotypes was developed. The phenol-extracted polysaccharide antigen was found to be suitable for the assay. When specific antisera were used, some weak cross reactions were found between serotypes 1, 9 and 11, as well as between serotypes 6 and 8. In addition, serotype 5(a) antiserum reacted with serotype 6 antigen. In the case of field samples, the OD values of the samples reacting with serotypes 9 and 11, 9 and 12, and 1 and 2 were correlated (p<0.01), indicating antigenic similarities between these serotypes or dual infection with different serotypes. Antibodies against various serotypes of A. pleuropneumoniae did not have an even distribution throughout Finland. Serotype 1, serotype 9 and serotype 12 antibodies were found most frequently in the west, serotype 3 most frequently in the south-west and serotype 4 and serotype 6 antibodies in central Finland. Antibodies against serotype 2 were distributed evenly throughout the country.

The disease-control herds were surveyed for A. pleuroneumoniae serotype 2 because this is the serotype which is isolated from clinical A. pleuropneumoniae outbreaks in Finland. This survey showed that A. pleuropneumoniae serotype 2 infection is more widely distributed in disease-control herds than had been
expected. Of the 154 herds tested, only 25 were totally free of *A. pleuropneumoniae* serotype 2 antibodies. Previously, according to the more established used disease-control methods, i.e. observing clinical signs and slaughterhouse inspection, only 1 or 2 herds were found to be diseased each year. Despite this low prevalence, *A. pleuropneumoniae* infections of unknown origin interrupted progeny tests in stations that took piglets only from disease-control herds. It was concluded, therefore, that the prevention of *A. pleuropneumoniae* infections is more complicated than was formerly considered. Clinical *A. pleuropneumoniae* infections were rare, due to the variability in the virulence of *A. pleuropneumoniae* strains.

An ELISA for the detection of *P. multocida* toxin antibodies was developed on the basis of a commercial test for detecting *P. multocida* toxin. Toxigenic *P. multocida* infection (progressive atrophic rhinitis) was found in one of the disease-control herds. The diagnosis was initially made on serological grounds, and thereafter confirmed clinically and bacteriologically. The diseased herd included imported pigs; these were the apparent source of the infection. An eradication programme for athrophic rhinitis has been successfully carried out in Finland for years. *P. multocida* toxin antibodies were not found in the disease-control herds, comprised only pigs of Finnish origin.

Antibody assays appeared to provide a reliable diagnostic methods at the herd level. In diagnosing the infection status of an individual animal, assessment of antibody status is not the most effective method, because it takes at least 2 weeks after infection
before the antibodies reach a measurable level. On the other hand, the animal continues to carry antibodies after it has expelled the infectious agent. In *M. hyopneumoniae* infection, antibodies were still detected by ELISA one year after inoculation, even though there were pathological lesion in the lungs and mycoplasmas were not detected (Bereiter et al. 1990). As herd tests, the antibody assays are perfect diagnostic methods for disease-control programs (Zimmermann and Tschudi 1989). In this study the sensitivity appeared to be close to 100%. As a result of these studies, *M. hyopneumoniae* antibody assays were included in the national disease-control program, and *P. multocida* toxin antibody assays are now required from herds applying for inclusion in the program.

According to the serological data available, *A. pleuropneumoniae* infections in Finnish Pig Health Scheme herds were so frequent that freedom from this agent could not be demanded. Most of the infections seemed to be mild, without any clear clinical signs. *A. pleuropneumoniae* has also spread within herds included in disease-control programs in other countries, and other methods for *A. pleuropneumoniae* disease control are therefore still required.
List of original articles

The present thesis is based on the following original articles, referred to in the text by the Roman numerals I to V.


Introduction

During the last three decades pig production has intensified dramatically. The increase in herd size sets serious demands for the control of infectious diseases. In several surveys infectious pneumonias have been shown to cause the greatest economic losses due to diseases in pig houses (Christensen and Mousing 1992, Straw et al. 1990). The spread of infectious diseases has led to the wide use of antibiotics as food additions. There is widening consumer concern about this kind of production method (Aarestrup et al. 1998). Although skilful management, proper housing and balanced feeding improve disease resistance, there are a number of infectious diseases which can also affect pigs in favourable conditions.

Fortunately, the structure of pig production allows many infectious diseases to be totally eliminated from pig herds. Elimination of infectious agents can be successfully achieved only if the agent in question can be accurately identified, the epidemiology pattern of the disease is known, and the spreading pathways can be effectively eliminated.

The virulent and easily transmissible diseases, which endanger human health or can ruin the economic outcome of pig farms, are controlled by government and EU programs. In addition to these diseases, there are a number of other diseases which harm the well-being of pigs and reduce the profitability of pig farming. In many countries, including Finland, voluntary disease-control programs for breeding herds have been
introduced. The Finnish Pig Health Scheme was established in 1963 (Schulman 1988), when 35 herds with the highest breeding value were included in the scheme. In 1984 the control programme was expanded and all the 200 active breeding herds joined the scheme. The diseases controlled were enzootic pneumonia (*Mycoplasma hyopneumoniae*), catarrhal pleuritis and pneumonia (*Actinobacillus pleuropneumoniae*), progressive atrophic rhinitis (toxigenic *Pasteurella multocida*), swine dysentery (*Serpulina hyodysenteriae*) and *Clostridium perfringens* type C enteritis of new born piglets.

The diagnostic methods used in the Finnish Pig Health Scheme initially consisted of farm visits by a veterinarian and inspection of organs and carcasses in slaughterhouse, and mix-match control in progeny testing stations that receive piglets only from the herds participating in the scheme.

The old screening methods have been found to be insufficient for disease control. More valid methods were called for by the local controlling veterinarians, who needed a good reference system for diagnosis. Serology was reported to be a promising diagnostic means for detecting subclinical diseases in breeding herds (Nicolet 1988, Nielsen 1988). In Switzerland and Denmark, serology has been successfully applied as a part of their disease-control programs (Nicolet 1988, Nielsen 1988). The application of enzyme-linked immunosorbent assays for the detection of antibodies dramatically increased the utilisation of serological methods in diagnostic laboratories during the 1990's. The assays were considered to be sensitive enough for the
measurement of weak immunoreactions typical of progressive atrophic rhinitis and enzootic pneumonia. The Finnish Ministry of Agriculture and Forestry decided to provide funds for the present study, the object of which is to develop and validate ELISAs for the control programmes of the Finnish Pig Health Scheme.
1. Review of the literature

1.1 The role of diagnostics in disease-control programs in pig farming

Contagious diseases have a negative effect on the well being of pigs and the economic outcome of pig farms. Therefore, disease-control programs have been introduced on a voluntary basis in several countries. The Finnish Pig Health Scheme was introduced by the Ministry of Agriculture and Forestry in 1963, and the Scheme has subsequently gained constantly growing significance.

Adequate diagnosis is of crucial importance in disease-control programs. The diagnostic methods adopted by the Finnish Pig Health Scheme were long established (Keller 1976, Goodwin 1977). The herds participating in the scheme were inspected every three months by the local official veterinarian. Carcasses and organs of five pigs per herd were inspected in slaughterhouses twice a year. In addition, the progeny testing stations, which take piglets only from disease control herds, served as a mix-match control. The diseases of concern were enzootic pneumonia (Mycoplasma hyopneumoniae), catarrhal pleuritis and pneumonia (Actinobacillus pleuropneumoniae), progressive atrophic rhinitis (toxigenic Pasteurella multocida), swine dysentery (Brachyspira hyodysenteriae) and Clostridium perfringens type C enteritis of new-born piglets. Routine laboratory testing was not included either in the original scheme or
the pig health scheme as renewed in 1984. Suspected disease outbursts were confirmed by bacterial cultivation or by typical pathological findings from slaughtered pigs.

*M. hyopneumoniae, A. pleuropneumoniae, P. multocida* and *B. hyodysenteriae* infections may exist subclinically in pig herds (Nicolet 1992, Ross 1992). These infections have spread within disease-control herds in other countries (Nielsen 1988). Subclinical infections were also suspected to occur in the Finnish Pig Health Scheme herds following the finding that *M. hyopneumoniae* and *A. pleuropneumoniae* caused epidemics in closed progeny testing stations rearing piglets produced by the Health Scheme breeders. Furthermore, doubt has been expressed about the efficiency of the traditional methods used for diagnosing diseases in control programs. Examination at slaughter has proved to be a poor indicator of lifetime pneumonia (Noyes et al. 1990, Wallgren et al. 1990), and surveillance using clinical herd inspection alone has failed to detect 30% of the infected herds (Sørensen et al. 1993).

Bacterial culture has subsequently been introduced as a diagnostic method into some disease-control programs. In Denmark, the isolation of *P. multocida* from nasal swabs and subsequent toxicity testing is included in the disease-control program (SPF) (Foged et al. 1988). However, the efficacy of bacterial cultivation is poor, and is not very applicable for *A. pleuropneumoniae* and *M. hyopneumoniae* diagnosis. *A. pleuropneumoniae* can only be isolated in the acute stage of the disease (Nielsen 1988), and *M. hyopneumoniae* often fails to
grow on laboratory media. Isolation is readily obscured by contaminating mycoplasma and bacteria (Armstrong 1994). Furthermore, samples suitable for *M. hyopneumoniae* isolation must be taken from a fresh lesion deep inside the lung lobes, a procedure which requires narcosis of the pig.

Serology is a promising diagnostic method for detecting subclinical infections as well as clinical cases. The development of the enzyme-linked immunosorbent assay, ELISA, which are adaptable to simple serological test procedures and can be extensively automated, contributed to the dramatic expansion of diagnostic procedures during the 1980's and 1990's (Engval and Perlmann, 1972). The assay is based on enzyme-labelled immunoreactants, usually antibodies and more recently on specific monoclonal antibodies. ELISA assays are available in a variety of modifications, the basic sandwich method and the competitive or blocking ELISA modifications being the most widely used for the determination of antibodies. In general, ELISA applications are considered to offer several advantages over the earlier diagnostic methods, including faster availability of the results, improved diagnostic sensitivity, and higher diagnostic specificity (Scarman et al., 1997). Large numbers of samples are easily obtained from living animals for serological testing, thus increasing the reliability of the diagnosis (Martin et al. 1987). For instance, the number of seropositive animals in the subclinical form of *M. hyopneumoniae* infection in an infected herd can be as low as 4% (Levonen et al. 1992). If freedom from disease has to be proven with 99% confidence on such a farm, the sample size required is high, 27 samples when the number of breeding
sows is 30 and the disease prevalence being below 5% (Martin et al. 1987).

The use of colostrum samples for antibody determination in pigs offers further diagnostic possibilities, because IgG is known to concentrate in the colostrum (Stokes and Bourne 1989, Wallgren et al. 1998). The IgG concentration is at its highest at the moment of parturition, and subsequently starts to decline. The higher antibody concentration in colostrum lasts for 7-11 hours (Sørensen et al. 1992, Nicolet 1988), after which colostrum turns to milk, which contains less IgG than serum.

Antibodies, as measured by ELISA, have a long half life (Bereiter et al. 1990). Once pigs have seroconverted, they most often remain seropositive throughout their life. Therefore the antibody concentration of sow’s colostrum reflects the long-term infectious status of a herd. This is clearly of benefit when confirmation of the freedom from disease is required. Other recognised advantages areas follows: the collection of colostrum samples causes very little stress to the sow, colostrum contains more antibodies than serum, and colostrum samples can be taken by the herd owner and frozen in a home freezer, pending assay. This saves veterinary costs in sample taking.

Although the control of some respiratory diseases of swine in a number of countries have included the use of serology, concern has been expressed at the specificity and sensitivity of the serological methods used (Nicolet 1988, Nielsen 1988). For example, \textit{M. hyopneumoniae} and \textit{P. multocida} toxin each evoke
only a weak humoral antibody response, thus requiring a high level of sensitivity from the serological test used, while *A. pleuropneumoniae* possesses 12 partially cross-reacting serotypes: thus heterogeneity causes problems in the interpretation of results (Nielsen 1988).

1.2 Enzootic pneumonia caused by *Mycoplasma hyopneumoniae*

1.2.1 The agent, *M. hyopneumoniae*

The causative agent of enzootic pneumonia, *M. hyopneumoniae* (Mare and Switzer 1965, Goodwin et al. 1965), belongs to the mycoplasmas, which are the smallest prokaryotes known. They do not have a cell wall, but are bounded by a single trilaminar membrane (Rosenbusch 1994). The most important swine mycoplasmas are *M. hyopneumoniae*, which causes enzootic pneumonia, *M. hyosynoviae*, which can cause arthritis, and the mainly non-pathogenic *M. hyorrhinis* and *M. flocculare*.

1.2.2 Enzootic pneumonia

*Mycoplasma hyopneumoniae* causes pneumonia characterised by a chronic non-productive cough (Groendaelen 1972, Armstrong 1994, Kobisch and Friis 1996, Maes et al. 1996). The disease causes retarded growth rate and inefficient feed utilisation, and adversely affects the economic outcome of the affected swine farms (Morris et al. 1995, Maes et al. 1999). In severe cases, and in outbreaks in previously unaffected herds, abortions and stillbirths can occur (Schulman 1988). Pigs infected with *M.*
hyopneumoniae are predisposed to secondary bacterial infections, especially to A. pleuropneumoniae and P. multocida (Ross 1992). M. hyopneumoniae and secondary infections result in the so-called enzootic pneumonia complex, which is the most common swine respiratory disease (Ross 1992) and which has a more severe clinical picture than mycoplasmal pneumonia alone. M. hyopneumoniae has been shown to potentiate porcine reproductive and respiratory syndrome virus (PRRSV) induced disease and the occurrence of lesions (Thacker et al. 2000). Pigs free of M. hyopneumoniae have been shown to have a better average daily weight gain and a lower mortality rate (Tuovinen et al. 1994 b).

1.2.3 Epidemiology of M. hyopneumoniae

M. hyopneumoniae spreads readily by pig-to-pig contact. Subclinical carrier swine play an important role in spreading the disease. Although airborne transmission has been proposed (Christensen and Mousing 1992), in most cases the source of the infection can be traced back to direct pig-to-pig contact. The incubation period of M. hyopneumoniae infection is 10-16 days (Ross 1992). In a herd, M. hyopneumoniae spreads slowly and periods of 6 months-2 years have been reported between the introduction of the infection in a herd and the overcome of clinical signs (Goodwin 1984, Keller 1976). In endemically infected herds, the piglets become infected after the protection provided by maternal antibodies has disappeared. Seroconversion is at its peak at the age of 4 months (Yagihashi et al. 1993). Most pigs affected recover from mycoplasmal pneumonia and, in the absence of secondary bacterial infections, the pneumonic lesions
may disappear before the normal slaughter age of 6 months (Noyes et al. 1990, Wallgren et al. 1990). Among feeder pigs secondary infections are, however, common and the losses incurred through respiratory diseases are considerable (Rautiainen et al. 1991) and sows as a rule recover from *M. hyopneumoniae* infections. Sows which have farrowed twice or more often rarely transmit the agent (Waldman and Radtke 1937).

Spontaneous recovery on the entire pig herd is rare, if the herd has more than 12 sows. Once infected with *M. hyopneumoniae*, there are always pigs of susceptible age which continue to harbour the infection. The disease agent cannot be eliminated by medication, and while vaccination only reduces pneumonic lesions, it does not prevent the infection (Djordjevic et al. 1997, Dohoo and Montgomery 1996, Zhang et al. 1994). Possible methods of eradicating the pathogen include the complete restocking or the adaption of a Waldman sanitation in which only the sows that have had two or more litters are retained (Waldman and Radtke 1937). One reason for the establishment of the Finnish Pig Health Scheme was the need to produce a replacement stock for the establishment of *M. hyopneumoniae* free herds with high breeding value.

Morbidity in mycoplasmal pneumonia is very high. In the major pig-producing countries most of the conventional pig herds are affected (Armstrong 1994). In the United States, a study of slaughter swine revealed that 99% of the herds had pigs with pneumonic lesions, and a screening of antibodies in breeding herds indicated that 60% of the herds were affected (Ross 1992).
In Finland, the disease status with respect to enzootic pneumonia is considerably better. The elite breeding herds are included in the disease-control program and thus are free of enzootic pneumonia. In south-western Finland only 10-15% of piglet-producing multiplying herds are affected (Schulman 1988, Tuovinen et al. 1994a), and in western Finland, 30% (Rautiainen 1998).

1.2.4 Diagnosis of *M. hyopneumoniae*

Diagnosis of *M. hyopneumoniae* infection has been traditionally based on the detection of clinical signs of pneumonia and the examination of lesions post-mortem (Keller 1976, Goodwin 1977). Recording of clinical signs alone is not, however, an especially accurate diagnostic method because, as mentioned earlier, surveillance through clinical herd inspection alone failed to detect 30% of the infected herds (Sørensen et al. 1993). Furthermore, periods of subclinical manifestation are common in *M. hyopneumoniae* infections (Nicolet 1988). On the other hand, environmental conditions such as dusty pig-house air can also lead to mis-diagnosis of the disease.

Typical, but not pathognomonic macroscopical pathological lesions of *M. hyopneumoniae* pneumonia consist of purple to grey lobular areas at the apical and diaphragmatic lobes of the lungs (Häni et al. 1976, Armstrong 1994). Histologically lesions are characterised by peribronchial and perivascular aggregates of lymphocytes (Häni et al. 1976). On a herd basis, diagnosis is routinely made at slaughter examination: however, the detection of lung lesions at slaughter has proved to be a poor indicator of

More refined post-mortem diagnosing methods which have been assessed include immunofluorescence and the isolation of *M. hyopneumoniae* (Armstrong 1994). Immunofluorescence has provided a specific diagnostic method for *M. hyopneumoniae*; however, this procedure often yields false negative results, thus lacking sensitivity (Armstrong 1994). Regarding culture techniques *M. hyopneumoniae* is a fastidious grower. Absolutely fresh lung samples are required, and isolation is often obscured by other mycoplasmas especially *M. hyorrhinis* (Armstrong 1994, Ross 1992). Immunofluorescence and the cultivation of *M. hyopneumoniae* are not readily adaptable for use as diagnostic methods within disease-control programs, due to the fact that they are time consuming and too often yield false negative results. Polymerase chain reaction (PCR) offers a rapid and precise means for *M. hyopneumoniae* diagnostics (Stemke et al. 1994, Harasawa et al. 1991, Mattson et al. 1995, Artiushin and Minion 1996, Blanchard et al. 1996, Stemke 1997). However, no PCR application suitable for routine diagnostic use has so far been developed.

Serological methods for detecting *M. hyopneumoniae* antibodies include indirect hemagglutination (IHA), hemagglutination inhibition (HI), complement fixation (CF) test and enzyme-linked immunosorbent assays (ELISA's) (Ross 1992). The ELISA is the most sensitive and specific serological

1.3 Catarrhal pneumonia and pleuritis caused by *Actinobacillus pleuropneumoniae*

1.3.1 *A. pleuropneumoniae*

*A. pleuropneumoniae* (formerly *Haemophilus pleuropneumoniae, Haemophilus parahaemolyticus*) is a small, gram-negative capsulated rod, with haemolytic activity on blood agar media (Pfeiffer 1892, Shope 1964). *A. pleuropneumoniae* has two biovars, of which biovar 1 is nicotine amide dinucleotide (NAD) dependent and biovar 2, which is NAD independent although it requires specific pyridine nucleotides or pyridine nucleotide precursors for its NAD biosynthesis. At present, 12 main serotypes of *A. pleuropneumoniae* biovar 1 have been identified (Nielsen 1988, Martin and Mulks 1999, Nielsen et al. 2000). Capsular polysaccharides and cellular lipopolysaccharides are responsible for the serotype. Pathogenicity varies between serotypes, and also between strains within one serotype (Rosendal et al. 1985, Brandrecht and Smith 1987). The serotypes 1, 5, 9, 10, and 11 are more virulent than the others (Nielsen 1982, Komal and Mittal 1990). In Finland, serotype 2 has been responsible for severe acute infections with high morbidity and mortality if the swine affected are not treated (Prof. A. Schulman, personal communication).

*A. pleuropneumoniae* secretes toxins which have been implicated as important virulence factors (Jansen et al. 1993,
Kamp et al. 1994, Schaller et al. 1999). The nomenclature of these toxins has been standardised as *A. pleuropneumoniae*-RTX-toxin I (ApxI), *A. pleuropneumoniae* RTX-toxin II (ApxII), and *A. pleuropneumoniae*-RTX-toxin III (ApxIII). Before standardisation, several names were used for these toxins, such as cytolysin, haemolysin and pleurotoxin. These toxins are not all secreted by all serotypes: ApxI is secreted by serotypes 1, 5, 9, 10, and 11, ApxII by all serotypes except serotype 10, and ApxIII by serotypes 2, 3, 4, 6, and 8.

1.3.2 Disease caused by *A. pleuropneumoniae*

Pathogenic *A. pleuropneumoniae* causes a severe necrotising pneumonia and pleuritis in pigs. In acute infection, a mortality rate of up to 100% is possible (Nicolet 1992). A chronic but less severe form of the disease is also known. This highly infectious pathogen is encountered world-wide and it is a major cause of morbidity and mortality in pigs (Olander 1963, Shope 1964, Nicolet and König 1966, Nielsen 1970, Little 1971, Thörne and Hakansson 1970, Schulman et al. 1975, Hsu et al. 1976, De Jong 1978). In effect, *A. pleuropneumoniae* has spread to all countries where intensive pig production is practiced (Nicolet 1992). The economic importance of the disease is principally due to the high mortality rate and the veterinary costs incurred in acute outbreaks. *A. pleuropneumoniae* infection may be complicated by other respiratory infections such as mycoplasmal, viral and other bacterial pneumonias, and is, therefore, a component of the respiratory disease complex problem.
The pathogenesis of pleuropneumonia associated with the infection is not fully understood (Nicolet 1992, Oswald et al. 1999). In addition to Apx-toxins, capsular polysaccharide, lipopolysaccharide and outer membrane proteins also act as virulence factors for *A. pleuropneumoniae* (Komal and Mittal 1990). *A. pleuropneumoniae* primarily infects neutrophils and alveolar macrophages (Min and Chae 1998). The pathogenicity is multifactorial, involving the virulence factors of the bacterium and endogenous host factors including induced immunity (Nicolet 1992). However, pleuropneumonia can be caused by Apx toxins alone (Kamp et al. 1997).

Passively transferred serum antibodies confer effective protection against pleuropneumonia (Bossé et al. 1992). The protection elicited by capsular polysaccharides is highly serotype specific, whereas antibodies to the outer membrane proteins are cross-protective (Thwaits and Kadis 1991, Martin and Mulks 1999).

The acute form of pneumonia caused by *A. pleuropneumoniae* is characterised by severe dyspnea, often with blood-tinged froth in the nostrils, and death (Bertram 1985, Nicolet 1992). The acute form of the disease resembles septic shock in humans. In the peracute form the incubation period is less than 24h, and an animal can die suddenly without preceding signs. The chronic form is characterised by a cough of varying intensity, and loss of appetite resulting in decreased weight gain. In chronically infected herds there are often subclinically infected
carrier animals that are reservoirs of the infection for new susceptible individuals.

1.3.3 Epidemiology of *A. pleuropneumoniae*
Direct pig-to-pig contact is the most common route of spread of *A. pleuropneumoniae*; however, it can also be spread by infectious aerosols within a short distance, e.g. pen to pen, or one room to another. Contaminated exudates from acutely affected pigs can be spread to a new pig population by people, transportation vehicles and other means (Nicolet 1992).

The predominant serotype of *A. pleuropneumoniae* differs between regions. Serotype 1 and 5 are the most widely spread in Canada and the United States (Mittal et al. 1982, Rapp et al. 1985). In the Netherlands, serotype 9 causes the most severe epidemics (Kamp et al. 1987). Serotype 2 is predominant in Sweden, Denmark and Schleswig-Holstein (Gunnarsson 1980, Kielstein and Wuthke 1998, Møller et al. 1992, Nielsen 1982). In Finland, only one the serotype, serotype 2, has been isolated from acute cases of pleuropneumonia to-date.

Once *A. pleuropneumoniae* infection has become established in a herd, it is difficult to eliminate the infectious agent, although the herd appear to be healthy (Nicolet 1992). The persistence of *A. pleuropneumoniae* in convalescent pigs contributes significantly to the distribution of the disease (Hennig et al. 1999).

Serological testing by CF followed by the removal of seropositive pigs has been proposed as a means for eliminating *A. pleuropneumoniae* infection from a herd (Nielsen 1982). This method has also been used in Finland. However, while in the short term the herd in question had appeared to be *A. pleuropneumoniae* free, the infection later reappeared, indicating that all the carrier animals had not been removed.

1.3.4 Diagnosis of *A. pleuropneumoniae*

In acute cases due to *A. pleuropneumoniae* infection, the clinical signs are characteristic and dramatic, whereas chronic pleuropneumonia, and infections with nonvirulent strains may appear in the subclinical form (Nicolet 1992). Typical pathological changes are an aid for the diagnosis of *A. pleuropneumoniae* related diseases. The predominant gross lesions are diffuse fibrinous pleuritis, extensive amounts of blood-tinged froth in the bronchial and tracheal lumina, pulmonary necrosis, haemorrhage, infiltration of lymphocytes and macrophages, vascular thrombosis and fibrin deposition in blood vessels and lymphatics (Bertram 1985). In the chronic form, there is focal chronic adhesive pleuritis mainly in the caudal areas of the lungs and yellow to grey-yellow necrotic masses (Häni et al. 1973). However, chronic lesions caused by *A. pleuropneumoniae* cannot be distinguished
on histopathological grounds from chronic pleuritis caused by other bacteria, such as *P. multocida* (Nicolet 1992).

Isolation of *A. pleuropneumoniae* requires NAD-enriched media and incubation preferably in a 5% CO₂ atmosphere (Gunnarson 1979, Gunnarson 1990). The bacterium grows well from samples obtained from acute lesions, but the specimens obtained from chronic lesions are problematic and *A. pleuropneumoniae* may be isolated occasionally from such cases.

Detection of serotype specific antibodies is widely used in *A. pleuropneumoniae* diagnosis (Nielsen 1982). The method most commonly used has been CF. Certain cross reactions are regularly detected by this method, namely 1-9-11, 3-6-8, and 4-7 (Nielsen 1988). The antibodies detectable by CF arise within two weeks after the infection, and they persist for 3-4 months. Antibodies detectable by various types of ELISA (Nicolet et al. 1981, Gunnarson 1980, Zimmermann et al. 1990, Nielsen et al. 1991, Bossé et al. 1990, Nielsen 1995, Stenbaek et al. 1997, Leiner et al. 1999) last for longer period of time, and in practice, for the lifetime of a sow.

A number of novel diagnostic methods have been recently published. Most of them have to-date been used mainly in research but, in the future, they can potentially be applied for the laboratory diagnosis of *A. pleuropneumoniae*. A specific PCR assay for the gene (*omlA*) coding for an outer membrane protein of *A. pleuropneumoniae* serotypes 1 and 5 has been described
(Gram and Ahrens 1998). Putative \textit{A. pleuropneumoniae} isolates can be verified by PCR (Moral et al. 1999). Various monoclonal antibodies (mAbs) for distinct \textit{A. pleuropneumoniae} strains have been raised (Stenbaek et al. 1997, Lebrun et al. 1999, Bouh and Mittal 1999). Restriction endonuclease analysis and plasmid profiling could be useful in epidemiological studies of porcine pleuropneumonia in the future (Wards et al. 1998).

1.4 Progressive atrophic rhinitis (PAR) caused by toxigenic \textit{P. multocida}

Progressive atrophic rhinitis is caused by toxigenic strains of \textit{Pasteurella multocida}. \textit{Bordetella bronchiseptica} and non-toxigenic strains of \textit{P. multocida} are also involved in the atrophic processes affecting the porcine conchae. A consensus has been reached on the definition of the names of these atrophic disorders (De Jong 1992). The name “atrophic rhinitis” includes all atrophic processes of porcine conchae, and the term “progressive atrophic rhinitis” (PAR) applies to a contagious disease caused by toxigenic strains of \textit{P. multocida}. This etiological definition of PAR facilitates the identification of subclinically infected herds that are capable of transmit this severe clinical disease.

1.4.1 Toxigenic \textit{P. multocida}

\textit{P. multocida} is a member of the genus \textit{Pasteurellaceae}, which consists of the genera \textit{Pasteurella}, \textit{Actinobacillus}, and \textit{Haemophilus}, along with several other groups of animal parasites and pathogens (Mutters et al. 1989). \textit{P. multocida} is an important
animal pathogen which is distributed throughout the world (Rimler and Rhoades 1989). The bacterium can also infect humans (Avril et al. 1990, Nielsen and Frederiksen 1990). Toxin-producing strains of *P. multocida* were first shown to cause non-reversible conchal atrophy by I'lina and Zasukhin (1975) and De Jong et al. (1980). Toxigenic strains of *P. multocida* can be found within the serotypes D and A (*P. multocida* has five capsule serotypes A,B,D,E, and F).

*P. multocida* does not colonise the intact respiratory epithelium. The epithelium is first damaged by another infectious agent or by exposure to high ammonium concentrations in the pig-house air (Hamilton et al. 1996). The most common primary invader in PAR is *Bordetella bronchiseptica* (De Jong 1992, Sakano et al. 1997). Apx-toxins of *A. pleuropneumoniae* render the infected animal more susceptible to secondary infections such as *P. multocida* through impaired function of porcine alveolar macrophages (PAM) (Cruijsen et al. 1992, Chung et al. 1993). Also *M. hyopneumoniae* infection can render the lungs susceptible to *P. multocida* colonisation (Amass et al. 1994). In challenge models, *Pasteurella multocida* toxin (PMT) has been demonstrated to be capable of causing progressive atrophic rhinitis alone (Foged 1988, Van Dieman et al. 1994).

PMT is a proteinous toxin the production of which is an essential component for the formation of irreversible conchal atrophy. The estimates of its molecular weight range from 112 to 160 kDa (Rimler and Rhoades 1989) and 143kDa (Foged et al. 1988). The toxin produces haemorrhage and is dermonecrotic in
guinea pig skin. The toxin is lethal for mice, rats, guinea pigs, swine and turkeys. Toxin-producing *P. multocida* strains have been found in swine, cattle, goats, rabbits, cats, dogs, turkeys, and man (Nielsen and Fredriksen 1990). Naturally occurring progressive atrophic rhinitis has also been described in goats (Rimler and Rhoades 1989, De Jong 1992). Undefined *Pasteurella* strains which produce a similar toxin have caused atrophic rhinitis in cattle (Kamp et al. 1990).

1.4.2 Progressive atrophic rhinitis (PAR)

Progressive atrophic rhinitis is a respiratory disease of pigs characterised by chronic progressive atrophy of the nasal conchae, sneezing and, in severe cases twisting or shortening of the snout, and nasal haemorrhage (De Jong 1992). The form of the disease varies from a subclinical manifestation of the disease to a severe form associated with considerable growth retardation.

The clinical signs of progressive atrophic rhinitis are associated with the toxic effects of PMT. PMT induces a rapid increase in the number of chondroclasts and osteoclasts (Martineau-Doizé et al. 1991, Gwaltney et al. 1997), and thus affects bone formation in the nasal conchae, causing even complete atrophy of conchae in some cases. Parenteral injections with PMT induced liver cirrhosis, renal failure, marked decrease of peripheral blood lymphocytes without lysis, and growth retardation (De Jong 1992). Systemic changes induced by PMT are weight loss, hepatic necrosis, leucosytosis, elevated complement titre and, in high doses, death (Pettit et al. 1993). The clinical signs seen in pigs kept under farming conditions include
sneezing, lachrymal secretion, nasal bleeding, distortion of the snout, poor growth resulting in overall poor appearance and uneven growth pattern of the pigs.

1.4.3 Epidemiology of toxigenic *P. multocida*

The most important route by which PAR is introduced into a previously uninfected herd is by carrier animals (De Jong 1992). However, infectious aerosols and fomites also play a role in the transmission of atrophic rhinitis (Thomson et al. 1992).

The clinical picture of progressive atrophic rhinitis differs depending on the housing conditions and management factors on the farm (De Jong 1992). High ammonia content in pig house air enhances colonization by toxigenic *P. multocida* on the nasal turbinates (Foged et al. 1989, Andreasen et al. 2000). Also previous respiratory infections such as enzootic pneumonia rends pigs more susceptible for progressive atrophic rhinitis (Ross 1992).

In an infected herd, the prevalence both of clinically diseased animals and non-clinical carriers of the toxigenic *P. multocida* bacterium varies over the course of time. Subclinical periods ranging from 3 months to 2 years are commonly reported between the periods when the disease is evident.

Vaccines against atrophic rhinitis are available. These are effective in preventing and alleviating the clinical disease, but they do not prevent infection by toxigenic *P. multocida*. 
1.4.4 Diagnosis of PAR
Traditionally diagnosis of atrophic rhinitis has been determined by estimating the degree of conchal atrophy post-mortem (Martineau-Doizé 1991). This method can be used to estimate the severity of atrophic rhinitis in a herd, but the presence of toxigenic *P. multocida* and thus progressive atrophic rhinitis cannot be determined by this means alone.

Isolation of *P. multocida* from progressive atrophic rhinitis cases in swine is best achieved by culturing nasal swabs or tonsil swabs or biopsies. Because other nasal flora may mask the presence of *P. multocida*, selective media should be used (Pedersen 1983). The toxicity of the isolate can be measured by the mice lethality test, guinea pig skin test, ELISA, by testing cytopathogenicity on embryonic bovine lung or vero cells, and by detecting the toxin production gene by means of DNA probes (De Jong et al. 1980, Pedersen 1983, Foged et al. 1988, Pennings and Storm 1984, Kamps et al. 1990, Rutter and Luther 1984). Of these methods, the ELISA has been found to be the best method for the determination of toxicity (Foged et al. 1988, De Jong 1992).

Due to a lack of sensitive tests, serology has not been used for the control of PAR in pig herds. However, an ELISA has been used for PMT antibody measurement in vaccine trials (Foged et al. 1989, Foged et al. 1990). A serum neutralisation test on embryonic bovine lung (EBL) cells has also been described (Bechmann and Schöss 1990, Alt et al. 1992).
When choosing a diagnostic method for detecting PAR in pig herds, especially in the case of disease control programs, the fact that interruptions between periods of clinical disease are typical for chronic PAR in a herd, should be taken into account. These subclinical phases can vary from 2 months to about two years (De Jong 1992). Herd monitoring based on clinical and pathological features alone cannot guarantee the absence of infectious PAR in a pig herd. Complementary monitoring of a herd, based on bacteriological and/or serological detection of the PAR-toxigenic \textit{P. multocida}, has been regarded as being necessary in order to obtain sufficient information about the PAR-infected or PAR-free status of the herd (De Jong 1992, Matscullat et al. 1994).
2. Aims of the study

1. To employ commercial antibody test kits for *M. hyopneumoniae* and to evaluate their usefulness in surveying the disease status in breeding herds using sow colostrum as a sample.

2. To create an enzyme-linked immunosorbent assay (ELISA) for the detection of *A. pleuropneumoniae* antibodies.

3. To modify a *P. multocida* toxin ELISA to detect *P. multocida* toxin antibodies and to assay the toxin antibodies in sow’s colostrum.

4. To determine the prevalence of antibodies to *M. hyopneumoniae, A. pleuropneumoniae* and *P. multocida* toxin in breeding sows.

5. To determine the prevalence of different serotypes of *A. pleuropneumoniae* in different areas of the country in order to be able to effectively target the national disease-control program.

6. To evaluate the usefulness of antibody assays in the disease-control program for swine breeding farms.
3. Materials and methods

3.1 Collection of colostrum and serum samples

For the *M. hyopneumoniae* (I), *A. pleuropneumoniae* (III) and *P. multocida* (V) toxin antibody surveys, the herd owners of the 193 farms participating in the Finnish Pig Health Scheme were requested to take colostrum samples from all their sows that farrowed during a period of six months. Sampling the same farm twice a year facilitated the follow-up of the disease situation. Between November 1989 and April 1991 a total of 6,256 colostrum samples were received. Eight farms did not participate in the survey.

In order to obtain reliable results from antibody assays of colostrum, the samples were required to be taken during farrowing or immediately afterwards. In order to control the right sampling time, the farrowing time (delivery of the first piglet) and the sampling time were recorded. The samples were kept frozen on the farms and sent to the laboratory in batches of 10 to 20.

*M. hyopneumoniae* samples

The final number of herds tested for *M. hyopneumoniae* antibodies (I) was 185. All the 5,830 samples received were tested for *M. hyopneumoniae* antibodies. When positive or suspicious colostra were recorded, the farmers were asked to send extra follow-up samples. A total of 426 such samples were received from 20 herds.
For testing the repeatability of Chekit®Hyoptest for detecting \textit{M. hyopneumoniae} antibodies (II), 60 colostrum samples were selected from the above mentioned samples and 20 serum samples were also examined. Samples from both infected and noninfected herds were included.

The diagnostic performances of two different commercial \textit{M. hyopneumoniae} ELISA test kits (II) were compared, using samples from 7 selected herds. The farms were chosen so that on 3 of the farms the pigs had clear signs of enzootic pneumonia, and on 4 farms the animals were non-clinical and in the latter case, only a single positive reactor was found in preliminary assays.

In this study a total of 153 colostrum samples were assayed with both Chekit® Hyoptest and DAKO \textit{M. hyopneumoniae} ELISA.

\textbf{A. pleuropneumoniae} samples

The amount of colostrum in some samples was sufficient only for the \textit{M. hyopneumoniae} survey. This resulted in fewer sows and herds being tested for \textit{A. pleuropneumoniae} serotype 2 antibodies (III): 5,477 colostrum samples from 182 herds were tested. All the sows in 154 herds were tested, and 17\% to 68\% of the sows in 28 herds.

In addition to colostra, serum samples for the \textit{A. pleuropneumoniae} 12 serotypes survey (IV) were randomly collected from sows in Finnish slaughterhouses. At the time of
this survey there were seven abattoirs slaughtering swine in Finland. The number of samples taken in each slaughterhouse was proportional to the sow population in the collection area. A total of 736 blood samples were collected. In some of the samples there was too little serum to test all the 12 serotypes, and therefore the number of samples assayed for the different serotypes varied as follows: 736 for serotypes 1 and 2, 718 for serotype 3, 690 for serotypes 4-6, and 675 for serotypes 7-12.

*P. multocida* samples

For *P. multocida* toxin antibodies (V) 5,650 colostrum samples from 188 herds were tested. Six further herds with applications pending for inclusion in the Scheme were allowed to participate in the PMT antibody survey. All the sows were tested in 166 herds, but 22 farmers sampled only some of their sows. Producers who had sent spoiled samples (31 batches, 453 samples) were asked to collect new colostra from successive farrowings.

In order to reveal the origin of the PAR infection on farm no. 20, serum samples, which were collected from 58 imported pigs during the quarantine period, were included in the survey and screened for *P. multocida* toxin antibodies (V).
3.2 Bacterial strains used for the production of *A. pleuropneumoniae* antigen and for immunisation of rabbits

The following *A. pleuropneumoniae* strains were used for ELISA antigen preparation and for immunising rabbits to obtain antisera for testing cross reactions: serotype 1: 4074, serotype 2: ATCC 27089, serotype 3: ATCC 27090, serotype 4: M 62, serotype 5a:K 17, serotype 5b: L 20, serotype 6: Femø, serotype 7: MF 83, serotype 8: 405, serotype 9: CVJ 13261, serotype 10: 3039, serotype 11: 56153, and serotype 12: 8329. The ATCC type strains were obtained from the American Type Culture Collection, and the other strains were generous gifts from Statens Veterinære Serumlaboratorium, Copenhagen, Denmark.

3.3 Control serums in *A. pleuropneumoniae* ELISA

The positive control serum used in *A. pleuropneumoniae* serotype 2 ELISA (III) was a field serum obtained from a herd of a progeny testing station previously free of *A. pleuropneumoniae*, but which had subsequently been acutely infected with *A. pleuropneumoniae* serotype 2. The serum was collected two months after the acute outbreak of the disease. The serum was tested with a complement fixation test (CF) (Nielsen 1982) and gave a titre of 1:64. Using a competitive ELISA (Nielsen et al. 1991) the titre was 1:32. Checkerboard titration was performed on
the sera and the antigen. A dilution of 1:1000 of the serum was chosen as the best working dilution for an antigen diluted to 1:50.

The negative control serum (III, IV) was a serum from a disease-control herd with a long known *A. pleuropneumoniae*–free history and presently having no clinical signs of pneumonia. The pigs that were bled had no pneumatic lesions in slaughterhouse examination. The serum was found to be negative for *A. pleuropneumoniae* antibodies with the CF assay and the competitive ELISA.

Rabbit antisera against each serotype was used for ELISA’s for the other serotypes (IV). In serotype 3 and 4 assays swine antisera against these serotypes were used. These sera were generous gifts from SVA, Sweden. Checkerboard titrations were performed to determine the suitable dilution for the control serums. The positive control serum was used in a dilution which gave an OD value of 0.5 after 10 min reaction.

3.4 Performing the *M. hyopneumoniae* ELISA

Colostral and antibodies against *M. hyopneumoniae* (I,II) were determined according to the manufacturer's instructions using a commercial ELISA kit, Checkit®Hyoptest, Dr. Bommeli AG, Bern, Switzerland. Diluted porcine or colostra (1:100) were added to the microtiter wells previously coated with Tween 20 solubilized crude *M. hyopneumoniae* antigen (Bommeli 1986, Nicolet et al. 1980). After 1.5 h incubation at room temperature
the plate was washed with PBS-Tween 20, and a rabbit anti-swine horseradish peroxidase (HRP) conjugate was added. After 1.5 h incubation at room temperature the plate was washed and then incubated with a chromogen solution (ABTS; 2,2'-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] diammonium). The colour reaction was stopped with 0.5% SDS (sodium dodecyl sulphate). The photometric results were obtained at a wavelength of 409 nm using a photometer (Multiscan MCC, Labsystems, Helsinki, Finland). Samples which gave an optical density (OD) 95% or over of the positive control serum (weak positive) were regarded as positive, and samples which gave an OD 70-95% of the positive control were regarded as suspicious.

To determine any cross-reactions between *M. hyorhinis* (I), which is commonly found in pigs in Finland (Schulman et al. 1970), and *M. hyopneumoniae*, rabbit hyperimmune sera were tested in serial dilution from 1:100 to 1: 3200 against *M. hyopneumoniae* antigen using the ELISA assay. The rabbit hyperimmune sera against *M. hyopneumoniae* and against *M. hyorhinis* were donated by Dr. Freund, Arhus, Denmark.

The Dako *M. hyopneumoniae* antibody ELISA (II) (DAKO A/S, Denmark) is a competitive ELISA in which a specific monoclonal antibody (mAb) competes with any comparable antibody in the samples (Feldt et al. 1992). The kit was a prototype, which had been sent to our Laboratory for evaluation of its performance. The samples were diluted 1:10 and incubated in microwells precoated with *M. hyopneumoniae* antigen for 1.5 h at room temperature. A specific peroxidase conjugated
monoclonal antibody to *M. hyopneumoniae* was added to the wells to compete with the samples. The conjugate and the samples were incubated together for 15 min, and thereafter the wells were washed. A chromogenic substrate (OPD) was added, the colour was allowed to develop for 10 min in the dark, and the colour reaction was stopped by adding sulphuric acid. The optical densities were read by means of a photometer (Multiscan MCC, Labsystems, Helsinki, Finland) at a wavelength of 492 nm. The results were interpreted as percentage of the buffer control wells (Buffer + conjugate + chromogen), and samples which had an OD of less than 50% of the buffer control were regarded as positive.

3.5 Immunisation of rabbits to obtain *A. pleuropneumoniae* antisera (III, IV)

Antibodies against 12 *A. pleuropneumoniae* serotypes were raised in rabbits. The immunisation process was performed as follows: 6 h cultures of *A. pleuropneumoniae* on PPLO agar (Difco, Detroit, MI, USA) were harvested in 1% formol saline, kept overnight, diluted with saline to the density of Mc Farland tube no.4, mixed with Freunds incomplete adjuvant (Difco Laboratories, Detroit, Michigan, USA) and injected intramuscularly into two sites (1ml + 1ml). Two rabbits were injected with each serotype. The booster injections (0.5 ml) were given intravenously twice a week for three weeks. The bacterial suspension for the first booster injection was of low density; Mc Farland tube no.1, thereafter the injections were of the density of Mc. Farland no.4. The first three booster injections were
suspended in 0.2% formol, and the last three were without formol. The rabbits were bled one week after the last i.v. injection, and the sera were stored at -70°C until use.

3.6 Preparation of *A. pleuropneumoniae* antigen

The antigen for the *A. pleuropneumoniae* ELISA (III, IV) was prepared according to Gunnarson (1979), with some modifications. Each of the above-mentioned *A. pleuropneumoniae* strains was grown on (pleuropneumonia like organisms) PPLO agar for 6 h. The bacterial growth was suspended in phosphate buffered saline (PBS, pH 7.4). The suspension was centrifuged at 4000xG for 20 min at 4°C. The sediment was weighed and suspended in distilled water to 20 times the weight of the sediment. An equal volume of 90% phenol was added and the mixture was heated under magnetic stirring for 20 min at 62°C. The mixture was then centrifuged (4000xG) for 20 min at 4°C. The supernatant was collected, the pellet was resuspended with 20 times its weight of water, heated under magnetic stirring, centrifuged (4000xG) and the supernatant was collected. The supernatants were dialysed against distilled water overnight at 4°C and used as an antigen in the *A. pleuropneumoniae* ELISA at a dilution of 1:50.

3.7 Coating of microwell plates with *A. pleuropneumoniae* antigen

For coating the microwell plates for *A. pleuropneumoniae* ELISA (III, IV) the antigen was diluted 1:50 with 0.05M sodium bicarbonate buffer, pH 9.5-9.6. 100 µl aliquots of the diluted antigen were
pipetted into 96 well microtiter plates (Nunc Immunoplate Maxisorb, Roskilde, Denmark). The plates were incubated in a humid chamber for 6h in room temperature and then washed with three times PBS+ 0.5% Tween 20. Gelatine solution (1%, blocking reagent) in PBS-Tween was added to the wells, which were then incubated overnight in room temperature in a humid chamber. Thereafter, the plates were washed three times with PBS-Tween and stored wrapped in plastic at 4°C until used.

3.8 *A. pleuropneumoniae* ELISA assay (III)

Colostrum samples were thawed and centrifuged for 6 min at 13,000 rpm (Microliter, Hettig Zentrifugen, Tuttlingen, Switzerland) and the cream fraction was discarded. The colostrum samples and the positive and negative control sera were diluted 1:1000 with PBS + 0.05% Tween20 + 10% fetal bovine serum (FBS). 100µl aliquots of diluted samples were pipetted into the coated microtiter wells and the plates were incubated for two hours in a humid chamber at room temperature, and thereafter washed three times with PBS-Tween. Peroxidase-conjugated rabbit anti-swine-Ig, heavy and light chain specific (Cappel, Organon Teknika Corporation, Cochranville, PA, USA) was diluted 1:4000 with PBS + 0.05% Tween20 + 10% FBS and added to the wells (100 µl). The plates were again incubated for 2 h in a humid chamber at room temperature and washed three times with PBS-Tween. A 2,2'-azino-bis diammonium tablet (10 mg) was dissolved in 45 ml of substrate buffer (0.1M citric acid +
0.1M Na₂HPO₄ added to a pH of 4.0) and 9ml of 33% H₂O₂ was added. 100 µl of substrate was pipetted into the wells and the colour reaction was allowed to develop for 5-10 min. 50 µl of sodium dodecyl sulphate (SDS) was added to stop the colour reaction. The optical densities (OD) were measured using a photometer (Multiscan MCC, Labsystems) at a wavelength of 560 nm. The samples which gave an OD equal to or above that of the positive control were regarded as positive.

3.9 The ELISA assay for 12 *A. pleuropneumoniae* serotypes (IV)

The ELISA assay used for the detection of 12 serotypes was a modification of that used for the serotype 2 assay. The samples and the negative control serum were diluted 1:1000 with PBS + 0.05% Tween 20 + 10% fetal bovine serum (FBS). Diluted samples (100 µl) were pipetted into the coated microtiter wells. The plates were incubated for 2 h in a humid chamber at room temperature and thereafter washed three times with PBS-Tween. Peroxidase conjugated rabbit anti-swine-Ig, heavy and light chain specific (Cappel, Organon Teknika Corporation), was diluted 1:4000 with PBS + 0.05% Tween20 + 10% FBS and added to the wells (100 µl). Peroxidase conjugated goat anti-rabbit-Ig (Dako A/S, Glostrup, Denmark) was diluted 1:750 and added to the positive control and buffer control wells. The plates were again incubated for two hours in a humid chamber at room temperature, and washed three times with PBS-Tween. A 2,2'-azino-bis diammonium tablet (10 mg) was diluted in 45ml of substrate
buffer (0.1M citric acid + 0.1M Na₂HPO₄ added to pH 4.0) and 9µl of 33% H₂O₂ was added. Substrate (100 µl) was pipetted into the wells and the colour reaction was allowed to develop until the OD of the positive control serum was approximately 0.5. The OD of the negative control serum should not exceed 0.2. The reaction was stopped by adding 50 µl of 0.5% sodium dodecyl sulphate (SDS). The optical densities (OD) were measured using a photometer (Multiscan MCC, Labsystems, Helsinki, Finland) at a wavelength of 560 nm. Samples giving an OD of 0.5 or more were regarded as positive.

3.10 Testing cross reactions in *A. pleuropneumoniae* ELISA (III, IV)

Rabbits were immunised against strains of serotypes 1, 2, 3, 4, 5a, 5b, 6, 7, 8, 9, 10, 11, and 12. Reactions with each serotype antigen were tested at 10 serum dilutions between 1:100 and 1:6000. Serotype 2 rabbit antiserum was further diluted to 1:8000, 1:12,000, 1:16,000, 1:20,000, and 1:24,000. The ELISA test was performed as described above, using peroxidase-conjugated goat anti-rabbit Ig-G as a conjugate.

3.11 Repeatability of the test in *A. pleuropneumoniae* ELISA (III)

To test the repeatability of the *A. pleuropneumoniae* serotype 2 ELISA, 44 colostrum samples were assayed twice using microtiter plates which had been coated on separate occasions.
Agreement between the assays was assessed according to Martin et al. (1987).

3.12 ELISA for detection of *P. multocida* toxin (PMT) antibodies (V)

The serological blocking ELISA was developed using the anti-PMT kit of Dako (Dako A/S, Glostrup, Denmark). Both monoclonal and polyclonal antibodies, which recognise different antigenic epitopes of PMT, were used in the ELISA. The wells of the ELISA plate were coated with the monoclonal antibody, while the polyclonal antibody was conjugated with horseradish peroxidase (HRPO). PMT was trapped on the solid phase by the MAb. The antibodies in the samples inhibited the binding of RPO conjugate to the PMT antigen. The amount of bound conjugate was measured spectrophotometrically after the addition of chromogen. The monoclonal antibody and the conjugated polyclonal antigen were included in the commercial test kit.

The next step was developed from the PMT ELISA in order to detect the anti-PMT-antibodies. The PMT and colostrum samples were first preincubated in a dummy plate. Equal amounts of PMT (0.2mg/ml) and colostrum, 40 ml of each, were placed in the wells (Foged 1988). Colostra were used in 1:2 dilution (dilution buffer PBS + 0.5% Tween 20 + 1% BSA); the sera were undiluted. The positive control used was antiserum, undiluted, obtained from a pig immunised with formaldehyde-treated PMT.
4. Results

4.1 The *M. hyopneumoniae* ELISA test

In the *M. hyopneumoniae* survey (I) 6,256 colostrum samples were tested for *M. hyopneumoniae* antibodies, and 61 positive or suspicious samples were found (Table 1). 33 of these samples came from herds in which no clinical signs of *M. hyopneumoniae* infection, no pathological findings indicative of *M. hyopneumoniae* infection and no further positive reactions in follow up samples were found. The herds were observed for two years after the first positive samples. Some samples were later found to be negative in confirmatory assays (II). The 33 herds which were found to have singleton reactors were considered to be non-infected, and the samples were regarded as false-positive samples.
Table 1. The results of serological, clinical and pathological examinations of 23 pig herds from which positive colostrum samples were obtained.

<table>
<thead>
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<th>Herd nr.</th>
<th>Sows</th>
<th>Samples</th>
<th>Positive</th>
<th>Suspected Signs</th>
<th>Pathology</th>
<th>Follow-up</th>
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<td>3 + EP</td>
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<td><strong>Group C. Follow-up still going on</strong></td>
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The OD values of positively reacting colostra in the positive herds varied between 0.243 and 2.000 (mean 0.678; SD ± 0.376). The mean OD value for the negative control serum was 0.064 (SD 0.052) and for negative colostra, 0.067 (SD 0.049).

The specificity of the test was 99.4%. This refers to the proportion of negative and suspicious colostra from the herds, which did not develop clinical or pathological enzootic pneumonia during the study period. Herd specificity based on herds without clinical enzootic pneumonia that tested negative, was 91.5%. The apparent sensitivity of the assay was 66.7%. The samples from two diseased herds gave ELISA ODs that were regarded as suspicious. The OD values were, however, near to the cut-off level of the positive control. If the suspicious reactions were to be categorised as positives, then the assay has a 100% sensitivity at the herd level.

4.2 Cross-reactions with *M. hyorhinis*

When specific, hyperimmune antisera against *M. hyopneumoniae* and *M. hyorhinis* were assayed using *M. hyopneumoniae* antigen, both reacted positively (I). With *M. hyopneumoniae* antiserum the strong reaction (OD 1.700) started to decline at dilutions between 1:2000 and 1:3200 (OD 1.102). The top level of *M. hyorhinis* antiserum was much weaker, OD 0.972, and the 1:1600 dilution (OD 0.312) was the last dilution that gave positive result.
4. 3 Repeatability of the Chekit®Hyoptest

When testing samples in two occasions with the Chekit®Hyoptest M. hyopneumoniae ELISA (II), samples found to be positive or suspicious in the first test tended to change to suspicious or negative respectively in the second assay. The negative samples remained negative. The observed proportion agreement was 75% (Martin et al. 1987). If suspicious samples were regarded as positive, then kappa was 0.65, and if suspicious samples were regarded as negative, kappa was 0.71, indicating good agreement.

4. 4 Comparison of the Chekit®Hyoptest and DAKO M. hyopneumoniae ELISA

The DAKO M. hyopneumoniae ELISA identified more positive animals (70/153) than the Chekit®Hyoptest (30/153) (II). Ten samples that were suspicious according to the Hyoptest were positive with the DAKO test. Due to the fact that both assays were used as herd tests, only herd sensitivity could be estimated. Both ELISAs were able to identify as positive the three M. hyopneumoniae diseased herds. The herds tested had been selected from known infected and noninfected herds (Table 1). Both ELISAs detected each of the three truly infected herds, indicating that the sensitivity is high enough for eradication requirements. The results of the study (II) suggested that the single reactors in noninfected herds were false positives. Based on these results the apparent specificity of the DAKO test was 99.3% and the apparent specificity of the Hyoptest was 98.0%.
4.5 Control sera in the *A. pleuropneumoniae* ELISA

The positive control serum for serotype 2 gave an average OD of 0.535 (SD ±0.126) for 143 determinations (III). In the 12 serotypes survey (IV) the mean OD value of the positive control serum was 0.640 (SD ±0.04), and varied between serotypes from 0.53 for serotype 5 to 0.7 for serotype 11.

The mean OD for the negative control serum was 0.1006 (SD ±0.048) in the serotype 2 assay (III) and 0.130 (SD ±0.080) in the 12 serotypes survey (IV).

The cut-off point of the ELISA for the 12 *A. pleuropneumoniae* serotypes (IV) was set at 0.5. This was chosen decided after testing different positive sera, serial dilutions of positive sera and sera from negative herds. In order to minimise the variability between assays, only positive control sera with an OD figure of 0.5 or higher were included. Because the OD values were assayed visually before stopping the reaction, the values varied only slightly from test to test.

4.6 Repeatability of the *A. pleuropneumoniae* ELISA

Of the 44 samples assayed twice in the *A. pleuropneumoniae* serotype 2 ELISA, 15 were coconsistently positive and 21 negative in both assays. In the agreement testing (Martin et al. 1987) kappa was 0.63, indicating good agreement.
4.7 Cross-reactions in *A. pleuropneumoniae* ELISA

The OD values of each serotype rabbit antiserum at a dilution of 1:1000 with each serotype antigen are presented in table 2. All these rabbit antisera reacted with the homologous antigen. In addition, serotypes 1, 9 and 11 cross-reacted. Serotypes 6 and 8 also cross-reacted, and serotype 5(a) antiserum reacted with serotype 6 antigen. Slightly different cross reactions were found when the field samples were studied: the OD values of the samples reacting with serotypes 9 and 11, 9 and 12, and 1 and 2 showed some correlation (p<0.01).

Table 2. Reactions of *A. pleuropneumoniae* serotype 1-12 sera against serotype 1-12 antigen. OD values with serum dilution 1:1000.

<table>
<thead>
<tr>
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<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5a</th>
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<td>0.04</td>
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<tr>
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<td>0.01</td>
<td>0.19</td>
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<td>0.68</td>
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<td>0.01</td>
<td>0.02</td>
<td>0.00</td>
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The cross-reactions were evident only at the initial, low serum dilutions. At higher dilutions the reactions were specific. The *A. pleuropneumoniae* serotype 2 antiserum reacted very strongly with serotype 2 antigen to a dilution of 1:6000. Thereafter the ODs began to decline, but even at the dilution of 1:24,000 the OD was still 0.371.

4.8 Positive samples in the anti-PMT ELISA

The mean titre of the positive colostrum samples was 61.0, with a SD of ±141.1, minimum 1.11, and maximum 473.8. Compared to the positive control serum, the mean ratio of the titre sample: titre positive control (Ts:Tp) was 1.66, with a SD of ±3.36. Single positive samples were found in 11 herds. Based on repeated laboratory tests combined with clinical and pathological evaluations, these findings were categorised as false positives. The average titre of the false positives was 1.740 (SD ±0.230) and the average Ts:Tp ratio 0.051 (SD ±0.052).

4.9 Specificity and sensitivity of the anti-PMT ELISA

As only 11 false positive samples were recorded, a specificity of 99.8% was determinated. Owing to the low number of positive samples and the lack of a reference method, only a crude estimate of the specificity of the test could be made, however.
In the infected herd, 11 of the 21 sows had measurable amounts of antibodies to PMT in their colostrum. The apparent sensitivity at the individual level in this case was therefore at least 52%, and at the herd level, 100%. The recovery rate of toxigenic *P. multocida* from nasal swabs was 55%.

Spoiled samples of colostrum having a foul odour were found in 31 batches (453 samples). These samples gave a positive reaction in the anti-PMT ELISA. The farms of origin were requested to send a fresh batch of samples, and when these samples of good quality were assayed, they gave negative results.

**4.10 *M. hyopneumoniae* survey**

In the *M. hyopneumoniae* survey 6,256 colostrum samples from 185 breeding swine herds were tested for *M. hyopneumoniae* antibodies. Altogether 61 positive, or suspect, samples were found in 23 herds: six of these herds had been certified as *M. hyopneumoniae* infected. All the herds participating in this survey were followed closely for two years after the first collection of colostra.

The percentage of positive samples in the six infected herds averaged 10.3%. If the suspect samples were to be clarified as positive, then the mean percentage of positives would be 18.2%. The percentage of combined positive and suspect reacting sows in these herds varied between 5.6% and 38.5%.
The antibody increase was detected earlier than were the clinical signs: In four of the truly infected herds the first positive colostrum was found from 4 days to 6 months before the detection of the first clinical signs.

In the positively reacting herds in which no enzootic pneumonia either on clinical grounds or post mortem examinations was found (n=17), usually 1 positive sample was detected. In some herds there were also suspect samples. These samples were clarified, following additional testing as false positive singleton reactors. False positives were sometimes positive also in the second, follow-up collection. Singleton reactors of this sort present on 3 farms repeatedly gave positive results in the ELISA.

In one disease-free herd, 10 out of 19 swine were positive in the ELISA antibody tests. This herd had been recently formed and consisted of gilts that had farrowed for the first time. However, no enzootic pneumonia or *M. hyopneumoniae* antibodies were detected in the offspring. It was therefore concluded that the sows had had *M. hyopneumoniae* infection previously but had already recovered before their farrowing and were no longer infectious. The positive reactions were thus old positive titers of recovered individuals. Thereafter the herd has since remained *M. hyopneumoniae* free.
4.11 *A. pleuropneumoniae* serotype 12 survey

More than 70% of the sows in 154 herds were tested. Of the herds, 25 were totally free of sows with *A. pleuropneumoniae* serotype antibodies. In 31 herds 1-10% of the colostral samples tested were positive, in 76 herds 11-50% of the sows had antibodies, and in 22 herds more than half of the sows were positive. The number of colostrum samples tested was 5,477, of which 1307 were positive.

The herds in which the sows did not have *A. pleuropneumoniae* antibodies were smaller (mean number of sows 21, SD ±9) than the herds in which more than 50% of the sows had antibodies in their colostra (mean number of sows 25, SD ±14). No difference in the age of the sows or breed (large white or landrace) were detected.

Clinical signs typical of *A. pleuropneumoniae* infection were rare in all the herds investigated. During this study chronic pleuritis was a problem in two disease-control herds. However, *A. pleuropneumoniae* was not isolated either of these outbreaks. In these herds, 16% and 25% of the sows had *A. pleuropneumoniae* serotype 2 antibodies in their colostrum. In one of the seven progeny testing stations, there was an *A. pleuropneumoniae* serotype 2 epidemic, indicating that subclinical infections do in fact exist, as these stations only admit piglets from disease control herds.
4.12 A. pleuropneumoniae 12 serotypes

In the serum samples collected from slaughter sows, antibodies against all serotypes of A. pleuropneumoniae were found. The proportions of positives for each serotype were as follows: serotype 1, 7%, serotype 2, 26%, serotype 3, 51%, serotype 4, 8%, serotype 5, 1%, serotype 6, 2%, serotype 7, 6%, serotype 8, 1%, serotype 9, 8%, serotype 10, 1%, serotype 11, 3% and serotype 12, 10%.

The number of negative samples was 251. Altogether 229 samples had antibodies against one A. pleuropneumoniae serotype, and 195 samples had antibodies against two or more serotypes. In the case of serotypes 5, 6, 8, and 9, no samples were found which reacted with these serotypes alone.

The pattern of distribution of A. pleuropneumoniae serotypes varied throughout Finland. Serotype 1, serotype 9 and serotype 12 antibodies were found most frequently in herds in the western part of the country (p<0.001). Serotype 3 was found most frequently in the south-west (p<0.001) and serotypes 4 and 6 in central Finland (p<0.001). Antibodies against serotype 2, which was the serotype most often isolated in acute epidemics, were distributed evenly in herds throughout the country. In the case of serotype 11, there were too few positive sera to statistically assess the distribution of infected herds.
4.13 Anti-PMT survey

The number of samples tested for anti-PMT antibodies was 5,650, and the number of herds tested was 188. Of the samples, 5,628 were negative and 22 were positive. Both toxigenic *P. multocida* infection and progressive atrophic rhinitis (PAR) was found in one herd.

Positive samples were found in 12 herds. In follow-up studies including confirmatory testing one truly infected herd, containing 11 seropositive animals, was found. Clinical signs of PAR, i.e. sneezing, deviation of the snout and retarded growth, were present. Toxigenic *P. multocida* was isolated from 11 of 29 nasal swabs taken from piglets. In this case the source of infection was identified as a consignment of pigs imported from a herd known to be infected.
5. Discussion

5.1 *M. hyopneumoniae* infections in disease-control herds

The herds included in this study were disease-control herds and were therefore expected to be free of *M. hyopneumoniae*. However, six infected herds were found among the 185 herds examined. In two of these herds the infection originated from the same source; in the other four herds the infection route was unknown. The diagnostic methods used were not capable of indicating if these herds had been subclinically infected for a longer period of time. The disease was usually introduced into a herd through the purchase of new breeding animals.

Prior to this study, one or two herds in the disease-control program had been diagnosed as suffering from enzootic pneumonia each year (Prof. A. Schulman personal communication). More *M. hyopneumoniae* outbreaks were identified by means of the new ELISA methods. This was apparently due to the use of a more sensitive diagnostic method. Some of the cases detected might have been due to subclinical infections, because some of the outbreaks had earlier been diagnosed at progeny testing stations which take piglets only from disease-control herds. Nevertheless, the small number of infected herds identified demonstrates that subclinical *M. hyopneumoniae* infections are not widely spread among the herds controlled under the Finnish Pig Health Scheme.
M. hyopneumoniae infections are considered to be the most widespread lung infection in pigs (Nicolet 1992). In the United States, a study of slaughter swine revealed that 99% of the herds had pigs with pneumonic lesions, and a study of CF antibodies in breeding herds indicated that 60% of the herds were affected (Ross 1992). In Finland, however, the prevalence of this disease has been low: with levels of 8% in south-western Finland and 30% in western Finland being recorded (Rautiainen 1998, Tuovinen et al. 1994a).

Antibody assays on colostrum facilitates the earlier diagnosis of mycoplasmal pneumonia in herds. During the survey (I), an increase of antibodies was observed in three of the five herds affected before there was any report of clinical signs or pathological changes. The interval from the introduction of M. hyopneumoniae into a herd to the onset of clinical signs can be as long as half a year, and even two years (Goodwin 1984). Rapid detection of the infection facilitates eradication, and transmission to other herds can also be prevented by this means.

Enzootic pneumonia is not fatal but the disease does retard the growth of pigs. Samples for M. hyopneumoniae culture, immunofluorescence or PCR, and routine pathological examination, can be obtained from a few pigs; however the methods are not sensitive enough for diagnostic purposes. Serology appears to be the most effective method of detecting enzootic pneumonia in living pigs, and the collection of samples is easy. Blood samples can be taken from the selected animals and,
also by measuring antibodies in colostra, the infection status of
the herd can be determined.

5.2 Specificity and sensitivity of the *M. hyopneumoniae* ELISA

Crude *M. hyopneumoniae* antigens have been described as
having a limited specificity (Armstrong et al. 1983, Feldt et al.
1992). In this study, however, reactions which could have been
due to cross-reactivity between different porcine mycoplasmas,
were not numerous among the field material of over 6000
samples. The number of positive-reacting pigs in herds without a
disease background was 23. These animals were mostly false
positive singleton reactors found on individual farms. Infection by
any mycoplasma spp. would likely have resulted in several
antibody positive individuals in any herd.

Thus cross-reactions are possible among mycoplasmas, and
were found in ELISA tests carried out on hyperimmune sera
obtained from immunised rabbits. The strong, inactivated
mycoplasma suspensions used for immunisation also contained
common antigenic determinants. Repeated boostering of test
animals resulted in a strong immune response to both specific
and shared antigens. The phenomenon is less evident in natural
infection were antibodies are directed strongly against specific
surface epitopes. In ELISA assays, field sera or colostra can be
diluted in order to minimise cross-reactions to common antigens,
while specific reactions are still measurable at the higher dilutions.
Tween 20 solubilised crude antigen, as used in the Chekit® Hyoptest, has been shown to be more specific than other crude antigens (Nicolet et al. 1980, Bereiter et al. 1990, Armstrong 1994). Cross-reactions with the related, mainly apathogenic *M. flocculare* have been reported (Nicolet 1988). In other studies these cross-reactions have not been marked (Bereiter et al. 1990). Our results were identical to the latter study.

The construction of DAKO *M. hyopneumoniae* ELISA is more complicated than that of Chekit® Hyoptest. The plates are coated with rabbit antiserum to *M. hyopneumoniae*, and the *M. hyopneumoniae* antigen is attached to this antiserum (Feldt et al. 1992). In the next step, samples compete with a monoclonal antibody to *M. hyopneumoniae*. The results of study (II) imply that DAKO *M. hyopneumoniae* ELISA may be more specific than the more simply structured Chekit® Hyoptest. Both tests had a high apparent specificity (DAKO *M. hyopneumoniae* ELISA, 99.3%, Chekit® Hyoptest, 98%) and they were therefore appropriate for *M. hyopneumoniae* serology as part of the disease control program.

In order to be of use in a disease-control program, a serological test should clarify all the infected herds which are capable of spreading the disease further. Thus 100% sensitivity at the herd level is required. In study (I), the sensitivity of the Chekit® Hyoptest at the herd level was only 66.7%. All the infected herds could have been found if the suspect samples had been classified as containing specific antibodies (I). In the infected herds (I) where no clear positive samples were found, the
number of suspicious samples was 3 and 5. In the negative herds all the samples were consistently negative. Suspect samples always evoke doubts in the minds of skilled laboratory personnel, and the herd of origin will be retested and examined further for clinical signs. A different the cut-off point would have made the test more sensitive. As a result of this study, *M. hyopneumoniae* ELISA had been routinely used as a diagnostic method in the disease-control program, and the cut-off point used in the Chekit®Hyoptest was changed.

While 100% sensitivity at the herd level is to object, all diseased animals, however, cannot be found. ELISA’s are essentially herd tests by nature. When testing individual animals, these tests do not give a reliable indication of the current infectious status of the individual or herd. All animals in an infected herd do not necessarily become infected by *M. hyopneumoniae*. Furthermore, antibody production in infected animals can be slow or even below the detectable level. Finally, once developed, *M. hyopneumoniae* antibodies persist over several farrowings, although the animals have already recovered. Therefore, assessment of the *M. hyopneumoniae* infectious status by measuring antibodies from a single animal alone, is not reliable.

The percentage of seropositive animals in the *M. hyopneumoniae* infected herds (I) was low, an 11%. This was probably related to the early phase of infection at the time of sampling. Sanitation procedures were introduced immediately after the serological diagnosis was confirmed by clinical and
pathological findings. Therefore, there was no time for most of the sows to develop antibodies. In study (II), in one herd infected by *M. hyopneumoniae* only one positive sample was found. Therefore singleton reactors cannot be automatically judged as false positives, but the sample and the entire herd need to be investigated further. In study (II) the DAKO *M. hyopneumoniae* ELISA identified more positive animals (70/153) than the Chekit®Hyoptest (30/153), and thus was more sensitive.

5.3 An exceptional herd in the *M. hyopneumoniae* survey

An exceptional herd was found in study (I). In this herd *M. hyopneumoniae* antibodies were found in 10 of 19 colostra tested (53%). Despite the high number of positive animals, no clinical signs were detected and no pneumatic lesions were found at post mortem examination. The colostral antibody status of the herd was further investigated by repeated sampling. Because the antibody titre gradually declined, and no new infected animals were found, there was no need to initiate restocking procedures. This herd had recently been restocked with purchased animals. The sows had obviously overcome the infection before the birth of the offspring. It has been reported that small herds of up to 12 sows can spontaneously recover from *M. hyopneumoniae* infection (Waldman and Radtke 1937); however in larger herds there is always a susceptible population present which is capable of carrying over the pathogen to the next generation.
5.4 The *A. pleuropneumoniae* serotype 2 survey (III)

According to this study, *A. pleuropneumoniae* serotype 2 appears to be widely spread in the herds in Finnish Pig Health Scheme. Acute epidemics have nevertheless been rare. Only one or two herds annually have been slaughtered as a result of acute contagious pleuropneumonia. Despite this, an unexpectedly large number of *A. pleuropneumoniae* serotype 2 antibody positive herds was found: most of the disease control herds included sows that were sero-positive, suggesting exposure to this pathogen.

Compared with other surveys, these results are not particularly surprising. Other studies also support the finding that *A. pleuropneumoniae* is much more widely spread than the acute disease it causes (Brandrecht and Smith 1987, Elbers et al. 1990, Larsen et al. 1990, Lariviere et al. 1990). Commercial piglet-producing herds in south-western Finland also generally have sows with *A. pleuropneumoniae* serotype 2 antibodies (Tuovinen et al. 1994a). This suggests that serotype 2 is not always highly virulent. Variations in virulence between strains of different serotypes of *A. pleuropneumoniae* have been reported (Komal and Mittal 1990, Møller et al. 1992). The virulence of different serotypes also seems to vary geographically. In Denmark, serotype 2 infections are usually acute, although subclinical infections have also been detected (Nielsen 1988, Møller et al. 1992). Serotype 2 is the most prevalent serotype in Sweden but, but acute epidemics are rare (Beskow et al. 1989). The rearing
conditions in the Finnish breeding farms are very good, and as *M. hyopneumoniae* infections are rare, these conditions may reduce the likelihood of acute infections caused by *A. pleuropneumoniae* serotype 2 (Ross 1992).

Only 25 disease-control herds appeared to be free of *A. pleuropneumoniae* serotype 2 antibodies. Some of these herds were very small closed herds, but there was no statistical difference between the size of the most infected herds and the clean herds. It has been reported that the number of seropositive animals is not reflected enter of in the clinical signs (Nielsen 1988, Zimmermann et al. 1990). In our study, however, the herds which had more than 50% positive colostra also had pneumonic symptoms, although *A. pleuropneumoniae* was not cultivated. In this study we found 30 herds in which 1-10% of the animals were positive. These reactions may have been false positives, but it is also possible that they represented reactions to some earlier, already eliminated infections.

According to the results of this study, the control of *A. pleuropneumoniae* infections in the Finnish Pig Health Scheme herds appears to be cumbersome. Because most of the herds have *A. pleuropneumoniae* serotype antibodies without any clinical problems, it is apparently not necessary to require complete freedom from this infection. There have been no records of breeding animals transferring clinical infectious pleuritis to new herds. Infections in progeny testing stations give rise to problems, and more knowledge is needed regarding the
pathogenesis and epidemiology of \textit{A. pleuropneumoniae}, in order to control this infection in the Finnish Pig Health Scheme.

The prevalence of pleuritis as seen at meat inspection does not necessarily reflect the level of \textit{A. pleuropneumoniae} infection the herd in question, because pleuritis can have multifactorial causes. Sample size should also be taken into account when interpreting slaughterhouse data (Martin et al. 1987, Pointon et al. 1990). Therefore, slaughter checks, as applied in the Finnish Pig Health Scheme are not a reliable means of determining the \textit{A. pleuropneumoniae} status of a herd.

The control of \textit{A. pleuropneumoniae} related disease might be more effective if the diagnostic tests and vaccinations were directed against virulence factors instead of serotypes (Kamp et al. 1994). However, there is not enough information about the nature of virulence factors involved and their occurrence in field strains. Capsular polysaccharides, lipopolysaccharides and Apx toxins (hemolysins and cytotoxins) have been suggested to play a role in virulence (Komal and Mittal 1990). Ribotyping has been introduced as a tool in epidemiological studies (Fussing et al. 1998), but its discrimination power has been low.

\textbf{5.5 \textit{A. pleuropneumoniae} 12 serotypes survey (IV)}

Serological reactions to serotype 3 were the most prevalent in this survey, as more than half of the samples had serotype 3 antibodies. The number of positive samples was again relatively high compared to the occurrence of acute pleuropneumonia.
Serotype 3 has been known to be present on pig farms in Finland (Dr A. Schulman personal communication), but it has not caused acute epidemics. This is typical for serotype 3 strains, which have previously been reported to cause only subclinical or mild infections (Nielsen 1982, Nicolet 1992, Møller et al. 1992, Beck et al. 1994).

The second most common serotype of *A. pleuropneumoniae* appeared to be the serotype 2. The proportion of *A. pleuropneumoniae* serotype 2 positive sera was 26% in slaughter sows. This is in good agreement with the serotype 2 survey based on sow colostrum (III) and with the result of an earlier survey in pig herds in south-western Finland (Tuovinen et al. 1994a). 24% of the sows in the Finnish Pig Health Scheme herds had serotype 2 antibodies, as did 20% of the sows in south-western Finland. The number of seropositive animals reported here is higher than would have been expected according to the prevalence of clinical *A. pleuropneumoniae* manifestations. This again reflects the differences in pathogenicity among *A. pleuropneumoniae* strains.

Many of the samples examined, 25% of the total number, had antibodies against more than one *A. pleuropneumoniae* serotype. This distribution has also been reported previously (Nielsen 1988). Because natural infection with one serotype may elicit some cross-immunity to all other serotypes, new serotypes can spread with low clinical morbidity in a chronically infected herd (Nielsen 1982, Nielsen 1988, Van Haesebrouk et al. 1996).
The pattern of distribution of serotypes in a particular area must be known if disease control measures are to be planned successfully. The vaccines which are currently available afford protection only against the infection by the homologous serotype (De Jong 1978, Nicolet 1992). Commercially available vaccines reduce mortality, but do not prevent the carrier state developing (Andresen 1997, Chiers et al. 1998, Furesz et al. 1998). Cross-serovar protection can be achieved by the latest mutant vaccine strains (Prideaux et al. 1999).

According to the ELISA results, the most prevalent serotypes in Finland are 2 and 3. At the present time, disease control in Finland should be targeted towards these serotypes. However, antibodies against serotypes 1, 9 and 12 have been found in increased numbers in western Finland, as well as antibodies against serotypes 4 and 6 in central districts. The serotype pattern of *A. pleuropneumoniae* tends to change over the course of time (Nielsen 1988). The antibody ELISA can be used to follow changes and to identify the serotypes which may play a role as important pathogens in the future.

5.6 The *A. pleuropneumoniae* ELISA

ELISA was chosen as a serological method for this study because of its reported specificity and sensitivity (Nicolet 1981). ELISA is a much more sensitive method than the widely used complement fixation test (CF) (Nicolet et al. 1981, Nielsen et al. 1991).
The antigen used in this study was purified by phenol extraction, which removes contaminating proteins, leaving only the highly serotype-specific capsular polysaccharide (Bossé et al. 1990, Thwaits and Kadis 1991, Radacovici et al. 1995).

In bacteriological serology several Gram-negative bacteria have been shown to share common antigens (Devenish et al. 1989). Likewise, cross-reactions have also caused problems in A. pleuropneumoniae serology and serotyping (Nicolet 1992), and therefore, purification of the antigen is very important.

Antigenic cross-reactions have been reported between A. pleuropneumoniae serotypes 1, 9, 11 and 3, 6, 8 and 4, 7 (Nielsen 1985a, Nielsen 1985b, Mittal 1990, Gutierrez et al. 1991, Nicolet 1992). The ELISA used in this study showed cross-reactions between serotypes 1, 9, 11 and 6, 8 when tested with rabbit antisera. However, when testing the samples collected from slaughter sows were tested, cross-reactions were found between the serotypes 9, 11 and 1, 2 and 9, 12. These results are contradictory and it is therefore suggested that cross-reactions between serotypes 1, 2 and 9, 12 might be due, in each case, to infections with both of these serotypes. Parallel infections with several serotypes of A. pleuropneumoniae is known to occur (Nielsen 1988). While phenol extraction removes cross-reacting proteins there are a number of structural similarities among lipopolysaccharide O antigens of A. pleuropneumoniae capsular serotypes 1, 9 and 11 (Beynon et al. 1992), which may explain this occurrence.
5.7 Progressive atrophic rhinitis (PAR) survey

The majority of epidemiological studies performed on PAR have compared herds with and without a history of the clinical disease. Serological surveys of PMT antibodies have not been carried out in these herds prior this study.

According to the ELISA assays, the Finnish Pig Health Scheme herds were free of sows with antibodies to *P. multocida* toxin (PMT). These results were in agreement with the clinical surveys and laboratory tests carried out on the isolated bacterial strains. During 1984-1991, 125 *P. multocida* strains were isolated from 103 pig herds including disease-contol and and conventional herds that had suffered from respiratory infections: only two of the isolates produced toxin. The ELISA revealed only one PAR positive swine herd: this herd had imported pigs from abroad.

The herd of origin had a disease free status at the time of export, but had developed PAR again soon after selling pigs to Finland. This export had been conducted without close veterinary supervision. Periods of subclinical appearance are typical of herds chronically infected with toxigenic *P. multocida*. Complementary monitoring of a herd for at least 2 years, based on bacteriological and/or serological detection of the PAR-toxigenic *P. multocida*, may be necessary in order to obtain sufficient information concerning the true PAR-infected or PAR-free status of the herd (De Jong 1992, Matschullat, G. et al. 1994). The herds in the Finnish Pig Health Scheme were monitored for
two years after this study and no new cases of PAR appeared in the disease-control herds.

5.8 Specificity and sensitivity of the blocking ELISA

The serological blocking ELISA was developed from commercial kit for the detection of PM toxin. Both monoclonal and polyclonal antibodies, which recognise different antigenic epitopes of PMT, were used in the ELISA. Due to the design of the anti PMT-ELISA excellent specificity was to be expected. The results of the study (V) confirm the expectations.

One disadvantage of the test was that contaminated and otherwise spoiled colostrum samples produced weak positive reactions. A considerably high number of spoiled samples was found, 453 out of 5,650. Spoilage of the samples was easily detected, and these samples were excluded on the basis of foul smell and coagulation. As could be expected, the spoiled colostra were obtained during the summer months. Nine of the farms from which fetid samples were sent were visited, and it was obvious that the only possible explanation for the weak positive reactions was the spoilage of the samples. Colostra of good quality provided an excellent sample for the ELISA PAR antibody assay. The *M. hyopneumoniae* and *A. pleuropneumoniae* ELISAs did not react with the spoiled samples in the same way.

Infection with toxigenic *P. multocida* leads to a weak systemic immune response (Chanter and Rutter 1989), and it may
take up to three months before anti-PMT antibodies reach detectable levels (Bording et al. 1992). Therefore one aim of this study was to determine if *P. multocida* infections could be detected for assaying antibodies. The results of this study showed that, like several other serological tests used for the detection of swine respiratory infections, the ELISA for PMT antibodies is not able to classify individual animals into a positive or negative disease status category, but can be used as a herd test for identifying infected herds (Stokes et Bourne 1989). When the anti-PMT-ELISA is used as a herd test, the presence of animals with high and low antibody titres indicates that the herd is infected with *P. multocida*. Toxigenic *P. multocida* is maintained in a pig herd by a small population of breeding females (De Jong 1992), and, therefore, infection within a herd is best be detected by assaying sows as a group.

5.9 Colostrum sample

Sow colostrum is an ideal sample for antibody assay. IgG is concentrated in the colostrum and, for 24 h from the beginning of farrowing, more IgG is found in colostrum than in serum (Nicolet 1988, Stokes and Bourne 1989). More positive animals in a herd are found when colostrum samples are assayed compared to serum samples (Nicolet 1988, Sørensen et al. 1993).

In testing the repeatability of the Chekit® Hyoptest, it was noted that a smaller number of positive samples was found in the second assay. After recurrent freezing and thawing, samples tend
to show less reactivity in antibody ELISA’s. On the other hand, assay of colostrum only partly corrected this problem.

The collection of a colostrum sample causes very little distress to the sow. The farmer can collect the samples himself. However, there are some risks involved when the herd owner takes the samples for disease control. For example, the same sow can be sampled in error several times, or the samples can be taken too late after farrowing. The Finnish Pig Health Scheme is a voluntary disease control program. The owners of the herds participating in the scheme are highly motivated to do their best in regard to disease control, so as to provide the best pig breeding material and healthy animals.
6. Conclusions

1. Commercially available ELISAs for detecting *M. hyopneumoniae* antibodies are sufficiently specific and sensitive for the diagnosis of *M. hyopneumoniae* infection within the scope of a disease-control program. The DAKO *M. hyopneumoniae* ELISA is more sensitive and specific. Using both tests singleton positive reactions could be verified.

2. An ELISA for detecting antibodies to 12 serotypes of *A. pleuropneumoniae* was developed. The test clearly distinguished between positive and negative control sera, and positive and negative field samples. The repeatability of the test was good. However, the following cross-reactions between serotypes were detected: 1, 9 and 11, 6 and 8, also serotype 5(a) antiserum also reacted with serotype 6 antigen. When the field samples collected for this survey were examined, different cross-reactions were found; the OD values of the samples reacting with serotypes 9 and 11, 9 and 12, and 1 and 2 correlated (p<0.01).

3. The ELISA test for detecting *P. multocida* toxin was modified to detect antibody against PMT. A preincubation step was added in which the samples were allowed to react with PMT in case there were PMT antibodies in the sample. Antibodies in the samples blocked the binding of HRP-conjugate to the toxin. The specificity of the test was calculated to be 99.8%. At the herd level, the sensitivity was 100%. In the one infected herd the PMT-antibody ELISA found 52% of the sow colostra to be positive. Because the actual number of seroconverted animals in the herd
was not known, the sensitivity for individual samples was at least 52%. In comparison, the recovery rate of toxigenic *P. multocida* bacterium from nasal swab samples was 55%. Spoiled colostrum samples characterised by a foul odour interfered with the test and gave false positive reactions. Good quality colostrum is to be regarded a satisfactory sample in the PMT-antibody assay.

4. Colostrum from sows in the 185 top breeding herds, which should have been *M. hyopneumoniae* free, were collected during a half year period. Altogether 6,256 samples were received. A total of 61 positive or suspect samples were found in 23 herds, of which six herds were found to be truly *M. hyopneumoniae* infected. The prevalence of *M. hyopneumoniae* in this group was therefore 3.2%. The proportion of positive and suspicious samples in the affected herds averaged 18.2%.

*A. pleuropneumoniae* serotype 2 antibodies were tested in pigs in 154 herds. Of the herds, 25 were totally free of *A. pleuropneumoniae* serotype 2 antibodies. In 31 herds 1-10% of the samples tested were positive, in 76 herds, 11-50% of the sows had antibodies, and in 22 herds more than half of the sows were positive. The number of colostrum samples tested was 5,477 and 1307 samples were positive. In one of the seven progeny testing stations, which take piglets only from the disease-control herds, there was an *A. pleuropneumoniae* serotype 2 epidemic, indicating that subclinical infections can occur under such conditions.
188 herds were tested for PMT antibodies. Of the 5,650 samples examined, 5,628 were negative and 22 were positive. Toxigenic \( P. \) \( multocida \) infection, progressive atrophic rhinitis (PAR), was confirmed in one herd.

5. In the samples collected from slaughter sows (\( n=675 \)) are proportionally representing the different regions of Finland, antibodies were found against all 12 serotypes of \( A. \) \( pleuropneumoniae \). The proportions of positive sera for each serotype were as follows: serotype 1, 7%, serotype 2, 26%, serotype 3, 51%, serotype 4, 8%, serotype 5, 1%, serotype 6, 2%, serotype 7, 6%, serotype 8, 1%, serotype 9, 8%, serotype 10, 1% serotype 11, 3% and serotype 12, 10%. The number of negative samples was 251. Altogether 229 samples had antibodies against one \( A. \) \( pleuropneumoniae \) serotype, and 195 samples had antibodies against two or more \( A. \) \( pleuropneumoniae \) serotypes.

The serotype pattern of \( A. \) \( pleuropneumoniae \) varied throughout Finland. Serotype 1, serotype 9 and serotype 12 antibodies were found most frequently in the western part of the country (\( p<0.001 \)). In the case of serotype 11, there were too few positive reactions to test differences. Serotype 3 was found most frequently in the south-west (\( p<0.001 \)) and serotype 4 and serotype 6 antibodies in central Finland (\( p<0.001 \)). Antibodies against serotype 2, the serotype most often isolated in acute epidemics, were evenly distributed throughout the country.

6. The \( M. \) \( hyopneumoniae \) antibody assay on colostrum appeared to work well in disease-control programs. The
laboratory test was sensitive and specific, the collection of samples was easy and, by assaying for antibodies, more infected herds were found and the diagnosis was established earlier than by traditional methods.

The serological test for *A. pleuropneumoniae* appeared to be sensitive and specific, but the disease situation concerning *A. pleuropneumoniae* infection was worse and different to that expected. The ELISA test could be used as a diagnostic tool in disease cases but, due to the vast prevalence of subclinical *A. pleuropneumoniae* infections, disease surveillance by serology is not a reasonable measure.

The ELISA for *P. multocida* toxin antibodies is suitable for disease-control purposes. However, because of the very low prevalence of toxigenic *P. multocida* infections in Finnish Pig Health Scheme herds, the cost effectiveness of continuing surveillance by this or other means should first be evaluated.
7. References


Sakano, T., Okada, M., Taneda, A., Mukai, T., and Sato, S. Effect of Bordetella bronchiseptica and serotype D Pasteurella multocida bacterin-toxoid on the occurrence of atrophic rhinitis after experimental infection with B.


Wallgren, P., Bölske, G., Gustafsson, S., Mattsson, S., and Fossum, C. Humoral immune responses to *Mycoplasma hyopneumoniae* in sows and


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