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***Mycoplasma hyopneumoniae* infection in pigs: duration of the disease and resistance to reinfection**

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Summary — Forty-eight hysterectomy produced piglets reared in a pathogen-free environment were housed in 4 separate units. Three groups were intratracheally challenged with *M hyopneumoniae* at 2 wk, or at 16 wk, or at 2 and 16 wk of age. The fourth group was uninfected and received broth medium. Coughing started 2 weeks after the first infection but the booster infection did not induce any coughing. Macroscopic lesions, typical of mycoplasmal pneumonia, were noted 1 wk post-infection. After 7–9 wk, piglets showed recovering lesions. In the group that was twice infected, only 1 pig had extensive pneumonia after the second infection. The remaining pigs only had recovering lesions (RL) (resulting from the first challenge). Histopathology and scanning electron microscopy confirmed the macroscopic observations. Antibodies were detected by ELISA and immunoblotting at 3–4 wk post-infection, peaking after 11–12 wk and then gradually decreasing. However, the antibody response increased after the second infection. These findings showed a noticeable recovery following infection with *M hyopneumoniae* and a clear resistance to pneumonia following the booster infection.

***Mycoplasma hyopneumoniae* / reinfection / resistance / pneumonia / antibodies**

Résumé — Évolution dans le temps de l'infection à *Mycoplasma hyopneumoniae* et étude de la résistance à cette infection. Quarante-huit porcelets nés par hystérectomie et maintenus à l'abri des contaminants, sont répartis dans 4 animaleries séparées. Trois groupes ont été infectés par *M hyopneumoniae* à 2, ou à 16 ou à 2 et à 16 semaines d'âge, par voie intratrachéale. Le quatrième groupe, non infecté, reçoit le milieu de culture. La toux est apparue 2 semaines après la première infection, mais la seconde infection n'a pas induit de symptômes. Des lésions pulmonaires typiques de l'infection à *M hyopneumoniae* ont été observées après une semaine. Entre 7 et 9 semaines après l'infection, les porcelets étaient porteurs de lésions en voie de cicatrisation. Dans le groupe infecté deux fois par *M hyopneumoniae*, un seul porc a présenté une pneumonie en pleine extension. Les autres porcs du lot n'ont montré que des lésions cicatrisées, résultant de la première infection. L'histologie et la microscopie électronique à balayage ont permis de confirmer les observations macroscopiques. Les anticorps sériques ont été détectés par Elisa et par immunoblotting, entre 3 et 4 se-

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maines après l'infection. Le taux d'anticorps augmente progressivement jusqu'à 11–12 semaines, puis décline lentement. On note une augmentation du taux moyen d'anticorps après une deuxième infection. Ces résultats montrent clairement l'évolution spontanée de la maladie vers la guérison, ainsi qu'une résistance de l'animal lors d'un second contact avec *M hyopneumoniae*.

***Mycoplasma hyopneumoniae* / réinfection / résistance / pneumonie / anticorps**

INTRODUCTION

Mycoplasma hyopneumoniae is the primary agent of enzootic pneumonia, one of the most important and chronic diseases in industrial pig herds. Previous pathological and immunological studies have characterized the disease caused by this agent (Ross, 1973; Whittlestone, 1973; Adegboye, 1978; Sutter *et al*, 1985; Messier *et al*, 1990). However, the duration of the infection and the conditions required to heal the lung lesions are not clearly understood. According to Etheridge and Lloyd (1982) the reason was the unsatisfactory method used to induce standardized lesions with *M hyopneumoniae*. However, Goodwin *et al* (1969) detected resistance to experimental exposure up to 60 wk after appearance of the disease. Livingston *et al* (1972) reported that with uncomplicated *M hyopneumoniae* infection, pneumonic lesions regressed after 5–6 wk, whereas Underdahl *et al* (1980) found that 16 wk after inoculation, there was no evidence of noticeable regression or recovery.

In field conditions, clinical mycoplasmal pneumonia is common in young animals but generally not seen in adult pigs, probably because the latter had a previous *M hyopneumoniae* infection at an earlier age which protected them against reinfection (Goodwin *et al*, 1969). However, this pattern cannot be the result of age resistance: when an enzootic-pneumonia-free herd becomes infected with *M hyopneu-*

monia, pigs of all ages develop acute pneumonia (Whittlestone, 1973).

The purpose of this trial was to determine whether there was a regression of pneumonic lesions in experimental conditions after *M hyopneumoniae* infection in SPF pigs and if there was any resistance after a booster infection. The serological patterns of these animals were studied by ELISA and immunoblot analysis.

MATERIALS AND METHODS

***Mycoplasma hyopneumoniae* strains**

A field strain of *M hyopneumoniae* (BQ 14) was isolated from an outbreak of enzootic pneumonia in France and identified by the disk growth inhibition test (identification confirmed by EA Freundt, FAO/WHO Collaborating Center for Animal Mycoplasma, Aarhus, Denmark). The strain had been passaged 10 times in hysterectomy-produced pigs and was cultivated once in Friis medium (Friis, 1975).

A type strain of *M hyopneumoniae* (J-25934 ATCC) was used for antigen preparations. Cells were harvested by centrifugation at 15 000 g for 15 min at 4 °C, dispersed into 0.05 M PBS, NaCl 0.15 M, pH 7.4 and washed twice in PBS.

Experimental piglets

Forty-eight hysterectomy-produced colostrum-deprived gnotobiotic piglets, reared in a patho-

gen-free environment as previously described (Kobisch *et al*, 1987) were used in this trial. The piglets were housed in 4 separate units: Group 1: 12 piglets were challenged with BQ 14 strain at 2 weeks of age; Group 2: 12 piglets were challenged at 2 and 16 wk of age; Group 3: 8 piglets were challenged at 16 wk of age; Group 4: 16 uninfected piglets were given Friis culture medium at 2 wk, at 16 wk or at 2 and 16 weeks of age.

Experimental infection

The piglets were infected intratracheally with 5 ml (2 wk of age) or 10 ml (16 wk of age) of broth cultures of *M hyopneumoniae* BQ 14 (10^9 CFU/ml). The control group received Friis culture medium in the same manner.

Clinical observations and pneumonia assessment

Rectal temperatures and clinical signs were recorded daily: coughing was counted for 15 min. Blood samples were taken once a wk. The piglets were sacrificed at random intervals from 1–22 wk after the first infection and then necropsied. *Post-mortem* examinations were carried out on each animal. The lung lesions were scored according to the method described by Hannan *et al* (1984). The maximum total score possible for each complete lung was 35.

Lung microscopy and M hyopneumoniae detection

Lung tissue of all the pigs was used for isolation of *M hyopneumoniae* and bacteria. *M hyopneumoniae* was also detected by the use of a direct immunofluorescent test (Kobisch *et al*, 1978).

Histology

Pieces of lung tissue from all the piglets were collected and fixed in 10% buffered formalin.

Thin sections were stained by hematoxylin and eosin and examined by light microscopy (Lagadic, 1987).

Scanning electron microscopy (SEM)

Samples of trachea and bronchi from each pig were collected and treated as previously described by Blanchard *et al* (1992).

Immunological studies

Enzyme-linked-immunosorbent assay (ELISA)

ELISA was performed for detection of *M hyopneumoniae* antibodies, according to the procedure of Nicolet *et al* (1980). The antigen used was Tween 20 extract. After washing, *M hyopneumoniae* cells were suspended in 0.25 M NaCl to a concentration of 2 mg protein/ml. The optimal antigen concentration for coating microtiter plates was 40 µg protein/ml. All dilutions of sera (1/100) were performed in Tris buffer 0.1 M, pH 7.5.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

SDS-PAGE was performed as described by Laemmli (1970), using a 12.5% resolving gel and a 4.8% stacking gel. The cellular proteins were solubilized with 1% SDS and reduced with 0.24 M 2-mercaptoethanol. The protein concentration was determined according to the method of Lowry *et al* (1951) with serum albumin in SDS as the standard. Antigen protein solution (10 µg/cm) was loaded onto gels. Western transfer was performed as described by Towbin *et al* (1979) in 48 mM Tris-39 mM glycine buffer containing 20% methanol. Screening of antibody labelled proteins was performed with dilutions of sera (1/10) according to the method of Hawkes *et al* (1982) using rabbit anti swine IgG (H + L) peroxidase labeled conjugate (Dako SA, Versailles, France).

Pooled serum samples collected from all the experimental pigs were analysed by ELISA and immunoblot.

RESULTS

Clinical symptoms

The body temperature of piglets from groups 1, 2 and 3 remained at 40–40.3 °C for 1 wk after the first infection (normal temperature: 39.5 °C). The second challenge did not increase rectal temperature in group 2. Coughing started 2 wk after infection in groups 1, 2 and 3, peaked after 5 wk and gradually declined. Twelve wk post-

infection, no clinical symptoms were noticeable in groups 1 and 2. The second infection did not induce any coughing in group 2. No clinical signs were observed in uninfected piglets (group 4) (fig 1).

Post-mortem findings

A survey of the *post-mortem* observation on piglets is presented in table I.

No lesions were observed in the negative control (group 4). Macroscopic lesions

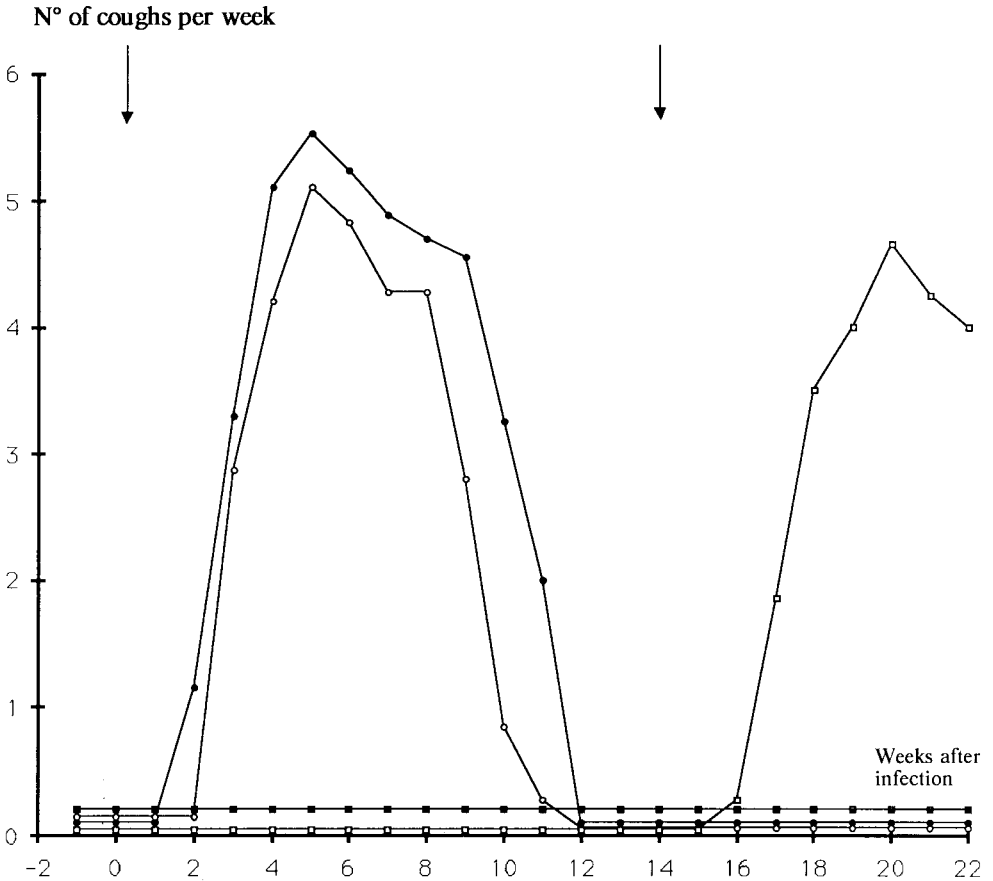


Fig 1. Coughing of piglets after intratracheal challenge with *M hyopneumoniae*. ○—: Group 1, infected at 2 wk; ●—: Group 2, infected at 2 and 16 wk; □—: Group 3, infected at 16 wk; ■—: Group 4 (negative control) N: number of coughs for each piglet per wk; → *M hyopneumoniae* infection.

Table 1. Post-mortem observations of infected piglets: mean of pneumonic scores in each group (maximum total score possible for each complete lung was 35).

Weeks after infection	Pigs infected at:		
	2 wk of age (12 pigs)	2 and 16 wk of age (12 pigs)	16 wk of age (8 pigs)
1	1.43 (++)	0.71 (-)	...
2	0.7 (+)	11.33 (++)	...
3	7.56 (++)	9.61 (++)	...
4	28.92 (+++)
5	...	19.19 (+++)	...
6	7.11 (+++)
7	...	16.58 (++)	...
8	11.38 (+++)
9	...	RL (+++)	...
10	RL (+++)
11	17.11 (+++)
13	...	RL (-)	...
15	9.85 (++)
17	RL (+)
18	...	7.21 (RL) (+++)	6.04 (++)
19	...	RL (-)	14.88 (++)
20	0 (-)	RL (-)	8.11 (++)
			15.32 (++)
21	0 (-)	RL (-)	RL (-)
22	0 (-)	RL (-)	2.85 (RL) (-)
			2.58 (RL) (+++)

RL: recovering lesions. Detection of *M hyopneumoniae* by direct immunofluorescent test (-, +, ++, +++).

typical of mycoplasmal pneumonia were generally observed from 1–11 wk post-inoculation in pigs infected at 2 wk of age and from 1–6 wk in pigs infected at 16 wk of age. These lesions were located in the apical and cardiac lobes, the anterior portion of the diaphragmatic lobes, and sometimes in the intermediate lobe of the lung. Recovering lesions (RL) consisting of fissures of collapsed alveoli adjoining areas of alveolar emphysema were observed after 7 wk in pigs infected at 16 weeks and after 9 wk in pigs infected at 2 or at 2 and

16 wk. In the latter group, only one piglet had extensive pneumonia and RL following the booster infection. The 4 last pigs had only chronic lesions. The immunofluorescent test performed on lung samples was positive for *M hyopneumoniae*, generally in association with extensive pneumonia. There was a good correlation between these results and the isolation of *M hyopneumoniae* from lung tissue. *M hyopneumoniae* was not isolated from pneumonia-free pigs. No other microorganism was isolated from the animals.

Histopathology

Pulmonary tissue modifications observed in the acute phase of infection showed bronchiolar and lymphoreticular hyperplasia and increased perivascular accumulation of mononuclear cells. Lymphocytes and plasmacytes were both detected, but the latter were much more abundant. In recovering lesions, extensive hyperplastic lymphoid nodules often compressing the lumina of the bronchioles were seen in all cases, including the 3 pigs in group 1, infected at 2 wk of age without macroscopic pneumoniae. In group 2, the pig necropsied 18 wk after the first infection and 4 wk after the booster revealed mixed lesions, suggesting both early and later infection. In control pigs, lung tissue did not present any lesions.

Scanning electron microscopy (SEM)

In the control pigs, normal tracheal and bronchial surfaces observed by SEM were composed of ciliated epithelial cells mixed with microvilli. In the 5 pigs killed at the first or second week after challenge, colonization of epithelial cells by *M hyopneumoniae* was noticeable. Two to 8 wk after inoculation, loss of cilia was observed. *M hyopneumoniae* covered the tufts of remaining cilia, and in some cases ciliated cells were seen to be exfoliating (fig 2). After this period, trachea and bronchi of pigs with recovering macroscopic lesions appeared normal with some mucus production (fig 3). Only one pig, necropsied 11 wk after *M hyopneumoniae* infection and with extensive pneumonia (score 17.11), presented a clear loss of cilia with areas corresponding to high mycoplasma concentrations. In group 2 (pigs infected at 2 and 16 wk of age), the only pig with acute and chronic lesions showed normal ciliated

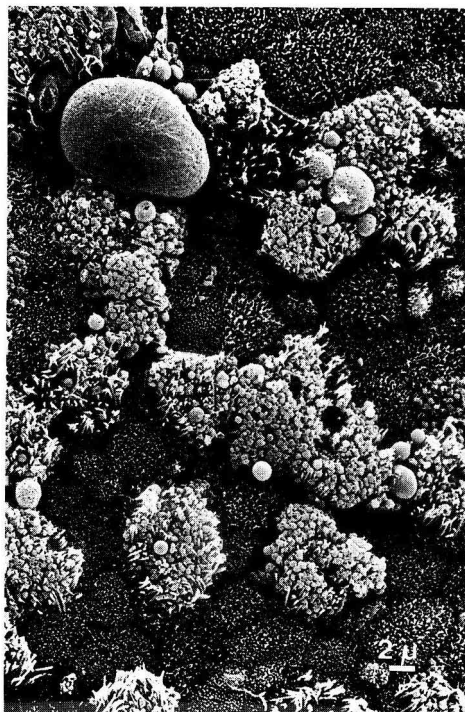


Fig 2. SEM of pig trachea, 6 wk post-infection with *M hyopneumoniae*. The loss of cilia of epithelial cells and *M hyopneumoniae* on the top of remaining cilia were observed; x 2 500.

cells, patches of cilia surrounded by mycoplasmas and areas without any cilia after the second challenge.

Serological results

ELISA

Sera of swine experimentally infected with *M hyopneumoniae* were tested by ELISA (fig 4). All inoculated pigs were seroposi-

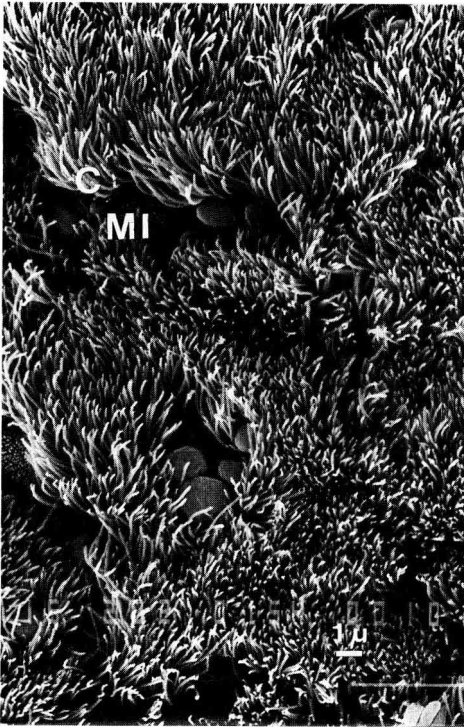


Fig 3. SEM of pig trachea, 13 wk post-infection with *M hyopneumoniae*. The epithelial cells appeared normal (ciliated cells: C and microvilli: MI). No mycoplasmas were observed; x 5 000.

tive 3 or 4 wk after challenge. In groups 1 and 2, the antibody response was similar in the earliest phase of the infection: peak titers were observed 10–12 wk post-inoculation and then declined regularly. But in group 2, ELISA titers clearly increased after the booster infection, then slowly declined. In group 3, ELISA titers were lower than in groups 1 and 2 although antibodies were detected at the same time after infection. Animals in group 4 were seronegative.

Immunoblot analysis

Pooled sera from piglets infected at 2 wk of age were analyzed by immunoblotting with *M hyopneumoniae* J as antigen (fig 5B). Four wk post-inoculation, (Pi) 6 immunogenic components of *M hyopneumoniae* were detected. Molecular weights of the 6 antigens were estimated to range from 36–138 kDa. Of these, the major antigen was 40 kDa. Eight weeks pi, antibodies against the 110-kDa antigen appeared and persisted during the entire experiment. The antibodies against the 36- and 62-kDa antigens could not be detected after 10 wk. Twenty wk pi the 40-, 45- and 72-kDa antigens were still clearly detectable. Pooled sera from animals infected at 16 wk of age (group 3) were analyzed (fig 5D). From the fifth week pi (wk 19 in figure 5D) until the end of the experiment, all immunogenic components of *M hyopneumoniae* were detected. Pooled sera from pigs infected at 2 and 16 wk of age were analyzed; the profiles are shown in figure 5C. Two weeks pi the 40-kDa antigen was weakly revealed and 2 wk later, new components estimated from 36–138 kDa were observed. After this period (the earliest phase of the infection) about 14 antigens were recognized and were still present at the end of the experiment. The booster infection intensified a band corresponding to the 72-kDa antigen. No components were detected in the uninfected group (data not shown).

DISCUSSION

Under the experimental conditions of this study, *M hyopneumoniae* induced clinical symptoms, essentially characterized by coughing and weak hyperthermia. The booster infection given 14 wk after the first challenge did not produce any symptoms.

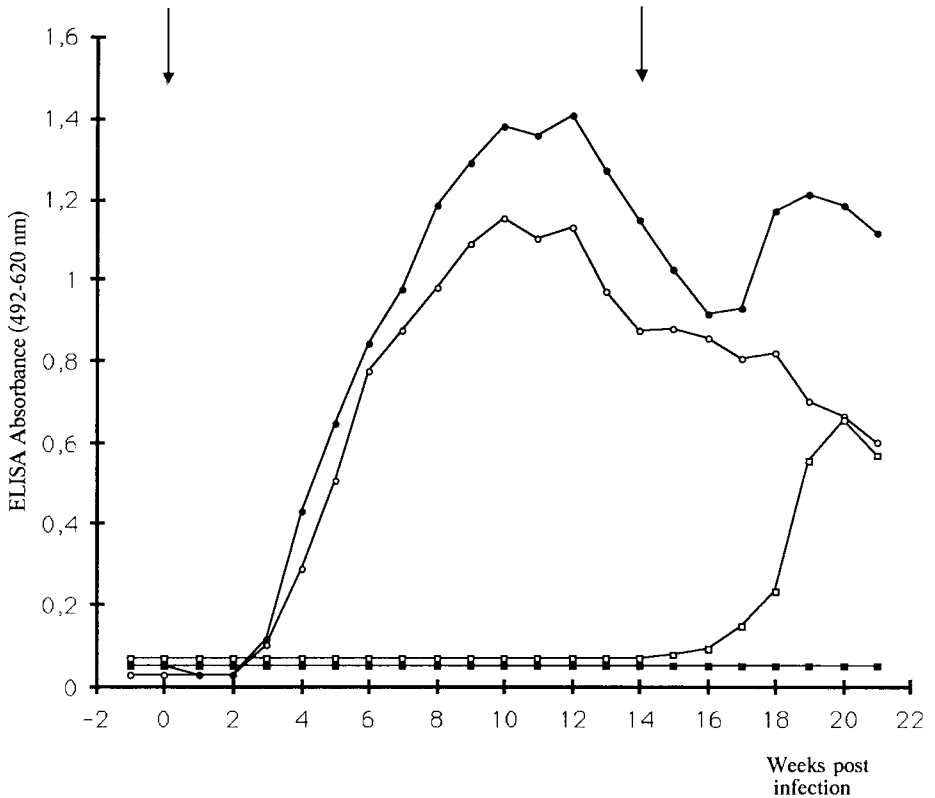


Fig 4. *M hyopneumoniae* antibody response in experimental pigs. ○-○: group 1, infected at 2 wk; ●-●: group 2, infected at 2 and 16 wk; □-□: group 3, infected at 16 wk; ■-■: group 4: negative control. ⇒ *M hyopneumoniae* infection.

A correlation was found between observation of coughing and the development of pneumonia which gradually resolved as clinical signs disappeared. No coughing was observed after the second challenge in twice-infected pigs. Among these, only one animal had extensive pneumonia. The remaining pigs had recovering lung lesions, probably resulting from the first challenge, and *M hyopneumoniae* was not isolated from lung tissue. Independent of individual sensitivity, there was no correlation between age and susceptibility of pigs

to *M hyopneumoniae*. Similar observations have been reported by Whittlestone (1979) and Piffer and Ross (1984).

Observation of the upper respiratory tract by SEM showed that the bronchial and the tracheal changes coincided with macroscopic pulmonary lesions and that regression of pneumonia corresponded with the disappearance of damage to epithelial cells. These results disagree with those of Underdahl *et al* (1980) who did not mention any regression as detected by SEM studies under similar experimental

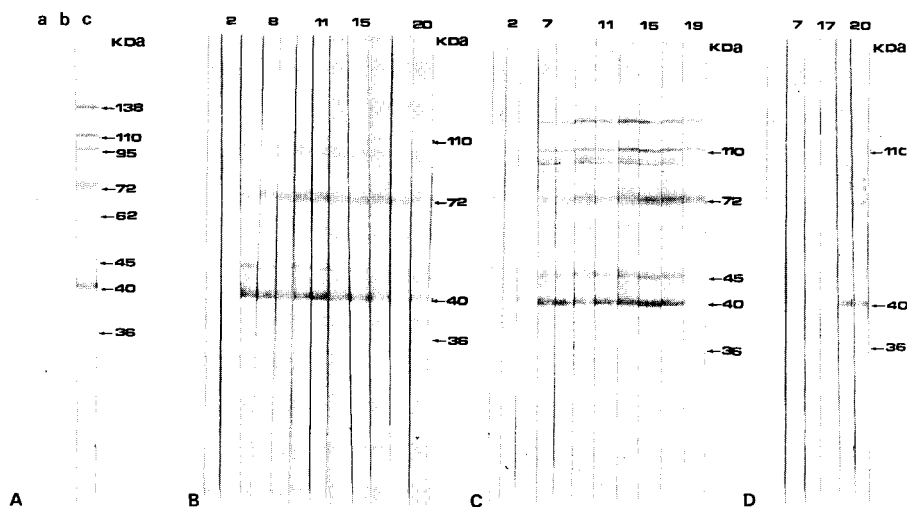


Fig 5. Immunoblot analysis of pooled sera from pigs infected with *M hyopneumoniae*. A. Controls; a: bovine serum albumin 1% in PBS; b: specific pathogen-free piglet sera (negative control); c: pooled sera from 6 pigs infected with *M hyopneumoniae* (positive control). B. Group 1: pooled sera from pigs infected at 2 wk of age. C. Group 2: pooled sera from pigs infected at 2 wk and 16 wk of age. D. Group 3: pooled sera from pigs infected at 16 wk of age. Weeks pi with *M hyopneumoniae* are indicated at the top. Positions and molecular weights (kDa) of the major immunogenic proteins of *M hyopneumoniae* are indicated on the positive control (A–c).

conditions. In contrast, our study suggested that the ciliated epithelium observed 15 weeks after *M hyopneumoniae* infection corresponded to regenerated tissue.

Pigs infected with *M hyopneumoniae* developed serum antibodies which were detectable by ELISA and immunoblotting. Among the antigens revealed, the 40- and 72-kDa species seemed to be the most immunogenic components. The immunoblotting results did not differ from those of Young and Ross (1987) and Strasser *et al* (1992). It is worthwhile noting that the signal of the P36 antigen which is claimed to be an early and persistent antigen in *M hyopneumoniae*-infected pigs (Strasser *et al* (1991) seems to decrease on the immunoblots 10 wk post-infection. Using an

ELISA test with non-denatured recombinant P36 protein as antigen, however, we detected circulating antibodies against P36 up to 20 wk post-infection in this experiment (Frey, unpublished results).

The antibodies reflected disease evolution, clinical symptoms and lung lesions, and confirmed the usefulness of the serodiagnostic technique in detecting *M hyopneumoniae* infection. In the twice-infected group, the booster infection induced a clear increase in antibody levels, including those in the pig with acute pneumonia. In this way there was no clear evidence that circulating antibodies alone could protect against a second challenge. These serological results confirmed previous observations following *M hyopneumoniae* infection

(Goodwin, 1969; Whittlestone, 1976; Adegboye, 1978).

Our study showed that under experimental conditions utilizing a severe challenge, SPF pigs acquired an immunity as the result of the first *M hyopneumoniae* infection. After the booster infection, histological examinations showed recovering lesions characterized by lymphoid nodules, except in the one pig with extensive pneumonia and where perivascular and peribronchiolar infiltrations were seen. These observations confirmed that cell-mediated immunity is very important in the pathogenesis of the disease, as previously suggested by Whittlestone (1976). According to Adegboye (1978) and Sutter *et al* (1985) lung lesions and the immune response after *M hyopneumoniae* could be the result of autoimmune reactions. Taylor and Howard (1981) demonstrated that in mice infected with *M pulmonis*, an intact immune system was necessary to induce lung lesions. These observations could explain the resistance observed here in twice-infected pigs.

The association of the pathomechanism of *M hyopneumoniae* and the immune reactions implicated in the disease could explain the resistance of pigs after a second challenge. Tajima and Tagihashi (1982) and Blanchard *et al* (1992) showed that *M hyopneumoniae* is closely associated with the upper part of ciliated cells but the real mechanisms involved in the adherence are not actually known, whereas in man, the knowledge of the mechanisms of adherence by *M pneumoniae* to target cells has greatly advanced: this mycoplasma is intimately combined with the hydrophobic membrane-associated regions of the respiratory epithelial cells. However, an immunocompetent host is not protected from a second *M pneumoniae* infection (Jacobs, 1991). A difference is therefore noted between the pathogenesis of respiratory mycoplasma infections in man and in pig.

The main conclusions of this study concerned the recovery from the disease after some weeks and the resistance induced in pigs after *M hyopneumoniae* infection. Moreover, the results indicated that the disease could be established independently of the age of the animals. Similar observations can be noticed in individual pigs in field conditions with uncomplicated *M hyopneumoniae* infection.

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