Acta vet. scand. 1985, *26*, 501—512.

From the State Veterinary Serumlaboratory, Copenhagen, Denmark.

SEROLOGICAL CHARACTERIZATION OF HAEMOPHILUS PLEUROPNEUMONIAE (ACTINOBACILLUS PLEUROPNEUMONIAE) STRAINS AND PROPOSAL OF A NEW SEROTYPE: SEROTYPE 9

By
Ragnhild Nielsen

NIELSEN, R.: *Serological characterization of Haemophilus pleuropneumoniae (Actinobacillus pleuropneumoniae) strains and proposal of new serotype: serotype 9.* Acta vet. scand. 1985, *26*, 501—512. — Ten strains of H. pleuropneumoniae isolated from 10 herd outbreaks of pleuropneumonia were studied by means of the slide agglutination test, the indirect haemagglutination (IHA) test and by gel diffusion. The strains were antigenically homogeneous and serologically distinct from serotypes 1 through 8. It is therefore proposed to refer these strains to a new serotype: serotype 9, with strain CVJ 13261 as the type strain.

In addition to the serotype-specific capsular antigens, capsular antigen of serotype 1 (strain 4074) could be demonstrated in the 10 strains by means of gel diffusion analyses.

In cross protection studies it was shown that the antigenic determinants shared by serotypes 9 and 1 were unable to yield a sufficient protection against disease. Thus, parenteral immunization with a killed 6-h culture of serotype 9 did not afford an acceptable protection against challenge with serotype 1 since only 3 of the 5 vaccinates were protected. The reverse experiment showed that parenteral immunization with serotype 1 only protected 1 out of 4 vaccinates.

indirect haemagglutination test; gel diffusion.

By agglutination and immunodiffusion tests *Nicolet* (1971) and *Gunnarsson* (1980) demonstrated that at least 2 type-specific capsular antigenic determinants were present in H. pleuropneumoniae, serotypes 1 through 5. *Branefors-Helander* (1972) reported that by immunodiffusion studies of Haemophilus influenzae more than 1 capsular serotype could be demonstrated in some strains. The presence of more than 1 serotype in H. pleuropneumoniae has recently been demonstrated in serotype 8 which, in

addition to serotype-specific capsular antigen, shares capsular antigen with serotypes 3 and 6 (*Nielsen & O'Connor* 1984). The close relationship between serotypes 8, 3 and 6 was further demonstrated in cross-protection experiments (*Nielsen* 1985). Studies of serotype 8 have indicated that the gel diffusion test and the indirect haemagglutination test (IHA) are the methods of choice for idenfication of unknown serotypes.

The purpose of this study was to report the serological and protective properties of 10 Dutch H. pleuropneumoniae strains isolated from outbreaks of pleuropneumonia in pigs.

MATERIAL AND METHODS

Ten Dutch strains (CVJ 13261, 87642, 89391, 89035, 89249, 89341, 97655, 94935, 5407 and 4219*) were examined.

The cultural and biochemical characteristics of the strains were consistens with earlier descriptions of H. pleuropneumoniae (*Kilian* 1976, *Biberstein et al.* 1977, *Nielsen* 1982).

Reference strains representing serotypes 1 through 8 were: Shope 4074, S 1536, S 1421, M 62, K 17, Femø, WF 83 and 405.

Antigens

The antigens for immunization of rabbits, for slide agglutination, for the indirect haemagglutination (IHA) and for gel diffusion were made as described earlier (*Nielsen & O'Connor* 1984). Heat treatment was made in a water bath at 100°C for 2 h.

Antisera

Rabbits were immunized as described earlier (*Nielsen & O'Connor* 1984) with serotype 1 (strain 4074), strain CVJ 13261 and strain 87642.

Cross protection experiments

A total of 11 nine-week-old pigs were used (9 vaccinates and and 2 controls). The pigs were derived from the laboratory SPF unit. None of the pigs had complement fixating antibodies to H. pleuropneumoniae serotypes 1 though 8 and strain CVJ 13261 at arrival. The pigs were divided into 2 groups (c.f. Tables 2 and 3).

* The strains were supplied by Laboratoria Dr. de Zeeuw, Utrecht and received by Dr. H.J. Riising, Nordisk Droge & Kemikalie, Copenhagen.

Vaccines

Vaccines were made from 6-h whole cells of H. pleuropneumoniae with Freund's incomplete adjuvant (3:1) as described earlier (*Nielsen* 1976).

Vaccination and challenge

The pigs were vaccinated twice with an interval of 3 weeks. Three weeks after the last vaccine injection the pigs were inoculated intranasally with 10^9 viable organisms. Three weeks after challenge the pigs were sacrificed and subjected to a full post mortem examination. Cultures were made from the lungs, tonsils and nasal cavity on 5 % calf-blood agar cross-inoculated with a non-haemolytic E. coli strain.

Serology

Blood samples were taken from the anterior vena cava before vaccination, before challenge and at sacrifice. Sera were tested by the modified complement fixation (CF) test (*Nielsen* 1974, 1982).

Indirect haemagglutination (IHA) test

The IHA test was made as described earlier (*Nielsen* 1974).

Gel diffusion

The technique used was described earlier (*Nielsen & O'Connor* 1984).

Absorption procedure

Absorption of antisera was made as described earlier (*Nielsen & O'Connor* 1984).

RESULTS

Slide agglutination

By the slide agglutination test none of the 10 strains revealed any serological relationship with serotypes 1 through 8. With rabbit antisera prepared against strain CVJ 13261 and 87642 flocculation was observed with all 10 strains.

IHA

Sheep red cells sensitized with saline washings or capsular extracts of the 10 strains were agglutinated to high titers by anti-

Table 1. IHA titers obtained with saline washings and capsular extracts of strains CVJ 13261 and 87642 against rabbit immune sera produced against 6-hour whole-cell antigens of the 2 strains.

	Antiserum					
Antigen	unabsorbed	absorbed with CVJ 13261	absorbed with 87642	87642 unabsorbed	absorbed with 87642	absorbed with CVJ 13261
CVJ 13261						
saline washings	10.240	—	—	2560	—	—
capsular extract	640	—	—	320	—	—
87642						
saline washings	10.240	—	—	5120	—	—
capsular extract	1280	—	—	640	—	—

Titers are given as reciprocals of the highest serum dilution giving positive reaction.
— = no reaction.

serum against strain CVJ 13261 and 87642. Homologous absorption removed all agglutinating activity from the sera. An example is given in Table 1.

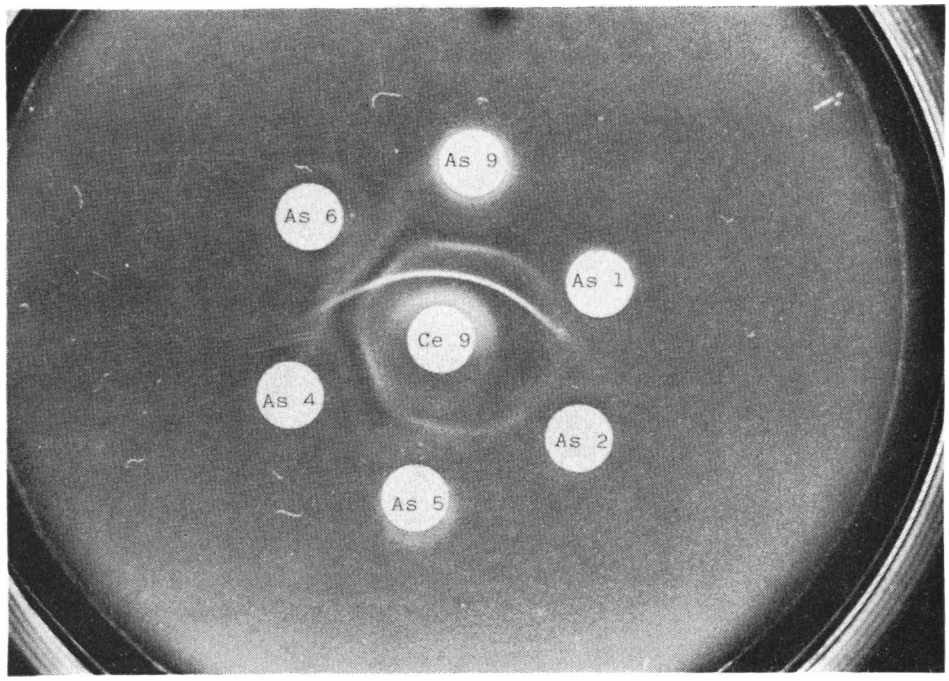
No cross reactions were observed between the 10 strains and serotypes 1 through 8.

Gel diffusion

When saline washings and capsular extracts of the 10 strains were used as antigen against antisera for strain CVJ 13261 and strain 87642 two precipitation lines were seen: one dense peripheral line and a second line close to the antigen well. Heat treatment of the antigen extracts did not remove the precipitation lines.

In addition to the homologous serotype-specific precipitates 1 or 2 precipitation lines showing reaction of identity between the 10 strains and serotypes 1 through 8 were observed. These precipitates were heat-labile.

Comparative analyses of capsular extracts from the 10 strains and strain 4074 (serotype 1) revealed a closer antigenic relationship between these strains. Thus, in addition to the 2 capsular precipitinogens characteristic of the serotype, capsular extract of the 10 strains contained precipitinogens which reacted with antiserum for strain 4074 (Fig. 1). Also, strain 4074 contained a precipitinogen which reacted with antiserum for strain CVJ



F i g u r e 1. Capsular extract of strain CVJ 13261 (Ce 9) in the center well. In the peripheral wells are antiserum for CVJ 13261 (As 9), strain 4074 (As 1), and serotypes 2, 5, 4 and 6 (As 2, 5, 4, 6).

13261 (Fig. 2). These precipitinogens were demonstrated as precipitation lines adjacent to the wells of the heterologous antigen preparation.

Cross absorption

Treatment of antiserum CVJ 13261 with its homologous antigen preparation resulted in complete absorption of the 2 serotype-specific precipitins in the antiserum. After heterologous absorption with antigen preparations of strain 4074 (serotype 1) the 2 precipitins could still be demonstrated in antiserum CVJ 13261 (Fig. 3). However, the precipitate situated close to the antigen well seemed broader before than after absorption with strain 4074.

The precipitin in antiserum CVJ 13261 which reacted with the precipitinogen of strain 4074 was removed both by homologous and heterologous absorption.

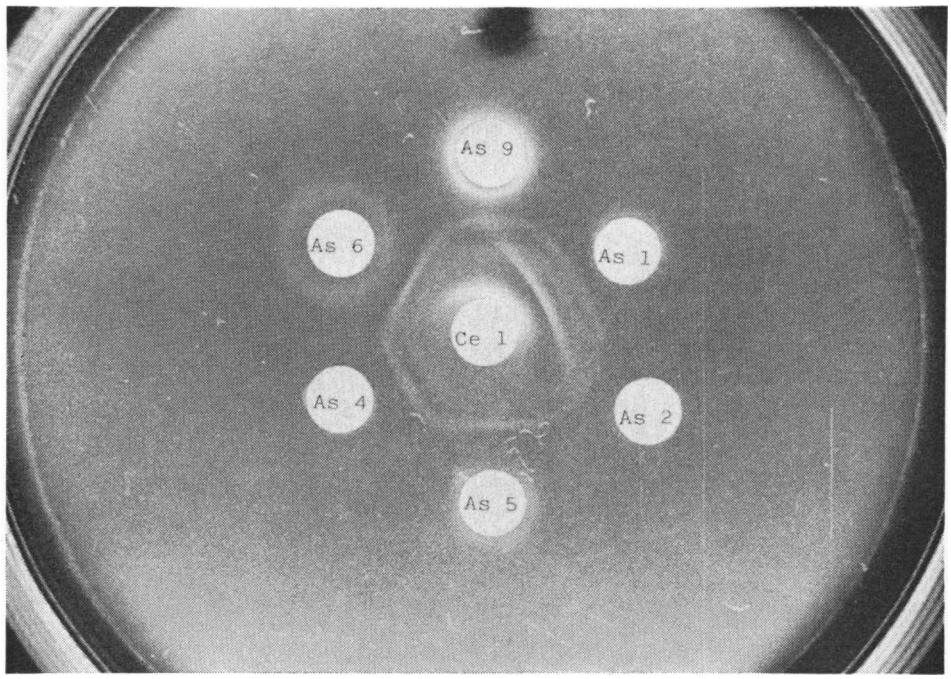
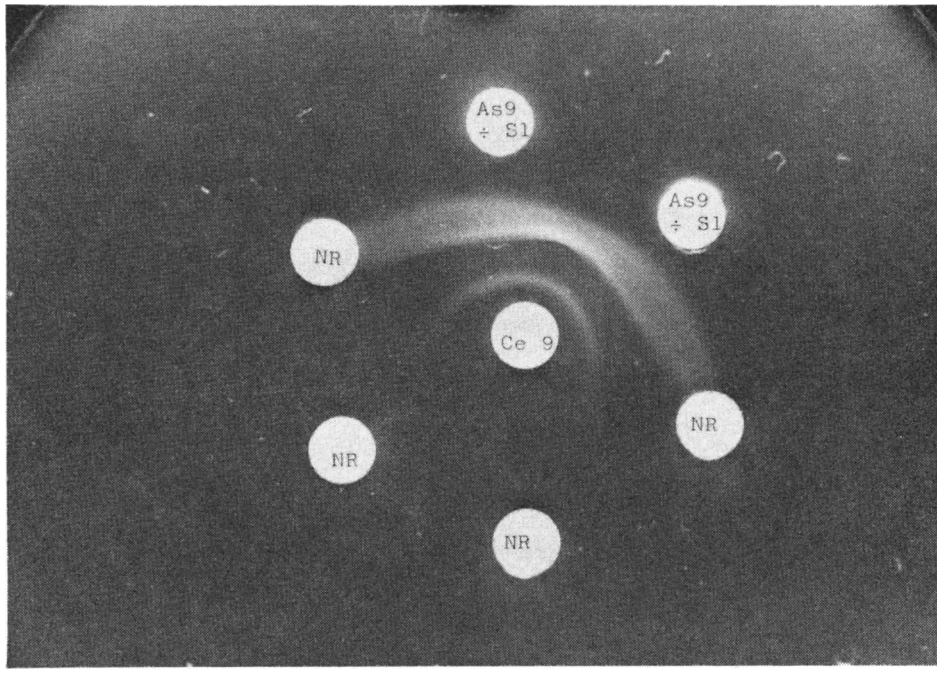


Figure 2. Capsular extract of strain 4074, serotype 1 (Ce 1) in the center well. In the peripheral wells are antiserum for CVJ 13261 (As 9), strain 4074 (As 1) and serotypes 2, 5, 4 and 6 (As 2, 5, 4, 6).

Treatment of antiserum 4074 (serotype 1) with its homologous antigen preparation removed the two serotype-specific precipitation lines (one peripheral and a second close to the antigen well). Heterologous absorption with an antigen preparation of strain CVJ 13261 removed completely the precipitation line seen close to the antigen well whereas the peripheral line remained unchanged.

The precipitin in antiserum 4074 which reacted with the precipitinogen of strain CVJ 13261 was removed both by homologous and heterologous absorption.

Thus, the results of the absorption experiments show that serotype 9, strain CVJ 13261, in addition to capsular precipitinogens characteristic of the serotype, contained capsular precipitinogens of serotype 1, strain 4074.



F i g u r e 3. Capsular extract of strain CVJ 13261 (Ce 9) in the center well. In the 2 upper peripheral wells are antiserum for CVJ 13261 (As 9) absorbed with strain 4074, serotype 1 (÷ S1). In the remaining 4 wells is serum from a non-immunized rabbit.

Cross protection experiments

G r o u p I. Five vaccinates, 1 control. Vaccine: 6-h culture of strain CVJ 13261. Challenge: serotype 1, strain 4074 (c.f. Table 2).

Three vaccinates (Nos. 1, 2, 5/70) showed no clinical symptoms and no lesions at necropsy. Two vaccinates (Nos. 3 and 4/70) and the control had severe respiratory distress 1 day after challenge (p.ch.). Necropsy revealed fibrous pleuritis and areas of necrosis which were more widespread in the control than in the vaccinates.

H. pleuropneumoniae, serotype 1 was re-isolated from the tonsils of all but 1 vaccinate and from the lungs of the 2 non-protected vaccinates and the control.

Thus, protection was obtained in 3 out of 5 vaccinates.

Post vaccination titers to strain CVJ 13261 were 1:256. Titers to serotype 1 were 1:32 to 1:128. A rise in titer to serotype 1 was

Table 2. Protective effect of a vaccine prepared from 6-h culture of serotype 9 (Strain CVJ 13261) with Freund's incomplete adjuvant (3:1). Dose of vaccine 2×2 ml s.c. Challenge inoculation was given intranasally with serotype 1 (Strain 4074) 3 weeks after the last vaccine injection. The pigs were sacrified 3 weeks after challenge.

Pig No.	Vaccine serotype	Challenge serotype	CF titers at challenge, Serotypes 9	1	CF titers at sacrifice, Serotypes 9	1	Clinical symptoms p.ch.*	Pleuropneumonia at necropsy	H. pleuropneumoniae serotype 1 re-isolated from:
1/70	9	1	256	128	128	64	none	o	tonsils
2/70	9	1	256	64	256	128	none	o	o
3/70	9	1	256	32	256	512	severe	+++	lung, tonsils
4/70	9	1	256	128	256	512	severe	+++	lung, tonsils
5/70	9	1	256	64	256	128	none	o	tonsils
6/70	control	1	o	o	64	64	severe	+++	lung, tonsils

* p.ch. = after challenge.
+++ = widespread pneumonic lesions.
Titers are given as reciprocals of the highest serum dilution giving positive reaction.

seen in the non-protected vaccinates. Titers to strain CVJ 13261 remained unchanged in all vaccinates p.ch. Before challenge the control pig was seronegative to strain CVJ 13261 and to strain 4074. Three weeks p.ch. it was seropositive to both strains with a titer of 1:64.

Table 3. Protective effect of a vaccine prepared from a 6-h culture of serotype 1 (strain 4074) with Freund's incomplete adjuvant (3:1). Dose of vaccine 2×2 ml s.c. Challenge inoculation was given intranasally with serotype 9 (strain 13261) 3 weeks after the last vaccine injection. The pigs were sacrificed 3 weeks after challenge.

Pig No.	Vaccine serotype	Challenge serotype	CF titers at challenge, Serotypes 1	9	CF titers at sacrifice, Serotypes 1	9	Clinical symptoms p.ch.*	Pleuropneumonia at necropsy	H. pleuropneumoniae serotype 9 re-isolated from:
6/69	1	9	128	128	256	512	severe	+++	lung, tonsils
7/59	1	9	128	64	256	512	severe	+++	lung, tonsils
8/69	1	9	64	128	128	256	mild	+	lung, tonsils
9/69	1	9	64	128	64	256	none	o	o
10/69	control	9	o	o	128	512	severe	+++	lung, tonsils

* p.ch. = after challenge.
+++ = widespread pneumonic lesions.
Titers are given as reciprocals of the highest serum dilution giving positive reaction.

Group II. Four vaccinates, 1 control. Vaccine: serotype 1 (strain 4074). Challenge: strain CVJ 13261 (c.f. Table 3).

One vaccinate (No. 9/69) showed no clinical symptoms and necropsy revealed no pulmonary lesions. Two vaccinates (Nos. 6 and 7/69) and the control had severe respiratory distress p.ch. Necropsy revealed fibrous pleuritis and areas of necrosis. The lesions were most severe in the control. One vaccinate (No. 8/69) had slight respiratory distress p.ch. At necropsy pulmonary lesions were much less severe than in the control.

H. pleuropneumoniae strain CVJ 13261 was re-isolated from the lungs and tonsils of the non-protected pigs and from the control.

Protection was obtained in 1 out of 4 vaccinates.

Post vaccination titers to serotype 1 were 1:64 to 1:128. No rise in titers was seen after challenge. Post vaccination titers to strain CVJ 13261 were 1:64 to 1:128. A rise in titer was seen in 2 of the non protected vaccinates (Nos. 6 and 7/69). Before antigens. As the 10 strains are antigenically homogeneous and strain 4074. Three weeks later it was seropositive to both strains with titers of 1:512 and 1:128, resp.

DISCUSSION

The 10 strains of Haemophilus pleuropneumoniae examined in the present study possessed 2 type-specific antigenic determinants of capsular origin as well as common species specific antigens. As the 10 strains are antigenically homogeneous and serologically distinct from serotypes 1 through 8 the strains are proposed to be referred to a new serotype, designated serotype 9, with strain CVJ 13261 as the type strain.

The results obtained in gel diffusion tests suggested a closer relationship between serotype 9 and 1 than between these serotypes and serotypes 2, 3, 4, 5, 6, 7 and 8. The cross-reactions observed between serotype 9 and 1 were shown by cross-absorption to be unrelated to type-specificity.

The presence of more than one capsular serotype in H. pleuropneumoniae has been described earlier (*Nielsen & O'Connor* 1984). Thus, in addition to serotype-specific capsular antigen, H. pleuropneumoniae serotype 8 share capsular antigens with serotypes 3 and 6. Results obtained with the IHA and gel diffusion tests indicated that the antigenic determinants shared by

serotypes 8 and 6 were of polysaccharide (PS) nature and those shared by serotypes 8 and 3 were of lipopolysaccharide (LPS) nature.

Since PS are the active principles in the sensitization of erythrocyte to the agglutinating effect of immune sera (*Keogh et al.* 1948) the finding in this study, that serotype 9 and 1 did not cross-react in the IHA test, indicated that the 2 serotypes do not share antigenic determinants of PS nature.

The antigenic determinants shared by serotypes 9 and 1 could be demonstrated only by gel diffusion. Heterologous precipitates were heat-stable and situated close to the antigen well which is consistent with a LPS nature of the precipitinogens (*Branefors-Helander* 1973).

In recent cross protection studies including serotypes 8, 3 and 6 the evidence obtained indicated that antibodies to type-specific capsular PS are important in the specific defence of the host against H. pleuropneumoniae infection. Also, the results suggested that antibodies to type-specific LPS play a role in the protection against this infection (*Nielsen* 1985). Thus, parenteral immunization with a killed 6-h culture of serotype 8 gave full protection against challenge with serotype 3. Likewise, pigs immunized with a killed 6-h culture of serotype 3 were fully protected against challenge with serotype 8.

In the present study parenteral immunization with a killed 6-h culture of serotype 9 did not afford an acceptable protection against challenge with serotype 1 since only 60 % of vaccinates were protected. The reverse experiment showed that parenteral immunization with serotype 1 only protected 25 % of vaccinates against challenge with serotype 9. These results show that the antigenic determinants shared by serotypes 9 and 1 are not able to yield a sufficient cross protection. Whether this is a question of quality or quantity cannot be ascertained until more is known about the antigenic structure of H. pleuropneumoniae serotypes.

The two control pigs inoculated intranasally with serotype 9 and 1 resp. developed complement fixating (CF) antibodies to both serotypes. In practice this means that it will not be possible to obtain an exact serotype diagnosis through blood testing of herds infected with these serotypes. Only by serotyping of isolated strains can a differentiation be accomplished. The experimental data presented above further sustains those obtained in recent studies (*Nielsen* 1985) that the serological and cross pro-

tective properties of H. pleuropneumoniae serotypes which share antigenic determinants with other serotypes should be identified before use in the CF test or in vaccines.

ACKNOWLEDGEMENT

The skilful technical assistance of Mrs. Tamara Plambeck is gratefully acknowledged.

REFERENCES

Branefors-Helander, P.: Serological studies of Haemophilus influenzae. I. Extracellular antigens of capsulated strains, types a to f, and two non-capsulated variants. Int. Arch. Allergy 1972, *43*, 533—547.

Branefors-Helander, P.: Serological studies of Haemophilus ifluenzae. III. The endotoxic effect of various antigen preparations and the relation between this effect and demonstrable precipitinogens. Int. Arch. Allergy 1973, *44*, 585—600.

Gunnarsson, A.: Haemophilus pleuropneumoniae syn parahaemolyticus. An antigenic and diagnostic study. Thesis, Uppsala 1980.

Keogh, E. V., E. A. North & M. F. Warburton: Adsorption of bacterial polysaccharides to erythrocytes. Nature 1948, *161*, 687—688.

Nicolet, J.: Sur l'hemophilose du porc. III. Différenciation sérologique de Haemophilus parahaemolyticus (Haemophilus infections in pigs. III. Serological studies on Haemophilus parahaemolyticus). Zbl. Bakt. 1971, *216*, 487—495.

Nielsen, R.: Serological and immunological studies of pleuropneumonia of swine caused by Haemophilus parahaemolyticus. Acta vet. scand. 1974, *15*, 80—89.

Nielsen, R.: Pleuropneumonia in swine caused by Haemophilus parahaemolyticus. Studies on the protection obtained by vaccination. Nord. Vet.-Med. 1976, *28*, 337—348.

Nielsen, R.: Haemophilus pleuropneumoniae infection in pigs. Thesis 1982. Commissioned by C. F. Mortensen A/S, Bülowsvej 5 C, DK-1870 Copenhagen V, Denmark.

Nielsen, R.: Haemophilus pleuropneumoniae (Actinobacillus pleuropneumoniae) serotypes 8, 3 and 6. Serological response and cross immunity in pigs. Nord. Vet.-Med. 1985, *37*, 217—227.

Nielsen, R. & P. J. O'Connor: Serological characterization of 8 Haemophilus pleuropneumoniae strains and proposal of a new serotype: Serotype 8. Acta vet. scand. 1984, *25*, 96—106.

SAMMENDRAG

Serologisk karakterisering af Haemophilus (Actinobacillus) pleuropneumoniae stammer og forelæggelse af en ny serotype: serotype 9.

Ti Haemophilus pleuropneumoniae stammer, isoleret fra hollandske besætningsudbrud af pleuropneumoni blev undersøgt serologisk

ved hjælp af objektglasagglutination, indirekte haemagglutination (IHA) og gel diffusion.

De 10 stammer udgjorde en homogen gruppe i antigen henseende og var serologisk forskellige fra de hidtil kendte serotyper 1 til 8. Gruppen er derfor blevet identificeret som en ny serotype: serotype 9 med stamme CVJ 13261 som typestamme.

Ved hjælp af gel diffusion blev det påvist, at de 10 stammer, foruden serotype specifikke kapselantigener, også indeholdt kapselantigener af serotype 1 (stamme 4074).

Krydsimmuniseringsforsøg viste, at en dræbt kultur af H. pleuropneumoniae serotype 9 kun beskyttede 3 af 5 vaccinerede grise mod challenge med serotype 1. Efter vaccination med en dræbt kultur af serotype 1 var kun 1 af 4 grise beskyttede mod challenge med serotype 9. Disse resultater viser, at de antigene determinanter, der er fælles for serotype 1 og 9, ikke alene er i stand til at give en tilstrækkelig beskyttelse mod pleuropneumoni.

(Received August 5, 1985).

Reprints may be requested from: Ragnhild Nielsen, the State Veterinary Serumlaboratory, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark.