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IMMUNOGLOBULINS IN NORMAL PORCINE TRACHEOBRONCHIAL SECRETIONS*

By
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HOLMGREN, NILS: *Immunoglobulins in normal porcine tracheobronchial secretions*. Acta vet. scand. 1973, 14, 366—380. — Methods of collecting sputum and tracheobronchial washings are described.

Proteins in normal porcine tracheobronchial secretions are studied by immunochemical qualitative and quantitative methods. Among the immunoglobulins, IgA, IgG and IgM are identified. Immuno-electrophoretic analysis of sputum shows albumin, transferrin and in the α -region 2 components not found in serum. The predominant immunoglobulin in tracheobronchial secretions is IgA, which accounted for about 85 % of the total immunoglobulin concentration in sputum and 61 % of the immunoglobulin concentration in tracheobronchial washings.

immunoglobulins; pigs; tracheobronchial secretions.

During the last decade the presence of a local immune system resident on mucosal surfaces has been studied intensely in different species. Since the work by *Hanson* (1961) in Sweden and *Chodirker & Tomasi* (1963) and *Tomasi et al.* (1965) in the United States it is well established that a local humoral immune system exists in human external secretions. An immunoglobulin analogous to human secretory IgA has been isolated from porcine colostrum and milk (*Porter 1969, Bourne 1969, Porter et al. 1970, Richardson & Kelleher 1970, Vaerman et al. 1970*), saliva (*Bourne*), gut mucosa and intestinal juice (*Bourne et al. 1971*).

The presence of immunoglobulins in the human tracheobronchial tree has mostly been studied in sputum during pathological conditions. The secretion from the normal tracheobronchial tree is scanty in amount and difficult to collect. Most in-

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vestigations have therefore been performed on tracheobronchial washings collected during anaesthesia (*Keimowitz 1964, Masson et al. 1965, Turgeon et al. 1969, 1971*).

Immunoglobulins have been studied in canine sputum collected undiluted in a surgically prepared tracheal pouch (*Lieberman et al. 1970*).

The aim of this study is to investigate the proteins in normal porcine tracheobronchial secretions by immunochemical qualitative and quantitative methods, with special reference to the immunoglobulins.

MATERIAL AND METHODS

Animals

Sputum and tracheobronchial washings were studied in 9 pigs of Swedish landrace. The pigs were about 6 months old. They were delivered from a stockfarm which for the last 15 years has been free of Swine Enzootic Pneumonia and other contagious respiratory diseases. All post-mortem examinations including microscopic investigations of the lungs from the 9 pigs showed normal conditions.

Collection and processing of specimens

The pigs were anaesthetized by intravenous injections of nembutal sodium and placed in a recumbent position on an operation table. An incision was made in the skin from the cartilago cariniformis to the larynx. The trachea and larynx were dissected in a blunt way in order to avoid bleedings. The trachea was lifted up and cut off behind the larynx. Sputum was collected by inserting a piece of cotton held by a forceps into the carina, where the cotton was soaked in sputum. The cotton was then immediately put in a plastic syringe (1 ml). The tip of the syringe was sealed with a rubber cork. The sputum samples were stored at -70°C until analysed. Sputum was collected by squeezing the cotton in a syringe.

Collection of tracheobronchial washings (TBW)

A rubber cork penetrated by a 15 cm plastic tube was fixed in the trachea with ligatures, so that the pig was breathing through the tube. Twenty ml of PBS (phosphate buffered saline, pH 7.4)

was injected through the plastic tube with the pig in a horizontal position. Then the operation table was lifted, so that the pig was placed in a vertical position with the head downwards. TBW was collected from the plastic tube into a 100 ml Erlenmeyer flask. The procedure was repeated 9 times. The TBW was homogenized in a Potter-Elvehjem homogenizer for 20 min. and centrifuged at 5700 r.p.m. for 20 min. The supernatant was concentrated with polyvinylpyrrolidone (PVP) about 50 times and stored at -70°C until analysed.

Preparation of IgA from sow's milk

The method of *Bourne* (1969) was used with some modifications. Milk was collected 12—15 days after partus from 3 different sows. The milk was pooled and the fat removed by centrifugation at 15,000 r.p.m. for 15 min. at 4°C . The casein was centrifuged down at 15,000 r.p.m. for 3 hrs. at 4°C . Lipoprotein was precipitated by adding 0.02 ml of a 10 % (w/v) dextran sulphate 500 (Pharmacia, Uppsala) solution followed by 0.1 ml 1 M- CaCl_2 to each ml of the milk. The precipitate was sedimented by centrifugation at 15,000 r.p.m. for 15 min. at 4°C . The supernatant was dialyzed against distilled water and then against 0.5 M Tris-HCl 0.15 M-NaCl buffer (pH 8.1). One hundred ml of this milk serum was separated on Sephadex G 200, column size 9×97 cm, with reverse flow 80 ml/hr. The fraction volume was 10 ml. 0.5 M Tris-HCl 0.15 M-NaCl buffer was used for elution. The second part of the first peak was pooled and dialyzed against distilled water and concentrated about 10 times with PVP. After dialysis against 0.15 M Tris-HCl buffer (pH 7.4), the concentrated fraction was chromatographed on Sephadex DEAE A 50, column size 2.5×46 cm, flow rate 16 ml/hr. and fraction volume 10 ml, with stepwise elution using Tris-HCl buffers 0.15 M, 0.2 M, 0.3 M, 0.5 M, and 0.7 M (pH 7.4). The peaks obtained at 0.2 and 0.3 molarity were concentrated with PVP and dialyzed against 0.5 M Tris-HCl 0.15 M-NaCl (pH 8.1). The peaks were run separately on Sephadex G 200, column size 2.5×46 cm, flow rate 10 ml/hr. and fraction volume 10 ml. The Tris-HCl buffer was used for elution. In both cases a single symmetrical peak was obtained. The second part of both peaks was pooled concentrated and re-separated under the same conditions. A single symmetrical peak was obtained. The second half of the peak was used for immunization purposes.

Preparation of rabbit anti-swine-IgA serum

On days 1 and 3, 0.3 ml of Freund's complete adjuvant was injected into the footpad of a hindleg. On day 7, when Igl. poplitalis was easily palpable, 0.3 ml of aluminium-adsorbed IgA (0.6 mg protein per ml as measured by u.v. absorption) was injected into the lymph node. This was repeated on days 11, 15 and 19. Aluminium-adsorbed IgA was then given i.v. 0.5 ml on days 27 and 28, and 1.0 ml on days 29 and 30. An antihistamine (Vetibenzamin, Ciba 0.5 ml i.m.) was given before the i.v. injections to inhibit anaphylactic reactions. Aluminium-adsorption of IgA was performed before each injection by adding some crystals of potassium aluminium sulphate followed by a few drops of 0.1 N-NaOH to make the solution basic. A heavy precipitate was formed.

Blood was drawn 1 week after the last injection.

Swine IgG and rabbit anti-swine-IgG serum were prepared according to *Martinsson* (1970). Pure swine IgM and a monospecific rabbit anti-swine-IgM serum were kindly supplied by Dr. A. Jönsson, Dept. Medicine I, Royal Veterinary College, Stockholm.

Pure swine transferrin and a rabbit anti-swine-transferrin serum were kindly donated by Dr. K. Martinsson and Dr. K. Thorén, Dept. Medicine I, Royal Veterinary College, Stockholm. A monospecific rabbit anti-swine-IgA serum was generously supplied by Dr. P. Porter, Unilever Research Laboratory, Colworth House, Scarnbrook, Bedford, Great Britain.

Preparation of rabbit anti-TBW serum

On days 1 and 3, 0.3 ml of Freund's complete adjuvant was injected into the footpad of a hindleg. On day 9, when Igl. poplitalis was easily palpable, 0.2 ml of concentrated TBW (0.2 mg protein per ml as measured by u.v. absorption) + 0.2 ml of Freund's incomplete adjuvant were injected into the lymph node. This was repeated on days 15, 21, 27, and 33. On days 43 and 45 2 ml of aluminium-adsorbed TBW was injected i.v. Antihistamine was given before the i.v. injections to inhibit anaphylactic reactions. Aluminium-adsorption of TBW was performed as with IgA.

Blood was drawn 6 days after the last injection.

Rabbit anti-TBW serum was absorbed with freeze dried swine serum for studies on sputum components not found in serum. Absorption was made until no reaction occurred with swine serum.

Immuno-electrophoretic analysis was performed according to the method of *Scheidegger* (1955).

The double diffusion-in-gel technique by *Ouchterlony* was used in its micromodification (*Wadsworth* 1957).

The single radial diffusion test was used essentially as described by *Mancini et al.* (1965). Each sample was determined in doubling dilutions in PBS, which gave 2—4 measurable values from which the mean values was calculated. IgM was measured by using the samples undiluted and diluted 1:2. Duplicate determinations were made for every TBW sample on different plates at different times, and the mean value was calculated. The volume of the sputum samples only permitted single determinations. At least 2 determinations of a control serum were made on each plate.

Reference antigens. IgG was obtained from swine serum according to *Martinsson*. The protein concentration of the IgG reference antigen was determined by the micro-Kjeldahl method, and the nitrogen content $\times 6.25$ was regarded as the protein concentration. IgA prepared from sow's milk was used as standard antigen. The protein concentration was determined by the Lowry method (*Lowry et al.* 1951). IgM was prepared according to *Jönsson* (to be published). The protein concentration of the IgM reference antigen was determined by the micro-Kjeldahl method, and the nitrogen content $\times 6.25$ was regarded as the protein concentration. The standard curves for the Mancini determinations of IgG, IgA and IgM were drawn with the use of dilutions of the reference immunoglobulins, and the areas of precipitates obtained were plotted against the corresponding concentrations.

Analytical errors. These were calculated from repeated determinations of a control serum on different plates and at different times. The following formula was used: $S^2 = \frac{n \sum x^2 - (\sum x)^2}{n(n-1)}$

RESULTS

Separation of IgA from milk

The results of separation on Sephadex G 200 of porcine milk collected 12—15 days after partus are shown in Fig. 1. Most IgA was eluted in the first peak together with some IgM and before IgG.

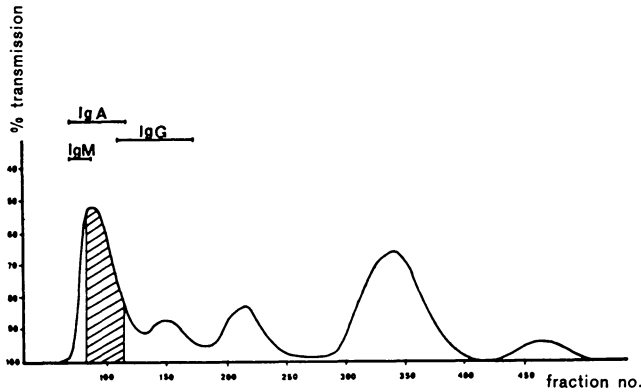


Figure 1. Gel filtration on Sephadex G 200 of porcine milk collected 12—15 days after partus. The curve represents the transmission at 280 m μ .

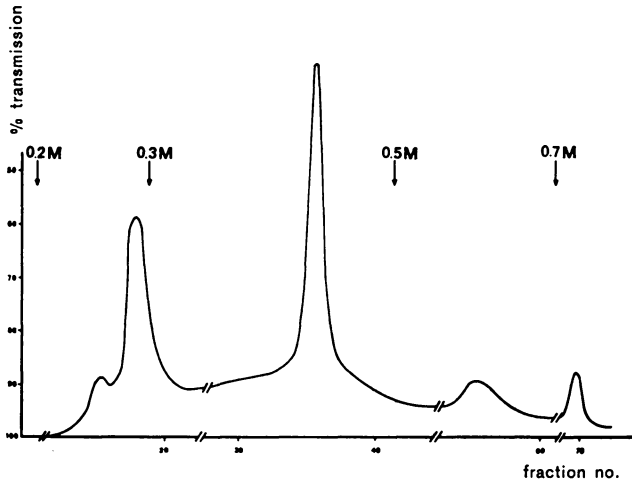


Figure 2. Elution pattern after chromatographic separation on DEAE-Sephadex A 50 of the IgA preparation from gel filtration on Sephadex G 200 (Fig. 1). The curve represents the transmission at 280 m μ .

The shaded area of the first peak was chromatographed on Sephadex DEAE A 50, and the elution pattern is shown in Fig. 2.

The peaks eluted at 0.2 and 0.3 molarity were run separately on Sephadex G 200, and a single symmetrical peak was obtained in both cases. The second part of these peaks was pooled and re-separated under the same conditions. The result of this final gel filtration is shown in Fig. 3.

Immunoelectrophoretic analyses and immunodiffusion tests

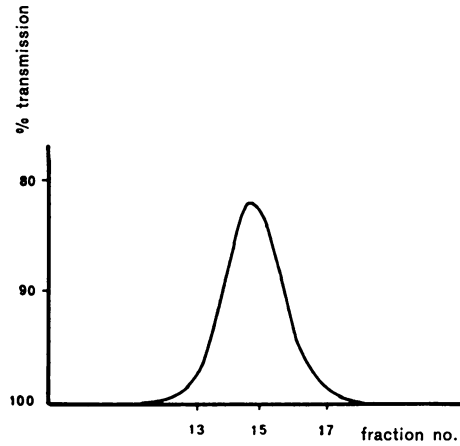


Figure 3. Gel filtration on Sephadex G 200 of the final IgA preparation. The curve represents the transmission at 280 m μ .

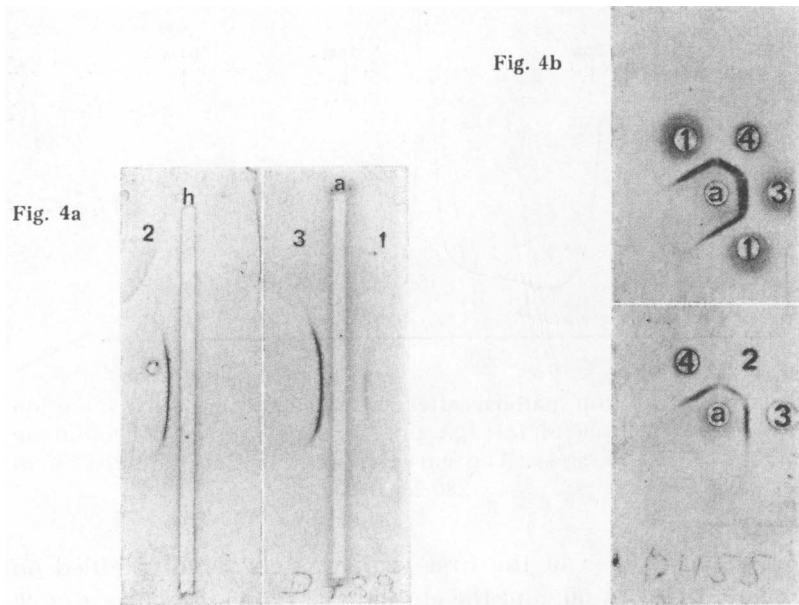


Figure 4a. Immuno-electrophoretic analyses of the IgA preparation (2), porcine milk (3) and swine serum (1) against rabbit anti-serum to porcine milk IgA (a) and rabbit anti-serum to swine serum (h).

Figure 4b. Immunodiffusion test of swine serum (1), milk IgA (2), milk (3), and sputum (4) against rabbit anti-porcine-milk IgA serum (a).

of the milk IgA are shown in Figs. 4 a and 4 b. The antiserum prepared against porcine milk IgA is showing a reaction of identity between milk IgA and serum IgA suggesting that the antiserum does not react with the secretory component. The rabbit anti-milk-IgA serum obtained with the immunization technique described did not react with IgG and IgM. The relationship of the isolated protein to sputum and milk and a reaction of identity between sputum and milk with monospecific anti-milk-IgA serum are shown in the same figures.

Qualitative immunochemical examination of tracheobronchial secretions

Immuno-electrophoretic analysis of sputum is shown in Fig. 5.

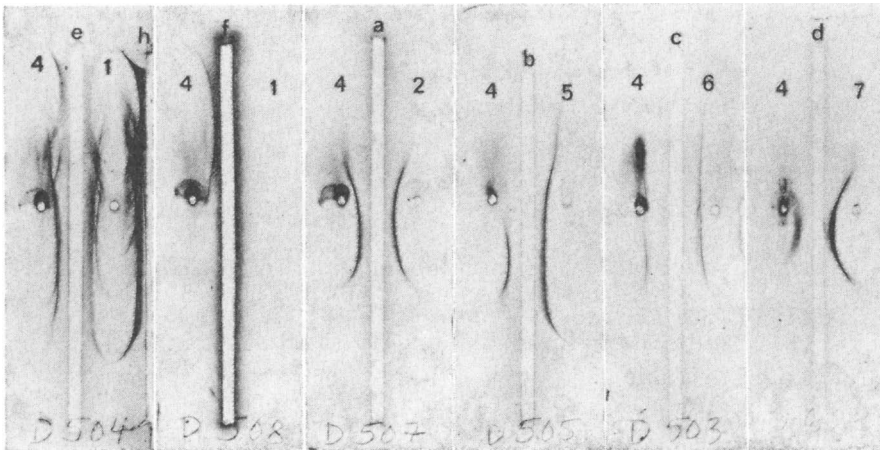


Figure 5. Immuno-electrophoretic analyses of sputum. The symbols refer to the following: 1 = swine serum, 2 = milk IgA, 4 = sputum, 5 = IgG, 6 = IgM, 7 = transferrin, a = rabbit antiserum to porcine milk IgA, b = IgG, c = IgM, d = transferrin, e = TBW, f = TBW absorbed with swine serum, and h = swine serum.

Among the immunoglobulins, IgG and IgA are easily detectable. IgM could be shown only by using a monospecific antiserum, probably because of low concentration of this immunoglobulin. Immuno-electrophoresis of sputum showed an arc that was electrophoretically similar to albumin in serum. Two arcs in the α -region remained after absorption of the anti-TBW serum with swine serum. The relationship of sputum to IgG, IgA, IgM and transferrin with monospecific antisera in the immunodiffusion test is shown in Fig. 6.

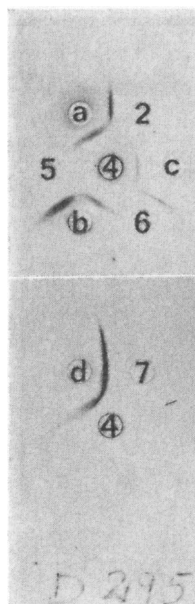


Figure 6. Immunodiffusion tests of sputum. The symbols are the same as in Fig. 5.

Quantitative estimation of immunoglobulins in tracheobronchial secretions

The results of the immunoglobulin quantitation in TBW are presented in Table 1.

Table 1. Immunoglobulin levels in TBW from 9 normal pigs.

Pig no.	mg/100 ml			Rel. %		
	IgA	IgG	IgM	IgA	IgG	IgM
1	29.1	26.2	2.2	50.6	45.6	3.8
2	20.4	29.6	2.2	50.2	56.7	4.2
3	42.4	28.8	6.2	54.8	37.2	8.0
4	142.6	11.9	15.1	84.1	7.0	8.9
5	26.4	8.1	2.4	66.7	22.0	6.5
6	29.9	9.7	1.3	73.1	23.7	3.2
7	54.1	30.1	4.1	61.3	34.1	4.6
8	66.4	58.1	7.3	50.4	44.1	5.5
9	21.3	10.8	2.5	61.6	31.2	7.2
Mean value \pm s				61.4 ± 11.7	33.5 ± 14.8	5.8 ± 2.0

With exception of pig no. 2, levels of IgA exceeded those of IgG, and the IgG exceeded the IgM values. Compared to the others, pig no. 4 had more IgA and IgM but less IgG. The mean values \pm s of relative percentage of the total immunoglobulin content are for IgA 61.4 ± 11.7 , IgG 33.5 ± 14.8 , and IgM 5.8 ± 2.0 .

The results of the immunoglobulin quantitation in sputum samples are shown in Table 2.

Table 2. Immunoglobulin levels in sputum from 9 normal pigs.

Pig no.	mg/100 ml			Total Ig conc.	Rel. %		
	IgA	IgG	IgM		IgA	IgG	IgM
1	541.7	69.8	17.5	629.0	86.1	11.1	2.8
2	nd	nd	nd	—	—	—	—
3	162.4	8.0	16.5	186.9	86.9	4.3	8.8
4	597.5	51.6	23.6	672.7	88.8	7.7	3.5
5	703.2	36.1	nd	—	—	—	—
6	277.6	50.3	9.0	327.9	84.7	15.3	2.8
7	540.6	69.0	21.3	630.9	85.7	10.9	3.4
8	nd	nd	nd	—	—	—	—
9	304.4	65.7	27.2	397.3	76.6	16.5	6.9
Mean	446.8	50.1	19.2	474.1	84.8	11.0	4.7
value \pm s	± 198.4	± 22.2	± 6.4	± 198.9	± 4.3	± 4.6	± 2.5

nd = not done.

The sputum samples show a great variation in the total immunoglobulin concentration. However, determination of the relative percentage of the total immunoglobulin content gives for IgA 84.4 ± 4.3 , IgG 11.0 ± 4.6 and IgM 4.7 ± 2.5 (mean values \pm s).

The IgA average is higher and the IgG average is lower than that found in TBW from the same pigs. The IgM average is low and about the same as that found in TBW.

The quotient between the relative percentage of the immunoglobulins gives an IgA/IgG average of 7.7 in sputum and 1.8 in TBW suggesting that IgA is the main immunoglobulin in normal porcine tracheobronchial secretions.

Analytical errors. For IgA the analytical error was calculated from 9 determinations of a control milk serum at different dilutions. The calculated mean value was 283.4 ± 9.8 mg per 100 ml of milk serum ($\bar{x} \pm s$) which means an analytical error of 3.5%. For IgG the analytical error was calculated from 26 determina-

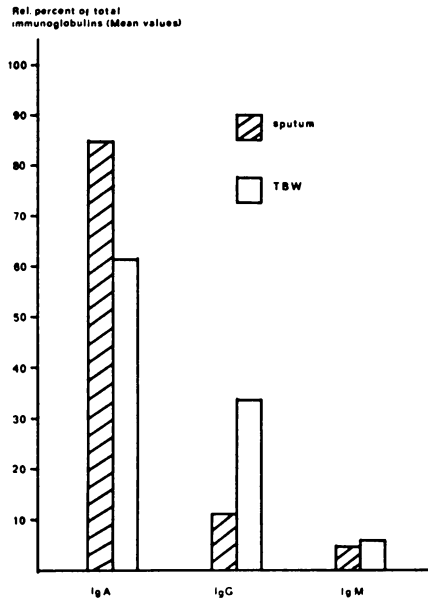


Figure 7. Mean values for IgA, IgG and IgM expressed in relative percentage of the total immunoglobulin concentration in sputum and TBW.

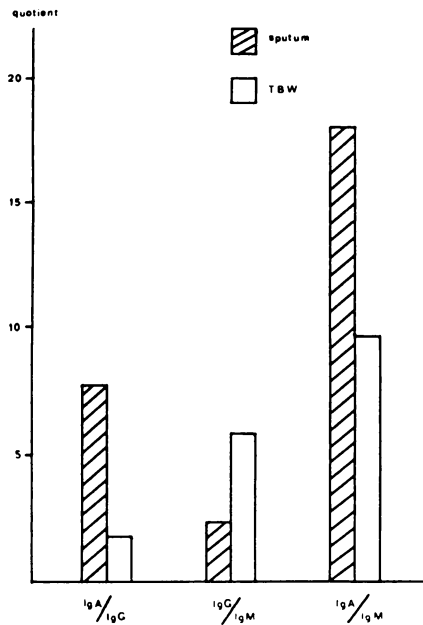


Figure 8. Quotients between the relative percentage of immunoglobulins in TBW and sputum.

tions of a control serum at different dilutions. The calculated mean values was 2301 ± 196 mg per 100 ml of serum ($\bar{x} \pm s$), which means an analytical error of 8.5 %. For IgM the analytical error was calculated from 12 determinations of a control serum at different dilutions. The calculated mean value was 125 ± 11.8 mg per 100 ml of serum ($\bar{x} \pm s$), which means an analytical error of 9.4 %

DISCUSSION

Compared to other external secretions⁵, little work has been done on the immunoglobulins in normal tracheobronchial secretions. This is probably due to great difficulties in collection of specimens. In normal individuals the sputum is scanty in amount and difficult to collect without injuring the mucous membrane. The method of collecting sputum specimens by a piece of cotton works well and has also been used by putting a piece of cotton through a bronchoscope into the carina. However, care must be taken to avoid traumatic lesions in trachea, which may result in contamination with blood. In these investigations no red blood corpuscles could be observed either in sputum or in samples of TBW.

All samples were taken during anaesthesia which seems to be necessary because shortly after death there is a transudation of serum into the tracheobronchial tree (Waldman *et al.* 1968).

Accurate quantitation of immunoglobulins in external secretions is complicated by technical problems when using precipitation techniques. For quantitation of IgA in secretions it is necessary to use a standard with similar antigenic properties as the tested antigen (Tomasi & Bienstock 1968). Use of milk IgA, which is homogenous in gel filtration, as a standard for quantitation of IgA in tracheobronchial secretions does not necessarily give absolute figures because there might be a heterogeneity in molecular size for IgA in sputum. However, it is not likely that this would change the finding of IgA as the predominant immunoglobulin in sputum and TBW.

The predominant immunoglobulin in tracheobronchial secretions was IgA which accounted for about 85 % in sputum and 61 % in TBW of the total immunoglobulin concentration. This is in agreement with the results obtained in other species (Keimowitz 1964, Masson *et al.* 1965, Waldman *et al.*, Turgeon *et al.*

1969, Lieberman *et al.* 1970), where the IgA levels exceeded those of IgG and IgM in tracheobronchial secretions.

The IgA/IgG ratio in sputum is 7.7:1 and in TBW 1.8:1. The corresponding quotient has been estimated as being 12:1 in porcine intestinal juice and 5:1 in porcine gut mucosa (Bourne *et al.* 1971). In porcine milk the IgA/IgG is about 3.5:1 (Vaerman *et al.* 1970).

The difference between the IgA/IgG quotient in sputum and TBW is difficult to explain. The sputum samples were collected at the carina. The TBW contained proteins also from the deeper part of the bronchial tree and there might be quantitative differences between the immunoglobulins at different levels of the tracheobronchial tree. On the other hand IgA has been shown to be a constituent of the insoluble bronchial mucous gel (Anzai *et al.* 1963). Rasche & Ullmer (1971) subjected sputum to centrifugation, ultrasonics, followed by freeze drying. The fraction obtained after centrifugation alone contained more IgG and less IgA compared to the IgG and IgA levels in the fraction obtained after ultrasonics and centrifugation. This finding indicates that difficulties in releasing IgA from the bronchial mucous gel with homogenization of TBW also may be of importance for the low IgA/IgG quotient in TBW.

By the use of immunodiffusion tests IgM could be detected in sputum and TBW of all pigs tested. In man IgM has infrequently been found in saline washings from the tracheobronchial tree (Keimowitz, Masson *et al.*, Waldman *et al.*, Turgeon *et al.*). This may be due to restricted possibilities in concentrating relatively small samples.

Besides the immunoglobulins, albumin and transferrin could be identified in tracheobronchial secretions. Two other precipitates could be seen in immunoelectrophoretic analysis using anti-TBW serum as antiserum. The absorption of rabbit anti-TBW serum with swine serum revealed 2 components in the α -region, which seemed to be specific for the bronchial secretions.

The origin of the proteins in sputum and TBW can not be determined judging from these investigations. However, the presence of albumin and transferrin indicates a passage from blood serum into the tracheobronchial tree. The occurrence of immunoglobulins may be due to both such a passage and also a local production, especially of IgA.

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SAMMANFATTNING

Immunoglobuliner i tracheobronchialsekret hos normala grisar.

Metoder att samla sputum och tracheobronchial spolvätska beskrivs. Proteiner i normala tracheobronchiala sekret hos svin studeras med immunokemiska kvalitativa och kvantitativa metoder. Bland immunoglobulinerna identifieras IgA, IgG och IgM. Immuno-elektroforetisk analys av sputum visar albumin, transferrin och i α -regionen två komponenter vilka ej kan påvisas i serum. Det dominerande immunoglobulinet i tracheobronchiala sekret är IgA, som utgör cirka 85 % av den totala immunoglobulinkoncentrationen i sputum och cirka 61 % av totala immunoglobulinkoncentrationen i tracheobronchial spolvätska.

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