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Variability of the Immunological State of Germfree Colostrum-Deprived Minnesota Miniature Piglets

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Minnesota miniature piglets obtained by hysterectomy and deprived of colostrum were examined for the presence of immunoglobulin by immunoelectrophoresis, double-gel diffusion, and radial immunodiffusion techniques with specific anti-immunoglobulin chain sera. A large amount of variability existed between different litters of piglets and between different piglets within the same litter, ranging from no detectable immunoglobulin in the serum to very high immunoglobulin levels approaching that of the adult pig. All known classes of porcine immunoglobulin including immunoglobulin G, immunoglobulin M, and immunoglobulin A could be found in the sera from litters where there was extensive placental damage. This contaminating immunoglobulin was shown to have antibody activity to actinophage MSP-2 even when present in very low concentrations. The low level contamination with immunoglobulin G, which was the most frequently encountered type of contaminant, was demonstrated to be similar to sow immunoglobulin G both antigenically and in its molecular size. The data demonstrates that individual piglets must be tested for immunoglobulin content rather than being assumed to be immunologically "virgin" and emphasizes the need for an intact placenta barrier to obtain piglets free from maternal immunoglobulin and devoid of antigenic stimulation.

Germfree colostrum-deprived Minnesota miniature piglets from pathogen-free swine herds have been shown to be free of all known classes of immunoglobulin, antibody, and antibody-producing cells (9, 10, 12). The fact that these animals can mount a detectable immune response within 48 h after injection of antigen indicates that they are highly immunocompetent but are kept immunologically "virgin" by a unique placental barrier in utero (9, 10, 12). These findings were consistent with those of other investigators (17) who were unable to detect antibody in germfree colostrum-deprived piglets using a bactericidal assay with a sensitivity of 10^{-6} μg of antibody N per ml.

Recently we have experienced an increase of the placental leakage of maternal immunoglobulin in the herd of Minnesota miniature swine. Therefore, it became necessary to reinvestigate the immunological status of individual colostrum-deprived Minnesota minature piglets obtained by hysterectomy. The frequency of contamination of piglets with maternal immunoglobulin and the nature of the contaminants were analyzed in these studies. (An abstract of part of this work has been presented elsewhere [T. M. Setcavage and Y. B. Kim, Fed. Proc. 33:599, 1974].)

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MATERIALS AND METHODS

Animals. Germfree colostrum-deprived Minnesota miniature piglets were obtained by hysterectomy 3 to 5 days before term as previously described (10).

Antibody titration. MSP-2 actinophage-neutralizing activity was determined as given elsewhere (4, 10).

Preparation of anti-chain sera. Heavy chains from sow immunoglobulin G (IgG) or immunoglobulin M (IgM) were obtained after reduction with dithiothreitol (7) and alkylation with iodoacetamide by Sephadex G-100 gel filtration in 1 N propionic acid. The first peak from these fractionation procedures was rechromatographed twice on the same column. Purified immunoglobulin chains were used to immunize rabbits by multiple site injection over a period of 5 weeks. Anti-gamma chain serum was highly specific. Anti-mu chain serum was made monospecific by absorbing with IgG bound to Sepharose 4B (15). Antiserum specific for alpha chains was made by immunizing with highly purified IgA and absorbing with glutaraldehydge aggregated IgG (2).

Radial immunodiffusion. Radial immunodiffusion experiments were conducted according to a modified procedure of Mancini et al. (13) for quanti-

tation of immunoglobulins. Briefly, Noble agar was melted in distilled water and diluted with an equal volume of 0.05 M veronal buffer. Anti-pig gamma chain antisera was added to the melted agar after the temperature was adjusted to 45 C to give a final dilution of 1:50. Twelve milliliters was dispensed into petri dishes, which were set on a level surface until solidified. Wells 2.5 mm in diameter were drilled and the agar was removed by suction. The bottom of each well was sealed with 1.5% agar by use of a 26-gauge needle and syringe. Ten microliters of sample was added to each well, and the plates were incubated at 37 C until fully developed. Each plate contained a minimum of four standard concentrations of purified sow IgG which were used to determine the slope of concentration versus diameter line. Pictures were taken of the developed plates and the diameters of the standard and sample precipitin rings were determined from the photographs.

Exclusion chromatography on Sephadex G-200. Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was swollen in tris(hydroxymethyl)aminomethane buffer (0.1 M Trizma base, 0.3 M NaCl adjusted to pH 7.8 at 4 C). Pharmacia water-jacketed columns K 50/100 (5.0 by 100 cm) were used for gel filtration procedures and fractions were collected in a refrigerated Buchler fraction collector (Buchler Instruments, Inc., Fort Lee, N. J.).

Samples which were previously dialyzed against tris(hydroxymethyl)aminomethane buffer overnight were applied to the bottom of the column by means of an LKB MultiPerpex pump (LKB Corporation, Stockholm, Sweden) and Pharmacia three-way valve. The elution was accomplished by upward flow of buffer through the column. The optical density of the fractions was measured with a Beckman Acta III spectrophotometer at 280 nm. Fractions were pooled and concentrated by pervaporation or were concentrated individually by Amicon macrosolute concentrators (Amicon Corp., Lexington, Mass.). Finally, the concentrated fractions were dialyzed against physiological saline at 4 C.

Molecular weight determinations by gel filtration. Molecular weight determinations were carried out on Sephadex G-200 columns. Protein standards and blue dextran obtained from Pharmacia Co. were filtered through the column in three separate column runs under exactly the same conditions as the test samples. The elution volumes for the standards were used to plot the selectivity curve (1). Pooled samples of whole piglet sera were chromatographed on the same column, and the IgG peak was located by radial immunodiffusion using anti-gamma chain antisera. The K_{av} value of this peak was used to determine the molecular weight of the IgG contaminant of piglet sera (1).

RESULTS

Colostrum-deprived Minnesota miniature piglets obtained by hysterectomy were bled by cardiac puncture shortly after the operation. Serum samples were analyzed by immunodiffusion techniques, and some piglets contained detectable amounts of immunoglobulin. The most frequently encountered class of immunoglobulin was IgG, which was commonly present in such small amounts that it was evidenced by only a shadow on an immunoelectrophoretic pattern. The barely discernible IgG usually appeared in the fast-moving γ_1 region on the interior of the pattern. Sera from a representative litter of eight piglets that demonstrated the same type of phenomenon were subjected to Ouchterlony analysis shown in Fig. 1. Highly specific anti-gamma chain antiserum in the center walls showed lines of precipitation with seven of the eight piglets. Purified sow IgG contained in two of the wells produced a line of identity with the piglet sera, substantiating the presence of IgG. It is also evident from this figure that different degrees of contamination were exhibited by members of the same litter. Po-1 did not show a precipitin band, whereas other piglet sera produced bands which were different distances from the center well and had different degrees of sharpness. These sera were tested by radial immunodiffusion in an attempt to quantitate the amount of IgG present in each sample. In each case the concentration of IgG was less than 50 μg/ml. Twenty-five milliliters of pooled piglet serum which contained this level of IgG contamination but no detectable immunoglobulins of the IgM or IgA class was chromatographed on Sephadex G-200. Fractions from the individual peaks were pooled, concentrated to 5.0 ml, and tested for antibody activity by the very sensitive technique of phage neutralization. The percentage of neutralization of each of the three peaks is shown graphically in Fig. 2. The second peak contained 70% of the total neutralizing capacity of the serum, the first peak contained 27%, and the third peak contained only 3%. The three peaks were also analyzed by immunoelectrophoresis as shown in Fig. 3. The small amount of IgG present in whole piglet sera eluted in the second peak of Sephadex G-200 chromatography.

An indirect molecular weight determination for this IgG contaminant was carried out on Sephadex G-200. The sample used for this experiment was pooled Po sera, each of which contained trace amounts of IgG (50 to 60 μg/ml). Twenty milliliters of sera was loaded onto a Sephadex G-200 column (70 by 5 cm), and 10.0-ml fractions were collected. Each fraction was concentrated to 1.0 ml and analyzed for IgG content by radial immunodiffusion with anti-gamma serum. Figure 4 shows the elution pattern obtained from the column with the radial immunodiffusion data superimposed. The molecular weight value for the IgG contaminant

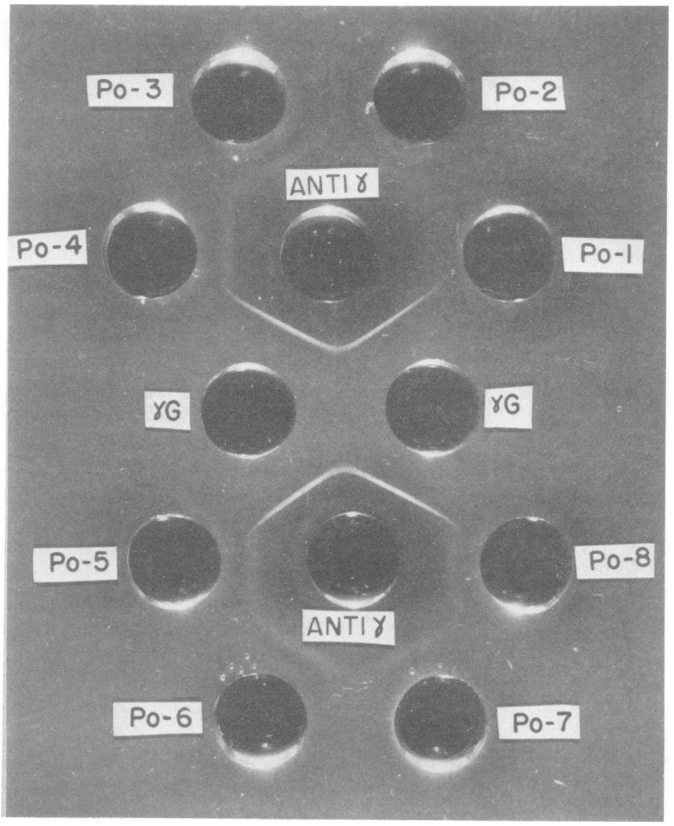

FIG. 1. *Double-gel diffusion pattern of the sera from eight piglets (Po) from the same litter. Anti-gamma chain sera in the center wells form a line of precipitation with Po-2 to Po-8. A line of identity is shown between piglet sera and purified sow IgG (γG, 0.5 mg/ml).*

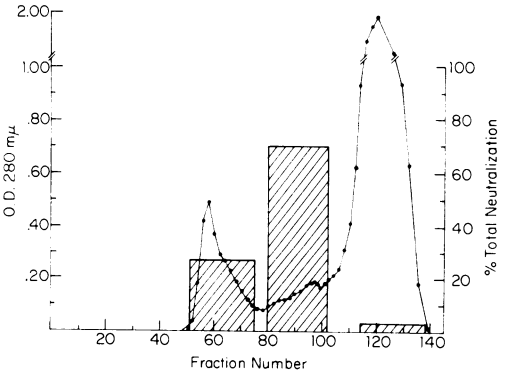

FIG. 2. *Distribution of antiphage activity in Po serum. Twenty-five milliliters of pooled Po serum was chromatographed on a Sephadex G-200 column. Eight-milliliter fractions were collected. The percentage of total amount of phage neutralization for each peak is represented by the vertical bars. Fractions 51 to 75 represent the first peak, fractions 80 to 102 represent peak 2, and fractions 114 to 138 were tested for peak 3.*

was 170,000, which was exactly the same as that obtained for sow IgG by the Yphantis sedimentation equilibrium technique (11).

An extreme example of variability in immunoglobulin contamination among the littermates is shown in Fig. 5. There was a high degree of contamination with immunoglobulin in one (Po-1, 265 μg of IgG/ml) out of three piglets. The serum from Po-3 contained a trace amount of IgG (<50 μg/ml), whereas Po-2 had no detectable immunoglobulin.

One exceptional litter of Minnesota miniature piglets was obtained from sow CCXVII. These piglets contained large amounts of ascites and pleural fluid and died within 1 h after birth. Immunoelectrophoretic analysis of ascites fluid, pleural fluid, and serum from these piglets revealed that high concentrations of immunoglobulin were present (Fig. 6). Radial immunodiffusion analyses of these samples indicated that they contained from 5.0 to 6.4 mg of IgG per ml.

The sera of 115 piglets from some of the re-

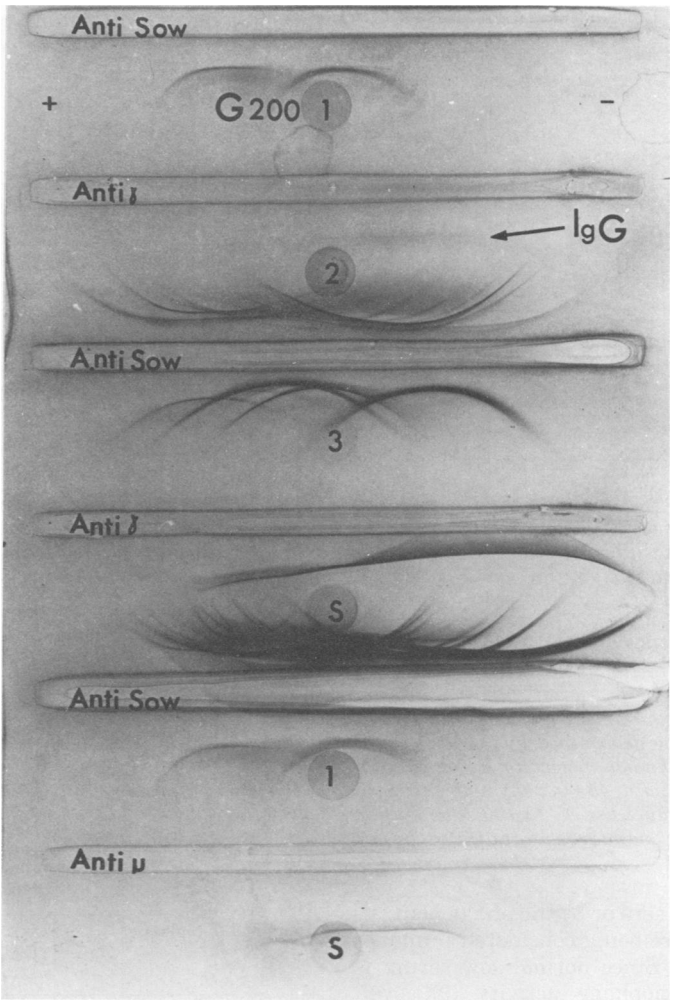

FIG. 3. *Immunoelectrophoretic pattern of peaks 1, 2, and 3 (G-200 1, 2, and 3) from Sephadex G-200 chromatography of piglet sera (Fig. 2). The second peak contains IgG, whereas peaks 1 and 3 do not. The first peak is also shown not to contain detectable quantities of IgM. The purity of anti-gamma and anit-mu sera are shown tested with sow serum (S) diluted 1:6 as controls. Anti-mu serum used here has some anti-α_2-macroglobulin activity.*

cent hysterectomies were examined for the presence of immunoglobulin by immunoelectrophoresis and/or Ouchterlony technique and radial immunodiffusion. The results are summarized in Table 1. Fifty-three percent of the piglets obtained showed detectable IgG in their sera, 5% contained IgM, and 3% contained IgA. Out of a total of 18 litters examined, in six litters all piglets were devoid of detectable immunoglobulin; in eight litters some were positive and some were negative; in four litters all piglets contained immunoglobulin in their sera.

DISCUSSION

These results demonstrate the variability that can exist in the immunological state of colostrum-deprived piglets obtained by hysterectomy. Individual piglets even within the same litter can have different classes and amounts of immunoglobulin owing to their separate compartmentalization. Piglet sera range from nondetectable to high levels of immunoglobulin as shown in several instances of gross leakage. Therefore, individual piglets must be tested for immunoglobulin content by use of

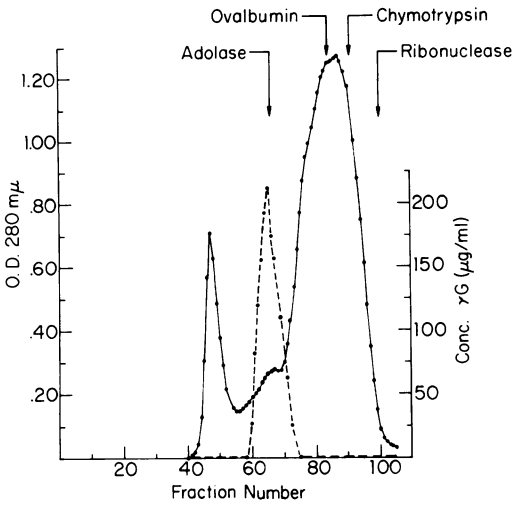

FIG. 4. *Molecular weight determination of IgG in Po serum. Twenty milliliters of pooled Po-piglet sera containing trace amounts of IgG was chromatographed on Sephadex G-200. The scale at the left shows optical density of each of the 10-ml fractions at 280 nm (solid line). The scale at the right measures IgG content of each fraction after it was concentrated to 1 ml as determined by radial immunodiffusion (dashed line). Thus the elution volume of the IgG in piglet serum could be determined by the use of protein standards with known molecular weights: aldolase, 158,000; ovalbumin, 45,000; chymotrypsinogen A, 25,000; and ribonuclease A, 13,700. The elution point of each of the standards is indicated by arrows.*

specific anti-chain sera or by the phage neutralization assay before being considered immunologically "virgin." Since normal sow serum is high in anti-actinophage activity (9), even small amounts of maternal immunoglobulin can be detected in piglet sera when placental leakage occurs. The most common type of contamination seen in our animals is of the IgG class, which is antigenically identical to sow IgG and possesses antibody activity. Radial immunodiffusion studies showed that all anti-gamma reactive components were located in a narrow portion of the Sephadex G-200 elution pattern corresponding to a molecular weight of 170,000, which is exactly the same as that obtained for sow IgG; furthermore, no 5S IgG or smaller IgG components such as those described by other investigators (6, 16) were detected in piglet serum.

The tissues from these contaminated piglets were obtained on the day of hysterectomy and examined histologically for evidence of antigenic stimulation. Piglets that had low levels of immunoglobulin in their sera (<50 μg of IgG/ml) had neither germinal centers, pyroninophilic cells, mature plasma cells, nor immunoglobulin-containing cells in their spleens or lymph nodes, indicating that the maternal circulation must be the source of this immunoglobulin. However, animals which had high levels of immunoglobulin in their sera, such as those in Fig. 6, resembled stimulated animals histologically, manifesting all the cellular changes associated with experimentally challenged piglets (12). In addition, gross leakage of maternal serum may be accompanied by leakage of other antigens, which the maternal sow has been exposed to in the environment, resulting in stimulation of the immunocompetent fetal piglets by these antigens.

These data indicate that even colostrum-deprived piglets obtained by hysterectomy cannot be assumed to be free of immunoglobulins or devoid of antigenic stimulation. If the placental barrier is damaged, it is possible that antibodies or immunoglobulins may appear in the fetus because of antigenic stimuli or material transfer of antibodies. Such damage can be induced by infection, endotoxemia, or mechanical or functional defects which would permit the transfer of macromolecules into the fetal system (9). The reason for some conflicting reports

TABLE 1. *Variability of the immunological state of germfree colostrum-deprived piglets at the day of hysterectomy*

Hysterectomy no.	No. of piglets	No. of immunoglobulin detected			No. of immunoglobulin-positive sera/total sera tested
		IgG	IgM	IgA	
CXCV	7	0	0	0	0/7
CXCVI	10	8	2	0	8/10
CXCVIII	6	2	0	0	2/6
CXCIX	4	4	4	3	4/4
CC	6	5	0	0	5/6
CCI	7	3	0	0	3/7
CCII	5	0	0	0	0/5
CCIII	5	0	0	0	0/5
CCIV	6	6	0	0	6/6
CCV	6	5	0	0	5/5
CCVI	7	7	0	0	7/7
CCVII	9	0	0	0	0/9
CCVIII	4	0	0	0	0/4
CCIX	4	0	0	0	0/4
CCX	11	7	0	0	7/11
CCXI	6	3	0	0	3/6
CCXII	8	5	0	0	5/7
CCXIII	7	6	0	0	6/6
Positive sera (%)		53	5	3	53

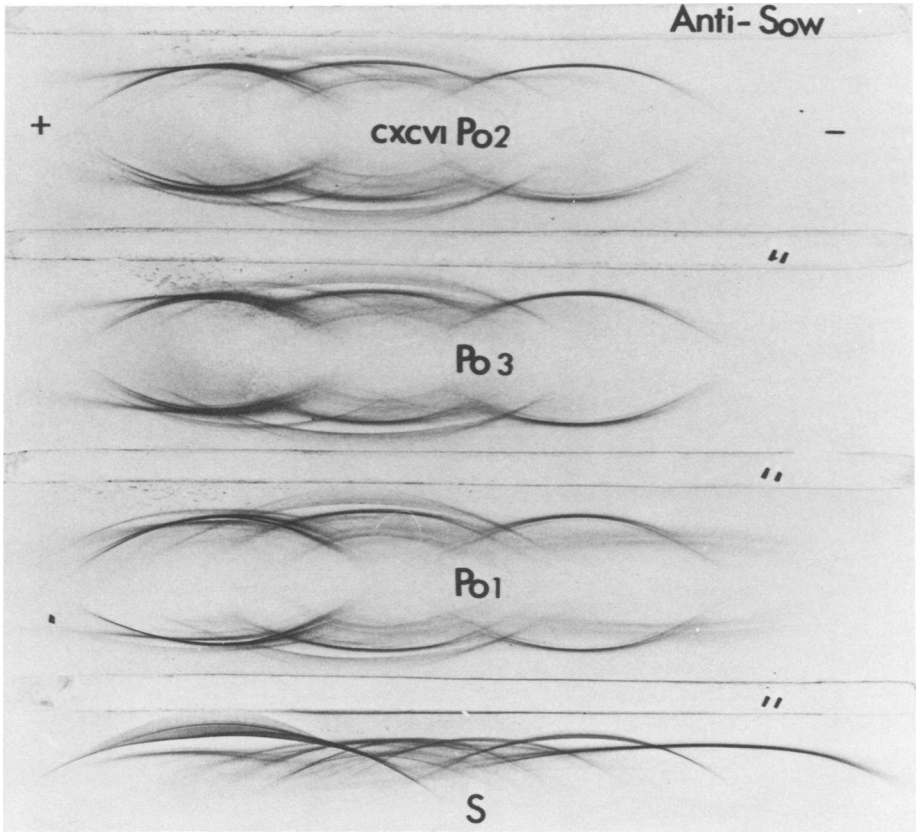

FIG. 5. *Immunoelectrophoretic analysis of three littermates' sera from sow CXCVI. Po-1 contains a large amount of immunoglobulin. Po-3 contains a trace amount. Po-2 has no detectable immunoglobulin. Sow serum (S) was diluted 1:8; all piglet sera were diluted 1:4.*

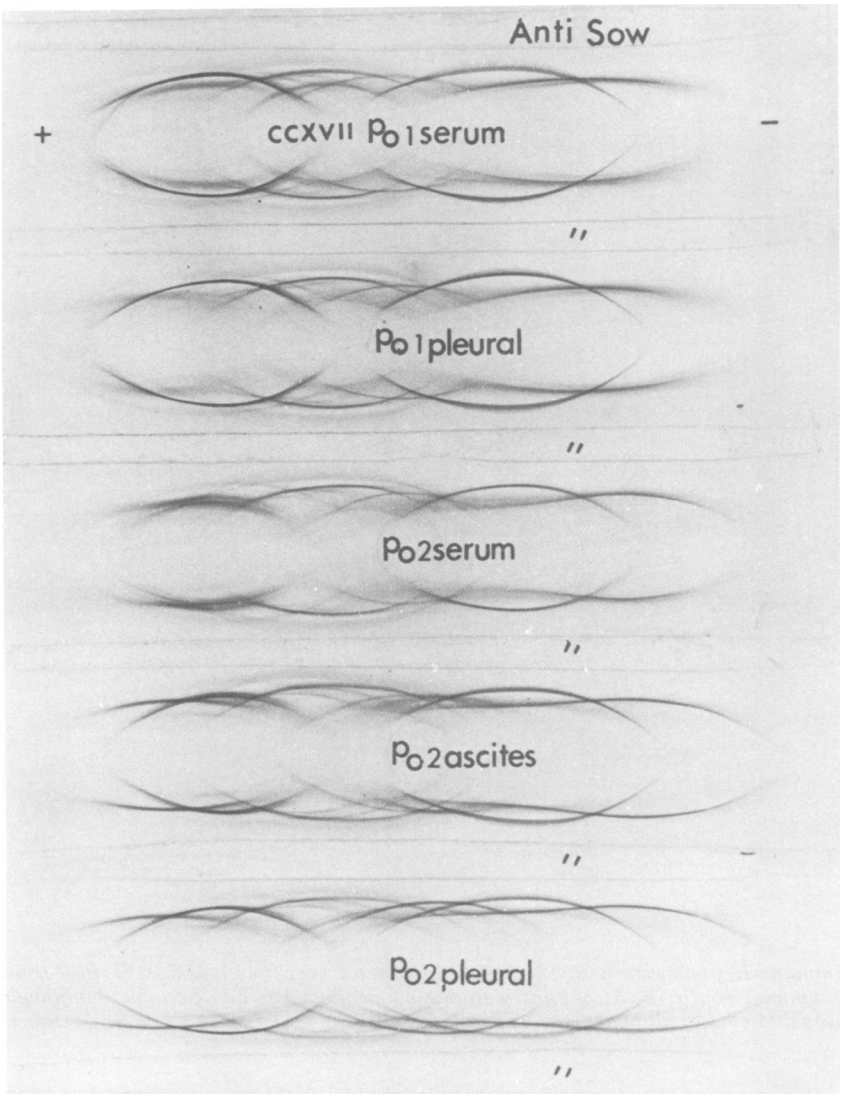

FIG. 6. *Immunoelectrophoretic patterns for samples taken from litter CCXVII. All troughs were filled with anti-whole sow serum. Serum samples were diluted 1:4. Pleural and ascites fluid were diluted 1:2.*

(3, 5, 6, 8, 14, 16) by different investigators may be due to the variability of the functional placental barriers of the different swine in different environments. Thus, the most important basic requirement for obtaining immunologically "virgin" piglets is healthy, specific pathogen-free pregnant sows with intact placental barriers. The increased problem of placental leakage in the Minnesota miniature swine herds appears to be due to difficulty in maintaining this requirement in a single large barn for a long period of time. Therefore, a new specific pathogen-free miniswine facility with multiple isolated compartments and vigorously controlled microbial environment is presently being constructed. This will provide the means with which a specific pathogen-free herd of Minnesota miniature swine can be reestablished to secure these unique research animals for the study of the ontogeny of the immune response.

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