STUDIES ON GERMFREE REARING OF PIGS

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An experiment to rear germfree pigs was performed at this laboratory for the first time in 1963. In this experiment, one germfree pig was delivered by hysterectomy and raised until 70 days of age. On the other hand, cesarean operation in germfree environment was performed in the second experiment which was conducted in February, 1964. Five germfree pigs were obtained, and some of them were raised up to 71 days of age. This communication deals with detailed results of these two experiments, especially the methods of surgical operation, rearing apparatus, diets, and growth rate of the animals, and also with some results of vitamin K deficiency in a germfree pig.

EXPERIMENT 1

The rearing apparatus employed was made of soft plastic, vinyl chloride, 150 cm in length and 90 cm both in width and in height (Fig. 1). It was placed on a wooden table, pulled up, and fixed to poles located at the edges of the table. The bottom of the isolator had a round opening, 30 cm in diameter, which was connected with a sleeve, 40 cm long. The sleeve was immersed into a germicidal trap, which measured 55 cm both in length and in width and 50 cm in height and which had been filled with Highpal No. 3 (Dainihon Pharmaceutical Co., Tokyo, Japan). Three pairs of neoprene gloves were attached to the lateral walls of the isolator through stainless steel flanges. Hard plastic tubes for inlet air and exhaust air were connected with glass-wool filters. A blower was attached to the filter for inlet air. The framework of a stainless steel pen was placed inside the isolator. It was 45 cm long, 41 cm wide, and 50 cm high when constructed.

The germicide employed for sterilization of the isolator was 3.0 per cent peracetic acid (Mitsubishi Edogawa Chemical Inc., Tokyo, Japan) to which had been added polyoxyethylene alkyl phenyl ether, a non-ion surface active agent, at a rate of 0.1 per cent. The amount of the germicide sprayed into the isolator was 1,360 ml per cubic meter. It was sprayed into the isolator through the outlet tube, as well as inside the isolator, by airless spray guns. The glass-wool filters which had already been sterilized by dry heat at 160°C for 90 minutes were fixed to the inlet tube, as well as the outlet tube, under the spray of peracetic acid. The isolator was allowed to stand in that state for the following 24 hours. Then a blower for air supply was put on, and the air within the isolator which had been filled with vapor of peracetic acid was flown out. The rearing apparatus was then ready for use.

Hysterectomy was planned in the first experiment. The operation was performed on a pregnant sow 113 days after the insemination, or two days before the anticipated

Table 1. Milk Formula for Germfree Pigs

Dry milk powder	100 g
Salt mix	6 g
Thiamine	5.0 mg
Riboflavin	1.8 mg
Pyridoxine	2.2 mg
Vitamin C	130.0 mg
Niacin	10.0 mg
Inositol	11.0 mg
PABA	5.0 mg
Folic acid	2.0 mg
Calcium pantothenate	7.0 mg
Biotin	0.1 mg
Choline	260.0 mg
Vitamin A	1,000 I.U.
Vitamin D	600 I.U.
Vitamin E	2.0 mg
Vitamin K	1.0 mg
Water	900 ml

Table 2. Diet for Germfree Pigs

Casein	80 g
Corn oil	40 g
Corn starch	40 g
Sucrose	16 g
Lactose	14 g
Salt mix	10 g
Thiamine	6.0 mg
Riboflavin	2.16 mg
Niacin	12.12 mg
Inositol	12.96 mg
PABA	6.0 mg
Folic acid	2.4 mg
Pyridoxine	2.64 mg
Calcium pantothenate	8.52 mg
Vitamin C	156.0 mg
Biotin	0.12 mg
Choline	312.0 mg
Vitamin A	1,200 I.U.
Vitamin D	720 I.U.
Vitamin E	2.4 mg
Vitamin K	1.2 mg
Water	1,000 ml

date of delivery. The animal which had been bound at the hind legs was lifted upside down by a crane and placed into a large bag containing solid carbon dioxide. A few minutes later when the animal got quiet, the bag was taken away. The skin of the abdominal wall which had been cleared of the hair with a hair clipper was washed with Osban, an antiseptic (Takeda Chemical Co., Osaka, Japan), followed by application of tincture of iodine. The operator wearing sterilized gloves quickly opened the abdominal cavity by a large surgical knife. It was originally planned to resect a horn of the uterus with fetuses inside and to bring it as a whole into the isolator through the germicidal trap. This procedure, however, was found to be impracticable for one operator to perform, because one horn of the uterus contained as many as seven fetuses the total weight of which amounted to more than ten kilograms. This circumstance made it impossible to carry out hysterectomy. Instead of this, an incision was made at each horn of the uterus, and the fetuses were taken out one by one and introduced into the isolator individually through the germicidal trap. It took a much longer time than had been expected. As a result, only one out of thirteen litter mates was successfully resuscitated.

This newborn piglet was found to be able to suck milk from a nursing bottle designed for human babies. The first drink administered was sterilized Tyrode's solution, which was given three hours after birth. Table 1 shows the milk formula which was fed to the germfree young from the first day through the 32nd day. The main component was dry milk powder (Morinaga Milk Industry Co., Tokyo, Japan) prepared for human babies. At the age of 33 days, the formula was changed to a synthetic diet which is shown in Table 2. As a source of unidentified nutrients, egg yolk was supplemented to the diet since eleven days of age, at a rate of two to four pieces per 450 ml of the milk diet. After the 33rd day of age when the animal was on the synthetic diet, the whole piece of egg yolk was given per day. Later, it was gradually decreased in amount and finally stopped at 64 days of age.

Chart 1. Growth Curve of a Germfree Pig

Chart 2.

The growth curve of the germfree pig is shown in Chart 1. It was thought that growth might have been retarded a little, as compared with that of conventional dam-fed pigs. When the animal attained the age of 70 days, it was too large to be kept in a pen and killed for examination. The mesenteric lymph node was underdeveloped, judging from the weight of the body.

EXPERIMENT 2

Cesarean operation in germfree environment was employed, instead of hysterectomy. Accordingly, an operative isolator was required, in addition to a rearing apparatus. The operative isolator was also made of vinyl film, 150 cm in length and 60 cm both in width and in height. It was placed on a wooden board which was 180 cm long and 70 cm wide. One half of this board was occupied by a plate of stainless steel which had an opening 40 cm in length and 28 cm in width. This opening was designed to expose the operative field; namely, when a pig was applied to the bottom of the operative isolator, its abdomen might bulge into the interior of the isolator through this opening. One lateral wall of the isolator was equipped with a pair of

neoprene gloves for the operator, while the opposite side had two pairs of them for assistants. The air inlet tube of hard plastic polyethylene was connected with a glass-wool filter, and a blower entered the isolator at the other lateral wall. On the opposite side was equipped a connecting link of stainless steel, 18 cm in diameter and 30 cm in length. It was designed to serve as a link to the sterile lock of the rearing apparatus, which is described below. The rearing apparatus was also made of vinyl film, 200 cm in length and 90 cm both in width and in height (Fig. 2). It was placed on a desk and fixed to poles located at the edges of the desk. Two pairs of neoprene gloves were attached to both lateral walls of the body via stainless steel flanges with O-rings. Another lateral wall was connected with the air outlet tube, which was in connection with a glass-wool filter. On the opposite wall were equipped the air inlet tube and a sterile lock, which was designed to serve as a connecting link for the operative isolator. Special attention was paid to the structure of the junction between the sterile lock and the body of the isolator, as is shown diagrammatically in Chart 2. The portion of the vinyl film directly surrounding the opening was inserted between a metal ring and the flange of the sterile lock via packing of neoprene, and compressed with screws. In order to reduce transmission of heat from the sterile lock to the body of the vinyl film, a tube for cooling water was installed inside the flange of the sterile lock.

The germicide employed was 2.5 per cent peracetic acid (Dainihon Pharmaceutical Co., Tokyo, Japan). Polyoxyethylene phenyl ether, a non-ion surface active agent, was added to it at a rate of 0.05 per cent. The germicide thus prepared was sprayed by a sprayer to the inner side of the inside door, to the junction between the gloves and the body, to the tubes for air inlet and outlet, and to various materials placed inside the isolator. Following this preliminary spraying, approximately one liter of the germicide was sprayed into the isolator for ten to fifteen minutes using an airless spray gun. Glass-wool air filters which had already been sterilized by dry heat at 160°C for 90 minutes were connected with the inlet and outlet tubes under spraying of the germicide. The isolator was allowed to stand overnight. The next morning, it was sprayed with the germicide again in the same way. Three hours later, the blower was put on, and the germfree air blown into the isolator for twelve hours. Then, the isolator was ready for use.

The animal employed in this experiment was a sow of the Yorkshire breed weighing 180 kilograms. The operation was performed two days prior to the anticipated date of delivery. The animal which had been bound at the hind legs was lifted by a crane and laid down on the back on an operating board. The operating board was made slightly concave, so that it might be adapted to the curvature of the vertebrae of the pig. The anterior legs were fixed at the position hanging downward, because they could not be lifted upward. After cleared of the hair by an electric hair clipper followed by application of a depilatory, the skin of the abdominal wall was washed with 0.1 per cent Osban, an antiseptic (Takeda Chemical Co., Osaka, Japan), followed by tincture of iodine. As a muscle relaxant, succinylcholine was injected into a vein of the ear at a dose of 80 mg. This dose was nine times as large as that for man. Thiopental sodium was injected intramuscularly on the inner side of the thigh at a dose of 7 g, which was seven times as large as that for man. During the operation, the animals were subjected to inhalation anesthesia with ether (Fig. 3).

The operating table with the anesthetized animal on it was brought under the operating tank and moved upward by a jack until the abdominal surface got in extensive contact with a vinyl film which had been sterilized with tincture of iodine and which was across the opening of the board.

Chart 3. Growth Curves of 2 Germfree Pigs Put on a Diet Deficient in Vitamin A and K, Respectively, since 30 Days of Age

Table 3. Blood Coagulation Test

Coagulation time	2'30"
Prothrombin time (QUICK's one stage method)	8.1"
Plasma prothrombin	110%
Thromboplastin screening test (HICKS-PIHEY method)	8.1"

The skin of the abdominal wall was incised together with the vinyl film across the opening of the operative stage. The skin and the vinyl film were held together at the edge of the incision by large Kocher's forceps. The subcutaneous tissue and the muscular layer were likewise incised and the peritoneum was approached. Small perforation was made in the peritoneum by the blunt end of large Kocher's forceps. From this small opening, Kocher's forceps were introduced into the abdominal cavity together with a spatula which had been applied below the forceps in order to protect the intestine at the time of incising the peritoneum. The peritoneum was incised at its portion covering Kocher's forceps. Thus, the abdominal cavity was open to the interior of the isolator. A horn of the uterus was brought to the operative field and given an incision by scissors. Fetuses were taken out, the amnion was separated, and the umbilical cord was clamped and amputated. The assistants tried to resuscitate the newborn infants. When successfully resuscitated, the piglets were transferred to the rearing apparatus through the connecting link. The animals were made clean by wiping the body surface with gauze, and put into a pen. Thus, five newborn pigs were transferred to the rearing apparatus, while the remaining three were taken out to the conventional environment to serve as controls.

The milk formula given to the young is shown in Table 1. Dry milk powder (Morinaga Milk Industry Co., Tokyo, Japan) for human babies was the main component. The concentration of milk was 10 per cent at the beginning, and increased to 15 per cent on the sixth day, to 20 per cent on the eighteenth day, and finally to 25 per cent on the 21st day. Egg yolk was supplemented to milk at a rate of two pieces per 1,000 ml of the milk diet. After autoclaved at 121°C for fifteen minutes, it was fed to the animals with a nursing bottle. By thirty days after birth, the animals had grown too large to be kept all in the same pen. Three of them were then transferred to another isolator and used for an experiment. The remaining two

which had attained 30 days of age were put on the same diet as shown in Table 2, with the following exception (Fig. 4). Namely, vitamin A was withdrawn from this formula in one animal, while vitamin K was eliminated in the other. The growth curves of these two animals are given in Chart 3. By the age of 71 days, or 41 days after the initiation of the deficient diets, the animals had grown too large to be kept in a pen any more. They were sacrificed after subjected to various examinations. The pig which had been maintained on the vitamin A-deficient diet weighed 10 kg at the time of autopsy, while a germfree pig which had been put on the complete formula was 16 kg in weight. According to the data of the Livestock Experiment Station, Prefecture of Aichi, conventional pigs of the Yorkshire breed of the same age weigh about 21 kg on an average. The germfree pig maintained on the vitamin K-deficient diet weighed 17 kg at the time of autopsy. Table 3 shows the results of hematological tests performed just before autopsy. Contrary to the expectation, these results were all within a normal range. Autopsy revealed no hemorrhagic lesions in the sites examined, including subcutaneous tissue, serosa, and various viscera. No evidences of vitamin K deficiency could be obtained in these examinations. The lack of such positive findings as indicating vitamin K deficiency is particularly interesting in relation to the work of YOUNG and UNDERDAHL⁵⁾ who regarded vitamin A and also vitamin K as important constituents of a milk diet for colostrum-deprived newborn piglets.

DISCUSSION

The methods of surgical operation performed in these two experiments were hysterectomy and cesarean section. WAXLER³⁾ pointed out that hysterectomy was a method adequate to producing specific pathogen-free and germfree swine. The present authors' experience indicates that it is difficult to expect a high rate of survival from this method, unless the operator is quite experienced in the technique. The greatest disadvantage of this method was the weight of a horn of the uterus containing several fetuses. It is very difficult, if not impossible, to resect a uterine horn containing several fetuses and transfer it as a whole into an isolator through a germicidal trap within a very short period of time. An alternative way is cesarotomy, which has been employed by WHITEHAIR and THOMPSON⁴⁾ in producing disease-free swine. It is composed of taking out fetuses one by one, not as a mass, from the sow which is hung upside down, and introducing them individually into an isolator through a germicidal trap. These processes cause a high risk of asphyxia, as well as microbial contamination, of the newborn. Cesarean section, on the contrary, has the following advantage. First, the danger of microbial contamination is much less, because the operation is done in germfree environment. Secondly, it is not necessary to do the operation in quite a hurry for the same reason. Thirdly, the risk of asphyxia of the newborn is much lower, because no process of transferring the animals through a germicidal trap is necessary. For these reasons, it is possible even for an unskilled operator to perform this operation. The authors are in agreement with STARKEY²⁾ and LANDY and SANDBERG¹⁾ in the method of the operation.

As for the diet, as well as the method of feeding, it is much easier to raise germfree pigs than germfree rats or guinea pigs. First, pigs are able to suck from a nursing bottle immediately after birth. Secondly, they are not strict in nutritional requirements. This does not mean, however, that there are no nutritional problems for them. On the contrary, the nutritional requirements of germfree pigs seem to be an important problem to be solved by further studies, as is indicated by the preliminary study on vitamin K deficiency conducted by the authors.

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ブタの無菌飼育

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私どもは、1963年に初めて無菌ブタの作出実験を実施し、子宮切断術によって、1頭のブタを無菌空間にとり出し、70日間無菌飼育することができた。さらに1964年に、無菌環境下において帝王切開術を実施し、5頭のブタを無菌空間に取り出し、最長71日まで無菌飼育することができた。本報告は、これら2回の実験における取得手術法、飼育装置、飼料、ブタの発育、ビタミンのKおよびA不足飼料投与の結果を取り扱った。

無菌ブタ取得法としては、帝王切開術は子宮切断術よりも、生残率の点でまさっていたのみならず、操作も容易であり、汚染の危険も少ないと判

断された。飼料については、ブタでは哺乳瓶を利用することができるので、粉乳を中心とした飼料で哺育が容易であり、成育後は、合成飼料でも雑飼料でも飼育容易であった。この点は、モルモットやネズミの場合と著しく相違するところである。

無菌ブタにおける飼料の要求量については、研究報告が少なく、未開発の分野ではあるが、従来、とくに初乳投与を欠く場合、K不足に敏感であるといわれていた幼ブタが、無菌動物化によって、不足症の発現を示さなかった点は注意してよいと思う。

EXPLANTATION OF PLATES

PLATE I

- Fig. 1. A flexible plastic isolator with a plastic sleeve, which is immersed into a germicidal trap.
Fig. 2. A flexible rearing plastic isolator with an operative plastic isolator.

PLTE II

- Fig. 3. Cesarean operation in germfree environment is performed, using a flexible operative plastic isolator.
Fig. 4. Two germfree pigs put on a diet deficient in vitamin A and vitamin K, respectively.