Army Surgeon General (Contract NONR-1623-04), by the National Institutes of Health, Grant A-566 (c8), and by the University of Notre Dame.

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A Bacillus of the Alcaligenes-Pseudomonas Group Highly Pathogenic for Chick Embryos

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(Received for publication December 3, 1963)

A STRAIN belonging to the group Alcaligenes-Pseudomonas (referred to in the text as AP) was first isolated from a preparation of Rous virus (containing streptomycin and penicillin) obtained from a sarcoma in one of a breed of White Leghorn chickens in which the Rous sarcoma was being maintained at the Department of Zoology, University of Malaya. It had been noted that when similar preparations of virus were inoculated on the chorioallantoic membrane (CAM) of chick embryos, of the same breed, for purposes of assay, some of the embryos died in 3–6 days and had a haemorrhagic appearance. The number of deaths increased with further passages of the virus through chickens and chick embryos. The organism was recovered in an abundant pure culture, in the first instance, and similarly from embryos which died after inoculations with material containing AP or with cultures of it. The deaths of embryos in earlier experiments were clearly due to the presence of this organism in the inocula.

A number of investigations on the effects of various bacteria and viruses on the developing chick embryo have been reviewed by Chute (1960). The chick embryo has been used extensively for propagating bacteria and fungi to produce larg quantitites of the organisms but not for purposes of identification. A study of the literature shows the lack of detailed studies on the actual pathological effects of the organisms on the chick embryo. In view of this, the present studies were car-

ried out to investigate the pathological effects of AP on chick embryos and young chicks.

CHARACTERS OF THE BACILLUS

Gram-negative bacillus; length about 2–5 μ., suggestion of bipolar staining; motile; grows on simple media; on lemco agar, overnight, colonies 1 mm. round translucent greyish; lactose, glucose, sucrose, mannitol, adonitol, dulcitol, salicin, inositol, not fermented; citrate utilized; urea−; indole−; M.R.−; V.P.−.

Grows better at 37°C. than at 25°C. Grows well on MacConkey's medium at 37°C.

It was examined at the National Collection of Type Cultures, London, and was excluded from the genus achromobacter because it was found to be oxidase positive. It was placed in the alcaligenes-pseudomonas group, further differentiation depending on the arrangement of its flagella.

On lemco agar, discs containing 10 μg. of streptomycin or 5 units of penicillin caused no inhibition of growth. Ten units of penicillin had at most a barely detectable effect.

MATERIALS AND METHODS

Chick embryos and young chicks were obtained from the strain of White Leghorns maintained at the Faculty of Agriculture, University of Malaya. The embryos were used at the 10th day of incubation. Chicks were inoculated when they were seven days old.

For the inoculation of chick embryos serial ten-fold dilutions of an overnight culture in peptone-water were made in sterile phosphate buffered saline and 0.1 ml. inoculated on the CAM by the artificial air sac method in the usual manner. Inoculated embryos were placed in a humidified bacterial incubator at 38°C.

TABLE 1—*Infectivity of cultures for chick embryos*

Expt. No.	Dilution of culture	No. of embryos dead/No. inoculated	Mean survival time (days)	Percentage mortality	ID_{50}*
1	0.1×10^{0}	6/6	1.0	100	
	0.1×10^{-1}	6/6	1.1	100	—
	0.1×10^{-2}	6/6	1.3	100	
	Controls	0/6	—	0	
2	0.1×10^{0}	6/6	1.1	100	
	0.1×10^{-1}	6/6	1.5	100	
	0.1×10^{-2}	6/6	1.3	100	
	0.1×10^{-3}	6/6	2.0	100	$10^{-6.0}$
	0.1×10^{-4}	5/6	2.0	83	
	0.1×10^{-5}	5/6	2.0	83	
	0.1×10^{-6}	4/6	2.2	66	
	Controls	0/8	—	0	
3	0.1×10^{-4}	5/6	4.2	83	
	0.1×10^{-5}	5/6	2.8	83	
	0.1×10^{-6}	2/6	4.4	33	$10^{-5.53}$
	0.1×10^{-7}	0/6	—	0	
	0.1×10^{-8}	†1/6	—	0	
	Controls	0/7	—	0	
4	0.1×10^{-4}	5/6	1.8	83	
	0.1×10^{-5}	5/6	2.2	83	
	0.1×10^{-6}	6/6	2.2	100	
	0.1×10^{-7}	5/6	2.8	83	
	0.1×10^{-8}	4/6	2.8	66	$10^{-7.82}$
	0.1×10^{-9}	0/6	—	0	
	0.1×10^{-10}	0/6	—	0	
	0.1×10^{-11}	†1/6	—	0	
	0.1×10^{-12}	0/6	—	0	
	Controls	0/10	—	0	

* Based on the percentage mortality at all dilutions according to the method of Reed and Muench (1938).

† Bacteriological tests on embryos showed absence of bacteria, hence these were considered normal deaths.

Inoculated embryos were candled daily and dead embryos were opened up immediately after detection. The CAM and various organs—liver, spleen, kidney and lungs—which looked abnormal were fixed in Bouin's fluid. Sections were stained with haematoxylin and eosin.

RESULTS

Infectivity for chick embryos. Table 1 shows the results of titrating the infectivity of peptone water cultures, incubated in 37°C. overnight. Viable counts of these cultures by the method of Miles and Misra were of the order of 9×10^{8}/ml. Most of the embryos died within 48 hours after inoculation but deaths up to 6 days were included. The high infectivity of this organism for 10 day embryos is apparent. On occasion, death resulted from an estimated dose of not more than 100 bacteria. In general

the majority of embryos were killed by a ten-fold larger number. The effect of age of embryo on susceptibility was not tested.

Twenty-five embryos including some which had been inoculated with high dilutions were cultured post mortem. Pure cultures of AP were readily obtained from any of the abdominal or thoracic tissues of all the embryos. Two embryos in experiments 2 and 4, inoculated with high dilutions were sterile, as shown in Table 1, and their death was considered to be due to other causes. Four control embryos, alive when harvested, were sterile.

Inoculation of young chicks. A culture similar to those used for infecting embryos was diluted 1 in 10 and 0.2 ml. inoculated intramuscularly into the thigh of 5 seven-day old chicks. None showed any reaction. One was killed after 2 weeks; no gross lesions were found. Cultures from the site of inoculation, liver, kidney and lungs did not yield the AP organism. The other 4 chicks were killed 4 weeks after inoculation and were free from any apparent pathological reaction to the bacteria.

Six similar chicks were inoculated into the wing vein with the same dose of culture as was given intramuscularly. None had any sign of reaction. One was killed after 2 weeks. No gross pathological abnormality was found. Cultures from the kidney, liver and lung did not yield the AP organism but it was recovered from the spleen. The remaining chicks were killed four weeks after inoculation and showed no gross lesions.

Since the bacteria were isolated originally from a Rous sarcoma, a third series of 6 chicks was inoculated intramuscularly with 0.2 ml. of a mixture containing equal amounts of a Rous virus preparation and the bacterial suspension. The chicks developed Rous sarcomas at the site of inoculation within two to three weeks. The tumor, liver and spleen from one chick were cultured two weeks after inoculation. All were negative for the AP organism. This indicates that although the bacterium originally was associated with the Rous sarcoma, it did not show any tendency to grow better with the Rous sarcoma. It is possible that the inoculated bacteria did not survive in the muscle and were destroyed before the tumor could develop.

Pathology of the CAM and various organs of the dead embryos. The chorioallantoic membrane: On opening the embryos, the infected area of the CAM was seen to consist of a large number of blood clots. These were usually small (0.5–1 mm. in diameter). They were usually restricted to the region of the CAM on which the inoculum was placed although occasionally the other parts of the membrane were slightly involved. Studies of sections of such membranes showed that the blood vessels were enlarged and full of clotted blood (Figure 1). Many blood passages also had no distinct wall, sometimes two or three of them becoming confluent.

Occasionally there was no general clotting on the CAM but large distinct red pocks (3–5 mm. in diameter) could be seen. When these were punctured, they were seen to be filled with pale-coloured blood. On sectioning, they were found to be highly enlarged blood vessels with no distinct wall (Figure 1.) They were completely filled with erythrocytes, some of which were abnormally enlarged.

The embryo: The body of the embryo also was covered with a large number of small blood clots (1 mm. in size). These were especially common on the fore and hind limbs. Frequently a large blood clot was present below the skin just above the mid-brain region.

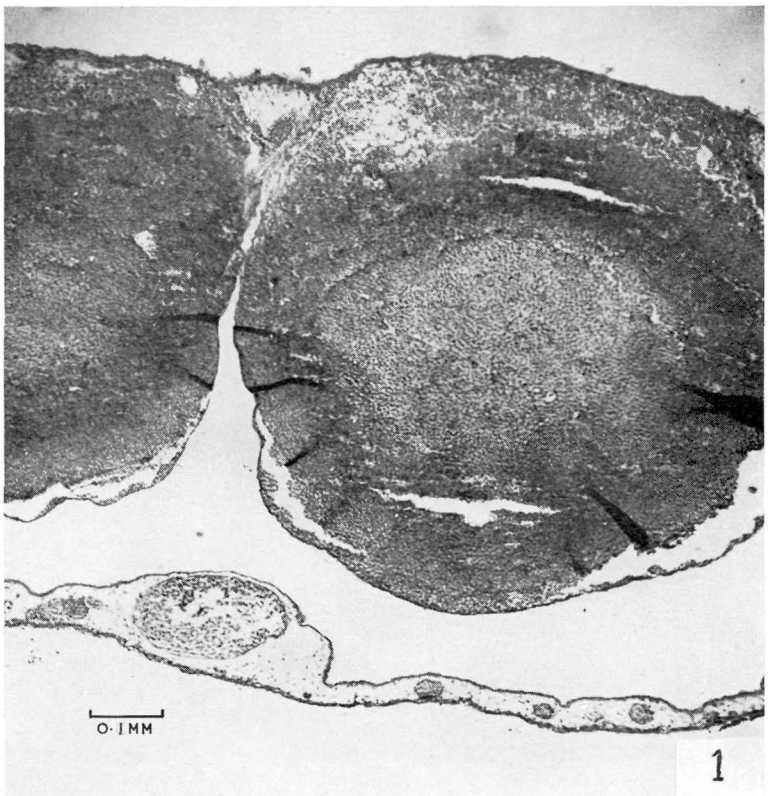

FIG. 1. Accumulation of blood in enlarged blood vessels on the CAM (L.P.).

On opening the embryo the peritoneal cavity was filled with a pale reddish ascites fluid. Smears of the ascites fluid stained with Leishman showed it to be filled with erythrocytes with very few lymphocytes. The erythrocytes were again abnormally enlarged.

The visceral organs and the lungs were involved to varying degrees depending on the severity of the infection.

Gross appearance of infected organs: Infected organs—lung, liver, kidney and spleen—were dark red-brown in colour and were softer in texture than the corresponding normal organs. The extent of infection ranged from all the internal organs to only a few of these. The kidneys and lungs were the commonest sites of infection.

Histopathology of infected organs: The characteristic feature of all the infected organs was the large accumulations of erythrocytes in the tissues. The extent to which this occurred varied according to the severity of the infection. The blood vessels frequently lacked definite walls and the blood cells infiltrated widely into the tissues. Very often it appeared as if nearly all the cells had moved out into the tissues, leaving cell-free areas in what appeared to be the vessels themselves (Figure 2).

The erythrocytes tended to lodge in between the liver lobules and the tubules of the kidney (Figure 3). Large accumulations of these replaced much of the tissue parenchyma, and caused slight enlargement of the organs. In extreme cases of

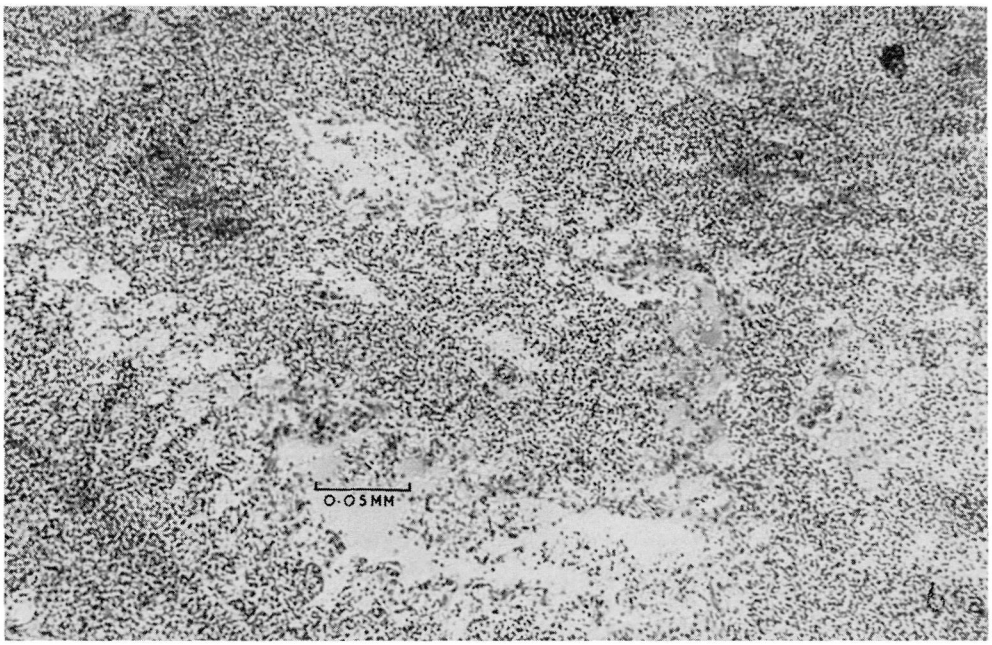

FIG. 2. Lung: no air sacs present, whole of lung being filled with red blood cells. Note lack of distinct blood vessels (L.P.).

infection the tissue parenchyma was completely replaced by the erythrocytes so that the whole organ in section appeared to be a mass of red blood cells. Figure 2 shows a section of a heavily infected lung in which practically no lung tissue is present, and Figure 4 shows a section of a heavily infected spleen. This accumulation of blood would account for the dark red colour of the various organs.

DISCUSSION

This study emphasizes the potentialities of using the chick embryo for the study of bacterial infection processes. It shows the susceptibility of the chick embryo to the AP organism. Most of the chick embryos inoculated with as little as 10–100 organisms succumbed to it. Weil and Valentino (1940) have shown that 3 types of *Shigella* spp. could grow on the CAM of a chick embryo and that 1–10 organisms were sufficient to produce a growth on the CAM. If the growth was small no gross changes occurred on the CAM. However, with intense infections greyish spots and haemorrhages (the degree of reaction depending on the amount of bacteria inoculated) were found on the CAM. In the present study even with low doses of bacteria haemorrhages were found on the CAM and on various organs of the chick embryo. The time taken for the embryo to die varied with the size of inoculum. Inoculation of over 10^3 bacteria killed the embryo within 2 days; lower doses took 3 to 5 days.

Finkelstein and Ransom (1960) carried out a number of experiments on the susceptibility of the chick embryo to *Vibrio cholerae*. They found that the 11-day old embryos were killed within 1 to 3 days with as little as 2 bacteria. However, approximately 5×10^8 bacteria were required to kill half the embryos within 3 days.

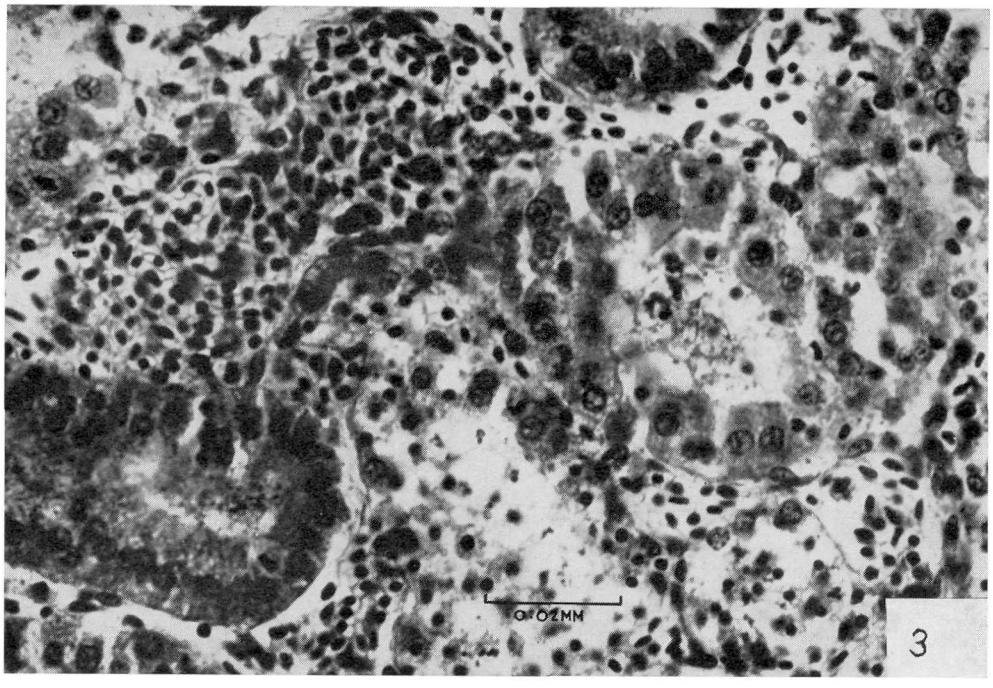

FIG. 3. Kidney: infiltration of erythrocytes among kidney tubules (H.P.).

Webster *et al.* (1953) found that *Vibrio fetus* was highly pathogenic to the chick embryo causing death in 84.01% of inoculated embryos. The bacteria produced arteriolar lesions on the CAM. There was only a slight increase in mortality with the older embryo.

No experiments were carried out on embryos of different ages in the present study. However, it is clear that a small number of bacteria had a severe lethal effect on the embryos. Since, the bacteria had no pathogenic effect on 7-day old chicks, it is possible that the older embryos would have been more resistant than the 10-day old embryos. The bacteria could be isolated from any of the abdominal or thoracic organs of chick embryos within 24 hours after inoculation indicating that the AP organism is capable of rapid invasion and propagation in the chick embryo.

It is somewhat surprising that the same strain of bacteria which is highly pathogenic for the chick embryo is completely non-pathogenic for the chick. The bacteria when inoculated intramuscularly or intravenously into 7-day old chicks could not be isolated from tissue at the site of inoculation or from any of the abdominal organs. The bacterium was isolated only from the spleen of one chick which was inoculated intravenously.

The pathological studies on chick embryos infected with the AP organism shows that it produces general haemorrhage in the embryo. It is capable of doing this rapidly. In embryos inoculated with 10^3 or more bacteria, haemorrhage occurred within 24 hours. The organism did not show a tendency to prefer any particular organ but produced general haemorrhage in various organs of the body.

The present study stresses the high susceptibility of the chick embryo to in-

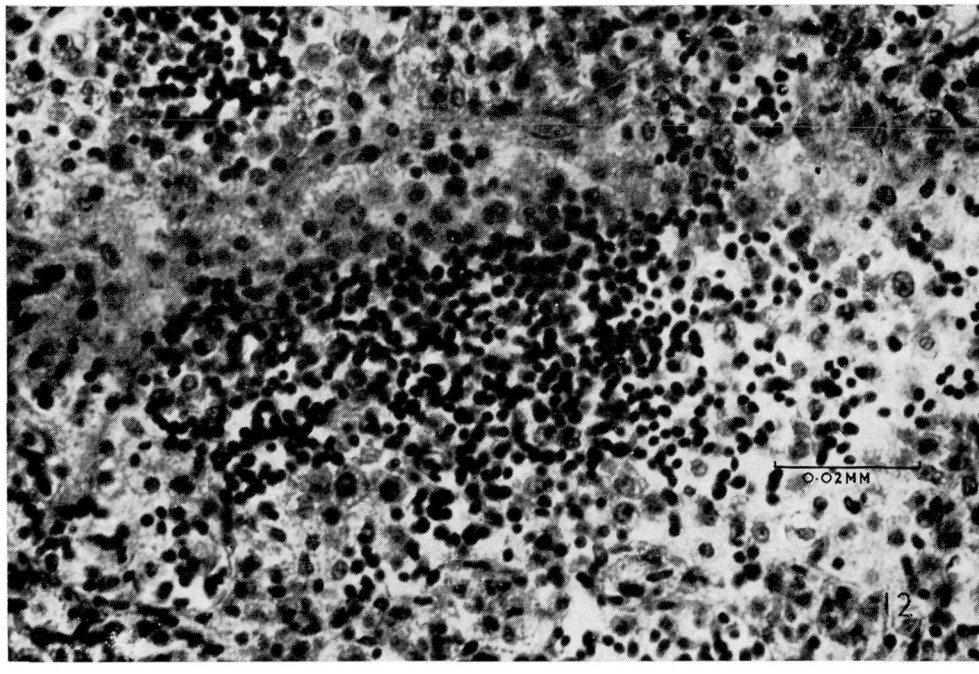

FIG. 4. Spleen of same embryo as in Figure 3. Note presence of erythrocytes all over spleen tissue (H.P.).

fection with a bacterium to which young chicks or adult fowl do not succumb. The complication which may be caused by the presence of such a bacterium in preparation of virus or other non-bacterial agents used for inoculating chick embryos is obvious. The pathological effects of such infections in the not fully differentiated chick embryo tissues may lack the features of specific localization and other characters which are associated with infectious diseases in adult fowls or animals.

SUMMARY

A strain of bacillus belonging to the alcaligenes-pseudomonas (AP) group was isolated from Rous sarcomas maintained in White Leghorn chicks. The characteristics of the bacillus are described.

AP was found to be highly lethal to 10-day old chick embryos when inoculated on the chorioallantoic membrane. It was found that a dose of 10^2 bacteria was sufficient to kill the embryo within 3 days. However, the AP organism had no pathogenic effect in the hatched chick (inoculations carried out in 7-day old chicks).

The AP organism could be isolated from any of the abdominal or thoracic organs of infected embryos within 24 hours of inoculation, indicating its powers of invasiveness. Pathologically, the organism induced general haemorrhage all over the embryo, especially in the peritoneum, abdominal and thoracic organs.

ACKNOWLEDGMENTS

The assistance of Dr. S. T. Cowan and Dr. K. J. Steel of the National Collection of Type Cultures, London, is gratefully acknowledged. We are grateful to Mr. Ang Whi Leng for technical assistance.

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Response of the Chicken Pancreas to Raw Soybeans

MORPHOLOGIC RESPONSES, GROSS AND MICROSCOPIC, OF THE PANCREASES OF CHICKENS ON RAW AND HEATED SOYBEAN DIETS

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(Received for publication December 6, 1963)

INTRODUCTION

CHERNICK *et al.* (1948) showed that the prolonged feeding of raw soybean (RS) diets to one-week-old chicks caused an enlargement of the pancreases with an increased proteolytic activity as compared with chicks fed heated soybean diets (HS). Methionine increased the growth of the chicks fed the raw soybean diets but did not counteract the hypertrophy of the pancreas nor the increase in proteolytic activity.

Subsequently it has been shown that chickens fed RS diet without prior adaptation to the RS diet showed greatly decreased proteases in the intestinal contents due to the action of the soybean trypsin inhibitor (Furuta and Lepkovsky, unpublished data). After adaptation to the RS for six weeks, the intestinal contents of these chickens contained as much proteases as that of those fed the HS diets. Presumably, the pancreases of the chickens adapted to the RS released enough proteases to maintain normal levels of proteases in the intestinal contents in spite of the losses caused by the action of the soybean tryspin inhibitor.

We wish to report additional gross microscopic and chemical observations obtained on the pancreases of chickens fed RS and HS diets without and with methionine.

EXPERIMENTAL

S. C. White Leghorn cockerels of the University of California flock were fed the U. C. stock mash (Lepkovsky and Furuta, 1960) until six weeks of age at which time they had an average weight of 600 gm. They were all transferred to heated soybean diet for two days. This diet consisted of 60 parts soybean (autoclaved 15 min. at 15 lbs. pressure); 36.5 cerelose; 1.0 vitamin-mineral mix (Lepkovsky and Furuta, 1960); 1.0 $CaCO_3$; 1.0 $Ca_3(PO_4)_2$; 0.5 NaCl.

Before adaptation. The chickens were fasted for 18 hours. One group was autopsied as the HS diet control. The rest of the chickens were divided into 4 groups and were given one feeding of the HS diet, the HS diet+0.5% methionine, the RS diet or the RS diet+0.5% methionine. After the chickens had been eating the diet for 2 hours, groups of 3 chickens