

STUDIES ON RESPIRATORY INFECTION

II. THE INFLUENCE OF AEROSOL PARTICLE SIZE ON INFECTION OF THE GUINEA-PIG WITH *PASTEURELLA PESTIS*

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(With 1 Figure in the Text)

INTRODUCTION

The influence of aerosol particle size on respiratory infection due to the inhalation of anthrax spores was described in a previous paper (Druett, Henderson, Packman & Peacock, 1953). It was shown that the number of spores necessary to cause infection rose sharply with particle size, and was also related to the fact that the majority of larger-sized particles are deposited in the upper respiratory tract. Similar studies have now been made in guinea-pigs with virulent *Pasteurella pestis*.

The objectives were to compare clouds of single organisms with clouds of 12μ diameter particles, in respect of the LD_{50} , the slope of the log-dose *v* probit mortality regression line and the course of the disease in guinea-pigs.

Pathological examination of animals exposed to clouds of single organisms and 12μ particles was also made, to see how far information about the type of disease produced would help to explain the experimental findings.

The techniques for the production of aerosols and their sampling and assessment proved as reliable with *Past. pestis* as with those organisms tested in previous work done during the development of the apparatus. However, some variation occurred in the virulence and stability of successive batches of *Past. pestis* suspensions. This complicated the experimental procedure, but we are satisfied that it in no way interfered with the general findings.

METHODS

(a) *Apparatus for the production of bacterial clouds.* For experiments with clouds of clusters of organisms, the apparatus of Druett & May (1952) was used. Clouds of single organisms were produced with the apparatus described by Henderson (1952).

(b) *Strain of Pasteurella pestis.* One strain (L. 37) was used throughout. It was isolated in North Africa in 1942 from a reputed case of pneumonic plague in man, and has been maintained over the years in the dry state on the gelatine-ascorbic-acid medium of Stamp (1947). It is highly virulent for the guinea-pig by the subcutaneous route, ten organisms or fewer forming a lethal dose.

(c) *Preparation of bacterial suspensions.* These were prepared from dried stock by culture and serial transfer on de-ionized casein partial hydrolysate (D.C.P.H.) medium at 28° C.

For use in the Henderson apparatus the suspension prepared on the D.C.P.H. medium was diluted in phosphate buffer (pH 7.6) to give the required number of organisms per ml. For the production of large particles in the Druett & May apparatus it was necessary to control both the number of organisms and the total solids per ml. of suspending fluid; this was done by adding D.C.P.H. medium and glass-distilled water until the required conditions were achieved. Thus, for example, in experiments with 12μ diameter particles the bulk of the particle consisted of dried medium and carried approximately 15 to 150 organisms according to the dosage level under test.

(d) *Cloud sampling and assessment.* The impinger technique previously described by Henderson (1952) was used for collecting samples. The pre-impinger (May & Druett, 1953) was used in all experiments with 12μ particles. The impingers carried 10 ml. and the pre-impingers 5 ml. of phosphate buffer. Viable bacterial assay was made by the method of Miles & Misra (1938), using tryptic meat agar medium containing 5% peptic sheep blood (Fildes, 1920) and 0.00025% crystal violet.

(e) *Estimation of particle size.* This was carried out by the technique previously described (Druett *et al.* 1953). The samples on slides were examined at the commencement of each experiment to show that the particles were of approximately the required size. The slides were then transferred to a desiccator, and a detailed size count was made on the following day. From the counts, the mean volume diameter was calculated. In no instance did this differ significantly from the value obtained by first inspection.

(f) *Definition of terms.* The terms used in expressing the parameters of the clouds of organisms to which the animals were exposed have been fully defined previously (Druett *et al.* 1953).

(g) *Experimental animals.* Guinea-pigs weighing 350–450 g. were used. In all experiments they were allocated to their groups by random sampling. After exposure to infection the animals were housed in groups of four to a cage, and to each cage one unexposed animal was added to find whether cross-infection might play a part in determining results; they were observed for a period of 21 days.

(h) *Pathology.* Diagnosis of death from plague was made by microscopic examination and culture of heart's blood for plague bacilli and macroscopic pathology of the animal.

The development of disease in animals exposed to clouds of single organisms or of 12μ diameter particles at a dosage calculated in each instance to give about a 95% death-rate was studied in more detail. Groups of animals were killed at different times after exposure. Blood cultures were made and tissues taken for detailed histological examination. Tissues were fixed in 4% formol saline, and sections stained with eosin-methylene blue. Animals that died from cross-infection were also examined in this way.

RESULTS

The experiments were designed so that with any one suspension of *Past. pestis*, groups of guinea-pigs exposed to single organism clouds were compared with groups exposed to 12μ particles. The results have been treated as previously

described (Druett *et al.* 1953), and the condensed data are given in Tables 1 and 2.

It is seen that the *LNt*50 dosage for 12 μ particles is about 2.5 times greater than with clouds of single organisms.

Table 1. *LNt*50 values. Comparison of the infectivity of single organisms and 12 μ diameter particles

Assay	Single organisms		12 μ particles		Ratio 12 μ / single at <i>LNt</i> 50
	No. of animals at risk	<i>LNt</i> 50 $\times 10^{-4}$	No. of animals at risk	<i>LNt</i> 50 $\times 10^{-4}$	
1	120	4-7-10	220	?-14-?	2
2	140	?-3-?	168	7-13-21	4.3
3	140	4-5-7	168	2-7-13	1.4

Weighted mean of ratios at *LNt* 50, 1.36-2.83-5.88.

Table 2. Slope values. Comparison of data from assays with single organisms and 12 μ diameter particles

Assay	Single organisms			12 μ particles		
	No. of animals at risk	Slope <i>b</i>	<i>V</i> (<i>b</i>)	No. of animals at risk	Slope <i>b</i>	<i>V</i> (<i>b</i>)
1	120	1.8	0.31	220	0.49	0.11
2	140	2.05	2.49	168	1.19	0.04
3	140	2.49	0.23	168	0.86	0.04
Combined data from above assays	400	2.16	0.26	556	0.8	0.03

The data obtained with clouds of single organisms gave regression line values not significantly different from the theoretical slope of 1.9 calculated on the basis of random chance (Druett, 1952).

Results with 12 μ particle clouds gave regression line values that are significantly different from those obtained with single organism clouds. They bear no relation to the theoretical slope made on the basis of random chance. A 2.5-fold difference in effectiveness between 12 μ diameter and single organism clouds is observed at the *LNt*50 level, whereas at the *LNt*75 level the difference is of the order of 9.5-fold. Possible explanations for this are discussed later.

Time incidence of death. This is shown in Tables 3 and 4. It is seen that over the range covered the time of death in any of the groups is independent of dosage. This is not surprising. For example, if the generation time *in vivo* of *Past. pestis* is of the order of 1 hr. it might be expected that the factor of 16 between the lowest and highest dosages used would vary the death time by about 4 hr. only. The twice-daily recording of death that was used in these experiments would not detect such differences. The first deaths occurred on the 3rd day after exposure in animals subjected to 12 μ particles. With three exceptions, no animals exposed to clouds of single organisms were found dead before the 4th day. The results are shown

graphically in Fig. 1; the number of deaths on each day after exposure is expressed as a percentage of the total number of deaths over the entire period of observation. It is clear that death in groups of animals exposed to 12μ particles starts earlier than in those exposed to clouds of single organisms. Further, the mortality curve is less widely spread and lacks the 'long tail' extending to the 20th day which is characteristic of the single organism cloud curve.

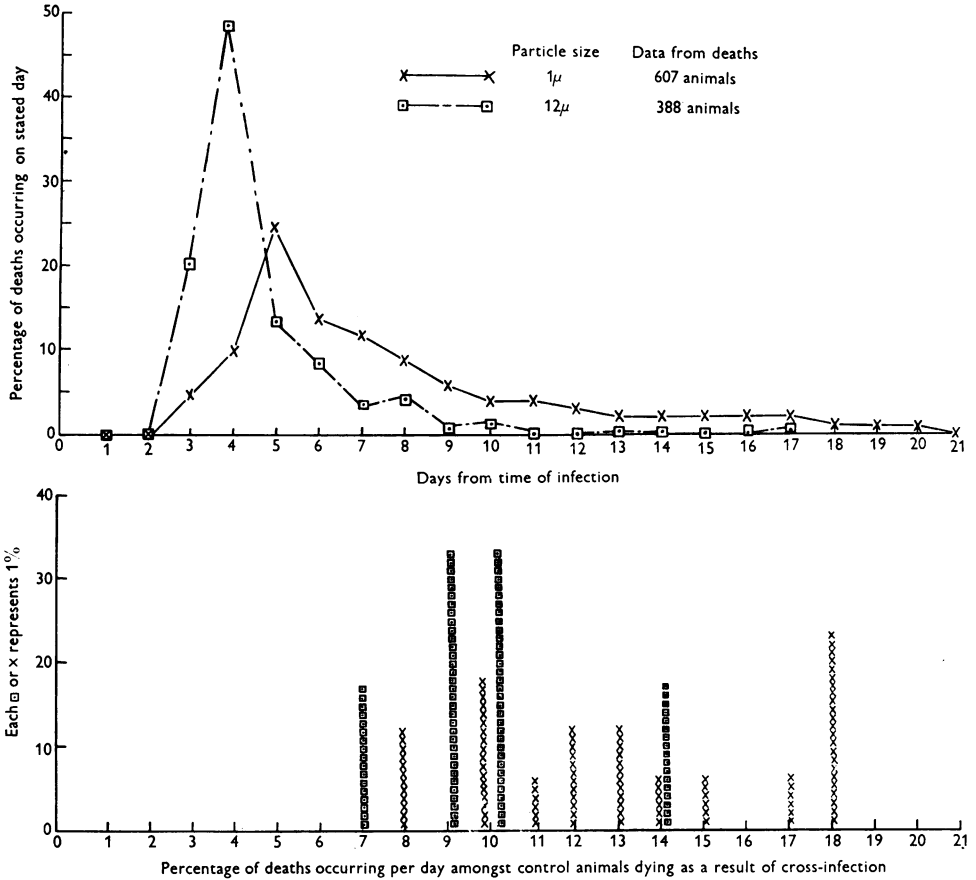


Fig. 1.

Cross-infection. In all experiments each cage carrying four exposed guinea-pigs housed also a 5th unexposed animal. This was done to examine first the possible influence on results of cross-infection and secondly, if such infection occurred, whether differences in degree were to be observed among animals exposed to large as distinct from small particles.

Table 5 summarizes the results. It shows that there was a positive correlation between the number of deaths in control animals and the number of dead or dying experimentally-infected animals with which they were in contact. There was no such positive correlation between the number of deaths in control animals and the dosage to which experimental animals were exposed. Table 5 also shows that the

particle size of the cloud infecting the experimental animals influences the cross-infection rate. This is clearly demonstrated by examining the combined data for cages in which one or more of the experimental animals died. Thus:

Experimental animals exposed to clouds of	Cross-infection of control animals	
	M/T	%
Single organisms	18/109	16.5
Particles of 12μ diameter	6/135	4.4

M = no. of deaths; T = total no. at risk.

Deaths in control animals are approximately four times greater after contact with those exposed to single organism clouds than to particles of 12μ diameter.

The days after exposure to experimental animals on which contact animals died are shown in the histogram in Fig. 1. No deaths were recorded before the 7th day after contact with animals exposed to 12μ diameter clouds, and before the 8th day after contact with animals exposed to single organism clouds. This is in nice agreement with the earlier onset of death in groups of animals exposed to 12μ

Table 5. *Deaths amongst cross-infection controls*

No. of deaths of exposed animals in cage	No. of deaths amongst controls			
	Single organism clouds		12μ clouds	
	Mortality/total	%	Mortality/total	%
4	13/50	26	4/53	7.6
3	3/25	12	1/33	3.2
2	2/21	9.5	1/29	3.4
1	0/13	0	0/20	0
0	0/5	0	0/5	0

clouds. Another point is that the period over which cross-infection was observed is much shorter in 12μ diameter cloud experiments than in tests with single organisms. It might be, therefore, that the 'long tail' in the mortality curve with animals exposed to these latter clouds is to be attributed in some part to death from cross-infection and not from the initial exposure. It is also pertinent to observe that the first deaths among control animals due to cross-infection occurred 4 days after the first death among experimental animals, which is the same as the most probable time between inhalation of large particles and death.

The degree of cross-infection observed in the various groups did not influence the general trend of results. However, it was of sufficient interest to warrant further test. Two identical experiments were planned. In each, guinea-pigs were housed in boxes $30 \times 30 \times 10$ in. deep. Twenty-four animals were maintained therein. The boxes were held on a carriage, one above the other, numbered 1-4 from top to bottom. During the experiments the temperature inside the boxes was $27-28^\circ\text{C}$. Room temperature was $17-18^\circ\text{C}$. and the relative humidity 50-60%. In boxes 1 and 2, twelve animals exposed to 7LD_{50} of single organisms were

housed with twelve control animals. Boxes 3 and 4 carried six animals exposed to the same cloud of *Past. pestis* and eighteen control guinea-pigs. In each box, as animals died, they were replaced by marked normal ones, thus maintaining twenty-four animals per cage until the experiment was ended 35 days after exposure.

The results are summarized in Table 6. They confirm the earlier observations and show approximately 18% cross-infection, but it is seen that the results from cage to cage are highly erratic. None of the fourteen animals added to replace those dead from plague contracted the disease. Animals surviving to the 35th day were killed; post-mortem examination failed to show any trace of infection with *Past. pestis*, and the culture of heart's blood was sterile. It is obvious that we failed to establish conditions which would lead to epizootic spread, although this point is discussed later in the light of differences in the pathological picture seen in animals exposed to single organisms and those exposed to 12 μ diameter particles.

Table 6. *Cross-infection tests*

		Experiment 1	Experiment 2
Cage 1	12 infected animals 12 controls	All dead by 8th day 3 deaths on 12th, 18th and 23rd days	All dead by 7th day 2 deaths on 7th and 11th days
Cage 2	12 infected animals 12 controls	All dead by 8th day No deaths	All dead by 7th day No deaths
Cage 3	6 infected animals 18 controls	All dead by 8th day 2 deaths on 8th day	All dead by 6th day 1 death on 8th day
Cage 4	6 infected animals 18 controls	All dead by 7th day 6 deaths between 12th and 18th day	All dead by 7th day No deaths

PATHOLOGY

Examination of animals used in the main experiments showed that by the time of death there was a marked difference in the appearance of animals exposed to aerosols of 12 μ particle size from those exposed to single organism aerosols, and that this difference did not seem to be altered by the level of dosage used in either case.

Post-mortem appearance. In animals exposed to large particles, there was oedema of neck tissues and gross enlargement of the deep cervical glands at the upper end of the trachea. The lungs showed engorgement and sometimes infarction and haemorrhage, but no pneumonic consolidation. Lymphatic glands throughout the body showed moderate enlargement. All internal organs were dark, soft and swollen.

Animals exposed to clouds of single organisms showed lesions in the lungs with enlargement of the bronchial glands. The lesions were sometimes unilateral and sometimes bilateral, and varied in extent. They consisted mainly of areas of pneumonic consolidation. Necrotic nodules were present at times and the general appearance was consistent with moderate septicaemia.

It was decided to follow the development of the disease by carrying out histo-

logical examination, blood cultures and white cell counts. Groups of animals were used as follows:

(1) Twenty guinea-pigs were exposed to an aerosol of 12μ particle size and 20 to an aerosol of single organisms. Five from each group were killed at 4, 24, 48 and 72 hr. after exposure. Blood cultures were made, post-mortems carried out and the histological appearance of the upper respiratory passages, lymphatic glands, lung, liver, spleen, kidney was examined to gain an overall picture of the disease.

(2) A second experiment was designed to follow the development of bacteraemia in the 12μ group of animals. Blood cultures were carried out in groups of three animals at hourly intervals during the first 12 hr., and in groups of four animals at 2-hourly intervals between 12 and 52 hr. after exposure. White blood cell counts were made and tissues from some of these animals examined histologically.

(3) A group of forty guinea-pigs was exposed to an aerosol of 1μ particle size, for the purpose of following the appearance of lesions in the lungs in a larger number of animals. They were killed between 44 and 76 hr. after exposure. Blood cultures and white blood cell counts were also made.

Blood cultures and blood counts. Animals exposed to 12μ diameter particles had positive blood culture by 32 hr. after exposure and by 48 hr. a marked bacteraemia was present, as evidenced by the microscopic demonstration of plague bacilli in the blood vessels seen in tissue sections.

The time of appearance of bacteraemia in animals exposed to single organisms was much more variable. It was usually present by 60 hr., but did not become demonstrable microscopically until after 72 hr.

Leucocytosis appeared about 12 hr. after the detection of bacteraemia in both groups of animals.

Histology. In animals exposed to 12μ diameter particles organisms were present in large numbers in the anterior part of the nose and in the nasopharynx by the 48th hour. They were found on the surface of the epithelium, between the epithelial cells and in the tissues just beneath the basement membrane, and were always extracellular. The organisms were seen in lymphatic channels in the mucosa, and the regional lymphatic glands were enlarged. Oedema was present with dilatation of lymphatic channels and polymorphonuclear cells were seen in large numbers. By 48 hr. masses of bacilli were present in the glands and the lymphatic channels in the cervical region were diffusely invaded. Necrosis was starting in the centre of the lymph glands, and gradually became more extensive giving rise to a classical bubo. There was thrombosis of vessels at the margin of these lesions, and damage to vascular epithelium was manifest. After 48 hr. secondary embolic foci and vascular damage were seen in other organs, including the lung. At no time was pneumonic consolidation present in the lung. Small areas of focal necrosis were sometimes found in the liver and spleen.

In animals exposed to single organism clouds there was no evidence of any local lesion in the cervical region. Some inflammatory changes were found in the upper respiratory passages but only at a late stage (72 hr.) when lesions in the lung were well advanced. It appeared that initial infection was in the terminal parts of the respiratory bronchioles. This was seen at 60 hr. when patches of bronchopneu-

monia had developed with large numbers of polymorphonuclear cells and plague bacilli in the alveolar exudate. Spreading peribronchial lymphangitis was present. Bronchial lymphatic glands were invaded by polymorphonuclear cells and plague bacilli. Between 60 and 72 hr. large confluent areas of bronchopneumonia were seen accompanied by pulmonary oedema. Masses of organisms were present in the oedema fluid. Phagocytosis of organisms was never found. In the terminal stages, when bacteraemia was established, the lungs sometimes showed infarction, haemorrhage into alveoli or abscess formation. Other organs showed congestion, and sometimes a few organisms in the blood vessels at a late stage, but no specific lesions.

Post-mortem and histological examination was made of animals which died from cross-infection as described earlier. It was found in all instances that they died from the upper respiratory type of infection characteristic of exposure to large particles.

DISCUSSION

The influence of particle size on the infectivity of *Past. pestis* by the respiratory route has been found to be governed in large measure by the level of infectivity at which the tests are made. Thus at the LNt50 level there is comparatively little difference in the effectiveness of 12μ particles and single organism clouds; the large particles are about $2\frac{1}{2}$ times less effective. On the other hand, when tested at the LNt75 level it is found that they are about $9\frac{1}{2}$ times less effective than a cloud of single organisms.

Regression line data reveal that the values obtained with single organisms are not significantly different from the theoretical slope of 1.9, made on the hypothesis that random chance determines the result (Druett, 1952). This has already been shown to hold in experiments with clouds of *Bacillus anthracis* and *Brucella suis*; two other organisms that we have studied in detail (Druett *et al.* 1953; Druett, Henderson & Peacock, 1956). On the other hand, the slope obtained with 12μ particles is significantly flatter than the hypothetical slope of 1.9. The same general tendency was observed with *B. anthracis* and *B. suis* but to a much lesser extent. No complete explanation of this finding has yet been found. It might be, however, that with *Past. pestis* the difference in portal of entry with the two types of particle plays a part. The primary lymphatic invasion via the nasal mucosa that occurs with 12μ particles could lead to a rapid stimulation of non-specific immune mechanisms in the host, particularly when large doses are given. Parry (1956), in experiments with rats, has shown that doses of *Past. pestis* over a critical level, injected intraperitoneally, lead at least to prolonged delay in death, and not infrequently to a higher survival rate than in animals given a lower dose. He has shown that this is the result of non-specific immunity stimulation due to the excess of bacterial protein in the larger dose. Such a phenomenon, while possibly applicable in experiments with 12μ particles, would be much less likely to happen in animals exposed to single organisms, for here the primary site of invasion is the lung and not lymphatic tissue. However, it is difficult to visualize the type of experiment that would effectively test this hypothesis.

From the physical point of view the importance of the site of deposition of inhaled particles in relation to infectivity has already been fully discussed (Harper & Morton, 1953; Druett *et al.* 1953), and it is evident that the factors involved will be applicable to all particles having similar physical properties. The subsequent behaviour of the deposited particles of *Past. pestis* has been found to differ markedly from those of either *B. anthracis* or *B. suis*. As noted above, when tested at the LNt_{50} level 12μ particles of *Past. pestis* are only about $2\frac{1}{2}$ times less effective than clouds of single organisms. This is in contrast to the corresponding 17-fold difference with *B. anthracis* (Druett *et al.* 1953) and about a 600-fold difference with *B. suis* (Druett *et al.* 1956). Further, as with inhaled anthrax spores, but in contrast to *B. suis*, the LD_{50} dose for guinea-pigs by the respiratory route is very much greater than by parenteral injection. The LD_{50} of inhaled single organisms of *Past. pestis* is approximately 1000-fold greater than by the subcutaneous route. Clearly the mechanisms of pathogenesis in the three diseases are widely different. (See Henderson (1955), Henderson, Peacock & Belton (1956) and Ross (1956) on anthrax; Druett *et al.* (1956) and Harper (1955) on brucellosis.)

Past. pestis proves to be a very labile organism when airborne. In large measure this may explain the discrepancy in the LD_{50} dose needed by the respiratory route as compared with the subcutaneous one. If organisms die in the process of becoming airborne and dried, as can be detected by immediate collection and assay on the best culture media, many growing thereon would die under less favourable conditions. Thus, for example, it can be shown that clouds held airborne for periods of less than 1 min. rapidly 'decay'. It seems highly probable, therefore, that there will be a high death-rate among particles deposited on the lining of the respiratory tract; only those sufficiently protected, or with enough vigour, would live to invade in the manner that histological and cultural examination has helped to reveal.

Pathological examination of animals exposed to, or dead from, inhaled *Past. pestis* has shown that two quite distinct forms of disease arise according to the site of deposition of particles. Where they are deposited on the epithelial lining of the small bronchioles, or on the alveolar wall (and survive) they appear to penetrate the tissue and quickly establish themselves, setting up a broncho-pneumonia. The disease eventually becomes septicaemic and death follows. Particles deposited in the region of the head penetrate local epithelium but seem more rapidly to reach the lymphatic system. This leads to a much earlier septicaemia than occurs with organisms deposited on the bronchiolar or alveolar wall. The animals die earlier with haemorrhagic infarction in the lung but no pneumonia. We examined the possibility that large particles deposited in the upper respiratory tract which are rapidly removed to the alimentary tract might there initiate disease. Doses greatly in excess of those used in respiratory experiments were introduced to the stomach by cannulation. Some deaths occurred, but post-mortem examination readily showed that trauma induced by the experimental technique was responsible. There was no evidence that the gastro-intestinal route of infection played any part in determining results in the present experiments.

The studies on cross-infection proved informative in several respects. Practic-

ally, they showed that the general trend of results was not significantly influenced by this type of infection. It was found also that cross-infection was much more frequent when the initial disease was characteristic for exposure to single organisms, namely a primary pneumonia. It was interesting also to find that cross-infected animals suffered from the form of disease characteristic of animals exposed to large particle clouds, i.e. septicaemia arising from a primary focus of infection in the cervical glands with infarction of lung but no pneumonia. This probably was one of the major factors responsible for the failure to establish the disease in epizootic form, for in the absence of insect vectors the animals would only occasionally be expected to be contagious. If this is so, then for purely physical reasons the guinea-pig is not a suitable animal for such study; its respiratory tract is too highly selective in preventing particles over about 4μ diameter reaching the lung (Harper & Morton, 1953). But another important reason why the guinea-pig may not be a suitable test animal for such studies is that neither form of the disease gives a pathological picture as described for pneumonic plague in man. A more satisfactory species might be the monkey where nose and/or mouth breathing is practised. We have made some experiments with monkeys, using single organism clouds and 12μ diameter particles. Both seem to produce a lobar pneumonia, although the disease takes longer to develop after exposure to large particles. However, much more information on the physical and biological properties of airborne particles carrying *Past. pestis* is probably needed before studies on the epizootic form of the disease could profitably be made.

SUMMARY

The LD₅₀ dose of *Past. pestis* is much greater when tested by the respiratory route than by subcutaneous challenge. This is probably due to trauma inflicted on the airborne particles.

Two forms of plague, both originating in the respiratory tract of the guinea-pig, can develop according to the size of the particle containing *Past. pestis* presented to the host. Small particles initiate a broncho-pneumonia which leads to septicaemia and death. Large particles establish a septicaemia, and death results more quickly without the development of a pneumonia.

Cross-infection to normal animals occurs irregularly when they are exposed to others developing plague by the respiratory route. Such incident is rare when the initially infected animals are exposed to large particles. Cross-infected animals suffer from the disease characteristic of exposure to large particles. Attempts to establish an epizootic by cross-respiratory infection were abortive, probably due, in some measure, to the type of disease developing in first cross-infections.

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