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Aerosol exposure of cattle to foot-and-mouth disease virus

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SUMMARY

Slight modifications of a small, plastic covered greenhouse provided a chamber for the exposure of cattle of all ages to aerosols of foot-and-mouth disease virus. Particle size distributions of aerosols were 76% < 3 μm, 17% 3–6 μm, and 7% > 6 μm immediately after the deVilbis no. 40 nebulizer used was turned off and 90% < 3 μm, 8% 3–6 μm, and 2% > 6 μm 20–30 min later. Pharyngeal virus growth curves and viremia patterns correlated well with the dose of virus to which test cattle were exposed and were similar to those of cattle inoculated intranasally.

INTRODUCTION

Once the airborne transmission of foot-and-mouth disease (FMD) had been established (Fogedby *et al.* 1960; Hyslop, 1965; McKercher, Dellers & Giordano, 1966), investigators studying the early growth of the virus, exposed experimental animals by placing them in indirect or direct contact with other infected animals (Burrows, 1968; Graves *et al.* 1971). Contact exposure entails many uncontrollable variables such as the total amount of virus to which the test animal is exposed, the particle size distribution of the aerosol, the air flow patterns, and the amount of direct viral contamination. We have shown, for example, that early pharyngeal growth patterns are greatly affected by the infectious state of the donor at the start of contact exposure (Sutmoller & McVicar, 1976*a*).

In order to control some of the variables inherent in contact exposure, investigators have used various combinations of aerosol generators and exposure configurations often involving considerable expenditure of laboratory resources (Henderson, 1952; Hyslop, 1965; Beard & Easterday, 1965; Lincoln *et al.* 1965; Lillie & Thompson, 1972). Large animals have usually been exposed by means of masks although in one study whole head exposure of calves was used (Lillie & Thompson, 1972). We report here on a relatively simple and inexpensive means of exposing cattle of all ages to aerosols of FMD virus.

MATERIALS AND METHODS

Cattle. Test subjects were grade Hereford cattle. Adults were 1½–2 year-old steers and heifers; male and female calves were 1–6 weeks of age.

Virus. Inocula consisted of suspensions of FMD virus type O_1 in cell culture

medium without serum. A South American (CANEFA-2) and a European (Brugge) strain were used but because no differences in results were observed the data were pooled.

Viral assays. Quantitative infectivity assays utilized low (2–5) passage bovine kidney (BK) cell cultures overlayered with gum tragacanth and incubated at 37 °C in a humidified 3 % CO_2 atmosphere. Results are expressed as plaque forming units (p.f.u.) per ml except in the case of air sampler specimens where the titre of the entire sample is reported. Qualitative infectivity assays were done by inoculating 1 ml of sample into low passage BK cell cultures in 75 cm^2 flasks. Cultures were examined daily for 5 days for cytopathic effect (CPE). Cultures showing CPE were frozen at −70 °C and thawed, then the decanted fluid was clarified by low speed (2000 rev/min) centrifugation. Harvested fluids were plaque assayed in BK cell cultures and inoculated into suckling mice. For the mouse assay, 0·1 ml of harvest was inoculated intra-abdominally into each member of two litters of 4–6 day-old mice. The mice of one litter had been inoculated 2 h previously with 0·1 ml of type specific antiserum subcutaneously. The death of all untreated and survival of all serum treated mice were taken together as evidence that the harvested fluid contained FMD virus type 0. Cultures negative for CPE after 5 days were frozen at −70 °C, thawed, and inoculated into freshly prepared BK cultures in flasks. If CPE was not observed during a subsequent 5-day incubation period, the original speciman was considered to have been free of FMD virus.

Aerosol chamber. The aerosol chamber was a commercially available greenhouse (Sears Roebuck Inc., USA) made of tubular aluminium covered with transparent heavy guage plastic sheeting (Fig. 1). The house was approximately 1·5 m wide, 1 m deep and 2·2 m high with a volume of 2350 l. Modifications required were the addition of a floor of rubberized sheeting held on with waterproof tape and the sealing of a louvred vent over the door. A 2·5 cm diameter hose was tape-sealed into the rear corner of the peak (Fig. 1, 1) and the free end was suspended with string near the exhaust vent of the isolation room in which the exposure was done. A small diameter hose was similarly sealed into the left rear corner at the bottom to supply air pressure to a no. 40 deVilbiss nebulizer (Fig. 1, 2). The nebulizer was held in a clamp so that the discharge tube was approximately 20 cm from and parallel to the floor. Air pressure was supplied by a portable pump located outside the chamber. Open Petri dishes used as air samplers (Fig. 1*a*, *b*, *c*) were placed on shelves 1 and 0·5 m from the floor and on the floor. A shelf was suspended under the peak of the chamber so that air could also be sampled 1·8 m from the floor (Fig. 1*d*). A thermometer was suspended along the back wall of the chamber as was the sensing element of an electronic hygrometer located outside the chamber.

Exposure of adult cattle. For the exposure of adult cattle, the 0·5 × 1·8 m door was removed and the top and bottom of the opening sealed with plastic sheeting leaving a 0·5 × 0·8 m opening at a height that would accommodate the test animal's head and neck. A sleeve of plastic sheeting approximately 1 m long and 0·8 m in diameter was taped to the opening (Fig. 1, 3). The nebulizer was filled with 10 ml of viral suspension and the air samplers were put in place as follows: open Petri dishes containing 20 ml of cell culture fluid (Hanks balanced salts with 0·5 % lactalbumin hydrolysate and 28 mM-HEPES) with 0·2 % bovine serum albumin, pH 7·3 (Sellers, Herniman & Gumm, 1977) were placed at four locations (Fig. 1*a*,

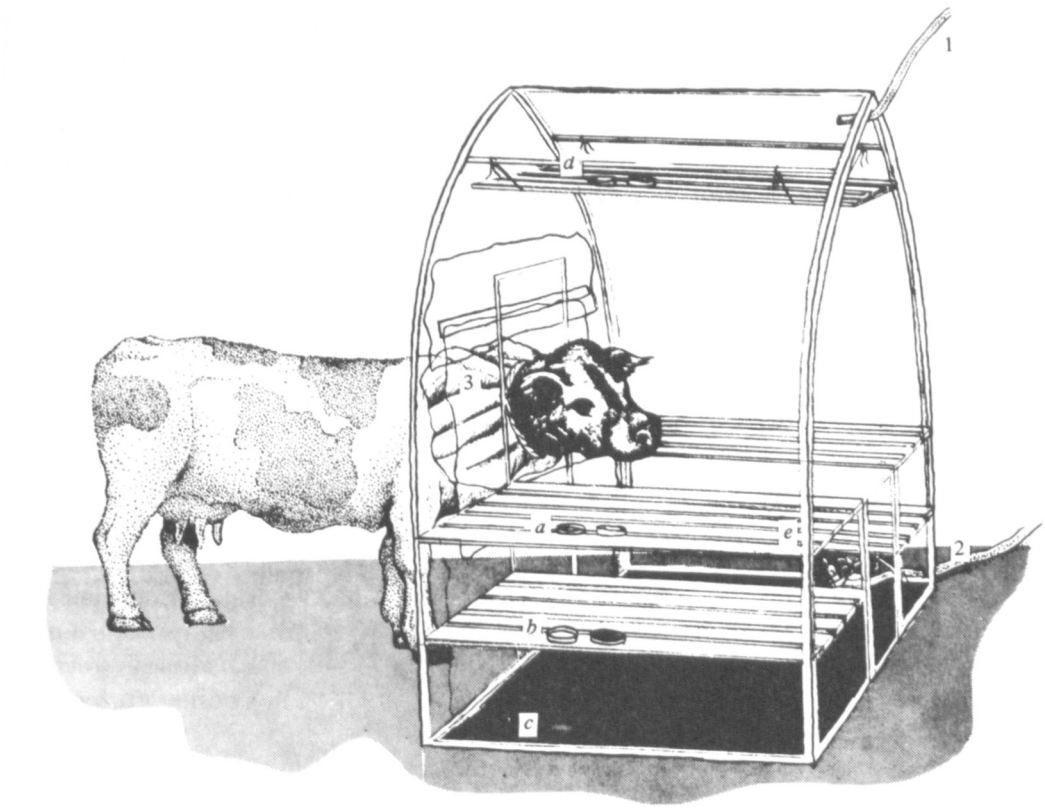

Fig. 1. Aerosol chamber in place for exposure of an adult bovine. 1 = exhaust hose. 2 = air pressure supply hose, 3 = neck sleeve of plastic sheeting, *a*, *b*, *c*, *d* = open Petri dish air samplers, *e* = location of May air sampler if used.

b, *c*, *d*) and a May sampler, when used, at another (Fig. 1 *e*). The latter was supplied by a vacuum hose let in through the side of the chamber. The test animal was confined in a 0·75 × 2 m chute with a rear restraint to ensure that the maximum amount of neck protruded through the head stock. The chamber was lifted slightly and moved toward the test animal while its head was directed into the open end of the sleeve. When the front of the chamber was against the head stock, the sleeve was gathered around the neck of the test animal and secured with an elastic ligature of thin latex tubing.

The breath of the test animal increased both the relative humidity (RH) and the temperature of the air in the chamber. The pump was started to begin the exposure when the RH had reached 70% at which time the temperature was usually between 22 and 24 °C. The pump was operated at a pressure of 20 lbs/sq in. and was turned off when the inoculum was gone; never longer than 10 min. Exposure was continued for a total of 30 min at which time the ligature was loosened and the chamber was moved away from the test animal until its head was out of the sleeve. The Petri dishes were then covered and removed from the chamber and the contents transferred to specimen vials. A small quantity of residual inoculum was removed from the nebulizer for assay if so desired. The

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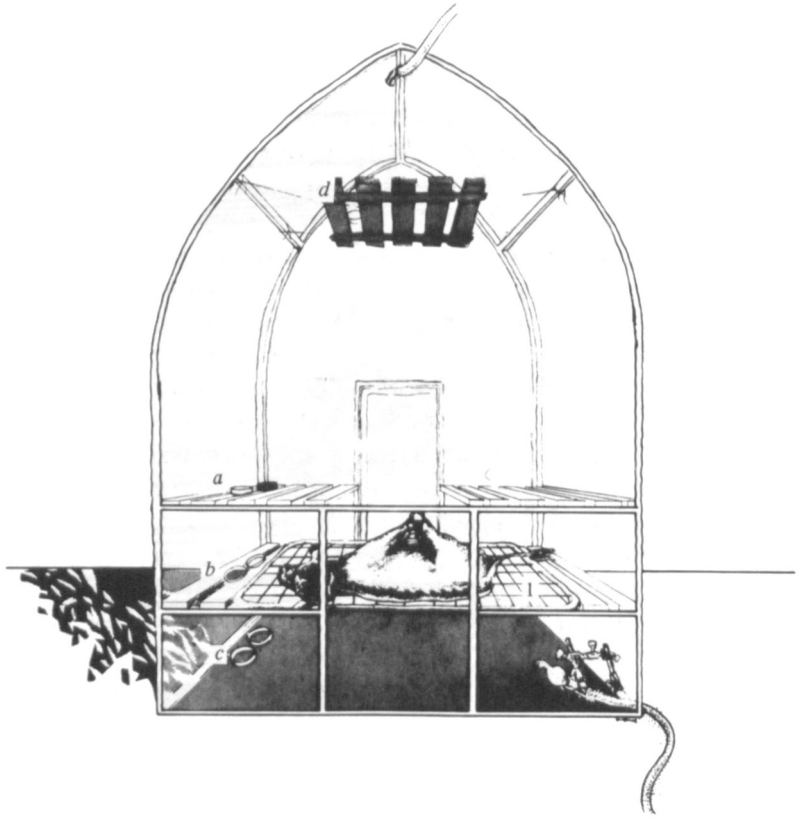

Fig. 2. Aerosol chamber in use for exposure of a calf. *a*, *b*, *c*, *d* = open Petri dish air samplers.

chamber was decontaminated by spraying the inside with an appropriate disinfectant and rinsing with water. The chamber easily fits into our animal isolation rooms in which the cattle remained confined during exposure and subsequent sampling. Fourteen adult cattle have been exposed by this method with the total amount of virus nebulized into the chamber ranging up to 10^{10} p.f.u.

Exposure of calves. For exposure of calves, the procedure was modified as follows. To provide humidity and warmth equivalent to that caused by the breath of adult cattle, a small amount of water was boiled on an electric hot plate placed in the rear of the chamber on the floor. A metal grid was placed across the lower shelves approximately 0·5 m above the floor (Fig. 2, 1). All four feet of the calf were tied together and the calf was put in a plastic bag with its head protruding and an elastic ligature around is neck. The calf was then placed on the metal grid and the door of the chamber was closed and sealed with tape. Open Petri dishes were located as during adult cattle exposure (Fig. 2, *a*, *b*, *c*, *d*). At the end of the exposure period (30 min), the calf was removed from the chamber and the plastic bag peeled away so as to prevent contact with the contaminated outside surface. If the calf was to be put in with other animals, its head was swabbed with 5% acetic acid. Sixteen calves have been exposed by this method with the total amount of virus nebulized into the chamber ranging from $10^{6\cdot6}$ to $10^{9\cdot2}$ p.f.u.

Pharyngeal virus growth curves. Nine adult cattle were exposed to total virus doses as follows: three cattle to $10^{9\cdot2}$ p.f.u. and one each to $10^{8\cdot2}$, $10^{7\cdot2}$, $10^{6\cdot2}$, $10^{5\cdot2}$ and $10^{4\cdot2}$ p.f.u. Assuming uniform distribution of the aerosol in the chamber the cattle were exposed to atmospheres containing from $10^{5\cdot8}$ p.f.u. to $10^{0\cdot8}$ p.f.u./l of air. Starting immediately at the end of the 30 min exposure period, samples of oesophageal-pharyngeal (OP) fluid were taken at 20 min intervals for 2 h, hourly up to 28 h, and at 48 and 72 h. Heparinized blood samples were taken every 2 h from 2 to 28 h and at 48 and occasionally 72 h. Samples were plaque assayed in BK cell cultures with results reported as p.f.u./ml of sample.

Early viremia study. Adult cattle were exposed to high levels of viral infectivity by nebulizing three times the usual amount of undiluted inoculum ($10^{8\cdot2}$/ml) and extending the exposure period to 90 min. The additional 10 ml amounts of inoculum were added to the nebulizer 20 and 40 min after the start of exposure with a cannula inserted through a small resealable hole in the chamber. Before the chamber was put in place, the left jugular vein was catheterized with polyethylene tubing as previously described (Sutmoller & McVicar, 1976*b*). The external part of the tubing, approximately 1 m long, was lead caudally out from under the neck sleeve and allowed blood samples to be taken as required. Blood was drawn into a heparinized syringe and immediately transferred to a specimen tube immersed in ice water. All samples were frozen at −70 °C and thawed before the qualitative assay described above. Blood samples were taken at 5 min intervals during the exposure period.

RESULTS

The test animals tolerated the chamber very well. Adult cattle did not resist when the chamber was moved into position and demonstrated no anxiety while their heads were in the chamber. Calves struggled briefly from time to time but never to the extent of inducing hyperventilation. Respiratory rates of cattle or calves did not change appreciably except for an occasional slight increase when nebulization started.

With adult cattle it took an average of 30 min for the RH to reach 70 % once the animals head was in the chamber. The RH continued to increase slowly throughout the exposure period but never went over 80 %. Chamber temperatures ranged from 23·4 to 26·2 °C during a series of exposures with an average increase of 0·5 °C during a single exposure period. Sometimes a fog-like condition developed before the end of the exposure period. With calves, the RH was between 65 and 70 % after 30 min with the chamber sealed and the water boiling. On some occasions the RH was augmented by the nebulization of 10 or 20 ml of sterile distilled water. Temperatures were in the same range as with adult cattle. The fog-like condition never developed while calves were being exposed.

The May sampler was used on five occasions, three times with no test animal in place and twice during the exposure of an adult bovine (Table 1). The mean percentages of particle size distributions when the sampling was done immediately after the nebulizer was turned off (0 min) were 76 % < 3 μm, 17 % 3–6 μm, and 7 % > 6 μm. On three occasions samples were taken 20–30 min later and the mean percentages were 90 % < 3 μm, 8 % 3–6 μm, and 2 % > 6 μm. At no time were less than 67 % of the particles < 3 μm in diameter.

Table 1. *Distribution of particle sizes in aerosol chamber after nebulization of foot-and-mouth disease virus O_1*

Trial	Time (min)	Particle sizes†		
		< 3 μm	3–6 μm	> 6 μm
1	0	4·2** (72)***	3·7 (23)	3·0 (5)
2	0	7·4 (68)	7·0 (27)	6·3 (5)
	5	7·5 (77)	6·9 (19)	6·2 (4)
	25	7·2 (90)	6·2 (9)	5·2 (1)
3	0	7·7 (92)	6·6 (7)	5·8 (1)
	30	6·5 (98)	5·4 (2)	4·9 (1)
4	0	6·6 (67)	6·0 (17)	6·0 (16)
	20	6·2 (83)	5·4 (13)	4·8 (4)
5	0	7·5 (79)	6·7 (13)	6·5 (8)

** Titre of sample in stage indicated ($\log_{10}$ p.f.u./sample).
*** Percentage of entire sample.
† Determined from a 3 stage impinger (May 1966).

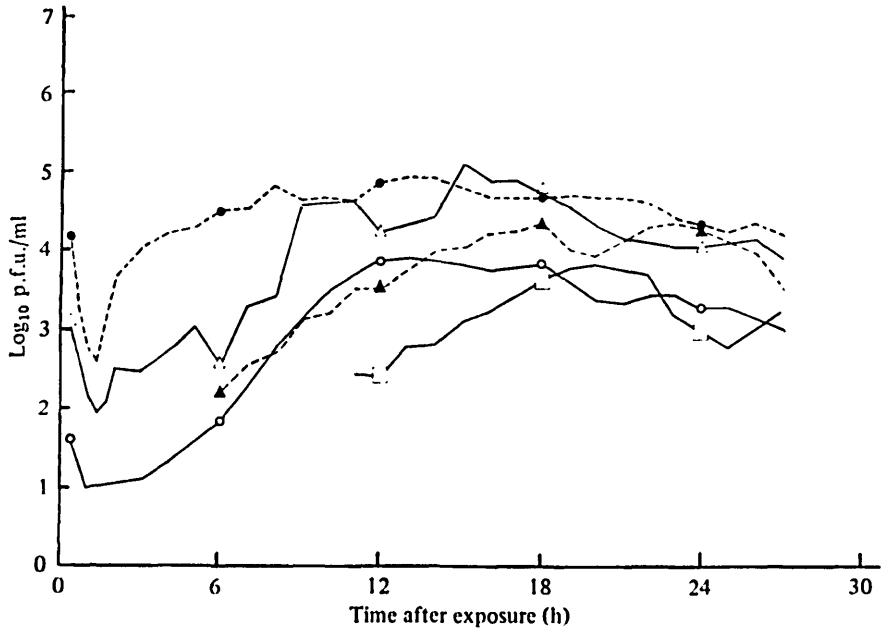

Fig. 3. Pharyngeal virus growth curves from five cattle exposed to various doses of foot-and-mouth disease virus, type 0. PFU of virus/l of air: ●, $10^{5\cdot8}$; △, $10^{4\cdot8}$; ○, $10^{3\cdot8}$; ▲, $10^{2\cdot8}$; □, $10^{1\cdot8}$.

Mean titres ($\log_{10}$ p.f.u.) of Petri dish samples from the four locations (*a–d*, Fig. 1) during exposures to 10-fold decreasing doses of aerosolized virus were: 7·3, 7·4, 7·9, 7·3; 6·0, 6·3, 6·6, 6·2; 5·2, 5·1, 5·7, 5·1; 4·1, 4·2, 4·9, 3·9; and 3·2, 3·1, 3·5, 2·9. During these 20 exposures, the titre of the Petri dish sample from the floor of the chamber (Fig. 1, *c*) was always higher than the mean of the other three samples (Fig. 1, *a*, *b*, *d*), the mean difference being 3-fold (range 1–10-fold). Statistical analysis of the data from 20 exposures showed this difference to be highly

significant ($P > 0{\cdot}001$) as was the similarity of titres between samples from the other three locations. The mean titre of these latter three samples closely approximated 1/1000 of the total amount of virus nebulized into the chamber.

Pharyngeal virus growth curves of adult cattle exposed to five different doses of virus are shown in Fig. 3. Viral growth was not detected in a steer exposed to the least amount of virus ($10^{0{\cdot}8}$ p.f.u./l of air). A graded response was observed in the other five animals. Cattle exposed to the three highest doses had virus in the first sample of OP fluid taken. The OP fluid virus titres then declined steadily going below detectable levels in the animal exposed to $10^{3{\cdot}8}$ p.f.u./l of air. A subsequent increase in titre of samples from cattle exposed to the two highest doses or the re-detection or first detection of virus in the other cattle signalled the appearance of progeny virus. This lag period was 2, 3, 4, 5 and 8 h in cattle exposed to increasing doses of virus. Viremia was first detected at 14, 18 and 24 h respectively, in the cattle exposed to the three highest doses of virus but not until the 48 h sample from the cattle exposed to the two lower doses. Not all cattle were held after the last sample was taken but those that were developed clinical signs typical of FMD starting 48–72 h after exposure to the higher doses of virus.

A pharyngeal virus growth curve done on one of the calves was similar to that of cattle exposed to the highest viral dose. Clinical signs and pathological findings suggested a massive exposure to virus. Most calves were febrile within 24 h and lethargic and anorectic a day later. At necropsy 2 or 3 days after exposure, scattered dark-red foci of varying size were seen in the lungs and some calves had larger areas that appeared atalectic.Ten of the 16 calves had gross or microscopic evidence of necrosis of the heart muscle.

Only one blood sample from one of the three cattle tested while being exposed to a high level infectious aerosol was positive for virus. This sample was taken 20 min after the start of exposure and contained too little virus to be titrated.

DISCUSSION

This type of chamber provides an efficient, simple and economical means of exposing cattle of all ages to infectious aerosols. It is large enough to allow sufficient air for comfortable breathing and yet light enough to be easily portable. The transparent covering permits observation of the test animal and of any equipment placed within. Adult cattle in particular are completely comfortable while being exposed as evidenced by the fact several started ruminating during this time. It was easy to achieve levels of RH expected to have the least deleterious effect on the viability of the viral aerosol (Barlow, 1972; Donaldson, 1972).

Sellers and his colleagues (1969, 1970, 1972, 1977), using a three stage air sampler (May 1966), reported that air in a box stall holding pigs infected with FMD has an infectious particle size distribution of 11 % < 3 μm, 21 % 3–6 μm, and 68 % > 6 μm. After the death or removal of the pigs the spectrum shifts rapidly so that after 5 min, the proportions are approximately equal, and after 1 h, 68 % of the particle sampled are < 3 μm in diameter (Sellers, Herniman & Donaldson, 1971). Size distribution data for cattle aerosols are less exact because of the relatively low amounts of virus excreted (Sellers, 1969) but appear to be approximately 25 % < 3 μm, 25 % 3–6 μm, and 50 % > 6 μm (A. I. Donaldson, personal

communication). The predicted settling velocity of particles > 6 μm in diameter is > 3 in/min in still air (Hatch, 1961). This fact together with the observations already cited for pigs would suggest that during indirect contact, cattle would be exposed to infectious aerosols with a predominant particle size of 3 μm or less, a condition established by the use of this chamber. Direct contact exposure is very difficult to simulate under experimentally controllable conditions because the amount of actual physical contact by licking or rubbing or close contact by sniffing are extremely variable. An important objective was to provide a high proportion of small droplets (< 3 m) and this was achieved by locating the nebulizer below the test animal's head. The deVilbiss no. 40 nebulizer has been shown to produce a high percentage of droplets in the 1–2 μm range (Palmer & Kingsbury, 1952). The sizes of particles, detected by the May sampler (Table 1) were in agreement with those of Larson, Young & Walker (1976) who reported that 84–95% of the airborn mass of aerosols generated by the DeVilbiss no. 40 was in particles < 5 μm in diameter. The larger amount of infectivity in the lowest Petri dish no doubt resulted from direct capture of the larger particles and the faster settling rate of 3–6 μm particles that were temporarily suspended in the turbulent air directly in front of the nebulizer. Particles < 3 μm in diameter would be expected to stay in suspension for considerably longer periods of time (Hatch, 1961).

The open Petri dishes proved to be a convenient means of assessing the total amount of virus nebulized and the uniformity of distribution of virus in the chamber. The distribution of the particle sizes was measured only at the level of the test animal's head (Fig. 2, level *a*) and the size of particles sampled by the other plates was unknown. The lack of variation of titres of Petri dish samples from all but the lowest level would suggest that the generated aerosol was rather uniformly distributed within the chamber. A similar uniform distribution was noted by Sellers, Herniman & Gumm (1977) in box stalls containing infected pigs.

In spite of the fact that 70–90% of the particles in the generated aerosols were < 3 μm in diameter, the shape of the pharyngeal virus growth curves, length of the lag periods, and time to onset of viremia resembled those of intranasally inoculated cattle (McVicar & Sutmoller, 1976). Indeed, cattle exposed to $10^{5.8}$ p.f.u. of FMD virus/litre of air in the chamber (total dose of $10^{9.2}$ p.f.u.) had growth curves very similar to those of cattle given 10^7 p.f.u. intranasally, a relationship that was retained through all of the doses tested. Approximately 10% of the particles were > 6 μm in diameter and a substantial part of these would be expected to lodge in the upper respiratory tract (Jericho & O'Connell, 1974; Lloyd & Etherridge, 1975; Knight, 1980; Morrow, 1980). If one assumes a tidal volume of 3 l and an average of 50 respirations/min, the maximum exposure to such large particles would be $10^{8.5}$ p.f.u. during a 30 min high dose exposure period. We have shown previously that only about 10% of intranasally instilled virus is retained in the upper respiratory tract (McVicar & Sutmoller, 1976) so only about 1% of the large particles in the aerosol would have been required to establish infection at the level of an intranasal dose of 10^7 p.f.u. Barlow (1972) showed that, even at a RH of 60%, approximately 90% of the infectivity of an FMD virus aerosol was lost in the first 5 min and so the efficiency of deposition in the upper respiratory tract may be even higher.

The fate of virus reaching the lower respiratory tract is less clear. There is good

evidence that a substantial percentage of particles $< 3\ \mu m$ reach the alveolar spaces (Hatch & Gross, 1964; Morrow, 1980; Knight, 1980). We have hypothesized that virus introduced in this manner might rather rapidly find its way into the circulation (Sutmoller & McVicar, 1976*a*). Lincoln *et al.* (1965) reported that bacilli were found in the lymphatics of monkeys as early as 1 h after exposure to aerosols of anthrax spores. They did not find, however, evidence of direct transfer of spores or bacilli into the blood. The fact that we were able to detect virus in only one of three cattle exposed to high levels of small particle aerosols would appear to argue against this route of entry but our single observation may be a significant one. We have shown that large amounts of virus must be injected intravenously to cause a detectable level of viremia in cattle (Sutmoller & McVicar, 1976*b*). Because of their blood volume, adult cattle have a dilution factor of approximately 10^{-4} and clearance is rapid in addition to this. In one trial, 10^6 p.f.u. of virus injected intravenously every 10 min only produced a relatively modest viremia of $10^{1.5}$–10^2 p.f.u./ml. It is entirely possible, therefore, that substantial amounts of virus could reach the blood stream and not be detected and that the single positive sample in this and in a previous study (Sutmoller & McVicar, 1976*a*) merely represented the tip of the viral iceberg. That circulating virus would rapidly make its way to predilection sites is suggested by the detection of virus in the OP fluid of cattle within minutes of its intravenous inoculation (Sutmoller & McVicar, 1976*b*). It is perhaps then a combination of local deposition of large particles and the delivery of virus from small particles via the circulation that caused the pharyngeal virus growth curves to appear like those of cattle inoculated intranasally.

Early virus growth patterns typical of cattle exposed by contact to other infected cattle (Sutmoller & McVicar, 1976*a*) were not produced by the use of the chamber. Such patterns are characterized by substantial fluctuations in viral titres in OP fluid and by the simultaneous appearance of viremia and sustained viral growth. Direct and indirect contact entail many uncontrollable variables but perhaps the most important feature is that, during cattle-to-cattle contact, exposure is to very low levels of virus (Sellers, 1969) over a long period of time, conditions difficult to simulate experimentally.

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