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A simple device for the exposure of animals to infectious microorganisms by the airborne route

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SUMMARY

In order to evaluate prophylaxis and therapy for individuals infected with pathogens by the airborne route, we have designed and built a simple apparatus in which small laboratory animals may be exposed to aerosols of infectious microorganisms. Animals are kept in a chamber closed by a HEPA filter and exposed to the pathogen aerosolized using a Collison nebulizer. Air in the exposure chamber may be sampled to show that the infectious agent is present but the dose of agent must be expressed as 50% effective doses determined by titration. An effective dose may be defined by whatever criteria are chosen to judge disease. Using this apparatus we have shown that St Louis encephalitis (SLE) virus is infectious for mice by the airborne route. These data support the idea that there may be significant hazard to personnel exposed to aerosols of infectious SLE after a laboratory accident.

INTRODUCTION

St Louis encephalitis (SLE) virus is a member of the family *Flaviviridae*. These small, single positive-stranded RNA viruses are usually transmitted to their natural vertebrate hosts by tick or mosquito vectors [1]. There are 22 human pathogens in the family [2] that cause diseases varying from a mild febrile illness (West Nile virus), to encephalitis (Central European tick-borne encephalitis, Japanese encephalitis) or severe haemorrhagic disease (yellow fever, haemorrhagic dengue). The annual incidence of disease may vary from only a few cases (Murray valley encephalitis in Australia) to hundreds of thousands of cases (dengue in South East Asia). Survivors of the encephalitic illnesses often have significant long term sequelae, and prevention and treatment of these diseases is of considerable interest.

SLE virus infection occurs throughout the American continent and is the leading cause of epidemic viral encephalitis in the USA [1, 2]. The virus is transmitted by mosquitoes, and the animal reservoir is in passeriform and columbiform birds, which develop viraemia without overt disease. Humans are tangential hosts. Approximately 1/1000 human infections leads to an encephalitis that may be life-threatening, is often more severe in the elderly and very young, and may produce prolonged sequelae; the case mortality rate is 5–15% (1). Since SLE virus was first isolated over 60 years ago at least four infections have been reported in laboratory workers engaged in diagnostic or research work [3]. The circumstances under which these have occurred raise the possibility that SLE virus may infect humans by the airborne route. The demonstration that SLE virus has significant stability in experimentally generated aerosols [4] supports this idea. Here we describe the design, construction and operation of a simple apparatus for the exposure of small laboratory animals to aerosols containing pathogenic micro-organisms and show for the first time that SLE may readily infect mice by the airborne route. We have

tested the infectivity of SLE virus in aerosol in order to examine the risk to laboratory personnel working with the virus. Our methods and these data may also be used to facilitate research into prophylaxis and therapy for individuals exposed to aerosols of infectious virus in the laboratory.

MATERIALS AND METHODS

Animals

Locally produced Porton outbred TO strain mice (age 3–4 weeks, weight < 18 g) were used for the virus exposure experiments and for the preparation of suckling mouse brain suspensions containing infectious SLE virus.

Cells, viruses and bacteria

We used the MS1-7 strain of SLE virus throughout (kindly supplied by Dr E. A. Gould of the NERC Institute of Virology, Oxford, UK). All virus samples were stored frozen at -70 °C. Infected mouse brain suspensions were prepared by standard procedures. Briefly suckling mice were inoculated intracerebrally with approximately 1000 pfu of SLE virus in 25 μl of Leibovitz L15 medium containing 2% foetal calf serum. Brains were harvested when the animals developed signs of paralysis (3–4 days) and a 10% w/v suspension prepared in L15 medium as above. Trehalose (Sigma UK) was added as a stabilizer to virus suspensions before aerosolization to a final concentration of 2%.

SLE virus was titrated by plaque assay in BHK21 cells grown on Eagles MEM supplemented with 10% foetal calf serum and 10% tryptose phosphate broth. The plaques were allowed to develop for 4 days under an overlay of L15 medium containing 1.5% carboxymethyl cellulose and 2% foetal calf serum before formalin fixation and staining of the residual cell sheet with crystal violet.

Spore suspensions were prepared from *Bacillus subtilis* var. *niger* cultured in nutrient broth and diluted as required in nutrient broth. Viable spores were estimated by titration and colony counts performed in quadruplicate on nutrient agar plates.

Aerosol exposure apparatus

The apparatus consisted of a box made of sheet aluminium measuring $40 \times 50 \times 40$ cm and enclosing a volume of 80 l ((Fig. 1). The inside of the box was visible through a hardened glass window on the top surface and access was through an airtight door measuring 20×20 cm on the front. Air within the box circulated by a small low speed electric fan mounted in one upper corner.

The aerosol was generated with a standard Collison spray [5] and fed into the box directly through a 10 mm port at one end. Air left the box through a 30 cm^2 HEPA filter mounted on the opposite end. A second port adjacent to the Collison inlet allowed the box to be flushed with air after each exposure period. The compressed air supply passed through filters to remove oil and water vapour and odour. Isolation and pressure control valves, and a flow rotameter directed a controlled flow of air to either the Collison spray or directly to the box. There were two 10 mm ports on the front of the box connected to an all glass impinger (AGI 30) in series with a control rotameter and a small pump. The impinger was charged with 60 ml glass beads and 40 ml of phosphate buffered saline (PBS) solution, so that air could be bubbled through it at low flow rates (usually 2 l/min) using the pump. Used in this manner an impinger operates at *c*. 70% efficiency. Impinger samples containing virus were diluted with an equal volume of L15 medium before freezing and storage at -70 °C prior to titration.

For exposure to aerosolized virus animals were placed free on paper bedding in the box and the door was closed and the air circulation fan switched on. The Collison spray was operated at a flow rate of 8 l/min by adjusting the air pressure in the feed line. Samples were taken using the impinger at a flow rate of 1 or 2 l/min (higher sampling flow rates would be expected to reduce the concentration of aerosol in the box). After an exposure time of 20 min the air supply was switched from the Collison spray to the direct line to flush out the box for a further 10 min and to reduce the concentration of aerosolized agent within. The air supply was then disconnected, the box opened and the animals removed. During all exposures of animals to virus the apparatus was operated in secondary containment to Category III level.

RESULTS

Calibration of the apparatus

To examine the concentration of aerosol produced inside the box the Collison spray was filled with *B. subtilis* var. *niger* spores ($10^{9\cdot2}$ spores/ml) and an

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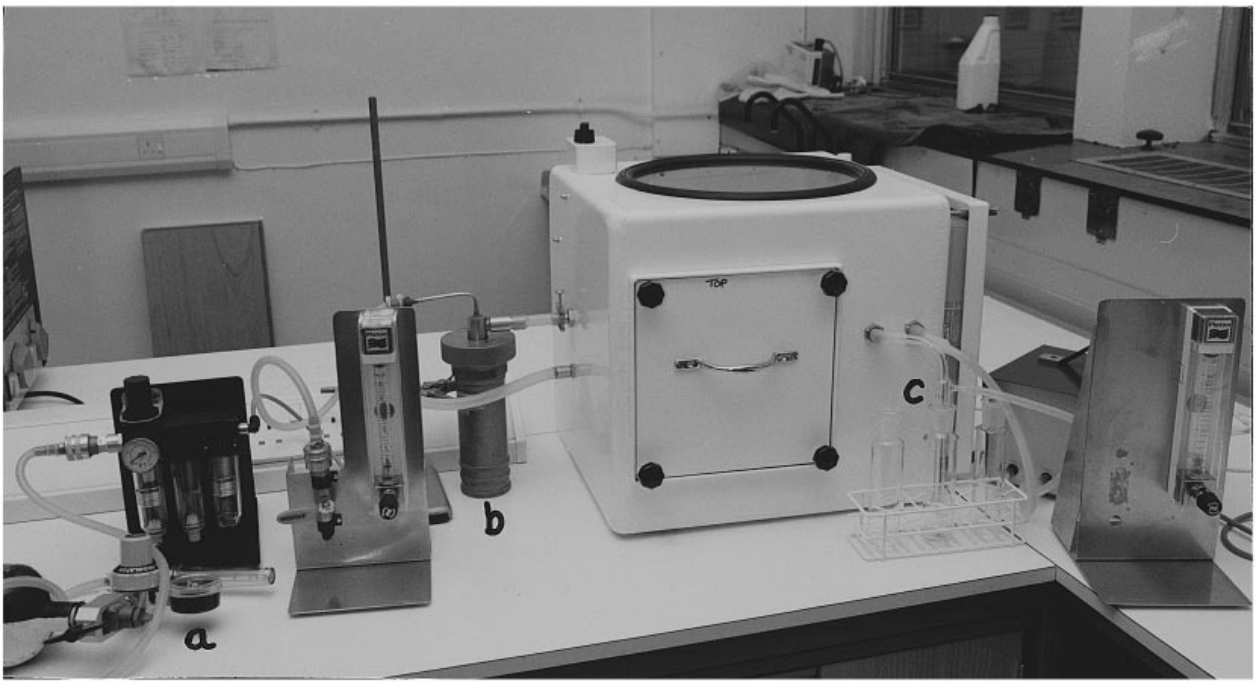

Fig. 1. The apparatus. The small animal exposure box shown with filtered air supply (*a*), Collison spray (*b*) and impinger sampler (*c*). All experiments with SLE virus were performed in secondary containment (UK ACDP category 3).

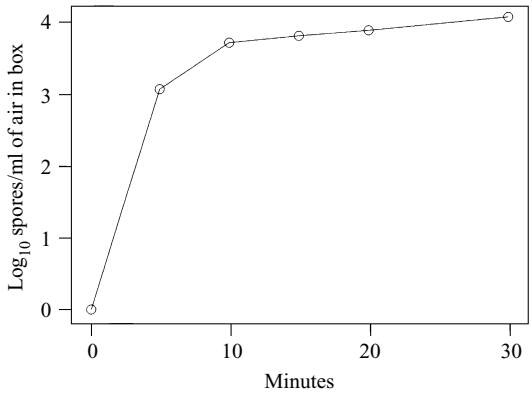

Fig. 2. Concentration of *B. globigii* spores in the exposure box at various times as determined from 5 min samples (ending at the times shown) during a 30 min period of aerosol generation (8 l/min). The impinger sampler ran at 2 l/min.

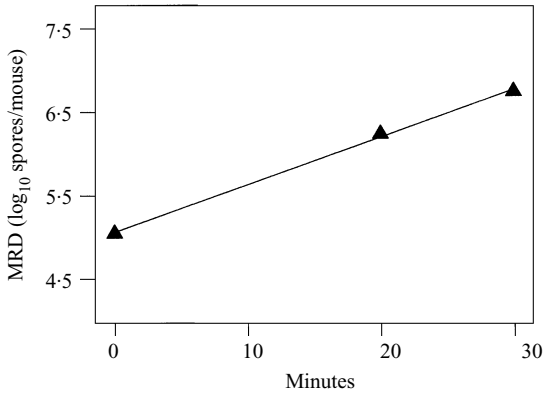

Fig. 3. Linear regression plot of spores/ml in spray fluid *vs.* MRD ($r = 0{\cdot}998$, $P < 0{\cdot}01$). The impinger sampler ran at 2 l/min for the entire 30 min period of the experiment. MRD was calculated from the quantity of viable spores in impinger samples assuming a respiratory rate for the mouse of 10 ml/min [6].

aerosol was generated for 30 min. Samples were taken by impinger every 5 min. Counts of the viable spores present in these samples showed that there was a stable concentration of spores in the box after 10–15 min (Fig. 2). No viable spores were detected in impinger samples taken after the 10 min flushing period (data not shown).

Clearly, either time of exposure or adjustment of the concentration of microorganisms in the spray fluid may be used to control the dose of inhaled micro-organisms received by an animal in the box. In a further experiment the concentration of spores in the spray fluid was varied, and viable spores present in the impinger fluid were estimated at the end of a 30 min interval (Fig. 3). The number of spores present in the box was expressed as mouse received doses (MRD) calculated using the mouse respiratory minute volume estimate of Guyton [6]. There was a direct linear relationship. A MRD of $10^{5{\cdot}0}$ spores/animal could be readily achieved when $10^{8{\cdot}5}$ spores/ml were present in the spray fluid.

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Table 1. *Mortality in mice exposed to airborne SLE virus*

Virus in spray fluid (log_{10} pfu/ml)	Mortality*	MRD log_{10} pfu/mouse†
7·6	10/10 (100)	2·53
6·6	10/10 (100)	1·6
5·6	10/10 (100)	1·16
4·6	9/10 (90)	< 1·0
3·6	2/10 (20)	< 1·0
2·6	2/10 (20)	< 1·0

* Numbers in parentheses indicate indicate % mortality.
† MRD was calculated from the quantity of viable virus in impinger samples assuming a respiratory rate for the mouse of 10 ml/min [6].

Mouse exposure experiments

Mice were exposed for 20 min to aerosolized SLE virus. Titrations of the spray fluid before and after each experiment indicated that a loss in titre of $< 10^{0·5}$ pfu/ml occurred during spraying (data not shown). Virus concentrations in the spray fluid, determined by back titration of the fluid after spraying, ranged from $10^{7·6}$ plaque forming units (pfu)/ml to $10^{2·6}$ pfu/ml. Severely ill mice with signs of severe infection (low mobility, hunched posture and paralysis) were culled before death in order to minimize suffering of the animals ('humane end-points'). Experience has shown that these signs are typical of SLE virus disease and that such animals will survive for only a few hours. The cause of death was confirmed by isolation of a virus, identified by immunofluorescence as SLE virus, from the brains of five affected mice (data not shown).

Mortality was 100% at the higher doses although deaths occurred at all the dose levels tested (Table 1). Using probit slope analysis the median lethal dose corresponded to $10^{3·6}$ pfu/ml of virus in the spray fluid (95% confidence limits 3·2–3·9). Titration of the infectious virus recovered in impinger samples, with values corrected for transport and freezing/thawing losses (determined experimentally, data not shown) suggested that between 1 and 100 pfu of SLE virus delivered to 3–4 week old Porton strain mice by the airborne route may be lethal.

DISCUSSION

Several methods have been used to expose animals to airborne microorganisms. The Henderson apparatus allows temperature and humidity to be controlled [5], and addition of a reservoir such as the Goldberg drum [5] allows ageing of the aerosol. These factors may affect the viability or pathogenic potential of the infecting organism [5]. As discussed elsewhere [5] these systems are subject to many variables and seem unnecessarily complex for simple exposure of an animal to airborne microorganisms. The system described here offers a simple solution which may require fewer staff to operate and cost less.

To show that a vaccine or antimicrobial treatment may protect against infection by the airborne route it seems only necessary to demonstrate that: (i) sufficient particles of small enough size to infect have been generated, (ii) the exposure is genuinely by the respiratory tract rather than the alimentary tract or any other portal of entry and (iii) an overwhelming dose of challenge organism was not given. The simple apparatus we have designed and used in the experiments described here readily meets these criteria.

A Collision spray produces 79% of its particles by mass in the size range $< 5\ \mu$m diameter which would penetrate and remain within the mouse lung [7]. However, as significant proportion of the aerosol mass is present in particles $> 7\ \mu$m diameter, some deposition in the nose was unavoidable [5] in our experiments as we used whole body exposure. Whole body exposure proved satisfactory for Venezuelan equine encephalomyelitis virus (VEE; [9]). Others have shown that the principle route of SLE virus entry to the brain after intraperitoneal or intranasal inoculation of weanling mice is via infection of the olfactory neuroepithelium [8]. The pathogenesis would be expected to be similar after lung exposure. SLE virus has been shown not to be infectious via the alimentary tract [10], suggesting that the majority of virus received by our mice was via the respiratory tract. Although exposure via the conjunctiva is possible, this seems unlikely in all but rare instances and would not have a significant impact on our experimental results. Experiments with another flavivirus, West Nile virus, have shown mice to be refractory to infection by this route [11].

There is considerable difficulty in calculating infectious doses delivered by the airborne route regardless of the apparatus used. Sampling an infectious aerosol by any method may reduce the viability of the organisms it contains [12]. Furthermore, estimates of viability made by *in vitro* methods may not agree with those derived from animal exposure data. The calculation of received dose from aerosol sample data using estimates of respiratory rate may only inaccurately indicate the retained dose of pathogen.

Although we sampled the aerosol present in our apparatus, data so obtained indicate only the generation of an infectious aerosol. Any estimate of the dose of pathogen received must be based upon titrations by airborne exposure and may be expressed as 50% effective doses.

We have designed and built a simple apparatus suitable for exposing small laboratory animals to airborne pathogens. Characterization of the apparatus showed stable concentrations of aerosolized microorganisms in the exposure chamber within 10–15 min from starting aerosol generation. The quantity of airborne microorganisms was directly proportional to their concentration in the spray fluid. Using this apparatus we have demonstrated for the first time that SLE is infectious by the airborne route for weanling mice. The apparatus can be used to challenge small laboratory animals by the airborne route to estimate the efficacy of prophylactic or therapeutic antimicrobial treatments against airborne infection.

Our approximation of the airborne lethal dose of SLE virus for weanling mice (1–100 pfu of virus) may be less than would be inhaled by a laboratory worker who accidentally dropped and so shattered a vessel containing infected cell culture fluid, the titre of which may range from 10^6 to 10^7 pfu/ml. Although the airborne infectious dose for humans of SLE virus is unknown our findings emphasise the need for caution when working with SLE virus.

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