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A comparison of aerosol and intragastric routes of infection with *Listeria* spp.

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SUMMARY

Aerosol infection (AI) of Porton outbred mice with *Listeria* species, exhibiting varying degrees of virulence, was compared with gastric intubation (GI) on the basis of numbers of deaths, 50% lethal dose (LD_{50}) and pattern of listerial infection. The AI route appeared to be more sensitive, efficient and consistent than GI in that it required 10^5 fewer micro-organisms to obtain infection and death then ensued within 4 days, with GI deaths usually occurring on day 7. All the virulent strains tested caused 100% mortality by AI, while virulent and avirulent strains were indistinguishable by GI. Bacterial counts in the livers and spleens of infected mice were consistent with the relative virulence of the infectious agent using AI but not in GI mice. There were higher numbers of micro-organisms and more widespread lesions in the organs of AI mice than in GI. Results indicate that AI is an accurate *in vivo* indicator of virulence in listeria and using AI, bacterial counts in the liver and spleen could replace LD_{50} tests, thereby reducing the number of animals required for *in vivo* virulence testing.

INTRODUCTION

Only four species of *Listeria* have been reported to cause infections in animals and man. These are *Listeria monocytogenes*, *L. ivanovii*, *L. innocua* and *L. seeligeri* [1]. The other species *L. grayi* and *L. welshimeri*, have not been implicated with naturally occurring infections. Moreover it has been reported that 98% of strains in humans and 87% of strains in animals causing disease are identified as *L. monocytogenes* [2]. In the last decade *L. monocytogenes* has been implicated with outbreaks of food-borne disease [3–6], and a few sporadic episodes [7]. There are 13 recognised serovars of *L. monocytogenes* and although they may all cause human listeriosis the majority of cases are associated with 3 serovars, namely 1/2a, 1/2b and 4b.

In recent years, the experimental infection of rodents has been used to investigate virulence in *L. monocytogenes*. Oral, intraperitoneal, subcutaneous or intravenous inoculation have all been used to cause infection [8] but these modes of infection have proved to produce variable results when testing for virulence.

The phenotypic expression of a micro-organism is known to be dependent on, and varies with, the environment [9]. We are interested in comparing the

expression of virulence determinants in *L. monocytogenes* when grown under different environmental conditions. Hence, together with various *in vitro* assays for virulence, we had need of an efficient and reproducible animal model to carry out *in vivo* virulence tests.

In this study we have modified an existing aerosol model of infection [10] in order to determine the relative pathogenicity of strains of listeria in immunocompetent mice. The comparative virulence of listeria strains have been assessed on the basis of numbers of deaths and 50% lethal dose (LD_{50}) in mice challenged by aerosol infection (AI) or gastric intubation (GI). We have also examined and compared the pattern of listeria infection by AI and GI.

METHODS

Bacterial strains and culture methods

The strains of listeria used in this study are listed in Table 1. *L. monocytogenes* EDG, NCTC 4485 and NCTC 11994 were included in the study because they were all involved in human infection and were therefore assumed to be virulent. Strain 11994_{HLY^-} was used because it is a non-haemolytic mutant of NCTC 11994 and therefore potentially avirulent. L3700 is a rough environmental mutant. NCTC 10357 was studied because it is the type stain of *L. monocytogenes* and *L. ivanovii*, *L. grayi*, *L. seeligeri* and *L. innocua* were included because they represent other species of the genus *Listeria*. For the preparation of standard innocula, strains were taken from stock cultures maintained at −70 °C in glycerol yeast-extract broth and grown up overnight at 37 °C, on Columbia Blood agar (CBA) (Oxoid Ltd) containing 7% horse blood. The resulting colonies were then suspended in 0·9% sterile saline to give a concentration of 10^{10} c.f.u. ml^{-1}.

Animals

Immunocompetent Porton outbred mice, 20 g in weight and approximately 4 weeks old, were used throughout this study. Mice were observed up to day 6 post challenge for comparison of progress of infection, and up to day 10 for all other experiments.

Aerosol infection

Aerosol infection (AI) was achieved using a three jet Collison spray at 90% relative humidity in a mobile Henderson apparatus [10], which generates aerosols containing particles 5 μm in diameter. Mice were exposed to bacterial aerosols containing 10^{10} c.f.u. ml^{-1} of listeria for 5 min. The numbers of listeria retained in the lungs (retained dose, RD) was determined by viable counts of lungs taken from animals immediately post infection. LD_{50}s for aerosol infected mice were determined according to the method of Reed and Muench [11]. At least three sets of duplicate mice were used in each test and some species of *Listeria* were tested in triplicate.

Gastric intubation

Animals challenged by gastric intubation (GI) received 0·5 ml of a 10^{10} c.f.u. ml^{-1} suspension directly into the stomach after overnight fasting.

Table 1. *Bacterial strains*

Species	Strain	Serotype	Isolated from:
L. monocytogenes	NCTC 10357* (ATCC 15313)	1/2a	Rabbit
L. monocytogenes	NCTC 10887	1/2a	Chinchilla
L. monocytogenes	EGD†	1/2a	Human
L. monocytogenes	NCTC 4885	4b	Human
L. monocytogenes	NCTC 11994	4b	Cheese‡
L. monocytogenes	11994_{HLY^-}§	4b	
L. monocytogenes	L3700‖	nt¶	Environmental
L. ivanovii	SLCC 3765†		Not specified
L. ivanovii	ATCC 19119*†		Sheep
L. grayi	NCTC 10815 (ATCC 25400)		Vegetation
L. seeligeri	NCTC 11856*		Soil
L. innocua	NCTC 11288* (ATCC 33090)	6a	Cow

* Type strain.
† Kindly supplied by Professor W. Goebel, Universität Würzburg, Würzburg, Germany.
‡ Soft cheese associated with a case of meningitis.
§ Haemolysin negative mutant, obtained by transposon mutagenesis of NCTC 11994.
‖ Kindly supplied by Dr J. McLauchlin, CPHL, London, UK.
¶ nt, not typed. L3700 cannot be typed because of its agglutinating properties (Dr J. McLauchlin, personal communication).

Feeding experiment

In order to determine whether micro-organisms could enter the respiratory tract during swallowing, mice were fed 0·2 ml of a 10^9 c.f.u. ml^{-1} suspension of either virulent, *L. monocytogenes*, NCTC 11994, or an avirulent environmental mutant, L3700. Feeding was accomplished by placing drops of bacterial suspension onto the tongue of the animal and then encouraging the animal to swallow by gently rubbing the throat. At least three sets of duplicate mice were used for each test. After 30 min their respiratory tract was removed and the viable count of listeria in the lungs and trachea determined.

Enumeration of micro-organisms

For culture from tissue, organs were macerated in 0·9% sterile saline and 0·1 ml samples of the appropriate dilution inoculated onto CBA. Total viable counts per organ, or per ml blood, were determined after 24 h incubation at 37 °C.

Histology

Brain, lungs, liver, spleen, and kidney were removed and fixed in 10% buffered neutral formalin. The tissues were processed by standard methods and embedded in paraffin wax. Sections were cut at 5 μm and stained by haematoxylin and eosin. Selected sections were also stained according to Gram's method.

Pathogenesis profile of L. monocytogenes

L. monocytogenes NCTC 11994 and L3700 were chosen to compare the pattern of infection for both AI and GI. A suspension of 10^9 c.f.u. ml^{-1} in sterile 0·9% saline was used for both routes of infection. Three animals infected with either

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strain, by AI or GI, were sacrificed at daily intervals over a period of 6 days following challenge, and blood, brain, lungs, liver, spleen and kidneys removed for bacterial enumeration and histological examination.

RESULTS

Comparison of aerosol and intragastric routes of infection

The aerosol route of infection appears to be a more sensitive means of determining virulence than the intragastric route. Mice infected by AI retained approximately 10^5 c.f.u. in the lungs (mean retained dose = $1{\cdot}86 \times 10^5$ c.f.u.) (Table 2) and it was possible to calculate LD_{50}s for certain strains by this route, namely; EGD, $LD_{50} = 10^{4{\cdot}0}$, 4885, $LD_{50} = 10^{3{\cdot}1}$, 11994, $LD_{50} = 10^{3{\cdot}3}$, L3700, $LD_{50} = 10^{5{\cdot}5}$. Animals challenged by GI received approximately 2×10^{10} c.f.u. directly into the stomach (mean dose = $2{\cdot}58 \times 10^{10}$ c.f.u.); however, we were unable to obtain an LD_{50} value for any of the strains tested.

AI was also found to be a more efficient method of determining virulence (Table 2). If a strain of listeria was virulent, death ensued within 4 days of AI. In comparison, mice infected by GI died on at least day 6, but more usually on day 7.

The aerosol route of infection gave more consistent results than those obtained by GI. All strains of *L. monocytogenes*, with the exception of L3700, 11994_{HLY^-} and 10357, caused 100% mortality in the mice infected by AI but not deaths were observed among animals infected with other species of listeria by this route (Table 2). Virulent and avirulent strains were indistinguishable by GI. Micro-organisms which had caused human infection and which should have tested virulent (e.g. 4885) were found to be relatively avirulent, and avirulent micro-organisms (e.g. L3700 and 19119) produced similar fatalities to those of virulent strains. Moreover AI produced consistent numbers of micro-organisms in the livers and spleens of infected mice. Animals infected with virulent listeria died by day 4 and were found to have high bacterial counts in their livers and spleens (means 8·08 and 7·55 $\log_{10}$ c.f.u., respectively) (Table 3). In contrast, for avirulent listeria, AI animals survived to day 10 when micro-organisms were only detected in low numbers, if at all, in the livers and spleens.

Feeding experiment

L. monocytogenes was found to be present in the trachea and lungs of mice after being fed with the micro-organisms (Table 4). The numbers of *L. monocytogenes* found in the lungs after swallowing were 1–3% relative to the retained aerosol dose and approximately 0·2% in the trachea.

Pathogenesis

The virulent strain of *L. monocytogenes*, 11994, caused 100% mortality by day 4 in AI infected animals, and approximately 30% mortality by day 7 in animals infected by GI. The mortality figure for GI infected mice did not increase after day 7. Mice infected with the less virulent strain of *L. monocytogenes*, L3700, displayed no symptoms of infection when challenged by AI or GI, and there were no mortalities in either the AI or GI group. Symptoms of infection observed included

Table 2. *Comparison of aerosol infection and gastric intubation*

	Aerosol infection			Gastric intubation		
Strain	RDL* (log_{10} c.f.u.)	No. of deaths	Day	RDS† (log_{10} c.f.u.)	No. of deaths	Day‡
10887	5·44	5/5	4	9·94	1/5	7
EGD	5·11	5/5	5	10·02	2/5	7
4885	5·46	6/6	4	10·18	3/5	7
11994	5·12	5/5	4	10·18	3/5	6
11994	5·19	5/5	4	10·57	2/4	7
11994	5·71	4/4	4	10·95	5/5	7
L3700	5·12	3/5	7	10·37	1/5	7
10357	4·65	0/5	0	9·85	0/5	0
11994_{HLY^-}	5·03	0/5	0	nd§	nd§	nd§
3765	5·25	0/3	0	10·18	0/5	0
19119	5·8	0/5	0	10·18	1/5	9
10815	2·77	0/5	0	10·02	0/5	0
11856	4·33	0/5	0	9·93	0/5	0
11288	4·15	0/5	0	9·00	0/3	0

* RD, Retained dose in the lung immediately post infection.
† RD, Retained dose in the stomach immediately post infection.
‡ Day of last animal death.
§ nd, not done.

Table 3. *Numbers of viable listeria recovered from the livers and spleens of aerosol-infected animals*

Strain	Day*	Liver (log_{10} c.f.u.)	Spleen (log_{10} c.f.u.)
10887	4D	7·63 (±0·14)	7·58 (±0·12)
EGD	4D	8·57 (±0·25)	7·60 (±0·15)
4885	4D	8·38 (±0·02)	7·61 (±0·08)
11994	4D	7·74 (±0·16)	7·40 (±0·05)
L3700	7	5·59 (±0·07)	4·27 (±0·12)
11994_{HLY^-}	7	3·85 (±0·04)	4·50 (±0·05)
10357	10	ND	ND
3765	10	ND	ND
19119	10	ND	ND
10815	10	ND	ND
11856	10	ND	ND
11288	10	ND	ND

* D, animal died; ND, not detected.

Table 4. *Entry of 11994 and L3700 into respiratory tract during swallowing*

	Lungs (c.f.u.)	% of RD*	Trachea (c.f.u.)	% of RD
11994	$2·52 \times 10^3$	1	$3·23 \times 10^2$	0·17
L3700	$6·59 \times 10^3$	3	$3·78 \times 10^2$	0·2

* % of RD, the viable counts of listeria from the lungs and trachea after feeding expressed as a percentage of the retained dose (RD) of viable listeria from the lungs after AI.

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weight loss and pyrexia, eventually leading to paralysis. In addition, 20% of GI mice, prior to death, developed a 'twisting motion' when the animal was held up by the tail.

In general, organs from AI mice contained higher numbers of micro-organisms than those from mice infected by GI, and 11994 was present in higher numbers than L3700 (Fig. 1). Histology of the organs showed that *L. monocytogenes* 11994 produced more widespread lesions in AI mice than in GI animals and that no lesions were observed with L3700 in any tissue regardless of the route of infection.

There follows a brief résumé of observations from microbiological counts and histological studies for each organ examined.

Blood

Microbiology. Similar numbers of *L. monocytogenes* 11994 and L3700 were isolated from the blood of AI mice. Strain 11994, although not detectable at day 1, was isolated in increasing numbers, reaching a maximum at day 4 (Fig. 1A). However, using GI, this micro-organism was only detected in high numbers at day 4.

L3700 was only found in high numbers in the blood from AI mice.

Brain

Microbiology. L. monocytogenes was isolated from the brains of mice in all four groups during the course of the study (Fig. 1B). There appeared to be no difference in the numbers of strain 11994 from aerosol or intragastric routes of infection, and both increased with time. Considerably lower numbers of micro-organisms were present in the brains of mice infected with L3700 via the aerosol route and this micro-organism was only detectable on day 3 from GI mice.

Histology. Histological examination revealed no lesions in the brains of any of the mice infected, regardless of the route or infectious agent.

Lung

Microbiology. AI infected animals exhibited continuously high numbers of listeria in their lungs throughout the study (Fig. 1C). The numbers of strain 11994 from AI and GI mice and of L3700 from AI mice were similar. GI L3700 numbers in the lungs were negligible until day 2 of the experiment.

Histology. Lesions in lungs of animal infected with 11994 via AI were situated in the alveolar walls. The lesions increased in size up to day 4, when they were predominantly lysed cells with masses of basophilic bacteria, many within macrophages. These were also present in the lung parenchyma, and oedema of interlobular septa and blood vessel walls was visible. Hilar lymph nodes draining the lungs also contained typical necrotic foci.

Lungs of 11994 GI mice contained scattered foci of polymorphonuclear leukocytes (PMNs) and monocytes, with localized areas of bronchopneumonia at day 1 post-infection.

L3700 infections produced no lesions.

Liver

Microbiology. Similar patterns of growth were observed in the livers of both AI and GI mice infected with 11994, although higher numbers were found in the

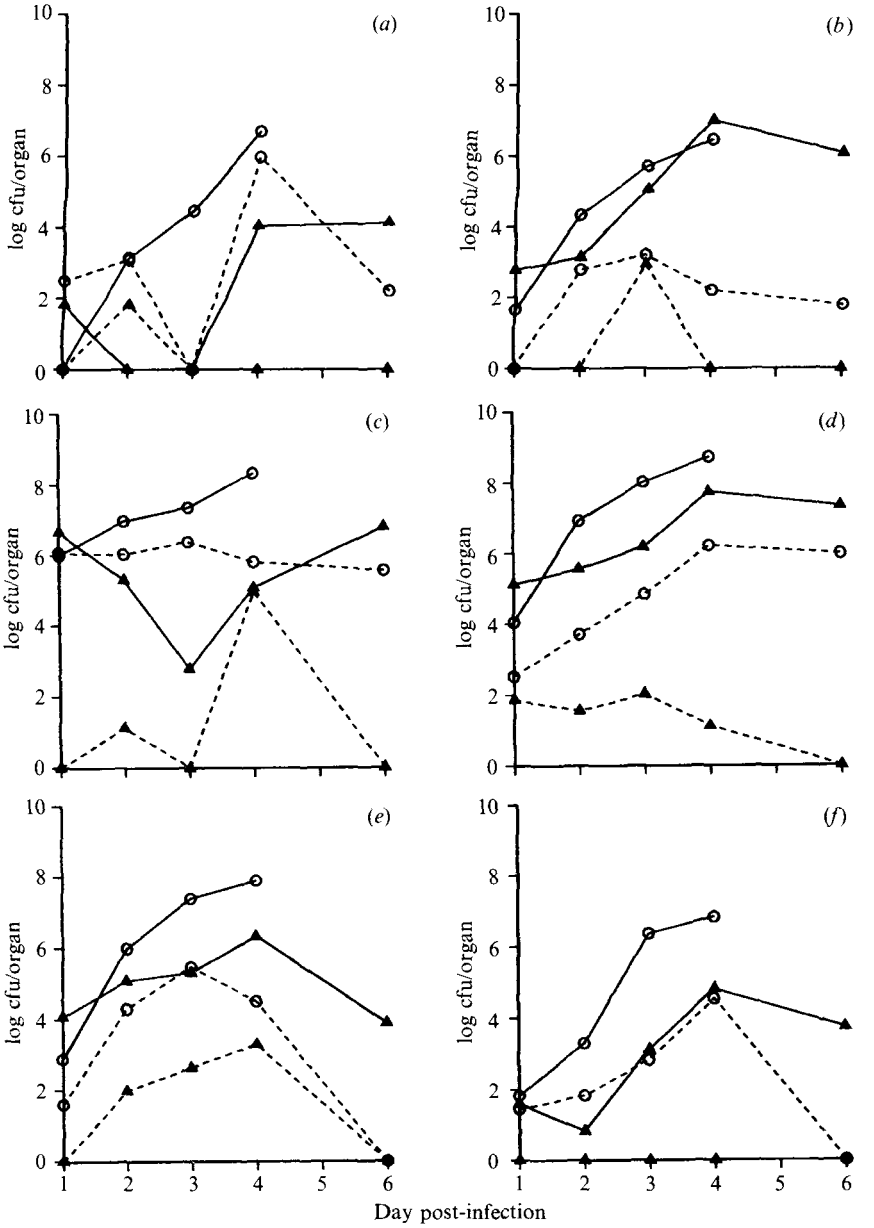

Fig. 1. Growth of 11994 and L3700 in mouse tissue following AI or GI. (A), Blood; (B), brain; (C), lung; (D), liver; (E), spleen; (F), kidney.

former (Fig. 1D). Growth of L3700 differed depending on the route of infection, showing a marked increase when AI was used compared to a decrease with GI.

Histology. Necrosis of hepatocytes and activation of Kupffer cells were observed in livers of AI mice infected with *L. monocytogenes* 11994. Bacteria-laden macrophages could be seen in the liver lobules at day 4. Livers from GI infected animals developed lesions from day 3 onwards.

No lesions were observed in AI or GI mice infected with L3700.

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Spleen

Microbiology. A similar pattern of growth for 11994 was observed in the spleens of AI and GI infected mice, although the latter group were found in lower numbers (Fig. 1E). This was also true for strain L3700, with numbers of this micro-organism increasing until day 4 then decreasing, until they were undetectable at day 6.

Histology. Lesions in the spleen involved both the red and white pulp. No lesions were observed with strain L3700.

Kidney

Microbiology. The pattern of growth in the kidney followed the general pattern observed for all organs in that there were higher numbers of strain 11994 than L3700, and AI facilitated greater infectivity than GI (Fig. 1F). Interestingly, the avirulent strain, L3700, was not detected in the kidneys of GI animals.

Histology. No lesions were found in the kidneys of mice regardless of the infectious agent or route.

DISCUSSION

Infection via the aerosol route is a sensitive, efficient and reproducible method for assessing the virulence of strains of listeria, particularly *L. monocytogenes*. A clear distinction between virulent, less virulent and avirulent strains of listeria was routinely observed using aerosol infection (AI) which was not possible using gastric intubation (GI) as the route of infection. GI was found to be less sensitive, less efficient, and less reproducible.

The sensitivity of AI was emphasized by observations that animals infected via this route received considerably fewer micro-organisms (10^5 c.f.u.) than those infected intragastrically, and yet virulent strains of *L. monocytogenes* consistently caused 100% mortality within 4 days of AI. Mortality varied between 20 and 100% among GI mice, with death occurring up to day 7 and in one instance on day 9. The variation in numbers of deaths observed with GI meant that it was not possible to obtain LD_{50} values for this route of infection.

The length of time needed to estimate virulence was less using AI and the dissemination of the micro-organisms throughout the animal was more efficient than that observed in GI. A similar retained dose of listeria evoked the same response, in terms both of numbers of animal deaths and bacterial counts in the liver and spleen, indicating that the AI method was reproducible. In contrast, the observed response to GI challenge varied in each experiment, even when the doses used were similar. Moreover, similar to the findings of Kautter and colleagues [12], a linear dose response was observed for AI (data not shown). The influence of variations in individual host susceptibility on the response to challenge is unclear; however, our findings indicate that this does not affect the response to AI.

The two haemolysin negative strains tested, namely, 11994_{HLY^-}, a transposon mutant produced in this laboratory, and 10357, proved to be completely avirulent by either AI or GI. Haemolysin is widely recognized as being an essential

factor in the pathogenesis of *L. monocytogenes*, and therefore the lack of virulence of these two strains is not unexpected [13, 14].

It is quite possible that death is the wrong criteria for assessing virulence of listeria strains, since the relatively high numbers of animals required renders LD_{50} determination impractical in some instances. However we have observed that numbers of micro-organisms in the liver or spleen following AI reflect the virulence of the strain tested, this might be better than mortality as an indicator of virulence (see Table 3).

Two strains were chosen to examine in greater depth the pathogenesis profile of *L. monocytogenes*: a virulent strain, 11994, currently being used in our study on the influence of growth environment on the expression of virulence determinants, and L3700, an environmental rough mutant, which shows considerably reduced virulence. Infection using fine particle aerosols such as those generated by the Collison spray enables micro-organisms to penetrate deep into the lungs of the infected animal; the rich blood supply to the lungs then accelerates the dissemination of bacteria throughout the body. Our observations reflect this phenomenon. Both 11994 and L3700 could be detected in the tissues of AI mice earlier, and in higher numbers, than in those of GI animals, despite receiving approximately 10^5 fewer micro-organisms (Fig. 1). The virulent strain, 11994, was detected in 90% (65/72) of tissues tested from AI mice, however it was only present in 65% (62/96) of those from GI mice. L3700 was detected in 70% (63/90) of organs from AI animals, but from only 21% (18/84) of those infected by GI.

In some instances, L3700 was isolated from the blood in similar numbers to those observed for 11994. However, it was always present in lower numbers in all of the organs tested, irrespective of the route of infection and failed to produce lesions in any tissue. This was thought to be due to a reduction in the invasive properties of the micro-organism; however, preliminary results from attachment and intra-cellular growth studies in 3T6 mouse embryonic fibroblasts appear to show no difference in the invasive properties of L3700 and 11994, the virulent strain. This work is currently in progress.

It has been previously reported that *L. monocytogenes* multiplies mainly in the spleen, bacterial counts per spleen being higher than those per liver [15]. However only one of the strains we examined exhibited organ tropism of this nature (11994_{HLY^-}), and in general bacterial counts in the liver were higher than those in the spleen (Fig. 1, D and E).

There is no evidence from our observations that AI caused meningitis or the encephalitic form of listeriosis, as no lesions were found in the brain. It is most likely that mice infected via the aerosol route died of an overwhelming septicaemic infection. The 'twisting motion', thought to be indicative of central nervous system involvement, was only observed in mice infected with 11994 by GI; however, histological examination surprisingly revealed no evidence of lesions in the brains of these animals. In fact we have only detected lesions in the brains of mice infected intranasally (data not shown).

The feeding experiment results indicate that micro-organisms can enter the respiratory tract during feeding, although the numbers involved are small in comparison to the retained dose following AI (Table 4). This may go some way to explaining the histological findings in the lungs at day 1 post GI; however, it is

more probable that this was due to inadvertent direct inoculation of bacteria into the lungs during gastric intubation.

As far as we are aware there have been a few reported incidents of listeriosis associated with aerosol infection in man or animals. Seeliger [16] suggested that the 'inhalation of infected dust' was involved in a case of human listeriosis and more recently Schlech [17] described a case of listeria sepsis following an aspiration pneumonia after eating contaminated coleslaw. The aim of this study, however, was not to develop a model which mimicked naturally occurring listeria infection, but to achieve an accurate *in vivo* indicator of virulence. Nevertheless the results reported here may be of interest to both the food industry and laboratory workers, where there is the potential to create aerosols. Similarly in farming communities, where the spraying of fields with raw sewage, sewage sludge or manure for fertilization is advocated, since listeria has been isolated from these environments [18]. The possible infection of livestock via this route should not be excluded.

Kautter and colleagues [12] studied the importance of routes of infection in relation to virulence in listeria and found that, in contrast to intraperitoneal, intravenous and intracerebral routes, the most consistent results were obtained by the respiratory route. We conclude that aerosol infection is an effective and reproducible method for determining virulence of strains of listeria, and that the numbers of micro-organisms in the organs of the mice, using this route of infection, was a good indicator of infection.

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