Evaluation of a Murine Model for Intranasal and Intraperitoneal Infection by *Haemophilus parasuis*: Short Report

Ana-Judith Martín de la Fuente, César-B. Gutiérrez Martín[*], Sonia Martínez Martínez, Rafael Frandoloso and Elías-F. Rodríguez Ferri

Department of Animal Health, Section of Microbiology and Immunology, Faculty of Veterinary Medicine, University of León, 24007-León, Spain

Abstract: NMRI mice were evaluated as model for experimental infection by *Haemophilus parasuis* by intranasal (IN) and intraperitoneal (IP) routes. No deaths, clinical signs and macroscopic lesions were noticed after receiving several doses (except when the mice were infected intraperitoneally with the highest dose), and the organism was cleared rapidly from spleen and lungs. These results suggest that NMRI mice are not a suitable model to reproduce experimentally Glässer's disease.

Keywords: *Haemophilus parasuis*, Glässer's disease, mouse, experimental model.

INTRODUCTION

Haemophilus parasuis, a non-haemolytic, nicotinamide adenine dinucleotide (NAD)-dependent Gram-negative rod belonging to the family *Pasteurellaceae*, is a commensal of the upper respiratory tract of healthy pigs [1]. However, it is also considered an important porcine pathogen and the etiological agent of Glässer's disease, which is characterized by fibrinous polyserositis, polyarthritis and meningitis. This disease is an emerging challenge in the pig-rearing industry worldwide today, often associated with significant financial losses [2]. In addition, *H. parasuis* is also related to septicemia, penumonia and myositis [3].

Experimental studies in the natural host are difficult because *H. parasuis*-free pigs are required. Caesarean-derived, colostrum-deprived (CDCD) piglets have been used successfully, but this model requires intense management, entailing a high cost. Both naturally farrowed, colostrum-deprived (NFCD) pigs [4, 5] and guinea pigs [6, 7] have been proposed to reproduce the disease, with lower costs and less handling cares. Although the mouse is not a natural host for *H. parasuis*, an alternative experimental model to pigs and guinea pigs for Glässer's disease, easy to be managed, would be of a great interest. In this respect, not many studies about *H. parasuis* infections of mice have been performed, showing contradictory results depending on the medium in which this organism was suspended [6].

The goal of this study was to examine the ability of Nagasaki strain of *H. parasuis* serotype 5 (considered highly virulent in pigs) to cause in mice a disease similar to that found in the natural host by intranasal (IN) and intraperitoneal (IP) routes, and thus to investigate the feasibility of using experimental infection of the mouse as a cost-effective alternative model of Glässer's disease for further virulence and protection studies.

MATERIALS & METHODOLOGY

The Nagasaki reference strain of serotype 5 (one of the most prevalent serotypes worldwide [1]) of *H. parasuis* was grown on chocolate-blood agar plates (bioMérieux, Barcelona, Spain), and incubated at 37ºC for 24-48 h in an atmosphere containing 5% CO_2. Bacterial cells were harvested in sterile saline, centrifuged, and resuspended in RPMI 1640 medium (Sigma-Aldrich, Madrid, Spain). In order to standardize the number of cells inoculated per animal, a viable count (colony-forming units -CFU-) of the suspension was determined by plating dilutions onto chocolate-blood agar and incubating overnight at 37ºC. The cells were kept at 4ºC up to the time of inoculation. A new viable count was carried out after it in order to check the inoculum viability and to discard contaminations. Similar counts were recorded before and after inoculation and other bacteria than *H. parasuis* were not isolated.

A total of 80 female NMRI mice (weighing 15 to 20 g) were housed in a controlled animal facility (22 ± 2ºC, 12-h light/12-h dark cycle), allowed food and water *ad libitum*, and acclimated no less than 7 days before starting of the experiment. All animal handling and procedures were approved by the University of León Ethical Committee and conducted under a licence, in accordance with the conditions approved by the Spanish Government. Mice were randomly assigned into 16 groups of five animals. Seven groups were inoculated in the nasal cavity and seven others were injected intraperitoneally. Ten-fold doses (from 2×10^3 to 2×10^9 CFU per mouse) were given in each inoculation route, one to each of the groups separated. Two other groups (one for each route), which remained as infection controls, were given RPMI alone instead of *H. parasuis* cells. Mice were anesthetized with isoflurane (Forano[®], Abbott Laboratories, Madrid, Spain) before inoculation. For intranasal (IN) infection, a volume of 50 µl of bacterial suspension was dropped onto the external nares, and for intraperitoneal (IP) injection, 200 µl were aseptically injected in the abdominal cavity. At five days post-inoculation, all surviving mice were sacrified under terminal anaesthesia.

*Address correspondence to this author at the Department of Animal Health, Section of Microbiology and Immunology, Faculty of Veterinary Medicine, University of León, 24007-León, Spain; Tel: 34 987 291 203; Fax: 34 987 291 304; E-mail: dsacgm@unileon.es

Clinical signs were recorded every hour for the first day and then daily until death or euthanasia. The number of dead mice was recorded and the LD_{50} was calculated by the method of Reed and Muench [8]. All surviving animals were necropsied and macroscopic lesions recorded. Samples were collected aseptically from the spleen, lungs, abdominal and thoracic cavities for bacteriological examination. Individual organs were ground in sterile homogenisers with 5 ml of RPMI, centrifuged and 100 µl of the supernatant were plated onto chocolate-blood agar. The suspected colonies were studied for NAD requirement by observing the satellite phenomenon on blood agar cross-streaked with a nurse strain of *Staphylococcus intermedius*.

For the bacterial elimination assays, 21 female NMRI mice were inoculated by the IN route with 2×10^9 CFU of *H. parasuis* suspended in RPMI. Three mice were sacrificed at 0, 4, 6, 8, 10, 12 and 24 hours after the exposure, and their lungs and spleen were removed for bacterial reisolation and identification as described previously. In addition, 100 µl of each supernatant were inoculated onto chocolate-blood agar plates in duplicate to determine the number of CFU.

RESULTS AND DISCUSSION

All mice inoculated by the IN route survived after the infection, and only those receiving the highest dose (2×10^9 CFU) showed tremor or ruffled coat within 24 h after bacterial exposure (Table **1**). None of the mice in the groups treated with lower doses and in the control group showed clinical signs. No remarkable macroscopic lesions were found at the necropsy in any group of mice, and *H. parasuis* could not be recovered from any of the 40 animals infected by this route when they were necropsied five days after infection. It was not possible to calculate the LD_{50} for the IN route because of the absence of mortality in any of the seven doses inoculated.

The IN route, through which *H. parasuis* preferentially infects the natural host [1], has hardly been assessed in mice. From our results, it can be concluded that NMRI mice were not susceptible to Nagasaki strain when they were inoculated intranasally, even using doses as high as 2×10^9 CFU. However, similar or lower doses have been proved to develop severe Glässer's disease (and even death rapidly after exposure) in CDCD [9] and NFCD [4] pigs by this route, as well as in conventional guinea pigs [7] and SPF pigs [10] inoculated intratracheally.

Concerning the results of the bacterial elimination assays, *H. parasuis* could be isolated in high numbers (more than 300 colonies) from the lungs of each of the three mice ne-

cropsied at 4, 6, 8 and 10 h post-inoculation, but the count decreased considerably (36.0 ± 12.7 CFU) 12 h after exposure and the organism was only recovered from two of the three mice in this group. *H. parasuis* was completely eliminated from the lungs after 12 h following the IN inoculation of 2×10^9 CFU of Nagasaki strain. On the other hand, *H. parasuis* colonized the spleen between 8 and 10 h post-infection, reaching substantially lower counts (28.5 ± 9.2 and 37.0 ± 4.2, respectively) than in lungs at the same time. The organism was cleared from the spleen after 10 h.

These results indicate that, although *H. parasuis* is capable to gain access to murine lungs and spleen after IN exposure, it is rapidly eliminated from both organs and, therefore, is unable to induce disease. One possible explanation might be the activation of the innate immunity, presumably through phagocytosis. This mechanism would have been able to effectively destroy *H. parasuis* before 24 h following inoculation. However, the use of mice strain with specific mutations of the innate immune system, such as the toll-like receptors, might have provided different results. It would be also speculated that the rapid bacterial clearance might be related to the inability of this organism of acquiring iron from murine transferrin, because this high-affinity iron uptake system seems to be rather specific for the natural host [11].

All mice infected intraperitoneally with 2×10^9 CFU of *H. parasuis* died 6 to 24 h following inoculation, with dullness, tremor, closed eyes, ruffled coat, and a severe congestion in the abdominal cavity (Table **1**). In contrast, the mice inoculated with doses lower than 2×10^9 CFU or with RPMI alone survived for all the exposure time, without clinical signs and gross lesions. *H. parasuis* was only recovered from the abdominal cavities of the five mice receiving 2×10^9 CFU. Other bacteria than *H. parasuis* were not isolated from these animals. For the IP route, the LD_{50} of Nagasaki strain was of 6.32×10^8 CFU.

Quite different to our results, other authors [6] observed no deaths among the mice inoculated intraperitoneally with the same infecting dose of strain no. 4 (belonging to serotype 1) suspended in physiological saline, with only unspecific clinical signs, no gross lesions and *H. parasuis* recovery from only one mouse; however, 58.3% of mice died when the same dose was suspended in a broth containing NAD instead of saline, and the recovery rate increased to 50%.

CONCLUSION

In spite of being pathogenic for mice by IP inoculation with 2×10^9 CFU of *H. parasuis* in our study and being recovered from all animals inoculated with this dose, the ab-

Table 1.   Results of Experimental Infection of Mice with Different Doses of *H. parasuis*, Strain Nagasaki (Serotype 5) by Intranasal (IN) or Intraperitoneal (IP) Inoculations

Dose (CFU)	Route	No. of Mice	No. of Dead Mice	Nº. of Mice with Clinical Signs	Nº of Mice with Lesions	Nº of Mice from which *H. parasuis* was Recovered
2×10^3, 2×10^4, 2×10^5, 2×10^6, 2×10^7 and 2×10^8	IN	5	0	0	0	0
	IP	5	0	0	0	0
2×10^9	IN	5	0	5	0	0
	IP	5	5	5	5	5

sence of clinical signs and macroscopic lesions resembling those produced in natural cases of disease strongly suggest that modeling Glässer's disease caused by Nagasaki strain of *H. parasuis* in NMRI mice is not a viable alternative to use of pigs and guinea pigs.

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