Development of luminescent *M. avium* subsp. *paratuberculosis* for rapid screening of vaccine candidates in mice

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ABSTRACT

*M. avium* subsp. *paratuberculosis* (MAP) is a slowly growing mycobacterial species requiring 6-8 weeks of culture before colonies can be counted visually. This seriously hampers diagnosis and experimental work with MAP. Snewin *et al.* (1999) previously reported on a luminescent *M. tuberculosis* H37Rv isolate expressing the *luxAB* genes of *Vibrio harveyi*. With this luminescent isolate, fastidious and expensive enumeration of CFU by plating on agar can be replaced by easy and inexpensive luminometry. Here, we report on the construction of two luminescent isolates of MAP, i.e. the reference ATCC 19698 strain and clinical isolate S-23 of bovine origin. BALB.B10 mice were infected intravenously with $10^6$ CFU of luminescent MAP and monitored for bacterial replication in the spleen by determining the number of Relative Light Units (RLU) at 5, 10 and 15 weeks post infection. Results show that luminescent MAP may be a valuable tool for the rapid screening of experimental paratuberculosis vaccines *in vivo*.

*Key words*: luminescence, mouse, plasmid, BALB.B, *Vibrio harveyi*.

INTRODUCTION

The current vaccines against *Mycobacterium avium* subsp. *paratuberculosis* (MAP) are based on killed or live attenuated whole bacterial preparations. Although these vaccines are partially effective through reducing fecal shedding and the number of clinically affected animals in a herd, they do not protect against infection. An efficient subunit vaccine that would not interfere with bovine tuberculosis diagnosis would be very valuable in the management of paratuberculosis, but requires the characterization of immunodominant and protective antigens. A number of immunogenic MAP proteins have been described (4), but they have mostly been analyzed for diagnostic purposes (Bannantine *et al.*, 2002) and little is known on their vaccine potential. Experimental MAP infection and vaccination in mice is seriously hampered by technical problems. Indeed, MAP is a slow growing mycobacterial species, requiring 6-8 weeks of culture before colonies can be counted visually. Determining the number of colony forming units (CFU) in organ homogenates has so far consisted of labor-intensive plating on expensive mycobactin-supplemented Middlebrook agar. Snewin *et al.* previously reported on the construction of a luminescent *M. tuberculosis* H37Rv isolate that expressed the *luxAB* genes of *Vibrio harveyi* introduced by transformation with the shuttle plasmid pSMT1 (Snewin *et al.*, 1999). With this luminescent isolate, fastidious enumeration of CFU can be replaced by easy and inexpensive luminometry. We have used this luminescent *M. tuberculosis* isolate successfully for the screening of plasmid based DNA vaccines against tuberculosis and have shown that reductions in $\log_{10}$ mRLU values correlate exactly with reductions in $\log_{10}$ CFU counts (D’Souza *et al.*, 2002; Tanghe *et al.*, 2001).

The same pSMT1 plasmid was used to transform two isolates of MAP i.e. the reference ATCC 19698 strain and a clinical bovine isolate S-23 of origin. These two isolates were compared for their replication in the spleen of BALB.B10 mice upon intravenous infection.
MATERIALS AND METHODS

Electroporation of M. avium ssp paratuberculosis
MAP strains ATCC 19698 (Merkal, 1979) and S-23 (Foley-Thomas et al., 1995) grown on Middlebrook 7H9 medium supplemented with OADC and mycobactin J were transformed with plasmid pSMT1 (10) (kindly given by D. Young, Imperial College London) using a previously described procedure (3). pSMT1 DNA was prepared from E. coli strains either by an alkaline lysis miniprep method (9) or with the Wizard® Miniprep Kit (Promega, Madison, WI, USA). Transformants were grown at 37°C for 5 weeks on Middlebrook 7H9 agar supplemented OADC, mycobactin J and 50 µg/ml hygromycin. This is the first report on the use of this drug marker to select MAP transformants.

Infection of mice
Female BALB.B10 mice were obtained from the animal facilities of the Pasteur Institute. They were offspring from breeding couples originally received from the Netherlands Cancer Institute. Mice were infected intravenously in a lateral tail vein with re 360 Relative Light Units RLU of ATCC 19698 strain or 415 RLU of S-23 strain grown in Middlebrook 7H9 medium supplemented with OADC, mycobactin J and hygromycin, to an optical density of 0.6.
The number of bioluminescent bacteria in spleen homogenates was determined using a bioluminescence assay with a Turner Design 20/20 luminometer and 1% n-decyl-aldehyde (Sigma) in ethanol as substrate. For statistical analysis (Student’s t test), mRLU values were converted to log_{10} values per organ per mouse. A comparison of mRLU and CFU values of an axenic MAP culture indicated that one milliRLU unit corresponded to 2.5 CFU units (unpublished data).

RESULTS

Bacterial replication of luminenscent MAP ATCC 19698 and S-23 strain following intravenous infection of BALB.B10 mice
As shown in Fig. 1, a sustained bacterial presence of the two luminescent MAP strains could be detected by luminometry in the spleen from infected mice. Mean log_{10} mRLU number of clinical MAP isolate S-23 increased in the mouse spleen between week 5 and 10 after infection, and subsequently mRLU values stabilized at week 15. The luminescent ATCC 19698 strain was slightly less virulent and mRLU values slowly declined between 5 and 15 weeks of infection.

DISCUSSION

MAP causes Johne’s disease, a severe gastroenteritis in ruminants, with a significant impact on the agricultural economy, particularly the dairy industry. Vaccines consisting of whole killed or attenuated live MAP bacilli can provide partial protection by delaying fecal shedding and reducing the number of clinically
affected animals, but they do not protect against infection. Animals immunized with these paratuberculosis vaccines develop positive reactions in the tuberculin skin test (the reference bovine tuberculosis detection method) and therefore paratuberculosis vaccination is subject to approval by the veterinary services. It is clear that the development of an efficient paratuberculosis sub-unit vaccine that would not interfere with bovine tuberculosis detection would offer a solution. However, few protective antigens against paratuberculosis have been characterized in detail.

Experimental MAP infection and vaccination studies are seriously hampered by technical problems. As a slow growing mycobacterial species, determining the number of MAP colony forming units (CFU) in organ homogenates is labor-intensive plating and expensive. The use of luminescent MAP isolates as reported here will be of great benefit for future experimental MAP studies. Besides enabling an easy *in vivo* screening of vaccine candidates, these luminescent bacteria could also be used for *in vitro* drug testing (Williams et al., 1999) and *in vitro* monitoring of bacterial replication in cell lines such as bovine epithelial cells.

The clinical S23 strain (isolated in 1995) was somewhat more virulent than the ATCC 19698 strain (dating back to 1979 and subjected to extensive *in vitro* passages); it replicated slightly in the spleen of BALB.B10 mice between week 5 and 10 after infection. The BALB.B10 mouse is a MHC congenic mouse with H-2b haplotype from the C57BL/10 strain on a BALB/c background. This mouse strain expresses the *bcg* allele (encoded by *nramp*, known to confer susceptibility to intravenous infection with BCG and *M. avium*). Using the luminescent MAP isolate, we demonstrated that the BALB.B10 mouse strain also expresses the susceptible allele with respect to infection with *M. paratuberculosis*. Analysis of various mouse strains expressing either the resistant or the susceptible allele of the *bcg* gene confirmed this finding (Rosseels, unpublished data).

**CONCLUSION**

These results show that luminescent MAP transformed with pSMT1 plasmid encoding the *luxAB* genes of *Vibrio harveyi* may be a valuable tool for the rapid screening of paratuberculosis vaccines in a murine experimental infection model.

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