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Short communication

Use of enoyl coenzyme A hydratase of *Mycobacterium avium* subsp. *paratuberculosis* for the serological diagnosis of Johne's disease

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ABSTRACT

Johne's disease (JD), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), remains difficult to control because of the lack of specific and sensitive diagnostic tests. In order to improve the specificity of sero-diagnosis for JD, the phage display library derived from genomic DNA of MAP was immunoscreened to identify novel antigenic targets. We selected a clone using antibodies from MAP experimentally infected cattle, and annotated its coding sequence as MAP1197 in the MAP genome, which encoded "echA12_2" in the MAP protein (Map-echA) belonging to Enoyl-CoA hydratase, known as a crotonase enzyme. The Map-echA was expressed in *Esherichia coli* and purified as a histidine-tag recombinant protein (rMap-echA), and the diagnostic potential of the protein was further evaluated by enzyme-linked immunosorbent assays (ELISA). Antibody responses to rMap-echA were higher in MAP-infected cattle than in uninfected cattle. The specificity of the Map-echA ELISA was also confirmed by evaluation with hyper-immune sera against various kinds of *Mycobacterium* species. Furthermore, in all experimentally infected cattle the antibody against rMap-echA was detected 2–7 months earlier than by a commercially available ELISA kit. These results suggested that Map-echA can be used as a specific and sensitive serological diagnostic antigen for the detection of MAP infection.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), characterized by a chronic granulomatous inflammatory bowel disease in ruminants. The disease is widespread in almost all countries, including Japan (Kobayashi et al., 2007). Control of JD has proven to be difficult due to the nature of MAP infection and the lack of sensitive and specific diagnostic tests to detect subclinically infected cattle. Early diagnosis is therefore important for identifying and removing potential faecal shedders for the control of JD (McDonald et al., 1999; Antognoli et al., 2007). The current diagnostic tests are based on the cell-mediated and humoral immune response or by the detection of MAP in faeces by bacterial culture or real-time PCR. The faecal culture test has been recognized as the gold standard for diagnosis, but it requires several months to obtain results. A real-time PCR assay targeting a MAP-specific gene such as IS900 is expensive and not applicable during the prepatent period of MAP infection (Cousins et al., 1999). Although current serological tests are useful for detecting cattle in the late phase of JD, the application of these tests to diagnose cattle in early phases of infection or in subclinical stages has proven to be of limited value because of the relatively late antibody rise during the course of the disease (Kreeger, 1991). The humoral immune responses are not usually induced until after the onset of shedding

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Table 1
Mycobacterium species and related bacteria tested for Map-echA ELISA in calves.

Species	Strains
Corynebacterium bovis	ATCC7715
M. bovis	BCG Tokyo
M. scrofulaceum serovar 41	Bridge
Mycobacterium sp.	2333
M. intracellulare serovar 7	Manten 157
'*M. avium* subsp. hominissuis' serovar 8	KMM-8
M. avium subsp. paratuberculosis	ATCC 19698

(Chiodini, 1996; Whitlock and Buergelt, 1996). Another limitation of serological tests is the potential reduction in specificity resulting from the cross-reaction with environmental mycobacterium species. The specificity of the most frequently used enzyme-linked immunosorbent assays (ELISA) is improved by removing cross-reacting antibodies by absorbing sera with *Mycobacterium phlei* (*M. phlei*) (Yokomizo, 1983; Ridge et al., 1991). However, in some cases this absorption with *M. phlei* may be insufficient for removing all cross-reacting antibodies induced by mycobacterium species other than MAP.

In this study, the phage displayed DNA library derived from MAP strain ATCC19698, which had been constructed using the Zap Express cloning vector (Nagata et al., 2005), was screened with sera from calves infected with MAP in order to detect the MAP antigens that elicit humoral immunity. The clone that strongly reacted with sera from infected calves was identified, and the recombinant protein was expressed in *Esherichia coli* (*E. coli*) and used for serological assays.

2. Materials and methods

2.1. Experimental animals and serum samples

The sensitivity and specificity of ELISA using the recombinant protein was evaluated with serum samples collected from 30 MAP-infected cattle confirmed by positive faecal culture, and 30 uninfected cattle that were free from MAP infection as confirmed by negative results in a commercial JD ELISA, faecal PCR, faecal culture and IFN-γ assay.

To test the cross-reactivity of the recombinant protein ELISA, serum samples were collected from 14 male Holstein calves that were divided into 7 groups, and 2 calves of each group were immunized intradermally with seven strains (Table 1) of heat-killed mycobacterium species (Nagata et al., 2010). Sera taken once every 14 days from these animals were tested with both a commercial ELISA kit (Institut Pourquier, Montpellier, France) as a reference serological assay and the recombinant protein ELISA.

For experimental infection, eight male Holstein calves were purchased from a farm with no history of JD. The calves #22, #25, #26, #27 and #28 were orally inoculated with 1.5 g wet weight of MAP isolate cells at the age of 4 days. The calves #29 and #30 were inoculated with 5.2×10^{10} CFU of intestinal tissue emulsion of diseased cattle at the age of 4 days, on the other, calf #42 was challenged with 2.1×10^{8} CFU of intestinal tissue emulsion at 1 month of age. MAP and serum samples were detected from all cattle by the faecal culture test done every 14 days. In the calves #22, #25, #26, #27, #28 and #42, MAP might be passed through their faeces shortly after inoculation. Then an incubation stage of 5–10 months (24 months in the case of #42) was observed, followed by MAP re-shedding. In contrast, MAP shedding from calves #29 and #30 began 2 weeks after the inoculation and continued throughout the observation period without a latent stage.

All experimental infections were induced in compliance with the guidelines of the Animal Care Committee of the National Institute of Animal Health.

2.2. Screening of the phage display library with sera from infected calves

The MAP DNA expression library was constructed essentially as previously described (Nagata et al., 2005). The sera used for screening of the phage display library were absorbed with plaques from non-recombinant phages, *E. coli* and *M. phlei* to remove cross-reacting antibodies. For the primary immunoscreening the library was plated out at 25,000 pfu per 150 mm plate using XL1 blue MRF' host cells. Then nitrocellulose filters incubated with 20 mM isopropylβ-D-thiogalacto-pyranoside (IPTG) for 10 min were overlaid onto the plates and then incubated for 3 h at 37 °C to induce prokaryotic expression. After incubation, their orientation was blocked for 1 h at RT in 2% gelatin/TBS. The pre-absorbed sera were then added to the filters and incubated for 1 h at RT. Membranes were washed three times with 0.01% TBS Tween-20 (TBST) for 5 min. Bound antibody was detected using a 1:5000 dilution of sheep anti-bovine IgG1 conjugated with horseradish peroxidase (HRP) (AbD Serotec, Kidlington, UK) in 0.2% gelatin/TBS overnight at 16 °C and TMB membrane peroxidase substrate (KPL, MD, USA). After about 4×10^{5} plaques were screened, we finally cloned one recombinant phage-expressing MAP antigen that reacted strongly with serum antibodies from infected calves. The MAP DNA insert cloned into the phage vector was excised out of the phage and inserted into the form of the phagemid vector with *E. coli* strain XLOLR, and a part of the 5′ end of the insert DNA was sequenced using the T3 universal primer.

2.3. Sequence analysis, expression and purification of the recombinant protein

Because the inserted DNA of the clone that reacted strongly with sera from infected calves was identified as part of the encoding "echA12_2" of the MAP gene, an enoyl-CoA hydratase protein (echA) (Accession No. 41407295), the genomic DNA of MAP ATCC19698 was used as a template in PCR for the amplification of the "echA12_2" with primers containing *Bam*HI and *Hind*III restriction endonuclease sites, forward 5′CTA GGG ATC CGT GAG TTT GGT ACT3′ and reverse 5′GAT CAA GCT TCT ATT TGT CGT CGG T3′. DNA amplification was carried out in a 50 μl reaction mixture containing 5 ng of MAP DNA, 400 μM of each dNTP, 0.3 μM of each primer, 1× PCR buffer for KOD FX and 1 unit of KOD FX (Taq polymerase, Toyobo, Co., Ltd., Osaka, Japan). The PCR product was cut with *Bam*HI and *Hind*III, and inserted into *pQE* 80 L (Qiagen, Hilden,