Detection of Humoral Response Using a Recombinant Heat Shock Protein 70, DnaK, of Mycoplasma haemofelis in Experimentally and Naturally Hemoplasma-Infected Cats

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Received 5 August 2010/Returned for modification 2 September 2010/Accepted 27 September 2010

Hemoplasmas is the trivial name given to a group of erythrocyte-parasitizing bacteria of the genus Mycoplasma. Of the feline hemoplasmas, Mycoplasma haemofelis is the most pathogenic, while “Candidatus Mycoplasma haemominutum” and “Candidatus Mycoplasma turicensis” are less pathogenic. Shotgun libraries of fragmented M. haemofelis genomic DNA were constructed, and random colonies were selected for DNA sequencing. In silico-translated amino acid sequences of putative open reading frames were compared to mass spectrometry data from M. haemofelis protein spots identified as being immunogenic by two-dimensional gel electrophoresis and Western blotting. Three of the spots matched the predicted sequences of a heat shock protein 70 (DnaK) homolog, elongation factor Ts, and a fragment of phosphoglycerate kinase found during library screening. A full-length copy of the M. haemofelis dnaK gene was cloned into Escherichia coli and recombinantly expressed. Recombinant M. haemofelis DnaK was purified and then used in Western blotting and an enzyme-linked immunosorbent assay (ELISA) to investigate the humoral immune response during acute infection in cats experimentally infected with M. haemofelis, “Ca. Mycoplasma haemominutum,” or “Ca. Mycoplasma turicensis”. The recombinant M. haemofelis DnaK ELISA also was used to screen clinical samples submitted for hemoplasma PCR testing to a commercial laboratory (n = 254). Experimentally infected cats became seropositive following infection, with a greater and earlier antibody response seen in cats inoculated with M. haemofelis than those seen in cats inoculated with “Ca. Mycoplasma haemominutum” or “Ca. Mycoplasma turicensis,” by both Western blotting and ELISA. Of the clinical samples, 31.1% had antibodies detected by the ELISA but only 9.8% were positive by PCR for one or more hemoplasmas.

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† Published ahead of print on 6 October 2010.
‡ The authors have paid a fee to allow immediate free access to this article.
acid fragment of *M. haemofelis* heat shock protein 70 (DnaK) was recombinantly expressed and used in preliminary Western blot analyses to detect the presence of anti-DnaK antibodies in three cats experimentally infected with *M. haemofelis*, “Ca. Mycoplasma haemominutum,” or “Ca. Mycoplasma turicensis” (5). The further use of this assay in cats at different time points of hemoplasma infection or on samples obtained from clinical cases has not been reported.

In the current study, we describe the detection and identification of immunogenic proteins of a feline hemoplasma, with the characterization of the immune response to one of these proteins. *M. haemofelis* genomic DNA (gDNA) shotgun libraries were constructed and random clones analyzed to generate partial genome sequence coverage. Three in silico–translated amino acids predicted from these libraries were found to match mass spectrometry data pertaining to three immunogenic *M. haemofelis* protein spots identified using two-dimensional electrophoresis and Western blotting. The genes encoding these proteins were cloned and expressed in *Escherichia coli*. Purified recombinant *M. haemofelis* DnaK subsequently was used in one-dimensional Western blot analyses and ELISAs for the detection of reactive anti-DnaK antibodies in experimentally infected cats during acute infection and in clinical samples submitted for hemoplasma quantitative PCR (qPCR) to a commercial laboratory.

**MATERIALS AND METHODS**

**Feline plasma samples.** Remaining plasma from samples collected from 16 specific-pathogen-free (SPF)-derived cats in a previous feline hemoplasma study were used in this study (14, 15). Ten cats had been infected experimentally with *M. haemofelis*, three with “Ca. Mycoplasma haemominutum,” and three with “Ca. Mycoplasma turicensis” (*M. haemofelis*-infected cats were designated HF1, HF2, HF3, HF4, HF6, HF7, HF8, HF9, HF10, and HF12; “Ca. Mycoplasma haemominutum”-infected cats were HM1, HM2, and HM4; “Ca. Mycoplasma turicensis”-infected cats were TU1, TU2, and TU4). The plasma samples had been derived from 1-ml samples of EDTA-anticoagulated whole blood by centrifugation at 2,200 × g for 3 min and had been stored at −20°C until use. Plasma samples were available for both pre- and postinfection time points; for all cats, plasma was available from 8, 15, 22, 29, 36, 43, 50, 57, 64, and 71 days postinfection (dpi) and, in addition, additional plasma was available for cats HF4 and HF8 from 130, 153, and 177 dpi.

EDTA-anticoagulated blood, available from samples submitted to the Diagnostic Laboratories, Langford Veterinary Services, University of Bristol, for feline hemoplasma qPCR testing between November 2009 and May 2010 were collected. Samples were centrifuged (2,200 × g, 3 min) and the plasma removed. The samples that generated at least 100 μl of plasma were stored at −20°C until use. Previously determined hemoplasma qPCR results were available for all blood samples. Hematology results were available for the experimental blood samples.

**Sources of *M. haemofelis* DNA and protein and feline red blood cell membrane ghosts.** Preparations of *M. haemofelis* had been previously purified from blood taken from cat HF14 at a high titer (14 dpi) (7). Briefly, *M. haemofelis* organisms were dislodged from the surface of phosphate-buffered saline (PBS; 137 mM NaCl, 1.47 mM KH2PO4, 10 mM Na2HPO4, 2.7 mM KCl, pH 7.0)–washed erythrocytes using 3% (wt/vol) EDTA and 0.15% (vol/vol) Tween 20 in PBS. The erythrocytes and debris were separated from the *M. haemofelis* organisms in suspension by low-speed centrifugation (600 × g, 30 min). The supernatant then was subjected to high-speed centrifugation to pellet the suspended hemoplasmas (40,000 × g, 30 min, 4°C). The resultant supernatant was determined, by qPCR (8), to be rich in *M. haemofelis* DNA, which was assumed to be the result of *M. haemofelis* cell lysis releasing free gDNA. This gDNA was purified and concentrated using the Macherey-Nagel Nucleopop mini-cell and blot module and NuPAGE transfer buffer per the manufacturer’s instructions.

**Cloning and expression of recombinant *M. haemofelis* DnaK.** The full-length *M. haemofelis* dnak gene sequence was cloned into pET101/D-TOPO and expressed in BL21 Star (DE3) *E. coli* as a fusion protein with a C-terminal 6-His tag using the Champion pET directional TOPO kit (Invitrogen). His-tagged recombinant *M. haemofelis* DnaK was purified under denaturing conditions using the Ni-NTA spin kit (Qiagen, Crawley, United Kingdom). Protein levels were quantified using the Quant-iT protein assay kit (Invitrogen). Protein levels were quantified using the Quant-iT protein assay kit (Invitrogen). Purified recombinant *M. haemofelis* DnaK was subjected to one-dimensional SDS-PAGE (NuPAGE Novex Bis-Tris gel system; Invitrogen) and stained with Coomassie blue R-250, and its mass was determined by comparison to a molecular size ladder (All Blue Precision Plus Protein Standards). The band at approximately 70 kDa, along with two minor bands at approximately 52 and 12 kDa, were excised from the gel and subjected to trypsin digestion and mass spectrometry (4700 MALDI-TOF/mass spectrometer).

**Investigation of the humoral immune response to recombinant *M. haemofelis* DnaK in experimentally infected cats using one-dimensional Western blotting.** The one-dimensional PAGE of recombinant *M. haemofelis* DnaK (35 ng/well) under denaturing conditions were analyzed by Western blotting using NuPAGE Novex 4 to 12% Bis-Tris precast gels, NuPAGE morpholinepropanesulfonic acid (MOPS) running buffer, and NuPAGE LDS sample buffer (all Invitrogen). Proteins were transferred to 0.45-μm-pore-size nitrocellulose membranes using the XCell mini-cell and blot module and NuPAGE transfer buffer per the manufacturer’s instructions.
instructions (all from Invitrogen). A pretainted molecular size standard (All Blue Precision Plus Protein Standards) was included on each gel to monitor transfer efficiency.

Western blotting was carried out as described previously with the following modifications: membranes were blocked for 2 h, followed by being probed with preinfection plasma from 10 cats (HF1, HF2, HF3, HF4, HF5, HF7, HF9, HF10, and HF12) and postinfection plasma from all 16 cats from 29 dpi at a 1:250 dilution.

Development of a recombinant M. haemofelis DnaK ELISA. Vinyl flat-bottom microtiter plates (Thermo Fisher Scientific) were coated with dilutions of recombinant M. haemofelis DnaK in a volume of 100 µl sodium carbonate buffer (0.05 M, pH 9.6) and incubated overnight at 4°C. Wells were filled with 692, 546, 173, 86.5, 43.3, 21.6, 10.8, or 5.4 ng recombinant M. haemofelis DnaK, with control wells containing no recombinant protein included on each test plate. Plates were washed with PBS containing 0.05% (vol/vol) fat-free milk powder (PBST-10 M; Marvel) for 2 h at room temperature with agitation and then washed with PBST. Wells of each recombinant M. haemofelis DnaK dilution were incubated in duplicate with 100 µl preinfection plasma (HF4) diluted 1:200, 1:400, and 1:800 in PBST-10 M for 2 h at room temperature with agitation and then washed with PBST. Wells then were incubated with 100 µl alkaline phosphatase-conjugated goat anti-cat IgG (H+L) at a 1:10,000 or 1:20,000 dilution in PBST-10 M for 2 h at room temperature with agitation and then washed with PBST. Wells then were washed with PBST containing 10% (wt/vol) fat-free milk powder (PBST-10 M; Marvel) for 2 h at room temperature with agitation and then washed with PBST. Wells of each recombinant M. haemofelis DnaK dilution were incubated in duplicate with 100 µl preinfection plasma (HF4) diluted 1:200 in PBST-10 M and 100 µl postinfection plasma (HF4; 174 dpi) diluted (1:200, 1:400, and 1:800) in PBST-10 M for 2 h at room temperature with agitation and then washed with PBST. Wells then were incubated with 100 µl alkaline phosphatase-conjugated goat anti-cat IgG (H+L) at a 1:10,000 or 1:20,000 dilution in PBST-10 M for 2 h at room temperature with agitation and then washed with PBST. Wells then were incubated with p-nitrophenyl phosphate (pNPP; 100 µl/well; 1 mg/ml in sodium carbonate buffer, 0.05 M, pH 9.6) in the dark. Optical density at 405 and 495 nm (OD405–495) were measured using a computer-assisted microplate reader (LabSystems Multiscan Ex Primary EIA v2.1-0 with Genesis v3.0, VWR International Ltd., Lutterworth, United Kingdom) at 30, 45, 60, 75, and 90 min.

Screening of feline plasma using an ELISA of recombinant M. haemofelis DnaK. To screen the plasma samples, ELISA was performed as described above with plates coated with 14 ng/well recombinant M. haemofelis DnaK, the secondary antibody used at a 1:10,000 dilution in PBST-10 M, and the plates read at 75 to 80 min. Pre- and postinfection plasma (8, 15, 22, 29, 36, 43, 50, 57, 64, and 71 dpi) from all 16 experimentally infected cats were tested, as well as plasma from the feline clinical samples.

Each plate contained wells to which (i) no plasma was added (in triplicate), (ii) preinfection plasma from HF4 (1:200, 1:400 and 1:800 dilutions) was added, and (iii) duplicate 2-fold serial dilutions of a strong hemoplasma-positive (HF6; pooled plasma at 15, 22, and 29 dpi) feline plasma standard (1:200 to 1:400,600) was added. Samples were deemed positive if their average OD405–495 value for the 1:200 dilution was greater than three standard deviations above the mean value of results obtained from the preinfection experimental cat plasma. At 75 min, OD45 values obtained from the samples were plotted against the log of the dilutions, and relative antibody levels (RAL) were calculated by comparison to the standard dilution series if sample results lay within the linear portion of the plot, with the undiluted standard being assigned a RAL of 10,000.

Data were explored by plotting each peak of hemoplasma copy number and nadir of packed cell volume (PCV) against maximum RAL for each M. haemofelis-infected cat, and correlation was assessed using the Spearman's rank correlation test, using Predictive Analytics SoftWare (PASW) Statistics Package, version 17. Significance was taken at P < 0.05.

RESULTS

M. haemofelis shotgun libraries. M. haemofelis gDNA was successfully extracted and shotgun cloned into E. coli. A random screening of the library and DNA sequencing identified a 1,809-bp sequence that encoded the entire gene of putative elongation factor Ts (EF-Ts; HQ267766) and a partial 5’ fragment of putative phosphoglycerate kinase (pgk; HQ267765). Both EF-Ts and pgk genes contained opal codons, unlike the M. haemofelis dnaK gene. The M. haemofelis pgk gene fragment had 78% identity to the pgk gene of Mycoplasma agalactiae (FP671138), while the M. haemofelis EF-Ts gene did not significantly match any mollicute sequences in the database. The translation of the M. haemofelis EF-Ts gene gave a 277-amino-acid protein with a predicted mass of 30.8 kDa and 37% identity (57% similarity) to EF-Ts of Mycoplasma gallisepticum (NP_852870). The translation of the partial M. haemofelis pgk sequence gave a 343-amino-acid peptide fragment with 50% identity (71% similarity) to pgk of Mesoplasma florum (YP_053819).

Immunogenic M. haemofelis proteins identified from 2-dimensional Western blot. A number of spots were present on the 2D M. haemofelis Western blot probed with postinfection plasma (Fig. 1) that were not present on the blot probed with preinfection plasma or on the RBC membrane ghost Western blot probed with postinfection plasma. Twenty-one of these spots corresponded to visible protein spots on the Sypro ruby-stained 2D gel and therefore could be analyzed by tandem mass spectrometry (Fig. 2). Peptide mass fingerprint data and tandem mass spectra for three spots significantly (score, >20)
matched predicted amino acid sequence data; one spot corre-
sponded to the predicted complete *M. haemofelis* DnaK (score
of 77, including spectra of two peptide mass peaks, at 1,744.9
and 1,581.8 Da), another to the complete *M. haemofelis*
EF-Ts (score of 85; one peptide mass peak, at 1,256.6 Da), and the
predicted partial fragment of pgk (score of 97; four peptide
mass peaks, at 2,154.0, 1,562.9, 1,057.6, and 1,012.6 Da).

**Expression of recombinant* M. haemofelis* DnaK.** Recombi-
nant *M. haemofelis* DnaK was successfully expressed in *E. coli*
and purified by Ni-NTA chromatography using the C-terminal
His tag. The recombinant protein was seen as a band at ap-
proximately 70 kDa on PAGE (Fig. 3) and was confirmed by
mass spectrometry to be DnaK (protein score, 962; a score of
/44 was significant). The two smaller bands at 32 and 12 kDa
also were identified by mass spectrometry as C-terminal break-
down fragments of DnaK. The expression of *M. haemofelis*
pgk (partial) and *M. haemofelis* EF-Ts was attempted following the
site-directed mutagenesis of both genes to remove opal codons
(data not shown). However, the expression efficiency was low,
resulting in insufficient protein being available for Western
blotting and ELISAs.

**Humoral immune response to recombinant* M. haemofelis* DnaK by experimentally infected cats on one-dimensional Western blotting.** Recombinant *M. haemofelis* DnaK did not react to Western blotting with preinfection plasma from any of the experimental cats tested. Postinfection plasma (29 dpi)

Western blotting of recombinant *M. haemofelis* DnaK demon-
strated positive bands at approximately 70 kDa (full-length
protein) and occasionally at 32 kDa (breakdown fragment) for
all 10 cats infected with *M. haemofelis* (Fig. 4 is a representa-
tive blot), as well as bands at approximately 70 kDa in all three
“Ca. Mycoplasma turicensis”-infected cats and in two out of
three cats infected with “Ca. Mycoplasma haemominutum”. The
“Ca. Mycoplasma haemominutum”-infected cat with a
negative Western blot at 29 dpi became positive by 36 dpi (data
not shown). Plasma from the *M. haemofelis*-infected cats pro-
duced a stronger signal than the plasma from “Ca. Myco-
plasma haemominutum”- and “Ca. Mycoplasma turicensis”-
infected cats (data not shown).

**Development and use of a recombinant* M. haemofelis* DnaK
ELISA to screen feline plasma.** The recombinant *M. haemofei-
lis* DnaK was successfully applied to the ELISA format. Quan-
tities of antigen of 21.6 and 10.8 ng per well gave the best
signal-to-noise ratio. A secondary antibody dilution of 1:10,000
gave the optimal signal at around 75 min after the application
of pNPP, with wells containing a 1:20,000 secondary antibody
dilution taking considerably longer to develop an acceptable
signal. Therefore, the final protocol included the following:
recombinant *M. haemofelis* DnaK at 14 ng per well (equivalent
to a 1:5,000 dilution of the purified protein stock), secondary
antibody at a 1:10,000 dilution, and the reading of the plates at
75 min.

All experimental cats were negative for anti-*M. haemofelis*
DnaK antibodies in preinfection plasma samples. The mean ± standard deviation OD for all of the preinfection samples was 0.059 ± 0.012. ELISA results were considered positive at ODs of >0.095. At 75 min, ODs obtained from the standard sample plotted against the log of the standard dilutions was linear, where OD = 0.4, equivalent to an approximate RAL of ≥250. All 10 *M. haemofelis*-infected cats showed a quantifiable recombinant *M. haemofelis* DnaK ELISA response at 15 dpi; at 8 dpi only unquantifiable positive (n = 2) or negative (n = 8) results occurred (Table 1). The appearance of a quantifiable antibody response coincided with the mean lowest PCV value (Table 1). However, no correlation was detected between nadir PCV and peak RAL (ρ = −0.250; P = 0.486), and there was no correlation detected between peak hemoplasma copy number and peak RAL (ρ = 0.372; P = 0.290) for cats infected with *M. haemofelis*. The “Ca. Mycoplasma haemominutum”- and “Ca. Mycoplasma turicensis”-infected cats (n = 3 for each) all gave positive recombinant *M. haemofelis* DnaK ELISA results by 36 dpi (Table 1); however, none produced quantifiable results. All but one of the experimental cats remained ELISA positive up to 71 dpi. Quantitative PCR data from the experimental cats used in this study (15) showed that they become positive by 2 dpi with *M. haemofelis* and “Ca. Mycoplasma haemominutum” infection and by 8 dpi for “Ca. Mycoplasma turicensis” infection, indicating a delay of at least 6 days between qPCR positivity and the production of detectable antibodies (Fig. 5 shows representative qPCR and ELISA results).

Of the clinical samples submitted for hemoplasma qPCR, 254 had plasma samples available for analysis by ELISA. Of these 254 samples, qPCR analysis found one to be positive for *M. haemofelis* alone (0.4%), one was dual positive for *M. haemofelis* and “Ca. Mycoplasma haemominutum” (0.4%), and 23 cats were positive for “Ca. Mycoplasma haemominutum” alone (9.1%). None were qPCR positive for “Ca. Mycoplasma turicensis.” The *M. haemofelis*-infected cat was positive (unquantifiable) using the ELISA, while the positive result for the dually *M. haemofelis*- and “Ca. Mycoplasma haemominutum”-infected cat was quantifiable (RAL of 1,398). Of the 23 cats infected with “Ca. Mycoplasma haemominutum” only, 16 (70.0%) were positive; of these, five were quantifiable (RALs of 303, 303, 414, 965, and 1,098). Of the 229 qPCR hemoplasma-negative samples, 61 (26.6%) were positive; of these, four were quantifiable (RALs of 254, 294, 323, and 353).

**TABLE 1. Recombinant M. haemofelis DnaK ELISA results for the experimentally infected cats**

| Cat  | Preinfection score | Score on dpi: | 8  | 15 | 22 | 29 | 36 | 43 | 50 | 57 | 64 | 71 |
|------|--------------------|---------------|----|----|----|----|----|----|----|----|----|----|
| HF1  | N (40.0)           | P (25.0)      | 1,614 (7.0) | 1,455 (19.0) | 410 (15.0) | 574 (23.5) | P (23.5) | P (30.0) | P (33.5) | P (31.0) | P (30.0) |
| HF2  | N (44.0)           | N (34.0)      | 1,179 (17.0) | 2,622 (21.0) | 2,120 (17.5) | 1,885 (27.0) | 664 (31.0) | 516 (47.0) | 454 (35.0) | 411 (35.0) | 345 (36.0) |
| HF3  | N (41.0)           | N (31.0)      | 1,608 (14.0) | 5,297 (9.0)  | 2,633 (24.0) | 2,558 (19.0) | 1,366 (26.0) | 865 (29.0) | 643 (35.0) | 590 (35.0) | 451 (32.5) |
| HF4  | N (41.0)           | N (35.5)      | 1,061 (18.5) | 4,391 (21.0) | 3,137 (23.5) | 2,007 (31.0) | 1,029 (34.0) | 643 (35.0) | 590 (35.0) | 451 (32.5) | 497 (38.0) |
| HF6  | N (42.0)           | P (30.0)      | 6,277 (6.0)  | 9,362 (10.0) | 10,725 (28.0) | 9,069 (31.0) | 5,165 (37.0) | 2,846 (32.0) | 1,793 (35.0) | 1,450 (32.5) | 726 (42.0) |
| HF7  | N (43.0)           | N (30.0)      | 486 (24.0)  | 2,071 (25.0) | 1,459 (21.0) | 463 (33.5)  | 342 (32.0)  | 284 (29.0) | 305 (35.0) | 329 (34.0) | 323 (35.0) |
| HF8  | N (41.5)           | N (32.0)      | 6,186 (11.5) | 8,342 (12.0) | 3,951 (17.5) | 1,532 (30.0) | 884 (33.5) | 503 (27.0) | 441 (29.5) | 407 (26.0) | 424 (36.0) |
| HF9  | N (41.5)           | N (28.0)      | 856 (7.5)   | 2,033 (21.0) | 7,110 (17.5) | 1,574 (24.0) | 879 (26.5) | 490 (29.0) | 466 (30.0) | 386 (33.5) | P (33.5) |
| HF10 | N (39.0)           | N (31.0)      | 488 (20.9)  | 7,859 (20.0) | 11,513 (18.0) | 4,011 (30.0) | 1,679 (35.0) | 699 (31.0) | 543 (33.5) | 473 (32.0) | 318 (34.0) |
| HF12 | N (41.0)           | N (31.0)      | 1,850 (17.0) | 1,519 (21.5) | 1,128 (20.0) | 579 (23.5)  | 450 (35.0) | 448 (35.0) | 466 (39.0) | 391 (36.0) | 307 (45.0) |

*For each time point the ELISA result was recorded as either negative (N), as an unquantifiable positive (P), or as a quantifiable positive (relative antibody level compared to a standard). Packed cell volume (PCV) is recorded for all *M. haemofelis*-infected cats in parentheses, and the nadir is highlighted in boldface. None of the “Ca. Mycoplasma haemominutum”- or “Ca. Mycoplasma turicensis”-infected cats became anemic (PCV < 25%). PCV reference range, 25 to 45%.*
the immune response to DnaK is triggered by the infecting hemoplasma species due to the severity of disease. In line with this idea, an earlier report on the hematological data acquired from the experimental cats used in this study (15) showed that *M. haemofelis* was the most pathogenic, inducing significant anemia and the production of both cold and warm reactive erythrocyte-bound antibodies, while “Ca. Mycoplasma haemominutum” and “Ca. Mycoplasma turicensis,” although causing a drop in RBC count, did not result in anemia or the production of detectable erythrocyte-bound antibodies. The same study also demonstrated that there was no significant difference in copy numbers between the *M. haemofelis*- and “Ca. Mycoplasma haemominutum”-infected cats used in this study, while the “Ca. Mycoplasma turicensis”-infected cats had significantly lower copy numbers than both *M. haemofelis*- and “Ca. Mycoplasma haemominutum”-infected cats, indicating that differences in response to the three hemoplasma species are not dependent on copy number alone. Additionally, our data did not show any correlation between peak *M. haemofelis* hemoplasma copy number or the nadir of PCV and peak RAL, indicating a degree of host variability in response to infection.

The percentage of cats qPCR positive for hemoplasmas (9.8%) in the samples submitted to the commercial laboratory was slightly lower than those previously reported for the United Kingdom (14.0 to 18.5%), as determined by PCR (8, 10). The finding that 31.1% of these samples were recombinant *M. haemofelis* DnaK ELISA positive suggests, however, that a significant proportion of the cats had been exposed to hemoplasmas despite not being qPCR positive at the time of testing. These cats could have either cleared hemoplasma DNA from the blood or been infected with levels of hemoplasma organisms below the sensitivity limit of qPCR detection. Importantly, this study suggests that reported hemoplasma prevalences based on PCR in the United Kingdom, and by inference world-wide, may have been significantly underestimated. Regarding the clinical samples, it is not known whether the recombinant *M. haemofelis* DnaK antibodies detected in some of the qPCR-positive cats in this study were produced in response to the current hemoplasma species detected by PCR or as a result of earlier infection with a different hemoplasma species that subsequently was cleared. The possibility of cross-reacting antibodies generated as a result of a nonhemoplasma infection in these cats also cannot be excluded. Interestingly, the single clinical *M. haemofelis* qPCR-positive cat, which had a weakly positive recombinant *M. haemofelis* DnaK ELISA result, had a high hemoplasma load documented by qPCR (2.2 × 10^9 copies per ml blood), which is consistent with the early humoral response to acute infection seen around 8 dpi in the experimentally infected cats. In contrast, the dually *M. haemofelis*- and “Ca. Mycoplasma haemominutum”-infected cat had moderate and low hemoplasma species loads (4.4 × 10^7 copies *M. haemofelis* and 200 copies “Ca. Mycoplasma haemominutum” per ml blood) but a quantifiable recombinant *M. haemofelis* DnaK ELISA-positive result, which is consistent with the established humoral response to *M. haemofelis* infection seen from 15 dpi onwards in the experimentally infected cats.

This is the first description of the use of shotgun clone-derived genomic sequences in the identification of an immunogenic protein of a feline hemoplasma from 2D proteome analysis. This protein subsequently was expressed and applied

![Graph](image-url)

**FIG. 5.** *Mycoplasma haemofelis* qPCR results (square boxes, dotted line) and recombinant *M. haemofelis* DnaK ELISA relative antibody levels (diamond boxes, dashed line) from two representative cats: HF4 (A) and HF12 (B). The horizontal dashed line (W) represents the quantification minimum limit of both assays.

**DISCUSSION**

We have identified the dnaK, pgk, and EF-Ts genes of *M. haemofelis* using the DNA sequencing of random clones from a gDNA shotgun library and *in silico* analysis. We have further shown these proteins to be immunogenic, corresponding to a spot on a Western blot using mass spectrometry. This is similar to findings for other mammals infected with mycoplasmas where housekeeper proteins, such as *M. suis* DnaK and glyceraldehyde-3-phosphate dehydrogenase and *Mycoplasma mycoides* subsp. mycoides small-colony DnaK, pgk, and elongation factors G and Tu were found to be immunogenic (3, 4).

Recombinant *M. haemofelis* DnaK has been expressed in *E. coli* and has been shown to adsorb reactive antibodies in cats infected with all three species of hemoplasma by both Western blotting and ELISA, indicating cross-reactivity between the hemoplasma species. Therefore, it is not useful in discriminating between infecting species. The relative reactivity to recombinant *M. haemofelis* DnaK on both the Western blotting and ELISA of cats, however, varied with the infecting species; *M. haemofelis* produced a greater response than “Ca. Mycoplasma turicensis,” which in turn produced a greater response than “Ca. Mycoplasma haemominutum.” This could be due to the humoral immune response being directed against conserved, hemoplasma clade-specific (8), and/or species-specific epitopes on *M. haemofelis* DnaK, or a measure of the degree to which...
to the serological study of cats experimentally infected with hemoplasmas, and clinical samples were submitted for diagnostic qPCR hemoplasma testing. Further testing is required to clarify its use in non-SPF-derived cats and assess any serological cross-reactivity as a result of other chronic bacterial infections, such as *Bartonella henselae*, *Chlamydia phila* felis, and *Mycoplasma felis*. In the future, the ELISA should be applied to experimentally infected cats beyond the acute infection period to determine whether recombinant *M. haemofelis* DNAK antibodies persist in chronically infected cats and/or in cats that generate qPCR-negative results following the suspected clearance of infection.

**ACKNOWLEDGMENTS**

The feline samples used in this study were generated from a study funded by the Wellcome Trust (Grant no 077718). E.N.B. was supported by a University of Bristol Postgraduate Research Scholarship and Pfizer Health Limited. R.H.-L. is the recipient of a professorship funded by the Wellcome Trust (Grant no 077718). E.N.B. was supported by a University of Bristol Postgraduate Research Scholarship.

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