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Originally published at:
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Abstract

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.
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.

Hemoplasmas is the trivial name given to a group of erythrocyte-parasitizing bacteria of the genus *Mycoplasma* within the Mollicutes class, following their reclassification from the *Haemobartonella* and *Eperythrozoon* genera (6). In the United Kingdom, three feline hemoplasmas have been documented: *Mycoplasma haemofelis*, “*Candidatus Mycoplasma haemominutum*,” and “*Candidatus Mycoplasma turicensis*” (8, 10, 19). Clinical signs of hemoplasma infection range from asymptomatic to mild pyrexia to life-threatening (and occasionally fatal) hemolytic anemia even in immunocompetent individuals (9). Of the feline hemoplasmas, *M. haemofelis* appears to be most significant in terms of inducing hemolysis. Cats can remain infected with hemoplasmas in the absence of clinical signs, for months to years, and therefore they can represent a reservoir of infection (2, 17). This “carrier” status appears to be more commonly encountered with “*Ca. Mycoplasma haemominutum*” and less so with *M. haemofelis*, which could contribute to the differences in prevalences reported for these hemoplasma species (11, 12, 18).

The diagnosis of hemoplasma infection originally was based on clinical signs and the visualization of erythrocytic bodies by light microscopic examination of blood smears. More recently, PCR technology has superseded cytology for diagnosis due to its superior sensitivity and specificity (13, 16). The diagnosis of hemoplasma infection status using PCR is, however, not completely optimal. PCR results become negative when hemoplasma copy numbers fall below the detection limit of the assay. This may occur despite cats still being hemoplasma infected, such as in some carrier cats or following antibiotic treatment (12, 14). PCR testing of blood also requires the submission of a blood sample to a specialist laboratory, with associated cost implications in terms of both time and money, whereas a number of enzyme-linked immunosorbent assay (ELISAs) have been developed for point-of-care use in general practice, for example, the SNAP ELISAs (Idexx Labs.).

The ideal serological assay for hemoplasma infection would be both specific and sensitive to identifying hemoplasma-infected cats, and it would differentiate acute from chronic infection. The lack of *in vitro* cultivation techniques for hemoplasmas has limited the use of whole-hemoplasma preparations in serological assays. Recently a 299-amino-
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 haemomininatum,” and three with “Ca. Mycoplasma turicensis” (*M. haemofelis*-infected cats were designated HF1, HF2, HF3, HF4, HF6, HF7, HF8, HF9, HF10, and HF12; and “Ca. Mycoplasma haemomininatum”-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. Plasma DNA was purified and concentrated using the Macherey-Nagel Nucleospin gDNA Blood kit (ABgene, Epson, United Kingdom) for use in genomic studies. The gDNA was purified and concentrated using the Macherey-Nagel Nucleospin gDNA Blood kit (ABgene, Epson, United Kingdom) for use in genomic studies. The gDNA was sheared by nebulization into 2- to 5-kb fragments, as determined by gel electrophoresis. These fragments then were blunt ended and ligated into pCR4Blunt-TOPO and transformed into one-shot chemically competent *E. coli*. Purified plasmids from randomly selected clones were sequenced using plasmid primers and, where the insert length was greater than 900 bp, internal sequencing primers. DNA sequencing was performed by DNA Sequencing and Services (University of Dundee, Scotland; www.dnaseq.co.uk) using Applied Biosystems Big-Dye version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer (Applied Biosystems, Foster City, CA). BLASTn and BLASTp analyses of nucleotide and corresponding predicted amino-acid sequences were performed to identify the origin of the insert sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (1). Gene sequences were translated using the Mycoplasma/Spiroplasma codon usage table. Custom primers designed to match the DNA sequences were used in conjunction with plasmid primers to obtain contiguous sequences by PCR using the *M. haemofelis* libraries as the template.

**Identification of *M. haemofelis* immunogenic proteins.** For proteomic studies, the *M. haemofelis* and RBC membrane ghost protein samples were prepared for two-dimensional (2D) SDS-PAGE using the 2D clean-up kit (GE Healthcare, Amersham Place, Little Chalfont, United Kingdom). Proteins were separated according to their isoelectric points on Immobilbe DryStrip pH 3-11 NL (GE Healthcare) and then according to mass (12.5% polyacrylamide gel) along the gel molecular weight marker (unstained or All Blue Precision Plus Protein Standards; Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom). Gels either were subjected to Western blotting on polyvinylidene difluoride (PVDF) membranes or stained using Sypro ruby protein gel stain (Invitrogen). Membranes were blocked by gentle agitation for 5 h in Tris-buffered saline (25 mM Tris, 150 mM NaCl, pH 7.6) with Tween 20 (0.1%, vol/vol) and 5% (vol/vol) nonfat milk powder (TBST-5 M, Marvel, Premier Foods). Membranes were incubated overnight with preinfection or postinfection (139 and 153 dpi) plasma samples. The plasma samples that generated at least 100% bands were subjected to one-dimensional SDS-PAGE and blotted to nitrocellulose membranes. Prior to use, membranes were washed in Tris-buffered saline (25 mM Tris, 150 mM NaCl) with Tween 20 (0.1% vol/vol) (TBST) between steps (20 min; two washes) or overnight (25 rpm and room temperature). Following the final washes, visualization was achieved by the exposure of Amersham Hyperfilm ECL (GE Healthcare) to membranes coated with Lumi-Phos WB chemiluminescent substrate (Pierce Biotechnology, Thermo Fisher Scientific, Cramlington, United Kingdom). From the Sypro ruby-stained gel, protein spots corresponding to spots of immunoreactivity were picked for tryptic digestion, and the resulting peptides were analyzed using the 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). Results were analyzed in Mascot (www.matrixscience.com) and compared to known *M. haemofelis* sequences derived from the shotgun libraries and to the NCBI protein database.

**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 *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 Quanti-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 32 and 12 kDa, was excised from the gel and subjected to tryptic 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 was transferred using NuPAGE Novex 4 × 12% Bis-Tris pregels, NuPAGE morpholinepropanesulfonylic 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
results (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, HF6, HF7, HF8, 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 incubated with 100 μl preinfection 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 preinfection 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. Plates were washed with PBS containing 0.05% (vol/vol) Tween 20 (PBST), blocked using 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 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 (OD 405–495) was 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 (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 1:409,600) was added. Samples were deemed positive if their average OD 495 nm 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, ODs 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 heat shock protein 70 (dnaK; HQ013311), with 99.2% identity to a partial fragment of the M. haemofelis dnaK gene (FJ463263), 82.1% identity to a partial fragment of Mycoplasma haemocanis dnaK (AY150992), and 72.8% identity to a partial fragment of M. haemofelis dnaK (YP_053819). The translation of this nucleotide sequence gave a 602-amino-acid protein (ADN05760) with a predicted mass of 65.6 kDa and 99.6% identity (99.6% similarity) to a partial fragment of M. haemofelis dnaK (AC007298), 91.3% identity (95.2% similarity) to a partial fragment of M. haemocanis dnaK (AAN37401), and 71.0% identity (84.8% similarity) to heat shock protein A1 of M. suis (CAK22359). Library screening also identified 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 Meso-plasma 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 corresponded 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.** Recombinant *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 approximately 70 kDa on PAGE (Fig. 3) and was confirmed by mass spectrometry to be full-length recombinant *M. haemofelis* DnaK (70 kDa) and two C-terminal breakdown fragments (32 and 12 kDa). All Blue Precision Plus Protein Standard (Bio-Rad) (weights marked for major bands) was used to estimate molecular weights.

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 demonstrated 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 representative 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 produced a stronger signal than the plasma from “Ca. Mycoplasma 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. haemofelis* DnaK was successfully applied to the ELISA format. Quantities 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 (RLAs 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 (RLAs of 254, 294, 323, and 353).

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

<table>
<thead>
<tr>
<th>Cat</th>
<th>Preinfection score</th>
<th>Score on dpi:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>HF1</td>
<td>N (40.0)</td>
<td>P (25.0)</td>
</tr>
<tr>
<td>HF2</td>
<td>N (44.0)</td>
<td>N (34.0)</td>
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<td>HF3</td>
<td>N (41.0)</td>
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<td>HF5</td>
<td>N (42.0)</td>
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<td>N (43.0)</td>
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<td>HF9</td>
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<td>HM2</td>
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</tbody>
</table>

*a 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 worldwide, 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 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 × 10⁸ 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 × 10⁷ 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

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*, *Chlamydophila 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 from the Swiss National Science Foundation (PP00P3-119136). We thank Ben Crossett at Sydney University Proteome Research Unit for his assistance in data analysis.

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