Identification, Characterization, and Application of a Recombinant Antigen for the Serological Investigation of Feline Hemotropic Mycoplasma Infections


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In felids, three hemotropic mycoplasma species (hemoplasmas) have been described: Mycoplasma haemofelis, "Candidatus Mycoplasma haemominutum," and "Candidatus Mycoplasma turicensis." In particular, M. haemofelis may cause severe, potentially life-threatening hemolytic anemia. No routine serological assays for feline hemoplasma infections are available. Thus, the goal of our project was to identify and characterize an M. haemofelis antigen (DnaK) that subsequently could be applied as a recombinant antigen in a serological assay. The gene sequence of this protein was determined using consensus primers and blood samples from two naturally M. haemofelis-infected Swiss pet cats, an experimentally M. haemofelis-infected specific-pathogen-free cat, and a naturally M. haemofelis-infected Iberian lynx (Lynx pardinus). The M. haemofelis DnaK gene sequence showed the highest identity to an analogous protein of a porcine hemoplasma (72%). M. haemofelis DnaK was expressed recombinantly in an Escherichia coli DnaK knockout strain and purified using Ni affinity, size-exclusion, and anion-exchange chromatography. It then was biochemically and functionally characterized and showed characteristics typical for DnaKs (secondary structure profile, thermal denaturation, ATPase activity, and DnaK complementation). Moreover, its immunogenicity was assessed using serum samples from experimentally hemoplasma-infected cats. In Western blotting or enzyme-linked immunosorbent assays (ELISAs) (10), M. haemofelis DnaK was recognized by sera from cats infected with M. haemofelis, "Ca. Mycoplasma haemominutum," and "Ca. Mycoplasma turicensis," respectively, but not from uninfected cats. This is the first description of a full-length purified recombinant feline hemoplasma antigen that can readily be applied in future pathogenesis studies and may have potential for application in a diagnostic serological test.

Hemotropic mycoplasmas (hemoplasmas) are small (0.3 to 0.8 μm) erythrocytic bacteria, which have been known as Haemobartonella and Eperythrozoon species. In felids, Mycoplasma haemofelis, "Candidatus Mycoplasma haemominutum," and "Candidatus Mycoplasma turicensis" have been described (5, 6, 19, 36). They vary in their pathogenicity, responsiveness to antimicrobial drugs, and probably in their ability to form a carrier state (5, 26, 36). M. haemofelis in particular may cause severe, potentially life-threatening hemolytic anemia (5).

Real-time PCR assays are the tools of choice for diagnosing and differentiating feline hemoplasma infections (29, 36). However, they may not detect all hemoplasma infections, e.g., due to fluctuating M. haemofelis bacteremia (29), reduced bacterial blood loads after antibiotic treatment (5), or chronic carrier status of infected animals with undetectable numbers of circulating hemoplasmas (28). To overcome the resulting diagnostic gap and to further characterize the course and pathogenesis of feline hemoplasma infections, a diagnostic assay based on serum antibody detection would be desirable.

To date, no routine serological assays for the diagnosis of feline hemoplasma infections are available. The development of such assays has been significantly hampered by the fact that hemoplasmas cannot be cultured in vitro, and therefore antigens have had to be produced by the experimental infection of cats with hemoplasmas. Experimental serological assays have been described using hemoplasma antigen either on blood smears (5) or purified from large volumes of blood (1) from infected cats. Western blot analyses of Haemobartonella felis antigen preparations resulted in the identification of five antigens recognized by sera from experimentally H. felis-infected cats (1). A recent study identified M. haemofelis antigens in crude antigen preparations from erythrocytes collected from an experimentally infected cat (21). Those antigens reacted with plasma antibodies of cats collected at different time points after experimental infection when applied in Western blot analyses. The first recombinant hemoplasma antigen, Mycoplasma suis HspA1, was developed during a study of experimentally M. suis-infected pigs for application in Western blotting and enzyme-linked immunosorbent assays (ELISAs) (10). This antigen belongs to the heat shock protein 70 (HSP70) family. It was found to be DnaK-like and present on the surface of M. suis (10). DnaKs are molecular chaperones consisting of an N-terminal nucleotide-binding domain (ATPase ac-
infected cats, such as cat Y (Table 1).

Ca.

M. haemofelis

experimentally

serum samples have been used during this study are listed in Table 1. For ELISA, pre- and postinfection samples from a total of 20 SPF cats were used, including the experimentally-infected cat QLA5, 8 “Ca. Mycoplasma haemominutum”-infected cats, such as cat Y (Table 1).

The experimental infections of the “Ca. Mycoplasma haemominutum”- and “Ca. Mycoplasma turicensis”-infected cats have been described earlier (7, 18).

The aim of this study was to identify the complete DnaK gene of M. haemofelis, to recombinantly produce, highly purify, and characterize the antigen, and to apply it in an ELISA as a serological tool for the detection and quantification of the humoral immune response during experimental feline hemoplasma infection.

MATERIALS AND METHODS

Animals, experimental hemoplasma infections, and samples. All animals from which samples have been used during this study are listed in Table 1. For sequencing purposes, samples from the following six hemoplasma-infected felids were used: the experimentally M. haemofelis-infected specific-pathogen-free (SPF) cat QLA5 (Liberty Research, Waverly, NY), the naturally M. haemofelis-infected Swiss domestic pet cats 1008 and 7415 (35), the free-living Iberian lynxes (Lynx pardinus) Dalia and Cicuta, which were naturally infected with M. haemofelis and “Ca. Mycoplasma haemominutum,” respectively (16), and the experimentally “Ca. Mycoplasma turicensis”-infected SPF cat Y (4.4 × 10^9 copies/ml blood), and from lynx Dalia (6.6 × 10^9 copies/ml blood) 10 days postinfection (dpi) were used for M. haemofelis DnaK gene amplification and sequencing. DNA from 1 ml of blood of cat QLA5 was extracted manually using the QIAamp DNA blood mini kit (Qiagen, Hombrechtikon, Switzerland). Total nucleic acids from cats 1008, 7415, and lynx Dalia were extracted from 200 µl of blood using the MagNa pure LC total nucleic acid isolation kit I (Roche Diagnostics, Reinach, Switzerland).

Determination of M. haemofelis DnaK gene sequence and phylogenetic analysis. Based upon the sequence information of M. haemofelis DnaK gene fragments AY150993 (303 bp) and FJ63263 (899 bp) and of all other mycoplasma DnaK gene sequences available from the GenBank database until June 2009, several consensus primer pairs were designed manually and tested for the amplification of the potential M. haemofelis DnaK gene. Using the primer pairs F1-35Mpf/R934-956Mhf and F600-623Var/R1746-1768Ms (Table 2), the M. haemofelis DnaK gene was amplified as two overlapping fragments of 976 and 1,307 bp, respectively. The following thermal cycling conditions were applied: initial denaturation at 98°C for 180 s, 35 cycles of 98°C for 10 s, 60 (976 bp) or 63°C (1,307 bp) for 30 s, and 72°C for 150 s, with final extension at 72°C for 5 min. The amplified fragments were ligated into pCR 2.1-TOPO and transformed into competent E. coli DH5α cells according to standard procedures.

The presence of correctly amplified DnaK gene fragments was determined by TaqMan real-time PCR (35), and had been stored at −80°C until use. All SPF cats were kept in groups (QLAS was kept together with a female neutered SPF companion cat) and examined clinically prior to the study, and their SPF status was verified as described previously (18).

After hemoplasma inoculation, EDTA-anticoagulated whole-blood samples were collected regularly, hemograms were generated using a Cell-Dyn 3500 (Abbott, Baar/Switzerland), and the quantification of hemoplasma blood loads was performed by TaqMan real-time PCR (35). Serum or plasma samples were collected for serological analyses (see below). Anemia was defined as a hemato-

RESULTS

The DnaK sequence of “Ca. Mycoplasma turicensis” could not be amplified.

Sequence (5’–3’)

Length (bp)

Product length (bp)

F1-35Mpf

GGCAAAAAAAGATTTTGGAGTTTAGGTACCTAGG

33

976

R934-956Mhf

CTTACCAAACTCTGATACCCGAC

23

F600-623Var

GTGGTGACGATTGGGATCAAGC

22

1,307

R1746-1768Ms

CTGATGCAGCTTGTCCTCCAGCA

23

FDnaKMhfET

CGACGCACTATGGCGAAAAAGAAATATTTAGGAAATTGACTTACAGCA

44

1,824

RDnaKMhfET

CGACGACTCGAGGAGATATGTTTATACCTACGTCTTACCTCCT

26

1,119

R1750-1783Mhf

TTAGATTGTITTATCTATCCAGTCTTATC

34

Table 2. Primers used for amplification and construction of the M. haemofelis DnaK and rDnaK genes

a Primer names indicate the forward (F) and reverse (R) orientation of the primers. All primers were used at a final concentration of 0.5 µM each.

b Boldface indicates the NdeI recognition site.

c Boldface indicates the XhoI recognition site.

Table 1. Animals from which samples have been used for sequencing and serology

<table>
<thead>
<tr>
<th>Identity*</th>
<th>Species, SPF status</th>
<th>Infectious agent</th>
<th>Infection type</th>
<th>Sample use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>QL5</td>
<td>Domestic cat, SPF</td>
<td>M. haemofelis</td>
<td>Exp</td>
<td>Sequencing, serology</td>
<td>Present study</td>
</tr>
<tr>
<td>1008</td>
<td>Domestic cat</td>
<td>M. haemofelis</td>
<td>Natural</td>
<td>Sequencing</td>
<td>35</td>
</tr>
<tr>
<td>7415</td>
<td>Domestic cat</td>
<td>M. haemofelis</td>
<td>Natural</td>
<td>Sequencing</td>
<td>35</td>
</tr>
<tr>
<td>Dalia</td>
<td>Iberian lynx</td>
<td>M. haemofelis</td>
<td>Natural</td>
<td>Sequencing</td>
<td>16</td>
</tr>
<tr>
<td>Cicuta</td>
<td>Iberian lynx</td>
<td>Ca. Mycoplasma haemominutum</td>
<td>Natural</td>
<td>Sequencing</td>
<td>16</td>
</tr>
<tr>
<td>Y</td>
<td>Domestic cat, SPF</td>
<td>“Ca. Mycoplasma turicensis”</td>
<td>Exp</td>
<td>Serology</td>
<td>7</td>
</tr>
<tr>
<td>09NFR2</td>
<td>Domestic cat, SPF</td>
<td>“Ca. Mycoplasma haemominutum”</td>
<td>Exp</td>
<td>Serology</td>
<td>18</td>
</tr>
<tr>
<td>Various (n = 10)</td>
<td>Domestic cat, SPF</td>
<td>“Ca. Mycoplasma turicensis”</td>
<td>Exp</td>
<td>Serology</td>
<td>18</td>
</tr>
<tr>
<td>Various (n = 7)</td>
<td>Domestic cat, SPF</td>
<td>“Ca. Mycoplasma haemominutum”</td>
<td>Exp</td>
<td>Serology</td>
<td>7</td>
</tr>
</tbody>
</table>

* The number of animals is indicated if it is more than one.

b The DnaK sequence of “Ca. Mycoplasma turicensis” could not be amplified.

c Only a partial DnaK sequence of “Ca. Mycoplasma haemominutum” could be amplified.
DnaK gene was amplified as two overlapping fragments from DNA. The rDnaK gene was obtained in two steps: first, the cDNA was calculated by the neighbor-joining method. Bootstrap support (1,000 replicates) was calculated using MEGA version 4 (27). Bootstrap values greater than 70% are given on their 16S rRNA gene (23, 34) or on their RNase P RNA gene sequences (hemoplasma and haemofelis groups) (22), are indicated. The sequence of E. coli served as an outgroup, establishing the root of the tree.

Gene construction and molecular cloning. The recombinant M. haemofelis DnaK gene sequence within pMG211 was confirmed by DNA sequencing before protein production. For the DnaK complementation assay, the gene of E. coli chorismate mutase (EcCM) (12, 25) was NdeI and Xhol digested and ligated to the Xhol-Ndel fragment of plasmid pMG211 as described above.

Prokaryotic expression and purification of recombinant M. haemofelis DnaK. Plasmid pMG211, containing the M. haemofelis rDnaK gene (naturally without UGA readthroughs), was transformed into the recA-deficient Escherichia coli strain XL1 blue (Stratagene, LaJolla, CA) for plasmid storage and multiplication. Cells were grown on LB agar containing 150 μg/ml ampicillin and in LB medium containing 200 μg/ml ampicillin at 37°C and 250 rpm, respectively. Plasmid DNA was purified using a Jetquick Plasmid Miniprep Spin kit (Genomed, Löhne, Germany). Transformed XL1 blue cells were stored as glycerol stocks at −80°C. For protein production, plasmid pMG211 M. haemofelis rDnaK was transformed into the kanamycin-resistant strain JW0013, an in-frame DnaK knockout mutant of E. coli K-12 (2). Preparative cultures were inoculated from overnight starter cultures and grown at 30°C and 250 rpm in LB medium containing 150 μg/ml ampicillin and 25 μg/ml kanamycin. Gene overexpression was induced with 1 mM salicylate at an optical density at 600 nm (OD600), and the culture was incubated for an additional 20 h at 25°C and 250 rpm. After cell lysis using 1 mg/ml lysosome and ultrasonication, protein was purified from the soluble fraction by affinity chromatography on Ni2+–nitrilotriacetic acid (NTA) agarose (Qiagen). The purification progress was assessed by SDS–PAGE analysis (see below) after each purification step. Fractions containing monomeric M. haemofelis DnaK were isolated by size-exclusion chromatography on a calibrated Superdex 200 10/300 GL column (Amersham Pharmacia Biotech, Uppsala, Sweden) in Tris-buffered saline (TBS), pH 7.4. Those fractions then were subjected to anion-exchange chromatography on a Mono Q HR 16/10 column (Amersham Pharmacia Biotech) in TBS, pH 7.4, using a salt gradient from 150 to 500 mM NaCl. Fractions containing protein with a molecular mass of about 66 kDa were combined and concentrated using Amicon Ultra Centrifugal Filter 10 K (Millipore, Carrigtwohill, Cork, Ireland), and their protein concentration was determined by the Bradford assay (Coomassie plus protein assay reagent calibrated with bovine serum albumin [BSA]; Thermo Scientific, Rockford, IL).
Molecular mass determination of recombinant M. haemofelis DnaK. The molecular mass of M. haemofelis DnaK protein was determined at the protein service unit of the Functional Genomics Center Zurich (FGCZ), University of Zurich, Zurich, Switzerland. The purified protein solution was analyzed using electrospray ionization mass spectrometry. The experimentally determined molecular mass then was compared to the mass calculated by the ProtParam tool (www.expasy.ch/tools/protparam.html) based on the deduced protein sequence of M. haemofelis DnaK.

Structure and stability determination. Circular dichroism (CD) spectroscopy was performed on an Aviv circular dichroism spectrometer model 202 (Aviv Instruments Inc., Lakewood, NJ) in quartz cuvettes of 0.2-cm path length (d). Far-UV spectra were recorded from 260 to 200 nm in 1-nm steps at 25°C and a 1 µM M. haemofelis DnaK protein concentration (c) in TBS, pH 7.4 (50 mM Tris base, 150 mM NaCl). For stability studies, KCl (100 mM) together with MgCl\(_2\) (2.5 mM) and/or ATP (0.1 mM) were added. Data were collected for 5 s at each step. Five scans were averaged, and buffer spectra determined under identical conditions were subtracted. The observed ellipticity (θ) at a wavelength λ was transformed into molar ellipticity per residue (θ\(_{222}\)) using equation 1 (where n is the number of residues), resulting in θ\(_{222}\) = θ\(_{0}\) (c·d·n).

Thermal denaturation experiments were performed in TBS, pH 7.4, at a 1 µM protein concentration by monitoring the CD signal at 222 nm between 10 and 95°C and reverse in 0.5 K steps with 60 s of temperature equilibration, 60 s of data collection, and a 1-K-per-min heating/cooling rate between temperature steps. T\(_{m}\) the melting point of the M. haemofelis DnaK ATPhase domain, was defined as the inflection point of the melting curve and was determined from the first derivative after curve smoothing using the SigmaPlot v11.0 software package (Systat Software Inc., Richmond, CA).

ATPase activity/ATPase. ATPase activity was measured using a spectrophotometric assay (32), which quantified the released amount of inorganic phosphate (P\(_i\)) during ATP hydrolysis. The reaction of 2-amino-6-mercaptop-7-methylpurine-ribonucleoside (MESG) with P was catalyzed by the purine nucleoside phosphorylase (PNP; Sigma-Aldrich, Buchs, Switzerland) and led to a measurable change in absorbance at 360 nm. MESG was synthesized and purified as previously published (32). All measurements were performed at 25°C and 1 U/ml PNP, the change in absorbance at 360 nm was subtracted to the PNP control (100 µM MESG using (190 µM) P and P(0, 1, 5, 25, and 50 µM from a 200 µM Na\(_2\)HPO\(_4\), solution). Michaelis-Menten kinetic measurements of M. haemofelis DnaK then performed under the following ATPhase assay conditions: 50 mM Tris, 100 mM KCl, 2.5 mM MgCl\(_2\), 400 µM purified M. haemofelis DnaK, and 380 µM MESG. The change in absorbance at 360 nm was measured in duplicate for 26 min at ATP concentrations of 10, 50, 100, 250, and 500 µM after an equilibration time of 4 min in a Lambda 35 spectrophotometer (PerkinElmer, Waltham, MA). Blank values measured without M. haemofelis DnaK were subtracted, and the reaction rates were calculated. The catalytic parameters k\(_c\) and k\(_w\) were determined from curve fitting to the Michaelis-Menten equation using the SigmaPlot v11.0 software package (Systat Software Inc.).

DnaK complementation assay. The E. coli DnaK knockout mutant strain JW0013 was transformed with plasmid pMG211 containing either M. haemofelis DnaK or the EcCM gene (as a negative control). Liquid cultures were prepared and protein transfer to nitrocellulose membranes were performed in a PhastSystem development unit, and the obtained sequences of M. haemofelis DnaK have been deposited in the GenBank database (HM594280, HM594281, and HM594283). Additionally, a partial "Ca. Mycoplasma haemominutum" DnaK sequence, acquired using identical methods, was submitted (HM594282).

RESULTS

M. haemofelis DnaK sequences. The obtained sequences of the potential M. haemofelis DnaK gene included, according to BLASTN search results, an open reading frame (ORF) most similar to mycoplasma DnaK gene sequences (data not shown). Compared to mycoplasma DnaK complete gene sequences from the GenBank database, the highest identities with this ORF were found for M. suis DnaK (HspA1; 72%) and Mycoplasma penetrans DnaK (69%). Identities with the two partial M. haemofelis DnaK gene sequences FJ463263 (899 bp) and AY150993 (303 bp) were 99 and 97%, respectively. Accordingly, the ORF was named the M. haemofelis DnaK gene.

Further comparisons of M. haemofelis DnaK gene sequences derived from different four M. haemofelis-infected animals resulted in three distinct M. haemofelis DnaK gene sequences: from cats 7415 and QLAs an identical sequence was obtained (HM594280). Compared to the 7415/QLA5 sequence, sequences obtained from cat 1008 (HM594283) and lynx Dalia (HM594281) shared the same 21 silent point mutations as well as one point mutation causing a serine-to-arginine exchange at position 580. The sequence from lynx Dalia (HM594281) additionally contained a point mutation causing a proline-to-
serine exchange at position 577. When the deduced M. haemofelis DnaK protein sequence (cats 7415 and QL5; HM594280) was compared to mycoplasma DnaK sequences from the GenBank database using the BLASTP search algorithm, again M. suis (70%) and M. penetrans (65%) shared highest identities. The phylogenetic analyses of the M. haemofelis DnaK protein sequence revealed that it clustered within the haemofelis group of the hemoplasmas, which is distinct from other mycoplasma groups (Fig. 1).

Expression and biochemical characterization of the recombinant M. haemofelis DnaK. Ni-affinity chromatography of crude extracts of salicylate-induced JW0013 pMG211 M. haemofelis rDnaK cells yielded one predominant protein band corresponding to a molecular mass of about 66 kDa (data not shown). After size-exclusion chromatography, anion-exchange chromatography, and protein concentration, M. haemofelis rDnaK was judged to be pure by SDS-PAGE (Fig. 2A).

Mass spectrometry analysis determined the molecular mass of M. haemofelis DnaK to be 66,406 Da, while the calculated molecular mass based on its deduced protein sequence was 66,537 Da. This difference in mass of 131 Da corresponds to the N-terminal loss of methionine during mass spectrometry analysis.

CD spectrum analysis of M. haemofelis rDnaK showed two distinct minima at 208 and 222 nm (Fig. 3A). The CD spectrum of M. haemofelis rDnaK recorded without ATP or K\(^+\) and Mg\(^{2+}\) ions was not markedly different from those recorded with additives. The temperature-dependent CD signals at 222 nm showed the thermally induced unfolding of M. haemofelis rDnaK with well-defined (\(T_{m1}\)) and less-well-defined (\(T_{m2}\)) temperature transitions (Fig. 3B). The melting temperature of the nucleotide-binding domain of M. haemofelis rDnaK without additives (\(T_{m1}\)) was determined to be 42°C, the addition of the nucleotide ATP increased it to a \(T_{m1,e}\) of 46°C, and ATP together with K\(^+\) and Mg\(^{2+}\) ions further increased it to a \(T_{m1,d}\) of 50°C. The addition of K\(^+\) and Mg\(^{2+}\) ions alone caused only a minimal increase to a \(T_{m1,0}\) of 43°C.

Enzymatic activity of the ATPase domain of M. haemofelis rDnaK was determined in a spectrophotometric assay. Fitting reaction rates at various substrate concentrations to the Michaelis-Menten equation yielded catalytic parameters for ATP hydrolysis (Fig. 3D).

During the DnaK complementation assay, the in vivo functional protein of DnaKs, to allow bacterial growth at cell-stressing temperatures by repairing damaged enzymes, was tested for M. haemofelis rDnaK. No difference in the extent of bacterial growth between M. haemofelis rDnaK and the EcCM transformant could be seen for any of the tested culture dilutions at 30°C (Fig. 3D). At 43°C, however, cells overexpressing EcCM and lacking DnaK protein grew only until a dilution of \(10^{-1}\) (20 colonies), while for rDnaK-expressing cells growth until a \(10^{-3}\) dilution (1 colony) was observed (Fig. 3D).

Immunogenicity of the recombinant M. haemofelis DnaK. Western blot analyses of M. haemofelis rDnaK showed that the protein was recognized by serum antibodies from cats experimentally infected with M. haemofelis (cat QL5), “Ca. Mycoplasma haemominutum” (cat 09NFR2), and “Ca. Mycoplasma turicensis” (cat Y). Preinfection serum or plasma samples from the same cats did not result in a positive Western blot signal (Fig. 2B).

For the M. haemofelis rDnaK ELISA, 50 ng M. haemofelis rDnaK/well and a dilution of 1:200 for sera from M. haemofelis-infected cats and 1:100 for sera from “Ca. Mycoplasma haemominutum” and “Ca. Mycoplasma turicensis”-infected cats were found to be the optimal conditions. OD\(_{415}\) values for serum samples from 20 SPF cats prior to hemoplasma infection ranged from 0.12 to 0.33 under these conditions. After experimental infection, for the M. haemofelis-infected SPF cats QL5 the OD\(_{415}\) peaked at 1.4. For the eight “Ca. Mycoplasma haemominutum”-infected SPF cats (Table 1), the OD\(_{415}\) peak values ranged from 0.5 to 1.2, and for the 11 “Ca. Mycoplasma turicensis”-infected SPF cats (Table 1), they were between 0.7 and 1.5. The signal-to-noise ratio of the M. haemofelis-, “Ca. Mycoplasma haemominutum”- and “Ca. Mycoplasma turicensis”-infected SPF cats reached a maximum of 10.4, 10.4, and 10.6, respectively. A signal-to-noise ratio of at least 1.5 was considered serologically positive for anti-M. haemofelis rDnaK antibodies.

Experimental M. haemofelis infection and quantification of anti M. haemofelis DnaK antibodies. The experimentally M. haemofelis-infected SPF cats showed the expected OD\(_{415}\) values (Table 1), indicating that the rDnaK was an immunogenic recombinant protein. The OD\(_{415}\) values of the experimentally infected SPF cats were significantly higher than those of the uninfected SPF cats (Table 1).
haemofelis-infected cat QLA5 turned *M. haemofelis* TaqMan real-time PCR positive within 4 dpi and became anemic within 10 dpi (Fig. 4A). On the day of infection (0 dpi) the cat was mildly anemic (hematocrit of 28%), probably due to a baseline blood collection of 26 ml 11 days prior to *M. haemofelis* infection. However, the cat recovered to hematocrit values within the reference range within a few days (7 to 9 dpi) before a decrease in the hematocrit was observed starting at 10 dpi. The minimum hematocrit value of 15% was measured 36 dpi (Fig. 4A). However, no severe clinical signs were observed that necessitated blood transfusion or antibiotic treatment during the course of infection, and the cat subsequently recovered from anemia. From 148 dpi (5.3 months postinfection [mpi]) onwards, the hematocrit values stayed within the reference range until the end of the experiment, 28.6 mpi (Fig. 4A and data not shown).

The peak *M. haemofelis* load in blood (2.2 × 10^8 copies/ml blood) was recorded at 29 dpi. Between 4 and 42 dpi the first marked *M. haemofelis* copy number fluctuations were observed; they ranged from 10^3 to 10^8 *M. haemofelis* copies/ml blood within a minimum of 2 days (Fig. 4A). From 3.8 to 8.3 mpi a second episode of copy number cycling was observed; the loads ranged from 10^2 to 10^5 copies/ml blood within a minimum of 8 days. Five distinct *M. haemofelis* load peaks were observed in 1- to 2-month intervals during this second cycling period. QLA5 stayed PCR negative from 260 dpi (9.3 mpi) until the end of the observation period at 28.6 mpi (Fig. 4A and B).

The seroconversion of cat QLA5, defined as a signal-to-noise ratio of at least 1.5, occurred between 8 (signal ratio, 1.3) and 14 dpi (signal ratio, 6.9) (Fig. 4B). QLA5 stayed serologically positive until the end of the observation period at 28.6 MPI. Twelve and 18 mpi the signal-to-noise ratio dropped to a minimum of 2.8, followed by a signal ratio increase to 6.0 without detectable amounts of *M. haemofelis* DNA in the cat’s blood (Fig. 4B). The reinfection of QLA5 by its SPF compa-
M. haemofelis (B) Anti- and 95% quantiles) of the hematocrit reference range (33 to 45%). The dashed lines indicate the lower and the upper limit (5
data not shown). The observed cross-reactivity is also in agreement with a previous study using whole feline hemoplasma antigen preparations (5). In the latter study, antigen derived from H. felis large form (today known as M. haemofelis) was tested with sera from cats infected with M. haemofelis and “Ca. Mycoplasma haemominutum” (formerly known as H. felis small form). M. haemofelis-derived whole hemoplasma antigen cross-reacted with sera from M. haemofelis and “Ca. Mycoplasma haemominutum”-infected cats, while “Ca. Mycoplasma haemominutum”-derived antigen was recognized only by sera from “Ca. Mycoplasma haemominutum”-infected cats.

The antigen M. haemofelis rDnaK was purified to homogeneity from potentially antigenic proteins originating from the production process to improve the signal quality of the serological assays and to minimize interbatch variations in antigen quality. Indeed, M. haemofelis rDnaK protein expression and purification was repeated with identical results. The identity of the protein was proven by the comparison of the observed and calculated molecular masses of M. haemofelis rDnaK. CD spectrum analysis of M. haemofelis rDnaK revealed two minima (at 208 and 222 nm), suggesting that it consisted mostly of α-helices (8), which is in good agreement with known DnaK structures, e.g., of E. coli (Protein Database identiy [PDB ID]: 2KH0) and G. kaustophilus (PDB ID: 2V7Y). The structure profile of M. haemofelis rDnaK was insensitive to a change in the presence of nucleotide, as has been shown before for Bacillus licheniformis DnaK (13), and also in the presence of K⁺ and Mg²⁺ ions. The thermal denaturation of M. haemofelis rDnaK was characterized by two temperature transitions. This corresponded well to an earlier study (17), where deletion mutants of E. coli DnaK were used. The authors demonstrated that the first transition (Tm1) was related to the unfolding of the DnaK N-terminal nucleotide-binding domain, while the second transition was related to the unfolding of the C-terminal substrate-binding domain. A raising of Tm in the presence of nucleotide, as also observed for M. haemofelis rDnaK, was reported for E. coli DnaK to be caused by a stabilizing effect occurring due to the ligand binding to the nucleotide-binding domain of the protein (20). As found for M. haemofelis rDnaK, to HspAl, the DnaK of M. suis (CAK22359); the latter protein was demonstrated to be expressed on the surface of M. suis cells and to have immunogenic potential (10). In analogy to this, we found that hemoplasma-infected cats readily produced antibodies to M. haemofelis rDnaK.

The protein cross-reacted with sera from cats experimentally infected with M. haemofelis, “Ca. Mycoplasma haemominutum” and “Ca. Mycoplasma turicensis,” but not with serum samples from SPF cats. However, the optimization of the ELISA resulted in higher sample dilutions for M. haemofelis samples than for “Ca. Mycoplasma haemominutum” and “Ca. Mycoplasma turicensis” samples, which indicates that the immunogenicity of M. haemofelis rDnaK is caused by conserved as well as species-specific epitopes of this antigen. This would be in agreement with the high identity (71%) that we found between the M. haemofelis DnaK and the partial “Ca. Mycoplasma haemominutum” DnaK gene sequence (1,304 bp; HM594282) but also could explain why we were unable to amplify the 3’ end of the “Ca. Mycoplasma haemominutum” DnaK gene sequence using consensus primers despite several attempts (data not shown). The observed cross-reactivity is also in agreement with a previous study using whole feline hemoplasma antigen preparations (5). In the latter study, antigen derived from H. felis large form (today known as M. haemofelis) was tested with sera from cats infected with M. haemofelis and “Ca. Mycoplasma haemominutum” (formerly known as H. felis small form). M. haemofelis-derived whole hemoplasma antigen cross-reacted with sera from M. haemofelis and “Ca. Mycoplasma haemominutum”-infected cats, while “Ca. Mycoplasma haemominutum”-derived antigen was recognized only by sera from “Ca. Mycoplasma haemominutum”-infected cats.

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this stability was supposed to be further enhanced in the presence of nucleotide together with K\(^+\) and Mg\(^2+\) ions, which mediate contacts between DnaK and nucleotide (15, 33). The kinetic constants for ATP hydrolysis by pure M. haemofelis rDnaK (K\(_{\text{cat}}\) = 0.015/min; K\(_{\text{m}}\) = 23 μM; V\(_{\text{cat}}\)/K\(_{\text{m}}\) = 650/M/min) (Fig. 3C) were comparable to those published for E. coli DnaK, which showed K\(_{\text{cat}}\) values ranging from 0.02 to 0.2/min (3) and K\(_{\text{m}}\) values ranging from 20 nM to 20 μM (4, 14). This indicated that M. haemofelis rDnaK possesses a typically low ATPase activity when evaluated without its cochaperones DnaJ and grPE. The DnaK complementation assay confirmed the molecular chaperone activity of M. haemofelis rDnaK in an E. coli DnaK knockout mutant at heat shock temperatures. This heat shock protein activity serves as another piece of true evidence for the identity of M. haemofelis DnaK.

We demonstrated for the first time that an experimentally M. haemofelis-infected cat mounted antibodies to M. haemofelis rDnaK within 8 to 14 days after experimental infection and shortly after the cat’s blood was M. haemofelis PCR positive. Moreover, we found a correlation between the M. haemofelis blood loads and antibody levels. This indicates that M. haemofelis DnaK is immunogenic and that the recombinant antigen is suited for use in quantitative serological assays and to demonstrate seroconversion in infected animals.

The experimentally M. haemofelis-infected cat stayed serologically positive for more than 2 years postinfection and for more than 1.5 years after turning PCR negative for M. haemofelis in the blood. So far, we have data from only one M. haemofelis-infected cat. However, earlier results from “Ca. Mycoplasma turicensis” infection (18) and preliminary follow-up data from these cats (M. Novacco, G. Wolf-Jäckel, H. Lutz, and R. Hofmann-Lehmann, unpublished data) confirm the persistence of anti-M. haemofelis rDnaK antibodies in the absence of PCR positivity in blood. This indicates that there is active antigen stimulation in the chronic phase of hemoplasma infection, possibly by antigen sequestered in the tissue. We have postulated that the decline of Western blot signal in two cats after the antibiotic treatment of experimental “Ca. Mycoplasma turicensis” infection could have been due to therapy-induced “Ca. Mycoplasma turicensis” clearance from blood and tissues (18), whereas “Ca. Mycoplasma turicensis” antigen sequestered in the tissues of 10 untreated cats could have resulted in the continuous low-level stimulation of the humoral immune system (18). The latter would be in agreement with the findings from two experimentally “Ca. Mycoplasma turicensis”-infected cats: in nearly all tissues collected from those two cats at 20 and 94 dpi, more “Ca. Mycoplasma turicensis” copies were found than expected to result from blood contamination alone, which suggested that “Ca. Mycoplasma turicensis” can become concentrated in organs of infected cats (S. Tasker, personal communication). However, for M. haemofelis no evidence for significant tissue sequestration has been found so far (30).

In conclusion, this study provides evidence that M. haemofelis rDnaK, the antigen we have identified, recombinantly produced, and characterized herein, has biochemical and molecular chaperone properties of the HSP70/DnaK family. It has been applied successfully to quantify anti-feline hemoplasma antibodies in samples from experimentally infected cats, as well as to monitor seroconversion after experimental infection when used in Western blot assays and ELISA. The antigen and the assays are prerequisites to gain more insight into the course of hemoplasma infections, e.g., by investigating hemoplasma pathogenesis in experimental infection setups. In addition, the described antigen may have the potential to be used in a rapid test for clinicians, supporting quick diagnosis and faster choice of adequate therapy of feline hemolytic anemia. However, further studies will be necessary to fully evaluate the cross-reactivity of this antigen with antibodies directed against other bacterial pathogens and its potential benefit for testing samples from naturally hemoplasma-infected cats.

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