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Wolf-Jäckel, G A; Jäckel, C; Museux, K; Hoelzle, K; Tasker, S; Lutz, H; Hofmann-Lehmann, R

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Abstract

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, it 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) epirhthrocytic bacteria, which previously have been known as "Haemobartonella" and "Eperythrozoon" species. In felids, Mycoplasma haemofelis, "Candidatus Mycoplasma haemominutum," and "Candidatus Mycoplasma turicensis" have been described. They vary in their pathogenicity, responsiveness to antimicrobial drugs, and probably in their ability to form a carrier state. M. haemofelis infections 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, it 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.
tivity) that generates the energy necessary to refold misfolded proteins in cell stress situations (9). Misfolded proteins bind to the C-terminal substrate-binding domain of DnaKs. Most recently, we developed a recombinant feline hemoplasma antigen to demonstrate the seroconversion of experimentally “Ca. Mycoplasma turicensis”-infected cats in preliminary Western blot analyses (18). The antigen described was a truncated M. haemofelis DnaK form recombinantly expressed in E. coli that was only partially purified, leading to large interbatch variations with regard to quality and purity. The described assay did not allow for the quantification of antibody levels.

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 (18). For the experimental infections of the “Ca. Mycoplasma haemominutum”- and “Ca. Mycoplasma turicensis”-infected cats have been described earlier (7, 18). For experimental M. haemofelis infection, the SPF cat QLA5 was inoculated intraperitoneally at the age of 2.7 years with 2 ml of dimethylsulfoxide (DMSO)-preserved (20%, vol/vol) M. haemofelis-positive blood from the experimentally infected cat HF3 (31). The inoculum contained 10^8 M. haemofelis copies/ml, as determined in our laboratory using identical methods and blood samples from 58 clinically healthy cats. EDTA-anticoagulated blood samples collected from cat QLA5 (4.4 × 10^6 copies/ml blood), cat 1008 (2.8 × 10^6 copies/ml blood), cat 7415 (8.0 × 10^6 copies/ml blood), and from lynx Dalia (6.6 × 10^6 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).

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

| Identity | Species, SPF status | Infectious agent | Infection type | Sample use | Reference |
|----------|---------------------|------------------|----------------|------------|-----------|
| QLA5     | Domestic cat, SPF   | M. haemofelis    | Exp 1          | Sequencing, serology | Present study |
| 1008     | Domestic cat        | M. haemofelis    | Natural        | Sequencing | 35        |
| 7415     | Domestic cat        | M. haemofelis    | Natural        | Sequencing | 35        |
| Dalia    | Iberian lynx        | M. haemofelis    | Natural        | Sequencing | 16        |
| Cicuta   | Iberian lynx        | Ca. Mycoplasma haemominutum* | Natural | Sequencing| 16 |
| Y        | Domestic cat, SPF   | “Ca. Mycoplasma turicensis” | Exp 1 | Sequencing, serology | 18 |
| 09NFRI2  | Domestic cat, SPF   | “Ca. Mycoplasma haemominutum” | Exp 1 | Serology | 7 |
| Various (n = 10) | Domestic cat, SPF | “Ca. Mycoplasma turicensis” | Exp 1 | Serology | 18 |
| Various (n = 7) | Domestic cat, SPF | “Ca. Mycoplasma haemominutum” | Exp 1 | Serology | 7 |

* The number of animals is indicated if it is more than one.
* a The DnaK sequence of “Ca. Mycoplasma turicensis” could not be amplified.
* b Only a partial DnaK sequence of “Ca. Mycoplasma haemominutum” could be amplified.

The experimental infections of the “Ca. Mycoplasma haemominutum”- and “Ca. Mycoplasma turicensis”-infected cats have been described earlier (7, 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-
crit value of less than 33% (equal to a 5% quantile of the reference range
determined in our laboratory using identical methods and blood samples from 58
clinically healthy cats). EDTA-anticoagulated blood samples collected from cat QLA5 (4.4 × 10^6 copies/ml blood), cat 1008 (2.8 × 10^6 copies/ml blood), cat 7415 (8.0 × 10^6 copies/ml blood), and from lynx Dalia (6.6 × 10^6 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).

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

| Primer name | Sequence (5′−3′) | Length bp | Product length bp |
|-------------|-----------------|-----------|------------------|
| F1-35Mpf    | GGCAGAAAAAGAAAATTTTAGGAAAGTTCATTAGG | 33 | 976 |
| R934-956Mhf | CTATACACACACCTCCTGAAAGCGCGCA | 23 |            |
| F600-623Var | GTGGTGACGATTGGGATCAAGC | 22 | 1,307 |
| R1746-1768Ms | CTTCTGAGCTTTGCTCTCAGCA | 23 |            |
| FDnaKMHipfET | GCAGCACTATAGGCGAAAAAGAAATTTTAGAATGCACTTTAGG | 44 | 1,824 |
| RDnaKMHipfET | GCAGCACTTGCGAGGATTTATGTTTATCTACCTCGTACCTTATCC | 45 |            |
| F666-691Mhf | GCACCTCAAGACTTACGAGGATGCCG | 26 | 1,119 |
| R1750-1783Mhf | TTAGATTCTTGTCTTCTACTCTGAGCTTTATCC | 34 |            |

* 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.
(1,307 bp) for 30 s, 72°C for 60 s, and then a final elongation at 72°C for 10 min. All DNA amplification steps of this study were performed using Phusion high-fidelity DNA polymerase (Finzymes, Espoo, Finland). The resulting PCR products of the expected lengths were extracted from agarose gels using the Nucleospin extract II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Gene sequences of the PCR products were determined by DNA sequencing (Microsynth, Balgach, Switzerland). The sequence at the nodes of the tree. GenBank accession numbers are indicated in parentheses. Mycoplasma groups, which previously have been classified based on their 16S rRNA gene (23, 34) or on their RNase P RNA gene sequences (hemoplasma and haemofelis groups) (22), are indicated. The sequence at the nodes of the tree. GenBank accession numbers are indicated in parentheses. Mycoplasma groups, which previously have been classified based ...

ClustalW2 (11). The phylogenetic tree of mycoplasma DnaK protein sequences was constructed using MEGA version 4 (27). Bootstrap support (1,000 replicates) was calculated by the neighbor-joining method.

Gene construction and molecular cloning. The recombinant M. haemofelis DnaK gene was obtained in two steps: first, the M. haemofelis DnaK gene was amplified as two overlapping fragments from DNA extracted from blood of cat QLA5 (using primer pairs F1-35Mp/R934-956Mhf; Table 2). Those primers also inserted NdeI and XhoI cleavage sites at the 5′ and 3′ ends of the gene, respectively. The following cycling conditions were used: 5 cycles with an annealing temperature of 85°C, followed by the addition of primers, and 35 cycles with an annealing temperature of 60°C (remaining cycling conditions were as described above). The resulting PCR product (1,324 bp) was extracted from an agarose gel, digested with restriction enzymes NdeI and XhoI (New England Biolabs, Ipswich, MA) according to the manufacturer’s instructions, and ligated to the 4,690-bp Xhol-Ndel fragment of vector pMG211 (24). The vector contained an ampicillin resistance gene, a salicylate-inducible promoter, and the genetic information for a C-terminal 6×His tag followed by a stop codon. The correct M. haemofelis rDNA 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 rDNA 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 rDNA 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) of 0.6, and the culture was incubated for an additional 20 h at 25°C and 250 rpm. After cell lysis using 1 mg/ml lysozyme 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 10/10 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). FIG. 1. Phylogenetic tree demonstrating the relationship of the deduced M. haemofelis DnaK protein sequences to mycoplasma DnaK protein sequences from the GenBank database. Phylogenetic relationships were calculated using the neighbor-joining algorithm. Evolutionary distances are shown to scale. The data set was resampled 1,000 times to generate bootstrap percentage values. Bootstrap values greater than 70% are given at the nodes of the tree. GenBank accession numbers are indicated in parentheses. Mycoplasma groups, which previously have been classified based 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.
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-mm 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 MgCl2 (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 wavelength λ was transformed into molar ellipticity per residue (θ222) using equation 1 (where n is the number of residues), resulting in θ222 = 0.3(n × 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 from 10 to 95°C (Pi) during ATP hydrolysis. The reaction of 2-amino-6-mercaptopurine-ribonucleoside (MESG) with Pi was catalyzed by the purine nucleoside phosphatase (PNP). The change in absorbance at 360 nm was measured in duplicate for 26 haemofelis rum or plasma samples from the experimentally infected cats QLA5 (ImmunoResearch Laboratories, West Grove, PA) and 1:200. Antigen amounts of 200, 100, 50, and 10 ng/well were tested in duplicate. Wells containing only antigen without serum served as blanks, and wells containing preinfection serum samples served as negative controls.

Recombinant antigen-based ELISA. M. haemofelis rDnaK was heated in coating buffer (100 mM NAc2O4, 0.1% [wt/vol] SDS, pH 9.6) to 100°C for 1 min and then diluted 1:20 in coating buffer without SDS. Flat-bottomed 96-well microwell plates with medium binding capacity (Greiner Bio-One, Frickenhausen, Germany) then were coated with 100 μl of this M. haemofelis rDnaK solution per well for 3 h at 37°C and overnight at 4°C. Plates subsequently were washed three times with 200 μl/well washing buffer (150 mM NaCl, 0.05% [vol/vol] Tween 20) and incubated for 1 h at 37°C with 100 μl/well blocking buffer (150 mM NaCl, 50 mM Tris base, 1 mM TryptiX III, 0.1% [wt/vol] BSA, 0.1% [vol/vol] Tween 20, pH 7.4). After being washed as described above, 100 μl of serum samples diluted in serum buffer (blocking buffer without BSA) was added per well and incubated for 1 h at 37°C. The plates were washed, and each well was filled with 100 μl of peroxidase-conjugated, affinity-purified goat anti-cat IgG antibodies (diluted 1:3,000 in serum buffer; Jackson ImmunoResearch Laboratories) and incubated for 1 h at 37°C. After being washed, 100 μl/well substrate solution [150 mM citric acid pH 4.0, 1% (vol/vol) H2O2, 2%, 1% (vol/vol) 2,2'-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] diammonium salt] was added, and plates were incubated at room temperature for 10 min. Absorbance then was measured at a wavelength of 415 nm (OD415) using a Spectramax Plus 348 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Optimal serum dilutions and recombinant antigen concentrations were established by a checkerboard titration using pre- and postinfection sera from the three experimentally infected cats QLA5 (M. haemofelis; prior to and after 1, 2, 1, and 0 dpi), 09NFR2C ("Ca. Mycoplasma haemominutum"; prior to and after 1, 2, and 1,200). Antigen amounts of 200, 100, 50, and 10 ng/well were tested in duplicate. Wells containing only antigen without serum served as blanks, and wells containing preinfection serum samples served as negative controls.

Statistical analyses. Statistical analyses were performed using the SigmaPlot v11.0 software package (Systat Software Inc., Richmond, CA).
serine exchange at position 577. When the deduced \textit{M. haemofelis} DnaK protein sequence (cats 7415 and QLA5; HM594280) was compared to mycoplasma DnaK sequences from the GenBank database using the BLASTP search algorithm, again \textit{M. suis} (70\%) and \textit{M. penetrans} (65\%) shared highest identities. The phylogenetic analyses of the \textit{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 \textit{M. haemofelis} DnaK.**

Ni-affinity chromatography of crude extracts of salicylate-induced JW0013 pMG211 \textit{M. haemofelis} DnaK 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, \textit{M. haemofelis} rDnaK was judged to be pure by SDS-PAGE (Fig. 2A).

Mass spectrometry analysis determined the molecular mass of \textit{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 \textit{M. haemofelis} rDnaK showed two distinct minima at 208 and 222 nm (Fig. 3A). The CD spectrum of \textit{M. haemofelis} rDnaK recorded without ATP or K\textsuperscript{+} and Mg\textsuperscript{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 \textit{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 \textit{M. haemofelis} rDnaK without additives (\(T_{m1}^{\text{adj}}\)) was determined to be 42°C, the addition of the nucleotide ATP increased it to a \(T_{m1}\) of 46°C, and ATP together with K\textsuperscript{+} and Mg\textsuperscript{2+} ions further increased it to a \(T_{m1}^{\text{adj}}\) of 50°C. The addition of K\textsuperscript{+} and Mg\textsuperscript{2+} ions alone caused only a minimal increase to a \(T_{m1}^{\text{adj}}\) of 43°C.

Enzymatic activity of the ATPase domain of \textit{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 \textit{in vivo} biological function of DnaKs, to allow bacterial growth at cell-stressing temperatures by repairing damaged enzymes, was tested for \textit{M. haemofelis} rDnaK. No difference in the extent of bacterial growth between \textit{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 \(10^{-3}\) (20 colonies), while for rDnaK-expressing cells growth until a \(10^{-3}\) dilution (1 colony) was observed (Fig. 3D).

**Immunogenicity of the recombinant \textit{M. haemofelis} DnaK.**

Western blot analyses of \textit{M. haemofelis} rDnaK showed that the protein was recognized by serum antibodies from cats experimentally infected with \textit{M. haemofelis} (cat QLA5), “\textit{Ca. Mycoplasma haemominutum}” (cat 09NFR2), and “\textit{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 \textit{M. haemofelis} rDnaK ELISA, 50 ng \textit{M. haemofelis} rDnaK/well and a dilution of 1:200 for sera from \textit{M. haemofelis}-infected cats and 1:100 for sera from “\textit{Ca. Mycoplasma haemominutum}”- and “\textit{Ca. Mycoplasma turicensis}”-infected cats were found to be the optimal conditions. OD\textsubscript{405} 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 \textit{M. haemofelis}-infected SPF cat QLA5 the OD\textsubscript{415} peaked at 1.4. For the eight “\textit{Ca. Mycoplasma haemominutum}”-infected SPF cats (Table 1), the OD\textsubscript{415} peak values ranged from 0.5 to 1.2, and for the 11 “\textit{Ca. Mycoplasma turicensis}”-infected SPF cats (Table 1), they were between 0.7 and 1.5. The signal-to-noise ratio of the OD\textsubscript{415} was 1.5. A signal-to-noise ratio of at least 1.5 was considered serologically positive for anti-\textit{M. haemofelis} rDnaK antibodies.

**Experimental \textit{M. haemofelis} infection and quantification of anti-\textit{M. haemofelis} DnaK antibodies.**

The experimentally \textit{M. haemofelis}-infected SPF cats and quantitative analysis of the anti-\textit{M. haemofelis} DnaK antibodies were used to determine the immune response to the rDnaK during infection. The OD\textsubscript{415} peak values ranged from 0.5 to 1.2, and for the 11 “\textit{Ca. Mycoplasma turicensis}”-infected SPF cats (Table 1), they were between 0.7 and 1.5. A signal-to-noise ratio of at least 1.5 was considered serologically positive for anti-\textit{M. haemofelis} rDnaK antibodies.
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 × 10⁸ 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 10³ to 10⁸ 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 10² to 10⁵ 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 compan-
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* rDnA 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.

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 identity [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 (T_m1) 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 $T_m$ 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,
this stability was supposed to be further enhanced in the presence of nucleotide together with K⁺ and Mg²⁺ ions, which mediate contacts between DnaK and nucleotide (15, 33). The kinetic constants for ATP hydrolysis by pure M. haemofelis rDnaK (kcat = 0.015/min; km = 23 μM; kcat/km = 650/M/min) (Fig. 3C) were comparable to those published for E. coli DnaK, which showed kcat values ranging from 0.02 to 0.2/min (3) and km 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”-infected cats: in nearly all tissues collected from those two cats at 20 and 94 dpi, more “Ca. Mycoplasma turicensis”-infected cats: in nearly all tissues collected from those two cats at 20 and 94 dpi, more “Ca. Mycoplasma turicensis”-infected cats: in nearly all tissues collected from those two cats at 20 and 94 dpi, more “Ca. Mycoplasma turicensis”-infected cats: in nearly all tissues collected from those two cats at 20 and 94 dpi, more “Ca. Mycoplasma turicensis”-infected cats: in nearly all tissues collected from those two cats at 20 and 94 dpi, more “Ca. 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