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(54) **Title**: METHODS OF DIAGNOSIS AND VACCINES

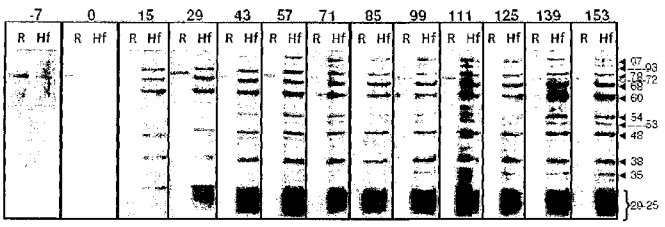


Figure 1

(57) **Abstract**: The invention relates to immunogenic polypeptides from Mycoplasma haemofelis (Mhf) and *'Candidatus* Mycoplasma haemominutum' (CMhm), and their use as vaccines and as markers of infection in the cat. The polypeptides are dnaK, gapA, pgk and EF-Ts.

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METHODS OF DIAGNOSIS AND VACCINES

The present invention relates to methods of diagnosis of haemoplasma infection, and vaccines for the prevention of the same. The methods and vaccines relate to the cat.

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

'Haemoplasmas' is the trivial name given to a group of erythrocyte parasitizing bacteria of the genus *Mycoplasma* within the Mollicutes class. They are most closely related to the *pneumoniae* group of mucosal mycoplasmas. Analysis of 16S ribosomal RNA gene sequences from haemoplasmas resulted in their recent reclassification from the *Haemobartonella* and *Eperythrozoon* genera. The haemoplasma group can be further divided into haemofelis and haemominutum clades based on 16S rRNA and Ribonuclease P ribosomal gene phylogeny.

Diagnosis of haemoplasma infection was originally based on clinical signs and visualisation of epierythrocytic bodies by light microscopic examination of blood smears. More recently PCR technology has superseded cytology for diagnosis due to its superior sensitivity and specificity. PCR assays have also allowed accurate determination of the prevalence of the various haemoplasma species within their respective hosts. Development of serological assays for the diagnosis of haemoplasmosis has been attempted in the past. While early attempts by one group were reported to have been promising (unpublished data), no further information has been submitted for publication (Turner and others 1986). These earlier studies may have been confounded by cytologically negative chronically infected haemoplasma carriers, resulting in positive results in cytologically negative cats. One group developing an enzyme-linked immunosorbent assay (ELISA) for the detection of the porcine haemoplasma *Mycoplasma suis* (Ms) antibodies in infected pigs reported significant quantities of porcine immunoglobulins as a contaminant of haemoplasma antigen preparation from blood, despite the inclusion of density gradient ultracentrifugation (Hoelzle and others 2006). The immunoglobulin contaminant displayed a marked ability to bind secondary conjugated antibodies, resulting in high background measurements.

Haemoplasmas have been found to infect a wide variety of mammalian species, from squirrel monkeys to llamas (Hoelzle 2008). And while they appear to be relatively host

specific, different haemoplasmas may exist within an individual host (Kenny and others 2004, Tasker and others 2003, Willi and others 2006a). Three haemoplasmas have been documented worldwide in both the domestic cat and wild felids: *Mycoplasma haemofelis* (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm), and '*Candidatus* Mycoplasma turicensis' (CMt) (Lobetti and Tasker 2004, Sykes and others 2007, Tasker and others 2003, Willi and others 2006b). A fourth feline haemoplasma, having close identity to the canine haemoplasma '*Candidatus* Mycoplasma haematoparvum' (CMhp), has been detected during a prevalence study conducted in the USA (Sykes and others 2007), but to date remains incompletely described and not reported elsewhere. Two haemoplasmas have been documented in the dog: *Mycoplasma haemocanis* (Mhc) and CMhp (Inokuma and others 2006, Kenny and others 2004, Sykes and others 2004, Wengi and others 2007).

Worldwide PCR based CMhm prevalence ranges from 8.5 to 46.6%, Mhf prevalence from 0.2 to 21.7%, and CMt prevalence from 0.5 to 26.1% (Bauer and others 2008, Fujihara and others 2007, Gentilini and others 2009, Kamrani and others 2008, Peters and others 2008, Roura and others 2010, Tasker 2006a, Willi and others 2006a, Willi and others 2006b). Prevalence of the canine haemoplasmas ranges are even greater with Mhc prevalence from 0.9 to 20.0% and CMhp prevalence from 0.3 to 33.4%, with occasional dual-haemoplasma infections (Barker and others 2008, Inokuma and others 2006, Kenny and others 2004, Roura and others 2010, Wengi and others 2008). However, differences exist between the populations examined, e.g. client owned vs. feral, sick vs. healthy, and PCR assays used, which prevents direct comparison.

A review of haemotropic mycoplasmas is found in Tasker (2010). Infections with haemoplasmas can range from being asymptomatic, through mild pyrexia, to life-threatening (and occasionally fatal) haemolytic anaemia even in immunocompetent individuals. Of the feline haemoplasmas Mhf appears to be most significant in terms of haemolysis (Tasker 2006b). Cats infected with CMhm are rarely clinically unwell, although a mildly lowered haematocrit is not uncommon when infected cats are compared to controls (George and others 2002, Tasker and others 2004, Tasker and others 2009b). A mildly lowered haematocrit was also seen in cats infected with CMt in a recent experimental study (Tasker and others 2009b). However, cats naturally infected with CMt are frequently found to be concurrently infected with one or more of the other haemoplasmas. In contrast to cats immunosuppression or splenectomy of the host appears to be required for disease to become clinically apparent in dogs infected with Mhc or CMhp (Kemming and others 2004, Lester and others 1995, Sykes and others

2004). Cats can remain infected with haemoplasmas, in the absence of clinical signs, for months to years, and therefore represent a source of infection (Berent and others 1998, Willi and others 2006a). This can present difficulties in terms of diagnosis and management, as they may be below the limit of PCR detection, and antibiotic treatment does not consistently clear infection. Carrier status is most commonly encountered with CMhm infection, less so with Mhf, which could account for difference in prevalence figures (Willi and others 2007). As one of the routes of infection remains the iatrogenic administration of blood products, the ability to identify these chronically infected animals is important during disease monitoring of potential blood donors (Hackett and others 2006).

In some host species haemoplasma infection has been shown to modify response to viral and malarial infections, as well as response to irradiation (Baker and others 1971, Contamin and Michel 1999). The potential role of haemoplasma infection in modifying the pathogenicity of other infections has yet to be fully elucidated in cats. However, CMhm infection has been associated with fatal myeloproliferative disease in chronically FeLV infected cats (George and others 2002), whilst other studies examining the effect of chronic FIV infection on acute Mhf or CMhm infection found no significant haematological differences between the retroviral positive and negative groups (Tasker and others 2006a, b). Long-term co-morbidity studies of natural cat populations have been limited by the presence of chronic infections and the lack of assays to determine exposure to haemoplasmas in the absence of a positive PCR result.

Expression and analysis of some mycoplasmal metabolic proteins e.g. DNA chaperone heat shock protein 70 (dnaK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; gapA), elongation factor thermo-unstable (EF-Tu), has shown that in addition to their cytosolic role they can also be surface expressed and may play a role in host cell attachment (Alvarez and others 2003, Balasubramanian and others 2009, Hoelzle and others 2007b, Hoelzle and others 2007c). GAPDH in *Mycoplasma genitalium* (MG301) has been shown to play a major role in binding to the host glycoprotein mucin (Alvarez and others 2003). EF-Tu of *Mycoplasma pneumoniae* has been shown to mediate binding to host fibronectin (Balasubramanian and others 2009). Some of these proteins are also immunogenic and have been suggested as vaccine candidates in particular species, such as GAPDH in the respiratory pathogen *Mycoplasma bovis* (Perez-Casal and Prysliak 2007).

A 40KDa immunoreactive GAPDH homolog of Ms, MSG1, has been shown to be expressed at both surface and cytosolic locations, have GAPDH activity and appeared to have a role in erythrocyte adhesion (Hoelzle and others 2007c). A 70KDa immunoreactive protein of Ms, HspA1, was determined to be a dnaK homolog; a finding that was supported by ATPase activity of the expressed recombinant HspA1 (Hoelzle and others 2007b). Recombinant MSG1 has been trialled as a vaccine candidate in the pig; however despite induction of anti-MSG1 antibodies they were not protective against a high-dose infection challenge (Hoelzle and others 2009). In an analysis of the immunoproteome of *Mycoplasma mycoides* subsp. *mycoides* which infects cattle a number of proteins were identified as being immunogenic, including gapA, dnaK, EF-Tu and phosphoglycerate kinase (pgk) (Jores and others 2009).

Recombinant forms of Ms proteins MSG1 and HspA1 have been generated and used in ELISA-based serological diagnostic assays for the detection of Ms infection in pigs and was found to be comparable to whole cell preparations (Hoelzle and others 2007a). A fragment of Mhf dnaK has also been expressed and used in western blots to detect the presence of anti-dnaK antibodies in cats experimentally infected with Mhf, CMhm or CMt (Museux and others 2009).

Before the present invention, no one has suggested that EF-Ts (Elongation Factor – Temperature sensitive) from mycoplasma is useful as an immunogenic marker or in a vaccine. Nor has anyone suggested that gapA (glyceraldehyde-3-phosphate dehydrogenase) or pgk (phosphoglycerate kinase) from Mhf or CMhm are useful as immunogenic marker proteins or in a vaccine for cats. GapA and pgk are both metabolic proteins found in the cytoplasm of bacteria playing an integral role in glycolytic metabolic pathways. Due to reductive evolution mycoplasmas have lost a significant number of genes, as a result some proteins have evolved to function in multiple roles within the cell. It is impossible to predict which of these metabolic proteins will be used in this way in individual species, more so whether they will play a role in the bacterial immunome.

Furthermore, before the present invention no-one had provided full length DNA and amino acid sequences for Mhf dnaK. Due to the absence of whole genome sequencing of any feline haemoplasmas, in fact any haemoplasmas *per se*, it is impossible to design amplification primers to the regions flanking the *dnaK* gene. Primers have to be designed to available sequences, or 'best guess' based on closely related bacterial species. Whilst this may allow the determination of partial fragments within a gene of interest the regions flanking a specific gene vary widely and cannot be predicted. In our

case genome fragmentation and cloning was required to determine full sequence of Mhf *dnaK*.

A first aspect of the invention provides a polypeptide comprising the amino acid sequence as set out in SEQ ID No 14 (Mhf EF-Ts) or a variant or fragment thereof. SEQ ID No 14 provides the native amino acid sequence of Mhf elongation factor temperature-sensitive (EF-Ts). EF-Ts forms part of a complex with elongation factors Tu and G, which together facilitate the events of translation (i.e. protein synthesis, where the DNA sequence is 'read' and amino-acids strung together). Specifically EF-Ts serves as the guanine nucleotide exchange factor for EF-Tu, catalyzing the release of GDP from EF-Tu, allowing a molecule of GTP to bind in its place, so permitting the entry of the next aminoacyl rRNA into the ribosome. This may be assayed by incorporation into an ELISA, probing a western blot with plasma/serum or via enzymatic assays.

A second aspect of the invention provides a polypeptide comprising the amino acid sequence as set out in SEQ ID No 10 (Mhf pgk) or SEQ ID No 26 (CMhm pgk) or a variant or fragment thereof. SEQ ID No 10 provides the partial native amino-acid sequence of Mhf phosphoglycerate kinase, and SEQ ID No 26 provides the native amino acid sequence of CMhm phosphoglycerate kinase. The Mhf pgk fragment comprised the N-terminal 344 amino-acids of what is suspected to be a total of 400-415 amino-acids of the complete protein. Pgk is an ubiquitous enzyme that catalyses the formation of ATP to ADP and vice versa acting as a transferase enzyme in the seventh step of glycolysis. This could be assayed by incorporation into an ELISA, probing a western blot with plasma/serum or via enzymatic assays.

A third aspect of the invention provides a polypeptide comprising the amino acid sequence as set out in SEQ ID No 6 (Mhf gapA) or SEQ ID No 22 (CMhm gapA) or a variant of fragment thereof. SEQ ID No 6 provides the native amino acid sequence of Mhf glyceraldehyde-3-phosphate dehydrogenase, and SEQ ID No 22 provides the native amino acid sequence of CMhm glyceraldehyde-3-phosphate dehydrogenase. GapA catalyzes the sixth step of glycolysis converting glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate. This could be assayed by incorporation into an ELISA, probing a western blot with plasma/serum or via enzymatic assays.

A fourth aspect of the invention provides a polypeptide comprising the amino acid sequence as set out in SEQ ID No 18 (CMhm dnaK) or a variant or fragment thereof. SEQ ID No 18 provides the native amino acid sequence of CMhm DNA chaperone heat

shock protein dnaK. The expression of heat shock proteins is up regulated in response to cell stressors such as heat or other environmental changes. More specifically heat shock protein 70, aka. dnaK, functions as an intra-cellular chaperone for other proteins permitting correct protein folding and preventing protein aggregation during unfavourable conditions. It may also be present at lower levels as a house-keeping protein fulfilling similar functions. This could be assayed by incorporation into an ELISA, probing a western blot with plasma/serum or via enzymatic assays.

A fifth aspect of the invention provides a polypeptide comprising the amino acid sequence as set out in SEQ ID No 2 (Mhf dnaK) or a variant thereof, or a fragment thereof with the amino acid sequence MSKKETIIG (SEQ ID No 29) or a fragment thereof from the sequence from position 309 to position 602 of Figure 3 (sequence B). SEQ ID No 2 provides the native amino acid sequence of Mhf DNA chaperone heat shock protein dnaK. Figure 3 indicates the amino-acid alignment between SEQ ID No 2 and the fragment of Mhf dnaK described by Museux and others (2009). Position 309 onwards starts with the sequence QILLVGG (SEQ ID No 30) and so on, and ends with the sequence KTEVDKTKS (SEQ ID No 31).

Preferably, the polypeptide is substantially free of other polypeptides with which it is naturally associated in the mycoplasma cell. Preferably the polypeptide is substantially pure. For example, the polypeptide may be in a composition where it accounts for at least 95% of the total polypeptide, such as at least 96%, 97%, 98%, 99% or 99.5% as judged by Coomassie blue staining of a preparation separated by SDS-PAGE.

By "variant" of the polypeptide we include polypeptides in which the sequence homology is at least 80% or 85% or 90%, more preferably at least 91% or 92% or 93% or 94% or 95% or 96% or 97% or 98% or 99% or 99.5%, the % homology being assessed using the BLASTp algorithm [http://blast.ncbi.nlm.nih.gov/Blast.cgi, BLASTp (protein-protein BLAST), NCBI non-redundant protein sequence database (Altschul and others 1990). Typically the variants include polypeptides in which 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 amino acids have been replaced with another amino acid or have been deleted. By variant, we also include the possibility that amino acids have been added to the polypeptide, for example in order to aid purification. Suitable tags to facilitate purification include poly-histidine additions. Typically, the variant retains the enzymatic active of the native polypeptide as given. Preferably, the variant is cross-reactive with the native polypeptide. In other words, the

variant is preferably able to bind antibodies that have been raised to the native polypeptide, which can be tested for, for example by using a suitable ELISA.

By "fragment" of the polypeptide we include fragments of the polypeptide that constitute 20% of the length of the native polypeptide, typically at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 97%. Typically the fragment is at least 20 amino acids, such as at least 30, 40, 50, 60, 70, 80 amino acids in length. Preferably, the fragment is cross-reactive with the native polypeptide. In other words, the fragment is preferably able to bind antibodies that have been raised to the native polypeptide, which can be tested for, for example by using a suitable ELISA. In relation to Mhf dnaK, the fragment is either MSKKETIIG (SEQ ID No 29) or is a fragment from position 309 to position 602 of Figure 3 (sequence B).

When the term "polypeptide" is used, unless the context does not allow it, the term includes the variants and fragments as defined including, in particular, the variants and fragments that are cross-reactive with the native polypeptide.

The polypeptides and variants and fragments are typically immunogenic in the cat, giving rise to an immune response that can be measured by detecting antibodies to the polypeptide in the serum of a cat that has been immunised with the polypeptide or variant or fragment, for example by using an ELISA.

Partial sequences for Mhf *dnaK* gene have previously been published (303bp in AY150993, 899bp in FJ463263). Sequence AY150993 covers nucleotides 622 to 924 of the complete gene with six mismatches to SEQ ID No 1. Sequence FJ463263 covers nucleotides 26 to 924 of the complete gene, with seven mismatches to SEQ ID No 1.

A sixth aspect of the invention provides a polynucleotide which encodes a polypeptide of any of the first, second, third, fourth or fifth aspects of the invention. The polynucleotide is typically DNA, but may be RNA. Preferred polynucleotides of the invention are SEQ ID No 1, SEQ ID No 3, SEQ ID No 5, SEQ ID No 7, SEQ ID No 9, SEQ ID No 11, SEQ ID No 13, SEQ ID No 15, SEQ ID No 17, SEQ ID No 19, SEQ ID No 21, SEQ ID No 23, SEQ ID No 25 and SEQ ID No 27. Codon usage refers to the triplet nucleotides that encode a specific amino-acid residue in a polypeptide chain or the termination of translation (stop codons). In *Mycoplasma* species the 'opal' codon UGA encodes for the amino-acid residue tryptophan, whilst in the majority of bacteria (including the *Escherichia coli*) UGA is a stop codon. To enable to complete expression of

Mycoplasma species genes that include opal codons, site-directed mutagenesis may be employed to convert the UGA codons to UGG, which also encodes the inclusion of tryptophan but is not translated as a stop codon in either the standard or mycoplasma codon usage tables. To enable the inclusion of a C-terminal poly-histidine motif in the expressed protein, the stop codon (where known) may be removed from the native gene and the blunt 3' end of the native gene arranged such as to allow 'in-frame' reading of an expression vector sequence, such as the pET plasmid sequence, containing the poly-histidine motif.

A seventh aspect of the invention provides an expression vector comprising the polynucleotide of the sixth aspect of the invention. The expression vector, when present in a suitable host cell, allows for the expression of the polypeptides of the invention. The expression vector may be a bacterial or yeast or insect cell or mammalian cell expression vector. It is preferred if the expression vector is a bacterial expression vector, such as an *E. coli* expression vector. Suitable expression vectors are known in the art, for example in Sambrook *et al* Molecular Cloning, a laboratory manual, 2nd edition, Cold Spring Harbor Laboraory Press, Card Spring Harbor, NY. Champion™ pET Directional TOPO® Expression Kits (Invitrogen) manual part no. 25-0400 describes the use of this expression vector

An eighth aspect of the invention provides a host cell comprising a polynucleotide or expression vector of the invention. The host cell may be any suitable host cell such as a bacterial cell, a yeast cell, an insect cell or a mammalian cell. Bacterial cells are preferred. Suitable host cells are known in the art, for example in Sambrook *et al.*

Molecular cloning techniques, DNA sequencing and PCR methods useful in the practice of the invention are well known, for example in Sambrook *et al.*

The polypeptides of the invention suitably are obtained by culturing a host cell which expresses the polypeptide by recombinant means, and then extracting the polypeptide from the host cell or culture medium (if secreted). The polypeptide may be purified using methods well known in the art, such as ion-exchange chromatography, size-exclusion chromatography, affinity chromatography and the like. For the proteins containing a poly-histidine motif, purification using Ni^{2+}-affinity chromatography may be used.

The polypeptides of the invention may be packaged and presented for use in medicine, in particular in veterinary medicine. The polypeptide or polypeptides of the invention may

be packaged and presented for use in medicine. Typically the polypeptide is prepared as a sterile formulation, with appropriate pharmaceutical excipients, if required. Typically, the polypeptide is substantially pure as described above. In particular, the polypeptides, either alone or in combination with other antigenic proteins, are useful as vaccines. Typically, the vaccine of the invention therefore includes one or more polypeptides of the invention, optionally with other antigenic proteins, such as other mycoplasma proteins, and further optionally with one or more adjuvants. Suitable adjuvants are known in the art and include, for example, alum.

A ninth aspect of the invention provides a method of vaccinating a cat against haemotropic mycoplasma infection, the method comprising administering to the cat one or more polypeptides according to any of the first, second, third, fourth or fifth aspects of the invention, or a vaccine according to the sixth aspect of the invention. For the avoidance of doubt, in this aspect of the invention the term "polypeptide" includes variants or fragments thereof that are cross-reactive with the native polypeptide, and which are able to give rise to an immune response against the native polypeptide. Typically, the polypeptide or polypeptides are administered by sub-cutaneous or intra-dermal or intra-muscular injection or by intra-nasal administration. The amount of polypeptide or polypeptide is sufficient to give rise to a protective immune response in the cat. Initially, western-blotting or ELISA may be used to determine whether the animal is able to produce an immune response to the recombinant protein itself. Then animals may be challenged with infective doses of the selected haemoplasma at various time points following vaccine or placebo administration, then monitored for haemoplasma infection (clinical signs, haematological parameters and blood haemoplasma copy numbers). Although a single immunization may be sufficient, typically, a booster immunization is given at a suitable period after the initial immunization. If more than one type of polypeptide is administered, they may be administered together or sequentially.

By "cat" we include any member of the cat (felidae) family, in particular the domestic cat or wild felids such as tiger or lion.

An tenth aspect of the invention provides a composition comprising one or more polypeptides according to any of the first, second, third, fourth, fifth aspects of the invention and optionally other antigens, and optionally an adjuvant, or a vaccine according to the sixth aspect of the invention, for vaccinating a cat against haemotropic mycoplasma infection.

The invention also includes the use of a composition comprising one or more polypeptides according to any of the first, second, third, fourth, fifth aspects of the invention, or a vaccine according to the sixth aspect of the invention, in the manufacture of a medicament for vaccinating a cat against haemotropic mycoplasma infection.

Typically, in all of the vaccination methods of the invention, the haemotropic mycoplasma infection is Mhf or CMhm or CMt.

An eleventh aspect of the invention provides an antibody directed to a polypeptide according to any of the first, second, third, fourth or fifth aspect of the invention. Antibodies may be prepared by methods well known in the art, for example as described in *"Monoclonal Antibodies: A manual of techniques"*, H Zola (CRC Press, 1988) and in *"Monoclonal Hybridoma Antibodies: Techniques and Applications"*, J G R Hurrell (CRC Press, 1982), by using an appropriate antigen such as a polypeptide of the invention or a variant or fragment thereof which is cross-reactive with the native polypeptide, or a mycoplasma EF-Ts polypeptide or a variant or fragment thereof which is cross-reactive with the native polypeptide. The antibody may be a polyclonal antibody or a monoclonal antibody or a recombinant antibody. By "antibody" we include fragment of antibodies such as Fab and Fv fragments, and synthetic antibody fragments such as scFv and dAb fragments. Preferably, the antibody is a specific antibody, in the sense that it binds the particular polypeptide but does not bind substantially to other polypeptides from the mycoplasma from which is may be obtained.

The antibody of the invention may be packaged and presented for use in medicine. Typically the antibody is prepared as a sterile formulation, with appropriate pharmaceutical excipients, if required.

A twelfth aspect of the invention provides a method of passively immunising a cat against haemotropic mycoplasma infection, the method comprising administering to the cat an antibody according to the eleventh aspect of the invention. A sufficient amount of antibody is administered so as to provide protection against subsequent infection with mycoplasma. Following administration of antibody the animal would be challenged with infective doses of the selected haemoplasma at various time points, then monitored for haemoplasma infection (clinical signs, haematological parameters and blood haemoplasma copy numbers).

The invention also includes an antibody according to the eleventh aspect of the invention for passively immunising a cat against haemotropic mycoplasma infection. The invention also includes the use of an antibody according to the eleventh aspect of the invention in the manufacture of a medicament for passively immunising a cat against haemotropic mycoplasma infection.

It will be appreciated that more than one type of antibody of the invention may be administered either together or sequentially.

It will be appreciated that cats that have been infected with haemotropic mycoplasma are likely to contain antibodies directed at immunogenic polypeptides of the infecting haemotropic mycoplasma. They may also contain some of the immunogenic haemotropic mycoplasma polypeptides themselves.

If the cat is diagnosed with haemotropic mycoplasma infection, typically it will be treated using antibiotics such as tetracyclines or fluoroquinolones. Supportive care may be given, such as fluid therapy if dehydrated, blood transfusion, or haemoglobin carrier solutions if severely anaemic. Appetite stimulation or nutritional support may be given if the cat is anorexic.

A thirteenth aspect of the invention provides a method of detecting whether a cat has been exposed to haemotropic mycoplasma infection, the method comprising determining whether a suitable sample from the cat is reactive with a polypeptide according to any of the first, second, third, fourth or fifth aspects of the invention. Typically, the sample is a fluid sample from the cat which is known to contain antibodies. Suitable samples include whole blood, plasma, serum, or saliva. Typically, the method comprises detecting the presence of antibodies in the sample which are reactive towards the polypeptide. Suitable methods for determining the presence of antibodies are known in the art, for example ELISA methods (enzyme-linked immunosorbent assay) as is discussed in more detail below.

A fourteenth aspect of the invention provides a method of detecting whether a cat has been exposed to haemotropic mycoplasma infection, the method comprising determining whether a sample from the cat contains one or more polypeptides according to the first, second, third, fourth or fifth aspects of the invention. Suitable samples include whole blood, plasma, serum, urine, tissues, faeces, or saliva.

Antibody-based methods useful for detecting polypeptides in a sample include immunoassays, such as the enzyme linked immunosorbent assay (ELISA), western-blot and the radioimmunoassay (RIA). For example, a polypeptide-reactive monoclonal antibody can be used both as an immunoadsorbent and as an enzyme-labeled probe to detect and quantify the polypeptide. The amount of polypeptide present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. Such an ELISA for detecting a tumour antigen is described in Lacobelli *et al*, *Breast Cancer Research and Treatment* 11: 19-30 (1988). In another ELISA assay, two distinct specific monoclonal antibodies can be used to detect polypeptide in a body fluid. In this assay, one of the antibodies is used as the immunoadsorbent and the other as the enzyme-labeled probe.

The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting a sample potentially containing the polypeptide with immobilized antibody and, without washing, contacting the mixture with the labeled antibody. The "two-step" assay involves washing before contacting the mixture with the labeled antibody. Other conventional methods may also be employed as suitable. It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed from the sample.

Suitable enzyme labels include, for example, those from the oxidase group, which catalyze the production of hydrogen peroxide by reacting with substrate. Glucose oxidase is particularly preferred as it has good stability and its substrate (glucose) is readily available. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labeled antibody/substrate reaction. Besides enzymes, other suitable labels include radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulfur ^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99}mTc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

It will be appreciated that detection of the polypeptide of the first aspect of the invention (Mhf EF-Ts), or an antibody thereto, in a sample is indicative of infection with Mhf.

It will be appreciated that detection of the polypeptide of the second aspect of the invention (Mhf pgk or CMhm pgk), or an antibody thereto, in a sample is indicative of infection with Mhf or CMhm.

It will be appreciated that detection of the polypeptide of the third aspect of the invention (Mhf gapA or CMhm gapA), or an antibody thereto, in a sample is indicative of infection with Mhf or CMhm. As is described in more detail in Example 2 below, in this embodiment it is possible to distinguish between infection with Mhf and CMhm since Mhf gapA polypeptide is reactive against serum from feline post-Mhf infection but not reactive against serum from feline post-CMhm infection. In contrast, CMhm gapA polypeptide is reactive against serum from feline post-CMhm infection but not reactive against serum from feline post-Mhf infection. Differentiation of infecting species is important as Mhf is pathogenic, resulting in significant disease during acute infection, whilst CMhm less frequently results in disease, but is less readily cleared from the infected animal. Animals infected with Mhf would most likely require antibiosis and supportive therapy (e.g. fluid therapy including blood transfusions, appetite stimulation) whilst those infected with CMhm rarely require therapy in the absence of concurrent disease unless attempted clearance of infection is desired.

It will be appreciated that detection of the polypeptide of the fourth aspect of the invention (CMhm dnaK), or an antibody thereto, in a sample is indicative of infection with CMhm.

It will be appreciated that detection of the polypeptide of the fifth aspect of the invention (Mhf dnaK), or an antibody thereto, in a sample is indicative of infection with Mhf or CMhm.

It will be appreciated that the methods of the thirteenth and fourteenth aspects of the invention may be considered to be methods of diagnosing whether a cat is or has been infected with haemotropic mycoplasma.

The invention also includes an immunosorbent assay for detecting anti-mycoplasma protein antibodies in a sample, the assay comprising a solid phase coated with a polypeptide according to any of the first, second, third, fourth or fifth aspects of the invention wherein the anti-mycoplasma protein antibodies in a sample exposed to the solid phase will bind to the polypeptide, and a detectable label conjugate which will bind to the anti-mycoplasma protein antibodies bound to the solid phase.

The invention also includes a solid substrate with a polypeptide according to any of the first, second, third, fourth or fifth aspects of the invention attached thereto.

Typically, the solid phase or solid substrate is a microtitre well or plate.

Preferably, the conjugate comprises an anti-cat antibody. Preferably, the conjugate comprises a detectable moiety such as an enzyme, for example horseradish peroxidise or alkaline phosphatase. Preferably, the immunosorbent assay also contains a substrate for the enzyme.

The assay comprises a solid phase coated with a suitable polypeptide (such as one of the polypeptides of the first or second or third or fourth or fifth aspect of the invention, or a mycoplasma EF-Ts polypeptide, wherein anti-mycoplasma antibodies in a sample exposed to the solid phase will bind to the polypeptide; and a detectable label conjugate which will bind to the anti-CRCV antibodies bound to the solid phase.

It is appreciated that an antigenic fragment of the polypeptide that coats the solid phase is of sufficient size to be bound by an anti-mycoplasma antibody.

Preferably, the haemotropic mycoplasma polypeptide, or antigenic variant or fragment thereof, that coats the solid phase is at least 10 amino acids in length. More preferably, the haemotropic mycoplasma polypeptide, or antigenic variant or fragment thereof, is at least 20, or at least 30, or at least 40, or at least 50, or at least 100 amino acids in length.

The invention also includes a kit of parts which include the components of the immunosorbent assays. The kit of parts may thus include a solid phase such as a microtitre plate, the appropriate haemotropic mycoplasma protein for coating the solid phase, a detectable label conjugate, such as an anti-cat antibody, which will bind to anti-mycoplasma antibodies bound to the solid phase. If the detectable label conjugate is an enzyme, the kit of parts may also include a substrate for the enzyme. The kit may also include a positive control sample that contains an anti-mycoplasma antibody, such as those described with reference to the eleventh aspect of the invention, and a negative control sample.

Typically, protein is coated on microtitre plates overnight at 4°C to 37°C, depending on the stability of the antigen. Unbound protein is washed off with a wash buffer such as phosphate buffered saline or Tris buffered saline. Serum or other samples are incubated on the plate, typically at 37°C for between 1 and several hours. Unbound material is washed off, the plates are incubated with enzyme-labelled (e.g. horseradish peroxidase or alkaline phosphatase) antibody, such as anti-host mammal (such as ant-feline) IgG or

IgM for plasma/whole blood/serum samples, or anti-host mammal (such as anti-feline) IgA for saliva, for 1 to several hours at 37°C. Unbound antibody is washed off and plates are incubated with a substrate such as OPD for about 10 min where horseradish peroxidase was used, or p-nitrophenyl phosphate for about 75 min where alkaline phosphatase was used, and the optical density measured in a photometer.

It will be appreciated that in the vaccination methods, immunological detection methods, kits of parts and solid substrates of the invention that the polypeptide is a native haemotropic mycoplasma polypeptide.

The invention will now be described by reference to the following non-limiting Examples and Figures wherein:

Figure 1 Western blot of RBC membrane antigen (R) and Mhf antigen (Hf) probed with plasma samples (1:250) collected pre-infection (-7 and 0) and post-infection (15, 29, 43, 57, 71, 85, 99, 111, 125, 139 and 153 DPI) from cat HF4. The calculated molecular mass for the major bands (kDa) are shown. The open triangle corresponds to the 71-72kDa band 4.2 protein identified in both RBC and Mhf antigen preparations.

Figure 2 A Comassie blue-stained PAGE gel (A) of RBC membrane antigen (R) and a Western blot (B) of a pooled plasma sample (P: days -7 and 0, all cats) and RBC membrane antigen (R) probed with the pooled plasma sample (1:250). The molecular mass (kDa) of each protein standard (S) is given on the left side. The calculated molecular mass (kDa) of each identified band is given on the right side with the likely RBC membrane proteins corresponding to the bands (3, 24). Three bands (*) were detected in the plasma sample (P) incubated with the secondary antibody alone and correspond to immunoglobulin light chains (29kDa), γ heavy chain of IgG (54kDa) and a faint band corresponding to μ heavy chain of IgM (75kDa).

Figure 3 Amino-acid alignment of Mhf dnaK (SEQ ID No 2) sequence (A) and the Mhf dnaK fragment (Accession number ACO07298) published by Museux and others (2009)(B) (SEQ ID No 32).

Figure 4 Two-dimensional electrophoresis of Mhf protein stained with Sypro Ruby. Immunogenic spots picked for mass-spectrometry indicated. Proteins were separated initially according to isoelectric point on a pH3-11NL IPG strip (horizontal),

then according to mass on a 8% poly-acrylamide gel (vertical; Precision Plus Protein standard on right hand side – major bands 75, 50, 25 kDa indicated).

Figure 5 Mhf protein preparation separated by two-dimensional electrophoresis and western blotted with post-infection plasma from two Mhf infected cats. The pH orientation and three molecular mass markers are indicated. The three matched spots are indicated in white.

Spot 12 = migrated at ~30kDa, 2 peaks (1911.9 and 1256.6 Da – including ion-spray) matched Mhf elongation factor-thermostable (predicted size 30.8kDa, pI: 5.94).

Spot 16 = migrated at ~45kDa, 4 peaks (2154.0, 1562.9, 1057.6, and 1012.6 Da – including ion spray) matched Mhf phosphoglycerate kinase. This gave a score of 97 (>20=significant)

Spot 23 = migrated 80kDa 2 peaks (1581.8 and 1744.9Da – including ion spray) matched Mhf heat shock protein 70 (*dnaK*) (predicted size 65.6kDa, pI: 5.02). This gave a score of 77.

Figure 6 Recombinant Mhf dnaK (rMhf dnaK) ELISA optical density results for plasma taken immediately prior to, and during, acute Mhf infection from cats HF6 (A) and HF7 (B). Prior to infection no reactions towards rMhf dnaK were detected. At 8 days post-infection the HF6 cat demonstrated a weak reaction to rMhf dnaK, and by days 15 to 29 post-infection both cats showed a strong reaction to rMhf dnaK.

Figure 7 Recombinant Mhf dnaK (rMhf dnaK) ELISA optical density results for plasma taken immediately prior to, and during, acute CMhm infection (HM2). Prior to infection no reactions towards rMhf dnaK were detected. At 29 days post-infection the cat demonstrated a weak reaction to rMhf dnaK, which decreased over the following 42 days.

Example 1: Antigen Specificity of the Humoral Immune Response to *Mycoplasma haemofelis* Infection

The aim of this study was to characterise the antigenic specificity of the humoral immune response made by cats infected with the feline haemoplasma, *Mycoplasma haemofelis* (Mhf). A crude Mhf antigen preparation was prepared from red blood cells (RBCs)

collected from a cat at a time of high bacteraemia. Plasma samples were collected from six cats pre- and post-experimental infection with Mhf, with regular sampling performed from 15 to 149 or 153 days post-infection (DPI). Pre-infection RBC membrane ghosts were prepared from these six cats, and used to identify erythrocyte proteins that may have contaminated the Mhf antigen preparation.

The Mhf antigen preparation comprised 11 protein bands. The immunodominant bands on western blotting with infected cat plasma were of molecular mass 78, 68, 60, 48 and 38kDa. Most cats (n=5) had plasma antibody that reacted with at least one band (always including 68kDa) at 15 DPI and all cats were seroreactive by 29 DPI. The maximum number of antibody specificities from an individual animal was identified in plasma collected between 57 and 99 DPI. Contamination of the Mhf antigen preparation with RBC membrane proteins was observed. The contaminating RBC proteins were of molecular mass 71-72kDa (consistent with band 4.2) and 261 and 238kDa (consistent with spectrin) and these were recognised by all plasma samples. A range of Mhf antigens are recognised by cats infected experimentally with the organism. These represent possible targets for immunoassays, but care must be taken to prevent false positive results due to host protein contamination.

We have recently investigated the kinetics and outcomes of experimental infection with each of the feline haemoplasma species, including Mhf (Tasker and others 2009a; Tasker and others 2009a). As part of those studies, plasma samples were collected for 22 weeks following Mhf infection, to enable more extensive characterisation of proteins recognised by the immune system of infected cats and to attempt to determine whether autoantibodies specific for erythrocyte membrane antigens were induced by the infection.

Materials and Methods:

Cats and Samples

Seven barrier-maintained, SPF derived domestic-shorthaired cats (7-month-old (n=6), 2-years-old (n=1); four neutered males and three entire females) were used in this study. All cats were infected experimentally with Mhf and details of the experimental procedures, inoculating doses administered and infection monitoring procedures have been described previously (Tasker and others 2009a; Tasker and others 2009b). The cats were designated as Cats HF1, HF2, HF4, HF6, HF8, HF12 and HF14, with the discontinuous numbering being due to the inclusion of additional cats in the parallel studies mentioned previously (Tasker and others 2009a; Tasker and others 2009b). Briefly, infection of the cats was carried out by obtaining heparinised blood from barrier-

maintained donor cats chronically infected with Mhf. The blood type of the donor cats and all recipients was predetermined to be Type A (RapidVet-H blood typing cards, DMS Laboratories Inc, New Jersey, US). Fresh blood was collected from donors and injected intravenously into the recipients. These inocula comprised 2 ml of heparinised blood (infective dose HF14: 7.21×10^7, others: 1.55×10^8 Mhf copies) given via pre-placed cephalic intravenous catheters within 5 min of collection from the donors. All procedures and experiments described were undertaken under a project license approved under the UK Animals (Scientific Procedures) Act 1986.

As described previously (Tasker and others 2009a; Tasker and others 2009b), EDTA-anticoagulated blood samples were regularly collected from all cats and subjected to real-time PCR (qPCR) to confirm that Mhf infection was absent before inoculation, and present post-infection (Tasker and others 2009a; Tasker and others 2009b). Seven days before infection (day -7), on the day of infection (day 0), and approximately every 2 weeks thereafter, until day 149 (HF2) or 153 (HF1, 4, 6, 8 and 12), an additional 1 ml volume of EDTA anticoagulated blood was collected from each cat, centrifuged (2200 × *g*, 3 min) and the plasma removed and stored at -20°C until use. A pooled plasma sample was made by mixing an equal volume of the pre-infection plasma samples collected during the study. Plasma samples, from EDTA blood (the RBC were used for other purposes e.g. Coombs' test), were used in order to minimize the total volume of blood collected from the animals and to comply with the terms of the studies' project license.

Antigen Preparation

M. haemofelis

Whole blood was obtained from cat HF14 at a time of high Mhf blood copy number as determined by qPCR ($4x10^9$ Mhf copies/ml, 11 days post-infection [DPI]) (Tasker and others 2009a). At the time of euthanasia, once deep general anaesthesia had been induced with intravenous pentobarbitone, approximately 100mls of blood was collected into Alsever's solution (1:1 v/v) (Sigma-Aldrich Ltd, Poole, Dorset, UK) by cardiac puncture prior to completion of the anaesthetic overdose. Mhf was isolated from the blood using the methods described by Hoelzle et al. (2003; 2006). Briefly, RBCs were sedimented by centrifugation at 600g for 10 min, plasma and buffy coat were aspirated, and the RBCs were washed and centrifuged in an equal volume of phosphate-buffered saline (PBS; 0.15M, pH 7.4). The PBS and remaining buffy coat was aspirated and the wash repeated twice more. The final packed, washed RBC were incubated in PBS containing 0.15% Tween-20 and 3% (w/v) EDTA, and incubated for 30 min at ambient

temperature, in a vertical shaker (speed 150rpm) to dislodge the Mhf. Debris and erythrocytes were pelleted by centrifugation at 600g for 10 min and the resultant supernatant was centrifuged at 40,000g for 30 min at 4°C to pellet the Mhf. The supernatant was removed, the pellet resuspended in 1ml PBS and centrifuged through 20% sodium diatrozoat meglumine and diatrozoat sodium (76% Urografin; Bayer plc, Newbury, Berkshire, UK) at 15,000rpm for 40min at 4°C, and the final pellet was resuspended in 1.0 ml PBS.

The resulting Mhf antigen preparation was depleted of any contaminating albumin or IgG using the ProteoExtract Albumin/IgG Removal Kit (Calbiochem, Merck Chemicals Ltd., Nottingham, UK) according to the manufacturer's instructions. The flow-through and the wash from the columns were added to a 20K iCON concentrator (Pierce Biotechnology, Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) and centrifuged at 6,000g for 1 hour. The protein concentration in the retained solution (approximately 1ml) was measured using a Qubit (Invitrogen Ltd., Paisley, Scotland) before storage at -80°.

A similar procedure was attempted using blood from cats infected with CMhm ($4.6x10^7$ CMhm copies/ml, 21 DPI) and CMt ($1x10^5$ CMt copies/ml, 17 DPI) but insufficient antigen was recovered, even when blood was collected at peak haemoplasma copy number for these species (unpublished observations).

RBC Membrane *Ghosts*

RBC membrane ghosts were prepared from 1ml EDTA anticoagulated blood from the six plasma source cats (HF1, 2, 4, 6, 8 and 12), prior to infection, using the methods described by Barker (Barker 1991). Briefly, RBCs from 1ml EDTA anticoagulated blood were washed six times in 10 volumes of PBS with aspiration of the supernatant and buffy-coat between washes. RBCs were lysed by adding 10 volumes of ice-cold lysis buffer (20mM Tris, pH 7.6) and pelleted by centrifugation at 40,000g for 30min at 4°C. The supernatant and opaque buttons of residual leucocytes were removed, using a pipette, leaving the translucent RBC membranes. The membranes were washed a minimum of four times in ice-cold lysis buffer until the supernatant was clear of haemoglobin. The concentration of protein in the final solution was measured by Qubit and stored at -80°C prior to use.

PAGE and Western Blot Analysis

Polyacrylamide gel electrophoresis (PAGE) was performed using the NuPAGE electrophoresis system (Invitrogen Ltd., Paisley, UK) under reducing conditions and

proteins were transferred to nitrocellulose membranes using the XCell mini-cell and Blot Module (Invitrogen, Paisley, UK) as per the manufacturer's instructions. NuPAGE Novex 4-12% Bis-Tris precast gels, NuPAGE MOPS running buffer, NuPAGE LDS sample buffer (all Invitrogen Ltd) and Mhf or RBC membrane antigen (20µg per well) were used for the investigation of the humoral immune responses. Protein transfer was performed using the NuPAGE transfer buffer (Invitrogen) and a pre-stained molecular weight standard (All Blue Precision Plus Protein Standard, Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) was included on each gel to monitor transfer efficiency and to allow calculation of molecular mass. The NuPAGE Large Protein Blotting Kit (Invitrogen Ltd.), which included 3-8% tris-acetate gels, the HiMark Pre-Stained High Molecular Weight Protein Standard and 0. 45 µm nitrocellulose membranes, was used to further investigate antigenic proteins in the RBC membrane preparations, as this gave better separation of the high molecular weight proteins.

After transfer, nitrocellulose membranes were blocked with 5% (w/v) non-fat milk in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH-7.6) for 2 hours. Membranes were then cut in to strips, each including a lane of Mhf antigen and RBC membrane ghosts from the individual cat. The strips were probed overnight with 1 in 250 dilution of plasma (D-7, 0, 15, 29, 43, 57, 71, 85, 99, 111, 125, 139 and 149 or 153) in TBST + 5% non-fat milk. After washing, binding of plasma antibody was identified by use of an alkaline phosphatase conjugated goat anti-cat IgG H+L Fab' antibody (Jackson ImmunoResearch Europe Ltd., Newmarket, Suffolk, UK) diluted 1 in 20,000 in TBST + 5% non-fat milk and applied for 2 hours. The membranes were washed three times with TBST between steps and all incubations were at room temperature on a rocking platform. Following the final washes, visualisation was achieved using Lumi-Phos WB Chemiluminescent Substrate (Pierce Biotechnology) and exposure to Kodak Bio-max Film (Sigma-Aldrich, Poole, Dorset, UK) for 5 min.

A negative control, omitting incubation with a plasma sample, was performed to control for reactivity between secondary antibody and the Mhf antigen and RBC membrane ghosts. The pooled plasma sample (10µl of 1:100 dilution), incubated with the secondary antibody, was used to identify bands resulting from plasma protein contamination of the antigen preparations.

Images of the western blots were captured using a document scanner and analysed using the TotalLab TL100 software (Nonlinear Dynamics Limited, Newcastle upon Tyne,

UK) in order to allow for correction of gel artefacts and comparison of banding patterns obtained from antigen preparations and different plasma samples.

Results:

Western blots were performed with RBC membrane ghosts and Mhf antigen preparations using plasma samples collected from 6 cats infected with MHf between 0 and 149 or 153 DPI. Antibodies were induced with specificity for 12 different bands in the Mhf antigen preparation (97, 93, 78, 72, 68, 60, 54, 53, 48, 38, 35 and 29-25kDa) and for a 71-72kDa band in the RBC membrane ghosts (Figure 1). The antibody specific for the 71-72kDa protein identified in the RBC ghosts and the Mhf antigen preparations was present in plasma collected pre- (days -7 and 0) and post-infection, although the intensity of this band varied when plasma samples taken at different time points were compared for the same animal. This band was not considered to be of Mhf origin and was excluded from the analysis of the immune response to Mhf. None of the 12 pre-infection plasma samples contained antibody to any of the other 11 Mhf bands. No protein bands were identified when the secondary antibody alone was incubated with the antigen preparations (data not shown).

Five of the six cats had developed antibody to at least one Mhf band (which always included the 68kDa band) at 15 DPI, and all six cats were had seroconverted by 29 DPI (Table 1). The maximum number of Mhf bands recognised by individual cats ranged from 8 to 11, with the 78, 68, 60, 48 and 38kDa bands recognised by plasma antibodies from all cats. These same five bands were those that tended to be recognised first. The maximum number of Mhf bands recognised by an individual was in samples collected between 57 and 99 DPI. There was some variation in band intensity with individual plasma samples after this time but this was not related to any changes in the infection kinetics or clinical parameters in that particular animal (Table 1). The band between 29 and 25kDa appeared as a single broad band in the majority of cats at most time points, but one cat had reactivity initially (15 DPI) at 29kDa only (Figure 1) and another had distinct bands within the 29 and 25kDa range at 15 DPI.

Table 1: Mhf antigens recognised during the humoral immune response in 6 Cats infected experimentally with the organism

	Number of animals reacting to individual bands at

	indicated DPI												
Calculated molecular mass of antigen band	**-7**	**0**	**15**	**29**	**43**	**57**	**71**	**85**	**99**	**111**	**125**	**139**	**153**
97kDa	0	0	0	1	2	4	4	4	3	4	3	4	4
93kDa	0	0	0	0	0	2	2	2	2	2	2	2	2
78kDa*	0	0	4	5	5	6	6	5	6	5	5	6	6
68kDa*	0	0	5	6	6	6	6	6	6	6	6	6	6
60kDa*	0	0	4	6	6	6	6	6	5	6	5	5	5
54kDa	0	0	1	1	3	4	3	3	4	6	6	6	6
53kDa	0	0	0	0	1	1	1	1	1	1	1	1	1
48kDa*	0	0	4	4	5	6	6	6	6	6	6	6	6
38kDa*	0	0	4	5	5	6	6	6	6	6	6	6	6
35kDa	0	0	0	0	0	4	3	4	4	4	5	5	5
29kDa 25kDa	0	0	1	6	6	6	6	6	6	6	6	6	6
Average number of bands detected	0	0	3.8	5.7	6.5	8.5	8.2	8.2	8.2	8.7	8.5	8.8	8.8

* These bands were recognised by all cats and tended to be recognised first, and were thus considered the major antigenic determinants.

The nature of the 71-72kDa band identified in the Mhf and RBC membrane antigen preparations by both pre-infection and post-infection plasma was further examined by comparison of feline RBC membrane proteins separated by PAGE and stained with Coomasie blue; a western blot of RBC membrane proteins probed with a pool of the plasma samples used in the study; and the pooled plasma sample probed with the secondary antibody (Figure 2). Bands at 54kDa and 29kDa were identified in the pooled plasma by the secondary antibody, corresponding to gamma heavy chain of IgG and immunoglobulin light chains. In addition, a faint band was present at 75kDa, which likely represents some cross-reactivity with the μ heavy chain of IgM. These bands were in contrast to the three bands (261, 238 and 71kDa) detected in the RBC membrane antigen preparation probed with the plasma pool (Figure 2) and the 78 individual plasma samples used in the study (data not shown). The high molecular mass bands (261 and 238) likely corresponded to α and β-spectrin bands that were visible on the Coomassie blue-stained RBC membrane ghost gel (Figure 2). The band at 71-72kDa did not correspond to the faint 75kDa band detected in the plasma pool but was the same weight as band 4.2 in the Coomassie blue-stained gel.

Discussion:

This study has characterised the specificity of the plasma antibody response to Mhf proteins made by cats infected experimentally with Mhf. This humoral immune response was monitored for 22 weeks following infection. To date, Mhf has not been cultured *in vitro* and the only means of producing antigen for such studies is by *in vivo* amplification and isolation from the blood of an infected cat (Alleman and others). Similar techniques have been applied to the preparation of antigen from Msu (Hoelze and others 2003; Hoelze andf others 2006) and *M. haemomuris* (Rikihisa and others 1997). Contamination of Mhf antigen preparations with host blood-derived proteins is a recognized problem and such contaminants may originate from the cellular components (RBC and white blood cells) and the plasma. In order to maximise the amount of antigen recovered, blood was collected from an experimentally infected cat at the time of presumptive peak Mhf copy number (11 DPI), and before the onset of anaemia and Coombs' test positivity (Tasker and others 2009b). Blood collected after the onset of anaemia and Coombs' positivity was susceptible to severe haemolysis during the isolation procedure, resulting in haemoglobin and RBC membrane contamination of the Mhf preparations (unpublished observations). An albumin and IgG depletion step was included in the Mhf antigen preparation procedure as immunoglobulin contamination of Msu preparations has been reported (Hoelze and others 2006). Bands consistent with the heavy and light chains from IgG were not detected when the Mhf antigen preparation

was incubated with the secondary antibody alone. The present study has used plasma harvested from EDTA blood samples collected for other assays rather than serum, in order to minimize the volume of blood collected from the animals. No problems were recognised with the use of plasma rather than serum in the western blotting procedure.

A 71-72kDa protein band was detected in the Mhf and RBC membrane antigen preparations by all pre and post-infection plasma samples, but was not detected when the secondary antibody was used alone. This protein band has molecular mass equivalent to the erythrocyte protein band 4.2 (Korsgren and others 1990; Steck and others 1974), and correlated with band 4.2 identified on a Commassie blue-stained PAGE gel of RBC membrane proteins. This band was not of a size that would be consistent with immunoglobulin light chains or γ or μ heavy chains identified in the blot of the pooled plasma sample. Therefore, reactivity involving the 71-72kDa band was considered to be the result of contamination of the Mhf antigen preparation by host erythrocyte proteins. These cats also had plasma antibody reactive with α and β-spectrin in the RBC membrane ghost preparations and this antibody was present both before and after Mhf infection. These bands were intermittently visible in the western blots with the Mhf antigen preparation, presumably due to their high molecular weight and inconsistent transfer when the standard blotting system was used. Naturally occurring autoantibodies to erythrocyte cytoskeleton proteins, including the spectrins, have been reported in a number of species including man (Ballas 1989; Lutz and Wipf 1982) and dogs (Barker and others 1991). The human anti-spectrin antibodies have been shown to cross-react with a protein migrating to an equivalent position as band 4.2 (Lutz and Wipf 1982). These physiological autoantibodies specific for erythrocyte proteins have a role in the removal of damaged and senescent RBC (reviewed by Kay 2005).

The plasma of all cats contained antibody reactive with a broad band of molecular mass 25-29kDa. This band appeared to be made up of a number of individual bands of similar size when two of the plasma samples collected at 15 DPI were used, but was present as a single band with all other positive plasma samples. A similar band was present in previous studies of antibody responses to Mhf (Alleman and others 1999) and Msu (Hoelze and others 2006) and corresponds to the immunoglobulin-containing area identified by two-dimensional SDS-PAGE/western blots of Msu (Hoelze and others 2007A). This broad protein band may therefore be composed of host-derived proteins. The size of this protein would be consistent with that of immunoglobulin light chain, but

the band was not recognized by the secondary antibody alone. Further work is required to determine the identity of this band.

The major antigenic determinants of Mhf, to which infected cats respond serologically, were of molecular mass 78, 68, 60, 48 and 38kDa. All cats produced antibodies reactive with all of these bands by 57 DPI. A previous study found significant reactivity to Mhf determinants of molecular mass 150, 52 and 14 kDa by 30 DPI (Alleman and others 1999). Antibodies reactive with Mhf proteins of 53 and 54kDa were found in the plasma of cats in the present study, but the former specificity was only identified in one cat and the latter was seen in only four cats by 57 DPI, which is the time point closest to the last sample (60 DPI) analysed in the study by Alleman *et al.*

Mice infected with *M. haemomuris* develop serum antibodies to *Mycoplasma* determinants of molecular mass 118, 65, 53, 45, and 40 kDa at 42 DPI (Rikihisa and others 1997). Pigs infected with Msu produce antibodies reactive with major MSu proteins of 70, 45 and 40kDa and minor proteins of 83, 73, 61, 57, and 33kDa. In these pigs, antibody specific for the major bands was present as early as 7 DPI and was well-established by 14 DPI (Hoelze and others 2006). The variation in the molecular mass of the Mhf determinants detected in our study compared with those from previous studies may be due to the experimental methods used or the haemoplasma species investigated. For instance, the major determinants described in the Msu study (70, 45 and 40kDa) may correspond to Mhf determinants of 68, 48 and 38kDa described in the present study, but use of a different acrylamide gel concentration (4-12% gradient vs 10%) and buffer system in the two studies may have resulted in slightly different migration rates, affecting the calculation of molecular mass. Alternatively, these differences may reflect variations between the *Mycoplasma* species as they reside in different clades within the haemoplasmas as determined by 16S rDNA and RNaseP phylogenetic analysis (Johansson and others 1999; Messick and others 2002; Neimark and others 2001; Peters and others 2008; Tasker and other 2003).

Later studies of the immune response to Msu identified the 70kDa protein (HspA1) as a DnaK-like protein (Hoelze and others 2007b) and the 40kDa protein (MSG1) as a surface-localised adhesion protein with properties similar to glyceraldehyde-3-phosphate dehydrogenase (Hoelze and others 2007c). These proteins have been evaluated for use in diagnostic immunoassays (Hoelze and others 2007b) and the latter as a potential vaccine candidate (Hoelze and others 2009). A recent study investigating CMt infection found that the majority of cats seroconverted 3 to 4 weeks following infection to a

recombinant, partial Mhf MSG1 protein by western blotting (Museux and others 2009). In addition, this study demonstrated reactivity to this recombinant protein in plasma collected from a cat infected with Mhf at 6 weeks post-infection and one infected with CMhm at 21weeks post-infection. Therefore, the results of that study agree with the present findings as five of the cats reported here seroconverted to the 68kDa protein by 29 DPI.

The cats utilised in the present study for the western blots, developed anaemia with positive Coombs' test results from 15 to 50 DPI (Tasker and others 2009b). There was no correlation between the Mhf antigenic determinants recognised and the development and subsequent disappearance of RBC-associated antibody. The first occasion on which the Coombs' test was positive correlated with the day on which the first plasma sample was collected for the analysis described in this study. Disappearance of the RBC-associated antibody did not correlate with the development or loss of reactivity to any particular Mhf antigenic determinant.

The results of the present study demonstrate that a range of *Mycoplasma* and erythrocyte antigenic determinants are recognised by circulating antibodies in cats with Mhf infection. The autoantibodies directed against the RBC membrane proteins α and β spectrin and the protein equivalent to band 4.2 were present in both pre-infection and post-infection samples. These likely represent physiological autoantibodies involved in the removal of senescent erythrocytes. It is of note that cats only become Coombs' test positive post-infection. It remains to be determined whether the erythrocyte-bound antibody (as opposed to the circulating antibody investigated here) is specific for surface Mhf antigens or erythrocyte autoantigens and whether the antigenic specificity of antibody eluted from the erythrocytes of Mhf-infected cats differs from that of the circulating antibody pool.

Example 2: Identification of antigens specific for the humoral immune response to Mhf, CMhm and CMt infection

Materials & Methods

Sources of haemoplasma DNA, haemoplasma protein, host protein and pre- & post-immune plasma

All procedures and experiments described were undertaken under a project license approved under the Animals in Scientific Procedures Act, 1986. Additional data from these experiments has been described in published reports (Peters and others 2010, Tasker and others 2009a, Tasker and others 2009b).

As part of another study (Peters and others 2010) blood was taken from either Mhf or CMhm infected SPF cats during periods of maximum parasitaemia. The erythrocytes were separated by centrifugation and the haemoplasma organisms dislodged from the erythrocyte surface using EDTA/Tween. The haemoplasma organisms were then separated from the erythrocytes and debris by low speed centrifugation. The supernatant containing the haemoplasmas was then subjected to high-speed centrifugation. The high-speed supernatant was determined by qPCR to be rich in haemoplasma DNA and was utilised in the generation of both haemoplasma genome libraries. The high-speed pellet of haemoplasmas was subjected to differential gradient centrifugation, and subsequently concentrated using a 9 KDa molecular weight cut-off filter. A similar extraction procedure from uninfected erythrocyte membranes was also performed to obtain a negative control.

As part of another study EDTA anti-coagulated blood was taken from barrier maintained SPF cats prior to and weekly thereafter following experimental infection with Mhf, CMhm or CMt. Excess EDTA anti-coagulated blood from samples submitted to Langford Veterinary Services Diagnostic Laboratories (LVS-DL) for companion animal haemoplasma PCR were also collected. Blood was centrifuged and separated. Plasma was stored at -20°C until required.

Partial sequencing of the haemoplasma genome

Haemoplasma genomic DNA was purified using Macherey-Nagel NucleoSpin® Blood kits, from the haemoplasma DNA rich supernatant. Extracted DNA was sheared by nebulisation into 2 to 5 Kbp fragments, as determined by gel electrophoresis. These fragments were ligated into pCR®4Blunt-TOPO® and transformed into One Shot® Chemically Competent *Escherichia coli* (TOPO® Shotgun Subcloning Kit; Invitrogen). Purified plasmids from randomly selected clones were sequenced using dye-terminator chemistry with plasmid and custom primers. BLASTn analysis of nucleotide sequences and BLASTp analysis of the corresponding amino-acid sequences was performed to identify the origin of the insert sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Target specific primers were used in conjunction with plasmid primers to obtain contiguous sequence by PCR using Mhf and CMhm libraries as template.

Identification of Mhf immunogenic proteins using two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (2D SDS-PAGE)

Mhf organisms and un-infected erythrocyte membrane protein extracts were prepared for 2D SDS-PAGE using the Amersham 2D-clean up kit (GE Life Sciences). Proteins were initially separated according to isoelectric point on Immobiline™ DryStrip pH 3-11 NL (GE Life Sciences), then according to mass (12.5% polyacrylamide gel). Gels were either western blotted onto a polyvinylidene difluoride (PVDF) membrane or stained using Sypro® Ruby Protein Gel Stain (Invitrogen). Membranes were blocked by gentle agitation for 5 hours in tris-buffered saline (25mM Tris, 150mM NaCl) with Tween-20 (0.1% v/v) and 5% (w/v) non-fat milk (TBST-5M). Membranes were incubated overnight with test plasma (1:500 dilution of plasma from each of two cats taken at different time point: 'pre-infection' – from 7 and 1 days pre-infection; 'post-infection' – from 139 and 153 days post-infection) in TBST-5M. Washed membranes were incubated for 2 hours at room temperature in TBST-5M containing 1:20,000 dilution of alkaline phosphatase conjugated goat anti-cat IgG (H+L) antibody. Membranes were washed in Tris-buffered saline (25mM Tris, 150mM NaCl) with Tween-20 (0.1% v/v) (TBST) between steps (20 minutes; two wash changes; orbital shaker at 25rpm; room temperature). Following the final washes, visualisation was achieved using Lumi-Phos WB™ Chemiluminescent Substrate and exposure to Amersham Hyperfilm ECL™. From the Sypro® Ruby stained gel protein spots corresponding to spots of immunoreactivity were cut out for trypsin-digestion and analysed using mass-spectrometry (4700 MALDI-TOF/TOF Mass Spectrometer; Applied Biosystems). Results were compared to known Mhf sequences and to the MSDB protein database.

Generation of recombinant haemoplasma proteins

The Mhf *gapA*, Mhf *dnaK* and CMhm *gapA* protein coding sequences were cloned into pET101/D-TOPO® and expressed in BL21 Star™ (DE3) *E. coli* as fusion proteins with C-terminal His-tags (Champion™ pET Directional TOPO® Expression Kit, Invitrogen). Prior to cloning the CMhm *gapA* coding sequence was subjected to site directed mutagenesis to convert opal stop codons (TGA) to tryptophan codons (TGG). His-tagged proteins were purified using the Ni-NTA Spin Kit (QIAgen). Proteins were separated using one-dimensional gel electrophoresis (NuPAGE® Novex® Bis-Tris Gel System) and western blotted onto nitrocellulose. Product size was determined by comparison to a molecular weight ladder (Precision Plus Protein™ Standards; Bio-Rad). Expressed protein bands were excised from the NuPAGE gels and subjected to trypsin-digestion and mass-spectrometry analysis to confirm their identity.

Immunoblots of recombinant haemoplasma proteins

Western-blot membranes were blocked in TBST-5M for 2 hours. The membranes were probed overnight with 1:250 dilution of test plasma in TBST-5M. After washing, alkaline phosphatase conjugated goat anti-cat IgG (H+L) or anti-dog IgG antibody (Jackson ImmunoResearch Europe Ltd.) diluted 1:20,000 in TBST-5M were added and incubated for 2 hours. The membranes were washed three times with TBST between steps and all incubations were at room temperature on a rocking platform. Following the final washes, visualisation was achieved using Lumi-Phos WB Chemiluminescent Substrate (Pierce Biotechnology) and exposure to Amersham Hyperfilm ECL (GE Life Sciences).

Screening of feline plasma using an ELISA of recombinant Mhf dnaK

Vinyl Flat Bottom Microtiter® plates (Thermo Fisher Scientific) were coated with rMhf dnaK (14ng/well) in a volume of 100µl sodium carbonate buffer (0.05M; pH 9.6) and incubated overnight at 4°C. The plates were washed with PBS (137mM NaCl, 1.47 mM KH_2PO_4, 10mM Na_2HPO_4, 2.7mM KCl; pH 7.0)-0.05% (v/v) Tween 20 (PBST), blocked using PBST containing 10% (w/v) fat-free milk powder (Marvel, Premier Foods; PBST-10M) for 2 hours at room temperature under agitation and then washed with PBST. Wells were incubated with plasma (duplicates of 1:200, 1:400 and 1:800 dilutions in 100µl/well PBST-10M) for 2 hours at room temperature under agitation then washed with PBST. Wells were then incubated with either anti-cat IgG conjugated with alkaline phosphatase (heavy and light chains; Jackson ImmunoResearch) or anti-dog IgG conjugated with alkaline phosphatase (heavy and light chains; Sigma) at 1:10,000 dilution in 100µl/well PBST-10M for 2 hours at room temperature under agitation and then washed with PBST. Wells were then incubated with p-nitrophenyl phosphate (100µl/well; 1mg/ml in sodium carbonate buffer, 0.05M, pH9.6) in the dark. Absorbance at 405nm and 495nm (to control for inter-well variation) was measured using a computer-assisted microplate reader (Labsystems Multiscan Ex Primary EIA v2.1-0 with Genesis v3.0) at 30, 60 & 75 minutes. Each plate contained wells to which (i) no plasma was added (triplicate), (ii) haemoplasma negative SPF feline plasma (1:200, 1:400 and 1:800 dilutions) was added, (iii) duplicate two-fold serial dilutions of a strong positive feline plasma (1:200 to 1:409,600) was added. A serum sample was deemed positive if its OD value was greater than the OD value of the 1:51,200 dilution of the positive control (typically an OD of 0.100 at ~75min). Equivocal results were those that were close to, but not greater than, the OD of the 1:51,200 dilution of the positive control sample, and demonstrated consistently reducing ODs in the plasma dilutions.

Pre- and post-infection (8, 15, 22, and 29 days post-infection for Mhf, CMhm & CMt; additional 36, 43, 50, 57, 64, 71 days post-infection for CMhm & CMt) plasma was available for cats experimentally infected with feline haemoplasmas. Plasma from 205 feline clinical samples submitted to LVS-DL for haemoplasma qPCR were available.

Results

Mhf immunogenic proteins identified using 2D SDS-PAGE immunoblot

A number of spots were seen on the 2D post-infection immunoblot, but not the pre-infection immunoblot. Twenty one of these post-infection spots corresponded to visible protein spots on the Sypro® Ruby stained SDS-PAGE gel and could therefore be analysed by tandem mass-spectroscopy (Figure 4 shows a 2 dimensional SDS-PAGE gel stained with SYPRO-Ruby - spots that were analysed by mass-spectrometry are indicated; Figure 5 shows the three matched spots on a post-infection immunoblot). Of these spots three corresponded to the Mhf proteins dnaK, phosphoglycerate kinase (pgk), and elongation factor thermo-stable (EF-Ts). Due to the immunoreactivity of post-infection plasma to these three proteins they were classified as immunogenic.

Expression of recombinant haemoplasma proteins

From the Mhf and CMhm genomic DNA shot-gun libraries the whole coding sequences for Mhf dnaK and gapA, and CMhm gapA were identified. The CMhm gapA coding sequence was successfully subjected to site-directed mutagenesis prior to cloning and expression. All three proteins were successfully expressed, purified and their identities confirmed by the visualisation of protein bands of the predicted size on 1D SDS-PAGE (Mhf dnaK ~75KDa; Mhf gapA ~40KDa; CMhm gapA ~40KDa) and by mass-spectrometry.

Immunoblots of recombinant haemoplasma proteins

Summary of reactivity (positive ✓ or negative ×) to recombinant haemoplasma proteins by plasma from experimental cats pre and post-infection with Mhf (n=2), CMhm (n=1), or CMt (n=1), and by plasma from a dog naturally infected with Mhc.

Test Plasma	rMhf dnaK	rMhf gapA	rCMhm gapA
Feline pre-infection	×	×	×
Feline post-Mhf infection (n=2)	✓	✓	×

Feline post-CMhm infection (n=1)	✓	×	✓
Feline post-CMt infection (n=1)	✓	✓	×
Canine post-Mhc infection (n=1)	✓	✓	×

ELISA of recombinant Mhf dnaK

All experimental cats (n=16) were negative for anti-Mhf dnaK antibodies in pre-infection plasma samples. All cats (n=10) infected with Mhf showed a marked production of anti-Mhf dnaK antibodies on day 15 post-infection, with equivocal or weakly positive (n=2) and negative (n=8) results on day 8 post-infection (for example see Figure 6). Cats infected with CMhm (n=3) became weakly positive for anti-Mhf dnaK antibodies on days 22, 29 and 43 that persisted until at least 71 days post-infection (for example see Figure 7). Cats infected with CMt (n=3) became weakly positive (albeit greater than those to CMhm) for anti-Mhf dnaK antibodies on days 22 and 29 (n=2), antibody levels then decreased by 71 days post-infection, to equivocal/very weakly positive levels in two cats.

Of the clinical samples one was positive for Mhf alone by qPCR (threshold cycle: 16.5), whilst 20 were positive for CMhm alone (threshold cycles: 25.1-44.6; unknown in 1 case). None of the samples tested were positive for CMt and there were no dual haemoplasma infections. The Mhf positive cat was also positive for anti-Mhf dnaK antibodies. Of the 20 CMhm positive cats, 12 were positive for anti-Mhf dnaK antibodies, 2 were equivocal and 6 were negative. Of the 184 haemoplasma qPCR negative samples, 33 were positive for anti-Mhf dnaK antibodies, 10 were equivocal and the remainder (n=141) were negative.

Titre	**-ve**	**Equiv.**	**1:51,200**	**1:25,600**	**1:12,800**	**1:6,400**	**1:3,200**	**1:1,600**	**1:800**
Mhf qPCR +ve (n=1)				1					
CMhm qPCR +ve (n=20)	6	2	3	3		4		1	1
Haemoplasma qPCR -ve (n=184)	141	10	17	7	6	2	1		

Discussion

Two haemoplasma proteins, gapA and dnaK, have been identified and expressed one of which (dnaK) has been confirmed to be immunogenic in cats using a combination of 2D PAGE of crude whole haemoplasma preparation and immunoblotting. Anti-Mhf dnaK antibodies were detected using 1D immunoblots in cats experimentally infected with Mhf, whilst cross-reactive antibodies were detected in cats experimentally infected with CMhm and CMt and in a dog naturally infected with Mhc. This suggests that an assay using rMhf dnaK could be used to detect exposure to haemotropic mycoplasmas in companion animals. In contrast, anti-rCMhm gapA antibodies were detected in a cat (n=1) experimentally infected with CMhm, whilst anti-rMhf gapA and cross-reactive antibodies were detected in cats (Mhf n=2; CMhm n=1) and dog (Mhc) infected with haemofelis clade haemoplasmas. No cross-reactive antibodies were detected against recombinant gapA from differing haemoplasma clades. This suggests that recombinant gapAs could be used to determine clade of infecting haemoplasma species in companion animals.

A further two haemoplasma proteins, pgk and EF-Ts, have been identified as immunogenic ie they are recognised with Mhf post-infection serum from cats, indicating suitability for use in a vaccine and as a marker of haemotropic mycoplasma infection.

Example 3: DNA and amino acid sequences of identified immunogenic proteins

As part of another study (Peters and others 2010) blood was taken from either Mhf or CMhm infected SPF cats during periods of maximum parasitaemia. The erythrocytes were separated by centrifugation and the haemoplasma organisms dislodged from the erythrocyte surface using EDTA/Tween. The haemoplasma organisms were then separated from the erythrocytes and debris by low speed centrifugation. The supernatant containing the haemoplasmas was then subjected to high-speed centrifugation. The high-speed supernatant was determined by qPCR to be rich in haemoplasma DNA and was utilised in the generation of both haemoplasma genome libraries.

Haemoplasma genomic DNA was purified using Macherey-Nagel NucleoSpin® Blood kits, from the haemoplasma DNA rich supernatant. Extracted DNA was sheared by nebulisation into 2 to 5 Kbp fragments, as determined by gel electrophoresis. These fragments were ligated into pCR®4Blunt-TOPO® and transformed into One Shot®

Chemically Competent *E. coli* (TOPO® Shotgun Subcloning Kit; Invitrogen). Purified plasmids from randomly selected clones were sequenced using dye-terminator chemistry with plasmid and custom primers. BLASTn analysis of nucleotide sequences and BLASTp analysis of the corresponding amino-acid sequences was performed to identify the origin of the insert sequence (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Target specific primers were used in conjunction with plasmid primers to obtain contiguous sequence by PCR using Mhf and CMhm libraries as template.

Native haemoplasma genes are translated using the *Mycoplasma/Spiroplasma* code. Expressed (pET) genes are translated using the standard *E. coli* code.

Mhf genes

Heat shock protein 70 (*dnaK*)

Native gene sequence (SEQ ID No 1)

ATGTCCAAAAAAGAAACAATAATCGGTATCGACCTAGGAACCACTAACTCCTGTGTTGCT
ATCGTTGAAAATGGTAATCCTAAGATCTTAGAAACAAATGAGGGTAAGAGAACTATTCCC
TCTGTTGTTTCTTTCAAAGGTGATGAGATTATTGTTGGTGATAGCGCTAAGCGTCAGATG
GTTACCAACAAGGATACTATTGTATCTATAAAGAGATTAATAGGTACTGGTAAGAAGGTA
AAGGCTAGAGGTAAGGAGTACACTCCAGAGGAGATCTCTGCTTATATTCTTAAACACATA
AAGAAATATGCCGAAGACAAGTTAGGGCACTCCGTTTCCAAGGCGGTTATTACTGTTCCA
GCTTACTTTAACGACTCTGAACGTCAAGCAACTAAGAATGCCGGAACTATTGCTGGATTG
GAAGTTGTAAGAATAGTTAACGAACCTACTGCTGCCGCTCTTGCTTACGGTCTAGACCAC
AGCGAGAAGGAACAGAAGATATTGGTTTATGACTTGGGGGGAGGTACCTTTGACGTGTCT
GTCCTTGATATGTCAGATGGTACTTTTGAAGTATTGGCTACTTCTGGTGATAACCATTTG
GGTGGTGACGATTGGGATCAAGCGTTAATAGATTGGCTATTAGAGGAAATCAAGAAGGAA
CACTCTATAGATCTTTCTTCAGATAATTTAGCACTTCAAAGACTTAAGGATGCCGCTGAG
AAGGCAAAGATAGAGCTTTCTTCCGTTACTCAAACTCAAATACTCCTTCCGTTTCTTTCT
ATGGTGGGTGGACAACCACTTAACATAGATAAAGTTGTTACTCGTGTTCAGTTTGAATCT
TTAACTAAGCATTTAATTGAGAAGACTAGGAAGCCCTTCTTGGATGCTTTGAAGGAATCT
AAGTTATCTGCTTCCGATATAGATCAAATTCTGTTGGTGGGTGGTTCTACCCGTATGCCT
GCCGTTCAGGAGTTGGTTAAGAGCCTTTCCGGGAAGACACCTAACTTGTCTATTAATCCC
GATGAAGTTGTTGCTTTAGGTGCTTCCGTTCAAGGTGCTATTCTTGCTGGGGATATTAAA
GATATCCTGTTATTGGACGTTACGCCTTTAACTCTAAGTATTGAGACTTTAGGTGGTGTA
GCAACTCCTTTAATTAAGAGAAATACTACTGTTCCTGTTGAAAAGACTCAGGTCTTCTCT

ACAGCTGCAGACAACCAACCTTCTGTGGATATTCATGTTGTTCAAGGTGAGAGACCGATG
GCAAATCAAAATAAATCATTGGGAATCTTTACTCTAGATGGAATTCAACCTGCTCCTAAA
GGCGTTCCTCAAATTCAAGTTACCTTCTCTATTGATGCTAACGGTATTCTAAAGGTTAAA
GCTGAGGATAAAGGGACAGGTAAGAGTAACTCTATTACCATAAACCAATCTTCTGGTTTG
AGTGATGAGGAGATTCAAAGAATTATCAAGGAGGCTGAAGAGAATGCAGAGAAGGATCAG
AAAGCAAAGGAGGCTATAGAGGTTAAGAATGAAGCGCAGTCTTGGATCTCAATAGTTGAA
AAACAACTCTCTGAATCTAATGCGACTGATGAGCAGAAAGAATCTGCTCAGAAGATGGTT
GATGAATTGAAGCTTCTTATTAAAGATGAGAAGATTGAGGAGTTAAAACAGAAGATGGAT
GCGATTAAAACCTTGAGTCAGGATATGACTAAGTACGCACAGGATAATCCTAAAGAAGAG
AGTGAGGAGGTTAAGGAAGCGGAGGTAGTAGAAGAGGATAAGACTGAGGTAGATAAAACT
AAATCCTAA

Native amino-acid sequence (SEQ ID No 2)

MSKKETIIGIDLGTTNSCVAIVENGNPKILETNEGKRTIPSVVSFKGDEIIVGDSAKRQM
VTNKDTIVSIKRLIGTGKKVKARGKEYTPEEISAYILKHIKKYAEDKLGHSVSKAVITVP
AYFNDSERQATKNAGTIAGLEVVRIVNEPTAAALAYGLDHSEKEQKILVYDLGGGTFDVS
VLDMSDGTFEVLATSGDNHLGGDDWDQALIDWLLEEIKKEHSIDLSSDNLALQRLKDAAE
KAKIELSSVTQTQILLPFLSMVGGQPLNIDKVVTRVQFESLTKHLIEKTRKPFLDALKES
KLSASDIDQILLVGGSTRMPAVQELVKSLSGKTPNLSINPDEVVALGASVQGAILAGDIK
DILLLDVTPLTLSIETLGGVATPLIKRNTTVPVEKTQVFSTAADNQPSVDIHVVQGERPM
ANQNKSLGIFTLDGIQPAPKGVPQIQVTFSIDANGILKVKAEDKGTGKSNSITINQSSGL
SDEEIQRIIKEAEENAEKDQKAKEAIEVKNEAQSWISIVEKQLSESNATDEQKESAQKMV
DELKLLIKDEKIEELKQKMDAIKTLSQDMTKYAQDNPKEESEEVKEAEVVEEDKTEVDKT
KS

Expression (pET) gene sequence (SEQ ID No 3)

ATGTCCAAAAAAGAAACAATAATCGGTATCGACCTAGGAACCACTAACTCCTGTGTTGCT
ATCGTTGAAAATGGTAATCCTAAGATCTTAGAAACAAATGAGGGTAAGAGAACTATTCCC
TCTGTTGTTTCTTTCAAAGGTGATGAGATTATTGTTGGTGATAGCGCTAAGCGTCAGATG
GTTACCAACAAGGATACTATTGTATCTATAAAGAGATTAATAGGTACTGGTAAGAAGGTA
AAGGCTAGAGGTAAGGAGTACACTCCAGAGGAGATCTCTGCTTATATTCTTAAACACATA
AAGAAATATGCCGAAGACAAGTTAGGGCACTCCGTTTCCAAGGCGGTTATTACTGTTCCA
GCTTACTTTAACGACTCTGAACGTCAAGCAACTAAGAATGCCGGAACTATTGCTGGATTG
GAAGTTGTAAGAATAGTTAACGAACCTACTGCTGCCGCTCTTGCTTACGGTCTAGACCAC

AGCGAGAAGGAACAGAAGATATTGGTTTATGACTTGGGGGGAGGTACCTTTGACGTGTCT
GTCCTTGATATGTCAGATGGTACTTTTGAAGTATTGGCTACTTCTGGTGATAACCATTTG
GGTGGTGACGATTGGGATCAAGCGTTAATAGATTGGCTATTAGAGGAAATCAAGAAGGAA
CACTCTATAGATCTTTCTTCAGATAATTTAGCACTTCAAAGACTTAAGGATGCCGCTGAG
AAGGCAAAGATAGAGCTTTCTTCCGTTACTCAAACTCAAATACTCCTTCCGTTTCTTTCT
ATGGTGGGTGGACAACCACTTAACATAGATAAAGTTGTTACTCGTGTTCAGTTTGAATCT
TTAACTAAGCATTTAATTGAGAAGACTAGGAAGCCCTTCTTGGATGCTTTGAAGGAATCT
AAGTTATCTGCTTCCGATATAGATCAAATTCTGTTGGTGGGTGGTTCTACCCGTATGCCT
GCCGTTCAGGAGTTGGTTAAGAGCCTTTCCGGGAAGACACCTAACTTGTCTATTAATCCC
GATGAAGTTGTTGCTTTAGGTGCTTCCGTTCAAGGTGCTATTCTTGCTGGGGATATTAAA
GATATCCTGTTATTGGACGTTACGCCTTTAACTCTAAGTATTGAGACTTTAGGTGGTGTA
GCAACTCCTTTAATTAAGAGAAATACTACTGTTCCTGTTGAAAAGACTCAGGTCTTCTCT
ACAGCTGCAGACAACCAACCTTCTGTGGATATTCATGTTGTTCAAGGTGAGAGACCGATG
GCAAATCAAAATAAATCATTGGGAATCTTTACTCTAGATGGAATTCAACCTGCTCCTAAA
GGCGTTCCTCAAATTCAAGTTACCTTCTCTATTGATGCTAACGGTATTCTAAAGGTTAAA
GCTGAGGATAAAGGGACAGGTAAGAGTAACTCTATTACCATAAACCAATCTTCTGGTTTG
AGTGATGAGGAGATTCAAAGAATTATCAAGGAGGCTGAAGAGAATGCAGAGAAGGATCAG
AAAGCAAAGGAGGCTATAGAGGTTAAGAATGAAGCGCAGTCTTGGATCTCAATAGTTGAA
AAACAACTCTCTGAATCTAATGCGACTGATGAGCAGAAAGAATCTGCTCAGAAGATGGTT
GATGAATTGAAGCTTCTTATTAAAGATGAGAAGATTGAGGAGTTAAAACAGAAGATGGAT
GCGATTAAAACCTTGAGTCAGGATATGACTAAGTACGCACAGGATAATCCTAAAGAAGAG
AGTGAGGAGGTTAAGGAAGCGGAGGTAGTAGAAGAGGATAAGACTGAGGTAGATAAAACT
AAATCCAAGGGCGAGCTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTC
GGTCTCGATTCTACGCGTACCGGTCATCATCACCATCACCATTGA

Expression (pET) amino-acid sequence (SEQ ID No 4)

MSKKETIIGIDLGTTNSCVAIVENGNPKILETNEGKRTIPSVVSFKGDEIIVGDSAKRQM
VTNKDTIVSIKRLIGTGKKVKARGKEYTPEEISAYILKHIKKYAEDKLGHSVSKAVITVP
AYFNDSERQATKNAGTIAGLEVVRIVNEPTAAALAYGLDHSEKEQKILVYDLGGGTFDVS
VLDMSDGTFEVLATSGDNHLGGDDWDQALIDWLLEEIKKEHSIDLSSDNLALQRLKDAAE
KAKIELSSVTQTQILLPFLSMVGGQPLNIDKVVTRVQFESLTKHLIEKTRKPFLDALKES
KLSASDIDQILLVGGSTRMPAVQELVKSLSGKTPNLSINPDEVVALGASVQGAILAGDIK
DILLLDVTPLTLSIETLGGVATPLIKRNTTVPVEKTQVFSTAADNQPSVDIHVVQGERPM
ANQNKSLGIFTLDGIQPAPKGVPQIQVTFSIDANGILKVKAEDKGTGKSNSITINQSSGL

SDEEIQRIIKEAEENAEKDQKAKEAIEVKNEAQSWISIVEKQLSESNATDEQKESAQKMV
DELKLLIKDEKIEELKQKMDAIKTLSQDMTKYAQDNPKEESEEVKEAEVVEEDKTEVDKT
KSKGELNSKLEGKPIPNPLLGLDSTRTGHHHHHH

Glyceraldehyde 3-phosphate dehydrogenase (*gapA*)

Native gene sequence (SEQ ID No 5)

ATGAAAAACATTGCGATCAACGGATTCGGAAGAATCGGAAGACTTGCTTTTAGAGAAATT
CTTAAAAATAAAGAATTAAAAGTTGTTGCCATCAACGACTTGACTGATCCTAAGACACTT
GCTCACCTTCTTAAATATGATACAGCTCATGGACCTGTTAGATGCTATGATATCAGTGTT
GAAGGTGACAGTATTGTTTTAGTTAATAAATGTAGTGGAGAAAAACAATCCTTCAAAGTT
ATTTCTGAAAGAGATCCTAAAGCTCTTCCTTGGAAGTCTTTAAATGTAGATTGCGTTCTT
GAATGTACTGGTCGTTTTACCGATAAAGATGCAGCTATGGCTCATGTTGAAGCGGGGGCT
AAGAAAGTAGTTATCTCCGCTCCAGCAAAAGGTGATTTAAAGACAATCGTTTACAACGTA
AACCATGGTACTTTAACTTCTTCTGATCAAGTTATCTCAGCAGCTTCCTGTACAACTAAC
GCTTTAGCTCCCGTTGTAGATGCTCTTCACAAGAAGTACAAAATTGTTTCTGGGTTTATG
ACAACCATTCATGCTTATACAGCGGATCAAAGACTTCAAGACTCTCCTCATAGCGATCTA
AGACGTTCAAGAGCGGCTGGTTCTTCAATCATCCCAACTTCAACAGGTGCTGCTGCAGCT
ATTGGTAAAGTTATTCCTGAACTTCTAGGTAAGTTAGATGGTGTTGCTCACCGTGTTCCA
ACTATTACAGGTTCCTTAGTAGATATGATATTAAGAATGGAATCTACTCCTACAGCTGAG
GAGATAAATGCAACTATTAAAGATGCTTCTTCTGAAACTCTATGTTACTGTGAAGATCCT
ATCGTTTCTTCCGATATTATTTCTCATACAGCGGCTTCTATCTTTGACTCTCTTTTAACT
AAGGTTCTTCCAACTGGAGAGGTTAAGCTTTACACTTGGTACGATAACGAATCTTCTTAT
GTGAATCAGCTTGTTAGAACTTTAAACTACTTTGCTTCTCTTTAA

Native amino-acid sequence (SEQ ID No 6)

MKNIAINGFGRIGRLAFREILKNKELKVVAINDLTDPKTLAHLLKYDTAHGPVRCYDISV
EGDSIVLVNKCSGEKQSFKVISERDPKALPWKSLNVDCVLECTGRFTDKDAAMAHVEAGA
KKVVISAPAKGDLKTIVYNVNHGTLTSSDQVISAASCTTNALAPVVDALHKKYKIVSGFM
TTIHAYTADQRLQDSPHSDLRRSRAAGSSIIPTSTGAAAAIGKVIPELLGKLDGVAHRVP
TITGSLVDMILRMESTPTAEEINATIKDASSETLCYCEDPIVSSDIISHTAASIFDSLLT
KVLPTGEVKLYTWYDNESSYVNQLVRTLNYFASL

Expression (pET) gene sequence (SEQ ID No 7)

ATGAAAAACATTGCGATCAACGGATTCGGAAGAATCGGAAGACTTGCTTTTAGAGAAATT
CTTAAAAATAAAGAATTAAAAGTTGTTGCCATCAACGACTTGACTGATCCTAAGACACTT
GCTCACCTTCTTAAATATGATACAGCTCATGGACCTGTTAGATGCTATGATATCAGTGTT
GAAGGTGACAGTATTGTTTTAGTTAATAAATGTAGTGGAGAAAAACAATCCTTCAAAGTT
ATTTCTGAAAGAGATCCTAAAGCTCTTCCTTGGAAGTCTTTAAATGTAGATTGCGTTCTT
GAATGTACTGGTCGTTTTACCGATAAAGATGCAGCTATGGCTCATGTTGAAGCGGGGGCT
AAGAAAGTAGTTATCTCCGCTCCAGCAAAAGGTGATTTAAAGACAATCGTTTACAACGTA
AACCATGGTACTTTAACTTCTTCTGATCAAGTTATCTCAGCAGCTTCCTGTACAACTAAC
GCTTTAGCTCCCGTTGTAGATGCTCTTCACAAGAAGTACAAAATTGTTTCTGGGTTTATG
ACAACCATTCATGCTTATACAGCGGATCAAAGACTTCAAGACTCTCCTCATAGCGATCTA
AGACGTTCAAGAGCGGCTGGTTCTTCAATCATCCCAACTTCAACAGGTGCTGCTGCAGCT
ATTGGTAAAGTTATTCCTGAACTTCTAGGTAAGTTAGATGGTGTTGCTCACCGTGTTCCA
ACTATTACAGGTTCCTTAGTAGATATGATATTAAGAATGGAATCTACTCCTACAGCTGAG
GAGATAAATGCAACTATTAAAGATGCTTCTTCTGAAACTCTATGTTACTGTGAAGATCCT
ATCGTTTCTTCCGATATTATTTCTCATACAGCGGCTTCTATCTTTGACTCTCTTTTAACT
AAGGTTCTTCCAACTGGAGAGGTTAAGCTTTACACTTGGTACGATAACGAATCTTCTTAT
GTGAATCAGCTTGTTAGAACTTTAAACTACTTTGCTTCTCTTAAGGGCGAGCTCAATTCG
AAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACCGGT
CATCATCACCATCACCATTGA

Expression (pET) amino-acid sequence (SEQ ID No 8)

MKNIAINGFGRIGRLAFREILKNKELKVVAINDLTDPKTLAHLLKYDTAHGPVRCYDISV
EGDSIVLVNKCSGEKQSFKVISERDPKALPWKSLNVDCVLECTGRFTDKDAAMAHVEAGA
KKVVISAPAKGDLKTIVYNVNHGTLTSSDQVISAASCTTNALAPVVDALHKKYKIVSGFM
TTIHAYTADQRLQDSPHSDLRRSRAAGSSIIPTSTGAAAAIGKVIPELLGKLDGVAHRVP
TITGSLVDMILRMESTPTAEEINATIKDASSETLCYCEDPIVSSDIISHTAASIFDSLLT
KVLPTGEVKLYTWYDNESSYVNQLVRTLNYFASLKGELNSKLEGKPIPNPLLGLDSTRTG
HHHHHH

Phosphoglycerate kinase (*pgk*)

Native gene sequence (partial) (SEQ ID No 9)

ATGGTTTACGATAAGGCGACGCTAAGGGATGTTTCCCTAGATGGAAAAAGAGTAGTAATT
CGTCTTGATTTAAACGTACCCGTTAAAGACGGAAAGATTACTGACAATAATAGGGTAGTT
CAAACTTTACCTACGGTTCGTTATCTTCTTGAGAAGGGTTGTAAATTAGTTGTTCTTAGT
CACTTCTCCAGAATTAAAGATATTTCTGAGGTTACTTCTGGAAAGAAATCTCTTAAAGTA
GTAGCTGAGGAATTTCAAAAACTACTTCCTGATAACAAGGTTGCCTTTGTTTCTGATATT
GACTTTAACAATGTTGCTTCTTTTGTTAATTCCAATACGGATGTGGATTTGTTTGTTCTT
GAGAACACTAGATATTATGACGTTGATGCCCATAACTCTTTAGTTAAATGGGAGTCTAAG
AATTATCCAGACTTATCTAAGTTTTGAGCTTCCCTTGGGGAGGTGTTTATTAATGATGCT
TTTGGTACCTCCCACAGAGCTCATGCTTCCAACGTAGGTATTGCGGGACATCTTCCTTCT
GCAATTGGATTATTGGTTGAGAAGGAGTTAGATATGCTTGGTAAAGCAGTTTCCTCTACA
GATTCTCCTAAGGTTTTAATCTTGGGCGGTTCTAAGGTATCCGATAAGTTGAAGTTAATA
AATGCGATCGCTCCCAAGGTAGATAAGCTTCTTATTGGTGGGGGTATGTCTTATACCTTC
CTTAAAGCACAAGGGAAGGAGGTTGGTAAGTCTATAGTGGAAACTGAAATGATTGAAGAA
TGTAAGGATCTTCTTCAGAGATATGCTGGCAAATTGGTTCTTCCCGTTGATCACGTTGTA
GCTCCCGAATTTAAAGATATAGCTGGGAGTGTGGTTGATTTAGAGGAGAAGAATTGGGGG
GATAATATGGCTTTAGATATTGGCCCTAAGACTGTTGAACTTTATAGAAGTGTTCTTGAG
AGTGCTAAAGTTGTTATTTGAAACGGTCCTATGGGAGTATTTGAATTTGATAACTTTGCA
GAGGGTACAAA

Native amino-acid sequence (partial) (SEQ ID No 10)

MVYDKATLRDVSLDGKRVVIRLDLNVPVKDGKITDNNRVVQTLPTVRYLLEKGCKLVVLS
HFSRIKDISEVTSGKKSLKVVAEEFQKLLPDNKVAFVSDIDFNNVASFVNSNTDVDLFVL
ENTRYYDVDAHNSLVKWESKNYPDLSKFWASLGEVFINDAFGTSHRAHASNVGIAGHLPS
AIGLLVEKELDMLGKAVSSTDSPKVLILGGSKVSDKLKLINAIAPKVDKLLIGGGMSYTF
LKAQGKEVGKSIVETEMIEECKDLLQRYAGKLVLPVDHVVAPEFKDIAGSVVDLEEKNWG
DNMALDIGPKTVELYRSVLESAKVVIWNGPMGVFEFDNFAEGT

Expression (pET) gene sequence (Gene fragment) (SEQ ID No 11)

ATGGTTTACGATAAGGCGACGCTAAGGGATGTTTCCCTAGATGGAAAAAGAGTAGTAATT
CGTCTTGATTTAAACGTACCCGTTAAAGACGGAAAGATTACTGACAATAATAGGGTAGTT
CAAACTTTACCTACGGTTCGTTATCTTCTTGAGAAGGGTTGTAAATTAGTTGTTCTTAGT
CACTTCTCCAGAATTAAAGATATTTCTGAGGTTACTTCTGGAAAGAAATCTCTTAAAGTA

GTAGCTGAGGAATTTCAAAAACTACTTCCTGATAACAAGGTTGCCTTTGTTTCTGATATT
GACTTTAACAATGTTGCTTCTTTTGTTAATTCCAATACGGATGTGGATTTGTTTGTTCTT
GAGAACACTAGATATTATGACGTTGATGCCCATAACTCTTTAGTTAAATGGGAGTCTAAG
AATTATCCAGACTTATCTAAGTTTTGGGCTTCCCTTGGGGAGGTGTTTATTAATGATGCT
TTTGGTACCTCCCACAGAGCTCATGCTTCCAACGTAGGTATTGCGGGACATCTTCCTTCT
GCAATTGGATTATTGGTTGAGAAGGAGTTAGATATGCTTGGTAAAGCAGTTTCCTCTACA
GATTCTCCTAAGGTTTTAATCTTGGGCGGTTCTAAGGTATCCGATAAGTTGAAGTTAATA
AATGCGATCGCTCCCAAGGTAGATAAGCTTCTTATTGGTGGGGGTATGTCTTATACCTTC
CTTAAAGCACAAGGGAAGGAGGTTGGTAAGTCTATAGTGGAAACTGAAATGATTGAAGAA
TGTAAGGATCTTCTTCAGAGATATGCTGGCAAATTGGTTCTTCCCGTTGATCACGTTGTA
GCTCCCGAATTTAAAGATATAGCTGGGAGTGTGGTTGATTTAGAGGAGAAGAATTGGGGG
GATAATATGGCTTTAGATATTGGCCCTAAGACTGTTGAACTTTATAGAAGTGTTCTTGAG
AGTGCTAAAGTTGTTATTTGGAACAAGGGCGAGCTCAATTCGAAGCTTGAAGGTAAGCCT
ATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACCGGTCATCATCACCATCACCAT
TGA

Expression (pET) amino-acid sequence (SEQ ID No 12)

MVYDKATLRDVSLDGKRVVIRLDLNVPVKDGKITDNNRVVQTLPTVRYLLEKGCKLVVLS
HFSRIKDISEVTSGKKSLKVVAEEFQKLLPDNKVAFVSDIDFNNVASFVNSNTDVDLFVL
ENTRYYDVDAHNSLVKWESKNYPDLSKFWASLGEVFINDAFGTSHRAHASNVGIAGHLPS
AIGLLVEKELDMLGKAVSSTDSPKVLILGGSKVSDKLKLINAIAPKVDKLLIGGGMSYTF
LKAQGKEVGKSIVETEMIEECKDLLQRYAGKLVLPVDHVVAPEFKDIAGSVVDLEEKNWG
DNMALDIGPKTVELYRSVLESAKVVIWNKGELNSKLEGKPIPNPLLGLDSTRTGHHHHHH

Elongation Factor-Thermo-stable (EF-Ts)

Native gene sequence (SEQ ID No 13)

ATGGCTATCGATAAAGACCTAATTTTAAAGCTTAGAAATGCAAGTCAAGCTGGTCTTGCT
GACTGTAAGAAGGCTTTAGAGGAGAATAATAATGATCTTGAGGCTTCTATTAAGTGACTT
CGTACAAAAGGAATAGCTAAGGCCACAAACAAGAACGCCTTAAGGGAGGCTAAGGAGGGG
AGTACCTTTGTTAAGAAAGATGGTAAGGGAGTTGTCATTATGGAGATGAATTCTGAAACT
GACTTTGTCGCTAATTCCAAAGAATTTACTGCTTTGGCAGACAGAATTATGGATTCTATT
TTGAATCTAGGGAAAGAGGATTTAGGAGATGTCAATAGTTTGAAGATGGATAGTGGAGAA

AGTGTAGCAGATGGTTGTCTTCACCTTTCCTCTATAACTGGAGAGAAGATTGTTTTAAGC
AGGGCTAAGTATATTTCTCTGGGAAGTGGGGAATCTGTTGCCTGCTATAGGCATAATAAC
GGAAGAATGTCTGCCTTTGTTATCCTTAATAAGGATCTTAAGGATGAAGATATTTATGGC
TTAGCAGTTCACTATGCAGCCAATAATCCTAAATTTATTTCTTCAGATCAGGTGGATGAA
TCATGAATTAATTCGGAGAGGGAGATAATTACTACTCTTCTAGAGAAGGAGAATAAGCCT
AAGGAGTTTCATGCAAATATTATTGAACAGAGAATTAAGAAATTGATTGCCCAAGAGGCT
TTCGTAGAGCAGCCTTATTTATATGATACTTCTAAGAAGATAAAGGATAGACTGAATGAC
TTAGGAGCTGAAGTTAAAATGGCTATGTATTTCGGATTAGGAGAATCTAAGTAG

Native amino-acid sequence (SEQ ID No 14)

MAIDKDLILKLRNASQAGLADCKKALEENNNDLEASIKWLRTKGIAKATNKNALRXAKEG
STFVKKDGKGVVIMEMNSETDFVANSKEFTALADRIMDSILNLGKEDLGDVNSLKMDSGE
SVADGCLHLSSITGEKIVLSRAKYISLGSGESVACYRHNNGRMSAFVILNKDLKDEDIYG
LAVHYAANNPKFISSDQVDESWINSEREIITTLLEKENKPKEFHANIIEQRIKKLIAQEA
FVEQPYLYDTSKKIKDRLNDLGAEVKMAMYFGLGESK

Expression (pET) gene sequence (Predicted) (SEQ ID No 15)

ATGGCTATCGATAAAGACCTAATTTTAAAGCTTAGAAATGCAAGTCAAGCTGGTCTTGCT
GACTGTAAGAAGGCTTTAGAGGAGAATAATAATGATCTTGAGGCTTCTATTAAGTGGCTT
CGTACAAAAGGAATAGCTAAGGCCACAAACAAGAACGCCTTAAGGGAGGCTAAGGAGGGG
AGTACCTTTGTTAAGAAAGATGGTAAGGGAGTTGTCATTATGGAGATGAATTCTGAAACT
GACTTTGTCGCTAATTCCAAAGAATTTACTGCTTTGGCAGACAGAATTATGGATTCTATT
TTGAATCTAGGGAAAGAGGATTTAGGAGATGTCAATAGTTTGAAGATGGATAGTGGAGAA
AGTGTAGCAGATGGTTGTCTTCACCTTTCCTCTATAACTGGAGAGAAGATTGTTTTAAGC
AGGGCTAAGTATATTTCTCTGGGAAGTGGGGAATCTGTTGCCTGCTATAGGCATAATAAC
GGAAGAATGTCTGCCTTTGTTATCCTTAATAAGGATCTTAAGGATGAAGATATTTATGGC
TTAGCAGTTCACTATGCAGCCAATAATCCTAAATTTATTTCTTCAGATCAGGTGGATGAA
TCATGGATTAATTCGGAGAGGGAGATAATTACTACTCTTCTAGAGAAGGAGAATAAGCCT
AAGGAGTTTCATGCAAATATTATTGAACAGAGAATTAAGAAATTGATTGCCCAAGAGGCT
TTCGTAGAGCAGCCTTATTTATATGATACTTCTAAGAAGATAAAGGATAGACTGAATGAC
TTAGGAGCTGAAGTTAAAATGGCTATGTATTTCGGATTAGGAGAATCTAAGAAGGGCGAG
CTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACG
CGTACCGGTCATCATCACCATCACCATTGA

Expression (pET) amino-acid sequence (Predicted) (SEQ ID No 16)

MAIDKDLILKLRNASQAGLADCKKALEENNNDLEASIKWLRTKGIAKATNKNALREAKEG
STFVKKDGKGVVIMEMNSETDFVANSKEFTALADRIMDSILNLGKEDLGDVNSLKMDSGE
SVADGCLHLSSITGEKIVLSRAKYISLGSGESVACYRHNNGRMSAFVILNKDLKDEDIYG
LAVHYAANNPKFISSDQVDESWINSEREIITTLLEKENKPKEFHANIIEQRIKKLIAQEA
FVEQPYLYDTSKKIKDRLNDLGAEVKMAMYFGLGESKKGELNSKLEGKPIPNPLLGLDST
RTGHHHHHH

CMhm genes

Heat shock protein 70 (*dnaK*)

Native gene sequence (SEQ ID No 17)

ATGTCAGTTAAAAAAGAAGTGATTCTAGGTATTGACCTAGGTACTACTAACTCTTGTGTA
GCAGTAATAGAGGGAGGTAAATCTAAGGTGCTAGAAACCCCAGAAGGAAAGAGAACTATT
CCTTCTATAGTAGCCTTCAAAGGAGAACAAATAATAGTAGGAGAAAGCGCTAAACGACAG
ATGGTTACAAATAAAAACACGATCTATTCTATTAAGAGGCTGATAGGTACTGACCAAAAG
GTAACTGCTCAGGGGAAAGAATATACACCTGAAGAAATATCTGCATATATTCTTTCCTAC
ATCAAAGAGTATTCTGAAAAGAAATTAGGTCATGATGTTCAGAAAGCAGTTATTACAGTA
CCAGCCTACTTTAATGATGGACAGAGACAATCTACAAAGAATGCAGGAAAAATAGCAGGA
CTTGAAGTAGTAAGAATAGTAAATGAACCTACTGCAGCTGCACTAGCTTATGGTCTTGAT
AAGAAAGATGGAGATCAGAAGATATTAGTTTATGACTTAGGAGGAGGAACATTTGATGTA
TCTATCTTAGAAATTGCTGAGGGAACATTTAAGGTGCTTTCTACCTCTGGAGATAATAAG
CTAGGAGGAGATGATTGAGACCAACGAATTATTAACTGATTACTTGAAACTATTAAAAAA
GAACATGGAGCTGATCTCTCAAAGGATAATCTTGTGTTACAAAGATTAAAAGAAGCAGCA
GAAAAAGCAAAAATAGAACTTTCTTCAGTGCAACAAACTCAAATTATGTTGCCCTTCCTA
ACTATGATTGGAGGAGAGCCTCTAAATGTAGACTTAACTCTTTCTAGAGCTCAATTTGAA
TTACTAACAAAAGATTTACTAGATAGAACAGTAAGACCTGTAGAAGATGCTGTCAAAGAA
TCTCAACTGAAATTAAGTGATATAGATCAAATACTTCTAGTAGGAGGCTCTACTAGGATG
CCAGCAGTGCAAGCACTTGTAGAAAAATTAACTGGAAAGAAACCTAATCTTTCAATAAAT
CCTGATGAGGTAGTAGCATTGGGAGCAGCTGTTCAAGCAGGAGTACTAGCAGGAGATGTA
AAAGATGTACTCTTGCTGGATGTAACTCCCCTAACCTTAAGTATTGAAACACTAGGGGGA
GTAGCTACTCCCCTAATACAACGGAATACTACTATACCTACAGAAAAGAAGCAGATTTTC

TCTACAGCAATAGATAATCAACCAAGTGTAGATATTCATGTAGTACAGGGAGAGCGACCT
ATGGCTGCAGATAACACTACTTTAGGTACTTTTGCACTGACTGGTATTAAGATGGCACCT
AAAGGCGTACCGAAAATAGAAGTTTCCTTCTCTATAGACGCTAATGGTATCTTAACTGTA
AGGGCAGAAGATAAAGATACAGGTAAGAGTAACAATATAGTAATTAAAAATGCATCAGGA
CTCTCAGAAGAAGAGATAAATAGAAAGATTAAGGAAGCTGAAGAAAATCTAGAAAAAGAT
AAAAAGGTAAGGGAAGAAGTTGAAACAGTAAATCAGGCTGAATCTTGAATTTCTATGCTG
GAAAAACAAATGGAAGACAAAAAAGAAGATGTTCCAGCTTCTCTAAAAGATGAGGCTAAA
AAACTAATAGATGAA

Native amino-acid sequence (SEQ ID No 18)

MSVKKEVILGIDLGTTNSCVAVIEGGKSKVLETPEGKRTIPSIVAFKGEQIIVGESAKRQ
MVTNKNTIYSIKRLIGTDQKVTAQGKEYTPEEISAYILSYIKEYSEKKLGHDVQKAVITV
PAYFNDGQRQSTKNAGKIAGLEVVRIVNEPTAAALAYGLDKKDGDQKILVYDLGGGTFDV
SILEIAEGTFKVLSTSGDNKLGGDDWDQRIINWLLETIKKEHGADLSKDNLVLQRLKEAA
EKAKIELSSVQQTQIMLPFLTMIGGEPLNVDLTLSRAQFELLTKDLLDRTVRPVEDAVKE
SQLKLSDIDQILLVGGSTRMPAVQALVEKLTGKKPNLSINPDEVVALGAAVQAGVLAGDV
KDVLLLDVTPLTLSIETLGGVATPLIQRNTTIPTEKKQIFSTAIDNQPSVDIHVVQGERP
MAADNTTLGTFALTGIKMAPKGVPKIEVSFSIDANGILTVRAEDKDTGKSNNIVIKNASG
LSEEEINRKIKEAEENLEKDKKVREEVETVNQAESWISMLEKQMEDKKEDVPASLKDEAK
KLIDE

Expression (pET) gene sequence (Gene fragment) (SEQ ID No 19)

ATGTCAGTTAAAAAAGAAGTGATTCTAGGTATTGACCTAGGTACTACTAACTCTTGTGTA
GCAGTAATAGAGGGAGGTAAATCTAAGGTGCTAGAAACCCCAGAAGGAAAGAGAACTATT
CCTTCTATAGTAGCCTTCAAAGGAGAACAAATAATAGTAGGAGAAAGCGCTAAACGACAG
ATGGTTACAAATAAAAACACGATCTATTCTATTAAGAGGCTGATAGGTACTGACCAAAAG
GTAACTGCTCAGGGGAAAGAATATACACCTGAAGAAATATCTGCATATATTCTTTCCTAC
ATCAAAGAGTATTCTGAAAAGAAATTAGGTCATGATGTTCAGAAAGCAGTTATTACAGTA
CCAGCCTACTTTAATGATGGACAGAGACAATCTACAAAGAATGCAGGAAAAATAGCAGGA
CTTGAAGTAGTAAGAATAGTAAATGAACCTACTGCAGCTGCACTAGCTTATGGTCTTGAT
AAGAAAGATGGAGATCAGAAGATATTAGTTTATGACTTAGGAGGAGGAACATTTGATGTA
TCTATCTTAGAAATTGCTGAGGGAACATTTAAGGTGCTTTCTACCTCTGGAGATAATAAG
CTAGGAGGAGATGATTGGGACCAACGAATTATTAACTGGTTACTTGAAACTATTAAAAAA
GAACATGGAGCTGATCTCTCAAAGGATAATCTTGTGTTACAAAGATTAAAAGAAGCAGCA

GAAAAGGCAAAAATAGAACTTTCTTCAGTGCAACAAACTCAAATTATGTTGCCCTTCCTA
ACTATGATTGGAGGAGAACCGCTAAATGTAGACTTAACTCTTTCTAGAGCTCAATTTGAA
TTATTAACAAAAGATTTACTAGATAGAACAGTAAGACCTGTAGAAGATGCTGTTAAAGAA
TCTCAACTGAAATTAAGTGATATAGATCAAATACTTCTAGTAGGAGGTTCTACTAGAATG
CCAGCTGTTCAAGCACTTGTAGAAAAACTGACTGGAAAGAAACCTAATCTTTCAATAAAT
CCTGATGAGGTAGTAGCATTGGGAGCAGCTGTTCAAGCAGGAGTACTAGCAGGAGATGTA
AAAGATGTACTCCTGCTTGATGTAACTCCCTTAACTCTAAGTATTGAGACACTAGGGGGA
GTAGCTACTCCCCTAATACAACGGAATACTACTATACCTACAGAAAAGAAGCAGATTTTC
TCTACAGCAATAGATAATCAACCAAGTGTAGATATTCATGTAGTACAGGGAGAGCGACCT
ATGGCTGCAGATAACACTACTTTAGGTACTTTTGCACTGACTGGTATTAAGATGGCACCT
AAAGGCGTACCGAAAATAGAAGTTTCCTTTTCTATAGACGCTAATGGTATCTTAACTGTA
AGGGCAGAAGATAAAGATACAGGTAAGAGTAACAATATAGTAATTAAAAATGCATCAGGA
CTCTCAGAAGAAGAGATAAATAGAAAGATTAAGGAAGCCGAAGAAAATTTAGAAAAAGAT
AAAAAGGTAAGGGAAGAAGTTGAAACAGTAAATCAGGCTGAATCTTGGATTTCTATGCTG
GAAAAGGGCGAGCTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGT
CTCGATTCTACGCGTACCGGTCATCATCACCATCACCATTGA

Expression (pET) amino-acid sequence (SEQ ID No 20)

MSVKKEVILGIDLGTTNSCVAVIEGGKSKVLETPEGKRTIPSIVAFKGEQIIVGESAKRQ
MVTNKNTIYSIKRLIGTDQKVTAQGKEYTPEEISAYILSYIKEYSEKKLGHDVQKAVITV
PAYFNDGQRQSTKNAGKIAGLEVVRIVNEPTAAALAYGLDKKDGDQKILVYDLGGGTFDV
SILEIAEGTFKVLSTSGDNKLGGDDWDQRIINWLLETIKKEHGADLSKDNLVLQRLKEAA
EKAKIELSSVQQTQIMLPFLTMIGGEPLNVDLTLSRAQFELLTKDLLDRTVRPVEDAVKE
SQLKLSDIDQILLVGGSTRMPAVQALVEKLTGKKPNLSINPDEVVALGAAVQAGVLAGDV
KDVLLLDVTPLTLSIETLGGVATPLIQRNTTIPTEKKQIFSTAIDNQPSVDIHVVQGERP
MAADNTTLGTFALTGIKMAPKGVPKIEVSFSIDANGILTVRAEDKDTGKSNNIVIKNASG
LSEEEINRKIKEAEENLEKDKKVREEVETVNQAESWISMLEKGELNSKLEGKPIPNPLLG
LDSTRTGHHHHHH

Glyceraldehyde 3-phosphate dehydrogenase (*gapA*)

Native gene sequence (SEQ ID NO 21)

ATGGAGTTTTACAGAGTTGCTATTAATGGATTTGGAAGAATAGGTAGGTTATTATTTAGA
AATTTGTTATCCTCACCTAGCGTTAATGTAGTAGCAGTTAATGATATTGTTGATGCATCG

GTTTTAGCTCATTTATTAAAGTATGACAGCTCTCAAGGAGTGTTGAAAGATTGAGAGGTT
AAAAGTGATGCAGAAAACATTTACTTAACTCACATTGATAGCGGAAAACAAAAAACAGTG
AAGGTCTTTAATTTCTTGAAAGAAAAGAGCTACCACTGGGGAGAGTTAGAAGTTGATTGC
GTGGTGGAATGTTCGGGACGTTTATTAACTAAAGATGCAGTGCAATGCCACTTGGATGCA
GGAGCTGAGAAAGTATTAATCTCAGCTCCTGCAAAAGATGATGCTATAAAAACTATTGTT
TTTAACGTAAATCATAATTCGATTAGCACTTCTGATACGGTAATTTCTGGAGCTTCTTGC
ACTACCAATGCATTGGCTCCTGTTGTTAAAGTGTTGCATAGAAAATTTGGAGTGCAGTCT
GGATTTATGACTACCATTCACGCCTTTACTTCAGACCAGAGACTACAAGATGCGCCTCAT
GCAGATTTAAGAAGAACTCGAGCAGCTGCCAATTCGATTATTCCAACTACCACCGGAGCA
GCTGCCGCTATAGGTAAAGTGATTCCTGACTTGAAAGGTAAGCTAGATGGAATTGCTCAT
AGAGTTCCAGTTTTGACAGGCTCATTAGTAGACTTGGTGGTACGTTTAGGTAAAAATGTT
ACAGCAGAGGAGGTCAACGCTGCTCTAGAATCGGCTCAGAATGAAACCATGTTATATTTG
AAAGATCCAATAGTGTCTTCAGATATTGTCGGAAGCACTTATGGGTCTATTTTTGACTCC
CTCTTAACTAAAGTGTTACCTACCGGAGAAGTGAAATTATATGCATGATATGATAATGAG
TCTTCTTATGTTAGCCAATTGTCTAGAACACTCCACTACTACATCTCTTTGTAG

Native amino-acid sequence (SEQ ID No 22)

MEFYRVAINGFGRIGRLLFRNLLSSPSVNVVAVNDIVDASVLAHLLKYDSSQGVLKDWEV
KSDAENIYLTHIDSGKQKTVKVFNFLKEKSYHWGELEVDCVVECSGRLLTKDAVQCHLDA
GAEKVLISAPAKDDAIKTIVFNVNHNSISTSDTVISGASCTTNALAPVVKVLHRKFGVQS
GFMTTIHAFTSDQRLQDAPHADLRRTRAAANSIIPTTTGAAAAIGKVIPDLKGKLDGIAH
RVPVLTGSLVDLVVRLGKNVTAEEVNAALESAQNETMLYLKDPIVSSDIVGSTYGSIFDS
LLTKVLPTGEVKLYAWYDNESSYVSQLSRTLHYYISL

Expression (pET) gene sequence (Gene fragment) (SEQ ID No 23)

CACCATGGAGTTTTACAGAGTTGCTATTAATGGATTTGGAAGAATAGGTAGGTTATTATT
TAGAAATTTGTTATCCTCACCTAGCGTTAATGTAGTAGCAGTTAATGATATTGTTGATGC
ATCGGTTTTAGCTCATTTATTAAAGTATGACAGCTCTCAAGGAGTGTTGAAAGATTGGGA
GGTTAAAAGTGATGCAGAAAACATTTACTTAACTCACATTGATAGCGGAAAACAAAAAAC
AGTGAAGGTCTTTAATTTCTTGAAAGAAAAGAGCTACCACTGGGGAGAGTTAGAAGTTGA
TTGCGTGGTGGAATGTTCGGGACGTTTATTAACTAAAGATGCAGTGCAATGCCACTTGGA
TGCAGGAGCTGAGAAAGTATTAATCTCAGCTCCTGCAAAAGATGATGCTATAAAAACTAT
TGTTTTTAACGTAAATCATAATTCGATTAGCACTTCTGATACGGTAATTTCTGGAGCTTC
TTGCACTACCAATGCATTGGCTCCTGTTGTTAAAGTGTTGCATAGAAAATTTGGAGTGCA

GTCTGGATTTATGACTACCATTCACGCCTTTACTTCAGACCAGAGACTACAAGATGCGCC
TCATGCAGATTTAAGAAGAACTCGAGCAGCTGCCAATTCGATTATTCCAACTACCACCGG
AGCAGCTGCCGCTATAGGTAAAGTGATTCCTGACTTGAAAGGTAAGCTAGATGGAATTGC
TCATAGAGTTCCAGTTTTGACAGGCTCATTAGTAGACTTGGTGGTACGTTTAGGTAAAAA
TGTTACAGCAGAGGAGGTCAACGCTGCTCTAGAATCGGCTCAGAATGAAACCATGTTATA
TTTGAAAGATCCAATAGTGTCTTCAGATATTGTCGGAAGCACTTATGGGTCTATTTTTGA
CTCCCTCTTAACTAAAGTGTTACCTACCGGAGAAGTGAAATTATATGCATGGTATGATAA
TGAGTCTTCTTATGTTAGCCAATTGTCTAGAACACTCCACTACTACATCTCTTTGAAGGG
CGAGCTCAATTCGAAGCTTGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTC
TACGCGTACCGGTCATCATCACCATCACCATTGA

Expression (pET) amino-acid sequence (SEQ ID No 24)

MEFYRVAINGFGRIGRLLFRNLLSSPSVNVVAVNDIVDASVLAHLLKYDSSQGVLKDWEV
KSDAENIYLTHIDSGKQKTVKVFNFLKEKSYHWGELEVDCVVECSGRLLTKDAVQCHLDA
GAEKVLISAPAKDDAIKTIVFNVNHNSISTSDTVISGASCTTNALAPVVKVLHRKFGVQS
GFMTTIHAFTSDQRLQDAPHADLRRTRAAANSIIPTTTGAAAAIGKVIPDLKGKLDGIAH
RVPVLTGSLVDLVVRLGKNVTAEEVNAALESAQNETMLYLKDPIVSSDIVGSTYGSIFDS
LLTKVLPTGEVKLYAWYDNESSYVSQLSRTLHYYISLKGELNSKLEGKPIPNPLLGLDST
RTGHHHHHH

Phosphoglycerate kinase (*pgk*)

Native gene sequence (SEQ ID No 25)

GTGAGGTACAATAAGGTCACTTTACGTGATCTTGAGCCTTCAGGAAAGACAGTTGTACTT
AGATTGGACCTAAATGTGCCTGTTAAAGATGGTCAGGTGATGAACAAAACTAGAATATTG
GGATCTATCGAGACTATTCAATATTTACTGGATAGAGAATGTAAGGTAGTGATATTGAGT
CATTTTGATCGAATTAAGAATTATGATGAAATTGCTGACGGCTCTAAAAGTTTATTGCCT
GTTGCCCAAGAAATTCAAAAAATATTTTTCAATAAGAAGGTACTTTTTATCAACACCACT
TCCTTTGAAAATGTGAAAGGAACTATTAAAAATAACCCTAAAGTGGATATTTTTGTATTG
GAAAATACAAGATACTATGATATAGATCCTCAAAGCAAGCAAAATGTCAAGTGAGAGAGT
GGAAATAATCCAGCTCTAGCACAATTTTATTCTGAAATTGGAGATATTTTTGTGAATGAC
GCTTTCGGCACTTCTCACAGAGCTCATGCTTCAAATGTGGGGGTAGCAGAGAGAAGCCAG
CAAAGCGCTGTAGGTTTTCTTATTGAAAAGGAATTAAAAGCCCTTAATTTTGTATTAGAT
GCTCCCCCAGACGGTAAAGTGATGATTTTGGGAGGTAGTAAGGTTTCAGATAAATTAAAG

CTCATTAAAGAGATTGTTAATCATGTTAGTACATTAATAATTGGGGGAGGAATGGCTTAC
ACATTTTTAAAAGCTCAGGGAAAATCTATAGGTAAATCTTTAGTCGAAGATGATTATTTA
GATGAATGTTCTAAGTTATTAAAAAATTACTCTGGTAAAATAGTTCTTCCCATTGATCAT
GTAGTGTCTGACTCTTTTGCAGATATTCCTGGACGTATTATTGAAGACAATTCCGATCAT
TGGGGAGTTGGGATGGCATTAGATATAGGTCCTAAAACTATTGAAAAGTTTTCCAAGATA
CTTGAAGGCGCTAAGATTGTGATCTGGAACGGACCTCTAGGGGTATTTGAAATGTCCAAT
TATTCAAAAGGCACTTTTGAAATTCTTAAAAAGTTATCTGATTTAACTGCTAGTAAAGGG
GTTTATTCTCTTATCGGTGGAGGAGATTCTGTGGCAGCTGCAGAAAAAAAGGGAATAGCG
GATAAATTTAGTTTTGTGTCAACGGGTGGTGGAGCTACTTTAACATTTCTAGAACGAAGT
AGTATGCCGGGAATAGAGGCCATTCAAAACAAATAG

Native amino-acid sequence (SEQ ID No 26)

MRYNKVTLRDLEPSGKTVVLRLDLNVPVKDGQVMNKTRILGSIETIQYLLDRECKVVILS
HFDRIKNYDEIADGSKSLLPVAQEIQKIFFNKKVLFINTTSFENVKGTIKNNPKVDIFVL
ENTRYYDIDPQSKQNVKWESGNNPALAQFYSEIGDIFVNDAFGTSHRAHASNVGVAERSQ
QSAVGFLIEKELKALNFVLDAPPDGKVMILGGSKVSDKLKLIKEIVNHVSTLIIGGGMAY
TFLKAQGKSIGKSLVEDDYLDECSKLLKNYSGKIVLPIDHVVSDSFADIPGRIIEDNSDH
WGVGMALDIGPKTIEKFSKILEGAKIVIWNGPLGVFEMSNYSKGTFEILKKLSDLTASKG
VYSLIGGGDSVAAAEKKGIADKFSFVSTGGGATLTFLERSSMPGIEAIQNK

Expression (pET) gene sequence (SEQ ID No 27)

ATGAGGTACAATAAGGTCACTTTACGTGATCTTGAGCCTTCAGGAAAGACAGTTGTACTT
AGATTGGACCTAAATGTGCCTGTTAAAGATGGTCAGGTGATGAACAAAACTAGAATATTG
GGATCTATCGAGACTATTCAATATTTACTGGATAGAGAATGTAAGGTAGTGATATTGAGT
CATTTTGATCGAATTAAGAATTATGATGAAATTGCTGACGGCTCTAAAAGTTTATTGCCT
GTTGCCCAAGAAATTCAAAAAATATTTTTCAATAAGAAGGTACTTTTTATCAACACCACT
TCCTTTGAAAATGTGAAAGGAACTATTAAAAATAACCCTAAAGTGGATATTTTTGTATTG
GAAAATACAAGATACTATGATATAGATCCTCAAAGCAAGCAAAATGTCAAGTGGGAGAGT
GGAAATAATCCAGCTCTAGCACAATTTTATTCTGAAATTGGAGATATTTTTGTGAATGAC
GCTTTCGGCACTTCTCACAGAGCTCATGCTTCAAATGTGGGGGTAGCAGAGAGAAGCCAG
CAAAGCGCTGTAGGTTTTCTTATTGAAAAGGAATTAAAAGCCCTTAATTTTGTATTAGAT
GCCCCCCCAGACGGTAAAGTGATGATTTTGGGAGGTAGTAAGGTTTCAGATAAATTAAAG
CTCATTAAAGAGATTGTTAATCATGTTAGTACATTAATAATTGGGGGAGGAATGGCTTAC
ACATTTTTAAAAGCTCAGGGAAAATCTATAGGTAAATCTTTAGTCGAAGATGATTATTTA

GATGAATGTTCTAAGTTATTAAAAAATTACTCTGGTAAAATAGTTCTTCCCATTGATCAT
GTAGTGTCTGACTCTTTTGCAGATATTCCTGGACGTATTATTGAAGACAATTCCGATCAT
TGGGGAGTTGGGATGGCATTAGATATAGGTCCTAAAACTATTGAAAAGTTTTCCAAGATA
CTTGAAGGCGCTAAGATTGTGATCTGGAACGGACCTCTAGGGGTATTTGAAATGTCCAAT
TATTCAAAAGGCACTTTTGAAATTCTTAAAAAGTTATCTGATTTAACTGCTAGTAAAGGG
GTTTATTCTCTTATCGGTGGAGGAGATTCTGTGGCAGCTGCAGAAAAAAAGGGAATAGCG
GATAAATTTAGTTTTGTGTCAACGGGTGGTGGAGCTACTTTAACATTTCTAGAACGAAGT
AGTATGCCGGGAATAGAGGCCATTCAAAACAAAAAGGGCGAGCTCAATTCGAAGCTTGAA
GGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACCGGTCATCATCAC
CATCACCATTGA

Expression (pET) amino-acid sequence (SEQ ID No 28)

MRYNKVTLRDLEPSGKTVVLRLDLNVPVKDGQVMNKTRILGSIETIQYLLDRECKVVILS
HFDRIKNYDEIADGSKSLLPVAQEIQKIFFNKKVLFINTTSFENVKGTIKNNPKVDIFVL
ENTRYYDIDPQSKQNVKWESGNNPALAQFYSEIGDIFVNDAFGTSHRAHASNVGVAERSQ
QSAVGFLIEKELKALNFVLDAPPDGKVMILGGSKVSDKLKLIKEIVNHVSTLIIGGGMAY
TFLKAQGKSIGKSLVEDDYLDECSKLLKNYSGKIVLPIDHVVSDSFADIPGRIIEDNSDH
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VYSLIGGGDSVAAAEKKGIADKFSFVSTGGGATLTFLERSSMPGIEAIQNKKGELNSKLE
GKPIPNPLLGLDSTRTGHHHHHH

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CLAIMS

1. A polypeptide comprising the amino acid sequence as set out in SEQ ID No 14 (Mhf EF-Ts) or a variant or fragment thereof.

2. A polypeptide comprising the amino acid sequence as set out in SEQ ID No 10 (Mhf pgk) or SEQ ID No 26 (CMhm pgk) or a variant or fragment thereof.

3. A polypeptide comprising the amino acid sequence as set out in SEQ ID No 6 (Mhf gapA) or SEQ ID No 22 (CMhm gapA) or a variant of fragment thereof.

4. A polypeptide comprising the amino acid sequence as set out in SEQ ID No 18 (CMhm dnaK) or a variant or fragment thereof.

5. A polypeptide comprising the amino acid sequence as set out in SEQ ID No 2 (Mhf dnaK) or a variant thereof, or a fragment thereof with the amino acid sequence MSKKETIIG or a fragment thereof from the sequence from position 309 to position 602 of Figure 3.

6. A polynucleotide encoding a polypeptide according to any of Claims 1 to 5.

7. An expression vector comprising a polynucleotide according to Claim 6.

8. A host cell comprising a polynucleotide according to Claim 6 or an expression vector according to Claim 7.

9. A polypeptide according to any of Claims 1 to 5 for use in medicine.

10. A vaccine comprising one or more of the polypeptides according to any of Claims 1 to 5 and optionally a further antigen and optionally an adjuvant.

11. A method of vaccinating a cat against haemotropic mycoplasma infection, the method comprising administering to the cat one or more polypeptides according to any of Claims 1 to 5, or a vaccine according to Claim 10.

12. A composition comprising one or more polypeptides according to any of Claims 1 to 5, or a vaccine according to Claim 10, for vaccinating a cat against haemotropic mycoplasma infection.

13. A method according to Claim 11, or a composition according to Claim 12, wherein the haemotropic mycoplasma infection is *Mycoplasma haemofelis* (Mhf) or '*Candidatus* Mycoplasma haemominutum' (CMhm) or '*Candidatus* Mycoplasma turicensis' (CMt).

14. An antibody directed to a polypeptide according to any of Claims 1 to 5.

15. An antibody according to Claim 14 for use in medicine.

16. A method of passively immunising a cat against haemotropic mycoplasma infection, the method comprising administering to the cat an antibody according to Claim 14.

17. An antibody according to Claim 14 for passively immunising a cat against haemotropic mycoplasma infection.

18. A method of detecting whether a cat has been exposed to haemotropic mycoplasma infection, the method comprising determining whether a suitable sample from the cat is reactive with a polypeptide according to any of Claims 1 to 5.

19. A method of detecting whether a mammal has been exposed to haemotropic mycoplasma infection, the method comprising determining whether a sample from the mammal contains a mycoplasma EF-Ts polypeptide.

20. An immunosorbent assay for detecting anti-mycoplasma protein antibodies in a sample, the assay comprising a solid phase coated with a polypeptide according to any of Claims 1 to 5 wherein the anti-mycoplasma protein antibodies in a sample exposed to the solid phase will bind to the polypeptide, and a detectable label conjugate which will bind to the anti-mycoplasma protein antibodies bound to the solid phase.

21. A solid substrate with a polypeptide according to any of Claims 1 to 5 attached thereto.

22. Any novel mycoplasma vaccine as herein disclosed.

23. Any novel method for detecting haemotropic mycoplasma infection as herein disclosed.

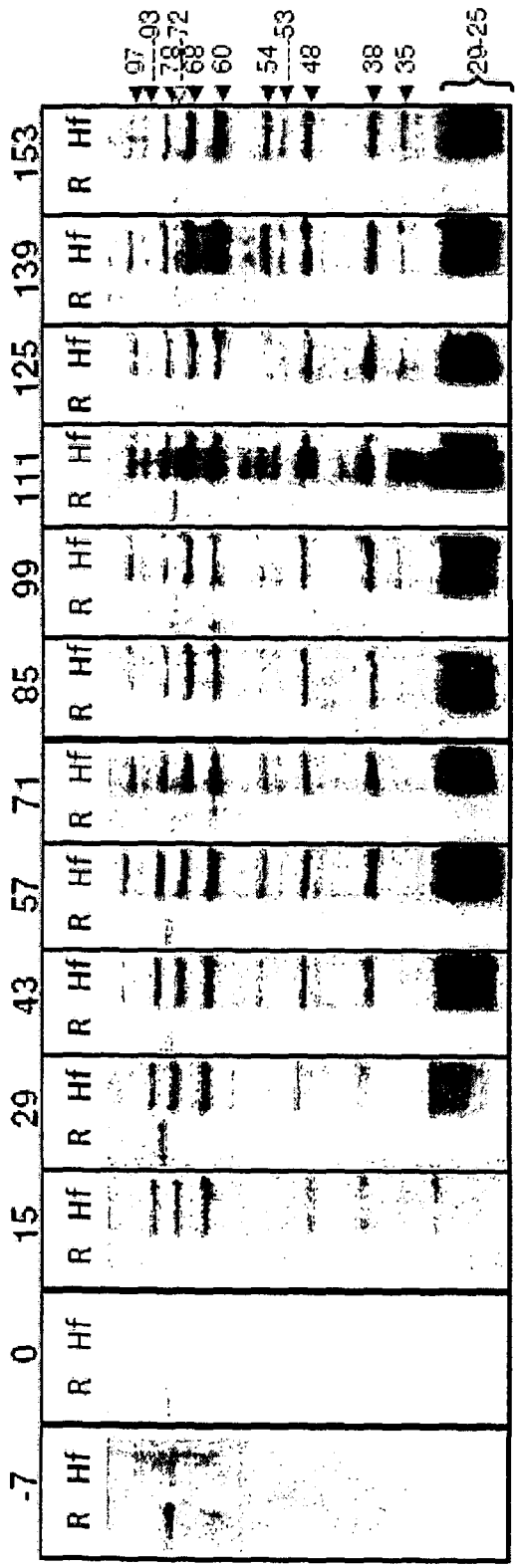

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Figure 1

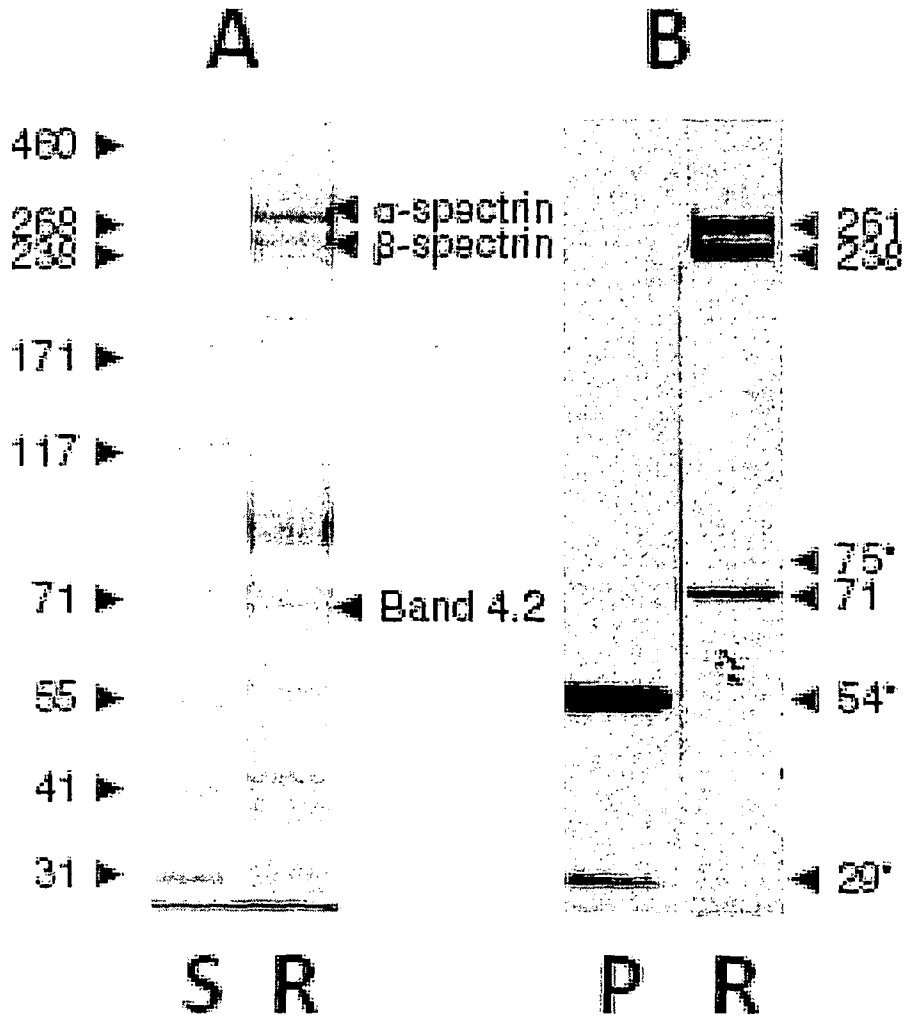

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Figure 2

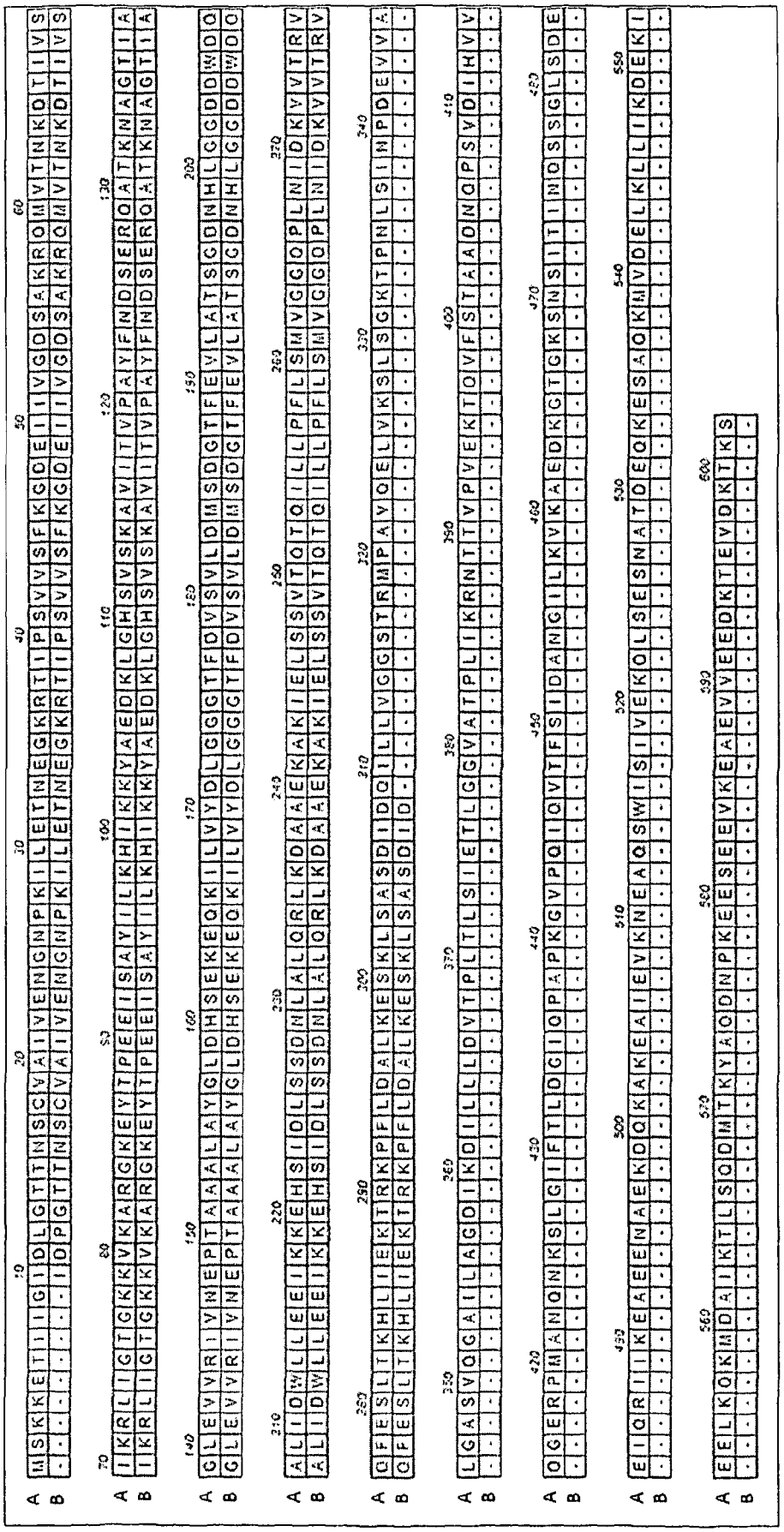


Figure 3

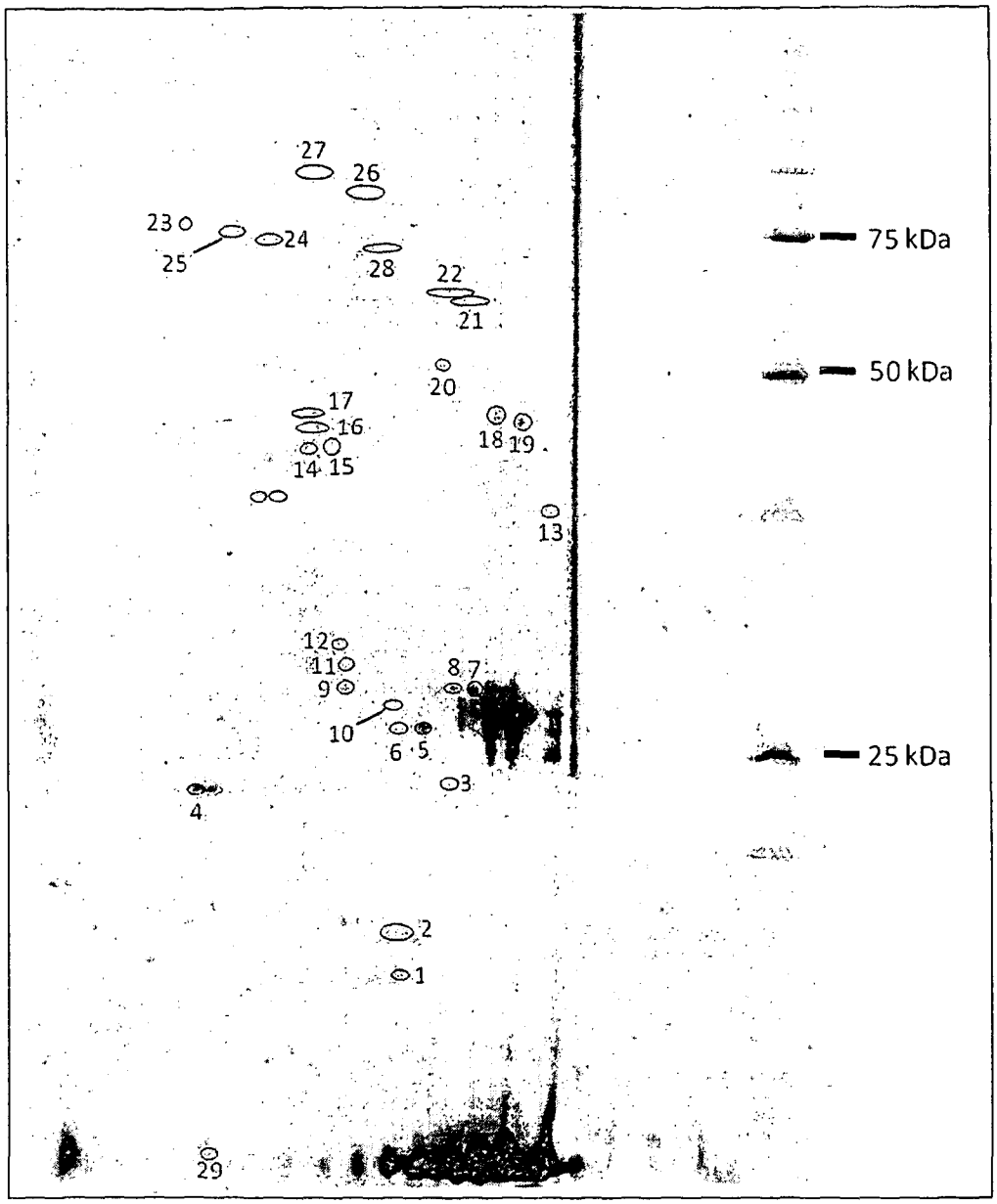

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Figure 4

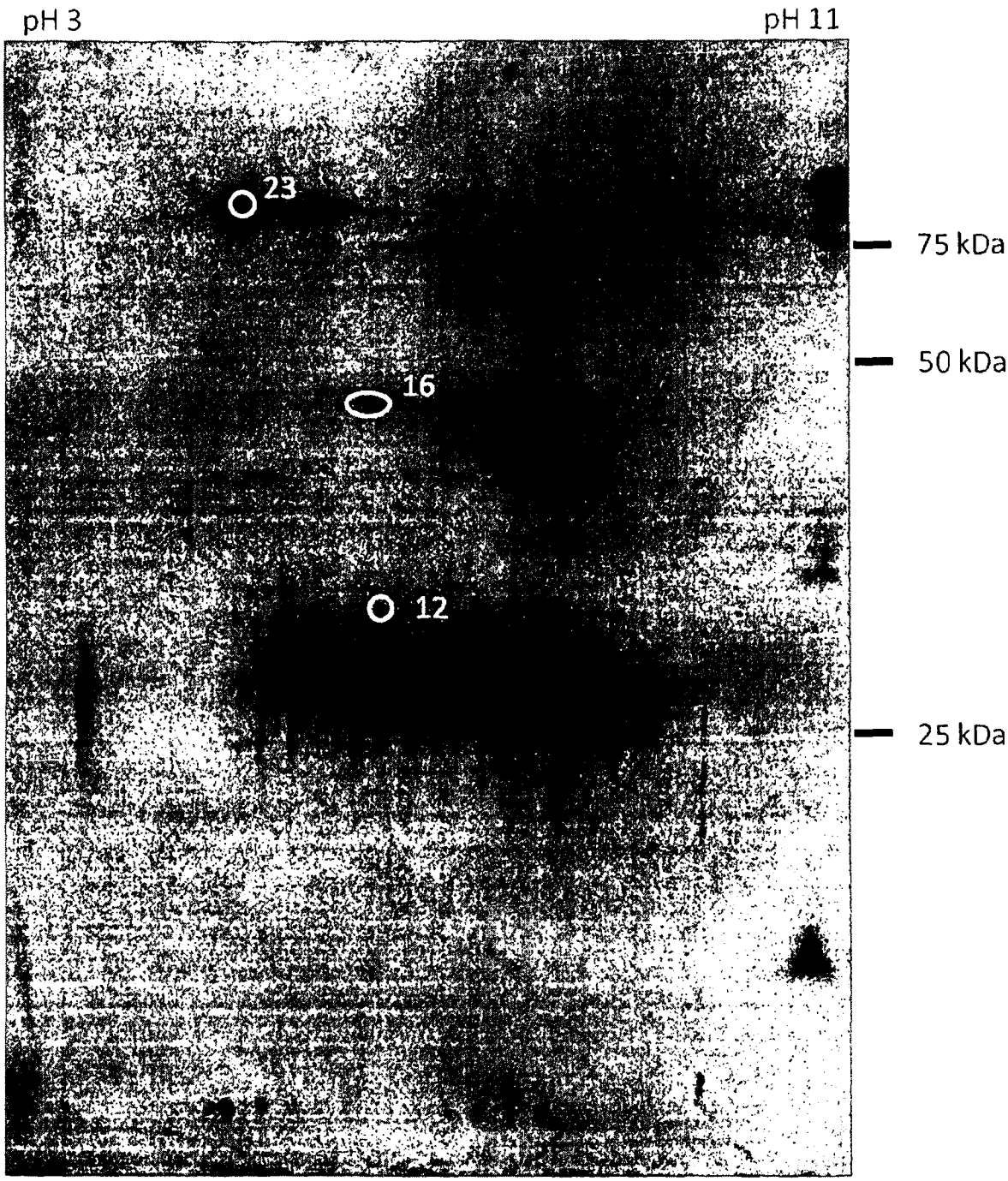


Figure 5


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Days post infection

Figure 6

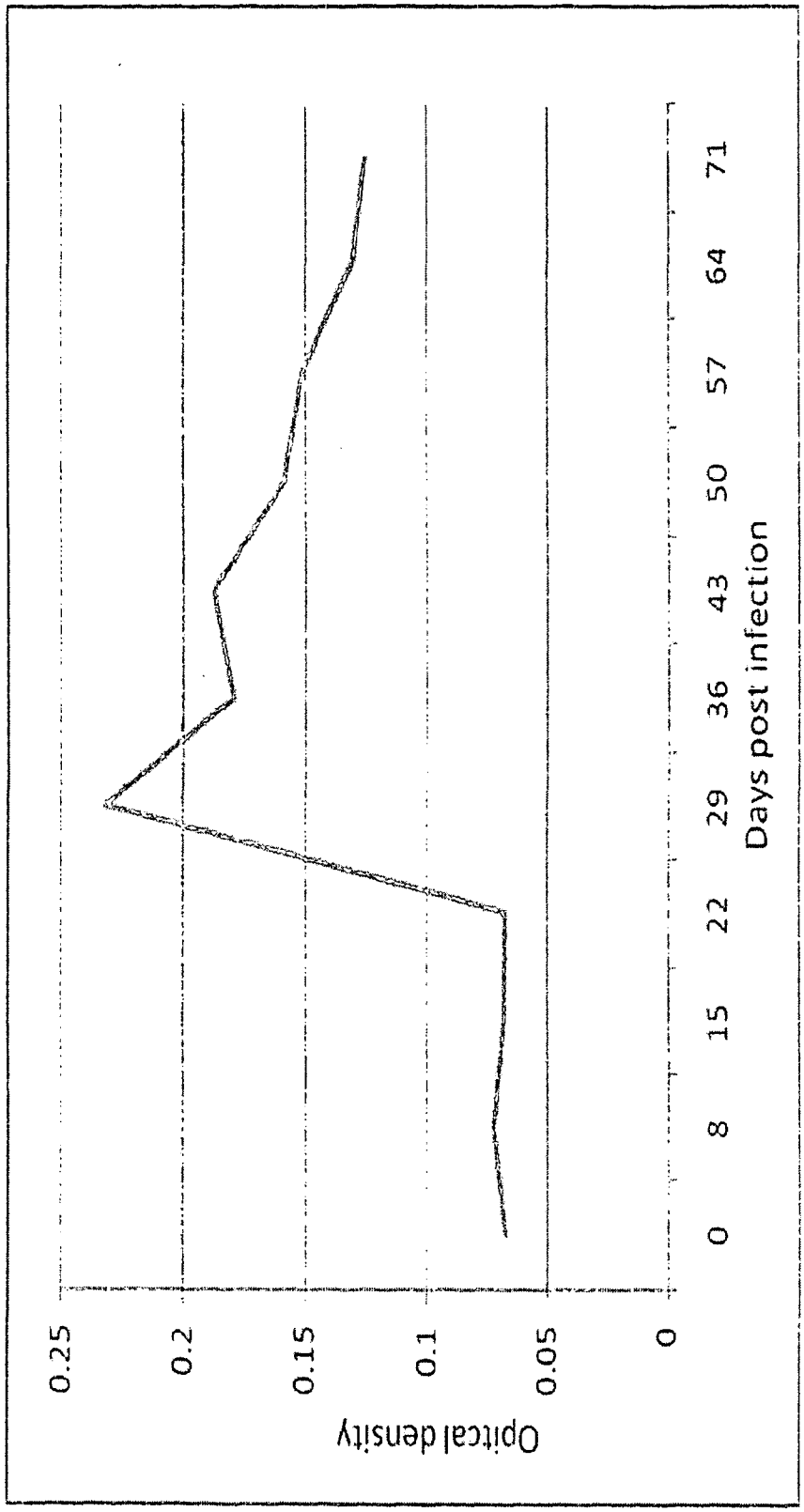

Optical density
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71
Days post infection

Figure 7