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(54) Titre : GENE CHIMERE FORME DE SEQUENCES D'ADN CODANT POUR LES SITES ANTIGENIQUES DE QUATRE PROTEINES DE L. INFANTUM, UTILE POUR LE DIAGNOSTIC SEROLOGIQUE DE LA LEISHMANIOSE CANINE, ET PROTEINE OBTENUE

(54) Title: CHIMERIC GENE FORMED OF THE DNA SEQUENCES THAT ENCODE THE ANTIGENIC DETERMINANTS OF FOUR PROTEINS OF L. INFANTUM, USEFUL FOR SEROLOGIC DIAGNOSIS OF CANINE LEISHMANIASIS AND PROTEIN OBTAINED

(57) **Abrégé/Abstract:**

Chimeric gene formed by the DNA sequences that encode the antigenic determinants of four proteins of L. infantum, useful for the serological diagnosis of canine Leishmaniosis and protein obtained, that consists of the prior employment of a cloning strategy. The patent describes the intermediate products generated during the process. A clone is achieved expressed in the protein rLiPO-Ct-Q (pPQI). To this initial vector, by means of the use of suitable restriction targets, DNA fragments are sequentially added that are encoded in other proteins and after each cloning step the correct orientation of each one of the inserts reduces the size of the expression products, the complete nucleotide sequence of the final pPQV clone being determined. A polypeptide is obtained with a molecular mass of 38 kD and with an isoelectric point of 7.37.



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ABSTRACT OF THE INVENTION

Chimeric gene formed by the DNA sequences that encode the antigenic determinants of four proteins of *L. infantum*, useful for the serological diagnosis of canine Leishmaniosis and protein obtained, that consists of the prior employment of a cloning strategy. The patent describes the intermediate products generated during the process. A clone is achieved expressed in the protein rLiPO-Ct-Q (pPQI). To this initial vector, by means of the use of suitable restriction targets, DNA fragments are sequentially added that are encoded in other proteins and after each cloning step the correct orientation of each one of the inserts reduces the size of the expression products, the complete nucleotide sequence of the final pPQV clone being determined. A polypeptide is obtained with a molecular mass of 38 kD and with an isoelectric point of 7.37.

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5

DESCRIPTION

**OBJECT OF THE INVENTION**

10 The present specification relates to an application  
for an Invention Patent, regarding a chimeric gene formed  
of the DNA sequences that encode the antigenic  
determinants of four proteins of L. infantum, useful for  
serologic diagnosis of canine leishmaniasis and protein  
15 obtained. The obvious purpose of this lies in using the  
protein obtained from the chimeric gene to perform an  
early diagnosis of canine leishmaniasis, that can be  
present in the body of a patient. This patient does not  
have to be a dog but can also be a human being who  
suffers from diseases that involve immuno-depression.  
20 This achieves an accurate diagnostic that avoids current  
diagnostic methods. These, in view of the fact that the  
antibodies present in animal and human serum to be  
analysed contain a large quantity of proteins can produce  
cross reactions, and can therefore give positive results  
25 when there is no real infection. Therefore existing  
types of analysis can give rise to uncontrolled false  
positive readings.

To summarise, with a view to minimising these  
problems, a chimeric gene will be produced that encodes a  
30 protein called MSPQ consisting of a chimeric product  
originating from an "in vitro" synthesis of a chimeric  
gene constructed "ad hoc", which contains five of the  
antigenic determinants of four different proteins. The  
product is configured as a highly sensitive and specific  
35 for the diagnosis of canine Leishmaniasis.

**FIELD OF THE INVENTION**

This invention is of utility within the industry



dedicated to the manufacture of pharmaceutical products in general.

#### **BACKGROUND OF THE INVENTION**

5 The parasitic protozoa of the Leishmania genus are the aetiological agents that cause Leishmaniasis, a range of diseases that have a world-wide distribution and that are characterised in that they give rise to a wide variety of clinical symptoms.

10 The main forms of Leishmaniasis are zoonotic in nature and humans are considered as secondary hosts.

The species denoted L. Infantum, widely distributed throughout many Mediterranean areas is the cause of visceral Leishmaniasis (LV) in humans and dogs.

15 In fact, dogs infected with L. infantum are the main animal reserve of this parasite, particularly during the long incubation period before the clinical symptoms can be observed.

20 The epidemiological data indicate that there is a direct correlation between the prevalence of canine Leishmaniasis and the transmission of the parasite to humans. For this reason, it is crucial to detect the disease or infection early on in campaigns undertaken to control the spread of the disease.

25 The parasite is transmitted to the host vertebrate as a flagellate promastigote, by means of a bite of a fly of the family "Phlebotominae", and the parasite enter the cells of the mononuclear phages where they differentiate and reproduce as amastigotes, within the phago-lisosomal structure.

30 The infected cells gather in certain tissues, mainly spleen, liver and lymph nodes. It is estimated that around 15 million people are infected with Leishmaniasis, and every year in the world 500,000 new clinical cases appear in the world, mainly in the underdeveloped and developing world.

35 In the south-western countries of Europe, Visceral Leishmaniosis (VL), is a zoonotic disease caused by the

L. Infantum species, as was mentioned earlier. Recent data derived from epidemiological studies indicate that there is an alarming incidence of this infection.

5 In Italy the reported data for incidence of VL ranges from 14.4% to 37% according to the region.

10 In Portugal, more particularly in the area around Lisbon, seropositive rates of 8.4% have been found and in the region of the French Maritime Alps different centres of prevalence have been found that vary between 3.2% and 17.1%.

15 In Spain, the prevalence of Leishmaniasis depends on the zone being studied. In Catalonia an average incidence rate of 9.3% has been observed although in some hot-spots a prevalence of infected dogs of up to 18% has been found.

On the Island of Mallorca, the incidence rate is 14%, and other rates that have been found are: 2.4% in Murcia, 8.8% in Granada, from 10 to 15% in Salamanca, 5.25% in the province of Madrid, and 14% in Caceres.

20 Although the number of cases of VL in humans caused by L. infantum can be considered relatively low, the high percentage of patients with immuno-depression that become infected by Leishmania could be related to the high level of this illness in dogs.

25 In fact, in the South of Europe, 50% of adults that are infected by Leishmaniasis are also patients infected by the HIV virus. On the other hand, according to these data of Leishmania-HIV co-infection, it has been estimated that the level of infection (by parasites) can be one or two orders of magnitude higher than this figure  
30 due to the existence of a large number of undetected infections.

35 A common characteristic of the different types of Leishmania infection is that it induces a strong humoral response in the host. Therefore, diagnostic methods based on serological techniques are currently the most widely used.



It has been described that these antibodies are detected even during the asymptomatic phase of the disease in natural and experimental infections.

5 The sensitivity and specificity of these methods depends on the type, source and purity of the antigen used. In immunological processes that are currently commercialised, complete promastigotes and preparations more or less prepared from these are used as a source of antigen. This method normally leads to cross-reactions  
10 with serum from patients suffering from leprosy, tuberculosis, African tripanosomiasis, Chagas disease, malaria and other parasitosis.

The sensitivity and specificity of the serologic methods depend on the type, source and purity of the  
15 employed antigen. During the last years a great number of Leishmania antigens have been characterised, some of them can be considered as proteins specific to the parasite.

Among these proteins specific to the parasite, the surface protease GP63, the surface glycoprotein gp46 and  
20 the lipophosphoglicane associated KMP-11 protein deserve a mention.

An additional group of Leishmania antigens are formed of evolutionarily conserved proteins, such as kinesine, thermal shock proteins, actin and tubulin.

25 As part of a strategy to develop a specific serological diagnostic system for Leishmaniasis canine, a laboratory based project has been undertaken to identify the antigens of *L. infantum*, by means of an immunodetection search of an expression library for genes of *L. infantum* using dog serum with active visceral  
30 Leishmaniasis.

It has been observed that most of the antigens isolated by this method belong to the family of proteins conserved during the course of evolution. The  
35 identification of the B epitopes of these antigens indicate, however, that in all cases the antigenic determinants were localised in regions that were not well

conserved.

In particular, the acidic ribosomal proteins LiP2a and LiP2b are recognised by more than 80% of the VL serums.

5 It has been confirmed that these proteins contain disease specific antigenic determinants, and that the recombinant proteins LiP2a and LiP2b, from which a fragment had been removed, could be used as a specific instrument able to distinguish between VL and Chages  
10 disease.

It, has also been shown that the PO ribosomal protein of L. infantum, very highly conserved on the evolutionary scale, is recognised by a high percentage of VL dog serums. Furthermore, the antigenic determinants  
15 are found exclusively on the C-terminus of the protein, that is to say, in the region that has been poorly conserved during the course of evolution.

It has been observed that in 78% of the VL dog serums, antigens against H2A protein are also present,  
20 and it has been confirmed that despite the sequence identity in all the H2A proteins among eukaryotic organisms, the humoral response to this protein in VL serums is particularly provoked by determinants specific to the Leishmania protein H2A.

25 The antigenic determinants recognised by the VL dog serums are found at both termini of the H2A protein.

The obvious solution to the problem currently encountered in this art would be to have an invention that would allow the assembly of a synthetic chimeric  
30 gene that contained the DNA regions encoding the antigenic determinants specific to the proteins LiP2a, LiP2b, LiPO, and H2A, with a view to constructing a protein rich in antigenic determinants.

35 However, as far as the applicant is aware, there is currently no invention that contains the characteristics described as ideal, with a view to reaching the desired aim. This aim is the construction of a protein rich in



antigenic determinants, arising from the assembly of a chimeric synthetic gene, that contains the DNA regions encoding the antigenic determinants specific to the aforementioned proteins.

5 **DESCRIPTION OF THE INVENTION**

In a first aspect, the invention relates to a chimeric gene formed by the DNA sequences that encode antigenic determinants of four proteins of *L.infantum*, useful for the serum diagnosis of canine Leishmaniasis.

10 In a further aspect, the invention relates to a protein encoded by said chimeric gene, containing one or more of the antigenic determinants of four proteins of *L.infantum* encoded by the chimeric gene.

The invention further relates to a diagnostic  
15 method for determining the presence of canine Leishmaniasis in a human being or an animal, in particular a dog, and/or in samples of biological fluids derived from humans or animals, such as a blood sample. In this diagnostic method, the chimeric gene of the  
20 invention or the protein encoded by it can be used.

Alternatively, in the diagnostic method, a nucleic acid probe sequences specific for the chimeric gene of the invention, or a part thereof, can be used, i.e. to establish the presence of canine Leishmaniasis in a  
25 patient or a sample.

Also, in the diagnostic method, antibodies against the protein encoded by the chimeric gene of the invention, or a antigenic part thereof such as an epitope, can be used.

30 The invention further relates to assays or other qualitative or quantitative methods for determining the presence of canine Leishmaniasis in a human being or an animal, in particular a dog, and/or in samples of biological fluids derived from humans or animals, such as  
35 a blood sample. Such assays can use the chimeric gene of the invention, the protein encoded by it, probes specific for the chimeric gene or part thereof, and/or antibodies



directed to the protein encoded by the chimeric gene of the invention, or any antigenic part thereof. Such assays can further be carried out in a manner known per se, for instance for probe-hybridization assays or immunoassays.

5 In a further aspect, the invention relates to diagnostic kits, at least comprising either a chimeric gene of the invention, a protein encoded by said chimeric gene, a probe specific for the chimeric gene of the invention, or an antibody directed to the protein of the  
10 invention. The kits can further contain all components for diagnostic kits and/or diagnostic assays known per se.

It should be noted that when herein, reference is made to the chimeric gene of the invention, this term  
15 also encompasses nucleic acid sequences that can hybridize with the sequence mentioned below under moderate or stringent hybridizing conditions.

In this context, heterologous hybridisation conditions can be as follows: hybridisation in 6 x SSC  
20 (20xSSC per 1000 ml : 175.3 g NaCl, 107.1 g sodium citrate.5H<sub>2</sub>O, pH 7.0), 0.1% SDS, 0.05% sodium pyrophosphate, 5\* Denhardt's solution (100 x Denhardt's solution per 500 ml : 10 g Ficoll™-400, 10 g polyvinyl-  
pyrrolidone, 10 g Bovine Serum Albumin (Pentax Fraction V)) and 20 µg/ml denatured herring sperm DNA at 56°C for  
25 18-24 hrs followed by two 30 min. washes in 5 x SSC, 0.1 % SDS at 56°C and two 30 min. washes in 2 x SSC, 0.1% SDS at 56°C.

For instance, sequences that can hybridize with the  
30 sequence mentioned below include mutant DNA sequences which encode proteins with the same biological function as the protein encoded by the sequence mentioned hereinbelow. Such mutant sequences can comprise one or more nucleotide deletions, substitutions and/or additions  
35 to the sequence mentioned below. Preferably, the mutant sequences still have at least 50%, more preferably at least 70%, even more preferably more than 90 % nucleotide

homology with the sequence given hereinbelow.

The term chimeric gene as used herein also encompasses nucleic acid sequences that comprise one or more parts of the sequence mentioned hereinbelow. Preferably, such sequences comprise at least 10%, more preferably at least 30%, more preferably at least 50% of the nucleotide sequence given hereinbelow. Such sequences may comprise a contiguous fragment of the sequence mentioned hereinbelow, or two or more fragments of the sequence given below that have been combined in and/or incorporated into a single DNA sequence.

It should be noted that when herein, reference is made to a protein encoded by the chimeric gene of the invention, this term also includes mutant proteins that still essentially have the same biological function. Such mutant proteins can comprise one or more amino acid deletions, substitutions and/or additions compared to the protein encoded by the sequence mentioned below. Preferably, the mutant proteins still have at least 50%, more preferably at least 70%, even more preferably more than 90 % amino acid homology with the sequence given hereinbelow.

The term protein also encompasses fragments of the protein encoded by the chimeric gene of the invention. Such fragments preferably still show the biological activity of the full protein. Preferably, such proteins comprise at least 30%, more preferably at least 50% of the amino acid sequence of the full protein. Also, two or more fragments of the full protein encoded by the chimeric gene of the invention may be combined to form a single protein.

Probes of the invention are such that they can - most preferably selectively- hybridize with the chimeric gene of the invention or part thereof, in particular under moderate as stringent hybridizing conditions, such as those mentioned above. Preferably, a probe of the invention will be essentially homologous with the



nucleotide sequence of the chimeric gene of the invention or a part thereof, i.e. show a homology of more than 80%, preferably more than 90%, more preferably more than 95%.

5 A skilled person will be able to select suitable probes. Usually, such probes will contain at least 15 bp, preferably more than 24 bp, of the sequence given hereinbelow.

10 The chimeric gene formed of the DNA sequences that encode the antigenic determinants of four proteins of *L. infantum*, useful for serological diagnosis of canine Leishmaniasis and protein obtained, that the invention proposes, in its own right constitutes an obvious novelty within its field of application, as according to the invention, a synthetic chimeric gene is produced that as  
15 it is obtained by assembly, containing the DNA region encoding the antigenic determinants specific to the proteins LiP2a, LiP2b, LiPO and H2A, thus constructing a protein rich in antigenic determinants. The chimeric gene obtained is expressed in *Escherichia coli* and the product  
20 has been analysed with respect to its antigenic properties. The results confirm that this chimeric protein maintains all the antigenic determinants of the parent proteins and that it constitutes a relevant diagnostic element for canine VL, with a sensibility that  
25 oscillates between 80% to 93%, and a specificity of between 96% to 100%.

More particularly, the chimeric gene formed by the DNA sequences that encode the antigenic determinants of four proteins of *L. infantum*, useful for the serological  
30 diagnostic of canine Leishmaniasis and protein obtained object of the invention, is produced by means of the following stages, namely:

- Construction of the chimeric gene. Methodology.  
Cloning strategy.  
35 Cloning of DNA sequences that encode antigenic determinants of the histone protein H2A.

Cloning of the sequences that encode rLiP2a-Q and

rLiP2b-Q.

Cloning of the sequence rLiPO-Q.

Cloning of the chimeric gene.

5 - Construction of the chimeric gene from the construction of intermediate products.

Cloning of epitopes specific to the L. infantum antigens.

- Construction of the final product

10 Construction of the chimeric gene that encodes a polypeptide that contains all the selected antigenic determinants.

- Evaluation of the final product.

Serums.

Purification of proteins

15 Electrophoresis of proteins and immuno-analysis.

Measurements by Fast-ELISA

- Evaluation of the final product.

Antigenic properties.

20 Sensitivity and specificity of the chimeric protein CP in the serum diagnosis of canine VL.

25 The strategy followed by the cloning of DNA sequences that encode each one of the selected antigenic determinants is the same in all cases, and in a first step, the sequence of interest is amplified by means of a PCR and the use of specific oligonucleotides that contain targets for restriction enzymes at the extremes.

30 For the cloning step, the amplified product is directed by means of the appropriate restriction enzyme and it is inserted in the corresponding restriction site of the plasmid pUC18.

35 After sequencing the DNA, the insert is recovered and sub-cloned to the corresponding restriction site of the modified plasmid denominated pMAL-c2. The modification is made by inserting a termination codon downstream of the target HindIII in the polylinker of pMal-c2, denominating the resulting plasmid pMAL-c2\*.

Regarding the cloning of the DNA sequence that



encodes the antigenic determinants of the histone protein H2A, it should be pointed out that the cDNA of the clone cL71, that encodes the histone H2A of *L. infantum*, is used as a template for the PCR reactions, and for the DNA amplification, that encodes the N-terminal region of the histone H2A, more exactly rLiH2A-Nt-Q, the following oligonucleotides are used: sense 5'- **CCTTTAGCTACTCCTCGCAGCGCCAAG**-3' (SEQ ID NO:1) (position 84-104 of the sequence cL71); antisense 5' **CCTGGGGGCGCCAGAGGCACCGATGCG**-3' (SEQ ID NO:2) (inverse and complimentary to position 204-224 of the sequence cL71).

The sequences that are included in the oligonucleotides for the cloning and that are not present in the parent sequence cL-71 are marked in boldface type.

The amplified DNA fragment is cloned directly from the restriction site XmnI of pMAI- c2\*.

The fragment is sequenced by means of the initiator #1234 maleE and the antigenic C-terminal region of histone H2A; in particular rLiH2A-Ct-Q, is amplified with the following oligonucleotides. These are:

Sense, 5'-GAATTCTCCGTAAGGCGGCCGCGCAG-3' (SEQ ID NO: 3) (position 276-296 of the sequence cL71).

Antisense, 5'-GAATTCGGGCGCGCTCGGTGTCGCCTTGCC-3' (SEQ ID NO:4) (inverse and complimentary to the positions 456-476 of the plasmid cL71).

A triplet that encodes proline (indicated as GGG after the underlined letters) is included in the antisense oligonucleotide, the restriction site *EccRI* that is included in both oligonucleotides for cloning is indicated by underlining.

Regarding the cloning of the sequences that encode rLiP2a-Q, it should be pointed out that the regions of interest are amplified by PCR from cDNAs encoding LiP2a and LiP2b.

The oligonucleotides that are used for constructing the expression clone LiP2a-Q, are the following.

Sense, 5'-GTCGACCCCATGCAGTACCTCGCCGCGTAC-3' (SEQ ID NO:5).

Anti-sense, 5'-GTCGACGGGGCCCATGTCATCATCGGCCTC-3' (SEQ ID NO:6).

It should be pointed out that the SalI restriction sites added to the 5' extremes of the oligonucleotides have been underlined.

When constructing the expression clone LiP2b-Q, the oligonucleotides used were:

Sense, 5'-TCTAGACCCGCCATGTCGTCGTCTTCCTCGCC-3' (SEQ ID NO:7).

Anti-sense, TCTAGAGGGGCCATGTCGTCGTCGGCCTC-3' (SEQ ID NO:8).

At the 5' extremes of the oligonucleotides the restrictions sites are included for the enzyme XbaI (underlined), and due to the cloning needs, an additional triplet, encoding a proline residue, is included downstream of the restriction site.

Regarding the cloning of the sequence rLiPO-Q, it should be pointed out that the cloning of the DNA sequence of the C-terminal region of the protein PO of *L. infantum* is carried out by amplifying a clone of cDNA called L27 and the following oligonucleotides:

Sense, 5'-CTGCAGCCCGCCGCTGCCGCGCCGGCCGCC-3' (SEQ ID NO:9) (positions 1-24 of the L27 cDNA) and the initiator of the pUC18 sequence (#1211), the amplified DNA is directed by the enzymes PstI+HindIII, with later insertion into the plasmid pMAL-c2.

The resulting clone is denominated pPQI and it should be noted that the restriction site PstI is included in the nucleotide with sense (underlined sequence) and that the restriction target HindIII is present in the cDNA L27.

Regarding the cloning of the chimeric gene, it should be pointed out that the DNA sequences that encode the five antigenic determinants are assembled into a chimeric gene, and this assembly is carried out on the clone pPQI, to which the codifying regions for the antigenic regions LiP2a-Q are added sequentially in the 3' direction (naming the results of cloning pPQ2), LiP2b-Q (clone pPQ3), LiH2a-Ct-Q (clone pPQ4) and LiH2A-Nt-Q (clone pPQ5).



Finally, the insert obtained after the SacI+HindIII digestion of the final clone pPQ5 is inserted into the pQE31 expression plasmid, naming the resulting clone pPQ.

#### DESCRIPTION OF THE DRAWINGS

5 To complete the description that is being made and with the aim of aiding the understanding of the characteristics of the invention, the present disclosure is accompanied, as an integral part thereof, by a set of plans of illustrative nature that are not limiting. The  
10 following is represented:

Figure number 1. Expression, purification, and antigenicity of the engineered Leishmania proteins. (A) SDS-PAGE of E. coli lysates transfected with MBP-LiP0-Ct-Q (lane 1), MBP-LiP2a-Q (lane 2), MBP-LiP2b-Q (lane  
15 3), MBP-LiH2A-Ct-Q (lane 4), and MBP-LiH2A-Nt-Q (lane 5). Mr: molecular mass markers. (B) SDS-PAGE of the corresponding recombinant proteins after purification through the amylose column. (C) Western blot of the  
20 reactivity of three pooled canine VL serum samples (final dilution, 1:100) against the purified proteins. (D) FAST-ELISA of the reactivities (means and SDs) of 26 VL serum samples against the purified antigens. Dilution of  
25 the sera 1:300.

Figure number 2. Sample of the different vectors considered to obtain the chimeric gene object of the invention, from which the pertinent protein destined to  
30 carry out an accurate diagnostic on animals or human beings that show symptoms of Leishmaniasis will be extracted.

Figure number 3. Corresponds to the identification of the protein obtained from the chimeric gene, the preparation of which is represented in Figure number 2.  
35

## 13a

Figure number 4. Expression, purification, and antigenicity of all the intermediates and the final chimeric protein. (A) SDS-PAGE of E. coli lysates transformed with PQI (lane 1), PQII (lane 2), PQIII (lane 3), PQIV (lane 4), and PQV (lane 5). Mr: molecular mass. (B) SDS-PAGE of the corresponding recombinant proteins after purification through the amylose column. (C) Western blot of the reactivity of three pooled canine VL serum samples (final dilution, 1:100) against the purified antigens. (D) SDS-PAGE of the pQE chimeric expression product. Lane 1, lysates of E. coli harboring the PQ protein; lane 2, the PQ protein after purification through a Ni-nitrilotriacetic acid column. Mr: molecular mass. (E) Western blot of the reactivity of a pool of three canine VL serum samples against the purified PQ protein. (F) FAST-ELISA of the reactivities (+ SDs) of 26 VL serum samples against each one of the intermediate proteins PQI to PQIV and final products PQV and PQ. Dilution of the sera 1:300 dilution.

Figure number 5. FAST-ELISA evaluation of the diagnostic value of the recombinant PQ protein. Mean O.D. values (plus SDs) of sera from dogs infected with L. infantum (group 1; n 59), sera from dogs infected with other pathogens or suffering from diseases other than leishmaniasis (group 2; n 49), and sera from healthy dogs (group 3; n 15). Dilution of sera 1:300



**PREFERRED EMBODIMENT OF THE INVENTION**

5 The chimeric gene formed from the DNA sequences that encode the antigenic determinants of four proteins of *L. infantum*, useful for the serological diagnosis of canine Leishmaniasis and the protein obtained that is being proposed are constituted from the construction of intermediate products. In a first instance, cloning of epitopes specific to the antigens of *L. infantum* is carried out, which is configured on the basis of earlier studies on the antigenic properties of four protein antigens of *L. infantum* (LiP2a, PiPO, LiP2b, LiH2a), which allow the existence of B epitopes to be defined for these proteins, and which are specifically recognised by the canine serums of VL.

10 With a view to improving the antigenic specificity of these antigens with respect to the proteins of *L. infantum*, the specific antigenic determinants are cloned from these proteins. After deleting certain regions of these proteins these can be recognised by serums from animals that are carriers of VL and other different diseases.

15 By using the specific oligonucleotides and amplification by PCR of regions specific to the genes LiP2a, LiP2b, PO and H2A, several clones are constructed that express the recombinant proteins rLiPO-Ct-Q, rLiP2a-Q, rLiP2b-Q, rLiH2A-Ct-Q and rLiH2A-Nt-Q, just has been detailed in the description of the invention relating to the methodology, where the cloning details are described.

20 The recombinant proteins used are the following:

- rLiPO-Ct-Q, which corresponds to the 30 C-terminal residues of the ribosomal protein LiPO.

- rLiP2a-Q and rLiP2b-Q, that are derived from the ribosomal proteins LiP2a and LiP2b respectively.

25 - Two sub-regions of the histone H2A, that correspond to the 46 N-terminus residues (xLiH2A-Nt-Q), and to the 67 C-terminus residues (residues (xLiH2A-Ct-

Q).

Each one of the recombinant proteins fused to the maltose binding protein (MBP) is expressed in *E. Coli*, as represented in Figure number 1, and they were purified by affinity chromatography on a amylose column. After the process of purification the electrophoresis was carried out on the recombinant proteins (lanes 1 to 5) in Figure number 3.

With the aim of analysing whether the recombinant proteins were recognised by VL canine serums, a Western blot was incubated, containing the recombinant proteins in a mixture of three VL canine serums. Given that all these proteins are recognised by the serums, it is concluded that the antigenic determinants present in the parent proteins are maintained in the recombinant proteins.

The antigenic properties of the recombinant proteins are compared with the antigenic determinants of the parent antigens by means of a FAST ELISA, testing against a collection of 26 VL canine serums, just as is shown in the section of Figure number 1, and the fact that the serums showed a similar reactivity value, both against the selected antigenic regions and the corresponding complete proteins, demonstrates that no alteration to the antigenic epitope has occurred during the cloning procedure.

In regard to the construction of the final product, more exactly of the chimeric gene that encodes a polypeptide that contains all the selected antigenic determinants, it should be pointed out that the cloning strategy, is indicated following Figure number 2 section A. The intermediate products generated during the process are shown.

A clone that expresses the proteins rLiPO-Ct-Q (pPQI) is used as the initial vector, and the fragments of DNA that encode the proteins rLiPO-Ct-Q, rLiP2a-Q, rLiP2b-Q, rLiH2A-Ct-Q and rLiH2A-Nt-Q are added



sequentially using appropriate restriction sites.

After each cloning step, the correct orientation of each one of the inserts is deduced from the size of the expression products, and finally the complete nucleotide sequence of the final clone pPQV is determined and the amino acid sequence deduced from the sequence represented in Figure number 3.

The polypeptide generated has a molecular mass of 38 kD, with an isoelectric point of 7.37, including spacer sequences encoding proline, underlined in Figure number 3. The aim of doing this is to efficiently separate the antigenic domains and avoid possible tertiary conformations that could interfere with the stability and antigenicity of the final product.

The expression and recovery of each of the intermediate products is shown in figure number 4, boxes A and B. As was expected, after each addition, the size of the expression product in the vector pMAL gradually increases until reaching a molecular weight of 80 kDa. Included in this are the sizes of the proteins rLiH2A-Ct and rLiH2A-Ct, observing a certain degree of rupture during purification.

The chimeric gene was also cloned in the plasmid pQE, a vector that allows the expression of proteins with a fragment of 6 histidines at the extreme N-terminus.

The resulting clone and the recombinant proteins are denominated pPQ and PQ respectively.

The level of expression of the protein in bacteria transformed with the pPQ plasmid and the purified proteins are shown in Figure number 4, referred to in particular with a D, with the protein PQ, purified by affinity chromatography in denaturising conditions is more stable than the recombinant protein pPQV represented in Figure number 4, in box E.

In order to evaluate the final product a series of materials were used, and obviously some techniques, as is described below.

Serums of VL obtained from dogs of different origins are used. The animals are evaluated clinically and analytically in the pertinent laboratory, generally in a Department of Parasitology, and all the positive serums are assayed for indirect immuno-fluorescence (IIF).

The presence of amastigotes of the parasites of these animals is confirmed by direct observation of the popliteal and pleescapular lymph nodes, and a second group of 33 serums of VL originating from other regions, were given a positive diagnosis in the ELISA against total protein extracts of the parasite and/or by IIF.

The serums of dogs affected by different diseases that were not VL are obtained from different origins. Within this group serums from the following infections are found:

Mesocestoides spp.  
Dyphylidium caninum  
Uncinaria stenocephala  
Toxocara canis  
Dipetalonema dranunculoides  
Demodex canis  
Babesia canis  
Ehrlichia canis  
Rickettsia rickettsiae.

The rest of the serums were obtained from dogs that exhibited various clinical symptoms that were not related to any infective process, and the serum controls were obtained from fifteen carefully controlled healthy animals.

Purification of the recombinant proteins expressed by the clones pMA1-c2 is carried out by affinity chromatography on amylose columns, and the purification of the recombinant protein expressed by the clone pPQ was performed on Ni-NTA resin columns in denaturising conditions (Qiagen).

For analysing the proteins electrophoresis on 10% polyacrimide gels in the presence of SDS was carried out



under standard conditions. Immunological analysis of the proteins separated by electrophoresis was carried out on nitrocellulose membranes to which the proteins had been transferred. The transferred proteins were blocked with  
5 dried 5% skimmed milk in a PBS buffer with 0.5% Tween™ 20.

The filters were sequentially brought into contact with primary and secondary anti-serum in blocking solutions and an immuno-conjugate labelled with  
10 peroxidase was used as second antibody, visualising the specific binding by means of an ECL system.

The Fast-ELISA was used instead of the classic ELISA, and the sensitisation of the antigen was carried out for 12 hours at room temperature.

15 The plates were sensitised with 100  $\mu$ l of antigen whose concentration in all cases was 2  $\mu$ g/ml.

After sensitising the wells the plates were incubated for 1 hour with blocking solution (0.5% powdered skimmed milk dissolved in PBS - 0.5% Tween™ 20.  
20 and the serums were diluted three hundred fold in blocking solution).

The wells were incubated with serum for 2 hours at room temperature, and after exposure to the antibody the wells were washed with PBS-Tween™ 20.

25 Antibodies labelled with peroxidase were used as second antibodies at a dilution of 1:2000 and the colour of the reaction was developed using the substrate ortho-phenylenediamine, measuring the absorption at 450 nm.

In regard to evaluation of the final product, it  
30 should be pointed out that the antigenic properties were determined by means of the pertinent study of the reactivity of the VL canine serums against the chimeric protein and against each one of the intermediate products in a "Western blot" assay. All the intermediate products  
35 maintained their antigenicity as well as did the final pPQV product, throughout the whole of the cloning process.

It should also be pointed out that the recombinant protein expressed by the pPQ plasmid was recognised by the VL serums. With a view to analysing with greater precision the antigenic properties of the chimeric protein and the intermediate products, an analysis of the reactivity of a wide variety of VL canine serums was performed by means of a fast-ELISA against the recombinant proteins, as is shown in the section F of Figure number 4. It can be highlighted that the absorption values and the sensitivity of the different intermediate products of cloning increases after each addition step. It should also be pointed out that the protein pQI is recognised by most of the VL serums and the protein PQII equally by most of the serums. This proportion is greater for the protein PQIII, and the proteins PQIV, PQV and PQ are recognised by practically all the serums.

According to what has been discussed above, the percentage of recognition shown by the serums was similar both in the case of assaying the chimeric proteins PQV and PQ, and of assaying a mixture of recombinant proteins rLiPO-Ct-Q, rLiP2a, rLiP2b and rLiH2A. It was seen that the antigenic properties of each one of the 5 selected antigenic regions are present in the PQ expression product, and therefore this product can be used for diagnosis instead of a mixture of the antigens expressed individually.

With a view to determining whether the chimeric protein can be used for canine VL serum diagnosis, and according to the pertinent analysis of a wide variety of canine serums against this protein, bearing in mind that according to the clinical characteristics of the animals, the canine serums have been classified into three groups. A first group consisted of serum of dogs with a real *L. infantum* infection. A second group was composed of serums of dogs that had various clinical symptoms without being infected with *Leishmania*, including dogs infected with



parasites different to Leishmania incorporated into this group. The rest of the serums originated from dogs that exhibit clinical symptoms that could be confused with those observed during Leishmaniasis.

5           The third group was made up of control serums, originated from serums of healthy dogs.

          In figure number 5 the average values of reactivity are shown for each group of serums, the reactivity of the VL serums reaching an average reactivity value of 0.8  
10       (S.D. = 0.4).

          Within this group the reactivity of 12 serums is less than 0.35, while the reactivity of 10 serums reaches values of between 0.35 and 0.5. It is observed that the reactivity over 23 serums varies between 0.5 and 1.0,  
15       with 14 serums showing a reactivity greater than 1.0.

          The average absorption value of the serums of the second group, that is to say, the group in which animals infected with Leishmania parasites and parasites different to Leishmania parasites, is 0.2 (S.D. = 0.05)  
20       and the reactivity of the control serums, that is to say, the third group, is 0.1 (S.D. = 0.003).

          The data presented above indicate that the chimeric protein PQ in the FAST ELISA has a sensitivity of 80% for the VL diagnosis, if the cut-off value is defined as the  
25       average reactivity value of the serums of group 2 plus three S.D.'s (that is to say 0.35).

          The sensitivity of the assayed group reaches 93%, if the cut-off value is defined by the reactivity values of the control group, and the data indicate that the  
30       protein CP has a specificity of 96% for the VL diagnosis, when the cut-off value is defined by the aforementioned serums of group 2, and only two serums from group 2 showed reactivity between 0.35 and 0.40. 100% specificity in the assay was reached when the reactivity  
35       values of healthy dogs were considered.

          The process to be used is the following:

1.- The microtitre plates are covered with

antibodies by incubated 100  $\mu$ l of a solution that contains 1  $\mu$ g/ml of antigen dissolved in a buffer PBS - 0.5% Tween 20 - 5% skimmed milk (Buffer A).

5 The incubation is performed for 12 hours at room temperature, and then the plates are washed three times with the same buffer containing no antigen. The dry antigenated plates could be maintained at room temperature.

10 2.- A first incubation of the wells was carried out with the serum of animal at a dilution of 1/200 in buffer A. The incubation lasts for 1 hour.

3.- The wells are washed with buffer A, as described in point 1, three times with a wash flask.

15 4.- They are incubated with a second antibody (IgG labelled with peroxide) diluted 1:2000 in buffer A, carrying out the incubation for 1 hour.

5.- The wells are washed once again with buffer A three times, as was indicated in the third section, that is to say with a wash flask.

20 6.- The reactivity is revealed using the substrate ortho-phenylenediamine and the absorption measured at 450 nm.

The protein used for the diagnosis extracted from the chimeric gene is identified as follows:

25 MBP IEGRPLATPRSAKKAVRKSGSKSAKCGLIFPVGRVGGMMRRGQYARRIGA 50  
SGAPRISEFSVKAAAQSGKKRCRLNPRTVMLAARHDDDIGTLLKNVTLSHSGVV 104  
 PNISKAMAKKKGGKKGKATPSAPEFGDSSRPMSTKYLAAYALASLSKASPSQAD 158  
 VEAICKAVHIDVDQATLAFVMESVTGRDVATLTAEGAAKMSAMPAASSGAAAGV 212  
 30 TASAAGDAAPAAAAAKKDEPEEEADDDMGPSVRDPMQYLAAYALVALSGKTPSK 266  
 ADVQAVLKAAGVAVDASRVDAVFQEVEGKSFDALVAEGRTKLVGSGSAAPAGAV 320  
 STAGAGAGAVAEAKKEEPEEEEEADDDMGPVLDLQPAAPAAAPSAAAKEEPEESD 374  
 35 EDDFGMGGLF (SEQ ID NO:10)

It is not considered necessary to extend this



description in order that someone skilled in the art can understand the scope of the invention and the advantages that it confers.

5 The materials, form, size and disposition of the elements are susceptible to change, provided it does not suppose a change in the essence of the invention.

The terms in which this disclosure has been written should always be considered as broad in nature and not limiting.

## SEQUENCE LISTING

(i) APPLICANT: C.B.F. Leti S.A.

(ii) TITLE OF INVENTION: Chimeric Gene Formed of the DNA Sequences that Encode the Antigenic Determinants of Four Proteins of L. Infantum, Useful for Serologic Diagnosis of Canine Leishmaniasis and Protein Obtained

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: BERESKIN & PARR
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- (E) COUNTRY: Canada
- (F) ZIP: M5H 3Y2

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: CA 2,256,125
- (B) FILING DATE: 23-DEC-1998
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Gravelle, Micheline
- (B) REGISTRATION NUMBER: 4189
- (C) REFERENCE/DOCKET NUMBER: 444-152

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (416) 364-7311
- (B) TELEFAX: (416) 361-1398

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTTTAGCTA CTCCTCGCAG CGCCAAG

27

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid



24

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCTGGGGGCG CCAGAGGCAC CGATGCG

27

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCTCCG TAAGGCGGCC GCGCAG

26

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAATTCGGGC GCGCTCGGTG TCGCCTTGCC

30

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTCGACCCCA TGCAGTACCT CGCCGCGTAC

30

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTCGACGGGG CCCATGTCAT CATCGGCCTC

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCTAGACCCG CCATGTCGTC GTCTTCCTCG CC

32

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCTAGAGGGG CCATGTCGTC GTCGGCCTC

29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGCAGCCCG CCGCTGCCGC GCCGGCCGCC

30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide



## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ile	Glu	Gly	Arg	Pro	Leu	Ala	Thr	Pro	Arg	Ser	Ala	Lys	Lys	Ala	Val
1				5					10					15	
Arg	Lys	Ser	Gly	Ser	Lys	Ser	Ala	Lys	Cys	Gly	Leu	Ile	Phe	Pro	Val
			20					25					30		
Gly	Arg	Val	Gly	Gly	Met	Met	Arg	Arg	Gly	Gln	Tyr	Ala	Arg	Arg	Ile
		35					40					45			
Gly	Ala	Ser	Gly	Ala	Pro	Arg	Ile	Ser	Glu	Phe	Ser	Val	Lys	Ala	Ala
	50					55					60				
Ala	Gln	Ser	Gly	Lys	Lys	Arg	Cys	Arg	Leu	Asn	Pro	Arg	Thr	Val	Met
65					70					75					80
Leu	Ala	Ala	Arg	His	Asp	Asp	Asp	Ile	Gly	Thr	Leu	Leu	Lys	Asn	Val
				85					90					95	
Thr	Leu	Ser	His	Ser	Gly	Val	Val	Pro	Asn	Ile	Ser	Lys	Ala	Met	Ala
			100					105					110		
Lys	Lys	Lys	Gly	Gly	Lys	Lys	Gly	Lys	Ala	Thr	Pro	Ser	Ala	Pro	Glu
		115					120					125			
Phe	Gly	Asp	Ser	Ser	Arg	Pro	Met	Ser	Thr	Lys	Tyr	Leu	Ala	Ala	Tyr
	130					135					140				
Ala	Leu	Ala	Ser	Leu	Ser	Lys	Ala	Ser	Pro	Ser	Gln	Ala	Asp	Val	Glu
145					150					155					160
Ala	Ile	Cys	Lys	Ala	Val	His	Ile	Asp	Val	Asp	Gln	Ala	Thr	Leu	Ala
				165					170					175	
Phe	Val	Met	Glu	Ser	Val	Thr	Gly	Arg	Asp	Val	Ala	Thr	Leu	Thr	Ala
			180					185					190		
Glu	Gly	Ala	Ala	Lys	Met	Ser	Ala	Met	Pro	Ala	Ala	Ser	Ser	Gly	Ala
		195					200					205			
Ala	Ala	Gly	Val	Thr	Ala	Ser	Ala	Ala	Gly	Asp	Ala	Ala	Pro	Ala	Ala
	210					215					220				
Ala	Ala	Ala	Lys	Lys	Asp	Glu	Pro	Glu	Glu	Glu	Ala	Asp	Asp	Asp	Met
225					230					235					240
Gly	Pro	Ser	Val	Arg	Asp	Pro	Met	Gln	Tyr	Leu	Ala	Ala	Tyr	Ala	Leu
				245					250					255	
Val	Ala	Leu	Ser	Gly	Lys	Thr	Pro	Ser	Lys	Ala	Asp	Val	Gln	Ala	Val
			260					265					270		
Leu	Lys	Ala	Ala	Gly	Val	Ala	Val	Asp	Ala	Ser	Arg	Val	Asp	Ala	Val
		275					280					285			
Phe	Gln	Glu	Val	Glu	Gly	Lys	Ser	Phe	Asp	Ala	Leu	Val	Ala	Glu	Gly
	290					295					300				

Arg	Thr	Lys	Leu	Val	Gly	Ser	Gly	Ser	Ala	Ala	Pro	Ala	Gly	Ala	Val
305					310					315					320
Ser	Thr	Ala	Gly	Ala	Gly	Ala	Gly	Ala	Val	Ala	Glu	Ala	Lys	Lys	Glu
				325					330					335	
Glu	Pro	Glu	Glu	Glu	Glu	Ala	Asp	Asp	Asp	Met	Gly	Pro	Val	Asp	Leu
			340					345					350		
Gln	Pro	Ala	Ala	Ala	Ala	Pro	Ala	Ala	Pro	Ser	Ala	Ala	Ala	Lys	Glu
		355					360					365			
Glu	Pro	Glu	Glu	Ser	Asp	Glu	Asp	Asp	Phe	Gly	Met	Gly	Gly	Leu	Phe
	370					375					380				



THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated nucleic acid molecule, comprising a recombinant cDNA molecule encoding a chimeric polypeptide which comprises at least one antigenic determinant, recognised by serum from dogs with visceral Leishmaniasis, from each of the LiP2a, LiP2b, LiH2a and LiPO proteins from *Leishmania infantum*, wherein said chimeric polypeptide has at least 70% sequence identity to SEQ ID NO: 10.
2. The isolated nucleic acid molecule of claim 1, wherein said at least one antigenic determinant from each of LiP2a, LiP2b, LiH2a and LiPO proteins are linked to each other in said chimeric polypeptide by spacer sequences.
3. The isolated nucleic acid molecule of claim 1 or 2, wherein said chimeric polypeptide has a molecular weight of 38 kD and an isoelectric point of 7.37.
4. The isolated nucleic acid molecule of any one of claims 1 to 3, wherein said chimeric polypeptide comprises the amino acid sequence of SEQ ID NO:10.
5. The isolated nucleic acid molecule of any one of claims 1 to 4, wherein said recombinant cDNA encodes said chimeric polypeptide fused to maltose binding protein.
6. The isolated nucleic acid molecule of any one of claims 1 to 5, wherein said at least one antigenic determinant from each of LiP2a, LiP2b, LiH2a and LiPO proteins are derived from rLiP2a-Q, rLiP2b-Q, the 46 N-terminal residues and the 67C- terminal residues of histone protein LiH2a, and the 30 C-terminal residues of ribosomal protein LiPO.
7. An isolated chimeric polypeptide encoded by the nucleic acid molecule of claim 1.
8. The isolated chimeric polypeptide of claim 7, which has a molecular weight of 38 kD and an isoelectric point of 7.37.

9. The isolated chimeric polypeptide of claim 7 or 8, comprising an amino acid sequence of SEQ ID NO:10.

10. The isolated chimeric polypeptide of any one of claims 7 to 9, wherein said at least one antigenic determinant from each of LiP2a, LiP2b, LiH2a and LiPO proteins are derived from rLiP2a-Q, rLiP2b-Q, the 46 N-terminal residues and the 67C-terminal residues of histone protein LiH2a, and the 30 C-terminal residues of ribosomal protein LiPO.

11. The isolated chimeric polypeptide of any one of claims 7 to 10, which is fused to maltose binding protein.

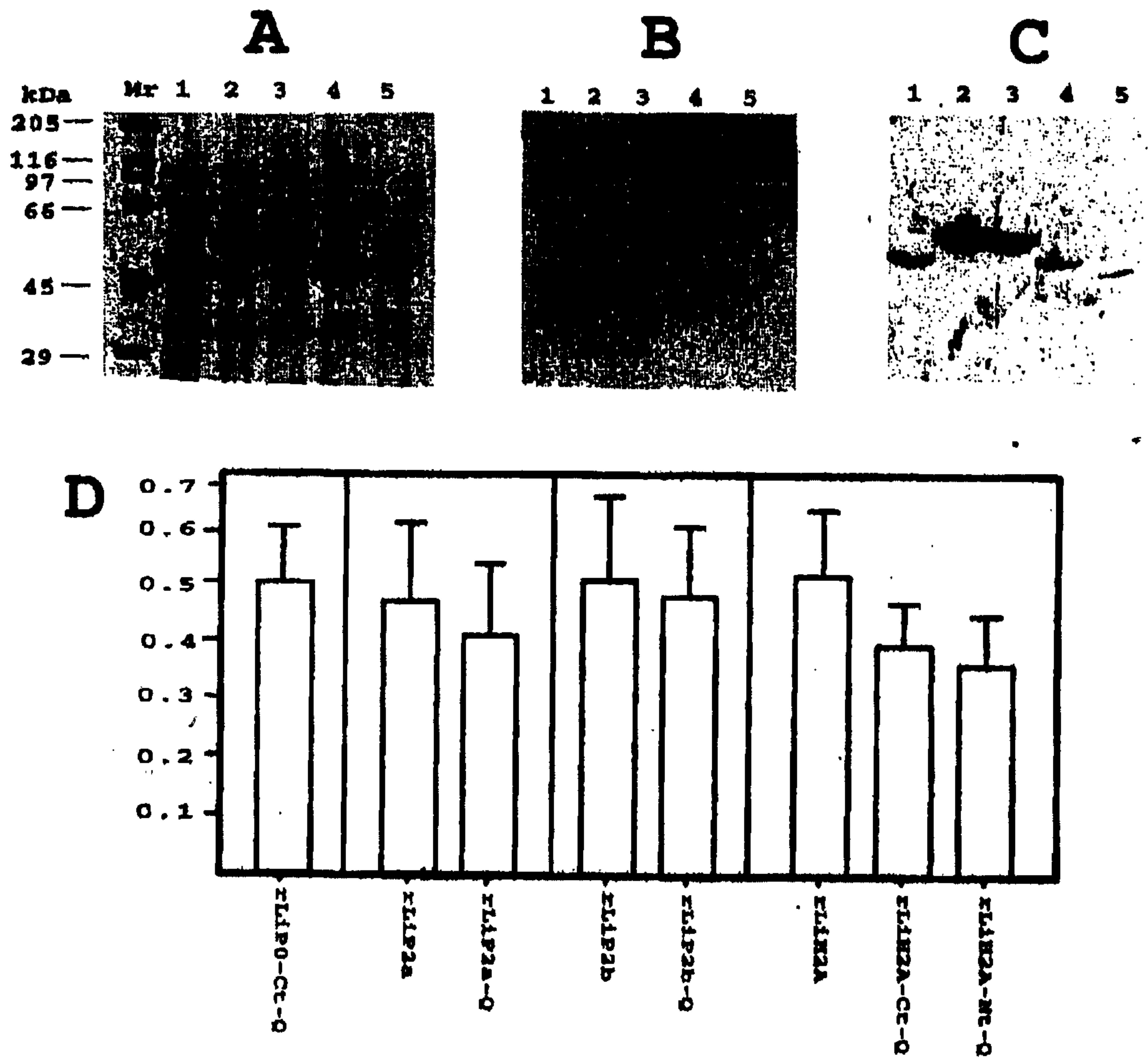
12. A composition comprising the polypeptide of any one of claims 7 to 11 and a carrier.

13. The composition of claim 12 which is a pharmaceutical composition.

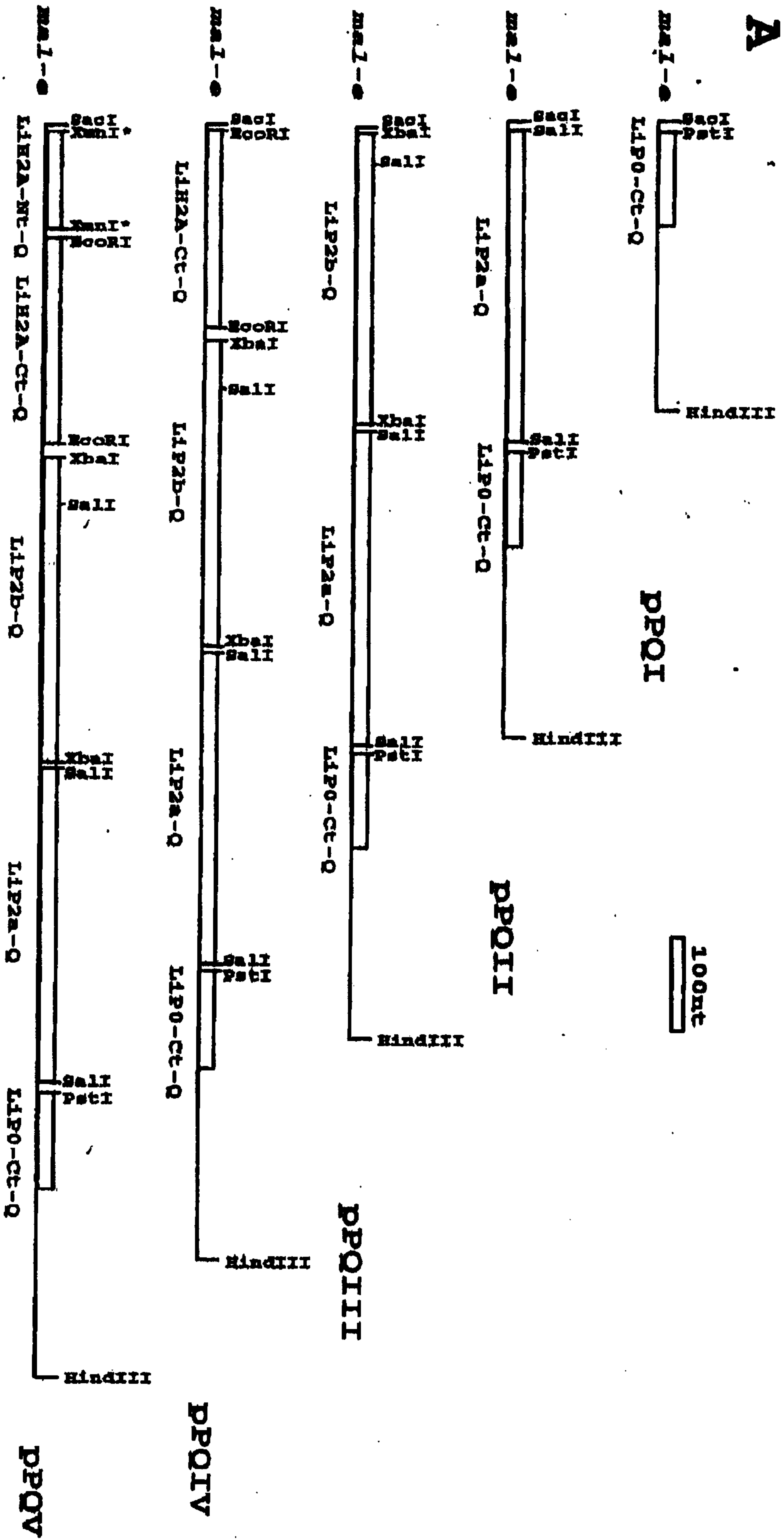
14. A method for detecting visceral Leishmaniasis, comprising: contacting the sample from the blood of a human or animal with the polypeptide of any one of claims 7 to 11; and determining the presence of visceral Leishmaniasis by detection of binding between the polypeptide and *Leishmania infantum* antibodies within the sample.

15. A use of a polypeptide according to any one of claims 7 to 11 for diagnosing visceral Leishmaniasis in a human or animal subject.





**FIG. 1**



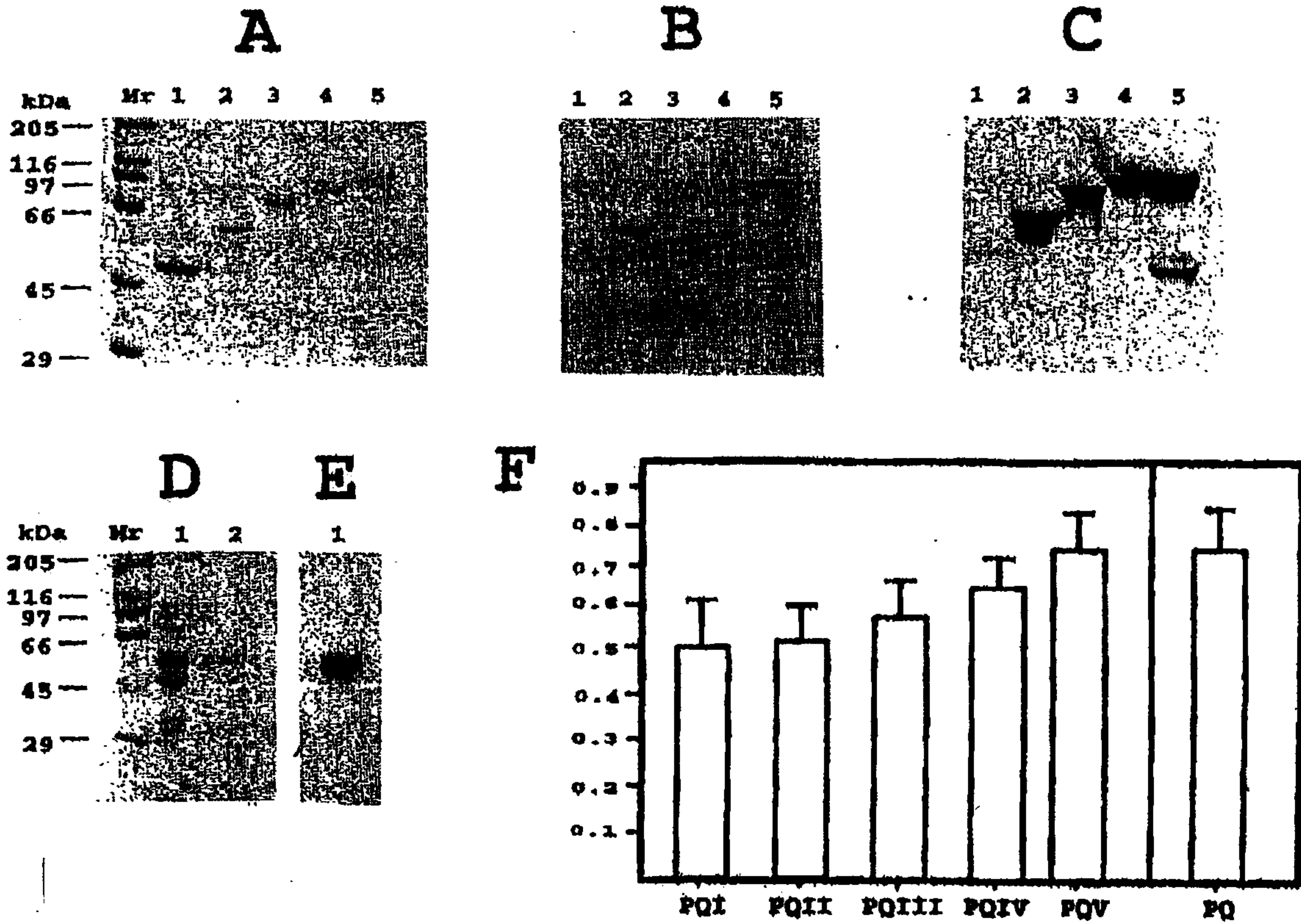
**FIG. 2**



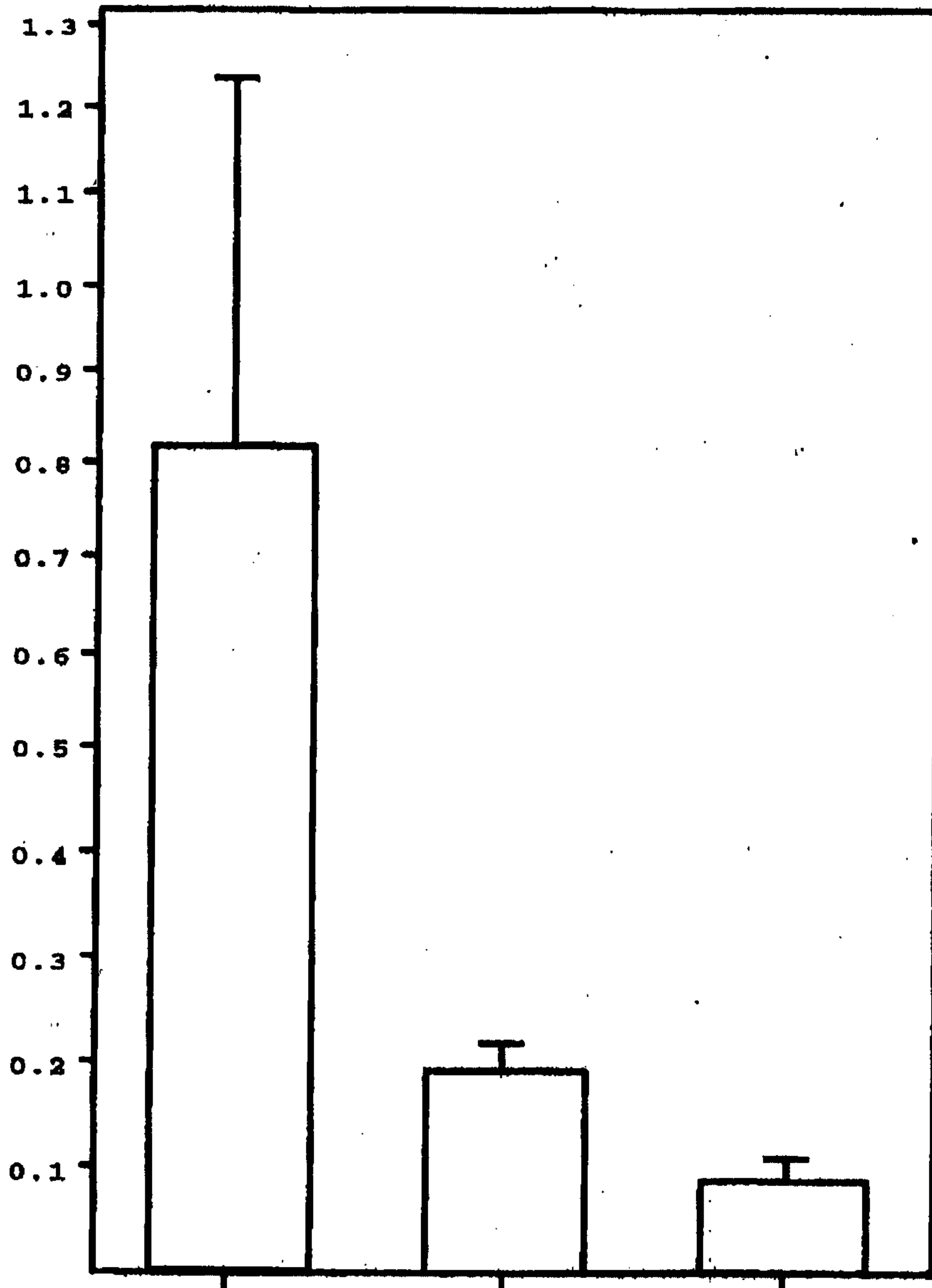
**B**

**MBP** IEGRPLATPRSAKKAVRKSGSKSAKCGLIFPVGRVGGMMRRGQYARRIGA 50  
 SGAPRISEFSVKAAAQSGKKRCRLNPRTVMLAARHDDDIGTLLKNVTLSHSGVV 104  
 PNISKAMAKKKGGKKGKATPSAPEFGDSSRPMSTKYLAAYALASLSKASPSQAD 158  
 VEAICKAVHIDVDQATLAFVMESVTGRDVATLTAEGAAKMSAMPAASSGAAAGV 212  
 TASAAGDAAPAAAAAKKDEPEEEADDDMGPSVRDPMQYLAAYALVALSGKTPSK 266  
 ADVQAVLKAAGVAVDASRVDAVFQEVEGKSFDALVAEGRTKLVGSGSAAPAGAV 320  
 STAGAGAGAVAEAKKEEPEEEEEADDDMGPVDLQPAAAAAPAAPSAAAKEEPEESD 374  
 EDDFGMGGLF

**FIG. 3**



**FIG. 4**



**FIG. 5**