



United States Patent [19]

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Knowles et al.

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[54] **SEROLOGICAL IDENTIFICATION OF CATTLE, SHEEP OR GOATS INFECTED WITH ANAPLASMA SPECIES**

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[73] **Assignees:** The United States of America as represented by the Secretary of Agriculture, Washington, D.C.; Washington State University Research Foundation, Pullman, Wash.

[21] **Appl. No.:** 730,995

[22] **Filed:** Oct. 16, 1996

Related U.S. Application Data

[63] **Continuation of Ser. No.** 156,426, Nov. 23, 1993, abandoned.

[51] **Int. Cl.⁶** G01N 33/53

[52] **U.S. Cl.** 435/7.93; 435/70.21; 435/340; 530/388.4

[58] **Field of Search** 435/7.1, 7.22, 435/7.9, 7.92, 7.93, 70.21, 332, 340; 436/518; 530/388.1, 388.2, 388.6, 388.4

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,134,792 1/1979 Boguslaski et al. .

OTHER PUBLICATIONS

Visser et al, *Infect Immun*, Dec. 1992, 60 (12):5139-5144.
Anderson, *Journal of Immunological Methods*, 1984, 74: 139-149.

Tebele et al, *Infect Immun*, Sep. 1991, 59(9): 3199-3204.
Harlow et al, "Antibodies: A Laboratory Manual", 1988 by Cold Spring Harbor Labs (N.Y) pp. 511-552.

Palmer et al, *International Journal For Parasitology*, 1988, 18(1):3149-3204.

Harlow et al, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press 1988 p. 342.

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[57] **ABSTRACT**

The subject invention concerns the use of monoclonal antibody ANAF16C1 and Anaplasma species major surface protein-5 in the competitive inhibition format for the serological identification of animals infected with Anaplasma species.

4 Claims, 2 Drawing Sheets

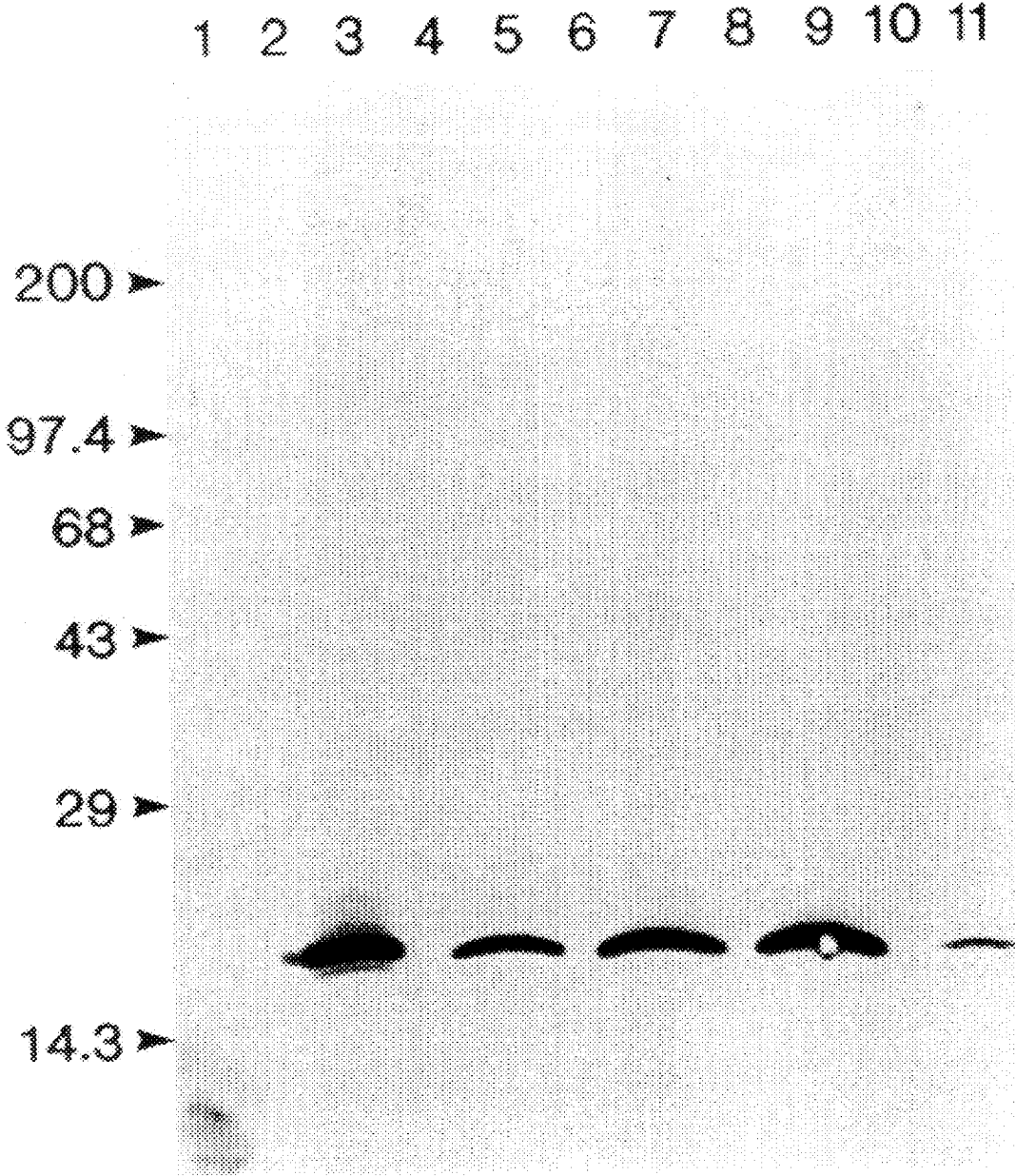


FIGURE 1

Competitive Inhibition ELISA

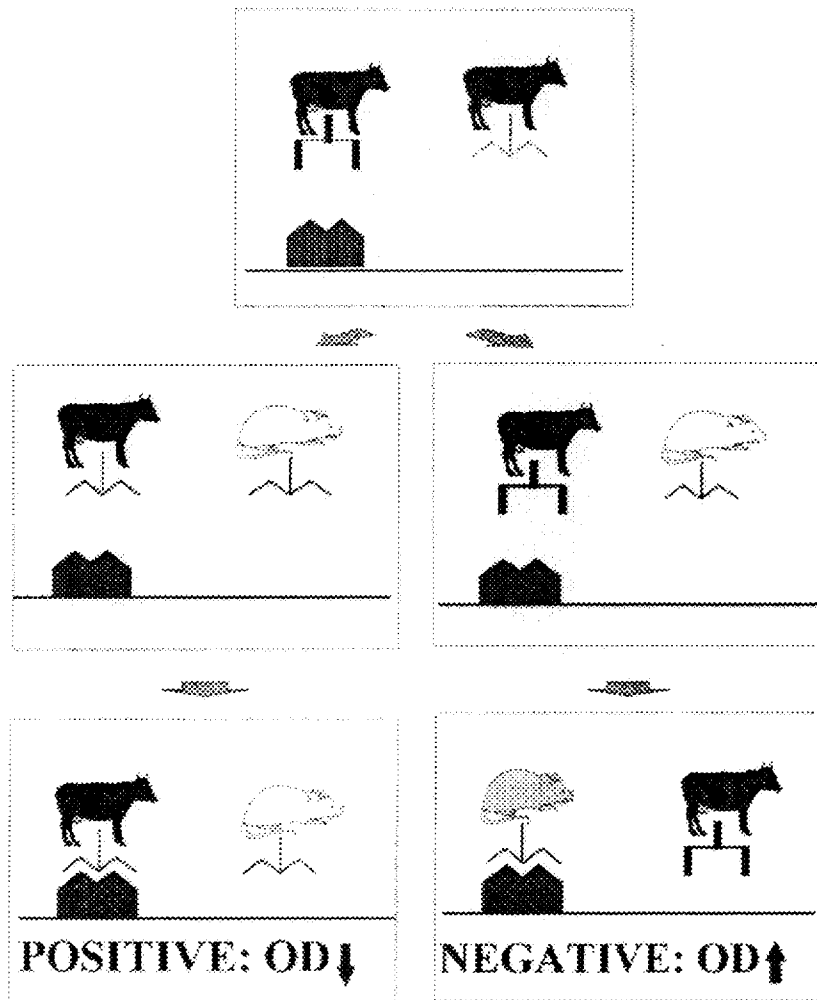


FIGURE 2

SEROLOGICAL IDENTIFICATION OF CATTLE, SHEEP OR GOATS INFECTED WITH ANAPLASMA SPECIES

This application is a continuation of application Ser. No. 08/156,426, filed Nov. 23, 1993 now abandoned.

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BACKGROUND OF THE INVENTION

Anaplasmosis, a vector-borne rickettsial disease of cattle, sheep and goats is caused by three species; *Anaplasma marginale*, *Anaplasma centrale* and *Anaplasma ovis*. Clinical disease is characterized by anemia, weight loss, abortion and death. Survivors are lifelong carriers of the rickettsia. Eventual control of *Anaplasma* species infection will require both an effective vaccine and identification of carrier cattle, sheep or goats. Two possible methods for routine carrier identification are a nucleic acid probe for hybridization of infected blood or the detection of *Anaplasma* species-specific antibody in serum. Hybridization of DNA extracted from blood with an *Anaplasma marginale*-specific nucleic acid probe does not always detect known carriers, because of cyclic changes in rickettsemia levels. Carrier identification by antibody requires that infected animals never clear the rickettsia. Indefinite persistence of *Anaplasma marginale* in infected cattle has been documented. Current serologic tests for anaplasmosis are not widely used, primarily because the error rate is high. One problem with current tests is false positive results caused by erythrocyte contamination of the *Anaplasma marginale* antigen used in the tests, and the presence of anti-erythrocyte antibody in the sera of some cattle.

Recently, progress has been made toward the characterization of a surface membrane protein of *Anaplasma marginale* for use in diagnosis (N. Tebele, T. C. McGuire, T. C., and G. H. Palmer, *Infect. Immun.* 59:3199-3204, 1991 and E. S. Visser, McGuire, T. C., Palmer, G. H., Davis, W. C., Shkap, V., Pipano, E. and D. P. Knowles, jr., *Infect. Immun* 60:5139-5144, 1992.). This protein, designated major surface protein 5 (MSP-5) and monoclonal antibody ANAF16C1 were shown to have utility when used together in the competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) format (Anderson, J. *Immunol. Meth.*, 74:139-149, 1984) for the diagnosis of cattle, sheep and goats infected with *Anaplasma marginale*, *Anaplasma centrale* or *Anaplasma ovis* (E. S. Visser, McGuire, T. C., Palmer, G. H., Davis, W. C., Shkap, V., Pipano, E. and D. P. Knowles, jr., *Infect. Immun* 60:5139-5144, 1992.).

BRIEF SUMMARY OF THE INVENTION

Disclosed and claimed here is a CI-ELISA using monoclonal antibody ANAF16C1 and the corresponding protein, *Anaplasma marginale* major surface protein-5, bound by monoclonal antibody ANAF16C1 for the identification of cattle, sheep or goats persistently infected with *Anaplasma marginale*, *Anaplasma centrale*, or *Anaplasma ovis*. This invention provides a means of identifying cattle, sheep; or goats that are persistently infected with *Anaplasma marginale*, *Anaplasma centrale* or *Anaplasma ovis*. This test is specific for *Anaplasma* species detection since the specificity of this CI-ELISA resides solely in monoclonal antibody ANAF16C1 and monoclonal antibody ANAF16C1 as been shown to specifically bind to only *Anaplasma* species MSP-5. Since MSP-5 is conserved in all known *Anaplasma*

species, it is logical to assert that MSP-5 is conserved in all isolates of *Anaplasma* species.

BRIEF DESCRIPTION OF THE DRAWINGS

SEQ ID NO:1 is the DNA sequence of *Anaplasma marginale* major surface protein 5.

SEQ ID NO:2 is the amino-acid sequence of *Anaplasma marginale* major surface protein 5.

FIG. 1 is an immunoblot demonstrating the binding of monoclonal antibody ANAF16C1 to major surface protein 5 of: Florida strain of *Anaplasma marginale* (lane 2); Israeli strain of *Anaplasma centrale* (lane 4); Israeli non-tailed strain of *Anaplasma marginale* (lane 6); Israeli tailed strain of *Anaplasma marginale* (lane 8), and Idaho strain of *Anaplasma ovis* (lane 10).

FIG. 2 is a diagram of the CI-ELISA using monoclonal antibody ANAF16C1 (mouse), *Anaplasma marginale* major surface protein 5 (▲), and test serum (cow).

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO. 1 is the DNA sequence of *Anaplasma marginale* major surface protein

SEQ ID NO. 2 is the amino acid sequence of *Anaplasma marginale* major surface protein 5.

DETAILED DESCRIPTION OF THE INVENTION

The subject invention pertains to the use of monoclonal antibody ANAF16C1 and *Anaplasma* major surface protein 5 in a CI-ELISA format (FIG. 2) for the serological detection of cattle, sheep or goats infected with *Anaplasma marginale*, *Anaplasma centrale* or *Anaplasma ovis*. Hybridoma ANAF16C1 which produces and secretes monoclonal antibody ANAF16C1 was deposited on Dec. 2, 1997, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852 USA, under terms of the Budapest Treaty, and has been assigned the accession number ATCC HB-12440.

The evidence that MSP-5 is effective in the CI-ELISA format for the diagnosis of animals infected with *Anaplasma* species are:

- (i) MSP-5 is conserved in all known *Anaplasma* species (FIG. 1);
- (ii) all immune sera tested from animals infected with *Anaplasma* species bind to MSP-5, and (iii) all immune sera tested from animals infected with *Anaplasma* species compete with monoclonal antibody ANAF16C1 for binding to MSP-5.

The evidence that monoclonal antibody ANAF16C1 is effective in the CI-ELISA format for the diagnosis of animals infected with *Anaplasma* species are: (i) monoclonal antibody ANAF16C1 binds to MSP-5 in all known *Anaplasma* species; (ii) monoclonal antibody ANAF16C1 binds to both native and recombinant MSP-5, and (iii) all immune sera tested from animals infected with *Anaplasma* species compete with monoclonal antibody ANAF16C1 for binding to MSP-5.

MATERIALS AND METHODS

The Florida strain of *Anaplasma marginale* from which native and recombinant MSP-5 were derived originated from a pooled blood sample collected from naturally infected cattle in various sections of Florida in 1955 (Ristic, M. and C. A. Carson, In L. H. Miller, J. A. Pino, and J. J.

McKelvey (ed.), Immunity to blood parasites of animals and man. Plenum Publishing Corp., New York, 1977).

Native MSP-5 was obtained from blood stabilates by differential centrifugation as described (Palmer, G. H. and T. C. McGuire, J. Immun. 133:1010-1015, 1984). Briefly, 20 milliliters of stabilize was thawed at 37° C. for 10 min and then washed 3 times by suspension in 40 ml of RPMI 1640 media (Flow Laboratories, McLean, Va.) containing 2 mM 1-Glutamine and 25 mM HEPES, with centrifugation at 27,000×G. The sediment was resuspended in 35 ml of media, disrupted by 2 min of sonication at 50 W (127×4 mm titanium probe, Braun-Sonic 1510; Braun Instruments, San Francisco, Calif.), and was washed two times at 1650 ×G for 15 min.

Recombinant MSP-5 (SEQ ID NO:2) was prepared from a 50-ml overnight culture of *E. coli* XL1-Blue containing pAM104 in LB broth with 50 ug of ampicillin per ml. Molecular clone pAM104 contains the *msp5* gene with the nucleotide sequence of MSP-5 presented in SEQ ID NO:1. A bacterial lysate prepared with PI buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 5 mM iodoacetamide, 0.1 mM N-a-p-tosyl-L-lysine chloromethyl ketone 1 mM phenylmethylsulfonyl fluoride, 1 mg of lysozyme per ml, 1% Nonidet P-40).

Monoclonal antibody ANAF16C1 was made by fusing X63-Ag8.653 murine myeloma cells (J. F. Kearney, Radbruch, A., Liesegang, B. and K. Rajewsky J. Immunol. 123:1548-1550, 1979) with spleen cells from BALB/c mice immunized with purified initial bodies of the Florida strain of *Anaplasma marginale*. An immunoglobulin G2a monoclonal antibody that immunoprecipitated a 19 kDa protein from 125I-surface-radiolabeled solubilized initial bodies was designated ANAF16C1. Monoclonal antibody ANAF16C1 was conjugated with horseradish peroxidase as described (A. G. Farr and P. K. Nakane, J. Immun. Meth. 47:129-144, 1981).

The CI-ELISA format was first described in 1984 (J. Anderson, J. Immunol. Meth., 74:139-149, 1984). An overview of the use of the CI-ELISA format for the detection of

animals infected with *Anaplasma* species is as follows: (i) Immulon 2 plates are coated with native or recombinant MSP-5. (ii) plate is incubated overnight at RT; (iii) the plate is rinsed and blocker is added (1 hour); (iv) test sera are incubated with antigen (15 min); (v) monoclonal antibody ANAF16C1 conjugated to horseradish peroxidase is added and incubated for 15 min; (vi) wells are rinsed and substrate (p-nitrophenyl phosphate) is added (10 min), and (vii) the reaction is stopped and the optical density is read at 490.

The specifics of the CI-ELISA using monoclonal antibody ANAF16C1 and MSP-5 for serological detection of animals infected with *Anaplasma* species are as follows. Preparation of all buffers and reagents are provided below. Wells of an Immulon 2 plate (Dynatech Laboratories, Chantilly, Va.) are coated with sufficient native or recombinant MSP-5 to provide an OD₄₉₀ reading of between 1.0 and 1.5. The appropriate dilution of initial body lysate (native *msp-5*) or bacterial lysate applied to an Immulon 2 plate (recombinant MSP-5) is determined by titration with monoclonal antibody ANAF16C1. After plates are coated with the appropriate amount of MSP-5 lysate, the Immulon 2 plates are sealed with acetate and incubated overnight at room temperature. The well contents are removed and the wells rinsed once with 200 ul of PBS/Tween. Coated plates are blocked by adding 200 ul blocking buffer, and incubating for 1 hr at room temperature. Blocker is removed from the plates and 40 ul of undiluted test serum is added to each well and incubated for 15 min at room temperature. Conjugated monoclonal antibody ANAF16C1 is then added at the appropriate concentration determined previously by titration. Conjugated monoclonal antibody ANAF16C1 is diluted such that the appropriate quantity of conjugated monoclonal antibody ANAF16C1 is added in 10 ul. The mixture is incubated for 15 min at room temperature. Contents are removed from the wells and the wells are rinsed twice with 200 ul of PBS/Tween and once with substrate buffer. Fifty ul of OPD substrate is added per well and incubated for 10 min at room temperature. The reaction is stopped by adding 25 ul of 3N HCl per well. The OD₄₉₀ is read.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 2

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1146 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(v) ORIGINAL SOURCE:

- (A) ORGANISM: *Anaplasma marginale*
- (C) INDIVIDUAL ISOLATE: Florida

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTATACTCAG TTGCGCCTGG CGCTTGACCA ACCTGGGCAT AGGTGCTACG ATCGCGCCTG 60
CICGTTTTGC CGTCCGGCAA TGTGGCGCAT TTTTGAGTGT TCGTTGGGGT GTGATAGATG 120

-continued

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AGAATTTTCA AGATTGTGTC TAACCTTCTG CTGTTTCGTTG CTGCCGTGTT CCTGGGGTAC      180
TCCTATGTGA ACAAGAAAAGG CATTTCAGC AAAATCGGCG AGAGGTTTAC CACTTCCGAA      240
GTTGTAAGTG AGGGCATAGC CTCCGCGTCT TTCAACAATT TGGTTAATCA CGAGGGGGGTC      300
ACCGTCAGTA GCGGGCATT TGGCGGCAAG CACATGTTGG TAATATTCGG CTTCTCAGCC      360
TGTAAGTACA CGTGCCCTAC CGAGTTAGGC ATGGCTTCTC AGCTCCTAAG TAAACTAGGC      420
GACCATGCCG ATAAGTTGCA AGTTGTGTTT ATAACGTGTTG ATCCGAAAAA TGACACCGTA      480
GCCAAGCTTA AAGAGTACCA CAAGTCTTTT GATGCGAGAA TTCAGATGCT CACAGGCGAA      540
GAAGCAGACA TAAAGAGCGT GGTTGAAAAAC TACAAGGTGT ATGTGGGCGA CAACAAGCCA      600
AGTGATGGTG ATATCGACCA CTCAACGTTT ATGTACCTCA TCAATGGGAA AGGCAGGTAT      660
GTCGGGCATT TTGCGCCAGA TTTAACGCG TCTGAGGGCC AAGGCGAGGA GCTGTTTAAAG      720
TTTGTCAGCG GTCACATGCT TAATTCTTAG TTAAGCATGG CAGTGGTACA GTTTCGIGTG      780
TCGGTCGTCC TTGTGAGGCA GTAGAAAAGTA TGGGGCTTTG GGGGCTTTC TTTGTGGCGT      840
TTGTGCGGCT TGC GTTAGGA GCTGGGGCTG ACCAGATCAG GGTGGTIGGC TCTTCCACCG      900
TGTTCCCATI TATCTCTTCT GTTGCCGAAG AGTTTGGTAG ATTCTCCGCC TATAGAACCC      960
CCGTCATAGA GTCCGTGGGA AGTGGCATGG GCTTTAACAT GTTTTGCCT GGCAGCAGCA     1020
GTGATACACC AGACATAGCC ATGTCTCTA GCGCATCAA GGATGCAGAA GTCGAACTTT     1080
GCGGCATGAA TGGCGTGAAG GACATGATCG AGATAGGTTCT GGGCTACGAC GGCATAGCCC     1140
GAATTC                                                                                   1146

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 210 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

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

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Met  Arg  Ile  Phe  Lys  Ile  Val  Ser  Asn  Leu  Leu  Leu  Phe  Val  Ala  Ala
 1          5          10
Val  Phe  Leu  Gly  Tyr  Ser  Tyr  Val  Asn  Lys  Lys  Gly  Ile  Phe  Ser  Lys
 20          25          30
Ile  Gly  Glu  Arg  Phe  Thr  Thr  Ser  Glu  Val  Val  Ser  Glu  Gly  Ile  Ala
 35          40          45
Ser  Ala  Ser  Phe  Asn  Asn  Leu  Val  Asn  His  Glu  Gly  Val  Thr  Val  Ser
 50          55          60
Ser  Gly  Asp  Phe  Gly  Gly  Lys  His  Met  Leu  Val  Ile  Phe  Gly  Phe  Ser
 65          70          75          80
Ala  Cys  Lys  Tyr  Thr  Cys  Pro  Thr  Glu  Leu  Gly  Met  Ala  Ser  Gln  Leu
 85          90          95
Leu  Ser  Lys  Leu  Gly  Asp  His  Ala  Asp  Lys  Leu  Gln  Val  Val  Phe  Ile
 100         105         110
Thr  Val  Asp  Pro  Lys  Asn  Asp  Thr  Val  Ala  Lys  Leu  Lys  Glu  Tyr  His
 115         120         125
Lys  Ser  Phe  Asp  Ala  Arg  Ile  Gln  Met  Leu  Thr  Gly  Glu  Glu  Ala  Asp
 130         135         140
Ile  Lys  Ser  Val  Val  Glu  Asn  Tyr  Lys  Val  Tyr  Val  Gly  Asp  Lys  Lys

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UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,798,219
DATED : August 25, 1998
INVENTOR(S) : Donald P. Knowles et al.

Page 1 of 1

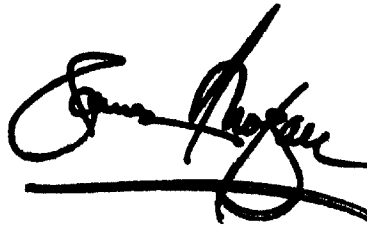
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Columns 5 and 6,

In SEQ ID NO:1, change nucleotide 594 from "C" to -- G --.

Signed and Sealed this

Seventeenth Day of June, 2003

A handwritten signature in black ink, appearing to read "James E. Rogan", with a long horizontal flourish underneath.

JAMES E. ROGAN
Director of the United States Patent and Trademark Office