APPLICATION FOR A STANDARD PATENT OR A STANDARD PATENT OF ADDITION

<table>
<thead>
<tr>
<th>NUMBER</th>
<th>COUNTRY</th>
<th>DATE OF APPLICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>674,449</td>
<td>U.S.A.</td>
<td>23rd November 1984</td>
</tr>
</tbody>
</table>

We, MILES LABORATORIES, INC., hereby apply for the grant of a Standard Patent for an invention entitled "AN IMPROVED PROCESS & PRODUCT" which is described in the accompanying complete specification.

For a Convention application - details of basic application(s) -

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We request that the Patent be granted as a Patent of Addition to Patent No. 674,449 in the name of MILES LABORATORIES, INC.

We request that the term of the Patent of Addition be the same as that for the main invention or so much of the term of the Patent for the main invention as is unexpired.

Our address for service is ARTHUR S. CAVE CO., Patent and Trade Mark Attorneys, 1 Alfred Street, Sydney, New South Wales, Australia 2000.

Dated this 18th day of November 1985.

MILES LABORATORIES, INC.
By Its Patent Attorneys
ARTHUR S. CAVE & CO.

To:
Commissioner of Patents

ARTHUR S. CAVE & CO.
PATENT AND TRADE MARK ATTORNEYS
SYDNEY

A.S.C. 1
A method of preparing a cell-free antigenic solution useful in immunizing horses against Streptococcus equi bacteria, the method comprising the steps of:

(a) growing Streptococcus equi bacteria under growth inducing conditions;

(b) exposing the bacteria of step (a) to a bacteriolytic enzyme under conditions sufficient to partially lyse the cell wall;

(c) exposing the partially lysed product of step (b) to an anionic detergent under conditions sufficient to extract immunogenic M-like protein(s) into a supernate;

(d) separating the soluble extracted M-like protein supernate from bacterial cells and cell debris;

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invention disclosed and that the inventions disclosed should be limited only by the following claims.
(e) sterilizing the product of step (d).
7. A solution prepared by the method of any one of the preceding claims.
8. A vaccine for in immunizing against Streptococcus equi bacteria comprising an immunogenic M-like proteinous material, the said material having been obtained from Streptococcus equi bacteria by enzymatic digestion and detergent extraction.
Short Title:

Int. Cl:

Application Number: 5022485

Lodged:

Complete Specification-Lodged:

Accepted:

Lapsed:

Published:

Priority:

Related Art:

TO BE COMPLETED BY APPLICANT:

Name of Applicant: MILES LABORATORIES, INC.

Address of Applicant: Fourth & Parker Streets, P.O. Box 1986, Berkeley, California 94701, U.S.A.

Actual Inventor: Karen K. BROWN, Sharon A. BRYANT & Kenneth S. LEWIS

Address for Service: ARTHUR S. CAVT & CO., Patent and Trade Mark Attorneys, 1 Alfred Street, Sydney, New South Wales, Australia, 2000.

Complete Specification for the invention entitled:

"AN IMPROVED PROCESS & PRODUCT"

The following statement is a full description of this invention, including the best method of performing it known to me:-
BACKGROUND OF THE INVENTION

Field: This disclosure is concerned with the preparation of an immunogenic protein material from *Streptococcus equi* bacteria using an enzymatic digestion and detergent treatment and use of the material as a vaccine against *Strep. equi* infection in equines.

Prior Art: *Streptococcus equi* is classified as a Lance-field Group C *Streptococcus*. See, for example, Bergey's Manual of Determinitive Bacteriology (8th Ed.), p. 498 (1974). It is recognized as the causative agent of a severe respiratory disease of horses referred to as "Strangles". The disease is endemic in most parts of the world and epidemic in the United States. Race and show horses are particularly susceptible to repeated infections due to the stress of travel and exposure to new contacts. The disease begins with a mucopurulent nasal discharge, temperatures of 103° - 106° F, and severe inflammation of the upper respiratory mucosa. It finally progresses to lymphadenitis and abscess formation which is sometimes severe enough to restrict air intake and cause suffocation of the animal. Strangles results in extensive loss of condition (loss of weight) as it often runs a course of 4 - 6 weeks.

Because of the debilitating and in some cases lethal effects of *Strep. equi* infections in horses, attempts have been made over the years to prepare *Strep. equi* vaccines which could be used for active immunization purposes.
and even abscess formation at the injection site. These known reactivities have tended to discourage the development and/or commercial use of immunizing Strep. equi products. Two commercially available Strangles vaccines do exist, however. The first commercial product was a whole culture, chemically inactivated Strep. equi preparation (supplied by Ft. Dodge Corporation). The second commercial product was a cell free M-protein vaccine (available from Burroughs Wellcome Co.) described as "a concentrated, aluminum hydroxide-absorbed suspension of purified antigens derived from Strep. equi". The method by which this vaccine is prepared is thought to be described in U.S. Patent No. 3,793,150 and U.S. Patent No. 3,852,420. The purification of such "M-like proteins" from Strep. equi is also described in an article by J. B. Woolcock, Infect. and Immun., July 1974, p. 116 - 122. As used herein, the expression "M-like protein" means the immunogenic protein(s) of the Strep. equi organism which appears similar in molecular weight and activity to the M-protein of group A streptococci.

The theory that a protein on the cell wall of the Strep. equi organism, referred to as an M-like protein, is the antigenic portion of the bacteria has been discussed in articles by S. K. Srivastava and D. A. Barnum in the Can. J. Comp. Med., Vol. 46, p. 51 - 56, 1982 and in the Am. J. Vet. Res., Vol. 44, p. 41 - 45, 1983 and in articles by E. D. Erickson and N. L. Norcross in the Can. J. Comp. Med., Vol. 39, p. 110 - 115, 1975. In all previous work, this M-like protein was extracted from the Strep. equi organism by subjecting the organism to low pH conditions (pH2) and high temperatures (95° - 100° C) for a given time (10 - 15 minutes). This has been referred to as a "heat extraction" method of preparing the M-like protein. The protein precipitates under these conditions and is solubilized by raising the pH of the solution to pH 7 or above.
We now have developed an improved method of removing the M-like protein from *Strep. equi* organisms, details of which are described herein.

We have now found that the antigenic M-like protein can be efficaciously removed from a *Strep. equi* culture in a two step process using lytic enzyme digestion followed by treatment with an anionic detergent and that this extract can be used to prepare a vaccine effective in immunizing horses against infection by *Strep. equi*. The potency of this antigen preparation has been determined using the method stated in above-cited Patent Application S.N. 454,906 entitled, Determining Potency of Streptococcal Preparations, and has been confirmed in a horse challenge study, described herein.

**SUMMARY OF THE INVENTION (non-limiting)**

The procedure for our enzymatic extraction of Streptococcal M-like protein involves growth of a Streptococcal culture under growth-inducing conditions (e.g. at 37°C in a suitable media) followed by concentration of the cells (e.g. by centrifugation or filtration). The cell concentrate is either diluted or washed in a suitable buffer. A bacteriolytic enzyme such as mutanolysin is then added to the cell concentrate and incubated at sufficient temperature and time for enzymatic lysis of part of the cell wall. Partial lysis means lysis sufficient to make the M-protein available for subsequent detergent extraction but without deleterious effect on the M-protein. In general, we found this can be accomplished by exposing the *Strep. equi* culture to the lytic enzyme at 37°C for no more than about 24 hours at an enzyme concentration of about 1 - 10 units per ml of original culture volume. An anionic detergent such as sodium lauryl sulfate or diocetyl sodium sulfosuccinate is then added to the cell concentrate and allowed
to incubate to complete the Strept. equi cell extraction treatment. Cells and cell debris are then removed by centrifugation or filtration and the final cell-free antigen solution sterilized by filtration or chemical treatment. The cell-free antigen solution is immunogenic and useful in immunizing horses against infection by Strept. equi organisms and has the following characteristics: a molecular weight ranging from 25,000 to 75,000 daltons; heat stability to about 95°C; and trypsin sensitivity.

**SPECIFIC EMBODIMENTS**

The preferred bacteriolytic enzyme used in our method is mutanolysin (N-acetylmuramidase) which is obtained from the culture filtrate of Streptomyces globisporus and which is commercially available from Sigma Chemical Co., St. Louis, Mo. 63178 and Dainippon Pharmaceutical Co., Ltd., Osaka, Japan. Studies using mutanolysin as a method of lysing Streptococcal cell walls have been conducted for purposes other than M-like protein retrieval. Articles of these studies have been written by K. Yohagawa, et al in Antimicrobial Agents and Chemotherapy, August 1974, p. 156-165, G. B. Calandar and R. M. Cole in Infect. Immun., June 1980, p. 1033-1037, and B. J. DeCueninck, et al, in Infect. Immun., Feb. 1982, p. 572-582. Mutanolysin and other bacteriolytic enzymes (glycosidases) such as egg white lysozyme are thought to act on linear sequences of N-acetylglycosamines and N-acetylmuramic acid residues of the bacterial cell walls.
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EXAMPLE

1. *Strep. equi* deposited with American Type Culture Collection, Rockville, Md. 20852 as A.T.C.C. No. 39,506 was used. Grow *Strep. equi* in chemically defined medium (I. van de Rijn, Infect. and Immun., 27: 444 - 448, 1980) at 37°C for 16 hours.

2. Concentrate *Strep. equi* cells to 10 - 50 fold using cross-flow filtration. Wash cells by addition of 0.1 M Trizma - HCl buffer with pH adjusted to 6.5 with NaOH.

3. Concentrate washed *Strep. equi* cells to 20 - 100 fold using cross-flow filtration.

4. Add mutanolysin as a 5,000 unit/ml solution to concentrated cells achieving a final enzyme concentration of 5 units per ml original culture volume. Incubate at 37°C for 16 hours.

5. Add 10% sodium lauryl sulfate to achieve a final concentration of .05%. Incubate at 37°C for 30 minutes.

6. Remove *Strep. equi* cells and cell debris by cross-flow filtration or centrifugation.

7. Sterile filter effluent through .2 micron filter and hold at 4°C.

Antigen prepared according to the example was tested for potency via the previously-mentioned mouse combining power assay. In this assay, the antigenicity of the vaccine is measured by the increase in LD50 over the LD50 of the Antiserum Control. The greater this increase in LD50, the greater the antigenicity of the vaccine. Table 1 shows the results of vaccines tested via this assay. It should be
To prove that the enzyme-detergent treatment was the key factor contributing to antigen retrieval, the following experiment was conducted:

1. *Strep. equi* culture was grown and the cells were then harvested by centrifugation.

2. The cells were resuspended in buffer and aliquots of the suspension treated in the following manner: (In all cases, cells were removed following treatment by centrifugation and supernates filtered thru a 0.2 micron sterile filter.)

   A. Suspension incubated at 50° C for 16 hours, then 0.05% sodium lauryl sulfate (SLS) added and suspension held at 37° C for 30 minutes.

   B. Suspension incubated at 37° C for 30 minutes after addition of 0.05% SLS.

<table>
<thead>
<tr>
<th>Antigen Dilution Used To Prepare Vaccine</th>
<th>Mouse LD50 (Log)</th>
<th>LD50 Increase over Antiserum Control (Log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:100</td>
<td>3.0</td>
<td>4.6</td>
</tr>
<tr>
<td>1:150</td>
<td>6.5</td>
<td>3.1</td>
</tr>
<tr>
<td>1:200</td>
<td>4.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Antiserum Control</td>
<td>3.4</td>
<td>-</td>
</tr>
<tr>
<td>Negative Control</td>
<td>8.2</td>
<td>-</td>
</tr>
</tbody>
</table>

Note that antigen diluted as much as 1:200 still demonstrates combining power.

TABLE 1

MOUSE COMBINING POWER RESULTS OF VACCINE PREPARATION

It should be noted that antigen diluted as much as 1:200 still demonstrates combining power.
Each of these preparations (A - F) were then tested in the mouse combining power assay to determine the amount of antigen released. The results (Table 2) show that detergent alone or in combination with heat (A, B, C) does not remove antigen. Mutanolysin incubated at 50°C (D) removes minimal quantity of antigen compared with the combination of Mutanolysin and detergent (E). In fact, this latter technique (E) appears to remove antigen as well as the hot acid extraction (heat extraction) method (F) described in U.S. Patent No. 3,793,150 and U.S. Patent No. 3,852,420 based on combining power results shown in this table.
Further work with the mutanolysin-detergent method has recently shown that antigen removal is even more effective at 37° C than at 50° C. Therefore, the current process used to prepare the vaccines shown tested in Table 1 has

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been established at this preferred lower temperature of 37° C. To confirm the antigenicity of vaccines as measured in the mouse combining power assay, a vaccine efficacy study was conducted in equines using the vaccines shown in Table 1. Horses were given two doses of vaccine at 3 week intervals and then challenged with virulent *Strep. equi*. The efficacy of the vaccines was measured by the reduction in clinical symptoms observed post challenge. Clinical index values were assigned to Strangles symptoms for the purpose of efficacy evaluation. These clinical index values are shown in Table 3 and were assigned to each horse for each day symptoms were observed post challenge. Observations of the horses were made every other day for 49 days post challenge.
The results of the vaccine efficacy study are shown in Table 4 as the accumulation of clinical index values assessed during the 49 day observation period. The reduction of clinical indices is also shown in Table 4 as the percent reduction of the indices of the vaccine groups as compared to the nonvaccinated control group. These data suggest that efficacious vaccines can be produced using enzyme-detergent extraction of *Strep. equi*.
### TABLE 4
Strep. Equi Efficacy Study
Clinical Indices

<table>
<thead>
<tr>
<th>Clinical Symptom</th>
<th>1:100</th>
<th>1:150</th>
<th>1:200</th>
<th>Non-Vaccinated Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Group Total</td>
<td>819</td>
<td>1235</td>
<td>1586</td>
<td>2453</td>
</tr>
<tr>
<td>Mean Value</td>
<td>28.2</td>
<td>42.6</td>
<td>56.6</td>
<td>81.8</td>
</tr>
<tr>
<td>% Reduction</td>
<td>64%</td>
<td>46%</td>
<td>28%</td>
<td>-</td>
</tr>
</tbody>
</table>

| Abscesses Group Total | 180   | 420   | 680   | 1240                    |
| Mean Value           | 6.2   | 14.5  | 24.3  | 41.3                    |
| % Reduction          | 85%   | 65%   | 41%   | -                       |

| WBC Group Total      | 375   | 470   | 585   | 820                     |
| Mean Value           | 12.9  | 16.2  | 20.9  | 7.3                     |
| % Reduction          | 53%   | 41%   | 23%   | -                       |

| Temperature Group Total | 179   | 230   | 211   | 222                     |
| Mean Value             | 6.2   | 7.9   | 7.5   | 7.4                     |
| % Reduction            | 16%   | 0%    | 0%    | -                       |

| Nasal Discharge Group Total | 85   | 115   | 110   | 70                      |
| Mean Value               | 2.9  | 4.0   | 3.9   | 2.3                     |
| % Reduction              | 0%   | 0%    | 0%    | -                       |

Given the above disclosure, it is thought variations will occur to those skilled in the art. Accordingly, it is intended that the above specific Example should be construed as illustrative of the best mode to date of the
invention disclosed and that the inventions disclosed should be limited only by the following claims.
WE CLAIM:
The claims defining the invention are as follows:

1. A method of preparing a cell-free antigenic solution useful in immunizing horses against Streptococcus equi bacteria, the method comprising the steps of:

(a) growing Streptococcus equi bacteria under growth inducing conditions;

(b) exposing the bacteria of step (a) to a bacteriolytic enzyme under conditions sufficient to partially lyse the cell wall;

(c) exposing the partially lysed product of step (b) to an anionic detergent under conditions sufficient to extract immunogenic M-like protein(s) into a supernate;

(d) separating the soluble extracted M-like protein supernate from bacterial cells and cell debris;

(e) sterilizing the product of step (d).

2. The method of claim 1 wherein the enzyme of step (b) is mutanolysin and the exposure is at 37° C for not more than about 24 hours at an enzyme concentration of 1 - 10 units per ml of original culture volume.

3. The method of claim 1 wherein the detergent of step (c) is sodium lauryl sulfate and the exposure is at 37° C for not more than about 60 minutes at a detergent concentration of 0.01 - 0.10%.

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4. The method of claim 1 wherein the separation of step (d) is via centrifugation or filtration.

5. The method of claim 1 wherein the sterilization of step (e) involves passing the product of step (d) through a 0.2 micron filter or by adding a suitable chemical sterilizing agent.

6. A method for preparing a cell-free antigenic solution substantially as herein described.

7. A solution prepared by the method of any one of the preceding claims.

8. A vaccine for use in immunizing against Streptococcus equi bacteria comprising an immunogenic M-like proteinous material, the said material having been obtained from Streptococcus equi bacteria by enzymatic digestion and detergent extraction.

9. The invention substantially as herein described.

DATED this 18th day of November 1985.

MILES LABORATORIES, INC.
By Its Patent Attorneys
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