



Señor Superintendente

Superintendencia de Industria y Comercio,
E. S. D.

Trámite : 011
Evento : 379
Actuación : 412

Referencia : Expediente No. 06006798
Asunto : Solicitud de patente para: "Lawsonia
intracellularis de origen europeo y vacunas,
agentes de diagnóstico y métodos de uso de
los mismos".
Solicitante : BOEHRINGER INGELHEIM VETMEDICA, INC
Fecha de solicitud: Enero 25 de 2006.

I. RECURSO VÍA GUBERNATIVA

Yo, **Luz Clemencia de Páez**, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, **BOEHRINGER INGELHEIM VETMEDICA, INC**, por el presente escrito atentamente manifiesto a usted que debidamente notificada de la Resolución No. **4245** del 31 de Enero de 2012, interpongo en su contra el recurso de **REPOSICIÓN**, para que sea revocada y en consecuencia se conceda el privilegio de patente de invención para "Lawsonia intracellularis de origen europeo y vacunas, agentes de diagnóstico y métodos de uso de los mismos".

II. OPORTUNIDAD DE LA IMPUGNACION

La Resolución No. **4245** del 31 de Enero de 2012, fue notificada mediante edicto No. 3405 desfijado el 27 de Febrero de dos mil doce (2012), por lo que el término para interponer el recurso de reposición vence el 5 de Marzo de 2012. En consecuencia, me encuentro dentro de la oportunidad legal.

III. LA DECISION RECURRIDA

3.1 Mediante la Resolución objeto de la presente impugnación, el Superintendente de Industria y Comercio decidió:





3.1.1 Negar el privilegio de patente para la invención "**Lawsonia intracellularis de origen europeo y vacunas, agentes de diagnóstico y métodos de uso de los mismos**".

3.2 En la parte motiva de la providencia, se mencionaron los siguientes fundamentos:

3.2.1 "La diferencia entre la invención, definida en la reivindicación 1 y el método para elaborar una vacuna contra *Lawsonia intracellularis* divulgado en la anterioridad D1 se encuentra dada por la cepa particular de bacteria que es empleada, puesto que la solicitud utiliza las cepas depósito ATCC PTA 4927 o ATCC PTA 4926 de origen europeo, mientras que el documento D1 utiliza la cepa ATCC 55783 de origen norteamericano".

3.2.2 "En ese orden de ideas, el efecto que logra el incluir las cepas de origen europeo de *Lawsonia intracellularis*, de acuerdo con el solicitante, es que estas cepas permiten propagar *Lawsonia intracellularis* atenuado, causando la reducción de excreción fecal en cerdos y permiten controlar la propagación de la infección. Por lo tanto, el problema técnico objetivo que pretende que pretende resolver esta invención es proveer un método de elaboración de una vacuna de *Lawsonia intracellularis* usando cepas atenuadas que permitan controlar la infección".

3.2.3 "No obstante lo anterior, a partir de la información divulgada en la anterioridad D1 y en la solicitud, no es evidente cómo las cepas de *Lawsonia intracellularis* de origen europeo permiten controlar la infección de forma mejorada. Lo anterior, teniendo en cuenta que el uso de una u otra cepa no implicó una diferencia en el método de aislamiento y elaboración de la vacuna, es decir, no hubo necesidad de una adaptación para llevar a cabo el método de la vacuna de la solicitud".

3.2.4 "De esta manera, se observa que la efectividad de las cepas de la presente invención no resulta evidente en factores tan importantes como la seroconversión, que miden la efectividad de la vacuna para que el animal desarrolle anticuerpo para la bacteria, el estado clínico de los animales y la ganancia de peso de los mismos. Por ello, a partir de las pruebas realizadas no resulta cierta la ventaja técnica que conlleva usar las cepas de la solicitud en cambio de las cepas conocidas en el estado del arte para elaborar la vacuna de *Lawsonia intracellularis*".

"En este orden de ideas, se tiene que únicamente la evaluación de presencia de heces con *Lawsonia* resultó favorable para las cepas de la solicitud, las cuales fueron negativas para la presencia de bacteria en el día 21 de inoculación. Sin perjuicio de lo anterior, frente al resto de parámetros medidos no resulta una ventaja significativa de la cepa utilizada. Lo anterior, teniendo en cuenta que la presencia de *Lawsonia* en las heces constituye un factor de riesgo en la infección de otros animales, pero la





La conversión o el estado clínico de los animales constituyen indicadores de la efectividad de protección de la vacuna y del efecto que cause la misma en los animales, respectivamente".

3.2.5 "Por otra parte, la motivación para emplear cepas de origen europeo o de otro origen para preparar vacunas está dada por la variación de la infección que ocasiona cada cepa. Sin embargo, la prueba de seroconversión evidencia que las cepas de origen europea no son una ventaja frente a cepas de origen americano para lograr la protección de los animales expuestos a infección con *Lawsonia intracellularis*".

"En este orden de ideas, en vista de que las cepas, además de ser fácilmente aisladas a partir de la naturaleza, no conceden un efecto técnico inesperado sobre la vacuna de *Lawsonia intracellularis* en el tratamiento animal, especialmente en cerdos, se considera que las sustituciones de las cepas formuladas en D1 por las empleadas en la solicitud estudiada son evidentes para una persona del oficio medianamente versada en la materia".

3.3 En cuanto a los argumentos presentados por mi representada en favor del nivel inventivo de la presente solicitud, ese Despacho mencionó lo siguiente:

3.3.1 "Por último, esta Oficina difiere del problema técnico formulado por la solicitante a partir de la divulgación del documento D1, toda vez que el problema técnico objetivo de la presente invención es proveer un método de elaboración de una vacuna de *Lawsonia intracellularis* usando cepas atenuadas que permitan controlar la infección, tal como se estableció anteriormente. De la misma forma, al ser este el problema a resolver, no se puede tener como único propósito el reducir la excreción fecal en cerdos post vacunados y dejar de lado una mejora o igual efectividad en la seroconversión de la vacuna. Por lo tanto, no se demuestra cual es la ventaja técnica de la invención que aquí se ha estudiado".

IV. OBJETO DEL RECURSO

De acuerdo con los argumentos que expongo a continuación, solicito atentamente a su Despacho lo siguiente:

- 4.1. Revocar la Resolución No. **4245** del 31 de Enero de 2012.
- 4.2. Conceder el privilegio de patente de invención para "**Lawsonia intracellularis de origen europeo y vacunas, agentes de diagnóstico y métodos de uso de los mismos**".





V. FUNDAMENTOS DEL RECURSO

Respetuosamente me permito disentir de lo expuesto por ese Despacho en la Resolución objeto del presente recurso, ya que la presente solicitud posee nivel inventivo a la luz del documento US 5,885,823 (D1).

La anterior afirmación tiene justificación jurídica y técnica por las siguientes razones:

De acuerdo con el artículo 18 de la Decisión 486:

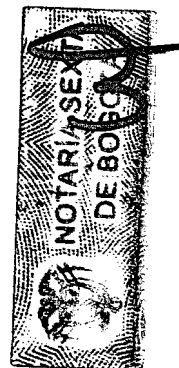
"Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica"

Así entonces, es posible concluir que para ser pertinentes en la evaluación de nivel inventivo de la presente invención, el documento D1 citado debería revelar sugerencias y enseñanzas claras para el técnico en la materia que lo lleven a obtenerla. Para este fin el Señor Examinador debe examinar si, de conformidad con dicho documento, una persona versada en la materia habría o no propuesto las características técnicas que distinguen la invención reivindicada y que permiten alcanzar los resultados obtenidos por dicha invención.

Ahora bien, teniendo en cuenta lo anterior, procederemos a continuación a exponer los argumentos por los cuales la presente invención es inventiva frente a la anterioridad citada.

El Examinador insiste que una persona versada en la materia derivaría de manera obvia u evidente la materia de invención acá reivindicada a partir de las enseñanzas reveladas en el documento D1, el cual es considerado el documento más cercano del estado de la técnica. El examinador reconoce que mientras la presente solicitud hace referencia a una cepa diferente de la revelada en el documento D1 depositada bajo ATCC PTA-55672 y correspondiente a *Lawsonia intracellularis* de origen **norteamericano**, la cepa que hace parte del método de la presente solicitud tiene origen **europeo**. Sin embargo, el Examinador no reconoce el efecto mejorado debido a esta diferencia técnica.

En particular, la materia de invención recitada en la reivindicación 1 se diferencia de aquella revelada en el documento D1, en que la presente solicitud emplea *Lawsonia intracellularis* depositada bajo ATCC No. PTA-4927 o ATCC No. PTA-4926. En la solicitud la *Lawsonia intracellularis* depositada bajo ATCC No. PTA-4926 también es designada como B903 (ver página 17, líneas 11-



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13 de la publicación internacional WO 2005/011731 A1) y como DK 15540 p80 (ver página 24 líneas 17-18 de WO 2005/011731 A1). De esta manera, la *Lawsonia intracellularis* depositada bajo ATCC No. PTA-4926 es designada como DK 15540 (ver página 16, líneas 23-28 de WO 2005/011731 A1).

El efecto técnico logrado por la diferencia arriba mencionada de la presente solicitud frente al documento D1, i.e. el uso de la cepa de *Lawsonia intracellularis* ATCC No. PTA-4926 (B3903, DK 15540 p80) o ATCC No. PTA-4927 (DK 15540), es que permite la producción de una vacuna modificada que no causa la excreción fecal en los días 14 a 21 luego de vacunar a los cerdos. La ausencia de excreción fecal es una ventaja clara de una vacuna en términos de seguridad, debido a que la contaminación fecal del ambiente es la forma más usual de transmisión de *Lawsonia intracellularis* de un animal a otro. Por lo tanto, la ausencia de excreción fecal en los días 14 a 21 luego de la vacunación en cerdos reduce el riesgo de propagación incontrolada de la bacteria a otros animales o de otros rebaños de animales con consecuencias imprevisibles (*Lawsonia intracellularis* no solo infecta cerdos sino que también se propaga en una amplia gama de otros mamíferos incluyendo caballos, perros, ratas, cobayas, conejos y hamsters).

De modo que, con las características distintivas de la presente invención frente a D1 lo que se logra es obtener o propagar *Lawsonia intracellularis* de forma atenuada, por medio de la vacuna causando la reducción de excreción fecal en cerdos en comparación con el documento D1, el cual no causa el mismo efecto en absoluto.

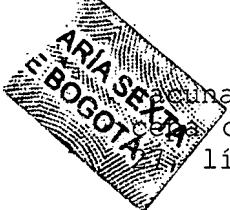
A partir del documento D1, el problema técnico objetivo que se busca solucionar con la presente solicitud es proporcionar un método que permita una vacuna mejorada para indicar una respuesta inmune a la bacteria *Lawsonia intracellularis*, en donde dicha vacuna no cause la propagación de materia fecal en los días 14 y 21 luego de aplicar la vacuna en cerdos. En este contexto, se dirige la atención del Señor Examinador a la página 2, líneas 20-21 de la publicación internacional (WO 2005/011731 A1), en donde uno de los objetivos de la invención se describe específicamente como la necesidad de proporcionar una vacuna de *Lawsonia intracellularis* mejorada.

Este problema técnico es solucionado usando cepas de *Lawsonia intracellularis* depositada bajo ATCC No. PTA-4926 (B3903, DK15540 p80) o ATCC No. PTA-4927 (DK 15540), las cuales son de origen Europeo. A este respecto, se dirige la atención del Señor Examinador a los datos experimentales del ejemplo 2 de la presente solicitud, en donde se muestra que la cepa DK 15540 p60 (obtenida mediante el procedimiento de la reivindicación 1 usando *Lawsonia intracellularis* ATCC No. PTA-4927, ver ejemplo 1 y página 24, líneas 21-22 del ejemplo 2) y el deposito ATCC No. PTA-4926, que no causan excreción fecal en los días 14 y 21 luego de la



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vacunación en cerdos, en comparación con la cepa N343 (la misma divulgada en D1), N101494 y DK15540p60 (ver tabla 5 y página líneas 26 hasta página 28, línea 2 de WO 2005/011731 A1).

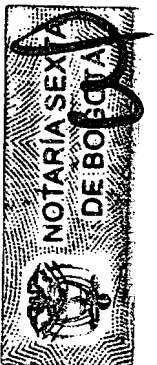
Dicha solución no es obvia para una persona versada en la materia a partir de las enseñanzas de D1 toda vez que este documento no enseña o sugiere que la cepa de *Lawsonia intracellularis* ATCC No. PTA-4927 (DK15540) o ATCC No. PTA-4926 (B3903, DK 15540p80) permitirían obtener una vacuna para inducir una respuesta inmune a *Lawsonia intracellularis*. En contraste, D1 únicamente se refiere al uso de la cepa de *Lawsonia intracellularis* N343 (Isi-1) para la elaboración de vacunas las cuales resultan, al menos parcialmente, en excreciones fecales en los días 14 y 21 post-vacunación de cerdos (ver columna 21, líneas 57-60 y columna 23, líneas 51-57 de D1; y la tabla 5 y página 27, líneas 26-29 y página 28, líneas 2-3 de WO 2005/01171 A1). Adicionalmente, D1 alejaría a una persona versada en la materia de la presente invención toda vez que en dicho documento únicamente se mencionan cepas de origen norteamericano.

En efecto, vale la pena resaltar nuevamente que D1 revela en su ejemplo 1 el origen de todas las muestras del microorganismo: "la muestra N24912 fue obtenida de una granja de **Iowa** (...) la muestra N72994 fue obtenida de una granja en **Minnesota** (...) la muestra N101494 fue obtenida de una granja en **Indiana**" (véase columna 9 línea 54 a columna 10, línea 6 de D1). Por su parte, el ejemplo 2 de D1 describe el uso de muestras intestinales del ejemplo 1 para cultivos en suspensión de *Lawsonia intracellularis* (véase columna 12, líneas 7 a 13 de D1). Igualmente el ejemplo 3 de D1 revela que se preparó un homogeneizado, tal como se describe en el ejemplo 2 y que se depositó una muestra de *Lawsonia intracellularis* a la cual se le asignó el número 55672 (véase columna 13, líneas 512-51 de D1). Por consiguiente, el depósito ATCC PTA-55672 y todos los derivados aislados de éste tienen origen **norteamericano**.

En contraste, la materia del pliego descriptivo de la presente solicitud hace referencia a los depósitos ATCC No. PTA-4927 y ATCC NO. PTA-4926, los cuales corresponden a *Lawsonia intracellularis* de origen **europeo**.

En este sentido, resulta revelante el ejemplo 2 de la presente solicitud, en donde se realizó un ensayo PCR para las heces de los cerdos y se demostró que los depósitos ATCC No. PTA-4927 o ATCC No. PTA-4926 (pases DK15540p60 y DK15540p80), ambos obtenidos con el método de las reivindicaciones 1 o 10, mostraron el efecto técnico mencionado (véase tabla 5 de la página 29 de la descripción). Tal como menciona la página 31, líneas 11 a 13:

"No se observó ninguna prueba de la presencia en los grupos de cepas clínicas de alto pase (DK15540 p60 y p80) usando una PCR a lo largo del estudio"



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Así entonces, debido a que el documento D1 no enseña o sugiere emplear el deposito de *Lawsonia intracellularis* ATCC No. PTA-4926 B3903, DK 15540p80) o ATCC No. PTA-4927 (DK15540), tal como SI lo hace la presente solicitud de patente, y dicho documento no menciona ninguna cepa de *Lawsonia intracellularis* de origen Europeo, el uso de las cepas mencionadas claramente no es obvio para una persona versada en la materia a partir de las enseñanzas de D1, y por tanto la presente solicitud tiene nivel inventivo.

Adicionalmente, vale la pena aclarar que en contraste a lo señalado por el Señor Examinador, a partir de los argumentos arriba mencionados es claro que las cepas de *Lawsonia intracellulairs* de origen Europeo permiten controlar la infección de forma mejorada. El efecto técnico logrado por el empleo de las cepas de origen Europeo, tal como se mencionó anteriormente, es una propagación de *Lawsonia intracellulairs* atenuada por medio de la vacuna causando la reducción de excreción fecal en cerdos. La materia reivindicada en la presente solicitud pone a disposición vacunas de *Lawsonia intracellularis* que permiten reducir y hasta eliminar la excreción fecal en cerdos, resultando más seguras:

- Dado que la Enteropatía Proliferativa Porcina PPE (por sus siglas en inglés) es transmitida por materia fecal, la presente invención permite controlar la propagación de la cepa atenuada de la vacuna evitando así la reversión de la virulencia de la cepa por mutaciones secundarias
- Dicho control permite que la cepa virulenta mutada no sea transmitida en la manada e incluso evita que salte a otras manadas

Este claro avance en la regla técnica resulta valioso y le confiere nivel inventivo a la presente solicitud.

Si bien es cierto que la mejora en la actividad de la vacuna del ejemplo 2 de la invención no presenta una mejora significativa en la infección en todos los posibles eventos en que se puede presentar la misma, es importante resaltar nuevamente que la ausencia de excreción fecal es una ventaja clara de una vacuna en términos de seguridad, debido a que la contaminación fecal del ambiente es la forma más usual de transmisión de *Lawsonia intracellularis* de un animal a otro. De manera que se debe reconocer que la efectividad del empleo de cepas de origen Europeo Si resulta evidente en factores tan importantes como la propagación de la infección por medio de la materia fecal de los animales.

De lo anterior, es claro que a partir de los datos experimentales divulgados en los ejemplos de la presente solicitud, es evidente la ventaja técnica que conlleva usar las cepas de la solicitud en lugar de las cepas ya conocidas en el estado de la técnica para la elaboración de la vacuna de *Lawsonia intracellularis*.





5.1 Conclusión.

Teniendo en cuenta que la solución técnica planteada en la presente solicitud, no resulta obvia para la persona medianamente versada en la materia en vista de las enseñanzas del documento D1, toda vez que no se revela o sugiere el uso de los depósitos de *Lawsonia intracellularis* ATCC No. PTA-4927 y ATCC No. PTA-4926 de origen europeo ni tampoco se sugiere el efecto técnico asociado, es claro que la presente solicitud de patente es inventiva frente a las enseñanzas de D1. De hecho, el documento D1 solamente enseña un método empleando cepas de *Lawsonia intracellularis* de origen norteamericano, en donde dicho método no resuelve el problema técnico de la presente solicitud y o sugiere el efecto acá reivindicado. Lo anterior toda vez que a pesar de la vacunación, los cerdos todavía se ven afectados por excreción fecal en los días 14 y 21 (ver columna 23, líneas 53 a 56 de D1). Por lo tanto, en vista del documento D1, la presente invención no puede resultar obvia y consecuentemente goza de nivel inventivo.

En efecto, deseamos poner de presente que no estamos de acuerdo con la metodología empleada por el Señor Examinador para el estudio de nivel inventivo toda vez que si bien deja clara las diferencias entre la presente solicitud y D1, no demuestra cómo una persona versada en la materia se vería motivada a emplear una cepa de *Lawsonia intracellularis* de origen Europeo a partir de dicho documento y/o predeciría la no excreción fecal de cerdos, tal como en la presente solicitud.

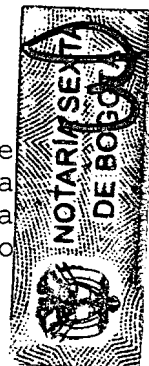
5.2 Por último, aunque la concesión de una solicitud de patente en otros países, equivalente a la solicitud Colombiana, no obliga a su Despacho a otorgar el privilegio de patente para la misma invención, sí constituye un antecedente favorable para el estudio de patentabilidad, respetuosamente solicitamos tener en cuenta que la presente invención ha sido otorgada en los siguientes países u oficinas de patentes:

- **Europa** con número de patente EP1651260.
- **Estados Unidos** con número de patente US7312065 y US7758870.

Así entonces, podemos concluir que la Resolución acá impugnada fue expedida con violación del artículo 18 de la Decisión 486 debido a que no era posible derivar de manera obvia la presente invención a partir del documento más cercano del estado del arte encontrado por su Despacho, que en el presente caso es D1.

VI. PETICIÓN

Por todo lo anterior, solicito a ese Despacho lo siguiente:



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Que al considerar las razones precedentes se revoque la decisión contenida en la Resolución No. **4245** del 31 de Enero de 2012, en cuando resuelve negar el privilegio de patente de invención.

6.2. Que se otorgue el privilegio de patente de la solicitud para: "**Lawsonia intracellularis de origen europeo y vacunas, agentes de diagnóstico y métodos de uso de los mismos**".

VII. ANEXO

- Copia simple de las patentes EP1651260, US7312065 y US7758870.

VIII. NOMBRE Y DIRECCION

El nombre y la dirección de la sociedad recurrente es el siguiente:

BOEHRINGER INGELHEIM VETMEDICA, INC.

St. Joseph

Missouri 64506-2002

Estados Unidos de América

ui

IX. NOTIFICACIONES

Atentamente manifiesto a usted que recibiré notificaciones en la Secretaria de su Despacho o en mi oficina de abogada situada en la Carrera 4ª. No. 72-35, piso 4º, de esta ciudad de Bogotá, D.C.

Señor Superintendente,

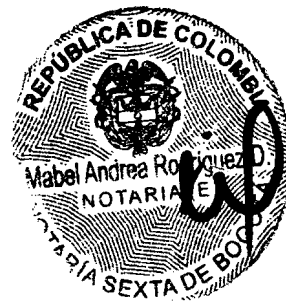
Luiz Clemencia de Paez
LUZ CLEMENCIA DE PAEZ
CC. 35.456.444 de Usaquén
T.P.A. No. 23.555

COLO-15149-841-001-9
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No. M-2012-212020\MGM



NOTARIA DE BOGOTÁ
R.D.C. CONSENTIMIENTO Y REPRESENTACION PERSONAL
Escriba, D.C.
Ante el **05 MAR 2012**
NOTARIO SEXTO (E) DEL CÍRCULO DE
BOGOTÁ, D.C.
Con el (los) (señor(es)) *Leiz*
la elegida Surseg
de la ef
3 5 7 9 2 4 6
7 8 2 3 5 7
Y declaró (así) que la(s) firma(s) que aparece(n)
en el presente documento es (son) la(s) suya(s) y
que el contenido del mismo es cierto.
En constancia se firma esta diligencia.

Leiz Clemente Sobres
TP 23555 055





US007758870B2

(12) **United States Patent**
Roof et al.

(10) **Patent No.:** **US 7,758,870 B2**
(45) **Date of Patent:** **Jul. 20, 2010**

(54) **LAWSONIA INTRACELLULARIS OF EUROPEAN ORIGIN AND VACCINES, DIAGNOSTIC AGENTS AND METHODS OF USE THEREOF**

(75) Inventors: **Michael B. Roof**, Ames, IA (US);
Jeremy J. Kroll, Urbandale, IA (US);
Jeffrey P. Knittel, Parkville, MO (US)

(73) Assignee: **Boehringer Ingelheim Vetmedica, Inc.**,
St. Joseph, MO (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **11/935,447**

(22) Filed: **Nov. 6, 2007**

(65) **Prior Publication Data**
US 2008/0112980 A1 May 15, 2008

Related U.S. Application Data

(62) Division of application No. 10/891,745, filed on Jul. 15, 2004, now Pat. No. 7,312,065.

(60) Provisional application No. 60/490,001, filed on Jul. 25, 2003.

(51) Int. Cl.
A61K 39/02 (2006.01)

(52) U.S. Cl. **424/234.1**; 424/93.4; 424/825;
435/252.1

(58) Field of Classification Search None
See application file for complete search history.

(56) **References Cited**
U.S. PATENT DOCUMENTS

5,714,375 A 2/1998 Knittel et al.
5,885,823 A 3/1999 Knittel et al.

OTHER PUBLICATIONS

J.J. Kroll et al; Efficacy of an Avirulent *Lawsonia intracellularis* Vaccine in Swine; Abstract of the General Meetings No. 101 May 23, 2001; Session No. 263/Z Abstract Z-40.
International Search Report for PCT/US2004/022704 mailed Nov. 3, 2004.
P.K. Holyoake et al; Enzyme-Linked Immunosorbent Assay for Measuring Ileal Symbiont Intracellularis-Specific Immunoglobulin G Response in Sera of Pigs; Journal of Clinical Microbiology Aug. 1994 vol. 32 No. 8 pp. 1980-1985; American Society for Microbiology.
NIH Guide; Multicomponent Vaccine Development; (1993) vol. 22, No. 28.
Fattom et al; Vaccine (1999) vol. 17, No. 2 pp. 126-133.

Primary Examiner—Jennifer E Graser
(74) *Attorney, Agent, or Firm*—Michael P. Morris;
Marry-Ellen M. Devlin; Joyce L. Morrison

(57) **ABSTRACT**

The present invention relates to *Lawsonia intracellularis* vaccines and methods for protecting against and diagnosing *L. intracellularis* infection. The products and processes of the invention are attainable, in part, as the result of an improved method for cultivating large scale supplies of *L. intracellularis*, including both a novel isolate of *L. intracellularis* of European origin and a method of preparing a lyophilized product containing the attenuated European isolate as vaccine product.

3 Claims, No Drawings

**LAWSONIA INTRACELLULARIS OF
EUROPEAN ORIGIN AND VACCINES,
DIAGNOSTIC AGENTS AND METHODS OF
USE THEREOF**

RELATED APPLICATIONS

This application is a divisional application of U.S. application Ser. No. 10/891,745, filed Jul. 15, 2004, which claims benefit to U.S. Provisional Application Ser. No. 60/490,001, filed Jul. 25, 2003, the contents of which are hereby incorporated by reference in their entirety.

This application claims benefit to U.S. Provisional Application Ser. No. 60/490,001, filed Jul. 25, 2003, the disclosure of which is incorporated by reference in its entirety.

BACKGROUND

The present invention relates to *Lawsonia intracellularis* vaccines and methods for protecting against and diagnosing *L. intracellularis* infection. The products and processes of the invention are attainable, in part, as the result of an improved method for cultivating large scale supplies of *L. intracellularis*, including both a novel isolate of *L. intracellularis* of European origin and a method of preparing a lyophilized product containing the attenuated European isolate as vaccine product.

L. intracellularis, the causative agent of porcine proliferative enteropathy ("PPE"), affects virtually all animals, including: rabbits, ferrets, hamsters, fox, horses, and other animals as diverse as ostriches and emus. *L. intracellularis* is a particularly great cause of losses in swine herds in Europe as well as in the United States.

A consistent feature of PPE is the occurrence of intracytoplasmic, non-membrane bound curved bacilli within enterocytes in affected portions of intestine. The bacteria associated with PPE have been referred to as "Campylobacter-like organisms." S. McOrist et al., Vet. Pathol., Vol. 26, 260-264 (1989). Subsequently, the causative bacteria have been identified as a novel taxonomic genus and species, vernacularly referred to as Ileal symbiont (IS) intracellularis. C. Gebhart et al., Int'l. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-538 (1993). More recently, these novel bacteria have been given the taxonomic name *Lawsonia* (*L.*) *intracellularis*. S. McOrist et al., Int'l. J. of Systemic Bacteriology, Vol. 45, No. 4, 820-825 (1995). These three names have been used interchangeably to refer to the same organism as further identified and described herein.

L. intracellularis is an obligate, intracellular bacterium which cannot be cultured by normal bacteriological methods on conventional cell-free media and has been thought to require attached epithelial cells for growth. S. McOrist et al., Infection and Immunity, Vol. 61, No. 19, 4286-4292 (1993) and G. Lawson et al., J. of Clinical Microbiology, Vol. 31, No. 5, 1136-1142 (1993) discuss cultivation of *L. intracellularis* using IEC-18 rat intestinal epithelial cell monolayers in conventional tissue culture flasks. In addition, H. Stills, Infection and Immunity, Vol. 59, No. 9, 3227-3236 (1991) discusses using Intestine 407 human embryonic intestinal cell monolayers and GPC-16 guinea pig colonic adenocarcinoma cell monolayers in conventional tissue culture flasks.

Recently, an *L. intracellularis* vaccine has been approved for use in the United States, which vaccine is based on *L. intracellularis* isolates described and claimed in U.S. Pat. Nos. 5,714,375 and 5,885,823, both of which patents are herein incorporated by reference in their entirety. The above-described vaccine is sold by Boehringer Ingelheim

Vetmedica, Inc., 2621 North Belt Highway, St. Joseph, Mo. 64506-2002, under the trademark ENTERISOL® Ileitis.

SUMMARY OF THE INVENTION

One object of the invention is to provide an improved *L. intracellularis* vaccine using an isolate of European origin.

Another object of the invention is to provide an improved method for cultivation of *L. intracellularis* on a large scale and improved techniques for production of *L. intracellularis* vaccines.

To achieve these and other objects, and in accordance with the purpose of the invention as embodied and broadly described herein, the present invention provides a newly isolated *L. intracellularis* from Europe, a method of attenuating such an isolate, and the attenuated isolate thereof. Also provided herein is a vaccine comprising the attenuated isolate. Also provided herein is a method for producing a vaccine comprising the attenuated isolate in lyophilized form for reconstitution at the time of administration and the lyophilized product thereof.

In one embodiment, the newly isolated *L. intracellularis* from Europe, isolate DK 15540, is deposit isolate ATCC accession No. PTA-4927. In another embodiment, the attenuated isolate derived from isolate DK 15540, is designated isolate B3903, ATCC accession No. PTA-4926.

DETAILED DESCRIPTION

As used herein, the term "*L. intracellularis*" means the intracellular, curved gram-negative bacteria described in detail by C. Gebhart et al., Int'l. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-538 (1993) and S. McOrist et al., Int'l. J. of Systemic Bacteriology, Vol. 45, No. 4, 820-825 (1995), each of which is incorporated herein by reference in their entirety, and includes but is not limited to the isolate designated DK 15540 which was deposited for patent purposes with the American Type Culture Collection ("ATCC"), 10801 University Boulevard, Manassas, Va. 20110-2209 on Jan. 9, 2003 and assigned ATCC accession number PTA-4927; the causative bacteria which can be obtained from PPE infected swine or other animals throughout the world given the knowledge in the art and the teachings herein; and variants or mutants of any of the above bacteria, whether spontaneously or artificially obtained.

As used herein, the term "attenuated isolate" means any *L. intracellularis* isolate that is prepared according to the cultivation and passaging techniques taught herein to achieve avirulence while maintaining immunogenic properties when administered to a host animal including but not limited to the attenuated isolate designated B-3903 which was deposited for patent purposes with the American Type Culture Collection ("ATCC"), 10801 University Boulevard, Manassas, Va. 20110-2209 on Jan. 9, 2003 and assigned accession number PTA-4926.

PTA-4927 and PTA-4926 were tested by the ATCC on Apr. 21, 2004, and were found viable. The date of conversion to Budapest Treaty was Oct. 16, 2007.

The attenuated isolate of the invention can be used as an immunogen in antimicrobial vaccines for animals, including birds, fish, and mammals such as cattle, swine, horses, and primates. Such vaccines can be prepared by techniques known to those skilled in the art and given the teachings contained herein. Such a vaccine would comprise an immunologically effective amount of the attenuated isolate in a pharmaceutically acceptable carrier. The vaccine could be administered in one or more doses. An immunologically

effective amount is determined by means known in the art without undue experimentation, given the teachings contained herein. The amount of avirulent bacteria should be sufficient to stimulate an immune response in disease-susceptible animals while still being avirulent. This will depend upon the particular animal, bacteria, and disease involved. The recommended dose to be administered to the susceptible animal is preferably about 3.0 TCID₅₀ (tissue culture infective dose 50% end point)/dose to about 6.0 TCID₅₀/dose and more preferably about 4.0 TCID₅₀/dose to about 5.0 TCID₅₀/dose. In a preferred embodiment, the titer of the vaccine is about 4.9 TCID₅₀/dose as determined by Tissue Culture Infective Dose 50% endpoint dilution assay (TCID₅₀). The carriers are known to those skilled in the art and include stabilizers and diluents. Such a vaccine may also contain an appropriate adjuvant. The vaccines of the invention may be used in combination with other vaccines, for example, as a diluent of another vaccine. The vaccine preparations may also be desiccated, for example, by freeze drying for storage purposes or for subsequent formulation into liquid vaccines.

Accordingly, the invention also comprises a method for inducing an immune response to virulent, wild-type *L. intracellularis* bacteria in an animal host for the purpose of protecting the host from such bacteria. The method comprises administering an immunologically effective amount of the attenuated bacteria or killed bacteria of the invention to the host and, preferably, administering the vaccine of the invention to the host.

As used herein, the term "large-scale cultivation" means a level of cultivation of *L. intracellularis* greater than approximately 2.0 to 3.0 liters and includes production on a scale of 100 liters or more. "Cultivation" as used herein, means the process of promoting the growth, reproduction and/or proliferation of *L. intracellularis*.

L. intracellularis can be cultivated by methods known in the art, preferably, according to U.S. Pat. Nos. 5,714,375 and 5,885,823. For example, culture cells may first be inoculated with an inoculum comprising *L. intracellularis* bacteria so as to infect the cells with the bacteria. Numerous cell lines can be used in practicing the invention, including, but not limited to, IEC-18 (ATCC 1589)-rat intestinal epithelial cells, HEP-2 (ATCC 23)-human epidermoid carcinoma cells, McCoy's (ATCC 1696)-mouse (non-specified) cells, BGJMK (Biowhitaker #71-176)-buffalo green monkey kidney cells, and swine intestinal epithelium cells. The preferred culture cells are HEP-2, McCoy's or IEC-18 cells.

If culture cells are used, prior to being inoculated, the cells may be in the form of a monolayer. To form a monolayer, the cells may be seeded into conventional flasks. Each flask is generally seeded with between about 1×10^5 cells to about 10×10^5 cells per 25, 75, 150, 850 cm² flask or roller bottle mixed with growth media. The growth media may be any media for cell cultivation which includes a nitrogen source, necessary growth factors for the chosen culture cells, and a carbon source, such as glucose or lactose. The preferred media is DMEM fortified with Ham's F 12 with 1-5% fetal bovine serum, although other commercially available media may be used with good results.

Successful cultivation of *L. intracellularis* is enhanced by maintaining the culture cells in a constant state of growth. Therefore, the culture cell monolayer should be at about 20 percent to about 50 percent confluency at the time of inoculation. Preferably, the cells should be at about 30 percent to about 40 percent confluency at the time of inoculation, most preferably at about 30 percent confluency.

Alternatively, the cells, prior to being inoculated, may be grown in suspension, as described infra. Preferably, the cells

are first grown to 100% confluency in the form of a monolayer in an adherent type system, e.g. a roller bottle system, and then transferred to 3-3000 liters and grown in suspension. Alternatively, the cells can be grown in suspension to the desired cell density, e.g. 2×10^5 cells/ml, within the 3-3000 liter vessel (bioreactor, Fermentor, spinner flask, etc.) using parameters suitable for growth within this system prior to inoculation.

The inoculum may be a pure culture of *L. intracellularis* obtained from infected swine or other animals. Preferably the inoculum may be a pure culture of *L. intracellularis* obtained from ATCC accession No. PTA-4927.

The inoculum can be an intestinal homogenate prepared by scraping the mucosa off of the ileum of a swine or other animal infected with PPE. When preparing an intestinal homogenate, ileal sections selected for culture should show severe lesions with gross thickening of the gut. Due to the fragile nature of the bacteria, samples should preferably be stored at -70° C. as quickly as possible after necropsy. An antibiotic to which *L. intracellularis* is resistant such as Vancomycin, Amphotericin B or members of the aminoglycoside group of antibiotics, including Gentamicin and Neomycin, to name a few, is preferably added to the inoculum to suppress contaminating bacteria while permitting *L. intracellularis* growth. Whether the inoculum is a pure culture or an intestinal homogenate, inoculation of the culture cells can be performed by various techniques known in the art, given the teachings herein.

The bacteria and/or inoculated culture cells are then incubated under a reduced dissolved O₂ concentration. At dissolved oxygen concentrations greater than 10% *L. intracellularis* growth is less than optimal with cessation of growth eventually occurring at oxygen concentrations outside this range. Preferably, the bacteria and/or inoculated culture cells are incubated in a dissolved oxygen concentration in the range of about 0% to about 10%. More preferably, the bacteria and/or cells are incubated in an oxygen concentration in the range of about 0% to about 8%, with an oxygen concentration of about 0% to about 3.0% being most preferred.

The proper concentration of carbon dioxide is also important to the proper growth of *L. intracellularis*. At carbon dioxide concentrations greater than 0% and less than 4%, non-optimum growth occurs with cessation of growth eventually occurring at carbon dioxide concentrations outside this range. Preferably, the carbon dioxide concentration is in the range from about 6% to about 10%, with a carbon dioxide concentration of about 8.8% being most preferred.

In addition, the cells are preferably incubated at a hydrogen concentration in the range from about 4% to about 10%. Most preferably, the cells are incubated in about 0 to about 8.0% O₂, about 8.8% CO₂, and about 4% H₂. Nitrogen is used as a "balance" in the gas mixture containing nitrogen (96%) and hydrogen (4%) or nitrogen (80%), carbon dioxide (10%) and hydrogen (10%) for growth of this organism. Cells are preferably incubated at a nitrogen concentration in the range from about 80% to 96%. Therefore, cells are most preferably incubated in about 0 to about 8.0% O₂, about 8.8% CO₂, about 4% H₂ and about 96% N₂.

Inoculated cells may be incubated in a dual gas incubator or other gas chambers which contain the proper hydrogen, oxygen and carbon dioxide concentrations and which allow the cells to be suspended during incubation. The chamber should comprise a means for maintaining the inoculated cells in suspension, and a gas monitor and supply source to supply and maintain the proper gas concentrations. The incubation temperature should be in the range of from 30° C. to about 45° C. and is more preferably in the range of from about 36° C. to

about 38° C. Most preferably, the temperature is about 37° C. The necessary equipment for cultivation and attenuation is readily available to those of ordinary skill in the art given the teachings herein. One example of equipment suitable for carrying out the present invention is a dual gas incubator, e.g., model 480 (Lab-Line, Melrose Park, Ill.) in conjunction with spinner flasks to maintain the cells in suspension. The presently preferred equipment comprises a fermentor, bioreactor, stir plate or rotary shaker containing at least about 2 liters media and capable of maintaining the culture cells in suspension via sparging gas of the appropriate concentration, or other means of mechanical agitation, and continuously monitoring dissolved O₂ levels in the media. New Brunswick, Braun and other companies make suitable fermentors and bioreactors for this purpose.

By maintaining the inoculated cells in a suspended state during incubation, maximum growth of the cells, and hence *L. intracellularis*, is achieved by increasing each individual cell's exposure to growth media and the proper mixture of hydrogen, oxygen and carbon dioxide. The culture cells can be agitated and maintained in suspension by a variety of methods known in the art including, for example, culture flasks, roller bottles, membrane cultures, biobags, WAVE™ bioreactor systems, fermentors and spinner flasks. The cells may be kept in suspension during incubation by incubating the cells in a spinner flask inside a dual gas incubator or similar apparatus. The term "spinner flask", as used herein, means a flask or other container which employs a paddle, propeller or other means to agitate the culture and keep the cells contained therein in suspension.

In a preferred embodiment, the inoculated cells are incubated until the cells reach confluency and then the cells are placed in a spinner flask containing growth media and incubated in a dual gas incubator while spinning the flask. Preferably, the inoculated cells are scraped or trypsinized and passaged into the spinner flask. This can be achieved by a variety of methods known in the art such as using a cell scraper to detach the cells. Once the cells are introduced into the spinner flask, the paddle of the spinner flask is typically rotated in the range of from about 5 to about 500 rpm on a magnetic stir plate in order to maintain the infected cells in suspension.

A portion of the cultivated *L. intracellularis* is then passaged to fresh culture to increase the production of *L. intracellularis* bacteria. The term "passaging" or variations thereof herein means the process of transferring a portion of the cultivated *L. intracellularis* to fresh culture cells in order to infect the fresh cells with the bacterium. The term "fresh", as used herein, means cells which have not yet been infected by *L. intracellularis*. Preferably such cells are on the average no more than approximately one day old.

The passage of *L. intracellularis* in suspension cultures may be accomplished by removing a portion of the original culture and adding it to a new flask containing fresh culture cells. If the original culture has a high number of bacteria/ml, for example, greater than about 10⁴ bacteria/ml, it is preferable to add between about 1 to 10% (volume to volume) of culture from the infected flask to a new flask containing fresh cells. This is preferably done when 50-100% of the cells are infected. If fewer than 50% of the cells are infected, passaging is preferably accomplished by splitting the culture 1:2 into a new flask and scaling-up the volume by adding fresh tissue culture cells and media. In either case, cell lysis and other steps are not required, in direct contrast to the passage of monolayer cultures, as in the prior art.

After sufficient growth of the culture cells and subsequent infection by *L. intracellularis*, as determined by indirect fluo-

rescent antibody (IFA) staining, TCID₅₀ or another comparable method, at least a portion of the cultivated *L. intracellularis* bacteria is then harvested. Harvesting is typically performed at cell infectivity of about 60% or higher; however, one skilled in the art knows that harvesting could be performed at a cell infectivity of less than 60%. The harvesting step may be performed by separating the bacteria from the suspension by various techniques known to those of ordinary skill in the art, given the teachings herein. Preferably, the *L. intracellularis* bacteria is harvested by centrifuging the contents of all or a portion of the suspension to pellet the culture cells, resuspending the resulting cell pellets, and lysing the infected cells. Typically, at least a portion of the contents is centrifuged at about 3000×g for about 20 minutes in order to pellet the cells and bacteria. The pellet may then be resuspended in, for example, a sucrose-phosphate-glutamate (SPG) solution and passed approximately 20 times through a 25 gauge needle in order to lyse the cells. If further purification is desired, the samples can be centrifuged at about 145×g for about five minutes to remove cellular nuclei and debris. The supernatant may then be centrifuged at about 3000×g for about twenty minutes and the resulting pellet resuspended in an appropriate diluent, such as SPG with fetal bovine serum (to prepare harvested bacteria suitable for lyophilization, freezing, or use as an inoculant) or growth media (to prepare harvested bacteria more suitable for passaging to fresh cells).

As previously mentioned, effective growth of *L. intracellularis* for large-scale production is enhanced by keeping the tissue cells actively growing. With monolayers, when cultures become confluent, the rate of cell division decreases substantially. Attempts to grow *L. intracellularis* on monolayer tissue cultures have had limited success and scale-up has not been possible. However, using suspension cultures greatly facilitates keeping the cells actively growing and permits continuous culture expansion and scale-up. Using a fermentor and between about 0 to 3% dissolved O₂ as explained above, enables growth of up to and greater than 10⁸ bacteria/ml.

When using IEC-18 cells, it is preferable to add gelatin, agarose, collagen, acrylamide or silica beads, such as Culti-sphere-G porous microcarriers (HyClone Laboratories, Logan Utah), along with the growth media. However, HEp-2 cells and others do not require microcarriers according to the methods used herein.

For culture maintenance purposes, with HEp-2 cultures, preferably 25% to 50% of the culture is removed and replaced with fresh media at weekly intervals. For cell cultures with microcarriers or beads, preferably 25% to 50% of the culture is removed and replaced with fresh media 1-2 times weekly. For scale-up purposes, an additional 25% to 50% of media, or media with microcarriers, may be added to the culture.

Depending upon the rate at which the culture cells become infected, passage to fresh cells generally occurs between about every 2 to about 7 days. Assuming that the culture cells become at least 70% infected within 2 to 7 days, preferably passage occurs between about every 5 to 7 days.

The present invention also provides vaccines and methods for producing vaccines against a novel isolate of *L. intracellularis* of European origin. Preferably, after maintaining the infected cells in suspension for an extended time (for example, 6-8 months), at least a portion of the cultivated *L. intracellularis* bacteria are harvested and monitored for potential attenuation. Such monitoring is preferably accomplished by host animal or animal model challenges to select for an attenuated isolate. Such attenuated isolates are used in vaccines according to the methods taught herein.

The present invention allows rapid culture expansion, an increase in yields of 100-1000 fold, and reduced cost for production of *L. intracellularis* of European origin. As a result, the abundant supply of *L. intracellularis* bacteria produced is readily attenuated for vaccine production purposes. The method of growing *L. intracellularis* in suspension greatly increases the ease, speed, and number of bacterium available for this purpose. The more cells and cell divisions which occur, the greater the level of mutations occurring which are advantageous in vaccine development. Thus, growth in suspensions increases the expression of important immunogens controlled by environmentally regulated genes and their expression products.

The resulting attenuated isolates can be cultivated in tissue culture monolayers but are preferably cultivated in suspension cultures. Other means of attenuation can include chemical attenuation by the use of, for example, N-methyl nitrosoguanidine and others known in the art. Whether by multiple passage or chemical means, an attenuated *L. intracellularis* is produced and selected for vaccine preparation. In a preferred embodiment, the resulting attenuated isolate is ATCC accession No. PTA-4926.

The vaccine antigen can be harvested by centrifugation or microfiltration as described above. The antigen is then standardized at a defined level based on the optimum host animal immune response, determined by a dose titration in the host animal species. The bacteria may be inactivated by methods known in the art, e.g., by using 0.3% formalin or other inactivating agents to prepare a killed vaccine. The antigen is then incorporated into a suitable adjuvant, such as aluminum hydroxide or mineral oil to enhance the immune response. The antigen is then used to vaccinate the host via intramuscular or subcutaneous infection, in the case of swine at about 3-4 weeks of age, with a booster dose if necessary.

Preferably, the bacteria is serially passaged to induce and select for an attenuated, avirulent live culture. The culture is tested in the host animal for signs of attenuation. The culture is harvested as described earlier and lyophilized. Swine, for example, are orally vaccinated with 1×10^4 to 1×10^6 bacteria. About twenty-eight days after vaccination, the swine are orally inoculated with about 1×10^7 organisms from a less passaged (less than 30 passages in vitro past the original isolation from the intestinal homogenate) virulent culture of *L. intracellularis*. Infected animals are necropsied 21 days after challenge and the small intestines observed for gross lesions as well as microscopic lesions. PCR, indirect fluorescent antibody (IFA) or immunohistochemistry (IHC) should also be performed. About eighty percent of the control animals will show gross or microscopic lesions and test positive for the presence of *L. intracellularis* in the mucosal cells of the intestines using either PCR, IFA or IHC testing methods. Vaccinated animals will have normal mucosal surfaces as determined by histological observations and will be negative by PCR testing 3 to 4 weeks post inoculation.

Generally, an attenuated immunogenic *L. intracellularis* isolate is produced after continuous culture for about 150 days to about 250 days, during which time the culture is passaged about 50-100 times. However, one skilled in the art knows that other attenuated cultures may be produced by varying these figures.

The vaccine product of the invention can be lyophilized. After harvesting, the isolate can be concentrated by various methods known in the art and can be mixed with a stabilizer, e.g. sucrose gelatin stabilizer. The vaccine product can then be subjected to freezing and drying (lyophilization). Generally, the freezing step comprises ramping to about $-45^\circ \text{C} \pm 3^\circ \text{C}$ and holding for about 150 minutes to about 480 minutes.

The drying step can comprise primary and secondary drying steps. For example, the primary drying step can comprise: (a) ramping to between about -30°C . to about -5°C . and holding for between about 120 minutes to about 1000 minutes, and, optionally (b) ramping to between about -5°C . to about 5°C . and holding for between about 150 minutes to about 2000 minutes. The secondary step generally comprises ramping to about $27^\circ \text{C} \pm 5^\circ \text{C}$. and holding for between about 330 minutes to about 1120 minutes. One skilled in the art knows that these ranges can be adjusted depending on conditions, e.g., starting volume.

A vaccine is then prepared comprising an immunologically effective amount of the attenuated *L. intracellularis* in a pharmaceutically acceptable carrier. In a preferred embodiment, a vaccine comprises ATCC accession No. PTA-4926 in a pharmaceutically acceptable carrier. The combined immunogen and carrier may be an aqueous solution, emulsion or suspension. An immunologically effective amount is determinable by means known in the art without undue experimentation given the teachings contained herein. In general, the quantity of immunogen will be between 5 and 5000 micrograms, and between $10^{2.0}$ and $10^{9.0}$ TCID₅₀, preferably between $10^{3.0}$ and $10^{6.0}$ TCID₅₀, more preferably between $10^{4.0}$ and $10^{5.0}$ TCID₅₀, when purified bacteria are used.

The present invention also encompasses combination vaccines comprising the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from at least one other pathogen, including but not limited to: *Salmonella* spp. (e.g., *Salmonella choleraesuis*, *Salmonella typhimurium*), *Erysipelothrix* spp. (e.g., *Erysipelothrix rhusiopathiae*), *Haemophilus* spp. (e.g., *Haemophilus parasuis*), *Mycoplasma* spp. (e.g., *Mycoplasma hyopneumoniae*), *Leptospira* spp., *Clostridium* spp. (e.g., *Clostridium perfringens*, *Clostridium difficile*), *Streptococcus* spp. (e.g., *Streptococcus suis*), *Brachyspira* spp. (e.g., *Brachyspira hyodysenteriae*), *Bordetella* (e.g., *Bordetella bronchiseptica*), *Pasteurella* spp. (e.g., *Pasteurella multocida*), circovirus (e.g., porcine circovirus type 2), porcine reproductive and respiratory syndrome (PRRS) virus, swine influenza virus (SIV), coronavirus (e.g., transmissible gastro-enteritis (TGE) virus, porcine respiratory corona virus), parvovirus, or *Escherichia coli*; and a pharmaceutically acceptable carrier.

In one embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis*, *Erysipelothrix* spp., *Clostridium* spp., *Brachyspira* spp., transmissible gastro-enteritis (TGE) virus, and *Escherichia coli*; and a pharmaceutically acceptable carrier. Antigenic material from *Clostridium* spp. can include, but is not limited to, *Clostridium perfringens* and *Clostridium difficile*. Antigenic material from *Erysipelothrix* spp. can include, but is not limited to, *Erysipelothrix rhusiopathiae*.

In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix* spp.; and a pharmaceutically acceptable carrier. In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix rhusiopathiae*; and a pharmaceutically acceptable carrier.

In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from at least one other pathogen, including but not limited to: *Clostridium* spp. e.g., *Clostridium tetani*, equine influenza

virus (EIV) (e.g., EIV-1, EIV-2), equine herpes virus (EHV) (e.g., EHV-1, EHV-2, EHV-3, EHV-4, EHV-5, EHV-6, EHV-7), alphavirus (e.g., eastern encephalitis virus, western encephalitis virus, Venezuelan encephalitis virus), or West Nile virus; and a pharmaceutically acceptable carrier.

The vaccines according to the invention are generally administered to susceptible animals, preferably swine, in one or more doses. The live or killed vaccine may be administered 1 or 2 times at 2 week intervals. For the attenuated, live vaccines, one dose is preferred. The preferred routes of administration of attenuated live isolates are intramuscular, oral or intranasal, but intramuscular and subcutaneous injection routes are most preferred for the killed vaccine.

Effective diagnosis of PPE has also been hindered by the time required to culture the causative bacteria. As a result of the present invention, development of diagnostic tools promoting rapid and accurate assays for the presence of *L. intracellularis* in biological samples taken from swine and other animals susceptible to PPE is now possible.

The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can be used as an antigen in an ELISA or other immunoassay, such as an immunofluorescent antibody test ("IFA"), to detect antibodies to *L. intracellularis* in the serum and other body fluids of animals suspected of being infected with the bacteria. The presently preferred immunoassay is an IFA as described in the example below. Alternatively, the bacteria of the instant invention can be used in a Western Blot assay.

The preferred ELISA protocol according to the invention is as follows:

1. Add 0.1 ml/well antigen diluted in coating buffer. Incubate for 18 hours at 4° C.
2. Wash 3 times with PBS.
3. Add 0.25 ml of blocking buffer to each well of plate. Incubate 1 to 2 hours at 37° C.
4. Wash 3 times with wash buffer.
5. Dilute serum in blocking buffer and add 0.1 ml to the first wells of plate. Make serial 1:2 dilutions across the plate. Incubate for 1 hour at 37° C.
6. Wash 3 to 5 times with wash buffer.
7. Dilute conjugate in blocking buffer and add 0.1 ml to wells of plate and incubate for 1 hour at 37° C.
8. Wash 3 to 5 times with wash buffer.
9. Add substrate.
10. Measure absorbance of light with a spectrophotometer.
11. Wells in which antigen was not added are used as blanks.
12. Positive and negative control swine serum should also be used with each test.

The preferred Western Blot protocol is as follows:

1. Run antigen on 12% SDS-PAGE and transfer to nitrocellulose membrane.
2. Place membrane in blocking buffer for 2 hours.
3. Remove blocking buffer and rinse with PBS for 1 minute.
4. Dilute serum in blocking buffer and add to membrane. Incubate for 2 hours at room temperature.
5. Wash 3 times with wash buffer (5 minutes for each wash).
6. Dilute conjugate in blocking buffer and add to membrane. Incubate for 1 hour at room temperature.
7. Wash 3 times with wash buffer.
8. Add substrate for 10 minutes or until strong banding occurs.
9. Rinse with PBS.
10. Air dry and store in the dark.

The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can also be used to prepare antiserum or antibodies for diagnostic, prophylactic, or therapeutic use. The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can be administered to a non-human animal in an amount effective to elicit an immune response and the antiserum or plasma containing antibodies to the *L. intracellularis* bacteria, or components derived from such bacteria, can be collected according to methods known in the art and described herein.

The present invention is further described in the following examples which are provided for illustrative purposes only and are not to be construed as limiting. Indeed, other variants of the invention will be readily apparent to one of ordinary skill in the art.

All publications and patents cited herein are incorporated by reference in their entireties.

Example 1

Production of *L. Intracellularis* Vaccine

Isolation and Attenuation of *L. intracellularis* from the Intestines of European Pigs with Porcine Proliferative Enteropathy (PPE):

L. intracellularis virulent isolate DK 15540 (DK 15540, DK-15540 and 15540 are used interchangeably herein) was isolated by the University of Minnesota from an ileal homogenate of a Danish pig infected with acute porcine hemorrhagic enteropathy. This isolate has been deposited under the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209 and assigned accession number PTA-4927. The isolation process included scraping the mucosa from the ileum, homogenizing, trypsinizing for 30 minutes, and passing through a tissue grinder. The ileal homogenate was then passed through a series of filters consisting of 5.0, 1.0, and 0.65 µm. The homogenate was diluted in sucrose phosphate glutamate buffer with 10% fetal bovine serum (FBS). Aliquots (6×1 ml) of homogenate were made and stored at less than -70° C. The homogenate was used as inoculum to infect T-75 cm² flasks of McCoy cells. Cultures were monitored daily for McCoy cell infection by scraping McCoy cell monolayers, lysing cells by potassium chloride treatment, and placing the concentrated cell pellet on microscope slides stained by IFA using monoclonal antibodies specific for *L. intracellularis*. After eleven passages on anchorage dependent cell cultures, inoculum from passage eleven was transferred into a 250 ml spinner flask containing McCoy cells and grown in suspension until harvest. The Danish isolate of *L. intracellularis* (ATCC accession No. PTA-4927) was attenuated by continuous in vitro passage in McCoy cells for 80 weeks and tested for identity by monoclonal antibodies. The attenuated isolate was designated B3903 (B3903, B 3903 and B-3903 are used interchangeably herein). Isolate B3903 was deposited under the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209 and assigned ATCC accession No. PTA-4926.

Cultures:

Identification

Characteristic growth requirements, PCR reactions, and monoclonal antibody reactions were used to identify *L. intracellularis* Master and Working Seed materials.

Purity

Purity of Master Seed and Working Seed of *L. intracellularis* was determined by examining cultures with monoclonal

antibody stains, conventional extraneous agents tests for bacteria and viruses and by sterility mycoplasma tests.

Virulence

Master Seeds of *L. intracellularis* were not virulent as demonstrated by the lack of ability of the Master Seed and Back passage inoculum to produce clinical signs and gross lesions that are observed in susceptible swine following exposure of virulent *L. intracellularis*, as further illustrated in Example 2, infra.

Range of Subcultures

Final harvested material from production of *L. intracellularis* did not exceed eleven passages from the Master Seed.

Medium Composition

EU McCoy Master Cell Stock (MCS) were grown and maintained in Dulbecco's Modified Eagle Medium with Ham's Fortified F12 (DMEM/F12) and 1-10% (v/v) newborn bovine serum (NBS) or fetal bovine serum (FBS) (cell growth and maintenance media).

Master and Working Seed were stored in DMEM/F12 with 1-10% (v/v) NBS or FBS and 5-15% (v/v) glycerol (master and working seed storage media).

The final harvest product was stored in sucrose gelatin stabilizer (SGS) (final product storage media).

Propagation

Master and Working Seed cultures of *L. intracellularis* were propagated in EU McCoy MCS using cell growth and maintenance media as described supra and stored at less than or about -35° C.

Source of Tissue

L. intracellularis seed and production organisms were grown in McCoy Master Cell Stock (ATCC accession number CRL 1696, batch number F-10422). The mast cell stock was identified as 3894MMCSS at passage X. The master cell stock was passaged an additional six times and identified as EU McCoy MCS X+0. EU McCoy MCS passage X+0 was stored at -70° C. ±5° C. or colder. Production vaccine was made in EU McCoy MCS cell line subcultures through the 40th passage.

Culture Containers

EU McCoy MCS were propagated in tissue culture flasks with 25-150 cm² surface area, Costar cell cubes, in roller bottles with 850 cm² to 2,2250 cm², in spinner flasks up to 40 L capacity, and in bioreactors having 3 L to 500 L capacity.

Seed cultures of *L. intracellularis* were grown in 250-40,000 ml spinner flasks, 850 cm² to 2,250 cm² roller bottles, tissue culture flasks with 25-150 cm² surface area, or in bioreactors having 3 L to 500 L capacity.

Production cultures of *L. intracellularis* were grown in 6 L to 40 L spinner flasks or in bioreactors having 3 L to 500 L capacity.

Methods of Preparing Suspensions for Seeding or Inoculation

Seeding Cultures

Frozen or fresh *L. intracellularis* Master or expanded Working Seeds were thawed at room temperature (25° C. ±3° C.) or at 37° C. ±2° C. Bioreactors, spinner flasks or bottles previously seeded with McCoy cells at a cell density of 50,000 to 500,000 cells/ml were infected with *L. intracellularis* at a concentration of 1 to 10% (v/v) or MOI of 0.08 to 1.0. The infected culture was incubated under reduced oxygen concentrations by overlaying with a gas mixture composed of 86% N₂, 4% H₂ and 10% CO₂. The culture was incubated for 3 to 10 days at 37° C. ±2° C., pH 6.7 to 7.3, and with continuous agitation (10 to 100 rpm) to maintain adequate mixing for the cells to remain in suspension.

Production Cultures

Frozen or fresh *L. intracellularis* Master or expanded Working Seeds were thawed at room temperature (25° C. ±3° C.) or at 37° C. ±2° C. Bioreactors or spinner flasks 3 L to 500 L capacity) previously seed (0 to 7 days) with McCoy cells at a cell density of 50,000 to 500,000 cells/ml were infected with *L. intracellularis* at a concentration of 1 to 10% (v/v) or MOI of 0.08 to 1.0. The infected culture was incubated under reduced oxygen concentrations by overlaying or sparging with a gas mixture composed of 96% N₂, 4% H₂. The culture was incubated for 3 to 8 days at 37° C. ±2° C., pH 6.7 to 7.3, and with continuous agitation (10 to 100 rpm) to maintain adequate mixing for the cells to remain in suspension.

Inoculation Techniques for Seed and Production Cultures

Seed Cultures

Up to 10% (v/v) of Master or Working Seed are inoculated (MOI=0.08 to 1.0) into bioreactors, spinner flasks or bottles seed with McCoy cells at 0-7 days in growth medium.

Production Cultures

Up to 10% (v/v) of Production Seed was inoculated (MOI=0.08 to 1.0) into an appropriate volume of growth medium seeded with McCoy cells at 0 to 7 days with the appropriate McCoy cell density in 3 L to 500 L capacity vessels.

Incubation of Microorganisms

The cultures were incubated at 37° C. ±2° C. for 3 to 10 days in a reduced oxygen atmosphere with agitation to maintain the suspension. Additional medium and/or McCoy cells can be added to continue the growth process.

Cultures were observed macroscopically during the incubation period for evidence of abnormal growth or signs of contamination.

Harvest:

Handling and Preparation of Cultures

Cultures were examined for signs of adequate bacterial growth by indirect fluorescent antibody (IFA) staining. Cultures that were ready for harvest exemplified 60 to 100% cell infectivity. Percent infectivity was determined by observation of at least three fields, each field containing enough McCoy cells to fill at least 80% of the area. To be considered infected, approximately 50% of the cell is filled with bacteria.

Potency of the harvest culture is tested by titration of the sample on McCoy cells which are fixed and stained using specific monoclonal antibody (anti-*L. intracellularis* monoclonal antibody VPM 53 Lot 31599 or equivalent; anti-mouse IgG-fluorescein conjugate (FITC) (ICN No. 55499) after 6 days of incubation at 37° C. ±2° C.

Cultures were examined visually for any obvious signs of contamination. Harvest occurred 3 to 10 days post inoculation.

Harvesting Techniques and Specifications

Cell and fluid contents in the production culture bioreactor, spinner flasks and bottle were partially or fully collected in a sterile receiving vessel. Each production culture bioreactor, spinner flask and bottle was harvested individually or the contents of several vessels were pooled with the addition of SGS and stored at 1° C. to 7° C. or colder. Harvested production culture was sampled for potency by TCID₅₀ and identification by IFA staining. Production cultures exhibited at least 4.9 TCID₅₀/ml by IFA staining and were free of any evidence of contamination upon microscopic observation.

Preparation of Vaccine Product:

Concentration Methods

The vaccine product can be concentrated by various methods, e.g. by allowing the culture to settle with subsequent decanting of the supernatant, by membrane filtration (0.22 μm or smaller), perfusion, or by centrifugation.

Sucrose Gelatin Stabilizer (SGS)

Hydrolyzed gelatin solution is prepared by mixing gelatin with deionized water or water for injection at approximately 25% final total volume of SGS batch size and hydrolyzing in an autoclave for 120 minutes at 121° C.

The hydrolyzed gelatin solution (40.0 g/L) was then mixed with deionized water or water for injection at approximately 75% final total volume of SGS batch size. Potassium hydroxide (AR) (0.548 g/L), L-glutamic acid (1.440 g/L), dipotas-
sium phosphate (AR) (2.508 g/L), potassium dihydrogen
phosphate (AR) (1.030 g/L), and sucrose (AR) (150.00 g/L)
were added and the solution was thoroughly mixed. The pH of
the stabilizer was then adjusted to 6.8 to 7.0 with hydrochloric
acid or sodium hydroxide solutions. Deionized water or water
for injection was added to 100% of desired final volume of the
SGS. The complete stabilizer was thoroughly mixed, and the
entire solution was sterilized by filtration through a 0.1
micron filter.

Example of Assembly of Units to Make a Serial is shown in
Table 1:

TABLE 1

<i>L. intracellularis</i>	200,000-300,000 ml
sucrose gelatin stabilizer (SGS)	100,000 ml (25% v/v)
DMEM/F12 (May be added to standardize the product)	0-150,000 ml
TOTAL VOLUME	400,000 ml

Volume of an average serial was 50 L to 500 L.

Lyophilization

The vaccine product was lyophilized according to the pro-
cedure outlined in Table 2 for a 10 dose cycle (6.0 ml fill) or
Table 3 for a 50/100 dose cycle (10.0 ml fill).

TABLE 2

STEPS	° C.	Rate (minutes)	Hold (minutes)	Pressure (mT)
PRE-COOL*	5°	Na	Na	Atm
FREEZE	-47° ± 3°	As fast as possible	150	Atm
1° DRYING, 1st	-15° ± 2°	120	120	100-150
1° DRYING, 2nd	0° ± 2°	120	180	100-150
2° DRYING, 1 st	32° ± 2°	240	180	60-80
2° DRYING, 2nd	26° ± 2°	240	As fast as possible	60-80

Total Time: 1352 minutes (22.5 hrs)

*Shelves are pre-cooled to 5° ± 2° C. during the loading of the lyophilizer.

TABLE 3

STEPS	° C.	Rate (minutes)	Hold (minutes)	Pressure (mT)
PRE-COOL*	5°	N/A	N/A	Atm
FREEZE	-48° ± 3°	60	90	Atm
1° DRYING	-15° ± 2°	60	1500	100-150
2° DRYING	26° ± 2°	60	600	60-80

Total Time: 2370 minutes (39.5 hrs)

*Shelves are pre-cooled to 5° ± 2° C. during the loading of the lyophilizer.

Example 2

Safety of *L. Intracellularis* Vaccine

Purpose:

The objectives of this study were two-fold. The first objec-
tive of this study was to observe and compare the incidence of

disease caused by three different low passage isolates (two of
U.S. origin and one of European origin) of *L. intracellularis*
in pigs at 6½ weeks of age. The second objective was to
observe the safety of two high passage isolates (both of Euro-
pean origin) of *L. intracellularis* in pigs at 6½ weeks of age.

Materials and Methods:

Test Substances

1. *L. intracellularis* low passage U.S. isolate N343
2. *L. intracellularis* low passage U.S. isolate N101494.
3. *L. intracellularis* low passage EU isolate DK 15540 p20
4. *L. intracellularis* high passage EU isolate DK 15540 p60
5. *L. intracellularis* high passage EU isolate DK 15540 p80
(Master Seed designation B3903).

Formulation of Test Substances

Low passage isolates were grown continuously for 10-20
weeks after isolation in McCoy cell suspension. High passage
isolates were grown continuously for 60-80 weeks after isola-
tion in McCoy cell suspension. All cultures were harvested
via centrifugation at 10,000 RPM for 15 minutes. The McCoy
cell culture pellets containing *Lawsonia* were resuspended in
Sucrose-Phosphate-Glutamine (SPG) solution with 10%
FBS.

Storage of Test Substances

Harvested cultures were stored at -70° C. until the day of
challenge. Challenge cultures of the same isolate but from
various harvest dates were thawed and combined into plastic
vaccine bottles, labeled, and stored at 4° C. or on ice until the
time of challenge.

Assay of Test Substances

TCID₅₀ was performed on all pooled challenge isolates at
the time of challenge (day 0). The average titers (n=3)
(TCID₅₀/ml) were as follows in Table 4:

TABLE 4

Test Substance	Average Titer (TCID ₅₀ /ml)
<i>L. intracellularis</i> N343	6.4
<i>L. intracellularis</i> N101494	6.1
<i>L. intracellularis</i> DK 15540 p20	6.2
<i>L. intracellularis</i> DK 15540 p60	6.87
<i>L. intracellularis</i> DK 15540 p80	7.4

Study Design

The study consisted of five experimental groups and one
control group. On day 0 of the study, Group 1 (10 pigs, 6½
weeks of age) received one 10 ml or equivalent intragastric
(IG) dose of *L. intracellularis* low passage U.S. isolate N343.
Group 2 (10 pigs, 6½ weeks of age) received one 10 ml or
equivalent IG dose of *L. intracellularis* low passage U.S.
isolate N101494. Group 3 (10 pigs, 6½ weeks of age)
received one 10 ml or equivalent IG dose of *L. intracellularis*
low passage EU isolate DK15540 p20. Group 4 (10 pigs, 6½
weeks of age) received one 10 ml or equivalent IG dose of *L.*
intracellularis high passage EU isolate DK15540 60 week.
Group 5 (20 pigs, 6 weeks of age) received one 10 ml or
equivalent dose of *L. intracellularis* high passage EU isolate
DK 15540 p80. Group 6 (10 pigs, 6½ weeks of age) desig-
nated as "Strict Controls" did not receive a treatment.

Daily health observations were made from initiation of
study to the day of challenge of appropriate test animals.
Clinical health (behavior, appetite, body condition, hair coat,
and stool consistency on a scale of 1 to 4 were scored daily
from day of challenge (day 0) to termination of study (day
21). Average daily weight gains (ADWG) were calculated
from day of challenge (day 0) to termination of study (day
21). Fecal shedding of *L. intracellularis* was evaluated on
days 0, 7, 14, and 21. The one animal that died (from Group

1) throughout the study was examined for gross and microscopic lesions. Death was determined to be due to lesions associated with PPE confirmed by histology and PCR analysis; the animal was not replaced. Qualitative analysis of *Lawsonia* content in feces was evaluated by PCR along with histological evaluation for *L. intracellularis* on the ileum and colon. Serum was collected on days 0, 7, 14, and 21 of the study.

Results:
Summary of Study Results

show no detectable seroconversion. Seroconversion to *Lawsonia* exposure increased in treatment groups receiving low passage *L. intracellularis* of both the U.S. and EU isolates day 21 of the study.

Fecal Shedding

PCR testing of the feces demonstrated shedding of *L. intracellularis* beginning on day 14 where 1/10 in the N343, 4/10 in the N101494, and 5/10 in the DK15540 p20 low passage animals tested positive. Both high passage treatment and strict control groups were PCR negative for day 14. On day 21, DK15540

TABLE 5

Group	Treatment	No. of Pigs	Titer (TCID ₅₀ /ml)	Serology	Fecal Shedding	PCR	FA	Histology	Gross Lesions	ADWG	Clinical Scores
1	N343	9	7.4	19%	11%	0%	11%	44%	1	0.61	5.23
2	N101494	10	7.1	10%	10%	10%	30%	30%	1.2	0.8	5.15
3	DK15540p20	10	7.2	18%	13%	30%	10%	20%	1.2	0.86	5.01
4	DK15540p60	10	7.87	5%	0%	0%	0%	10%	1	0.9	5
5	DK15540p80	20	8.4	0%	0%	0%	0%	0%	1	1.05	5
6	Strict Controls	10	0	0%	0%	0%	0%	0%	1.05	0.91	5

General Observations

Daily health observations were made until challenge. The clinical condition of the animals was evaluated daily following challenge for the duration of the study. Observations included: behavior, appetite, body condition, hair coat, and stool consistency. The clinical conditions of these animals were evaluated based on a numerical point system, which reflects the severity of illness. Scores ranged from 1 to 4 for each parameter. A score of 1 was given to an animal with a normal, healthy appearance, a score of 3 for an animal demonstrating severe clinical signs, and a score of 4 for an animal that has deceased. The average daily score for the strict controls, DK15540 p60, and DK15540 p80 was 5.0. The average daily scores for low passage material was N343 (5.23), N101494 (5.15), and DK15540 p20 (5.01). Statistical analysis on these results indicated no differences among treatment and control groups using Kruskal-Wallis Rank Sum Test.

Average Daily Weight Gains (ADWG)

Average daily weight gains were calculated from the time of challenge (day 0) to the termination of the study (day 21). The average weight gain per day for the strict control group was 0.9 pounds. The average weight gain for the low passage treatment groups was only 0.6 (N343), 0.8 (N101494), and 0.86 (DK15540 p20) pounds/day. The high passage treatment groups revealed the same or increased average weight gain per day compared to the strict control group that didn't receive challenge with 0.9 pounds/day (DK15540 p60) and 1.05 pounds/day (DK15540 p80) respectively. The mean difference in average daily weight gains was significantly lower in the N343 treatment group compared to higher passage treatment groups (DK15540 p60 and p80) and strict control group at day 21 of the study. (Pearson Chi-square p<0.05).

Seroconversion

Seroconversion to *Lawsonia* exposure in pigs was measured by testing for the presence of anti-*Lawsonia* antibodies using an IFAT assay. On day 0, only the N343 treatment group observed detectable seroconversion in 2/10 pigs. Day 7 observed 2/10 pigs (N343) and 1/10 pigs (DK15540 p20) IFA positive for *Lawsonia* antibodies. On day 14, 3/10 pigs were IFA positive in N343 treatment group, 1/10 in DK15540 p20 and p60 respectively. Day 21 revealed 1/10 pigs (N343), 4/10 pigs (N101494) and 5/10 pigs (DK15540 p20) were IFA positive while high passage treatment groups (DK15540 p60 and p80)

p20 treatment group had 1 animal PCR positive while all other treatment and control groups were PCR negative. No evidence of shedding was observed in high passage isolate groups (DK15540 p60 and p80) using PCR throughout the study. PCR positive animals indicating active shedding of *Lawsonia* in their feces were more significant in the low passage isolate groups (N343, N101494, and DK15540 p20) on day 14 of the study than high passage isolate groups and strict controls (Pearson Chi-square p<0.05).

PCR at Day 21: Ileums and Colons

PCR testing of mucosal scrapings from ileums and colons was performed after necropsy (day 21). Samples that were PCR positive for *L. intracellularis* colonization were (2/10 colons) in N101494 and (2/10 ileums, and 4/10 colons) in DK15540 p20 low passage isolate groups. All other treatment and control groups were PCR negative in tissues day 21 of the study. Results indicated ileums and colons of pigs in DK15540 p20 were significantly more colonized with *L. intracellularis* compared to all treatment and control groups (*Pearson Chi-square p<0.05).

Histology

Sections of the terminal ileum and colon were collected at necropsy (day 21) and placed in buffered formalin for histological analysis. Presence of intracellular bacteria and crypt hyperplasia was observed in tissues stained with Hematoxylin and Eosin (H&E) and arthin-Starry silver reagents of 1/10 pigs (N343), 3/10 pigs (N101494), 2/10 pigs (DK15540 p60) and 1/20 pigs (DK15540 p80). Lesion development was confirmed by florescent antibody staining using monoclonal antibodies against *Lawsonia intracellularis* in 1/10 pigs (N343), 3/10 pigs (N101494), and 1/10 pigs (DK15540 p60). FA detected no lesions caused by *Lawsonia* in the colons of all treatment and control groups. FA results indicated significant lesion development in ileums of pigs in N101494 treatment group compared to all treatment and control groups. H&E/silver staining showed significant lesion development associated with PPE in N343 treatment group compared to all treatment and control groups. (Pearson Chi-square p<0.05).

Gross Scores

Ileums and colons were scored at the time of necropsy (day 21) for lesions associated with PPE. Tissues were given a score of 1 for normal appearance (no lesion development), a score of 2 for lesions demonstrating mild thickening, 3 for

moderate thickening, and a score of 4 for severe thickening. Strict controls had an average clinical score of 1.05, N343 (1.0), N101494 (1.2), DK15540 p20 (1.2), and DK15540 p60 and p80 (1.0). Average gross lesion scores indicated no statistical difference between control and treatment groups using ANOVA test for multiple comparisons.

Conclusions:

Based on the data collected, this study demonstrated that pigs challenged with a low passage dose of N343, N101494, and DK15540 with a TCID₅₀ greater than 1×10⁷ bacteria/dose increases the incidence of PPE in these animals. High passage isolates (DK15540 p60 and p80) given to pigs of the same age with a TCID₅₀ greater than 1×10⁷ were proven safe and show reduction of colonization and lesion development associated with PPE. This conclusion was based on PCR on the mucosa of the ileum, histopathology, and FA stains of tissue sections.

A reduction of shedding *L. intracellularis* in the feces determined by PCR was evident in high passage isolates compared to low passage isolates. Average daily weight gains calculated for all treatment and control groups support this conclusion by demonstrating positive uniform daily weight gain in groups given the high passage isolate and strict controls compared to groups given low passage isolates especially animals in the N343 treatment group. This observation indicates reduction of weight gains in animals given low passage material which supports adequate and similar grow performance of animals given high passage material with animals receiving no challenge material. Compared to the strict controls, the high passage isolates showed no negative impact on weight gain and overall health based on clinical scores.

Example 3

Efficacy and Minimal Protective Titer

Purpose:

The objectives of this study were to determine the minimal protective titer of a vaccine comprising isolate B3903 (lyophilized) (DK 15540, passage 80) ("B3903 (Lyophilized) vaccine" and "B3903 vaccine" are used interchangeably herein) administered by oral drench in pigs 3 weeks of age and to demonstrate efficacy against a virulent heterologous pure culture challenge with low passage *L. intracellularis*, the causative agent of Porcine Proliferative Enteropathy (PPE) in swine.

Materials and Methods:

Test Substance: Attenuated Live Culture of *L. intracellularis*, Isolate B3903

Formulation of B3903 (Lyophilized) Vaccine

DK 15540 isolates were grown continuously for 80 weeks after isolation in McCoy cell suspension. All cultures were harvested via centrifugation at 10,000 RPM for 15 minutes. The McCoy cell culture pellets containing *Lawsonia* were resuspended in Sucrose-Phosphate-Glutamine (SPG) solution with 10% FBS. Desiccation was performed as described in Example 1, supra. The lyophilized product is reconstituted in water, q.s. ad 2.0 ml, for injections.

Storage of B3903 (Lyophilized) Vaccine

The vaccine was stored at 2° C.-8° C. until ready for use. After resuspension, the vaccine was stored on ice until administration.

Doses of B3903 (Lyophilized) Vaccine

- 1. High dose (Treatment Group 1): 1×2 mL (6.0 logs/dose) via direct oral drench on day 0 of the study.

- 2. Medium dose (Treatment Group 2): 1×2 mL (4.9 logs/dose) via direct oral drench on day 0 of the study.
- 3. Low dose (Treatment Group 3): 1×2 mL (3.8 logs/dose) via direct oral drench on day 0 of the study.

Test Substance: Placebo

A placebo consisting of uninfected McCoy tissue culture cells suspended in DMEM/F12 growth medium fortified with 5% NBS was given to treatment Groups 4 (challenge control) and 5 (negative control) on day 0 of the study. This substance was administered to piglets in treatment Group 4 by direct oral drench and given 1×2 mL of placebo per test animal.

Test Substance: *L. Intracellularis* N101494 Virulent Challenge

L. intracellularis N101494 was obtained from the intestines of a 12 week old pig from an Indiana farm (U.S. Pat. No. 5,714,375).

Formulation of *L. Intracellularis* N101494 Virulent Challenge

L. intracellularis challenge material was grown continuously in McCoy cell suspension no more than 30 passes after initial isolation from infected gut tissue. Active cultures (2×3 L) identified as SF 1422 and SF 1423 in addition to (IL) SF 1421 were grown in McCoy cell suspension for 7 days to 15-30% McCoy cell infection. On the day of challenge (day 21), active cultures were harvested via centrifugation at 10,000 RPM for 15 minutes and cell pellets resuspended in 350 mls total volume with SPG stabilizer. Harvested active culture was pooled with 300 mLs of frozen 10× to 20× concentrated challenge stocks of low passage N101494 at various passages (pass 24 to 27 post isolation).

Storage of *L. Intracellularis* N101494 Virulent Challenge
Final formulations ready for challenge were stored at 2° C. to 8° C. or on ice until inoculation.

Pre/Post-Vaccination and Challenge Titers

Results from the TCID₅₀ assay verified the amount of live *L. intracellularis* administered to each test animal per dose during vaccination and challenge. The average titers (n=5) of pre and post titrations for the B3903 vaccine and the challenge material (*L. intracellularis* N101494) were as follows in Table 6:

TABLE 6

Group	Treatment (logs/dose)	Average logs/dose (2 ml) Pre-vaccination (TCID ₅₀ /ml)	Average logs/dose (2 ml) Post-vaccination (TCID ₅₀ /ml)	Total Average logs/dose
1	B3903 vaccine high dose	6.07	5.89	6.0
2	B3903 vaccine medium dose	4.94	4.84	4.9
3	B3903 vaccine low dose	3.9	3.5	3.8
4	Challenge	7.85	7.57	7.71
	Controls			

Study Design

Sixty-five healthy *L. intracellularis* negative weaned piglets at 3 weeks of age were randomly divided into 5 treatment groups and housed separately throughout the study. On day 0, treatment Group 1 (15 pigs) received a 2 mL dose (6.0 logs/dose) of B3903 vaccine by direct oral drench. Treatment Group 2 (15 pigs) received 1×2 mL dose of B3903 vaccine titrated at 4.9 logs/dose by direct oral drench. Treatment Group 3 (15 pigs) received 1×2 mL dose of B3903 vaccine titrated at 3.8 logs/dose by direct oral drench.

Treatment Groups 4 and 5 (10 pigs/group) received 1×2 mL dose of placebo by direct oral drench.

On day 21 of the study (3 weeks post vaccination), test pigs in treatment Groups 1-4 received 1x10 mL dose of virulent low passage pure culture *L. intracellularis* heterologous isolate N101494 by gastric gavage.

On day 42 of the study (3 weeks post challenge), all treatment Groups (1-5) were euthanized and necropsied for gross and microscopic lesion analysis for PPE.

Daily health observations were made from study initiation to the day of challenge for each test animal. Clinical health (diarrhea, behavior, and body condition) were scored daily from day of challenge (day 21) to termination of study (day 42). Weights were taken on day of vaccination (day 0), day of challenge (day 21) and on day of study termination (day 42) to calculate average daily weight gains of each treatment group. Fecal shedding of *L. intracellularis* was evaluated by polymerase chain reaction (PCR) by testing fecal swabs (f-PCR) on days 0, 7, 14, 21, 28, 35, and 42 of the study. All animals euthanized at study termination (day 42) were examined for gross and microscopic lesions. Qualitative analysis of bacterial content in tissues was evaluated by PCR (t-PCR) along with histological evaluation for *L. intracellularis* in the ileum, colon, tonsil, and mesenteric lymph node day 42 of the study. Serum was collected on days 0, 7, 14, 21, 28, 35, and 42 of the study. Serum was tested using the indirect florescent antibody test (IFAT) to detect anti-*Lawsonia* antibodies in test animals. Treatment group comparisons were made by data analysis of average daily weight gains post vaccination and post challenge, clinical scores, seroconversion rates (IFAT), colonization (t-PCR), fecal shedding (f-PCR), gross lesion, and microscopic lesion development by immunohistochemistry (IHC).

Results:
Summary of Primary Results

TABLE 7

Treatment Group	Pigs per Group	Treatment. Group ID (logs/dose)	Average Gross Lesion Scores (ileum)	Average Gross Lesion Scores (colon)	Average Micro-lesion scores (ileum)	Average Micro-lesion Scores (colon)
1	15	B3903 vaccine high dose (6.0)	1.5 ab	1.2 a	0.2 ab	0.1 a
2	15	B3903 vaccine medium dose (4.9)	1.2 ab	1.0 a	0.4 ab	0.0 a
3	15	B3903 vaccine low dose (3.8)	2.5 b	1.5 b	1.0 b	0.3 a
4	10	Challenge Controls	3.6 c	2.2 b	2.4 c	1.5 b
5	10	Strict Controls	1.0 a	1.0 a	0.0 a	0.0 a

*Like letters indicate no significant difference (p < 0.05)

Final test results for each treatment group revealed significant gross and microscopic lesion development of the ileum and colon in non-vaccinated, challenge control pigs (Group 4) compared to vaccinated pigs regardless of dose (high, medium, and low) in Groups 1, 2, and 3 (p<0.05). Average gross lesion scores of the vaccine-low dose colons were not significantly different than challenge controls. The vaccine-low dose group (Group 3) is the only treatment group that received vaccine and had significant lesion development (gross and microscopic) in comparison to the strict control group which did not receive a vaccination, placebo, or challenge throughout the study.

Clinical Scores

Clinical scores were recorded for each animal daily from day of challenge (day 21) to necropsy (day 42). Clinical scores were calculated to obtain an average daily clinical

score reflecting the severity and duration of sickness among treatment groups due to receiving a virulent challenge of *L. intracellularis*. Average clinical scores for each treatment group are summarized in Table 8.

TABLE 8

Treatment Group	Group Identification	Average Clinical Score
1	B3903 vaccine-high dose	3.1 a
2	B3903 vaccine-medium dose	3.0 a
3	B3903 vaccine-low dose	3.0 a
4	Challenge Control	3.0 a
5	Strict Control	3.0 a

*Like letters denote no significant differences among average clinical scores from the day of challenge to necropsy (p < 0.05).

Statistical analysis of clinical scores was accomplished by reviewing the data in relation to treatment groups receiving average clinical scores using 1-way ANOVA. There was no evidence of significant differences among non-vaccinated, challenge controls and strict controls compared to the high, medium, or low dose vaccine groups.

Average Daily Weight Gains

Average daily weight gains (ADWG) were calculated from the time of vaccine administration (day 0), to challenge administration (day 21), to the termination of the study (day 42). Statistical analysis of weight gain differences (lbs.=pounds) between vaccine-high dose and non-vaccine, challenge control treatment groups revealed evidence of a significant difference from the time of challenge administration to necropsy (p<0.05). The weight data is summarized in Table 9.

TABLE 9

Treat-ment Group	Group Identifi-cation	Average Initial Weight (lbs.)	ADWG (day 0-21) (lbs.)	ADWG (day 21-42) (lbs.)	ADWG Total (lbs.) (Vaccination to Necropsy)
1	B3903 vaccine high dose	17.8 a	0.94 a	1.63 a	1.29 a
2	B3903 vaccine medium dose	17.7 a	1.00 a	1.60 ab	1.30 a
3	B3903 vaccine low dose	18.0 a	0.94 a	1.48 ab	1.21 a
4	Challenge Control	17.7 a	0.96 a	1.45 b	1.21 a
5	Strict Control	17.7 a	0.94 a	1.63 ab	1.28 a

*Like letters denote no significant differences between treatment groups in ADWGs from day of vaccination to challenge and from day of challenge to necropsy (p < 0.05).

Seroconversion (IFAT)

Serum samples were collected weekly from all test animals in each treatment group and tested for presence of anti-*Lawsonia* IgG antibodies on days 0, 7, 14, 21, 28, 35, and 42 of the study. Positive and negative IFAT control samples were 100% accurate in all assays performed in this study. On days 0 through 21 (3 weeks post vaccination), all test animals in each treatment group were IFA negative. On day 28 (1 week post challenge) of the study, 1/5 (6.7%) test animals in the vaccine-high dose treatment group were IFA positive while all others tested IFA negative. On day 35 (2 weeks post challenge) of the study, 4/5 (26.7%) pigs in the vaccine-high dose and medium dose treatment groups and 1/10 (10%) pigs in the non-vaccine, challenge control treatment group were IFA positive for *Lawsonia* antibodies. Both vaccine-low dose and strict control

treatment groups were IFA negative on day 35. On day 42 (3 weeks post challenge) of the study, 8/10 (80%) pigs in the challenge control group, 4/5 (40%) pigs in the vaccine-medium dose group, 3/5 (33.3%) pigs in the vaccine-low dose group, and 2/5 (20%) pigs in the vaccine-high dose group were IFA positive. The strict control treatment group was IFA negative at study termination (day 42).

Seroconversion data was analyzed using the Chi-square statistic. For results obtained in the strict control treatment group, a chi-square statistic was not computed due to the 100% negative responses found for each test animal throughout the study. In treatment groups receiving a vaccine or placebo, IFAT results were compared using Chi-square statistic with an estimation of exact p-value (Monte-Carlo). Positive IFAT results obtained from challenge groups receiving a virulent pure culture challenge were significantly higher than those receiving vaccine-high dose and vaccine-low dose (p<0.05) at day 42 (3 weeks post challenge) of the study.

Fecal Shedding of *L. Intracellularis* (PCR)

Fecal swabs were collected weekly from all test animals in each treatment group and tested for the presence of *L. intracellularis* by PCR on days 0, 7, 14, 21, 28, 35, and 42 of the study. Positive and negative DNA extraction and PCR reaction controls were 100% accurate for each assay conducted in this study. All test animals in each treatment group were fecal PCR negative for *L. intracellularis* from day 0 (vaccination) to day 21 (challenge). Pigs in the strict control group remained fecal PCR negative for *L. intracellularis* in their feces throughout the study. Fecal shedding of *L. intracellularis* (fecal PCR positive) was evident in various treatment groups each week after challenge inoculation. These results are summarized in Table 10.

TABLE 10

Treatment Group	Group Identification	Day 28 (1 week post challenge)	Day 35 (2 weeks post challenge)	Day 42 (3 weeks post challenge)
1	B3903 vaccine high dose	0/15 (0%) a	2/15 (13.3%) a	0/15 (0%) a
2	B3903 vaccine Medium dose	6/15 (40%) ab	5/15 (33.3%) a	0/15 (0%) a
3	B3903 vaccine low dose	4/15 (26.7%) ab	8/15 (53.3%) ab	2/15 (13.3%) ab
4	Challenge Control	4/10 (40%) b	7/10 (70%) b	4/10 (40%) b
5	Strict Control	0/10 (0%)	0/10 (0%)	0/10 (0%)

* Like letters denote no significant difference among treatment groups receiving a challenge (p < 0.05).

Statistical analysis using Chi-square statistic with an estimation of the exact p-value (Monte Carlo) compared the positive responses in each vaccine treatment group to the non-vaccine, challenge control group only. Results from the strict control group were withdrawn from group comparisons because each pig was 100% fecal PCR negative for *L. intracellularis* throughout the study. On day 28 (1 week post challenge), pigs receiving a high dose of vaccine had significantly less shedding of *L. intracellularis* than non-vaccinated, challenged pigs (p=0.017). On day 35 (2 weeks post challenge), pigs receiving a high dose (p=0.0024) and medium dose (p=0.041) of vaccine had significantly less shedding of *L. intracellularis* in their feces than non-vaccinated challenged pigs. On day 42 (study termination), again,

pigs receiving a high and medium dose of vaccine (p=0.017) had significantly less shedding of *L. intracellularis* in their feces compared to the non-vaccinated, challenged pigs. Pigs in the low dose group were not significantly less PCR positive than the challenge controls on any day post challenge.

L. Intracellularis Tissue Colonization (PCR)

Polymerase Chain Reaction testing of tissue sections of the terminal ileum, colon, tonsil, and mesenteric lymph node were performed after necropsy (day 42 of the study). In the vaccine-high dose group, 1/15 (6.7%) pigs were PCR positive for *L. intracellularis* colonization in the tonsil while all other treatment groups were PCR negative. Ileitis PCR testing of mesenteric lymph tissue revealed 3/10 (30%) pigs in the non-vaccinated, challenge controls were positive for *L. intracellularis* colonization while all other treatment groups were PCR negative. Ileitis PCR testing of mucosal scrapings of the terminal ileum revealed 4/10 (40%) pigs in the challenge controls, 4/15 (26.7%) pigs in the vaccine-low dose group, 2/15 (13.3%) pigs in the vaccine-medium dose group, and 1/15 (6.7%) pigs in the vaccine-high dose group PCR positive for *L. intracellularis* colonization. Ileitis PCR testing of the colon revealed 3/10 (30%) pigs in the challenge controls, 5/15 (33.3%) pigs in the vaccine-low dose group, 1/15 (6.7%) pigs in the vaccine-medium dose group, and 2/15 (13.3%) pigs in the vaccine-high dose group PCR positive for *L. intracellularis* colonization. No evidence of *L. intracellularis* colonization was seen in tissues of the strict control group.

Statistical analysis of Ileitis PCR positive results were compared among treatment groups using Chi-square statistic with Monte Carlo approximation of the exact p-value. Mesenteric lymph node PCR results indicated evidence of a significant difference among non-vaccinated, challenge controls and all treatment groups receiving a vaccine treatment (p=0.054). No statistical significance was evident among treatment groups in *L. intracellularis* colonization in the ileum, colon, or tonsil at day 42 of the study.

Histology (IHC/H&E)

Sections 2 to 4 cm in length of tonsil, mesenteric lymph node, terminal ileum, and colon were collected at necropsy (day 42) and placed in 10% buffered formalin for histological analysis. *Lawsonia intracellularis* was not detected by IHC staining of tonsil sections in all treatment groups at necropsy. The strict control group was negative for *L. intracellularis* by IHC in all tissue samples taken at necropsy. Presence of *L. intracellularis* and microscopic lesions associated with PPE were found in the ileums of 9/10 (90%) pigs of the non-vaccinated, challenge controls, 6/15 (40%) pigs of the vaccine-low dose group, 3/15 (20%) pigs of the vaccine-medium dose group, and 1/15 (6.7%) pigs of the vaccine-high dose group respectively. Microscopic lesions were evident in the colon of 8/10 (80%) pigs in the challenge control group, 3/15 (20%) pigs in the vaccine-low dose group, and 1/15 (6.7%) pigs in the vaccine-high dose group. The vaccine group receiving a medium dose was IHC negative for *L. intracellularis* in the colon. Stained sections of mesenteric lymph node produced only 1/10 (10%) pigs in the challenge control that had *L. intracellularis*. Positive IHC results in sections of the ileum and colon among treatment groups correlated well to the presence of marked crypt hyperplasia demonstrated by H&E methodology.

Statistical analysis of quantitative IHC data was accomplished using 1-way ANOVA and Kruskal-Wallis Rank Sum tests followed by specific contrasts of p-values between the challenge group and strict control groups to each vaccine dosage group. Statistical analysis reveal evidence of a significant lesion development due to *L. intracellularis* in the ileum and colon of the challenge control group compared to vacci-

nated pigs regardless of dose on day 42 of the study ($p<0.001$). Statistical significance was not evident in average IHC lesion scores of the ileum and colon in vaccinated animals receiving a high or medium dose of vaccine compared to the strict control treatment group ($p>0.05$). Pigs receiving a low dose of vaccine had significantly higher mean microscopic lesion scores due to *L. intracellularis* in the ileum compared to the strict control group ($p<0.05$).

Gross Scores

The ileum and colon of each test animal were scored at the time of necropsy (day 42) for gross lesions associated with PPE. Tissues from test pigs in the strict control were normal, did not contain lesions, and received average lesion scores of 1.1 (ileum) and 1.0 (colon). Tissues of test animals in the non-vaccinated, challenge control group received the highest average gross lesion score for the ileum (3.6) and colon (2.0) among treatment groups. Test pigs in the vaccine-low dose group received average gross lesion scores of 2.5 (ileum) and 1.5 (colon). Vaccine-medium dose test pigs received average gross lesion scores of 1.5 (ileum) and 1.0 (colon). Tissues of the vaccine-high dose group received average gross lesion scores of 1.5 (ileum) and 1.2 (colon).

Statistical analysis of average gross lesion scores among treatment groups was accomplished by using 1-way ANOVA. Average gross lesion scores indicated evidence of a significant difference between ileums in non-vaccinated, challenge control group and vaccine-medium and high dose groups respectively ($p<0.001$). Evidence of a significant difference was observed among average gross ileum scores of the challenge control compared to the vaccine-low dose group ($p<0.001$). Average gross lesion scores of the colon were significantly higher in the challenge control group compared to the vaccine-medium and high dose groups respectively ($p<0.05$). In addition, significant gross lesion development was observed in the ileum of the vaccine-low dose group compared to the strict control group ($p<0.001$). No evidence of a statistical significance in average gross lesion scores (ileum and colon) were evident in vaccine-medium and high dose groups compared to the strict control treatment group.

Conclusions

This study demonstrated protection in 3-week-old pigs against PPE was accomplished when given an oral 2 mL dose of B3903 (Lyophilized) vaccine containing a minimum of 4.9 logs of live *L. intracellularis* per dose. Similar if not slightly better protection was evident in the vaccine-high dose treat-

ment group that received 6.0 logs/dose 3 weeks prior to challenge. The low dose vaccine group (3.8 logs/dose) did not indicate adequate protection against a virulent pure culture heterologous challenge compared to the test animals receiving a high or medium dose of the experimental vaccine. Statistical differences were evident among the vaccine-low dose group and non-vaccinated, challenge controls regarding the severity of microscopic lesions in the ileum and colon ($p<0.001$) and average gross lesion scores of the ileum ($p<0.01$). However, significant PPE lesions were observed in the ileum (microscopic lesions) and colon (gross lesions) of this group compared to the strict control group that did not receive vaccine or challenge.

In summary, the data from this study demonstrated that: (1) the minimum protective titer of a single oral administration of B3903 (Lyophilized) vaccine to 3 week old pigs is 4.9 logs/dose; (2) B3903 (Lyophilized) vaccine is efficacious against a virulent low passage pure culture *L. intracellularis*, heterologous isolate N101494; and (3) B3903 (Lyophilized) vaccine aids in the reduction of gross and microscopic lesions, tissue colonization, and fecal shedding of *L. intracellularis* in vaccinated pigs compared to non-vaccinated pigs.

What is claimed is:

1. A method for eliciting an immune response to *Lawsonia intracellularis* in a non-human animal, comprising administering to said non-human animal an immunogenic composition containing an avirulent isolate of *Lawsonia intracellularis* of European origin, wherein said avirulent isolate of European origin is *Lawsonia intracellularis* deposit isolate ATCC No. PTA-4926, wherein said avirulent *Lawsonia intracellularis* of European origin does not cause fecal shedding at day 14 post vaccination.

2. The method according to claim 1 wherein the immunogenic composition comprises about 10^3 TCID₅₀ to about 10^6 TCID₅₀ of said avirulent *Lawsonia intracellularis* per dose.

3. A method for producing antisera against *Lawsonia intracellularis* in a non-human animal comprising:

(a) administering an avirulent *Lawsonia intracellularis* of European origin, wherein said avirulent isolate is *Lawsonia intracellularis* deposit isolate ATCC No. PTA-4926 to a non-human animal in an amount effective to elicit an immune response; and

(b) collecting antiserum or plasma containing antibodies to said *Lawsonia intracellularis*.

* * * * *



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(12) **United States Patent**
Roof et al.

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(54) **LAWSONIA INTRACELLULARIS OF EUROPEAN ORIGIN AND VACCINES, DIAGNOSTIC AGENTS AND METHODS OF USE THEREOF**

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See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to *Lawsonia intracellularis* vaccines and methods for protecting against and diagnosing *L. intracellularis* infection. The products and processes of the invention are attainable, in part, as the result of an improved method for cultivating large scale supplies of *L. intracellularis*, including both a novel isolate of *L. intracellularis* of European origin and a method of preparing a lyophilized product containing the attenuated European isolate as vaccine product.

7 Claims, No Drawings

**LAWSONIA INTRACELLULARIS OF
EUROPEAN ORIGIN AND VACCINES,
DIAGNOSTIC AGENTS AND METHODS OF
USE THEREOF**

RELATED APPLICATION

This application claims benefit to U.S. Provisional Application Ser. No. 60/490,001, filed Jul. 25, 2003, the disclosure of which is incorporated by reference in its entirety.

BACKGROUND

The present invention relates to *Lawsonia intracellularis* vaccines and methods for protecting against and diagnosing *L. intracellularis* infection. The products and processes of the invention are attainable, in part, as the result of an improved method for cultivating large scale supplies of *L. intracellularis*, including both a novel isolate of *L. intracellularis* of European origin and a method of preparing a lyophilized product containing the attenuated European isolate as vaccine product.

L. intracellularis, the causative agent of porcine proliferative enteropathy ("PPE"), affects virtually all animals, including: rabbits, ferrets, hamsters, fox, horses, and other animals as diverse as ostriches and emus. *L. intracellularis* is a particularly great cause of losses in swine herds in Europe as well as in the United States.

A consistent feature of PPE is the occurrence of intracytoplasmic, non-membrane bound curved bacilli within enterocytes in affected portions of intestine. The bacteria associated with PPE have been referred to as "Campylobacter-like organisms." S. McOrist et al., Vet. Pathol., Vol. 26, 260-264 (1989). Subsequently, the causative bacteria have been identified as a novel taxonomic genus and species, vernacularly referred to as *Ileal symbiont* (IS) *intracellularis*. C. Gebhart et al., Int'l. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-538 (1993). More recently, these novel bacteria have been given the taxonomic name *Lawsonia* (*L.*) *intracellularis*. S. McOrist et al., Int'l. J. of Systemic Bacteriology, Vol. 45, No. 4, 820-825 (1995). These three names have been used interchangeably to refer to the same organism as further identified and described herein.

L. intracellularis is an obligate, intracellular bacterium which cannot be cultured by normal bacteriological methods on conventional cell-free media and has been thought to require attached epithelial cells for growth. S. McOrist et al., Infection and Immunity, Vol. 61, No. 19, 4286-4292 (1993) and G. Lawson et al., J. of Clinical Microbiology, Vol. 31, No. 5, 1136-1142 (1993) discuss cultivation of *L. intracellularis* using IEC-18 rat intestinal epithelial cell monolayers in conventional tissue culture flasks. In addition, H. Stills, Infection and Immunity, Vol. 59, No. 9, 3227-3236 (1991) discusses using Intestine 407 human embryonic intestinal cell monolayers and GPC-16 guinea pig colonic adenocarcinoma cell monolayers in conventional tissue culture flasks.

Recently, an *L. intracellularis* vaccine has been approved for use in the United States, which vaccine is based on *L. intracellularis* isolates described and claimed in U.S. Pat. Nos. 5,714,375 and 5,885,823, both of which patents are herein incorporated by reference in their entirety. The above-described vaccine is sold by Boehringer Ingelheim Vetmedica, Inc., 2621 North Belt Highway, St. Joseph, Mo. 64506-2002, under the trademark ENTERISOL® Ileitis.

SUMMARY OF THE INVENTION

One object of the invention is to provide an improved *L. intracellularis* vaccine using an isolate of European origin.

Another object of the invention is to provide an improved method for cultivation of *L. intracellularis* on a large scale and improved techniques for production of *L. intracellularis* vaccines.

To achieve these and other objects, and in accordance with the purpose of the invention as embodied and broadly described herein, the present invention provides a newly isolated *L. intracellularis* from Europe, a method of attenuating such an isolate, and the attenuated isolate thereof. Also provided herein is a vaccine comprising the attenuated isolate. Also provided herein is a method for producing a vaccine comprising the attenuated isolate in lyophilized form for reconstitution at the time of administration and the lyophilized product thereof.

In one embodiment, the newly isolated *L. intracellularis* from Europe, isolate DK 15540, is deposit isolate ATCC accession No. PTA-4927. In another embodiment, the attenuated isolate derived from isolate DK 15540, is designated isolate B3903, ATCC accession No. PTA-4926.

DETAILED DESCRIPTION

As used herein, the term "*L. intracellularis*" means the intracellular, curved gram-negative bacteria described in detail by C. Gebhart et al., Int'l. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-538 (1993) and S. McOrist et al., Int'l. J. of Systemic Bacteriology, Vol. 45, No. 4, 820-825 (1995), each of which is incorporated herein by reference in their entirety, and includes but is not limited to the isolate designated DK 15540 which was deposited for patent purposes with the American Type Culture Collection ("ATCC"), 10801 University Boulevard, Manassas, Va. 20110-2209 on Jan. 9, 2003 and assigned ATCC accession number PTA-4927; the causative bacteria which can be obtained from PPE infected swine or other animals throughout the world given the knowledge in the art and the teachings herein; and variants or mutants of any of the above bacteria, whether spontaneously or artificially obtained.

As used herein, the term "attenuated isolate" means any *L. intracellularis* isolate that is prepared according to the cultivation and passaging techniques taught herein to achieve avirulence while maintaining immunogenic properties when administered to a host animal including but not limited to the attenuated isolate designated B-3903 which was deposited for patent purposes with the American Type Culture Collection ("ATCC"), 10801 University Boulevard, Manassas, Va. 20110-2209 on Jan. 9, 2003 and assigned accession number PTA-4926.

PTA-4927 and PTA-4926 were tested by the ATCC on Apr. 21, 2004, and were found viable. The date of conversion to Budapest Treaty was Oct. 16, 2007.

The attenuated isolate of the invention can be used as an immunogen in antimicrobial vaccines for animals, including birds, fish, and mammals such as cattle, swine, horses, and primates. Such vaccines can be prepared by techniques known to those skilled in the art and given the teachings contained herein. Such a vaccine would comprise an immunologically effective amount of the attenuated isolate in a pharmaceutically acceptable carrier. The vaccine could be administered in one or more doses. An immunologically effective amount is determined by means known in the art without undue experimentation, given the teachings contained herein. The amount of avirulent bacteria should be

sufficient to stimulate an immune response in disease-susceptible animals while still being avirulent. This will depend upon the particular animal, bacteria, and disease involved. The recommended dose to be administered to the susceptible animal is preferably about 3.0 TCID₅₀ (tissue culture infective dose 50% end point)/dose to about 6.0 TCID₅₀/dose and more preferably about 4.0 TCID₅₀/dose to about 5.0 TCID₅₀/dose. In a preferred embodiment, the titer of the vaccine is about 4.9 TCID₅₀/dose as determined by Tissue Culture Infective Dose 50% endpoint dilution assay (TCID₅₀). The carriers are known to those skilled in the art and include stabilizers and diluents. Such a vaccine may also contain an appropriate adjuvant. The vaccines of the invention may be used in combination with other vaccines, for example, as a diluent of another vaccine. The vaccine preparations may also be desiccated, for example, by freeze drying for storage purposes or for subsequent formulation into liquid vaccines.

Accordingly, the invention also comprises a method for inducing an immune response to virulent, wild-type *L. intracellularis* bacteria in an animal host for the purpose of protecting the host from such bacteria. The method comprises administering an immunologically effective amount of the attenuated bacteria or killed bacteria of the invention to the host and, preferably, administering the vaccine of the invention to the host.

As used herein, the term "large-scale cultivation" means a level of cultivation of *L. intracellularis* greater than approximately 2.0 to 3.0 liters and includes production on a scale of 100 liters or more. "Cultivation" as used herein, means the process of promoting the growth, reproduction and/or proliferation of *L. intracellularis*.

L. intracellularis can be cultivated by methods known in the art, preferably, according to U.S. Pat. Nos. 5,714,375 and 5,885,823. For example, culture cells may first be inoculated with an inoculum comprising *L. intracellularis* bacteria so as to infect the cells with the bacteria. Numerous cell lines can be used in practicing the invention, including, but not limited to, IEC-18 (ATCC 1589)-rat intestinal epithelial cells, HEp-2 (ATCC 23)-human epidermoid carcinoma cells, McCoys (ATCC 1696)-mouse (non-specified) cells, BG MK (Biowhittaker #71-176)-buffalo green monkey kidney cells, and swine intestinal epithelium cells. The preferred culture cells are HEp-2, McCoys or IEC-18 cells.

If culture cells are used, prior to being inoculated, the cells may be in the form of a monolayer. To form a monolayer, the cells may be seeded into conventional flasks. Each flask is generally seeded with between about 1×10⁵ cells to about 10×10⁶ cells per 25, 75, 150, 850 cm² flask or roller bottle mixed with growth media. The growth media may be any media for cell cultivation which includes a nitrogen source, necessary growth factors for the chosen culture cells, and a carbon source, such as glucose or lactose. The preferred media is DMEM fortified with Ham's F 12 with 1-5% fetal bovine serum, although other commercially available media may be used with good results.

Successful cultivation of *L. intracellularis* is enhanced by maintaining the culture cells in a constant state of growth. Therefore, the culture cell monolayer should be at about 20 percent to about 50 percent confluency at the time of inoculation. Preferably, the cells should be at about 30 percent to about 40 percent confluency at the time of inoculation, most preferably at about 30 percent confluency.

Alternatively, the cells, prior to being inoculated, may be grown in suspension, as described infra. Preferably, the cells are first grown to 100% confluency in the form of a monolayer in an adherent type system, e.g., a roller bottle

system, and then transferred to 3-3000 liters and grown in suspension. Alternatively, the cells can be grown in suspension to the desired cell density, e.g. 2×10⁵ cells/ml, within the 3-3000 liter vessel (bioreactor, Fermentor, spinner flask, etc.) using parameters suitable for growth within this system prior to inoculation.

The inoculum may be a pure culture of *L. intracellularis* obtained from infected swine or other animals. Preferably the inoculum may be a pure culture of *L. intracellularis* obtained from ATCC accession No. PTA-4927.

The inoculum can be an intestinal homogenate prepared by scraping the mucosa off of the ileum of a swine or other animal infected with PPE. When preparing an intestinal homogenate, ileal sections selected for culture should show severe lesions with gross thickening of the gut. Due to the fragile nature of the bacteria, samples should preferably be stored at -70° C. as quickly as possible after necropsy. An antibiotic to which *L. intracellularis* is resistant such as Vancomycin, Amphotericin B or members of the aminoglycoside group of antibiotics, including Gentamicin and Neomycin, to name a few, is preferably added to the inoculum to suppress contaminating bacteria while permitting *L. intracellularis* growth. Whether the inoculum is a pure culture or an intestinal homogenate, inoculation of the culture cells can be performed by various techniques known in the art, given the teachings herein.

The bacteria and/or inoculated culture cells are then incubated under a reduced dissolved O₂ concentration. At dissolved oxygen concentrations greater than 10% *L. intracellularis* growth is less than optimal with cessation of growth eventually occurring at oxygen concentrations outside this range. Preferably, the bacteria and/or inoculated culture cells are incubated in a dissolved oxygen concentration in the range of about 0% to about 10%. More preferably, the bacteria and/or cells are incubated in an oxygen concentration in the range of about 0% to about 8%, with an oxygen concentration of about 0% to about 3.0% being most preferred.

The proper concentration of carbon dioxide is also important to the proper growth of *L. intracellularis*. At carbon dioxide concentrations greater than 0% and less than 4%, non-optimum growth occurs with cessation of growth eventually occurring at carbon dioxide concentrations outside this range. Preferably, the carbon dioxide concentration is in the range from about 6% to about 10%, with a carbon dioxide concentration of about 8.8% being most preferred.

In addition, the cells are preferably incubated at a hydrogen concentration in the range from about 4% to about 10%. Most preferably, the cells are incubated in about 0 to about 8.0% O₂, about 8.8% CO₂, and about 4% H₂. Nitrogen is used as a "balance" in the gas mixture containing nitrogen (96%) and hydrogen (4%) or nitrogen (80%), carbon dioxide (10%) and hydrogen (10%) for growth of this organism. Cells are preferably incubated at a nitrogen concentration in the range from about 80% to 96%. Therefore, cells are most preferably incubated in about 0 to about 8.0% O₂, about 8.8% CO₂, about 4% H₂ and about 96% N₂.

Inoculated cells may be incubated in a dual gas incubator or other gas chambers which contain the proper hydrogen, oxygen and carbon dioxide concentrations and which allow the cells to be suspended during incubation. The chamber should comprise a means for maintaining the inoculated cells in suspension, and a gas monitor and supply source to supply and maintain the proper gas concentrations. The incubation temperature should be in the range of from 30° C. to about 45° C. and is more preferably in the range of from about 36° C. to about 38° C. Most preferably, the tempera-

ture is about 37° C. The necessary equipment for cultivation and attenuation is readily available to those of ordinary skill in the art given the teachings herein. One example of equipment suitable for carrying out the present invention is a dual gas incubator, e.g., model 480 (Lab-Line, Melrose Park, Ill.) in conjunction with spinner flasks to maintain the cells in suspension. The presently preferred equipment comprises a fermentor, bioreactor, stir plate or rotary shaker containing at least about 2 liters media and capable of maintaining the culture cells in suspension via sparging gas of the appropriate concentration, or other means of mechanical agitation, and continuously monitoring dissolved O₂ levels in the media. New Brunswick, Braun and other companies make suitable fermentors and bioreactors for this purpose.

By maintaining the inoculated cells in a suspended state during incubation, maximum growth of the cells, and hence *L. intracellularis*, is achieved by increasing each individual cell's exposure to growth media and the proper mixture of hydrogen, oxygen and carbon dioxide. The culture cells can be agitated and maintained in suspension by a variety of methods known in the art including, for example, culture flasks, roller bottles, membrane cultures, biobags, WAVE® bioreactor systems, fermentors and spinner flasks. The cells may be kept in suspension during incubation by incubating the cells in a spinner flask inside a dual gas incubator or similar apparatus. The term "spinner flask", as used herein, means a flask or other container which employs a paddle, propeller or other means to agitate the culture and keep the cells contained therein in suspension.

In a preferred embodiment, the inoculated cells are incubated until the cells reach confluency and then the cells are placed in a spinner flask containing growth media and incubated in a dual gas incubator while spinning the flask. Preferably, the inoculated cells are scraped or trypsinized and passaged into the spinner flask. This can be achieved by a variety of methods known in the art such as using a cell scraper to detach the cells. Once the cells are introduced into the spinner flask, the paddle of the spinner flask is typically rotated in the range of from about 5 to about 500 rpm on a magnetic stir plate in order to maintain the infected cells in suspension.

A portion of the cultivated *L. intracellularis* is then passaged to fresh culture to increase the production of *L. intracellularis* bacteria. The term "passaging" or variations thereof herein means the process of transferring a portion of the cultivated *L. intracellularis* to fresh culture cells in order to infect the fresh cells with the bacterium. The term "fresh", as used herein, means cells which have not yet been infected by *L. intracellularis*. Preferably such cells are on the average no more than approximately one day old.

The passage of *L. intracellularis* in suspension cultures may be accomplished by removing a portion of the original culture and adding it to a new flask containing fresh culture cells. If the original culture has a high number of bacteria/ml, for example, greater than about 10⁴ bacteria/ml, it is preferable to add between about 1 to 10% (volume to volume) of culture from the infected flask to a new flask containing fresh cells. This is preferably done when 50-100% of the cells are infected. If fewer than 50% of the cells are infected, passaging is preferably accomplished by splitting the culture 1:2 into a new flask and scaling-up the volume by adding fresh tissue culture cells and media. In either case, cell lysis and other steps are not required, in direct contrast to the passage of monolayer cultures, as in the prior art.

After sufficient growth of the culture cells and subsequent infection by *L. intracellularis*, as determined by indirect fluorescent antibody (IFA) staining, TCID₅₀ or another comparable method, at least a portion of the cultivated *L. intracellularis* bacteria is then harvested. Harvesting is typically performed at cell infectivity of about 60% or higher; however, one skilled in the art knows that harvesting could be performed at a cell infectivity of less than 60%. The harvesting step may be performed by separating the bacteria from the suspension by various techniques known to those of ordinary skill in the art, given the teachings herein. Preferably, the *L. intracellularis* bacteria is harvested by centrifuging the contents of all or a portion of the suspension to pellet the culture cells, resuspending the resulting cell pellets, and lysing the infected cells. Typically, at least a portion of the contents is centrifuged at about 3000×g for about 20 minutes in order to pellet the cells and bacteria. The pellet may then be resuspended in, for example, a sucrose-phosphate-glutamate (SPG) solution and passed approximately 20 times through a 25 gauge needle in order to lyse the cells. If further purification is desired, the samples can be centrifuged at about 145×g for about five minutes to remove cellular nuclei and debris. The supernatant may then be centrifuged at about 3000×g for about twenty minutes and the resulting pellet resuspended in an appropriate diluent, such as SPG with fetal bovine serum (to prepare harvested bacteria suitable for lyophilization, freezing, or use as an inoculant) or growth media (to prepare harvested bacteria more suitable for passaging to fresh cells).

As previously mentioned, effective growth of *L. intracellularis* for large-scale production is enhanced by keeping the tissue cells actively growing. With monolayers, when cultures become confluent, the rate of cell division decreases substantially. Attempts to grow *L. intracellularis* on monolayer tissue cultures have had limited success and scale-up has not been possible. However, using suspension cultures greatly facilitates keeping the cells actively growing and permits continuous culture expansion and scale-up. Using a fermentor and between about 0 to 3% dissolved O₂ as explained above, enables growth of up to and greater than 10 bacteria/ml.

When using IEC-18 cells, it is preferable to add gelatin, agarose, collagen, acrylamide or silica beads, such as Culti-sphere-G porous microcarriers (HyClone Laboratories, Logan Utah), along with the growth media. However, HEp-2 cells and others do not require microcarriers according to the methods used herein.

For culture maintenance purposes, with HEp-2 cultures, preferably 25% to 50% of the culture is removed and replaced with fresh media at weekly intervals. For cell cultures with microcarriers or beads, preferably 25% to 50% of the culture is removed and replaced with fresh media 1-2 times weekly. For scale-up purposes, an additional 25% to 50% of media, or media with microcarriers, may be added to the culture.

Depending upon the rate at which the culture cells become infected, passage to fresh cells generally occurs between about every 2 to about 7 days. Assuming that the culture cells become at least 70% infected within 2 to 7 days, preferably passage occurs between about every 5 to 7 days.

The present invention also provides vaccines and methods for producing vaccines against a novel isolate of *L. intracellularis* of European origin. Preferably, after maintaining the infected cells in suspension for an extended time (for example, 6-8 months), at least a portion of the cultivated *L. intracellularis* bacteria are harvested and monitored for potential attenuation. Such monitoring is preferably accom-

plished by host animal or animal model challenges to select for an attenuated isolate. Such attenuated isolates are used in vaccines according to the methods taught herein.

The present invention allows rapid culture expansion, an increase in yields of 100-1000 fold, and reduced cost for production of *L. intracellularis* of European origin. As a result, the abundant supply of *L. intracellularis* bacteria produced is readily attenuated for vaccine production purposes. The method of growing *L. intracellularis* in suspension greatly increases the ease, speed, and number of bacterium available for this purpose. The more cells and cell divisions which occur, the greater the level of mutations occurring which are advantageous in vaccine development. Thus, growth in suspensions increases the expression of important immunogens controlled by environmentally regulated genes and their expression products.

The resulting attenuated isolates can be cultivated in tissue culture monolayers but are preferably cultivated in suspension cultures. Other means of attenuation can include chemical attenuation by the use of, for example, N-methyl nitrosoguanidine and others known in the art. Whether by multiple passage or chemical means, an attenuated *L. intracellularis* is produced and selected for vaccine preparation. In a preferred embodiment, the resulting attenuated isolate is ATCC accession No. PTA-4926.

The vaccine antigen can be harvested by centrifugation or microfiltration as described above. The antigen is then standardized at a defined level based on the optimum host animal immune response, determined by a dose titration in the host animal species. The bacteria may be inactivated by methods known in the art, e.g., by using 0.3% formalin or other inactivating agents to prepare a killed vaccine. The antigen is then incorporated into a suitable adjuvant, such as aluminum hydroxide or mineral oil to enhance the immune response. The antigen is then used to vaccinate the host via intramuscular or subcutaneous infection, in the case of swine at about 3-4 weeks of age, with a booster dose if necessary.

Preferably, the bacteria is serially passaged to induce and select for an attenuated, avirulent live culture. The culture is tested in the host animal for signs of attenuation. The culture is harvested as described earlier and lyophilized. Swine, for example, are orally vaccinated with 1×10^4 to 1×10^6 bacteria. About twenty-eight days after vaccination, the swine are orally inoculated with about 1×10^7 organisms from a less passaged (less than 30 passages in vitro past the original isolation from the intestinal homogenate) virulent culture of *L. intracellularis*. Infected animals are necropsied 21 days after challenge and the small intestines observed for gross lesions as well as microscopic lesions. PCR, indirect fluorescent antibody (IFA) or immunohistochemistry (IHC) should also be performed.

About eighty percent of the control animals will show gross or microscopic lesions and test positive for the presence of *L. intracellularis* in the mucosal cells of the intestines using either PCR, IFA or IHC testing methods. Vaccinated animals will have normal mucosal surfaces as determined by histological observations and will be negative by PCR testing 3 to 4 weeks post inoculation.

Generally, an attenuated immunogenic *L. intracellularis* isolate is produced after continuous culture for about 150 days to about 250 days, during which time the culture is passaged about 50-100 times. However, one skilled in the art knows that other attenuated cultures may be produced by varying these figures.

The vaccine product of the invention can be lyophilized. After harvesting, the isolate can be concentrated by various

methods known in the art and can be mixed with a stabilizer, e.g. sucrose gelatin stabilizer. The vaccine product can then be subjected to freezing and drying (lyophilization). Generally, the freezing step comprises ramping to about $-45^\circ \text{C} \pm 3^\circ \text{C}$ and holding for about 150 minutes to about 480 minutes. The drying step can comprise primary and secondary drying steps. For example, the primary drying step can comprise: (a) ramping to between about -30°C to about -5°C and holding for between about 120 minutes to about 1000 minutes, and, optionally (b) ramping to between about -5°C to about 5°C and holding for between about 150 minutes to about 2000 minutes. The secondary step generally comprises ramping to about $27^\circ \text{C} \pm 5^\circ \text{C}$ and holding for between about 330 minutes to about 1120 minutes. One skilled in the art knows that these ranges can be adjusted depending on conditions, e.g., starting volume.

A vaccine is then prepared comprising an immunologically effective amount of the attenuated *L. intracellularis* in a pharmaceutically acceptable carrier. In a preferred embodiment, a vaccine comprises ATCC accession No. PTA-4926 in a pharmaceutically acceptable carrier. The combined immunogen and carrier may be an aqueous solution, emulsion or suspension. An immunologically effective amount is determinable by means known in the art without undue experimentation given the teachings contained herein. In general, the quantity of immunogen will be between 5 and 5000 micrograms, and between $10^{2.0}$ and $10^{9.0}$ TCID₅₀, preferably between $10^{3.0}$ and $10^{6.0}$ TCID₅₀, more preferably between $10^{4.0}$ and $10^{5.0}$ TCID₅₀, when purified bacteria are used.

The present invention also encompasses combination vaccines comprising the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from at least one other pathogen, including but not limited to: *Salmonella* spp. (e.g., *Salmonella choleraesuis*, *Salmonella typhimurium*), *Erysipelothrix* spp. (e.g., *Erysipelothrix rhusiopathiae*), *Haemophilus* spp. (e.g., *Haemophilus parasuis*), *Mycoplasma* spp. (e.g., *Mycoplasma hyopneumoniae*), *Leptospira* spp., *Clostridium* spp. (e.g., *Clostridium perfringens*, *Clostridium difficile*), *Streptococcus* spp. (e.g., *Streptococcus suis*), *Brachyspira* spp. (e.g., *Brachyspira hyodysenteriae*), *Bordetella* (e.g., *Bordetella bronchiseptica*), *Pasteurella* spp. (e.g., *Pasteurella multocida*), circovirus (e.g., porcine circovirus type 2), porcine reproductive and respiratory syndrome (PRRS) virus, swine influenza virus (SIV), coronavirus (e.g., transmissible gastro-enteritis (TGE) virus, porcine respiratory corona virus), parvovirus, or *Escherichia coli*; and a pharmaceutically acceptable carrier.

In one embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis*, *Erysipelothrix* spp., *Clostridium* spp., *Brachyspira* spp., transmissible gastro-enteritis (TGE) virus, and *Escherichia coli*; and a pharmaceutically acceptable carrier. Antigenic material from *Clostridium* spp. can include, but is not limited to, *Clostridium perfringens* and *Clostridium difficile*. Antigenic material from *Erysipelothrix* spp. can include, but is not limited to, *Erysipelothrix rhusiopathiae*.

In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix* spp.; and a pharmaceutically acceptable carrier. In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926

and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix rhusiopathiae*; and a pharmaceutically acceptable carrier.

In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from at least one other pathogen, including but not limited to: *Clostridium* spp. e.g., *Clostridium tetani*), equine influenza virus (EIV) (e.g., EIV-1, EIV-2), equine herpes virus (EHV) (e.g., EHV-1, EHV-2, EHV-3, EHV-4, EHV-5, EHV-6, EHV-7), alphavirus (e.g., eastern encephalitis virus, western encephalitis virus, Venezuelan encephalitis virus), or West Nile virus; and a pharmaceutically acceptable carrier.

The vaccines according to the invention are generally administered to susceptible animals, preferably swine, in one or more doses. The live or killed vaccine may be administered 1 or 2 times at 2 week intervals. For the attenuated, live vaccines, one dose is preferred. The preferred routes of administration of attenuated live isolates are intramuscular, oral or intranasal, but intramuscular and subcutaneous injection routes are most preferred for the killed vaccine.

Effective diagnosis of PPE has also been hindered by the time required to culture the causative bacteria. As a result of the present invention, development of diagnostic tools promoting rapid and accurate assays for the presence of *L. intracellularis* in biological samples taken from swine and other animals susceptible to PPE is now possible.

The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can be used as an antigen in an ELISA or other immunoassay, such as an immunofluorescent antibody test ("IFA"), to detect antibodies to *L. intracellularis* in the serum and other body fluids of animals suspected of being infected with the bacteria. The presently preferred immunoassay is an IFA as described in the example below. Alternatively, the bacteria of the instant invention can be used in a Western Blot assay.

The preferred ELISA protocol according to the invention is as follows:

1. Add 0.1 ml/well antigen diluted in coating buffer. Incubate for 18 hours at 4° C.
2. Wash 3 times with PBS.
3. Add 0.25 ml of blocking buffer to each well of plate. Incubate 1 to 2 hours at 37° C.
4. Wash 3 times with wash buffer.
5. Dilute serum in blocking buffer and add 0.1 ml to the first wells of plate. Make serial 1:2 dilutions across the plate. Incubate for 1 hour at 37° C.
6. Wash 3 to 5 times with wash buffer.
7. Dilute conjugate in blocking buffer and add 0.1 ml to wells of plate and incubate for 1 hour at 37° C.
8. Wash 3 to 5 times with wash buffer.
9. Add substrate.
10. Measure absorbance of light with a spectrophotometer.
11. Wells in which antigen was not added are used as blanks.
12. Positive and negative control swine serum should also be used with each test.

The preferred Western Blot protocol is as follows:

1. Run antigen on 12% SDS-PAGE and transfer to nitrocellulose membrane.
2. Place membrane in blocking buffer for 2 hours.
3. Remove blocking buffer and rinse with PBS for 1 minute.
4. Dilute serum in blocking buffer and add to membrane. Incubate for 2 hours at room temperature.

5. Wash 3 times with wash buffer (5 minutes for each wash).
6. Dilute conjugate in blocking buffer and add to membrane. Incubate for 1 hour at room temperature.
7. Wash 3 times with wash buffer.
8. Add substrate for 10 minutes or until strong banding occurs.
9. Rinse with PBS.
10. Air dry and store in the dark.

The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can also be used to prepare antiserum or antibodies for diagnostic, prophylactic, or therapeutic use. The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can be administered to a non-human animal in an amount effective to elicit an immune response and the antiserum or plasma containing antibodies to the *L. intracellularis* bacteria, or components derived from such bacteria, can be collected according to methods known in the art and described herein.

The present invention is further described in the following examples which are provided for illustrative purposes only and are not to be construed as limiting. Indeed, other variants of the invention will be readily apparent to one of ordinary skill in the art.

All publications and patents cited herein are incorporated by reference in their entireties.

EXAMPLE 1

Production of *L. Intracellularis* Vaccine

Isolation and Attenuation of *L. intracellularis* from the Intestines of European Pigs with Porcine Proliferative Enteropathy (PPE):

L. intracellularis virulent isolate DK 15540 (DK 15540, DK-15540 and 15540 are used interchangeably herein) was isolated by the University of Minnesota from an ileal homogenate of a Danish pig infected with acute porcine hemorrhagic enteropathy. This isolate has been deposited under the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209 and assigned accession number PTA-4927. The isolation process included scraping the mucosa from the ileum, homogenizing, trypsinizing for 30 minutes, and passing through a tissue grinder. The ileal homogenate was then passed through a series of filters consisting of 5.0, 1.0, and 0.65 µm. The homogenate was diluted in sucrose phosphate glutamate buffer with 10% fetal bovine serum (FBS). Aliquots (6×1 ml) of homogenate were made and stored at less than -70° C. The homogenate was used as inoculum to infect T-75 cm² flasks of McCoy cells. Cultures were monitored daily for McCoy cell infection by scraping McCoy cell monolayers, lysing cells by potassium chloride treatment, and placing the concentrated cell pellet on microscope slides stained by IFA using monoclonal antibodies specific for *L. intracellularis*. After eleven passages on anchorage dependent cell cultures, inoculum from passage eleven was transferred into a 250 ml spinner flask containing McCoy cells and grown in suspension until harvest. The Danish isolate of *L. intracellularis* (ATCC accession No. PTA-4927) was attenuated by continuous in vitro passage in McCoy cells for 80 weeks and tested for identity by monoclonal antibodies. The attenuated isolate was designated B3903 (B3903, B 3903 and B-3903 are used interchangeably herein). Isolate B3903 was deposited under the Budapest Treaty with the

American Type Culture Collection, 10801 University Boulevard, Manassas, Va. 20110-2209 and assigned ATCC accession No. PTA-4926.

Cultures:

Identification

Characteristic growth requirements, PCR reactions, and monoclonal antibody reactions were used to identify *L. intracellularis* Master and Working Seed materials.

Purity

Purity of Master Seed and Working Seed of *L. intracellularis* was determined by examining cultures with monoclonal antibody stains, conventional extraneous agents tests for bacteria and viruses and by sterility mycoplasma tests.

Virulence

Master Seeds of *L. intracellularis* were not virulent as demonstrated by the lack of ability of the Master Seed and Back passage inoculum to produce clinical signs and gross lesions that are observed in susceptible swine following exposure of virulent *L. intracellularis*, as further illustrated in Example 2, infra.

Range of Subcultures

Final harvested material from production of *L. intracellularis* did not exceed eleven passages from the Master Seed.

Medium Composition

EU McCoy Master Cell Stock (MCS) were grown and maintained in Dulbecco's Modified Eagle Medium with Ham's Fortified F12 (DMEM/F12) and 1-10% (v/v) newborn bovine serum (NBS) or fetal bovine serum (FBS) (cell growth and maintenance media).

Master and Working Seed were stored in DMEM/F12 with 1-10% (v/v) NBS or FBS and 5-15% (v/v) glycerol (master and working seed storage media).

The final harvest product was stored in sucrose gelatin stabilizer (SGS) (final product storage media).

Propagation

Master and Working Seed cultures of *L. intracellularis* were propagated in EU McCoy MCS using cell growth and maintenance media as described supra and stored at less than or about -35° C.

Source of Tissue

L. intracellularis seed and production organisms were grown in McCoy Master Cell Stock (ATCC accession number CRL 1696, batch number F-10422). The mast cell stock was identified as 3894MMCSS at passage X. The master cell stock was passaged an additional six times and identified as EU McCoy MCS X+0. EU McCoy MCS passage X+0 was stored at -70° C.±5° C. or colder. Production vaccine was made in EU McCoy MCS cell line subcultures through the 40th passage.

Culture Containers

EU McCoy MCS were propagated in tissue culture flasks with 25-150 cm² surface area, Costar cell cubes, in roller bottles with 850 cm² to 2,225 cm², in spinner flasks up to 40 L capacity, and in bioreactors having 3 L to 500 L capacity.

Seed cultures of *L. intracellularis* were grown in 250-40,000 ml spinner flasks, 850 cm² to 2,250 cm² roller bottles, tissue culture flasks with 25-150 cm² surface area, or in bioreactors having 3 L to 500 L capacity.

Production cultures of *L. intracellularis* were grown in 6 L to 40 L spinner flasks or in bioreactors having 3 L to 500 L capacity.

Methods of Preparing Suspensions for Seeding or Inoculation

Seeding Cultures

Frozen or fresh *L. intracellularis* Master or expanded Working Seeds were thawed at room temperature (25° C.±3° C.) or at 37° C.±2° C. Bioreactors, spinner flasks or bottles previously seeded with McCoy cells at a cell density of 50,000 to 500,000 cells/ml were infected with *L. intracellularis* at a concentration of 1 to 10% (v/v) or MOI of 0.08 to 1.0. The infected culture was incubated under reduced oxygen concentrations by overlaying with a gas mixture composed of 86% N₂, 4% H₂ and 10% CO₂. The culture was incubated for 3 to 10 days at 37° C.±2° C., pH 6.7 to 7.3, and with continuous agitation (10 to 100 rpm) to maintain adequate mixing for the cells to remain in suspension.

Production Cultures

Frozen or fresh *L. intracellularis* Master or expanded Working Seeds were thawed at room temperature (25° C.±3° C.) or at 37° C.±2° C. Bioreactors or spinner flasks 3 L to 500 L capacity) previously seed (0 to 7 days) with McCoy cells at a cell density of 50,000 to 500,000 cells/ml were infected with *L. intracellularis* at a concentration of 1 to 10% (v/v) or MOI of 0.08 to 1.0. The infected culture was incubated under reduced oxygen concentrations by overlaying or sparging with a gas mixture composed of 96% N₂, 4% H₂.

The culture was incubated for 3 to 8 days at 37° C.±2° C., pH 6.7 to 7.3, and with continuous agitation (10 to 100 rpm) to maintain adequate mixing for the cells to remain in suspension.

Inoculation Techniques for Seed and Production Cultures

Seed Cultures

Up to 10% (v/v) of Master or Working Seed are inoculated (MOI=0.08 to 1.0) into bioreactors, spinner flasks or bottles seed with McCoy cells at 0-7 days in growth medium.

Production Cultures

Up to 10% (v/v) of Production Seed was inoculated (MOI=0.08 to 1.0) into an appropriate volume of growth medium seeded with McCoy cells at 0 to 7 days with the appropriate McCoy cell density in 3 L to 500 L capacity vessels.

Incubation of Microorganisms

The cultures were incubated at 37° C.±2° C. for 3 to 10 days in a reduced oxygen atmosphere with agitation to maintain the suspension. Additional medium and/or McCoy cells can be added to continue the growth process.

Cultures were observed macroscopically during the incubation period for evidence of abnormal growth or signs of contamination.

Harvest:

Handling and Preparation of Cultures

Cultures were examined for signs of adequate bacterial growth by indirect fluorescent antibody (IFA) staining. Cultures that were ready for harvest exemplified 60 to 100% cell infectivity. Percent infectivity was determined by observation of at least three fields, each field containing enough McCoy cells to fill at least 80% of the area. To be considered infected, approximately 50% of the cell is filled with bacteria.

Potency of the harvest culture is tested by titration of the sample on McCoy cells which are fixed and stained using specific monoclonal antibody (anti-*L. intracellularis* monoclonal antibody VPM 53 Lot 31599 or equivalent; anti-

mouse IgG-fluorescein conjugate (FITC) (ICN No. 55499) after 6 days of incubation at 37° C.±2° C.

Cultures were examined visually for any obvious signs of contamination. Harvest occurred 3 to 10 days post inoculation.

Harvesting Techniques and Specifications

Cell and fluid contents in the production culture bioreactor, spinner flasks and bottle were partially or fully collected in a sterile receiving vessel. Each production culture bioreactor, spinner flask and bottle was harvested individually or the contents of several vessels were pooled with the addition of SGS and stored at 1° C. to 7° C. or colder. Harvested production culture was sampled for potency by TCID₅₀ and identification by IFA staining.

Production cultures exhibited at least 4.9 TCID₅₀/ml by IFA staining and were free of any evidence of contamination upon microscopic observation.

Preparation of Vaccine Product:

Concentration Methods

The vaccine product can be concentrated by various methods, e.g., by allowing the culture to settle with subsequent decanting of the supernatant, by membrane filtration (0.22 µm or smaller), perfusion, or by centrifugation.

Sucrose Gelatin Stabilizer (SGS)

Hydrolyzed gelatin solution is prepared by mixing gelatin with deionized water or water for injection at approximately 25% final total volume of SGS batch size and hydrolyzing in an autoclave for 120 minutes at 121° C.

The hydrolyzed gelatin solution (40.0 g/L) was then mixed with deionized water or water for injection at approximately 75% final total volume of SGS batch size. Potassium hydroxide (AR) (0.548 g/L), L-glutamic acid (1.440 g/L), dipotassium phosphate (AR) (2.508 g/L), potassium dihydrogen phosphate (AR) (1.030 g/L), and sucrose (AR) (150.00 g/L) were added and the solution was thoroughly mixed. The pH of the stabilizer was then adjusted to 6.8 to 7.0 with hydrochloric acid or sodium hydroxide solutions. Deionized water or water for injection was added to 100% of desired final volume of the SGS. The complete stabilizer was thoroughly mixed, and the entire solution was sterilized by filtration through a 0.1 micron filter.

Example of Assembly of Units to Make a Serial is shown in Table 1:

TABLE 1

<i>L. intracellularis</i>	200,000-300,000 ml
sucrose gelatin stabilizer (SGS)	100,000 ml (25% v/v)
DMEM/F12 (May be added to standardize the product)	0-150,000 ml
TOTAL VOLUME	400,000 ml

Volume of an average serial was 50 L to 500 L.

Lyophilization

The vaccine product was lyophilized according to the procedure outlined in Table 2 for a 10 dose cycle (6.0 ml fill) or Table 3 for a 50/100 dose cycle (10.0 ml fill).

TABLE 2

STEPS	° C.	Rate (minutes)	Hold (minutes)	Pressure (mT)
PRE-COOL*	5°	Na	Na	Atm
FREEZE	-47° ± 3°	As fast as possible	150	Atm
1° DRYING, 1st	-15° ± 2°	120	120	100-150

TABLE 2-continued

STEPS	° C.	Rate (minutes)	Hold (minutes)	Pressure (mT)
1° DRYING, 2nd	0° ± 2°	120	180	100-150
2° DRYING, 1 st	32° ± 2°	240	180	60-80
2° DRYING, 2nd	26° ± 2°	240	As fast as possible	60-80

10 Total Time: 1352 minutes (22.5 hrs)
*Shelves are pre-cooled to 5° ± 2° C. during the loading of the lyophilizer.

TABLE 3

STEPS	° C.	Rate (minutes)	Hold (minutes)	Pressure (mT)
PRE-COOL*	5°	N/A	N/A	Atm
FREEZE	-48° ± 3°	60	90	Atm
1° DRYING	-15° ± 2°	60	1500	100-150
2° DRYING	26° ± 2°	60	600	60-80

15 Total Time: 2370 minutes (39.5 hrs)
*Shelves are pre-cooled to 5° ± 2° C. during the loading of the lyophilizer.

EXAMPLE 2

Safety of *L. Intracellularis* Vaccine

30 Purpose:

The objectives of this study were two-fold. The first objective of this study was to observe and compare the incidence of disease caused by three different low passage isolates (two of U.S. origin and one of European origin) of *L. intracellularis* in pigs at 6½ weeks of age. The second objective was to observe the safety of two high passage isolates (both of European origin) of *L. intracellularis* in pigs at 6½ weeks of age.

40 Materials and Methods:

Test Substances

1. *L. intracellularis* low passage U.S. isolate N343
2. *L. intracellularis* low passage U.S. isolate N101494.
3. *L. intracellularis* low passage EU isolate DK 15540 p20
4. *L. intracellularis* high passage EU isolate DK 15540 p60
5. *L. intracellularis* high passage EU isolate DK 15540 p80 (Master Seed designation B3903).

Formulation of Test Substances

Low passage isolates were grown continuously for 10-20 weeks after isolation in McCoy cell suspension. High passage isolates were grown continuously for 60-80 weeks after isolation in McCoy cell suspension. All cultures were harvested via centrifugation at 10,000 RPM for 15 minutes. The McCoy cell culture pellets containing *Lawsonia* were resuspended in Sucrose-Phosphate-Glutamine (SPG) solution with 10% FBS.

Storage of Test Substances

Harvested cultures were stored at -70° C. until the day of challenge. Challenge cultures of the same isolate but from various harvest dates were thawed and combined into plastic vaccine bottles, labeled, and stored at 4° C. or on ice until the time of challenge.

Assay of Test Substances

TCID₅₀ was performed on all pooled challenge isolates at the time of challenge (day 0). The average titers (n=3) (TCID₅₀/ml) were as follows in Table 4:

TABLE 4

Test Substance	Average Titer (TCID ₅₀ /ml)
<i>L. intracellularis</i> N343	6.4
<i>L. intracellularis</i> N101494	6.1
<i>L. intracellularis</i> DK 15540 p20	6.2
<i>L. intracellularis</i> DK 15540 p60	6.87
<i>L. intracellularis</i> DK 15540 p80	7.4

Study Design

The study consisted of five experimental groups and one control group. On day 0 of the study, Group 1 (10 pigs, 6½ weeks of age) received one 10 ml or equivalent intragastric (IG) dose of *L. intracellularis* low passage U.S. isolate N343. Group 2 (10 pigs, 6½ weeks of age) received one 10 ml or equivalent IG dose of *L. intracellularis* low passage U.S. isolate N101494. Group 3 (10 pigs, 6½ weeks of age) received one 10 ml or equivalent IG dose of *L. intracellularis* low passage EU isolate DK15540 p20. Group 4 (10 pigs, 6½ weeks of age) received one 10 ml or equivalent IG dose of *L. intracellularis* high passage EU isolate DK15540 60 week. Group 5 (20 pigs, 6 weeks of age) received one 10 ml or equivalent dose of *L. intracellularis* high passage EU isolate DK 15540 p80. Group 6 (10 pigs, 6½ weeks of age) designated as "Strict Controls" did not receive a treatment.

Daily health observations were made from initiation of study to the day of challenge of appropriate test animals. Clinical health (behavior, appetite, body condition, hair coat, and stool consistency on a scale of 1 to 4 were scored daily from day of challenge (day 0) to termination of study (day 21). Average daily weight gains (ADWG) were calculated from day of challenge (day 0) to termination of study (day 21). Fecal shedding of *L. intracellularis* was evaluated on days 0, 7, 14, and 21. The one animal that died (from Group 1) throughout the study was examined for gross and microscopic lesions. Death was determined to be due to lesions associated with PPE confirmed by histology and PCR analysis; the animal was not replaced. Qualitative analysis of *Lawsonia* content in feces was evaluated by PCR along with histological evaluation for *L. intracellularis* on the ileum and colon. Serum was collected on days 0, 7, 14, and 21 of the study.

Results:

Summary of Study Results

TABLE 5

Group	Treatment	No. of Pigs	Titer (TCID ₅₀ /ml)	Serology	Fecal Shedding	PCR	FA	Histology	Gross Lesions	ADWG	Clinical Scores
1	N343	9	7.4	19%	11%	0%	11%	44%	1	0.61	5.23
2	N101494	10	7.1	10%	10%	10%	30%	30%	1.2	0.8	5.15
3	DK15540p20	10	7.2	18%	13%	30%	10%	20%	1.2	0.86	5.01
4	DK15540p60	10	7.87	5%	0%	0%	0%	10%	1	0.9	5
5	DK15540p80	20	8.4	0%	0%	0%	0%	0%	1	1.05	5
6	Strict Controls	10	0	0%	0%	0%	0%	0%	1.05	0.91	5

General Observations

Daily health observations were made until challenge. The clinical condition of the animals was evaluated daily following challenge for the duration of the study. Observations included: behavior, appetite, body condition, hair coat, and stool consistency. The clinical conditions of these animals were evaluated based on a numerical point system, which reflects the severity of illness. Scores ranged from 1 to 4 for each parameter. A score of 1 was given to an animal with a normal, healthy appearance, a score of 3 for an animal demonstrating severe clinical signs, and a score of 4 for an animal that has deceased. The average daily score for the strict controls, DK15540 p60, and DK15540 p80 was 5.0. The average daily scores for low passage material was N343 (5.23), N101494 (5.15), and DK15540 p20 (5.01). Statistical analysis on these results indicated no differences among treatment and control groups using Kruskal-Wallis Rank Sum Test.

Average Daily Weight Gains (ADWG)

Average daily weight gains were calculated from the time of challenge (day 0) to the termination of the study (day 21). The average weight gain per day for the strict control group was 0.9 pounds. The average weight gain for the low passage treatment groups was only 0.6 (N343), 0.8 (N101494), and 0.86 (DK15540 p20) pounds/day. The high passage treatment groups revealed the same or increased average weight gain per day compared to the strict control group that didn't receive challenge with 0.9 pounds/day (DK15540 p60) and 1.05 pounds/day (DK15540 p80) respectively. The mean difference in average daily weight gains was significantly lower in the N343 treatment group compared to higher passage treatment groups (DK15540 p60 and p80) and strict control group at day 21 of the study. (Pearson Chi-square p<0.05).

Seroconversion

Seroconversion to *Lawsonia* exposure in pigs was measured by testing for the presence of anti-*Lawsonia* antibodies using an IFAT assay. On day 0, only the N343 treatment group observed detectable seroconversion in 2/10 pigs. Day 7 observed 2/9 pigs (N343) and 1/10 pigs (DK15540 p20) IFA positive for *Lawsonia* antibodies. On day 14, 2/9 pigs were IFA positive in N343 treatment group, 1/10 in DK15540 p20 and p60 respectively. Day 21 revealed 1/10 pigs (N343), 4/10 pigs (N101494) and 6/10 pigs (DK15540 p20) were IFA positive while high passage treatment groups (DK15540 p60 and p80) show no detectable seroconversion. Seroconversion to *Lawsonia* exposure increased in treatment groups receiving low passage *L. intracellularis* of both the U.S. and EU isolates day 21 of the study.

Fecal Shedding

PCR testing of the feces demonstrated shedding of *L. intracellularis* beginning on day 14 where 4/9 in the N343,

4/10 in the N101494, and 5/10 in the DK15540 p20 low passage animals tested positive. Both high passage treatment and strict control groups were PCR negative for day 14. On day 21, DK15540 p20 treatment group had 1 animal PCR positive while all other treatment and control groups were PCR negative. No evidence of shedding was observed in high passage isolate groups (DK15540 p60 and p80) using PCR throughout the study. PCR positive animals indicating active shedding of *Lawsonia* in their feces were more significant in the low passage isolate groups (N343, N101494, and DK15540 p20) on day 14 of the study than high passage isolate groups and strict controls (Pearson Chi-square $p < 0.05$).

PCR at Day 21: Ileums and Colons

PCR testing of mucosal scrapings from ileums and colons was performed after necropsy (day 21). Samples that were PCR positive for *L. intracellularis* colonization were (2/10 colons) in N101494 and (2/10 ileums, and 4/10 colons) in DK 15540 p20 low passage isolate groups. All other treatment and control groups were PCR negative in tissues day 21 of the study. Results indicated ileums and colons of pigs in DK15540 p20 were significantly more colonized with *L. intracellularis* compared to all treatment and control groups (*Pearson Chi-square $p < 0.05$).

Histology

Sections of the terminal ileum and colon were collected at necropsy (day 21) and placed in buffered formalin for histological analysis. Presence of intracellular bacteria and crypt hyperplasia was observed in tissues stained with Hematoxylin and Eosin (H&E) and Warthin-Starry silver reagents of 4/9 pigs (N343), 3/10 pigs (N101494), 2/10 pigs (DK15540 p60) and 1/20 pigs (DK15540 p80). Lesion development was confirmed by florescent antibody staining using monoclonal antibodies against *Lawsonia intracellularis* in 1/9 pigs (N343), 3/10 pigs (N101494), and 1/10 pigs (DK15540 p60). FA detected no lesions caused by *Lawsonia* in the colons of all treatment and control groups. FA results indicated significant lesion development in ileums of pigs in N101494 treatment group compared to all treatment and control groups. H&E/silver staining showed significant lesion development associated with PPE in N343 treatment group compared to all treatment and control groups. (Pearson Chi-square $p < 0.05$).

Gross Scores

Ileums and colons were scored at the time of necropsy (day 21) for lesions associated with PPE. Tissues were given a score of 1 for normal appearance (no lesion development), a score of 2 for lesions demonstrating mild thickening, 3 for moderate thickening, and a score of 4 for severe thickening. Strict controls had an average clinical score of 1.05, N343 (1.0), N101494 (1.2), DK15540 p20 (1.2), and DK15540 p60 and p80 (1.0). Average gross lesion scores indicated no statistical difference between control and treatment groups using ANOVA test for multiple comparisons.

Conclusions

Based on the data collected, this study demonstrated that pigs challenged with a low passage dose of N343, N101494, and DK15540 with a TCID₅₀ greater than 1×10^7 bacteria/dose increases the incidence of PPE in these animals. High passage isolates (DK15540 p60 and p80) given to pigs of the same age with a TCID₅₀ greater than 1×10^7 were proven safe and show reduction of colonization and lesion development associated with PPE. This conclusion was based on PCR on the mucosa of the ileum, histopathology, and FA stains of tissue sections.

A reduction of shedding *L. intracellularis* in the feces determined by PCR was evident in high passage isolates

compared to low passage isolates. Average daily weight gains calculated for all treatment and control groups support this conclusion by demonstrating positive uniform daily weight gain in groups given the high passage isolate and strict controls compared to groups given low passage isolates especially animals in the N343 treatment group. This observation indicates reduction of weight gains in animals given low passage material which supports adequate and similar grow performance of animals given high passage material with animals receiving no challenge material. Compared to the strict controls, the high passage isolates showed no negative impact on weight gain and overall health based on clinical scores.

EXAMPLE 3

Efficacy and Minimal Protective Titer

Purpose:

The objectives of this study were to determine the minimal protective titer of a vaccine comprising isolate B3903 (lyophilized) (DK 15540, passage 80) ("B3903 (Lyophilized) vaccine" and "B3903 vaccine" are used interchangeably herein) administered by oral drench in pigs 3 weeks of age and to demonstrate efficacy against a virulent heterologous pure culture challenge with low passage *L. intracellularis*, the causative agent of Porcine Proliferative Enteropathy (PPE) in swine.

Materials and Methods:

Test Substance: Attenuated Live Culture of *L. intracellularis*, Isolate B3903

Formulation of B3903 (Lyophilized) Vaccine

DK 15540 isolates were grown continuously for 80 weeks after isolation in McCoy cell suspension. All cultures were harvested via centrifugation at 10,000 RPM for 15 minutes. The McCoy cell culture pellets containing *Lawsonia* were resuspended in Sucrose-Phosphate-Glutamine (SPG) solution with 10% FBS. Desiccation was performed as described in Example 1, supra. The lyophilized product is reconstituted in water, q.s. ad 2.0 ml, for injections.

Storage of B3903 (Lyophilized) Vaccine

The vaccine was stored at 2° C.-8° C. until ready for use. After resuspension, the vaccine was stored on ice until administration.

Doses of B3903 (Lyophilized) Vaccine

1. High dose (Treatment Group 1): 1×2 mL (6.0 logs/dose) via direct oral drench on day 0 of the study.
2. Medium dose (Treatment Group 2): 1×2 mL (4.9 logs/dose) via direct oral drench on day 0 of the study.
3. Low dose (Treatment Group 3): 1×2 mL (3.8 logs/dose) via direct oral drench on day 0 of the study.

Test Substance: Placebo

A placebo consisting of uninfected McCoy tissue culture cells suspended in DMEM/F12 growth medium fortified with 5% NBS was given to treatment Groups 4 (challenge control) and 5 (negative control) on day 0 of the study. This substance was administered to piglets in treatment Group 4 by direct oral drench and given 1×2 mL of placebo per test animal.

Test Substance: *L. intracellularis* N101494 Virulent Challenge

L. intracellularis N101494 was obtained from the intestines of a 12 week old pig from an Indiana farm (U.S. Pat. No. 5,714,375).

Formulation of *L. intracellularis* N101494 Virulent Challenge

L. intracellularis challenge material was grown continuously in McCoy cell suspension no more than 30 passes after initial isolation from infected gut tissue. Active cultures (2x3 L) identified as SF 1422 and SF 1423 in addition to (1 L) SF 1421 were grown in McCoy cell suspension for 7 days to 15-30% McCoy cell infection. On the day of challenge (day 21), active cultures were harvested via centrifugation at 10,000 RPM for 15 minutes and cell pellets resuspended in 350 mls total volume with SPG stabilizer. Harvested active culture was pooled with 300 mLs of frozen 10x to 20x concentrated challenge stocks of low passage N101494 at various passages (pass 24 to 27 post isolation).

Storage of *L. intracellularis* N101494 Virulent Challenge

Final formulations ready for challenge were stored at 2° C. to 8° C. or on ice until inoculation.

Pre/Post-Vaccination and Challenge Titers

Results from the TCID₅₀ assay verified the amount of live *L. intracellularis* administered to each test animal per dose during vaccination and challenge. The average titers (n=5) of pre and post titrations for the B3903 vaccine and the challenge material (*L. intracellularis* N101494) were as follows in Table 6:

TABLE 6

Group	Treatment (logs/dose)	Average logs/dose (2 ml) Pre-vaccination (TCID ₅₀ /ml)	Average logs/dose (2 ml) Post-vaccination (TCID ₅₀ /ml)	Total Average logs/dose
1	B3903 vaccine high dose	6.07	5.89	6.0
2	B3903 vaccine medium dose	4.94	4.84	4.9
3	B3903 vaccine low dose	3.9	3.5	3.8
4	Challenge Controls	7.85	7.57	7.71

Study Design

Sixty-five healthy *L. intracellularis* negative weaned piglets at 3 weeks of age were randomly divided into 5 treatment groups and housed separately throughout the study. On day 0, treatment Group 1 (15 pigs) received a 2 mL dose (6.0 logs/dose) of B3903 vaccine by direct oral drench. Treatment Group 2 (15 pigs) received 1x2 mL dose of B3903 vaccine titrated at 4.9 logs/dose by direct oral drench. Treatment Group 3 (15 pigs) received 1x2 mL dose of B3903 vaccine titrated at 3.8 logs/dose by direct oral drench.

Treatment Groups 4 and 5 (10 pigs/group) received 1x2 mL dose of placebo by direct oral drench.

On day 21 of the study (3 weeks post vaccination), test pigs in treatment Groups 1-4 received 1x10 mL dose of virulent low passage pure culture *L. intracellularis* heterologous isolate N101494 by gastric gavage.

On day 42 of the study (3 weeks post challenge), all treatment Groups (1-5) were euthanized and necropsied for gross and microscopic lesion analysis for PPE.

Daily health observations were made from study initiation to the day of challenge for each test animal. Clinical health (diarrhea, behavior, and body condition) were scored daily from day of challenge (day 21) to termination of study (day 42). Weights were taken on day of vaccination (day 0), day of challenge (day 21) and on day of study termination (day

42) to calculate average daily weight gains of each treatment group. Fecal shedding of *L. intracellularis* was evaluated by polymerase chain reaction (PCR) by testing fecal swabs (f-PCR) on days 0, 7, 14, 21, 28, 35, and 42 of the study. All animals euthanized at study termination (day 42) were examined for gross and microscopic lesions. Qualitative analysis of bacterial content in tissues was evaluated by PCR (t-PCR) along with histological evaluation for *L. intracellularis* in the ileum, colon, tonsil, and mesenteric lymph node day 42 of the study. Serum was collected on days 0, 7, 14, 21, 28, 35, and 42 of the study. Serum was tested using the indirect florescent antibody test (IFAT) to detect anti-*Lawsonia* antibodies in test animals. Treatment group comparisons were made by data analysis of average daily weight gains post vaccination and post challenge, clinical scores, seroconversion rates (IFAT), colonization (t-PCR), fecal shedding (f-PCR), gross lesion, and microscopic lesion development by immunohistochemistry (IHC).

Results:

Summary of Primary Results

TABLE 7

Treat-ment Group	Pigs per Group	Treatment Group ID (logs/dose)	Average Gross Lesion Scores (ileum)	Average Gross Lesion Scores (colon)	Average Micro-lesion scores (ileum)	Average Micro-lesion Scores (colon)
1	15	B3903 vaccine high dose (6.0)	1.5 ab	1.2 a	0.2 ab	0.1 a
2	15	B3903 vaccine medium dose (4.9)	1.2 ab	1.0 a	0.4 ab	0.0 a
3	15	B3903 vaccine low dose (3.8)	2.5 b	1.5 b	1.0 b	0.3 a
4	10	Challenge Controls	3.6 c	2.2 b	2.4 c	1.5 b
5	10	Strict Controls	1.0 a	1.0 a	0.0 a	0.0 a

*Like letters indicate no significant difference (p < 0.05)

Final test results for each treatment group revealed significant gross and microscopic lesion development of the ileum and colon in non-vaccinated, challenge control pigs (Group 4) compared to vaccinated pigs regardless of dose (high, medium, and low) in Groups 1, 2, and 3 (p<0.05). Average gross lesion scores of the vaccine-low dose colons were not significantly different than challenge controls. The vaccine-low dose group (Group 3) is the only treatment group that received vaccine and had significant lesion development (gross and microscopic) in comparison to the strict control group which did not receive a vaccination, placebo, or challenge throughout the study.

Clinical Scores

Clinical scores were recorded for each animal daily from day of challenge (day 21) to necropsy (day 42). Clinical scores were calculated to obtain an average daily clinical score reflecting the severity and duration of sickness among treatment groups due to receiving a virulent challenge of *L. intracellularis*. Average clinical scores for each treatment group are summarized in Table 8.

TABLE 8

Treatment Group	Group Identification	Average Clinical Score
1	B3903 vaccine - high dose	3.1 a
2	B3903 vaccine - medium dose	3.0 a

TABLE 8-continued

Treatment Group	Group Identification	Average Clinical Score
3	B3903 vaccine - low dose	3.0 a
4	Challenge Control	3.0 a
5	Strict Control	3.0 a

*Like letters denote no significant differences among average clinical scores from the day of challenge to necropsy (p < 0.05).

Statistical analysis of clinical scores was accomplished by reviewing the data in relation to treatment groups receiving average clinical scores using 1-way ANOVA. There was no evidence of significant differences among non-vaccinated, challenge controls and strict controls compared to the high, medium, or low dose vaccine groups.

Average Daily Weight Gains

Average daily weight gains (ADWG) were calculated from the time of vaccine administration (day 0), to challenge administration (day 21), to the termination of the study (day 42). Statistical analysis of weight gain differences (lbs.=pounds) between vaccine-high dose and non-vaccine, challenge control treatment groups revealed evidence of a significant difference from the time of challenge administration to necropsy (p<0.05). The weight data is summarized in Table 9.

TABLE 9

Treatment Group	Group Identification	Average Initial Weight (lbs.)	ADWG (day 0-21) (lbs.)	ADWG (day 21-42) (lbs.)	ADWG Total (Vaccination to Necropsy) (lbs.)
1	B3903 vaccine high dose	17.8 a	0.94 a	1.63 a	1.29 a
2	B3903 vaccine medium dose	17.7 a	1.00 a	1.60 ab	1.30 a
3	B3903 vaccine low dose	18.0 a	0.94 a	1.48 ab	1.21 a
4	Challenge Control	17.7 a	0.96 a	1.45 b	1.21 a
5	Strict Control	17.7 a	0.94 a	1.63 ab	1.28 a

*Like letters denote no significant differences between treatment groups in ADWGs from day of vaccination to challenge and from day of challenge to necropsy (p < 0.05).

Seroconversion (IFAT)

Serum samples were collected weekly from all test animals in each treatment group and tested for presence of anti-*Lawsonia* IgG antibodies on days 0, 7, 14, 21, 28, 35, and 42 of the study. Positive and negative IFAT control samples were 100% accurate in all assays performed in this study. On days 0 through 21 (3 weeks post vaccination), all test animals in each treatment group were IFA negative. On day 28 (1 week post challenge) of the study 1/15 (6.7%) test animals in the vaccine-high dose treatment group were IFA positive while all others tested IFA negative. On day 35 (2 weeks post challenge) of the study, 4/15 (26.7%) pigs in the vaccine-high dose and medium dose treatment groups and 1/10 (10%) pigs in the non-vaccine, challenge control treatment group were IFA positive for *Lawsonia* antibodies. Both vaccine-low dose and strict control treatment groups were IFA negative on day 35. On day 42 (3 weeks post challenge) of the study, 8/10 (80%) pigs in the challenge control group, 6/15 (40%) pigs in the vaccine-medium dose group, 5/15 (33.3%) pigs in the vaccine-low dose group, and 3/15 (20%) pigs in the vaccine-high dose group were IFA positive. The strict control treatment group was IFA negative at study termination (day 42).

Seroconversion data was analyzed using the Chi-square statistic. For results obtained in the strict control treatment group, a chi-square statistic was not computed due to the 100% negative responses found for each test animal throughout the study. In treatment groups receiving a vaccine or placebo, IFAT results were compared using Chi-square statistic with an estimation of exact p-value (Monte-Carlo). Positive IFAT results obtained from challenge groups receiving a virulent pure culture challenge were significantly higher than those receiving vaccine-high dose and vaccine-low dose (p<0.05) at day 42 (3 weeks post challenge) of the study.

Fecal Shedding of *L. intracellularis* (PCR)

Fecal swabs were collected weekly from all test animals in each treatment group and tested for the presence of *L. intracellularis* by PCR on days 0, 7, 14, 21, 28, 35, and 42 of the study. Positive and negative DNA extraction and PCR reaction controls were 100% accurate for each assay conducted in this study. All test animals in each treatment group were fecal PCR negative for *L. intracellularis* from day 0 (vaccination) to day 21 (challenge). Pigs in the strict control group remained fecal PCR negative for *L. intracellularis* in their feces throughout the study. Fecal shedding of *L. intracellularis* (fecal PCR positive) was evident in various treatment groups each week after challenge inoculation. These results are summarized in Table 10.

TABLE 10

Treatment Group	Group Identification	Day 28 (1 week post challenge)	Day 35 (2 weeks post challenge)	Day 42 (3 weeks post challenge)
1	B3903 vaccine high dose	0/15 (0%) a	2/15 (13.3%) a	0/15 (0%) a
2	B3903 vaccine Medium dose	6/15 (40%) ab	5/15 (33.3%) a	0/15 (0%) a
3	B3903 vaccine low dose	4/15 (26.7%) ab	8/15 (53.3%) ab	2/15 (13.3%) ab
4	Challenge Control	4/10 (40%) b	7/10 (70%) b	4/10 (40%) b
5	Strict Control	0/10 (0%)	0/10 (0%)	0/10 (0%)

*Like letters denote no significant difference among treatment groups receiving a challenge (p < 0.05).

Statistical analysis using Chi-square statistic with an estimation of the exact p-value (Monte Carlo) compared the positive responses in each vaccine treatment group to the non-vaccine, challenge control group only. Results from the strict control group were withdrawn from group comparisons because each pig was 100% fecal PCR negative for *L. intracellularis* throughout the study. On day 28 (1 week post challenge), pigs receiving a high dose of vaccine had significantly less shedding of *L. intracellularis* than non-vaccinated, challenged pigs (p=0.017). On day 35 (2 weeks post challenge), pigs receiving a high dose (p=0.0024) and medium dose (p=0.041) of vaccine had significantly less shedding of *L. intracellularis* in their feces than non-vaccinated challenged pigs. On day 42 (study termination), again, pigs receiving a high and medium dose of vaccine (p=0.017) had significantly less shedding of *L. intracellularis* in their feces compared to the non-vaccinated, challenged pigs. Pigs in the low dose group were not significantly less PCR positive than the challenge controls on any day post challenge.

L. intracellularis Tissue Colonization (PCR)

Polymerase Chain Reaction testing of tissue sections of the terminal ileum, colon, tonsil, and mesenteric lymph node

were performed after necropsy (day 42 of the study). In the vaccine-high dose group, 1/15 (6.7%) pigs were PCR positive for *L. intracellularis* colonization in the tonsil while all other treatment groups were PCR negative. Ileitis PCR testing of mesenteric lymph tissue revealed 3/10 (30%) pigs in the non-vaccinated, challenge controls were positive for *L. intracellularis* colonization while all other treatment groups were PCR negative. Ileitis PCR testing of mucosal scrapings of the terminal ileum revealed 4/10 (40%) pigs in the challenge controls, 4/15 (26.7%) pigs in the vaccine-low dose group, 2/15 (13.3%) pigs in the vaccine-medium dose group, and 1/15 (6.7%) pigs in the vaccine-high dose group PCR positive for *L. intracellularis* colonization. Ileitis PCR testing of the colon revealed 3/10 (30%) pigs in the challenge controls, 5/15 (33.3%) pigs in the vaccine-low dose group, 1/15 (6.7%) pigs in the vaccine-medium dose group, and 2/15 (13.3%) pigs in the vaccine-high dose group PCR positive for *L. intracellularis* colonization. No evidence of *L. intracellularis* colonization was seen in tissues of the strict control group.

Statistical analysis of Ileitis PCR positive results were compared among treatment groups using Chi-square statistic with Monte Carlo approximation of the exact p-value. Mesenteric lymph node PCR results indicated evidence of a significant difference among non-vaccinated, challenge controls and all treatment groups receiving a vaccine treatment ($p=0.054$). No statistical significance was evident among treatment groups in *L. intracellularis* colonization in the ileum, colon, or tonsil at day 42 of the study.

Histology (IHC/H&E)

Sections 2 to 4 cm in length of tonsil, mesenteric lymph node, terminal ileum, and colon were collected at necropsy (day 42) and placed in 10% buffered formalin for histological analysis. *Lawsonia intracellularis* was not detected by IHC staining of tonsil sections in all treatment groups at necropsy. The strict control group was negative for *L. intracellularis* by IHC in all tissue samples taken at necropsy. Presence of *L. intracellularis* and microscopic lesions associated with PPE were found in the ileums of 9/10 (90%) pigs of the non-vaccinated, challenge controls, 6/15 (40%) pigs of the vaccine-low dose group, 3/15 (20%) pigs of the vaccine-medium dose group, and 1/15 (6.7%) pigs of the vaccine-high dose group respectively. Microscopic lesions were evident in the colon of 8/10 (80%) pigs in the challenge control group, 3/15 (20%) pigs in the vaccine-low dose group, and 1/15 (6.7%) pigs in the vaccine-high dose group. The vaccine group receiving a medium dose was IHC negative for *L. intracellularis* in the colon. Stained sections of mesenteric lymph node produced only 1/10 (10%) pigs in the challenge control that had *L. intracellularis*. Positive IHC results in sections of the ileum and colon among treatment groups correlated well to the presence of marked crypt hyperplasia demonstrated by H&E methodology.

Statistical analysis of quantitative IHC data was accomplished using 1-way ANOVA and Kruskal-Wallis Rank Sum tests followed by specific contrasts of p-values between the challenge group and strict control groups to each vaccine dosage group. Statistical analysis reveal evidence of a significant lesion development due to *L. intracellularis* in the ileum and colon of the challenge control group compared to vaccinated pigs regardless of dose on day 42 of the study ($p<0.001$). Statistical significance was not evident in average IHC lesion scores of the ileum and colon in vaccinated animals receiving a high or medium dose of vaccine compared to the strict control treatment group ($p>0.05$). Pigs receiving a low dose of vaccine had significantly higher

mean microscopic lesion scores due to *L. intracellularis* in the ileum compared to the strict control group ($p<0.05$).

Gross Scores

The ileum and colon of each test animal were scored at the time of necropsy (day 42) for gross lesions associated with PPE. Tissues from test pigs in the strict control were normal, did not contain lesions, and received average lesion scores of 1.1 (ileum) and 1.0 (colon). Tissues of test animals in the non-vaccinated, challenge control group received the highest average gross lesion score for the ileum (3.6) and colon (2.0) among treatment groups. Test pigs in the vaccine-low dose group received average gross lesion scores of 2.5 (ileum) and 1.5 (colon). Vaccine-medium dose test pigs received average gross lesion scores of 1.5 (ileum) and 1.0 (colon). Tissues of the vaccine-high dose group received average gross lesion scores of 1.5 (ileum) and 1.2 (colon).

Statistical analysis of average gross lesion scores among treatment groups was accomplished by using 1-way ANOVA. Average gross lesion scores indicated evidence of a significant difference between ileums in non-vaccinated, challenge control group and vaccine-medium and high dose groups respectively ($p<0.001$). Evidence of a significant difference was observed among average gross ileum scores of the challenge control compared to the vaccine-low dose group ($p<0.01$). Average gross lesion scores of the colon were significantly higher in the challenge control group compared to the vaccine-medium and high dose groups respectively ($p<0.05$). In addition, significant gross lesion development was observed in the ileum of the vaccine-low dose group compared to the strict control group ($p<0.001$). No evidence of a statistical significance in average gross lesion scores (ileum and colon) were evident in vaccine-medium and high dose groups compared to the strict control treatment group.

CONCLUSIONS

This study demonstrated protection in 3-week-old pigs against PPE was accomplished when given an oral 2 mL dose of B3903 (Lyophilized) vaccine containing a minimum of 4.9 logs of live *L. intracellularis* per dose. Similar if not slightly better protection was evident in the vaccine-high dose treatment group that received 6.0 logs/dose 3 weeks prior to challenge. The low dose vaccine group (3.8 logs/dose) did not indicate adequate protection against a virulent pure culture heterologous challenge compared to the test animals receiving a high or medium dose of the experimental vaccine. Statistical differences were evident among the vaccine-low dose group and non-vaccinated, challenge controls regarding the severity of microscopic lesions in the ileum and colon ($p<0.001$) and average gross lesion scores of the ileum ($p<0.01$). However, significant PPE lesions were observed in the ileum (microscopic lesions) and colon (gross lesions) of this group compared to the strict control group that did not receive vaccine or challenge.

In summary, the data from this study demonstrated that: (1) the minimum protective titer of a single oral administration of B3903 (Lyophilized) vaccine to 3 week old pigs is 4.9 logs/dose; (2) B3903 (Lyophilized) vaccine is efficacious against a virulent low passage pure culture *L. intracellularis*, heterologous isolate N101494; and (3) B3903 (Lyophilized) vaccine aids in the reduction of gross and microscopic lesions, tissue colonization, and fecal shedding of *L. intracellularis* in vaccinated pigs compared to non-vaccinated pigs.

What is claimed is:

- 1. An avirulent isolate of *Lawsonia intracellularis*, wherein said avirulent isolate is *Lawsonia intracellularis* deposit isolate ATCC No. PTA-4926.
- 2. A vaccine for the immunization of an animal, comprising a pharmaceutically effective amount of the avirulent isolate of *Lawsonia intracellularis* according to claim 1, and a pharmaceutically acceptable carrier.
- 3. The vaccine according to claim 2 wherein the pharmaceutically effective amount of the avirulent isolate of *Lawsonia intracellularis* is about 10^3 TCID₅₀ to about 10^6 TCID₅₀ per dose.
- 4. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from at least one of the following pathogens: *Salmonella* spp., *Erysipelothrix* spp., *Haemophilus* spp., *Mycoplasma* spp., *Leptospira* spp., *Clostridium* spp., *Streptococcus* spp., *Brachyspira* spp., circovirus, porcine reproductive and respiratory syndrome (PRRS) virus, swine influenza virus (SIV), transmissible gastro-enteritis (TGE) virus, parvovirus, or *Escherichia coli*; and a pharmaceutically acceptable carrier.

- 5. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from *Salmonella choleraesuis*, *Erysipelothrix* spp. transmissible gastro-enteritis (TGE) virus, *Escherichia coli*, *Clostridium* spp., and *Brachyspira* spp.; and a pharmaceutically acceptable carrier.
- 6. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix* spp.; and a pharmaceutically acceptable carrier.
- 7. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from at least one of the following pathogens: *Clostridium* spp., equine influenza virus (EIV), equine herpes virus (EHV), alphavirus, and West Nile virus; and a pharmaceutically acceptable carrier.

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(54) **LAWSONIA INTRACELLULARIS OF EUROPEAN ORIGIN AND VACCINES, DIAGNOSTIC AGENTS AND METHODS OF USE THEREOF**

LAWSONIA INTRACELLULARIS EUROPÄISCHEN URSPRUNGS UND VAKZINE, DIAGNOSTIKA UND ANWENDUNGSVERFAHREN DAFÜR

LAWSONIA INTRACELLULARIS D'ORIGINE EUROPEENNE, VACCINS, AGENTS DIAGNOSTIQUES ET PROCEDES D'UTILISATION DE CEUX-CI

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Description

BACKGROUND

5 [0001] The present invention relates to *Lawsonia intracellularis* vaccines and discloses methods for protecting against and diagnosing *L. intracellularis* infection. The products and processes of the invention are attainable, in part, as the result of an improved method for cultivating large scale supplies of *L. intracellularis*, including both a novel isolate of *L. intracellularis* of European origin and a method of preparing a lyophilized product containing the attenuated European isolate as vaccine product.

10 [0002] *L. intracellularis*, the causative agent of porcine proliferative enteropathy ("PPE"), affects virtually all animals, including: rabbits, ferrets, hamsters, fox, horses, and other animals as diverse as ostriches and emus. *L. intracellularis* is a particularly great cause of losses in swine herds in Europe as well as in the United States.

[0003] A consistent feature of PPE is the occurrence of intracytoplasmic, non-membrane bound curved bacilli within enterocytes in affected portions of intestine. The bacteria associated with PPE have been referred to as "Campylobacter-like organisms." S. McOrist et al., Vet. Pathol., Vol. 26, 260-264 (1989). Subsequently, the causative bacteria have been identified as a novel taxonomic genus and species, vernacularly referred to as *Ileal symbiont* (IS) intracellularis. C. Gebhart et al., Int'l. J. of Systemic Bacteriology, Vol. 43, No. 3, 533-538 (1993). More recently, these novel bacteria have been given the taxonomic name *Lawsonia* (*L.*) *intracellularis*. S. McOrist et al., Int'l. J. of Systemic Bacteriology, Vol. 45, No. 4, 820-825 (1995). These three names have been used interchangeably to refer to the same organism as further identified and described herein.

20 [0004] *L. intracellularis* is an obligate, intracellular bacterium which cannot be cultured by normal bacteriological methods on conventional cell-free media and has been thought to require attached epithelial cells for growth. S. McOrist et al., Infection and Immunity, Vol. 61, No. 19, 4286-4292 (1993) and G. Lawson et al., J. of Clinical Microbiology, Vol. 31, No. 5, 1136-1142 (1993) discuss cultivation of *L. intracellularis* using IEC-18 rat intestinal epithelial cell monolayers in conventional tissue culture flasks. In addition, H. Stills, Infection and Immunity, Vol. 59, No. 9, 3227-3236 (1991) discusses using Intestine 407 human embryonic intestinal cell monolayers and GPC-16 guinea pig colonic adenocarcinoma cell monolayers in conventional tissue culture flasks.

25 [0005] Recently, an *L. intracellularis* vaccine has been approved for use in the United States, which vaccine is based on *L. intracellularis* isolates described and claimed in United States Patent Nos. 5,714,375 and 5,885,823. The above-described vaccine is sold by Boehringer Ingelheim Vetmedica, Inc., 2621 North Belt Highway, St. Joseph, Missouri 64506-2002, under the trademark ENTERISOL® Ileitis, and includes live attenuated *L. intracellularis* bacteria of North American origin.

SUMMARY OF THE INVENTION

35 [0006] One object of the invention is to provide an improved *L. intracellularis* vaccine using an isolate of European origin.

[0007] Another object of the invention is to provide an improved method for cultivation of *L. intracellularis* on a large scale and improved techniques for production of *L. intracellularis* vaccines.

40 [0008] To achieve these and other objects, and in accordance with the purpose of the invention as embodied and broadly described herein, the present invention provides a newly isolated *L. intracellularis* from Europe, a method of attenuating such an isolate, and the attenuated isolate thereof. Also provided herein is a vaccine comprising the attenuated isolate. Also provided herein is a method for producing a vaccine comprising the attenuated isolate in lyophilized form for reconstitution at the time of administration and the lyophilized product thereof.

45 [0009] In one embodiment, the newly isolated *L. intracellularis* from Europe, isolate DK 15540, is deposit isolate ATCC accession No. PTA-4927. In another embodiment, the attenuated isolate derived from isolate DK 15540, is designated isolate B3903, ATCC accession No. PTA-4926.

DETAILED DESCRIPTION

50 [0010] As used herein, the term "*L. intracellularis*" means the intracellular, curved gram-negative bacteria described in detail by C. Gebhart et al., Int'l. J. of Systemic Bacteriology, Vol. 43, No.3, 533-538 (1993) and S. McOrist et al., Int'l. J. of Systemic Bacteriology, Vol. 45, No. 4, 820-825 (1995), each of which is incorporated herein by reference in their entireties, and includes but is not limited to the isolate designated DK 15540 which was deposited under the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 on January 9, 2003 and assigned ATCC accession number PTA-4927; the causative bacteria which can be obtained from PPE infected swine or other animals throughout the world given the knowledge in the art and the teachings herein; and variants or mutants of any of the above bacteria, whether spontaneously or artificially obtained.

55 [0011] As used herein, the term "attenuated isolate" means any *L. intracellularis* isolate that is prepared according to

the cultivation and passaging techniques taught herein to achieve avirulence while maintaining immunogenic properties when administered to a host animal including but not limited to the attenuated isolate designated B-3903 which was deposited under the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 on January 9, 2003 and assigned accession number PTA-4926.

[0012] The attenuated isolate of the invention can be used as an immunogen in antimicrobial vaccines for animals, including birds, fish, and mammals such as cattle, swine, horses, and primates. Such vaccines can be prepared by techniques known to those skilled in the art and given the teachings contained herein. Such a vaccine would comprise an immunologically effective amount of the attenuated isolate in a pharmaceutically acceptable carrier. The vaccine could be administered in one or more doses. An immunologically effective amount is determined by means known in the art without undue experimentation, given the teachings contained herein. The amount of avirulent bacteria should be sufficient to stimulate an immune response in disease-susceptible animals while still being avirulent. This will depend upon the particular animal, bacteria, and disease involved. The recommended dose to be administered to the susceptible animal is preferably about 3.0 TCID₅₀ (tissue culture infective dose 50% end point)/dose to about 6.0 TCID₅₀/dose and more preferably about 4.0 TCID₅₀/dose to about 5.0 TCID₅₀/dose. In a preferred embodiment, the titer of the vaccine is about 4.9 TCID₅₀/dose as determined by Tissue Culture Infective Dose 50% endpoint dilution assay (TCID₅₀). The carriers are known to those skilled in the art and include stabilizers and diluents. Such a vaccine may also contain an appropriate adjuvant. The vaccines of the invention may be used in combination with other vaccines, for example, as a diluent of another vaccine. The vaccine preparations may also be desiccated, for example, by freeze drying for storage purposes or for subsequent formulation into liquid vaccines.

[0013] Accordingly, the disclosure also comprises a method for inducing an immune response to virulent, wild-type *L. intracellularis* bacteria in an animal host for the purpose of protecting the host from such bacteria. The method comprises administering an immunologically effective amount of the attenuated bacteria of the invention to the host and, preferably, administering the vaccine of the invention to the host.

[0014] As used herein, the term "large-scale cultivation" means a level of cultivation of *L. intracellularis* greater than approximately 2.0 to 3.0 liters and includes production on a scale of 100 liters or more. "Cultivation" as used herein, means the process of promoting the growth, reproduction and/or proliferation of *L. intracellularis*.

[0015] *L. intracellularis* can be cultivated by methods known in the art, preferably, according to United States Patent Nos. 5,714,375 and 5,885,823. For example, culture cells may first be inoculated with an inoculum comprising *L. intracellularis* bacteria so as to infect the cells with the bacteria. Numerous cell lines can be used in practicing the invention, including, but not limited to, IEC-18 (ATCC 1589)-rat intestinal epithelial cells, HEP-2 (ATCC 23)-human epidermoid carcinoma cells, McCoys (ATCC 1696)-mouse (non-specified) cells, BGMK (Biowhittaker #71-176)-buffalo green monkey kidney cells, and swine intestinal epithelium cells. The preferred culture cells are HEP-2, McCoys or IEC-18 cells.

[0016] If culture cells are used, prior to being inoculated, the cells may be in the form of a monolayer. To form a monolayer, the cells may be seeded into conventional flasks. Each flask is generally seeded with between about 1x10⁵ cells to about 10x10⁵ cells per 25, 75, 150, 850 cm² flask or roller bottle mixed with growth media. The growth media may be any media for cell cultivation which includes a nitrogen source, necessary growth factors for the chosen culture cells, and a carbon source, such as glucose or lactose. The preferred media is DMEM fortified with Ham's F 12 with 1-5% fetal bovine serum, although other commercially available media may be used with good results.

[0017] Successful cultivation of *L. intracellularis* is enhanced by maintaining the culture cells in a constant state of growth. Therefore, the culture cell monolayer should be at about 20 percent to about 50 percent confluency at the time of inoculation. Preferably, the cells should be at about 30 percent to about 40 percent confluency at the time of inoculation, most preferably at about 30 percent confluency.

[0018] Alternatively, the cells, prior to being inoculated, may be grown in suspension, as described *infra*. Preferably, the cells are first grown to 100% confluency in the form of a monolayer in an adherent type system, *e.g.*, a roller bottle system, and then transferred to 3-3000 liters and grown in suspension. Alternatively, the cells can be grown in suspension to the desired cell density, *e.g.* 2 x 10⁵ cells/ml, within the 3-3000 liter vessel (bioreactor, Fermentor, spinner flask, etc.) using parameters suitable for growth within this system prior to inoculation.

[0019] The inoculum may be a pure culture of *L. intracellularis* obtained from infected swine or other animals. Preferably the inoculum may be a pure culture of *L. intracellularis* obtained from ATCC accession No. PTA-4927.

[0020] The inoculum can be an intestinal homogenate prepared by scraping the mucosa off of the ileum of a swine or other animal infected with PPE. When preparing an intestinal homogenate, ileal sections selected for culture should show severe lesions with gross thickening of the gut. Due to the fragile nature of the bacteria, samples should preferably be stored at -70°C as quickly as possible after necropsy. An antibiotic to which *L. intracellularis* is resistant such as Vancomycin, Amphotericin B or members of the aminoglycoside group of antibiotics, including Gentamicin and Neomycin, to name a few, is preferably added to the inoculum to suppress contaminating bacteria while permitting *L. intracellularis* growth. Whether the inoculum is a pure culture or an intestinal homogenate, inoculation of the culture cells can be performed by various techniques known in the art, given the teachings herein.

[0021] The bacteria and/or inoculated culture cells are then incubated under a reduced dissolved O₂ concentration.

At dissolved oxygen concentrations greater than 10% *L. intracellularis* growth is less than optimal with cessation of growth eventually occurring at oxygen concentrations outside this range. Preferably, the bacteria and/or inoculated culture cells are incubated in a dissolved oxygen concentration in the range of about 0% to about 10%. More preferably, the bacteria and/or cells are incubated in an oxygen concentration in the range of about 0% to about 8%, with an oxygen concentration of about 0% to about 3.0% being most preferred.

[0022] The proper concentration of carbon dioxide is also important to the proper growth of *L. intracellularis*. At carbon dioxide concentrations greater than 0% and less than 4%, non-optimum growth occurs with cessation of growth eventually occurring at carbon dioxide concentrations outside this range. Preferably, the carbon dioxide concentration is in the range from about 6% to about 10%, with a carbon dioxide concentration of about 8.8% being most preferred.

[0023] In addition, the cells are preferably incubated at a hydrogen concentration in the range from about 4% to about 10%. Most preferably, the cells are incubated in about 0 to about 8.0% O₂, about 8.8% CO₂, and about 4% H₂. Nitrogen is used as a "balance" in the gas mixture containing nitrogen (96%) and hydrogen (4%) or nitrogen (80%), carbon dioxide (10%) and hydrogen (10%) for growth of this organism. Cells are preferably incubated at a nitrogen concentration in the range from about 80% to 96%. Therefore, cells are most preferably incubated in about 0 to about 8.0% O₂, about 8.8% CO₂ about 4% H₂ and about 96% N₂.

[0024] Inoculated cells may be incubated in a dual gas incubator or other gas chambers which contain the proper hydrogen, oxygen and carbon dioxide concentrations and which allow the cells to be suspended during incubation. The chamber should comprise a means for maintaining the inoculated cells in suspension, and a gas monitor and supply source to supply and maintain the proper gas concentrations. The incubation temperature should be in the range of from 30°C to about 45°C and is more preferably in the range of from about 36°C to about 38°C. Most preferably, the temperature is about 37°C. The necessary equipment for cultivation and attenuation is readily available to those of ordinary skill in the art given the teachings herein. One example of equipment suitable for carrying out the present invention is a dual gas incubator, e.g., model 480 (Lab-Line, Melrose Park, IL) in conjunction with spinner flasks to maintain the cells in suspension. The presently preferred equipment comprises a fermentor, bioreactor, stir plate or rotary shaker containing at least about 2 liters media and capable of maintaining the culture cells in suspension via sparging gas of the appropriate concentration, or other means of mechanical agitation, and continuously monitoring dissolved O₂ levels in the media. New Brunswick, Braun and other companies make suitable fermentors and bioreactors for this purpose.

[0025] By maintaining the inoculated cells in a suspended state during incubation, maximum growth of the cells, and hence *L. intracellularis*, is achieved by increasing each individual cell's exposure to growth media and the proper mixture of hydrogen, oxygen and carbon dioxide: The culture cells can be agitated and maintained in suspension by a variety of methods known in the art including, for example, culture flasks, roller bottles, membrane cultures, biobags, WAVE™ bioreactor systems, fermentors and spinner flasks. The cells may be kept in suspension during incubation by incubating the cells in a spinner flask inside a dual gas incubator or similar apparatus. The term "spinner flask", as used herein, means a flask or other container which employs a paddle, propeller or other means to agitate the culture and keep the cells contained therein in suspension.

[0026] In a preferred embodiment, the inoculated cells are incubated until the cells reach confluency and then the cells are placed in a spinner flask containing growth media and incubated in a dual gas incubator while spinning the flask. Preferably, the inoculated cells are scraped or trypsinized and passaged into the spinner flask. This can be achieved by a variety of methods known in the art such as using a cell scraper to detach the cells. Once the cells are introduced into the spinner flask, the paddle of the spinner flask is typically rotated in the range of from about 5 to about 500 rpm on a magnetic stir plate in order to maintain the infected cells in suspension.

[0027] A portion of the cultivated *L. intracellularis* is then passaged to fresh culture to increase the production of *L. intracellularis* bacteria. The term "passaging" or variations thereof herein means the process of transferring a portion of the cultivated *L. intracellularis* to fresh culture cells in order to infect the fresh cells with the bacterium. The term "fresh", as used herein, means cells which have not yet been infected by *L. intracellularis*. Preferably such cells are on the average no more than approximately one day old.

[0028] The passage of *L. intracellularis* in suspension cultures may be accomplished by removing a portion of the original culture and adding it to a new flask containing fresh culture cells.

If the original culture has a high number of bacteria/ml, for example, greater than about 10⁴ bacteria/ml, it is preferable to add between about 1 to 10% (volume to volume) of culture from the infected flask to a new flask containing fresh cells. This is preferably done when 50-100% of the cells are infected. If fewer than 50% of the cells are infected, passaging is preferably accomplished by splitting the culture 1:2 into a new flask and scaling-up the volume by adding fresh tissue culture cells and media. In either case, cell lysis and other steps are not required, in direct contrast to the passage of monolayer cultures, as in the prior art.

[0029] After sufficient growth of the culture cells and subsequent infection by *L. intracellularis*, as determined by indirect fluorescent antibody (IFA) staining, TCID₅₀ or another comparable method, at least a portion of the cultivated *L. intracellularis* bacteria is then harvested. Harvesting is typically performed at cell infectivity of about 60% or higher; however, one skilled in the art knows that harvesting could be performed at a cell infectivity of less than 60%. The harvesting step

may be performed by separating the bacteria from the suspension by various techniques known to those of ordinary skill in the art, given the teachings herein. Preferably, the *L. intracellularis* bacteria is harvested by centrifuging the contents of all or a portion of the suspension to pellet the culture cells, resuspending the resulting cell pellets, and lysing the infected cells. Typically, at least a portion of the contents is centrifuged at about 3000xg for about 20 minutes in order to pellet the cells and bacteria. The pellet may then be resuspended in, for example, a sucrose-phosphate-glutamate (SPG) solution and passed approximately 20 times through a 25 gauge needle in order to lyse the cells. If further purification is desired, the samples can be centrifuged at about 145xg for about five minutes to remove cellular nuclei and debris. The supernatant may then be centrifuged at about 3000xg for about twenty minutes and the resulting pellet resuspended in an appropriate diluent, such as SPG with fetal bovine serum (to prepare harvested bacteria suitable for lyophilization, freezing, or use as an inoculant) or growth media (to prepare harvested bacteria more suitable for passaging to fresh cells).

[0030] As previously mentioned, effective growth of *L. intracellularis* for large-scale production is enhanced by keeping the tissue cells actively growing. With monolayers, when cultures become confluent, the rate of cell division decreases substantially. Attempts to grow *L. intracellularis* on monolayer tissue cultures have had limited success and scale-up has not been possible. However, using suspension cultures greatly facilitates keeping the cells actively growing and permits continuous culture expansion and scale-up. Using a fermentor and between about 0 to 3% dissolved O₂ as explained above, enables growth of up to and greater than 10⁸ bacteria/ml.

[0031] When using IEC-18 cells, it is preferable to add gelatin, agarose, collagen, acrylamide or silica beads, such as Cultisphere-G porous microcarriers (HyClone Laboratories, Logan Utah), along with the growth media. However, HEp-2 cells and others do not require microcarriers according to the methods used herein.

[0032] For culture maintenance purposes, with HEp-2 cultures, preferably 25% to 50% of the culture is removed and replaced with fresh media at weekly intervals. For cell cultures with microcarriers or beads, preferably 25% to 50% of the culture is removed and replaced with fresh media 1-2 times weekly. For scale-up purposes, an additional 25% to 50% of media, or media with microcarriers, may be added to the culture.

[0033] Depending upon the rate at which the culture cells become infected, passage to fresh cells generally occurs between about every 2 to about 7 days. Assuming that the culture cells become at least 70% infected within 2 to 7 days, preferably passage occurs between about every 5 to 7 days.

[0034] The present invention also provides vaccines and methods for producing vaccines against a novel isolate of *L. intracellularis* of European origin. Preferably, after maintaining the infected cells in suspension for an extended time (for example, 6-8 months), at least a portion of the cultivated *L. intracellularis* bacteria are harvested and monitored for potential attenuation. Such monitoring is preferably accomplished by host animal or animal model challenges to select for an attenuated isolate. Such attenuated isolates are used in vaccines according to the methods taught herein.

[0035] The present invention allows rapid culture expansion, an increase in yields of 100-1000 fold, and reduced cost for production of *L. intracellularis* of European origin. As a result, the abundant supply of *L. intracellularis* bacteria produced is readily attenuated for vaccine production purposes. The method of growing *L. intracellularis* in suspension greatly increases the ease, speed, and number of bacterium available for this purpose. The more cells and cell divisions which occur, the greater the level of mutations occurring which are advantageous in vaccine development. Thus, growth in suspensions increases the expression of important immunogens controlled by environmentally regulated genes and their expression products.

[0036] The resulting attenuated isolates can be cultivated in tissue culture monolayers but are preferably cultivated in suspension cultures. Other means of attenuation can include chemical attenuation by the use of, for example, N-methyl nitrosoguanidine and others known in the art. Whether by multiple passage or chemical means, an attenuated *L. intracellularis* is produced and selected for vaccine preparation. In a preferred embodiment, the resulting attenuated isolate is ATCC accession No. PTA-4926.

[0037] The vaccine antigen can be harvested by centrifugation or microfiltration as described above. The antigen is then standardized at a defined level based on the optimum host animal immune response, determined by a dose titration in the host animal species. The bacteria may be inactivated by methods known in the art, e.g., by using 0.3% formalin or other inactivating agents to prepare a killed vaccine. The antigen is then incorporated into a suitable adjuvant, such as aluminum hydroxide or mineral oil to enhance the immune response. The antigen is then used to vaccinate the host via intramuscular or subcutaneous infection, in the case of swine at about 3-4 weeks of age, with a booster dose if necessary.

[0038] Preferably, the bacteria is serially passaged to induce and select for an attenuated, avirulent live culture. The culture is tested in the host animal for signs of attenuation. The culture is harvested as described earlier and lyophilized. Swine, for example, are orally vaccinated with 1x10⁴ to 1x10⁶ bacteria. About twenty-eight days after vaccination, the swine are orally inoculated with about 1x10⁷ organisms from a less passaged (less than 30 passages *in vitro* past the original isolation from the intestinal homogenate) virulent culture of *L. intracellularis*. Infected animals are necropsied 21 days after challenge and the small intestines observed for gross lesions as well as microscopic lesions. PCR, indirect fluorescent antibody (IFA) or immunohistochemistry (IHC) should also be performed. About eighty percent of the control

animals will show gross or microscopic lesions and test positive for the presence of *L. intracellularis* in the mucosal cells of the intestines using either PCR, IFA or IHC testing methods. Vaccinated animals will have normal mucosal surfaces as determined by histological observations and will be negative by PCR testing 3 to 4 weeks post inoculation.

[0039] Generally, an attenuated immunogenic *L. intracellularis* isolate is produced after continuous culture for about 150 days to about 250 days, during which time the culture is passaged about 50-100 times. However, one skilled in the art knows that other attenuated cultures may be produced by varying these figures.

[0040] The vaccine product of the invention can be lyophilized. After harvesting, the isolate can be concentrated by various methods known in the art and can be mixed with a stabilizer, e.g. sucrose gelatin stabilizer. The vaccine product can then be subjected to freezing and drying (lyophilization). Generally, the freezing step comprises ramping to about $-45^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and holding for about 150 minutes to about 480 minutes. The drying step can comprise primary and secondary drying steps. For example, the primary drying step can comprise: (a) ramping to between about -30°C to about -5°C and holding for between about 120 minutes to about 1000 minutes, and, optionally (b) ramping to between about -5°C to about 5°C and holding for between about 150 minutes to about 2000 minutes. The secondary step generally comprises ramping to about $27^{\circ}\text{C} \pm 5^{\circ}\text{C}$ and holding for between about 330 minutes to about 1120 minutes. One skilled in the art knows that these ranges can be adjusted depending on conditions, e.g., starting volume,

[0041] A vaccine is then prepared comprising an immunologically effective amount of the attenuated *L. intracellularis* in a pharmaceutically acceptable carrier. In a preferred embodiment, a vaccine comprises ATCC accession No. PTA-4926 in a pharmaceutically acceptable carrier. The combined immunogen and carrier may be an aqueous solution, emulsion or suspension. An immunologically effective amount is determinable by means known in the art without undue experimentation given the teachings contained herein. In general, the quantity of immunogen will be between 5 and 5000 micrograms, and between $10^{2.0}$ and $10^{9.0}$ TCID₅₀, preferably between $10^{3.0}$ and $10^{6.0}$ TCID₅₀, more preferably between $10^{4.0}$ and $10^{5.0}$ TCID₅₀, when purified bacteria are used.

[0042] The present invention also encompasses combination vaccines comprising the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from at least one other pathogen, including but not limited to: *Salmonella* spp. (e.g., *Salmonella choleraesuis*, *Salmonella typhimurium*), *Erysipelothrix* spp. (e.g., *Erysipelothrix rhusiopathiae*), *Haemophilus* spp. (e.g., *Haemophilus parasuis*), *Mycoplasma* spp. (e.g., *Mycoplasma hyopneumonia*), *Leptospira* spp., *Clostridium* spp. (e.g., *Clostridium perfringens*, *Clostridium difficile*), *Streptococcus* spp. (e.g., *Streptococcus suis*), *Brachyspira* spp. (e.g., *Brachyspira hyodysenteriae*), *Bordetella* (e.g., *Bordetella bronchiseptica*), *Pasteurella* spp. (e.g., *Pasteurella multocida*), circovirus (e.g., porcine circovirus type 2), porcine reproductive and respiratory syndrome (PRRS) virus, swine influenza virus (SIV), coronavirus (e.g., transmissible gastro-enteritis (TGE) virus, porcine respiratory corona virus), parvovirus, or *Escherichia coli*; and a pharmaceutically acceptable carrier.

[0043] In one embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis*, *Erysipelothrix* spp. *Clostridium* spp., *Brachyspira* spp., transmissible gastro-enteritis (TGE) virus, and *Escherichia coli*; and a pharmaceutically acceptable carrier. Antigenic material from *Clostridium* spp. can include, but is not limited to, *Clostridium perfringens* and *Clostridium difficile*. Antigenic material from *Erysipelothrix* spp. can include, but is not limited to, *Erysipelothrix rhusiopathiae*.

[0044] In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix* spp.; and a pharmaceutically acceptable carrier. In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix rhusiopathiae*; and a pharmaceutically acceptable carrier.

[0045] In another embodiment, the combination vaccine comprises the attenuated *L. intracellularis* isolate designated ATCC accession No. PTA-4926 and antigenic material from at least one other pathogen, including but not limited to: *Clostridium* spp. (e.g., *Clostridium tetani*), equine influenza virus (EIV) (e.g., EIV-1, EIV-2), equine herpes virus (EHV) (e.g., EHV-1, EHV-2, EHV-3, EHV-4, EHV-5, EHV-6, EHV-7), alphavirus (e.g., eastern encephalitis virus, western encephalitis virus, Venezuelan encephalitis virus), or West Nile virus; and a pharmaceutically acceptable carrier.

[0046] The vaccines according to the invention are generally administered to susceptible animals, preferably swine, in one or more doses. The live or killed vaccine may be administered 1 or 2 times at 2 week intervals. For the attenuated, live vaccines, one dose is preferred. The preferred routes of administration of attenuated live isolates are intramuscular, oral or intranasal, but intramuscular and subcutaneous injection routes are most preferred for the killed vaccine.

[0047] Effective diagnosis of PPE has also been hindered by the time required to culture the causative bacteria. As a result of the present invention, development of diagnostic tools promoting rapid and accurate assays for the presence of *L. intracellularis* in biological samples taken from swine and other animals susceptible to PPE is now possible.

[0048] The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can be used as an antigen in an ELISA or other immunoassay, such as an immunofluorescent antibody test ("IFA"), to detect antibodies to *L. intracellularis* in the serum and other body fluids of animals suspected of being infected with the bacteria. The presently preferred immunoassay is an IFA as described in the example below. Alternatively, the bacteria of the instant invention can be used in a Western Blot assay.

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[0049] The preferred ELISA protocol according to the invention is as follows:

1. Add 0.1 ml/well antigen diluted in coating buffer. Incubate for 18 hours at 4°C.
2. Wash 3 times with PBS.
3. Add 0.25 ml of blocking buffer to each well of plate. Incubate 1 to 2 hours at 37°C.
4. Wash 3 times with wash buffer.
5. Dilute serum in blocking buffer and add 0.1 ml to the first wells of plate. Make serial 1:2 dilutions across the plate. Incubate for 1 hour at 37.
6. Wash 3 to 5 times with wash buffer.
7. Dilute conjugate in blocking buffer and add 0.1 ml to wells of plate and incubate for 1 hour at 37°C.
8. Wash 3 to 5 times with wash buffer.
9. Add substrate.
10. Measure absorbance of light with a spectrophotometer.
11. Wells in which antigen was not added are used as blanks.
12. Positive and negative control swine serum should also be used with each test.

[0050] The preferred Western Blot protocol is as follows:

1. Run antigen on 12% SDS-PAGE and transfer to nitrocellulose membrane.
2. Place membrane in blocking buffer for 2 hours.
3. Remove blocking buffer and rinse with PBS for 1 minute.
4. Dilute serum in blocking buffer and add to membrane. Incubate for 2 hours at room temperature.
5. Wash 3 times with wash buffer (5 minutes for each wash).
6. Dilute conjugate in blocking buffer and add to membrane. Incubate for 1 hour at room temperature.
7. Wash 3 times with wash buffer,
8. Add substrate for 10 minutes or until strong banding occurs.
9. Rinse with PBS.
10. Air dry and store in the dark.

[0051] The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can also be used to prepare antiserum or antibodies for diagnostic, prophylactic, or therapeutic use. The *L. intracellularis* bacteria of European origin of the instant invention, or components derived from such bacteria, can be administered to a non-human animal in an amount effective to elicit an immune response and the antiserum or plasma containing antibodies to the *L. intracellularis* bacteria, or components derived from such bacteria, can be collected according to methods known in the art and described herein.

[0052] The present invention is further described in the following examples which are provided for illustrative purposes only and are not to be construed as limiting. Indeed, other variants of the invention will be readily apparent to one of ordinary skill in the art.

EXAMPLE 1

PRODUCTION OF *L. INTRACELLULARIS* VACCINE

Isolation and Attenuation of *L. intracellularis* from the Intestines of European Pigs with Porcine Proliferative Enteropathy (PPE):

[0053] *L. intracellularis* virulent isolate DK 15540 (DK 15540, DK-15540 and 15540 are used interchangeably herein) was isolated by the University of Minnesota from an ileal homogenate of a Danish pig infected with acute porcine hemorrhagic enteropathy. This isolate has been deposited under the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 on January 9, 2003 and assigned accession number PTA-4927. The isolation process included scraping the mucosa from the ileum, homogenizing, trypsinizing for 30 minutes, and passing through a tissue grinder. The ileal homogenate was then passed through a series of filters consisting of 5.0, 1.0, and 0.65 μ m. The homogenate was diluted in sucrose phosphate glutamate buffer with 10% fetal bovine serum (FBS). Aliquots (6x1ml) of homogenate were made and stored at less than -70°C. The homogenate was used as inoculum to infect T-75 cm² flasks of McCoy cells. Cultures were monitored daily for McCoy cell infection by scraping McCoy cell monolayers, lysing cells by potassium chloride treatment, and placing the concentrated cell pellet on microscope slides stained by IFA using monoclonal antibodies specific for *L. intracellularis*. After eleven passages on anchorage dependent cell cultures, inoculum from passage eleven was transferred into a 250 ml spinner flask

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containing McCoy cells and grown in suspension until harvest. The Danish isolate of *L. intracellularis* (ATCC accession No. PTA-4927) was attenuated by continuous *in vitro* passage in McCoy cells for 80 weeks and tested for identity by monoclonal antibodies. The attenuated isolate was designated B3903 (B3903, B 3903 and B-3903 are used interchangeably herein). Isolate B3903 was deposited under the Budapest Treaty with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209 on January 9, 2003 and assigned ATCC accession No. PTA-4926.

Cultures:

Identification

[0054] Characteristic growth requirements, PCR reactions, and monoclonal antibody reactions were used to identify *L. intracellularis* Master and Working Seed materials.

Purity

[0055] Purity of Master Seed and Working Seed of *L. intracellularis* was determined by examining cultures with monoclonal antibody stains, conventional extraneous agents tests for bacteria and viruses and by sterility mycoplasma tests.

Virulence

[0056] Master Seeds of *L. intracellularis* were not virulent as demonstrated by the lack of ability of the Master Seed and Back passage inoculum to produce clinical signs and gross lesions that are observed in susceptible swine following exposure of virulent *L. intracellularis*, as further illustrated in Example 2, *infra*.

Range of Subcultures

[0057] Final harvested material from production of *L. intracellularis* did not exceed eleven passages from the Master Seed.

Medium Composition

[0058] EU McCoy Master Cell Stock (MCS) were grown and maintained in Dulbecco's Modified Eagle Medium with Ham's Fortified F 12 (DMEM/F12) and 1-10% (v/v) newborn bovine serum (NBS) or fetal bovine serum (FBS) (cell growth and maintenance media).

[0059] Master and Working Seed were stored in DMEM/F12 with 1-10% (v/v) NBS or FBS and 5-15% (v/v) glycerol (master and working seed storage media).

[0060] The final harvest product was stored in sucrose gelatin stabilizer (SGS) (final product storage media).

Propagation

[0061] Master and Working Seed cultures of *L. intracellularis* were propagated in EU McCoy MCS using cell growth and maintenance media as described *supra* and stored at less than or about -35°C.

Source of Tissue

[0062] *L. intracellularis* seed and production organisms were grown in McCoy Master Cell Stock (ATCC accession number CRL 1696, batch number F-10422). The mast cell stock was identified as 3894MMCSS at passage X. The master cell stock was passaged an additional six times and identified as EU McCoy MCS X+0. EU McCoy MCS passage X+0 was stored at -70°C ± 5°C or colder. Production vaccine was made in EU McCoy MCS cell line subcultures through the 40th passage.

Culture Containers

[0063] EU McCoy MCS were propagated in tissue culture flasks with 25-150 cm² surface area, Costar cell cubes, in roller bottles with 850 cm² to 2,2250 cm², in spinner flasks up to 40L capacity, and in bioreactors having 3L to 500L capacity.

[0064] Seed cultures of *L. intracellularis* were grown in 250-40,000ml spinner flasks, 850 cm² to 2,250 cm² roller bottles, tissue culture flasks with 25-150 cm² surface area, or in bioreactors having 3L to 500L capacity.

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[0065] Production cultures of *L. intracellularis* were grown in 6L to 40L spinner flasks or in bioreactors having 3L to 500L capacity.

Methods of Preparing Suspensions for Seeding or Inoculation

Seeding Cultures

[0066] Frozen or fresh *L. intracellularis* Master or expanded Working Seeds were thawed at room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$) or at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Bioreactors, spinner flasks or bottles previously seeded with McCoy cells at a cell density of 50,000 to 500,000 cells/ml were infected with *L. intracellularis* at a concentration of 1 to 10% (v/v) or MOI of 0.08 to 1.0. The infected culture was incubated under reduced oxygen concentrations by overlaying with a gas mixture composed of 86% N_2 , 4% H_2 and 10% CO_2 . The culture was incubated for 3 to 10 days at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$, pH 6.7 to 7.3, and with continuous agitation (10 to 100 rpm) to maintain adequate mixing for the cells to remain in suspension.

Production Cultures

[0067] Frozen or fresh *L. intracellularis* Master or expanded Working Seeds were thawed at room temperature ($25^{\circ}\text{C} \pm 3^{\circ}\text{C}$) or at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Bioreactors or spinner flasks (3L to 500L capacity) previously seed (0 to 7 days) with McCoy cells at a cell density of 50,000 to 500,000 cells/ml were infected with *L. intracellularis* at a concentration of 1 to 10% (v/v) or MOI of 0.08 to 1.0. The infected culture was incubated under reduced oxygen concentrations by overlaying or sparging with a gas mixture composed of 96% N_2 , 4% H_2 . The culture was incubated for 3 to 8 days at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$, pH 6.7 to 7.3, and with continuous agitation (10 to 100 rpm) to maintain adequate mixing for the cells to remain in suspension.

Inoculation Techniques for Seed and Production Cultures

Seed Cultures

[0068] Up to 10% (v/v) of Master or Working Seed are inoculated (MOI=0.08 to 1.0) into bioreactors, spinner flasks or bottles seed with McCoy cells at 0-7 days in growth medium.

Production Cultures

[0069] Up to 10% (v/v) of Production Seed was inoculated (MOI=0.08 to 1.0) into an appropriate volume of growth medium seeded with McCoy cells at 0 to 7 days with the appropriate McCoy cell density in 3L to 500L capacity vessels.

Incubation of Microorganisms

[0070] The cultures were incubated at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 3 to 10 days in a reduced oxygen atmosphere with agitation to maintain the suspension. Additional medium and/or McCoy cells can be added to continue the growth process.

[0071] Cultures were observed macroscopically during the incubation period for evidence of abnormal growth or signs of contamination.

Harvest:

Handling and Preparation of Cultures

[0072] Cultures were examined for signs of adequate bacterial growth by indirect fluorescent antibody (IFA) staining. Cultures that were ready for harvest exemplified 60 to 100% cell infectivity. Percent infectivity was determined by observation of at least three fields, each field containing enough McCoy cells to fill at least 80% of the area. To be considered infected, approximately 50% of the cell is filled with bacteria.

[0073] Potency of the harvest culture is tested by titration of the sample on McCoy cells which are fixed and stained using specific monoclonal antibody (anti-*L. intracellularis* monoclonal antibody VPM 53 Lot 31599 or equivalent; anti-mouse IgG-fluorescein conjugate (FITC) (ICN No. 55499) after 6 days of incubation at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

[0074] Cultures were examined visually for any obvious signs of contamination. Harvest occurred 3 to 10 days post inoculation.

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Harvesting Techniques and Specifications

[0075] Cell and fluid contents in the production culture bioreactor, spinner flasks and bottle were partially or fully collected in a sterile receiving vessel. Each production culture bioreactor, spinner flask and bottle was harvested individually or the contents of several vessels were pooled with the addition of SGS and stored at 1 °C to 7°C or colder. Harvested production culture was sampled for potency by TCID₅₀ and identification by IFA staining.

[0076] Production cultures exhibited at least 4.9 TCID₅₀/ml by IFA staining and were free of any evidence of contamination upon microscopic observation.

Preparation of Vaccine Product:

Concentration Methods

[0077] The vaccine product can be concentrated by various methods, e.g., by allowing the culture to settle with subsequent decanting of the supernatant, by membrane filtration (0.22 µm or smaller), perfusion, or by centrifugation.

[0078] Hydrolyzed gelatin solution is prepared by mixing gelatin with deionized water or water for injection at approximately 25% final total volume of SGS batch size and hydrolyzing in an autoclave for 120 minutes at 121°C.

Sucrose Gelatin Stabilizer (SGS)

[0079] The hydrolyzed gelatin solution (40.0 g/L) was then mixed with deionized water or water for injection at approximately 75% final total volume of SGS batch size. Potassium hydroxide (AR) (0.548 g/L), L-glutamic acid (1.440 g/L), dipotassium phosphate (AR) (2.508 g/L), potassium dihydrogen phosphate (AR) (1.030 g/L), and sucrose (AR) (150.00 g/L) were added and the solution was thoroughly mixed. The pH of the stabilizer was then adjusted to 6.8 to 7.0 with hydrochloric acid or sodium hydroxide solutions. Deionized water or water for injection was added to 100% of desired final volume of the SGS. The complete stabilizer was thoroughly mixed, and the entire solution was sterilized by filtration through a 0.1 micron filter.

[0080] Example of Assembly of Units to Make a Serial is shown in Table 1:

Table 1

<i>L. intracellularis</i>	200,000-300,000 ml
sucrose gelatin stabilizer (SGS)	100,000 ml (25% v/v)
DMEM/F12 (Maybe added to standardize the product)	0-150,000 ml
TOTAL VOLUME	400,000 ml
Volume of an average serial was 50L to 500L.	

Lyophilization

[0081] The vaccine product was lyophilized according to the procedure outlined in Table 2 for a 10 dose cycle (6.0 ml fill) or Table 3 for a 50/100 dose cycle (10.0 ml fill).

Table 2

STEPS	°C	Rate (minutes)	Hold (minutes)	Pressure (mT)
PRE-COOL *	5°	Na	Na	Atm
FREEZE	-47° ± 3°	As fast as possible	150	Atm
1° DRYING, 1st	-15° ± 2°	120	120	100-150
1° DRYING, 2nd	0° ± 2°	120	180	100-150
2° DRYING, 1st	32° ± 2°	240	180	60-80

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(continued)

STEPS	°C	Rate (minutes)	Hold (minutes)	Pressure (mT)
2° DRYING, 2nd	26° ± 2°	240	As fast as possible	60-80
Total Time: 1352 minutes (22.5 hrs) *Shelves are pre-cooled to 5°C ± 2°C during the loading of the lyophilizer.				

Table 3

STEPS	°C	Rate (minutes)	Hold (minutes)	Pressure (mT)
PRE-COOL*	5°	N/A	N/A	Atm
FREEZE	-48° ± 3°	60	90	Atm
1° DRYING	-15° ± 2°	60	1500	100-150
2° DRYING	26° ± 2°	60	600	60-80
Total Time: 2370 minutes (39.5 hrs) *Shelves are pre-cooled to 5° ± 2°C during the loading of the lyophilizer.				

EXAMPLE 2

SAFETY OF *L. INTRACELLULARIS* VACCINE

Purpose:

[0082] The objectives of this study were two-fold. The first objective of this study was to observe and compare the incidence of disease caused by three different low passage isolates (two of U.S. origin and one of European origin) of *L. intracellularis* in pigs at 6 ½ weeks of age. The second objective was to observe the safety of two high passage isolates (both of European origin) of *L. intracellularis* in pigs at 6 ½ weeks of age.

Materials and Methods:

Test Substances

[0083]

1. *L. intracellularis* low passage U.S. isolate N343
2. *L. intracellularis* low passage U.S. isolate N101494.
3. *L. intracellularis* low passage EU isolate DK 15540 p20
4. *L. intracellularis* high passage EU isolate DK 15540 p60
5. *L. intracellularis* high passage EU isolate DK 15540 p80 (Master Seed designation B3903).

Formulation of Test Substances

[0084] Low passage isolates were grown continuously for 10-20 weeks after isolation in McCoy cell suspension. High passage isolates were grown continuously for 60-80 weeks after isolation in McCoy cell suspension. All cultures were harvested via centrifugation at 10,000 RPM for 15 minutes. The McCoy cell culture pellets containing Lawsonia were resuspended in Sucrose-Phosphate-Glutamine (SPG) solution with 10% FBS.

Storage of Test Substances

[0085] Harvested cultures were stored at -70°C until the day of challenge. Challenge cultures of the same isolate but

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from various harvest dates were thawed and combined into plastic vaccine bottles, labeled, and stored at 4°C or on ice until the time of challenge.

Assay of Test Substances

[0086] TCID₅₀ was performed on all pooled challenge isolates at the time of challenge (day 0). The average titers (n=3) (TCID₅₀/ml) were as follows in Table 4:

Table 4

Test Substance	Average Titer (TCID ₅₀ /ml)
<i>L. intracellularis</i> N343	6.4
<i>L. intracellularis</i> N101494	6.1
<i>L. intracellularis</i> DK 15540 p20	6.2
<i>L. intracellularis</i> DK 15540 p60	6.87
<i>L. intracellularis</i> DK 15540 p80	7.4

Study Design

[0087] The study consisted of five experimental groups and one control group. On day 0 of the study, Group 1 (10 pigs, 6 ½ weeks of age) received one 10ml or equivalent intragastric (IG) dose of *L. intracellularis* low passage U.S. isolate N343. Group 2 (10 pigs, 6 ½ weeks of age) received one 10ml or equivalent IG dose of *L. intracellularis* low passage U.S. isolate N101494. Group 3 (10 pigs, 6 ½ weeks of age) received one 10ml or equivalent IG dose of *L. intracellularis* low passage EU isolate DK15540 p20. Group 4 (10 pigs, 6 ½ weeks of age) received one 10ml or equivalent IG dose of *L. intracellularis* high passage EU isolate DK15540 60 week. Group 5 (20 pigs, 6 weeks of age) received one 10ml or equivalent dose of *L. intracellularis* high passage EU isolate DK 15540 p80. Group 6 (10 pigs, 6 ½ weeks of age) designated as "Strict Controls" did not receive a treatment.

[0088] Daily health observations were made from initiation of study to the day of challenge of appropriate test animals. Clinical health (behavior, appetite, body condition, hair coat, and stool consistency on a scale of 1 to 4 were scored daily from day of challenge (day 0) to termination of study (day 21). Average daily weight gains (ADWG) were calculated from day of challenge (day 0) to termination of study (day 21). Fecal shedding of *L. intracellularis* was evaluated on days 0, 7, 14, and 21. The one animal that died (from Group 1) throughout the study was examined for gross and microscopic lesions. Death was determined to be due to lesions associated with PPE confirmed by histology and PCR analysis; the animal was not replaced. Qualitative analysis of Lawsonia content in feces was evaluated by PCR along with histological evaluation for *L. intracellularis* on the ileum and colon. Serum was collected on days 0, 7, 14, and 21 of the study.

Results:

Summary of Study Results

[0089]

Table 5											
Group	Treatment	No.of Pigs	Titer (TCID ₅₀ /ml)	Serology	Fecal Shedding	PCR	FA	Histology	Gross Lesions	ADWG	Clinical Scores
1	N343	9	7.4	19%	11%	0%	11%	44%	1	0.61	5.23
2	N101494	10	7.1	10%	10%	10%	30%	30%	1.2	0.8	5.15
3	DK15540p20	10	7.2	18%	13%	30%	10%	20%	1.2	0.86	5.01
4	DK15540p60	10	7.87	5%	0%	0%	0%	10%	1	0.9	5
5	DK15540p80	20	8.4	0%	0%	0%	0%	0%	1	1.05	5
6	Strict Controls	10	0	0%	0%	0%	0%	0%	1.05	0.91	5

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General Observations

[0090] Daily health observations were made until challenge. The clinical condition of the animals was evaluated daily following challenge for the duration of the study. Observations included: behavior, appetite, body condition, hair coat, and stool consistency. The clinical conditions of these animals were evaluated based on a numerical point system, which reflects the severity of illness. Scores ranged from 1 to 4 for each parameter. A score of 1 was given to an animal with a normal, healthy appearance, a score of 3 for an animal demonstrating severe clinical signs, and a score of 4 for an animal that has deceased. The average daily score for the strict controls, DK15540 p60, and DK15540 p80 was 5.0. The average daily scores for low passage material was N343 (5.23), N101494 (5.15), and DK15540 p20 (5.01). Statistical analysis on these results indicated no differences among treatment and control groups using Kruskal-Wallis Rank Sum Test.

Average Daily Weight Gains (ADWG)

[0091] Average daily weight gains were calculated from the time of challenge (day 0) to the termination of the study (day 21). The average weight gain per day for the strict control group was 0.9 pounds. The average weight gain for the low passage treatment groups was only 0.6 (N343), 0.8 (N101494), and 0.86 (DK15540 p20) pounds/day. The high passage treatment groups revealed the same or increased average weight gain per day compared to the strict control group that didn't receive challenge with 0.9 pounds/day (DK15540 p60) and 1.05 pounds/day (DK15540 p80) respectively. The mean difference in average daily weight gains was significantly lower in the N343 treatment group compared to higher passage treatment groups (DK15540 p60 and p80) and strict control group at day 21 of the study. (Pearson Chi-square $p < 0.05$).

Seroconversion

[0092] Seroconversion to Lawsonia exposure in pigs was measured by testing for the presence of anti-Lawsonia antibodies using an IFAT assay. On day 0, only the N343 treatment group observed detectable seroconversion in 2/10 pigs. Day 7 observed 2/9 pigs (N343) and 1/10 pigs (DK15540 p20) IFA positive for Lawsonia antibodies. On day 14, 2/9 pigs were IFA positive in N343 treatment group, 1/10 in DK15540 p20 and p60 respectively. Day 21 revealed 1/10 pigs (N343), 4/10 pigs (N101494) and 6/10 pigs (DK15540 p20) were IFA positive while high passage treatment groups (DK15540 p60 and p80) show no detectable seroconversion. Seroconversion to Lawsonia exposure increased in treatment groups receiving low passage *L. intracellularis* of both the U.S. and EU isolates day 21 of the study.

Fecal Shedding

[0093] PCR testing of the feces demonstrated shedding of *L. intracellularis* beginning on day 14 where 4/9 in the N343, 4/10 in the N101494, and 5/10 in the DK15540 p20 low passage animals tested positive. Both high passage treatment and strict control groups were PCR negative for day 14. On day 21, DK15540 p20 treatment group had 1 animal PCR positive while all other treatment and control groups were PCR negative. No evidence of shedding was observed in high passage isolate groups (DK15540 p60 and p80) using PCR throughout the study. PCR positive animals indicating active shedding of Lawsonia in their feces were more significant in the low passage isolate groups (N343, N101494, and DK15540 p20) on day 14 of the study than high passage isolate groups and strict controls (Pearson Chi-square $p < 0.05$).

PCR at Day 21: Ileums and Colons

[0094] PCR testing of mucosal scrapings from ileums and colons was performed after necropsy (day 21). Samples that were PCR positive for *L. intracellularis* colonization were (2/10 colons) in N101494 and (2/10 ileums, and 4/10 colons) in DK 15540 p20 low passage isolate groups. All other treatment and control groups were PCR negative in tissues day 21 of the study. Results indicated ileums and colons of pigs in DK15540 p20 were significantly more colonized with *L. intracellularis* compared to all treatment and control groups (*Pearson Chi-square $p < 0.05$).

Histology

[0095] Sections of the terminal ileum and colon were collected at necropsy (day 21) and placed in buffered formalin for histological analysis. Presence of intracellular bacteria and crypt hyperplasia was observed in tissues stained with Hematoxylin and Eosin (H&E) and Warthin-Starry silver reagents of 4/9 pigs (N343), 3/10 pigs (N101494), 2/10 pigs (DK15540 p60) and 1/20 pigs (DK15540 p80). Lesion development was confirmed by florescent antibody staining using

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monoclonal antibodies against *Lawsonia intracellularis* in 1/9 pigs (N343), 3/10 pigs (N101494), and 1/10 pigs (DK15540 p60). FA detected no lesions caused by *Lawsonia* in the colons of all treatment and control groups. FA results indicated significant lesion development in ileums of pigs in N101494 treatment group compared to all treatment and control groups. H&E / silver staining showed significant lesion development associated with PPE in N343 treatment group compared to all treatment and control groups. (Pearson Chi-square $p < 0.05$).

Gross Scores

[0096] Ileums and colons were scored at the time of necropsy (day 21) for lesions associated with PPE. Tissues were given a score of 1 for normal appearance (no lesion development), a score of 2 for lesions demonstrating mild thickening, 3 for moderate thickening, and a score of 4 for severe thickening. Strict controls had an average clinical score of 1.05, N343 (1.0), N101494 (1.2), DK15540 p20 (1.2), and DK15540 p60 and p80 (1.0). Average gross lesion scores indicated no statistical difference between control and treatment groups using ANOVA test for multiple comparisons.

Conclusions:

[0097] Based on the data collected, this study demonstrated that pigs challenged with a low passage dose of N343, N101494, and DK15540 with a TCID₅₀ greater than 1×10^7 bacteria/dose increases the incidence of PPE in these animals. High passage isolates (DK15540 p60 and p80) given to pigs of the same age with a TCID₅₀ greater than 1×10^7 were proven safe and show reduction of colonization and lesion development associated with PPE. This conclusion was based on PCR on the mucosa of the ileum, histopathology, and FA stains of tissue sections.

[0098] A reduction of shedding *L. intracellularis* in the feces determined by PCR was evident in high passage isolates compared to low passage isolates. Average daily weight gains calculated for all treatment and control groups support this conclusion by demonstrating positive uniform daily weight gain in groups given the high passage isolate and strict controls compared to groups given low passage isolates especially animals in the N343 treatment group. This observation indicates reduction of weight gains in animals given low passage material which supports adequate and similar grow performance of animals given high passage material with animals receiving no challenge material. Compared to the strict controls, the high passage isolates showed no negative impact on weight gain and overall health based on clinical scores.

EXAMPLE 3

EFFICACY AND MINIMAL PROTECTIVE TITER

Purpose:

[0099] The objectives of this study were to determine the minimal protective titer of a vaccine comprising isolate B3903 (lyophilized) (DK 15540, passage 80) ("B3903 (Lyophilized) vaccine" and "B3903 vaccine" are used interchangeably herein) administered by oral drench in pigs 3 weeks of age and to demonstrate efficacy against a virulent heterologous pure culture challenge with low passage *L. intracellularis*, the causative agent of Porcine Proliferative Enteropathy (PPE) in swine.

Materials and Methods:

Test Substance: Attenuated Live Culture of *L. intracellularis*, Isolate B3903

Formulation of B3903 (Lyophilized) Vaccine

[0100] DK 15540 isolates were grown continuously for 80 weeks after isolation in McCoy cell suspension. All cultures were harvested via centrifugation at 10,000 RPM for 15 minutes. The McCoy cell culture pellets containing *Lawsonia* were resuspended in Sucrose-Phosphate-Glutamine (SPG) solution with 10% FBS. Desiccation was performed as described in Example 1, *supra*. The lyophilized product is reconstituted in water, q.s. ad 2.0 ml, for injections.

Storage of B3903 (Lyophilized) Vaccine

[0101] The vaccine was stored at 2°C-8°C until ready for use. After resuspension, the vaccine was stored on ice until administration.

Doses of B3903 (Lyophilized) Vaccine

[0102]

1. High dose (Treatment Group 1): 1 x 2mL (6.0 logs/dose) via direct oral drench on day 0 of the study.
2. Medium dose (Treatment Group 2): 1 x 2mL (4.9 logs/dose) via direct oral drench on day 0 of the study.
3. Low dose (Treatment Group 3): 1 x 2mL (3.8 logs/dose) via direct oral drench on day 0 of the study.

Test Substance: Placebo

[0103] A placebo consisting of uninfected McCoy tissue culture cells suspended in DMEM/F12 growth medium fortified with 5% NBS was given to treatment Groups 4 (challenge control) and 5 (negative control) on day 0 of the study. This substance was administered to piglets in treatment Group 4 by direct oral drench and given 1 x 2mL of placebo per test animal.

Test Substance: *L. intracellularis* N101494 Virulent Challenge

[0104] *L. intracellularis* N101494 was obtained from the intestines of a 12 week old pig from an Indiana farm (United States Patent 5,714,375).

Formulation of *L. intracellularis* N101494 Virulent Challenge

[0105] *L. intracellularis* challenge material was grown continuously in McCoy cell suspension no more than 30 passages after initial isolation from infected gut tissue. Active cultures (2 x 3L) identified as SF 1422 and SF 1423 in addition to (1L) SF 1421 were grown in McCoy cell suspension for 7 days to 15-30% McCoy cell infection. On the day of challenge (day 21), active cultures were harvested via centrifugation at 10,000 RPM for 15 minutes and cell pellets resuspended in 350mL total volume with SPG stabilizer. Harvested active culture was pooled with 300mL of frozen 10X to 20X concentrated challenge stocks of low passage N101494 at various passages (pass 24 to 27 post isolation).

Storage of *L. intracellularis* N101494 Virulent Challenge

[0106] Final formulations ready for challenge were stored at 2°C to 8°C or on ice until inoculation.

Pre/ Post-Vaccination and Challenge Titers

[0107] Results from the TCID₅₀ assay verified the amount of live *L. intracellularis* administered to each test animal per dose during vaccination and challenge. The average titers (n=5) of pre and post titrations for the B3903 vaccine and the challenge material (*L. intracellularis* N101494) were as follows in Table 6:

Table 6

Group	Treatment (logs/dose)	Average logs/dose (2mL) Pre-vaccination (TCID ₅₀ /mL)	Average logs/dose (2mL) Post-vaccination (TCID ₅₀ /mL)	Total Average logs/dose
1	B3903 vaccine high dose	6.07	5.89	6.0
2	B3903 vaccine medium dose	4.94	4.84	4.9
3	B3903 vaccine low dose	3.9	3.5	3.8
4	Challenge Controls	7.85	7.57	7.71

Study Design

[0108] Sixty-five healthy *L. intracellularis* negative weaned piglets at 3 weeks of age were randomly divided into 5 treatment groups and housed separately throughout the study. On day 0, treatment Group 1 (15 pigs) received a 2mL dose (6.0 logs/dose) of B3903 vaccine by direct oral drench. Treatment Group 2 (15 pigs) received 1 x 2mL dose of

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B3903 vaccine titrated at 4.9 logs/dose by direct oral drench. Treatment Group 3 (15 pigs) received 1 x 2mL dose of B3903 vaccine titrated at 3.8 logs/dose by direct oral drench.

[0109] Treatment Groups 4 and 5 (10 pigs/group) received 1 x 2mL dose of placebo by direct oral drench.

[0110] On day 21 of the study (3 weeks post vaccination), test pigs in treatment Groups 1-4 received 1 x 10mL dose of virulent low passage pure culture *L. intracellularis* heterologous isolate N101494 by gastric gavage.

[0111] On day 42 of the study (3 weeks post challenge), all treatment Groups (1-5) were euthanized and necropsied for gross and microscopic lesion analysis for PPE.

[0112] Daily health observations were made from study initiation to the day of challenge for each test animal. Clinical health (diarrhea, behavior, and body condition) were scored daily from day of challenge (day 21) to termination of study (day 42). Weights were taken on day of vaccination (day 0), day of challenge (day 21) and on day of study termination (day 42) to calculate average daily weight gains of each treatment group. Fecal shedding of *L. intracellularis* was evaluated by polymerase chain reaction (PCR) by testing fecal swabs (f-PCR) on days 0, 7, 14, 21, 28, 35, and 42 of the study. All animals euthanized at study termination (day 42) were examined for gross and microscopic lesions. Qualitative analysis of bacterial content in tissues was evaluated by PCR (t-PCR) along with histological evaluation for *L. intracellularis* in the ileum, colon, tonsil, and mesenteric lymph node day 42 of the study. Serum was collected on days 0, 7, 14, 21, 28, 35, and 42 of the study. Serum was tested using the indirect florescent antibody test (IFAT) to detect anti-Lawsonia antibodies in test animals. Treatment group comparisons were made by data analysis of average daily weight gains post vaccination and post challenge, clinical scores, seroconversion rates (IFAT), colonization (t-PCR), fecal shedding (f-PCR), gross lesion, and microscopic lesion development by immunohistochemistry (IHC).

Results:

Summary of Primary Results

[0113]

Table 7

Treatment Group	Pigs per Group	Treatment. Group ID (logs/dose)	Average Gross Lesion Scores (ileum)	Average Gross Lesion Scores (colon)	Average Micro-Lesion scores (ileum)	Average Micro-Lesion Scores (colon)
1	15	B3903 vaccine high dose (6.0)	1.5 ab	1.2 a	0.2 ab	0.1 a
2	15	B3903 vaccine medium dose (4.9)	1.2 ab	1.0 a	0.4 ab	0.0 a
3	15	B3903 vaccine low dose (3.8)	2.5 b	1.5 b	1.0 b	0.3 a
4	10	Challenge Controls	3.6 c	2.2 b	2.4 c	1.5 b
5	10	Strict Controls	1.0 a	1.0 a	0.0 a	0.0 a
*Like letters indicate no significant difference (p<0.05)						

[0114] Final test results for each treatment group revealed significant gross and microscopic lesion development of the ileum and colon in non-vaccinated, challenge control pigs (Group 4) compared to vaccinated pigs regardless of dose (high, medium, and low) in Groups 1, 2, and 3 (p<0.05). Average gross lesion scores of the vaccine-low dose colons were not significantly different than challenge controls. The vaccine-low dose group (Group 3) is the only treatment group that received vaccine and had significant lesion development (gross and microscopic) in comparison to the strict control group which did not receive a vaccination, placebo, or challenge throughout the study.

Clinical Scores

[0115] Clinical scores were recorded for each animal daily from day of challenge (day 21) to necropsy (day 42). Clinical scores were calculated to obtain an average daily clinical score reflecting the severity and duration of sickness among treatment groups due to receiving a virulent challenge of *L. intracellularis*. Average clinical scores for each treatment group are summarized in Table 8.

Table 8

Treatment Group	Group Identification	Average Clinical Score
1	B3903 vaccine - high dose	3.1 a
2	B3903 vaccine - medium dose	3.0 a
3	B3903 vaccine - low dose	3.0 a
4	Challenge Control	3.0 a
5	Strict Control	3.0 a
*Like letters denote no significant differences among average clinical scores from the day of challenge to necropsy (p<0.05).		

[0116] Statistical analysis of clinical scores was accomplished by reviewing the data in relation to treatment groups receiving average clinical scores using 1-way ANOVA. There was no evidence of significant differences among non-vaccinated, challenge controls and strict controls compared to the high, medium, or low dose vaccine groups.

Average Daily Weight Gains

[0117] Average daily weight gains (ADWG) were calculated from the time of vaccine administration (day 0), to challenge administration (day 21), to the termination of the study (day 42). Statistical analysis of weight gain differences (lbs. = pounds) between vaccine-high dose and non-vaccine, challenge control treatment groups revealed evidence of a significant difference from the time of challenge administration to necropsy (p<0.05). The weight data is summarized in Table 9.

Table 9

Treatment Group	Group Identification	Average Initial Weight (lbs.)	ADWG (day 0 - 21) (lbs.)	ADWG (day 21 - 42) (lbs.)	ADWG Total (lbs.) (Vaccination to Necropsy)
1	B3903 vaccine high dose	17.8 a	0.94 a	1.63 a	1.29a
2	B3903 vaccine medium dose	17.7 a	1.00 a	1.60 ab	1.30a
3	B3903 vaccine low dose	18.0 a	0.94 a	1.48 ab	1.21a
4	Challenge Control	17.7 a	0.96 a	1.45 b	1.21a
5	Strict Control	17.7 a	0.94 a	1.63 ab	1.28a
*Like letters denote no significant differences between treatment groups in ADWGs from day of vaccination to challenge and from day of challenge to necropsy (p<0.05).					

Seroconversion (IFAT)

[0118] Serum samples were collected weekly from all test animals in each treatment group and tested for presence of anti-Lawsonia IgG antibodies on days 0, 7, 14, 21, 28, 35, and 42 of the study. Positive and negative IFAT control

samples were 100% accurate in all assays performed in this study. On days 0 through 21 (3 weeks post vaccination), all test animals in each treatment group were IFA negative. On day 28 (1 week post challenge) of the study, 1/15 (6.7%) test animals in the vaccine-high dose treatment group were IFA positive while all others tested IFA negative. On day 35 (2 weeks post challenge) of the study, 4/15 (26.7%) pigs in the vaccine-high dose and medium dose treatment groups and 1/10 (10%) pigs in the non-vaccine, challenge control treatment group were IFA positive for Lawsonia antibodies. Both vaccine-low dose and strict control treatment groups were IFA negative on day 35. On day 42 (3 weeks post challenge) of the study, 8/10 (80%) pigs in the challenge control group, 6/15 (40%) pigs in the vaccine-medium dose group, 5/15 (33.3%) pigs in the vaccine-low dose group, and 3/15 (20%) pigs in the vaccine-high dose group were IFA positive. The strict control treatment group was IFA negative at study termination (day 42).

[0119] Seroconversion data was analyzed using the Chi-square statistic. For results obtained in the strict control treatment group, a chi-square statistic was not computed due to the 100% negative responses found for each test animal throughout the study. In treatment groups receiving a vaccine or placebo, IFAT results were compared using Chi-square statistic with an estimation of exact p-value (Monte-Carlo). Positive IFAT results obtained from challenge groups receiving a virulent pure culture challenge were significantly higher than those receiving vaccine-high dose and vaccine-low dose (p<0.05) at day 42 (3 weeks post challenge) of the study.

Fecal Shedding of *L. intracellularis* (PCR)

[0120] Fecal swabs were collected weekly from all test animals in each treatment group and tested for the presence of *L. intracellularis* by PCR on days 0, 7, 14, 21, 28, 35, and 42 of the study. Positive and negative DNA extraction and PCR reaction controls were 100% accurate for each assay conducted in this study. All test animals in each treatment group were fecal PCR negative for *L. intracellularis* from day 0 (vaccination) to day 21 (challenge). Pigs in the strict control group remained fecal PCR negative for *L. intracellularis* in their feces throughout the study. Fecal shedding of *L. intracellularis* (fecal PCR positive) was evident in various treatment groups each week after challenge inoculation. These results are summarized in Table 10.

Table 10

Treatment Group	Group Identification	Day 28 (1 week post challenge)	Day 35 (2 weeks post challenge)	Day 42 (3 weeks post challenge)
1	B3903 vaccine high dose	0/15 (0%) a	2/15 (13.3%) a	0/15 (0%) a
2	B3903 vaccine Medium dose	6/15 (40%) ab	5/15 (33.3%) a	0/15 (0%) a
3	B3903 vaccine low dose	4/15 (26.7%) ab	8/15 (53.3%) ab	2/15 (13.3%) ab
4	Challenge Control	4/10 (40%) b	7/10 (70%) b	4/10 (40%) b
5	Strict Control	0/10 (0%)	0/10 (0%)	0/10 (0%)
* Like letters denote no significant difference among treatment groups receiving a challenge (p<0.05).				

[0121] Statistical analysis using Chi-square statistic with an estimation of the exact p-value (Monte Carlo) compared the positive responses in each vaccine treatment group to the non-vaccine, challenge control group only. Results from the strict control group were withdrawn from group comparisons because each pig was 100% fecal PCR negative for *L. intracellularis* throughout the study. On day 28 (1 week post challenge), pigs receiving a high dose of vaccine had significantly less shedding of *L. intracellularis* than non-vaccinated, challenged pigs (p = 0.017). On day 35 (2 weeks post challenge), pigs receiving a high dose (p = 0.0024) and medium dose (p = 0.041) of vaccine had significantly less shedding of *L. intracellularis* in their feces than non-vaccinated challenged pigs. On day 42 (study termination), again, pigs receiving a high and medium dose of vaccine (p = 0.017) had significantly less shedding of *L. intracellularis* in their feces compared to the non-vaccinated, challenged pigs. Pigs in the low dose group were not significantly less PCR positive than the challenge controls on any day post challenge.

L. intracellularis Tissue Colonization (PCR)

[0122] Polymerase Chain Reaction testing of tissue sections of the terminal ileum, colon, tonsil, and mesenteric lymph

node were performed after necropsy (day 42 of the study). In the vaccine-high dose group, 1/15 (6.7%) pigs were PCR positive for *L. intracellularis* colonization in the tonsil while all other treatment groups were PCR negative. Ileitis PCR testing of mesenteric lymph tissue revealed 3/10 (30%) pigs in the non-vaccinated, challenge controls were positive for *L. intracellularis* colonization while all other treatment groups were PCR negative. Ileitis PCR testing of mucosal scrapings of the terminal ileum revealed 4/10 (40%) pigs in the challenge controls, 4/15 (26.7%) pigs in the vaccine-low dose group, 2/15 (13.3%) pigs in the vaccine-medium dose group, and 1/15 (6.7%) pigs in the vaccine-high dose group PCR positive for *L. intracellularis* colonization. Ileitis PCR testing of the colon revealed 3/10 (30%) pigs in the challenge controls, 5/15 (33.3%) pigs in the vaccine-low dose group, 1/15 (6.7%) pigs in the vaccine-medium dose group, and 2/15 (13.3%) pigs in the vaccine-high dose group PCR positive for *L. intracellularis* colonization. No evidence of *L. intracellularis* colonization was seen in tissues of the strict control group.

[0123] Statistical analysis of ileitis PCR positive results were compared among treatment groups using Chi-square statistic with Monte Carlo approximation of the exact p-value.

Mesenteric lymph node PCR results indicated evidence of a significant difference among non-vaccinated, challenge controls and all treatment groups receiving a vaccine treatment ($p = 0.054$). No statistical significance was evident among treatment groups in *L. intracellularis* colonization in the ileum, colon, or tonsil at day 42 of the study.

Histology (IHC / H&E)

[0124] Sections 2 to 4cm in length of tonsil, mesenteric lymph node, terminal ileum, and colon were collected at necropsy (day 42) and placed in 10% buffered formalin for histological analysis. *Lawsonia intracellularis* was not detected by IHC staining of tonsil sections in all treatment groups at necropsy. The strict control group was negative for *L. intracellularis* by IHC in all tissue samples taken at necropsy. Presence of *L. intracellularis* and microscopic lesions associated with PPE were found in the ileums of 9/10 (90%) pigs of the non-vaccinated, challenge controls, 6/15 (40%) pigs of the vaccine-low dose group, 3/15 (20%) pigs of the vaccine-medium dose group, and 1/15 (6.7%) pigs of the vaccine-high dose group respectively. Microscopic lesions were evident in the colon of 8/10 (80%) pigs in the challenge control group, 3/15 (20%) pigs in the vaccine-low dose group, and 1/15 (6.7%) pigs in the vaccine-high dose group. The vaccine group receiving a medium dose was IHC negative for *L. intracellularis* in the colon. Stained sections of mesenteric lymph node produced only 1/10 (10%) pigs in the challenge control that had *L. intracellularis*. Positive IHC results in sections of the ileum and colon among treatment groups correlated well to the presence of marked crypt hyperplasia demonstrated by H&E methodology.

[0125] Statistical analysis of quantitative IHC data was accomplished using 1-way ANOVA and Kruskal-Wallis Rank Sum tests followed by specific contrasts of p-values between the challenge group and strict control groups to each vaccine dosage group. Statistical analysis reveal evidence of a significant lesion development due to *L. intracellularis* in the ileum and colon of the challenge control group compared to vaccinated pigs regardless of dose on day 42 of the study ($p < 0.001$). Statistical significance was not evident in average IHC lesion scores of the ileum and colon in vaccinated animals receiving a high or medium dose of vaccine compared to the strict control treatment group ($p > 0.05$). Pigs receiving a low dose of vaccine had significantly higher mean microscopic lesion scores due to *L. intracellularis* in the ileum compared to the strict control group ($p < 0.05$).

Gross Scores

[0126] The ileum and colon of each test animal were scored at the time of necropsy (day 42) for gross lesions associated with PPE. Tissues from test pigs in the strict control were normal, did not contain lesions, and received average lesion scores of 1.1 (ileum) and 1.0 (colon). Tissues of test animals in the non-vaccinated, challenge control group received the highest average gross lesion score for the ileum (3.6) and colon (2.0) among treatment groups.

Test pigs in the vaccine-low dose group received average gross lesion scores of 2.5 (ileum) and 1.5 (colon). Vaccine-medium dose test pigs received average gross lesion scores of 1.5 (ileum) and 1.0 (colon). Tissues of the vaccine-high dose group received average gross lesion scores of 1.5 (ileum) and 1.2 (colon).

[0127] Statistical analysis of average gross lesion scores among treatment groups was accomplished by using 1-way ANOVA. Average gross lesion scores indicated evidence of a significant difference between ileums in non-vaccinated, challenge control group and vaccine-medium and high dose groups respectively ($p < 0.001$). Evidence of a significant difference was observed among average gross ileum scores of the challenge control compared to the vaccine-low dose group ($p < 0.01$). Average gross lesion scores of the colon were significantly higher in the challenge control group compared to the vaccine-medium and high dose groups respectively ($p < 0.05$). In addition, significant gross lesion development was observed in the ileum of the vaccine-low dose group compared to the strict control group ($p < 0.001$). No evidence of a statistical significance in average gross lesion scores (ileum and colon) were evident in vaccine-medium and high dose groups compared to the strict control treatment group.

Conclusions

[0128] This study demonstrated protection in 3-week-old pigs against PPE was accomplished when given an oral 2mL dose of B3903 (Lyophilized) vaccine containing a minimum of 4.9 logs of live *L. intracellularis* per dose. Similar if not slightly better protection was evident in the vaccine-high dose treatment group that received 6.0 logs/dose 3 weeks prior to challenge. The low dose vaccine group (3.8 logs/dose) did not indicate adequate protection against a virulent pure culture heterologous challenge compared to the test animals receiving a high or medium dose of the experimental vaccine. Statistical differences were evident among the vaccine-low dose group and non-vaccinated, challenge controls regarding the severity of microscopic lesions in the ileum and colon ($p < 0.001$) and average gross lesion scores of the ileum ($p < 0.01$). However, significant PPE lesions were observed in the ileum (microscopic lesions) and colon (gross lesions) of this group compared to the strict control group that did not receive vaccine or challenge.

[0129] In summary, the data from this study demonstrated that: (1) the minimum protective titer of a single oral administration of B3903 (Lyophilized) vaccine to 3 week old pigs is 4.9 logs/dose; (2) B3903 (Lyophilized) vaccine is efficacious against a virulent low passage pure culture *L. intracellularis*, heterologous isolate N101494; and (3) B3903 (Lyophilized) vaccine aids in the reduction of gross and microscopic lesions, tissue colonization, and fecal shedding of *L. intracellularis* in vaccinated pigs compared to non-vaccinated pigs.

Claims

1. An avirulent *Lawsonia intracellularis* isolate, wherein said avirulent isolate is *Lawsonia intracellularis* deposit isolate ATCC No. PTA-4926 or any avirulent *Lawsonia intracellularis* isolate of European origin, characterized in that said avirulent *Lawsonia intracellularis* does not cause fecal shedding at day 14 post vaccination.
2. A vaccine for the immunization of an animal, comprising a pharmaceutically effective amount of an avirulent *Lawsonia intracellularis* according to claim 1, and a pharmaceutically acceptable carrier.
3. The vaccine according to claim 2 wherein the pharmaceutically effective amount of the avirulent isolate of *Lawsonia intracellularis* is 10^3 TCID₅₀ to 10^6 TCID₅₀ per dose.
4. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from at least one of the following pathogens: *Salmonella* spp., *Erysipelothrix* spp., *Haemophilus* spp., *Mycoplasma* spp., *Leptospira* spp., *Clostridium* spp., *Streptococcus* spp., *Brachyspira* spp., circovirus, porcine reproductive and respiratory syndrome (PRRS) virus, swine influenza virus (SIV), transmissible gastro-enteritis (TGE) virus, parvovirus, and *Escherichia coli*; and a pharmaceutically acceptable carrier.
5. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from *Salmonella choleraesuis*, *Erysipelothrix* spp, transmissible gastro-enteritis (TGE) virus, *Escherichia coli*, *Clostridium* spp., and *Brachyspira* spp.; and a pharmaceutically acceptable carrier.
6. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from *Salmonella choleraesuis* and *Erysipelothrix* spp.; and a pharmaceutically acceptable carrier.
7. A vaccine for the immunization of an animal, comprising the avirulent isolate of *Lawsonia intracellularis* according to claim 1 and antigenic material from at least one of the following pathogens: *Clostridium* spp., equine influenza virus (EIV), equine herpes virus (EHV), alphavirus, and West Nile virus; and a pharmaceutically acceptable carrier.
8. The avirulent isolate of *Lawsonia intracellularis* according to claim 1 for use as a vaccine.
9. The avirulent isolate of *Lawsonia intracellularis* according to claim 8 for use as vaccine for protecting an animal against porcine proliferative enteropathy.
10. A method for making a vaccine for inducing an immune response to *Lawsonia intracellularis* bacteria in an animal, comprising the steps of: (a) incubating a *Lawsonia intracellularis* isolate according to claim 1 in culture cells which are in suspension at an oxygen concentration of less than 10 percent to cultivate said bacteria; and (b) mixing said cultivated bacteria with a pharmaceutically acceptable carrier.

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11. A method for cultivating *Lawsonia intracellularis* according to claim 1 comprising:

- (a) infecting cultured cells with an inoculum comprising the avirulent *Lawsonia intracellularis* according to claim 1,
- (b) incubating said infected cells at an oxygen concentration of less than 10 percent while maintaining said infected cells in suspension by agitation of said cells, and
- (c) harvesting at least a portion of said *Lawsonia intracellularis*.

12. The method according to claim 11 wherein the oxygen concentration is in the range of 0 percent to 8 percent.

13. The method according to claim 12 wherein the oxygen concentration is in the range of 0 percent to 3 percent.

14. The method according to claim 11 wherein said incubation occurs at a carbon dioxide range of 6 percent to 10 percent.

15. The method according to claim 11 wherein said cultured cells are selected from the group consisting of: HEp-2, McCoy, and IEC-18 cells.

16. The method according to claim 11 wherein said IEC-18 cells are cultured on microcarriers.

17. The method according to claim 11 wherein the infected cells are incubated according to step (b) for a period of 2 to 10 days.

18. The method according to claim 11 further comprising a step (d) of lyophilizing said harvested bacteria.

19. The method according to claim 18 wherein said lyophilizing comprises a primary drying step and a secondary drying step.

20. A method for the diagnosis of porcine proliferative enteropathy comprising performing an assay to detect antibodies to *Lawsonia intracellularis* in a non-human animal subject comprising:

- (a) contacting a sample from the subject with the *Lawsonia intracellularis* isolate according to claim 1 to form an antigen-antibody complex; and
- (b) detecting the formation of said complex,

wherein the presence of said complex correlates positively with a diagnosis of porcine proliferative enteropathy.

21. The method according to claim 20 wherein the assay comprises an assay selected from the group consisting of: radioimmunoassay, enzyme-linked immunosorbent assay, fluorescence immunoassay, and immunoelectrophoresis assay.

22. A method for producing antisera against *Lawsonia intracellularis* in a non-human animal comprising:

- (a) administering the *Lawsonia intracellularis* isolate of claim 1 to a non-human animal in an amount effective to elicit an immune response; and
- (b) collecting antiserum or plasma containing antibodies to said *Lawsonia intracellularis*.

Patentansprüche

1. Avirulentes *Lawsonia intracellularis*-Isolat, wobei es sich beim avirulenten Isolat um das *Lawsonia intracellularis*-Hinterlegungsisolat ATCC Nr. PTA-4926 oder um ein beliebiges avirulentes *Lawsonia intracellularis*-Isolat europäischen Ursprungs handelt, **dadurch gekennzeichnet, dass** das avirulente *Lawsonia intracellularis* 14 Tage nach einer Impfung keine fäkale Ausscheidung verursacht.
2. Impfstoff zur Immunisierung eines Tiers, umfassend eine pharmazeutisch wirksame Menge eines avirulenten *Lawsonia intracellularis* nach Anspruch 1 und einen pharmazeutisch verträglichen Träger.
3. Impfstoff nach Anspruch 2, wobei die pharmazeutisch wirksame Menge des avirulenten Isolats von *Lawsonia intracellularis* 10^3 TCID₅₀ bis 10^6 TCID₅₀ pro Dosis beträgt.

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4. Impfstoff zur Immunisierung eines Tiers, umfassend das avirulente Isolat von *Lawsonia intracellularis* nach Anspruch 1 und antigenes Material von mindestens einem der folgenden Pathogene: *Salmonella* spp., *Erysipelothrix* spp., *Haemophilus* spp., *Mycoplasma* spp., *Leptospira* spp., *Clostridium* spp., *Streptococcus* spp., *Brachyspira* spp., Circovirus, porcines reproduktives und respiratorisches Syndrom (PRRS)-Virus, Schweineinfluenzavirus (SIV), Virus der übertragbaren Gastro-enteritis (TGE), Parvovirus und *Escherichia coli*; und einen pharmazeutisch verträglichen Träger.
5. Impfstoff zur Immunisierung eines Tiers, umfassend das avirulente Isolat von *Lawsonia intracellularis* nach Anspruch 1 und antigenes Material von *Salmonella choleraesuis*, *Erysipelothrix* spp., Virus der übertragbaren Gastroenteritis (TGE), *Escherichia coli*, *Clostridium* spp. und *Brachyspira* spp.; und einen pharmazeutisch verträglichen Träger.
6. Impfstoff zur Immunisierung eines Tiers, umfassend das avirulente Isolat von *Lawsonia intracellularis* nach Anspruch 1 und antigenes Material von *Salmonella choleraesuis* und *Erysipelothrix* spp.; und einen pharmazeutisch verträglichen Träger.
7. Impfstoff zur Immunisierung eines Tiers, umfassend das avirulente Isolat von *Lawsonia intracellularis* nach Anspruch 1 und antigenes Material von mindestens einem der folgenden Pathogene: *Clostridium* spp., equines Influenzavirus (EIV), equines Herpesvirus (EHV), Alphavirus und West Nile-Virus; und einen pharmazeutisch verträglichen Träger.
8. Avirulentes Isolat von *Lawsonia intracellularis* nach Anspruch 1 zur Verwendung als Impfstoff.
9. Avirulentes Isolat von *Lawsonia intracellularis* nach Anspruch 8 zur Verwendung als Impfstoff zum Schutz eines Tiers gegen porcine proliferative Enteropathie.
10. Verfahren zur Herstellung eines Impfstoffes zur Induktion einer Immunantwort gegen *Lawsonia intracellularis*-Bakterien bei einem Tier, umfassend folgende Stufen: (a) Inkubieren eines *Lawsonia intracellularis*-Isolats nach Anspruch 1 in Kulturzellen, die sich in Suspension bei einer Sauerstoffkonzentration von weniger als 10 % befinden, um die Bakterien zu züchten; und (b) Mischen der gezüchteten Bakterien mit einem pharmazeutisch verträglichen Träger.
11. Verfahren zur Züchtung von *Lawsonia intracellularis* nach Anspruch 1, umfassend die folgenden Stufen:
- (a) Infizieren von gezüchteten Zellen mit einem Inoculum, das das avirulente *Lawsonia intracellularis* nach Anspruch 1 umfasst,
- (b) Inkubieren der infizierten Zellen bei einer Sauerstoffkonzentration von weniger als 10 %, wobei die infizierten Zellen durch Bewegen der Zellen in Suspension gehalten werden, und
- (c) Ernten mindestens eines Teils des *Lawsonia intracellularis*.
12. Verfahren nach Anspruch 11, wobei die Sauerstoffkonzentration im Bereich von 0 bis 8 % liegt.
13. Verfahren nach Anspruch 12, wobei die Sauerstoffkonzentration im Bereich von 0 bis 3 % liegt.
14. Verfahren nach Anspruch 11, wobei die Inkubation bei einem Kohlendioxidbereich von 6 bis 10 % erfolgt.
15. Verfahren nach Anspruch 11, wobei die gezüchteten Zellen aus der Gruppe ausgewählt werden, die aus Hep-2-, McCoy- und IEC-18-Zellen bestehen.
16. Verfahren nach Anspruch 11, wobei die IEC-18-Zellen auf Mikroträgern gezüchtet werden.
17. Verfahren nach Anspruch 11, wobei die infizierten Zellen für einen Zeitraum von 2 bis 10 Tagen gemäß Stufe (b) inkubiert werden.
18. Verfahren nach Anspruch 11, ferner umfassend eine Stufe (d) der Lyophilisation der geernteten Bakterien.
19. Verfahren nach Anspruch 18, wobei die Lyophilisation eine primäre Trocknungsstufe und eine sekundäre Trocknungsstufe umfasst.
20. Verfahren zur Diagnose von porciner proliferativer Enteropathie, umfassend die Durchführung eines Tests zum

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Nachweis von Antikörpern gegen *Lawsonia intracellularis* bei einem nicht-humanen Tiersubjekt, umfassend:

- (a) das Kontaktieren einer Probe des Subjekts mit *Lawsonia intracellularis*-Isolat nach Anspruch 1 zur Bildung eines Antigen-Antikörper-Komplexes; und
- (b) das Nachweisen der Bildung dieses Komplexes,

wobei die Anwesenheit des Komplexes positiv mit einer Diagnose von porciner proliferativer Enteropathie korreliert.

21. Verfahren nach Anspruch 20, wobei der Test einen Test umfasst, der aus der Gruppe ausgewählt ist, die besteht aus: Radioimmunoassay, enzymgebundener Immunosorbenttest, Fluoreszenzimmunoassay und Immunoelktrophoreseassay.

22. Verfahren zur Herstellung von Antiseren gegen *Lawsonia intracellularis* in einem nicht-humanen Tier, umfassend:

- (a) das Verabreichen des *Lawsonia intracellularis*-Isolats nach Anspruch 1 an ein nicht-humanes Tier in einer Menge, die die Herbeiführung einer Immunantwort bewirkt; und
- (b) das Gewinnen von Antiserum oder Plasma mit einem Gehalt an Antikörpern gegen *Lawsonia intracellularis*.

Revendications

1. Isolat de *Lawsonia intracellularis* avirulent, où ledit isolat avirulent est l'isolat de dépôt de *Lawsonia intracellularis* (ATCC n°. PTA-4926 ou tout isolat de *Lawsonia intracellularis* avirulent d'origine européenne, **caractérisé en ce que** ledit isolat de *Lawsonia intracellularis* avirulent ne provoque pas de propagation fécale le jour 14 après la vaccination.
2. Vaccin pour l'immunisation d'un animal comprenant une quantité pharmaceutiquement efficace d'un *Lawsonia intracellularis* avirulent selon la revendication 1 et un vecteur pharmaceutiquement acceptable.
3. Vaccin selon la revendication 2 où la quantité pharmaceutiquement efficace de l'isolat avirulent de *Lawsonia intracellularis* est 10^3 TCID₅₀ à 10^6 TCID₅₀ par dose.
4. Vaccin pour l'immunisation d'un animal comprenant l'isolat avirulent de *Lawsonia intracellularis* selon la revendication 1 et un produit antigénique issu d'au moins l'un des pathogènes suivants : *Salmonella* spp., *Erysipelothrix* spp., *Haemophilus* spp., *Mycoplasma* spp., *Leptospira* spp., *Clostridium* spp., *Streptococcus* spp., *Brachyspira* spp., le circovirus, le virus du syndrome dysgénésique et respiratoire porcin (PRRS), le virus de la grippe porcine (SIV), le virus de gastro-entérite transmissible (TGE), le parvovirus et *Escherichia coli* ; et un vecteur pharmaceutiquement acceptable.
5. Vaccin pour l'immunisation d'un animal comprenant l'isolat avirulent de *Lawsonia intracellularis* selon la revendication 1 et un produit antigénique issu de *Salmonella choleraesuis*, *Erysipelothrix* spp., virus de gastro-entérite transmissible (TGE), *Escherichia coli*, *Clostridium* spp., et *Brachyspira* spp., et un vecteur pharmaceutiquement acceptable.
6. Vaccin pour l'immunisation d'un animal comprenant l'isolat avirulent de *Lawsonia intracellularis* selon la revendication 1 et un produit antigénique issu de *Salmonella choleraesuis* et *Erysipelothrix* spp. ; et un vecteur pharmaceutiquement acceptable.
7. Vaccin pour l'immunisation d'un animal comprenant l'isolat avirulent de *Lawsonia intracellularis* selon la revendication 1 et un produit antigénique issu d'au moins l'un des pathogènes suivants : *Clostridium* spp., le virus de la grippe équine (EIV), l'herpèsvirus équin (EHV), l'alphavirus, et le virus West Nile ; et un vecteur pharmaceutiquement acceptable.
8. Isolat avirulent de *Lawsonia intracellularis* selon la revendication 1 destiné à être utilisé comme vaccin.
9. Isolat avirulent de *Lawsonia intracellularis* selon la revendication 8 destiné à être utilisé comme vaccin pour protéger un animal contre l'entéropathie proliférative porcine.

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- 5 10. Procédé pour produire un vaccin pour induire une réponse immunitaire à des bactéries *Lawsonia intracellularis* chez un animal, comprenant les étapes de : (a) incuber un isolat de *Lawsonia intracellularis* selon la revendication 1 dans des cellules en culture qui sont en suspension à une concentration d'oxygène inférieure à 10 pourcent pour cultiver lesdites bactéries ; et (b) mélanger lesdites bactéries cultivées avec un vecteur pharmaceutiquement acceptable.
11. Procédé pour cultiver *Lawsonia intracellularis* selon la revendication 1 comprenant :
- 10 (a) l'infection de cellules cultivées avec un inoculum comprenant le *Lawsonia intracellularis* avirulent selon la revendication 1,
(b) l'incubation desdites cellules infectées à une concentration d'oxygène inférieure à 10 pourcent tout en maintenant lesdites cellules infectées en suspension par agitation desdites cellules, et
(c) la récolte d'au moins une partie dudit *Lawsonia intracellularis*.
- 15 12. Procédé selon la revendication 11 où la concentration d'oxygène est dans la plage de 0 pourcent à 8 pourcent.
13. Procédé selon la revendication 12 où la concentration d'oxygène est dans la plage de 0 pourcent à 3 pourcent.
- 20 14. Procédé selon la revendication 11 où ladite incubation a lieu avec une plage de dioxyde de carbone de 6 pourcent à 10 pourcent.
15. Procédé selon la revendication 11 où lesdites cellules cultivées sont choisies dans le groupe consistant en : les cellules HEp-2, McCoy et IEC-18.
- 25 16. Procédé selon la revendication 11 où lesdites cellules IEC-18 sont cultivées sur des microsupports.
17. Procédé selon la revendication 11 où les cellules infectées sont incubées selon l'étape (b) pendant une durée de 2 à 10 jours.
- 30 18. Procédé selon la revendication 11 comprenant en outre une étape (d) de lyophilisation desdites bactéries récoltées.
19. Procédé selon la revendication 18 où ladite lyophilisation comprend une étape de séchage primaire et une étape de séchage secondaire.
- 35 20. Procédé pour le diagnostic d'entéropathie proliférative porcine comprenant la mise en oeuvre d'une analyse pour détecter des anticorps contre *Lawsonia intracellularis* chez un sujet animal non humain comprenant :
- 40 (a) la mise en contact d'un échantillon issu du sujet avec l'isolat de *Lawsonia intracellularis* selon la revendication 1 pour former un complexe antigène-anticorps ; et
(b) la détection de la formation dudit complexe,
- où la présence dudit complexe est corrélée positivement avec un diagnostic d'entéropathie proliférative porcine.
- 45 21. Procédé selon la revendication 20 où l'analyse comprend une analyse choisie dans le groupe consistant en: une radioimmunoanalyse, une analyse à immunosorbant lié à une enzyme, une immunoanalyse à fluorescence et une analyse par immunoélectrophorèse.
22. Procédé pour produire des antisérums contre *Lawsonia intracellularis* chez un animal non humain comprenant :
- 50 (a) l'administration de l'isolat de *Lawsonia intracellularis* selon la revendication 1 à un animal non humain en une quantité efficace pour déclencher une réponse immunitaire ; et
(b) la collecte d'antisérum ou de plasma contenant des anticorps contre ledit *Lawsonia intracellularis*.
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REFERENCES CITED IN THE DESCRIPTION

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