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(54) Title: NOVEL BACTERIAL POULTRY DISEASE

(57) Abstract: The invention belongs to the field of animal health and in particular the causative agent of a new bacterial poultry disease, *Pasteurella trehalosi* and/or *Mannheimia haemolytica*. The invention provides said *Pasteurella trehalosi* and/or*Mannheimia haemolytica* bacteria, vaccine comprising inactivated *Pasteurella trehalosi* and/or *Mannheimia haemolytica*, and a method of immunizing to prevent the disease in chicken.

Novel bacterial poultry disease

Field of the invention

The invention belongs to the field of animal health and in particular the causative agents of a new bacterial poultry disease, *Pasteurella trehalosi and/or Mannheimia haemolytica*. The invention provides said *Pasteurella trehalosi and/or Mannheimia haemolytica* bacteria, a vaccine comprising inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica*, and a method of immunizing chicken to prevent said disease in chicken.

Background of the invention

During the last decade, intensive poultry farming methods to increase productivity, has resulted in an increase of disease manifestation throughout all major poultry producing countries. This has caused an increasing need for new and better vaccines and vaccination programs to control these diseases. Nowadays, most animals are immunized against a number of diseases of viral and bacterial origin.

Examples of viral diseases in poultry are Newcastle Disease, Infectious Bronchitis, Avian Pneumovirus, Fowlpox, Infectious Bursal Disease etc.

Examples of bacterial diseases are Avian Coryza caused by *Haemophilus paragallinarum* (upper respiratory tract), *Bordetella avium* (upper respiratory tract), *Ornithobacterium rhinotracheale* (lower respiratory tract), *Salmonella* infections (digestive tract), *Pasteurella multocida*, which is the causative agent of fowl cholera (septicemic), and *E. coli* infections.

Therefore, the technical problem underlying this invention was to identify a new bacterial poultry disease, to provide the causative agent of said disease and a to provide a vaccine to prevent said disease.

Figure legends

A) From field outbreaks

FIG. 1) Broilers: Nasal discharge and swollen areas around the eye.

FIG. 2) Broilers: Haemorrhage in heart and coronary fat.

FIG. 3) Broilers: Conjuctivitis and inflammation around the eye.

FIG. 4) Layers: Nasal discharge and displaced comb with cyanosis.

FIG. 5) Layers: Inflammation and haemorrhage around the eye.

FIG. 6) Layers: Haemorrhage in the dermal tissue behind entrance to auditory orifice.

FIG. 7) Layers: Inflammation of kidneys.

FIG. 8) Layers: Haemorrhages in oviduct.

FIG. 9) Layers: Deformed ovarian follicles.

FIG. 10) Layers: Haemorrhage in the junction between proventriculus and gizzard.

FIG. 11) Layers: Congestion and haemorrhage in oviduct.

B) Experimental Infection

FIG. 12) Layers: Inflammation and haemorrhage in kidney.

FIG. 13) SPF: Prostration.

FIG. 14) Layers: Haemorrhage in joint and muscle.

FIG. 15) Layers: Nasal discharge and pale comb.

FIG. 16) Layers: Haemorrhage in muscle.

FIG. 17) SPF: Haemorrhage in heart and coronary fat.

FIG. 18) Layers: Healthy bird on the left and sick bird on the right with ruffled feathers.

FIG. 19) Layers: Greenish diarrhea.

FIG. 20) SPF: Haemorrhage in muscle.

FIG. 21) SPF: Prostration (locomotive problems) and greenish diarrhea.

FIG. 22) Layers: Enlarged liver with haemorrhage.

Disclosure of the invention

Definitions of terms used in the description:

Before the embodiments of the present invention it must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a *Pasteurella trehalosi*" includes a plurality of such *Pasteurella trehalosi* bacteria reference to the "cell" is a reference to one or more cells and equivalents thereof known

to those skilled in the art, and so forth. It is irrelevant whether a word is capitalized or not, therefore both "Arabinose" and "arabinose" have the same meaning, unless indicated otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the cell lines, vectors, and methodologies as reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Surprisingly, a new bacterial poultry disease has been observed by the present inventors, which occurs primarily in layers and less frequent in broilers. The disease was seen in chicken that had been vaccinated against the bacterium *Haemophilus paragallinarum* (causative agent of avian Coryza), and *Pasteurella multocida* (causative agent of fowl cholera). The symptoms of this new disease differ from the specific symptoms of Coryza. Given the fact that the newly discovered disease clearly shows the clinical signs of a upper respiratory tract infection as described below, *H. paragallinarum* could be ruled out as the causative agent.

The present invention relates in a first aspect to Gram-negative, facultative anaerobic, pleomorphic rod-shaped bacteria causing a novel disease of the upper respiratory and of the reproductive tract of poultry, wherein said bacteria are selected from the group of *Pasteurella trehalosi* and/or *Mannheimia haemolytica*.

Said bacteria may be isolated from infected trachea, palatine cleft, liver, heart, kidney and gonads (broilers). They can be identified as *Pasteurella trahalosi and/or Mannheimia haemolytica* according to the invention based on the tests listed below:

Beta haemolysis	+
Gram stain	-
Oxidase	+

Catalase	+
Urease	-
Nitrate	+
Indole	-

The bacterial isolates may be purified and biotyped according to the method described by Jaworski et al. (1). This method is also exemplified in the examples. Important method to classify bacteria are DNA-DNA hybridization, REA (restriction enzyme analysis see e.g. J. Clinical Microbiol, 1993, 31: 831-835) and ribotyping. Said methods may be applied by the artisan to find out whether bacteria are within the scope of the present invention. A challenge model to validate Koch's postulates is also exemplified in the examples.

Thus, an important aspect of the present invention are *Pasteurella trehalosi and/or Mannheimia haemolytica*, wherein said *Pasteurella and/or Mannheimia are* beta(β)-haemolysis-positive, Gram-negative, oxidase-positive, catalase-positive, urease-negative, nitrate-positive and indole-negative. Preferably, said *Pasteurella trehalosi and/or Mannheimia haemolytica* are also MacConkey-positive. Even more preferred, said *Pasteurella trehalosi and/or Mannheimia haemolytica* are additionally Sucrose-positive, Mannitol-positive, Arabinose-negative, Celobiose-negative, Xylose-positive, Salicin-negative, Ornithine-negative, Esulin-negative, alpha-Fucosidase-negative, beta-Galactosidase-positive. Most preferred are *Pasteurella trehalosi*, wherein said Pasteurella are also Arabinose-negative and Trehalose-positive. Preferably also, said *Pasteurella trehalosi* are also beta(β)-Glucosidase-negative or –positive, depending on the biotype. Also most preferred are *Mannheimia haemolytica*, wherein said Mannheimia are furthermore Arabinose-negative and Trehalose-negative. Preferably also, said *Mannheimia haemolytica* are also beta-Glucosidase negative.

These characteristic properties of the bacteria may render the bacteria novel over other known bacterial poultry pathogens (Diseases of Poultry, Tenth Edition, Edited by B.W. Calnek, Iowa State University Press, Iowa, U.S.A. 1997).

Another preferred embodiment is *Pasteurella trehalosi and/or Mannheimia haemolytica*, wherein said poultry is selected from the group of chicken, turkey, duck, goose, dove, pigeon and quail.

Disclosed herein is a Gram-negative, facultative anaerobic, pleomorphic rod-shaped bacteria, said bacteria being characterized by the bacteria deposited at the American Type Culture Collection (ATCC), 1081, University Boulevard, Manassas, VA 20110 – 2009, USA, under the following deposit numbers:

- *Pasteurella trehalosi* ATCC No. PTA-3667 (internal designation BIV-4985);
- *Pasteurella trehalosi* ATCC No. PTA-3668 (internal designation BIV-AVICOR);
- *Mannheimia haemolytica* ATCC No. PTA-3669 (internal designation BIV-07990).

The date of deposit was August 22, 2001.

Thus, a second aspect of the present invention is *Pasteurella trehalosi* as deposited at the under accession number ATCC No. PTA-3667. These bacteria are further exemplified in table 3 of example 1.

A third aspect of the present invention is *Pasteurella trehalosi* as deposited at the under accession number ATCC No. PTA-3668. These bacteria are further exemplified in table 2 of example 1.

A fourth aspect of the present invention is *Mannheimia haemolytica* as deposited at the under accession number ATCC No. PTA-3669. These bacteria are further exemplified in table 1 of example 1.

The results of tests from sections A and B in the examples (Tables 1, 2 and 3) confirms the bacteria BIV- 4895; ATCC No. PTA-3667 and BIV-AVICOR; ATCC No. PTA-3668 to belong to the family Pasteurellaceae (*Pasteurella trehalosi*, which are Trehalose positive and arabinose negative), while the bacteria BIV-07990; ATCC No. PTA-3669 belong to the family Mannheimia (*Mannheimia haemolytica*, which are Trehalose negative and arabinose negative).

Also disclosed herein is a microbiological culture comprising bacteria as disclosed above. The culture may be made by growing said bacteria at a temperature of between 35° and 37°C. The bacteria may be grown under normal atmospheric oxygen pressure. The bacteria can be grown in a variety of different general-purpose bacterial growth promoting media known to the skilled person, e.g. Tryptose Broth (TB), Soy Trypticasein Broth or Brain Heart Infusion broth or any

enriched media. The bacteria may also be grown on sheep blood agar incubated at 37°C for 24 hours.

Various physical and chemical methods of bacterial inactivation are known in the art. Examples of physical inactivation are UV- radiation, X-ray radiation, gamma-radiation and heating. Examples of inactivating chemicals are beta-propiolactone, glutaraldehyde, beta-ethyleneimine and formaldehyde.

Preferably the bacteria are inactivated with formaldehyde. Surprisingly, the use of formaldehyde at a final concentration of 0.2% is an excellent method to inactivate the bacteria.
Thus, in a fifth aspect, the invention relates to a method for inactivation of a *Pasteurella trehalosi and/or Mannheimia haemolytica* according to the invention comprising the use of formaldehyde at a final concentration of 0.2%.

Said bacteria which are inactivated by the methods disclosed *supra* and by other methods of inactivating the bacteria known to the skilled person are embodied in the present invention. Therefore, a sixth aspect is inactivated *Pasterurella trehalosi and/or Mannheimia haemolytica* obtainable by a method according to the invention or by a method known in the art. Preferably, said inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* are selected from the group of *Pasteurella trehalosi* ATCC No. PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA-3669.

Therefore, a seventh aspect is live attenuated *Pasterurella trehalosi and/or Mannheimia haemolytica* wherein the *Pasterurella trehalosi and/or Mannheimia haemolytica* are attenuated. Preferably, said live attenuated *Pasterurella trehalosi and/or Mannheimia haemolytica* are selected from the group of *Pasterurella trehalosi* ATCC No. PTA-3667, *Pasterurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA-3669. Said *Pasterurella trehalosi and/or Mannheimia haemolytica* can be attenuated by multiple passages in appropriate culture media or by any other method known in the art.

Inactivated as understood herein means, that the *Pasteurella trehalosi and/or Mannheimia haemolytica* are killed without possible replication to cause clinical disease.
Attenuated as understood herein means, that the *Pasteurella trehalosi and/or Mannheimia haemolytica* are live bacteria with possible replication to cause clinical disease.

Disclosed herein are fractions or fragments of *Pasterurella trehalosi and/or Mannheimia haemolytica* obtainable by a method known in the art. Said fragments may be prepared by detergent solubilization of *Pasteurella trehalosi and/or Mannheimia haemolytica* according to the invention or by any other method known in the art.
Preferably, said fractions or fragments are purified antigens of said *Pasteurella trehalosi and/or Mannheimia haemolytica*. Preferably, said fractions/fragments are outer membrane proteins of *Pasterurella trehalosi and/or Mannheimia haemolytica*.
A „fragment" can be any immunogenic subunit of a said *Pasterurella trehalosi and/or Mannheimia haemolytica*, i.e. any polypeptide subset.

Thus, an eighth aspect relates to fragments or fractions containing at least one antigen of *Pasterurella trehalosi and/or Mannheimia haemolytica* according to the invention. Most preferably, said fragments are containing at least one antigen of bacterial selected from the group of *Pasterurella trehalosi* ATCC No. PTA-3667, *Pasterurella trehalosi* ATCC No. PTA-3668 and/or Mannheimia haemolytica ATCC No. PTA-3669. Said fragment may comprise whole bacterial cells of said strain(s), bacterial extracts, Outer Membrane Fractions, bacterial exo- and/or endotoxins, and purified proteins. Antigenic polypeptides or fragments thereof may for example be obtained from purified bacterial proteins or by expression of the corresponding genetic material in some prokaryotic or eukaryotic expression system or by organo-chemical synthesis. Said methods are known to the skilled person.

Disclosed herein is live, and/or live attenutated, and/or inactivated *Pasterurella trehalosi and/or Mannheimia haemolytica* according to the invention and/or

fractions of said *Pasteurella trehalosi and/or Mannheimia haemolytica* for use in a vaccine.

Also disclosed herein is a vaccine derived from the newly identified bacteria disclosed above. Thus, in a ninth aspect, the invention further relates to a vaccine composition comprising a live, and/or live attenuated, and/or inactivated *Pasterurella trehalosi and/or Mannheimia haemolytica* and/or fragments or fractions of said *Pasterurella trehalosi and/or Mannheimia haemolytica.*

The term "vaccine" as understood herein is a vaccine for veterinary use comprising antigenic substances and is administered for the purpose of inducing a specific and active or passive immunity against a disease provoked by said *Pasteurella trehalosi and/or Mannheimia haemolytica.* The live or live attenuated *Pasteurella trehalosi and/or Mannheimia haemolytica* may confer active immunity that may be transferred passively via maternal antibodies against the immunogens it contains and sometimes also against antigenically related organisms. The inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* and/or fractions of said *Pasterurella trehalosi and/or Mannheimia haemolytica* may confer passive immunity.

Additional components to enhance the immune response can be constituents commonly referred to as adjuvants, like e.g. aluminium hydroxide, mineral or other oils or ancillary molecules added to the vaccine or generated by the body after the respective induction by such additional components, like but not restricted to interferons, interleukins or growth factors.

In a preferred embodiment, said vaccine comprises inactivated bacteria.

Preferably, the vaccine refers to a vaccine as defined above, wherein one immunologically active component is a live *Pasteurella trehalosi and/or Mannheimia haemolytica*. The term "live vaccine" refers to a vaccine comprising a particle capable of division/multiplication.

Preferably also, the vaccine comprises attenuated *Pasterurella trehalosi and/or Mannheimia haemolytica* according to the invention and a pharmaceutically acceptable carrier or excipient. Said vaccine may also be administered as a combined vaccine comprising two or more strains of said live, and/or

live attenuated, and/or inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* and/or fractions of two or more strains of said *Pasteurella trehalosi and/or Mannheimia haemolytica*. Most preferably said live, and/or live attenuated, and/or inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* and/or fractions of said *Pasterurella trehalosi and/or Mannheimia haemolytica* in the vaccine are selected from the group of *Pasteurella trehalosi* ATCC No. PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA-3669.

Preferably also, the vaccine comprises inactivated *Pasterurella trehalosi and/or Mannheimia haemolytica* and a pharmaceutically acceptable carrier or excipient. Said vaccine may also be administered as a combined vaccine comprising two or more strains of said inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica*.

Furthermore, fractions of whole cells may also be used as the relevant immunogen in the vaccine according to the invention. Therefore, preferably the vaccine comprises fractions of *Pasterurella trehalosi and/or Mannheimia haemolytica* and a pharmaceutically acceptable carrier or excipient. Said vaccine may also be administered as a combined vaccine comprising two or more strains of said inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica*. In particular, the vaccines may comprise fragments which contain at least one antigen of *Pasterurella trehalosi and/or Mannheimia haemolytica*. Most preferably, the vaccines may comprise fragments which contain at least one antigen of bacteria selected from the group of *Pasteurella trehalosi* ATCC No. PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA-3669. Said fragment may comprise whole bacterial cells, bacterial extracts, Outer Membrane Fractions, bacterial exo- and/or endotoxins, and purified proteins. Antigenic polypeptides or fragments thereof may for example be obtained from purified bacterial proteins or by expression of the corresponding genetic material in some prokaryotic or eukaryotic expression system or by organo-chemical synthesis. Said methods are known to the skilled person.

Preferably, the vaccine may also comprise an adjuvant. Therefore, the vaccine composition may further comprise one or more suitable adjuvant(s) and/or excipients(s) and/or carrier(s).

Adjuvants as used herein can comprise substances that boost the immune response of the injected animal. A number of different adjuvants are known in the art. Adjuvants as used herein may include Freund's Complete and Incomplete adjuvant, vitamin E. non-ionic block polymers, muramyldipeptides, Quil A, mineral and non-mineral oil, vegetable oil, and Carbopol (a homopolymer). In a preferred embodiment, the vaccine bacterin may comprise a water-in-oil emulsion adjuvant. Said vaccine can also be called bacterin comprising inactivated (killed) bacteria and a water-in-oil emulsion adjuvant. Other ways of adjuvating the bacteria known to the skilled person are also embodied in the disclosed embodiments.

Also preferably, the vaccine may comprise one or more suitable emulsifiers, e.g. Span or Tween.

Preferably also, said live, and/or live attenuated, and/or inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* and/or fractions of said *Pasteurella trehalosi and/or Mannheimia haemolytica* in the vaccine can be selected from the group of *Pasterurella trehalosi* ATCC No. PTA-3667, *Pasterurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA-3669.

Preferably, the vaccine comprises at least one antigen of bacteria selected from the group of *Pasterurella trehalosi* ATCC No. PTA-3667, *Pasterurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA- 3669. Said vaccine may comprise whole bacterial cells of said strain(s), bacterial extracts, Outer Membrane Fractions, bacterial exo- and/or endotoxins, and purified proteins. Antigenic polypeptides or fragments thereof may for example be obtained from purified bacterial proteins or by expression of the corresponding genetic material in some prokaryotic or eukaryotic expression system or by organo-chemical synthesis. Said methods are known to the skilled person.

Disclosed herein is a vaccine composition, further comprising at least one other antigen from a virus or microorganism pathogenic to poultry. Preferably, said antigen is in the form of live, attenuated or inactivated viruses or microorganisms or fragments thereof. Said fragment may comprise whole bacterial cells or viral particles, bacterial extracts, viral antigens, viral subunits, Outer Membrane Fractions, bacterial exo- and/or endotoxins, and purified proteins. Antigenic polypeptides or fragments thereof may for example be obtained from purified bacterial proteins or by expression of the corresponding genetic material in some prokaryotic or eukaryotic expression system or by organo-chemical synthesis. Said methods are known to the skilled person.

Also disclosed herein is a vaccine composition, further comprising at least one other antigen from a virus or microorganism pathogenic to poultry, wherein said virus or microorganism is selected from, but not restricted to, the group consisting of Infectious Bronchitis Virus, Newcastle Disease Virus, Infectious Bursal Disease Virus (disease: Gumboro), Chicken Anaemia agent, Avian Reovirus, *Mycoplasma gallisepticum*, Avian Pneumovirus, *Haemophilus paragallinarum* (disease: Coryza), Chicken Poxvirus, Avian Encephalomyelitis virus, *Pasteurella multocida* and *E. coli*.

A "pharmaceutical composition" essentially consists of one or more ingredients capable of modifying physiological e.g. immunological functions of the organism it is administered to, or of organisms living in or on the organism. The term includes, but is not restricted to antibiotics or antiparasitics, as well as other constituents commonly used to achieve certain other objectives like, but not limited to, processing traits, sterility, stability, feasibility to administer the composition via enteral or parenteral routes such as oral, intranasal, intravenous, intramuscular, subcutaneous, intradermal or other suitable route, tolerance after administration, controlled release properties. Thus, in also disclosed herein is a pharmaceutical composition comprising a live, and/or live attenuated, and/or inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* and/or fractions of said *Pasteurella trehalosi and/or Mannheimia haemolytica*.

Disclosed herein is a method of treating a *Pasteurella trehalosi and/or Mannheimia haemolytica*-infected animal (e.g. the live bacteria as described above) belonging to the group of poultry wherein said live, attenuated, inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* and/or fractions and/or fragments thereof as described supra, are administered to the poultry animal in need thereof at a suitable doses as known to the skilled person and the reduction of symptoms caused by said *Pasteurella trehalosi and/or Mannheimia haemolytica* infection is monitored. Said treatment preferably may be repeated.

Also disclosed herein is a method of immunizing poultry against the disease of the respiratory and reproductive tract caused by a *Pasteurella trehalosi and/or Mannheimia haemolytica* (e.g. the live bacteria as described above) comprisinig administration of an immunologically effective amount of a vaccine as described above and the reduction of symptoms caused by said *Pasteurella trehalosi and/or Mannheimia haemolytica* infection is monitored.

A tenth aspect provides the use of an inactivated *Pasteurella trehalosi andlor Mannheimia haemolytica* according to the invention and/or live *Pasteurella trehalosi and/or Mannheimia haemolytica* according to the invention and/or live attenuated *Pasteurella trehalosi andlor Mannheimia haemolytica* according to the invention and/or fragments or fractions of said *Pasteurella trehalosi andlor Mannheimia haemolytica* according to the invention in the manufacture of a vaccine for the prophylaxis of *Pasteurella trehalosi andlor Mannheimia haemolytica* infections.

An eleventh aspect relates to a method of diagnosis of a disease caused by comprising the steps of obtaining a sample from poultry, wherein said sample is selected from the group of blood, serum, plasma, tissue scrapings, washings, swabs, tissue, analysing said sample for the presence of *Pasteurella trehalosi andlor Mannheimia haemolytica* according to the invention.

In a preferred embodiment the presence of *Pasteurella trehalosi and/or Mannheimia haemolytica* is determined by an immune test. An immune test may use monoclonal antibodies or polyclonal antisera specific to *Pasteurella trehalosi and/or Mannheimia haemolytica*. The generation of monoclonal antibodies is known in the art (3,4).

Immune tests may include the methods of detection known in the art such as the ELISA test (enzyme-linked immuno-sorbent assay) or the so-called sandwich-ELISA test, dot blots, immunoblots, radioimmuno tests (radioimmunoassay RIA), diffusion-based Ouchterlony test or rocket immunofluorescent assays) or agglutination tests (rapid plate or micro-plate agglutination tests). Another immune test may be the so-called Western blot (also known as Western transfer procedure or Western blotting). The purpose of Western blot is to transfer proteins or polypeptides separated by polyacrylamide gel electrophoresis onto a nitrocellulose filter or other suitable carrier and at the same time retain the relative positions of the proteins or polypeptides obtained from the gel electrophoresis. The Western blot is then incubated with an antibody which specifically binds to the protein or polypeptide under consideration. These methods of detection can be used by the average skilled person to perform the invention described herein. Literature references in which the skilled person can find the above-mentioned methods and other detection methods are listed as follows: *An Introduction to Radioimmunoassay and Related Techniques*, Elsevier Science Publishers, Amsterdam, The Netherlands (1986); Bullock *et al.*, *Techniques in Immunocytochemistry*, Academic Press, Orlando, FL Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, *Practice and Theory of Enzyme Immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1985).

In another, most particular embodiment, the sample as disclosed *supra* is incubated with antibodies which are specific to *Pasteurella trehalosi and/or Mannheimia haemolytica* and the antigen/antibody complex thereby formed is determined.

In a particularly preferred embodiment of the method according to the invention, the presence of *Pasteurella trehalosi and/or Mannheimia haemolytica* in a sample as disclosed *supra* is determined by molecular biology methods. Molecular biology methods as used herein means detection methods which include, for example, polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR) or may be Northern or Southern blots which the skilled person can find in the standard reference books (e.g. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York and Bertram, S. and Gassen, H.G. Gentechnische Methoden, G. Fischer Verlag, Stuttgart, New York, 1991).

In a twelfth aspect, the invention relates a diagnostic test kit comprisinig all the necessary elements for detecting *Pasteurella trehalosi and/or Mannheimia haemolytica*.

A diagnostic test kit is a collection of all the components for carrying out a method of diagnosis. Some examples (not an exhaustive list) of other elements for performing a method according to the invention include containers such as 96-well plates or microtitre plates, test tubes, other suitable containers, surfaces and substrates, membranes such as nitrocellulose filter, washing reagents and buffers. A diagnostic test kit may also contain reagents which may detect bound antibodies, such as for example labelled secondary antibodies, chromophores, enzymes (e.g. conjugated with antibodies) and the substrates thereof or other substances which are capable of binding antibodies.

Disclosed herein is a diagnostic test kit which may contain all the necessary elements for carrying out a PCR or RT-PCR to detect *Pasteurella trehalosi- and/or Mannheimia haemolytica*-specific DNA or RNA. Said kit may contain, but is not limited to in addition to test tubes or 96-well plates or microtitre plates, other suitable containers, surfaces and substrates, membranes such as nitrocellulose filters, washing reagents and reaction buffers (which may vary in pH and magnesium concentrations), sterile water, mineral oil, BSA (bovine serum albumin), $MgCl_2$, $(NH_4)_2SO_4$, DMSO (dimethylsulphoxide), mercaptoethanol, nucleotides (dNTPs), enzymes such as *Taq*-polymerase and reverse transcriptase and, as the DNA matrix, the DNA or cDNA specific for *Pasteurella trehalosi and/or Mannheimia haemolytica*, oligonucleotides specific for a *Pasteurella trehalosi and/or Mannheimia haemolytica* DNA or RNA, control template, DEPC-water, DNAse, RNAse and further compounds known to the skilled artisan. Oligonucleotides are short nucleic acid molecules from about 15 to about 100 nucleotides long, which bind under stringent conditions to the nucleic acid sequence which is complementary to a *Pasteurella trehalosi and/or Mannheimia haemolytica* protein. By stringent conditions the skilled person means conditions which select for more than 85%, preferably more than 90% homology (cf. Sambrook etal. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York and Bertram, S. and Gassen, H.G. Gentechnische Methoden, G. Fischer Verlag, Stuttgart, New York, 1991).

The following examples serve to further illustrate the present invention; but the same should not be construed as limiting the scope of the invention disclosed herein.

Example 1

Field disease outbreaks associated with Pasteurella trehalosi and Mannheimia haemolytica

Clinical signs

From field observations

Layers	Broilers
Mild upper respiratory / reproductive	Severe upper respiratory / reproductive
Age : 22 weeks of age	Age : 7 weeks
Nasal discharge	Sneezing with rales
Swollen areas around the eye	Swollen head
Low feed consumption	Low feed consumption
Whitish diarrhea	Depression
Decreased egg production	Non uniform growth
Low mortality	Ruffled feathers
Comb displaced	Prostration
Cyanosis of comb	Mortality 8%

Gross Lesions	
Ovarian atrophy with haemorrhages and regression	Haemorrhages in gonads
Ovarian follicles deformed	Upper part of trachea with haemorrhages
Enlarged liver	Enlarged liver with haemorrhages
Inflammation of kidneys	Airsacculitis
Haemorrhages in abdominal fat	Enlarged spleen with haemorrhages
Haemorrhages in thoracic cavity	Haemorrhages in muscle
Haemorrhages in oviduct	Haemorrhages in the heart and hydropericardium
Haemorrhages of coronary fat	Haemorrhages in the thoracic cavity

Three strains of a novel type of Gram-negative, facultative anaerobic, pleomorphic rod-shaped bacteria, were deposited at the American Type Culture Collection (ATCC), 1081, University Boulevard, Manassas, VA 20110 – 2209, USA, under deposit number

: ATCC No. PTA – 3667 for BIV-4985 Pasteurella trehalosi; ATCC No. PTA-3668 for BIV-AVICOR Pasteurella trehalosi and ATCC No. PTA-3669 for BIV-07990, Mannheimia haemolytica. The date of deposit being August 22, 2001.

The deposited bacteria were typed according to standard determination methods, using Bergey's Manual of Systematic Bacteriology Volume I (1984. Williams and Wilkins, 428 East Preston Street. Baltimore, USA.)

TABLE 1

Mannheimia haemolytica : BIV – 07990 (Biotype 1); ATCC No. PTA-3669

Macroscopic morphology

Colonies grown on Sheep Blood Agar for 24 hours, range from 1.0 to 1.5 mm in diameter, bright translucent, low convex, smooth and creamy, β hemolysis.

Microscopic morphology

Gram-negative , non-motile, pleomorphic rods, often exhibit bipolar staining.

Biochemical and other tests

Section A

TEST	REACTION
Oxidase	+
Catalase	+
Indole	-
Glucose	+
Sucrose	+
MacConkey	+
Urease	-
Nitrate	+

Section B

Maltose	+
Mannitol	+
Arabinose	-
Celobiose	-
Sorbitol	+
Xilose	+
Treahalose	-
Salicin	-

Ornithine	-
Esculine	-
β Glucosidase	-
α - Fucosidase	-
β Galactosidase	+

TABLE 2

Pasteurella trehalosi BIV-AVICOR (Biotype 2); ATCC No. PTA-3668

Macroscopic morphology

Colonies grown on Sheep Blood Agar for 24 hours, range from 1.0 to 1.5 mm in diameter, bright translucent, low convex, smooth and creamy, β hemolysis.

Microscopic morphology

Gram-negative , non-motile, pleomorphic rods, often exhibit bipolar staining.

Biochemical and other tests

Section A

TEST	REACTION
Oxidase	+
Catalase	+
Indole	-
Glucose	+
Sucrose	+
MacConkey	+
Urease	-
Nitrate	+

Section B

Maltose	+
Mannitol	+
Arabinose	-
Celobiose	-
Sorbitol	+
Xylose	+
Trehalose	+
Salicin	-
Ornithine	-
Esculin	-
β Glucosidase	+ [α]
α - Fucosidase	-
β Galactosidase	+

[α] 80% are positive

TABLE 3

Pasteurella trehalosi : BIV – 4895 (Biotype 4); ATCC No. PTA-3667

Macroscopic morphology

Colonies grown on Sheep Blood Agar for 24 hours, range from 1.0 to 1.5 mm in diameter, bright translucent, low convex, particulate and dry, β hemolysis.

Microscopic morphology

Gram-negative , non-motile, pleomorphic rods, often exhibit bipolar staining.

Biochemical and other tests

Section A

TEST	REACTION
Oxidase	+
Catalase	+
Indole	-
Glucose	+
Sucrose	+
MacConkey	+
Urease	-
Nitrate	+

Section B

Maltose	-
Mannitol	+
Arabinose	-
Celobiose	-
Sorbitol	+
Xylose	+
Trehalose	+
Salicin	-
Ornithine	-
Esculin	-
β Glucosidase	-
α - Fucosidase	-
β Galactosidase	+

The results of tests from Section A and B (Tables 1, 2 and 3) confirms the bacteria BIV-4895; ATCC No. PTA-3667 and BIV- AVICOR; ATCC No. PTA- 3668 belong to the family Pasteurellaceae, (*Pasteurella trehalosi*, which are Trehalose positive and arabinose negative), while the bacteria BIV- 07990; ATCC No. PTA- 3669 belong to the

family Mannheimia (*Mannheimia haemolytica*, which are Trehalose negative and arabinose negative).

Identification of the causative agent

Bacteria were isolated form infected trachea, palatine cleft, ovary, liver, heart, kidney and gonads (broilers). They were identified as *Pasteurella trehalosi* and *Mannheimia haemolytica* based on the tests listed below :

Beta haemolysis	+
Gram stain	-
Oxidase	+
Catalase	+
Mac Conkey	+
Urease	-
Nitrate	+
Indole	-

No isolations of virus or any other bacteria were made.

Biotyping:

Bacterial isolates were purified and biotyped according to the method described by Jaworski et al.(1). Three different biotypes (4, 2, 1) were identified.

Briefly, from the purified isolates, a single colony was inoculated into tubes containing 3 ml of Tryptose Broth and incubated at 37°C for 8 hours. A loop of inoculum (20 µl) from the tube was then transferred into another tube containing 3 ml of 1% sugar to be tested and incubated for 7 days at 37°C before results were recorded

Challenge Model:

Following purification of the bacteria, isolates were grown in tryptose media to obtain large quantities of pure pathogens. In order to validate Koch postulates, 3 different groups (20 birds per group) of specific pathogen free (SPF) chicken 13 weeks of age were infected with each biotype (0.2 ml / bird; $3x10^8$ CFU / ml) by intravenous route. The birds were observed daily for 3 days for morbidity and mortality. At the end of the

3rd day, all birds were sacrificed, post-mortem lesions recorded and organ samples (liver and gonads) were collected for re-isolation. Post-mortem lesions of birds that died were also recorded.

Results

Clinical signs: Prostration, Lameness, Displaced comb, Ruffled feathers, Cyanosis at the tip of the comb.

Lesions:

Lesion	BIV- 4895 (Biotype 4)	BIV- Avicor (Biotype 2)	BIV- 07990 (Biotype 1)
Heart edema	73 %	37 %	90 %
Heart haemorrhages	90 %	—	70 %
Haemorrhages in coronary fat	90 %	37 %	50 %
Pericarditis	73 %	46 %	30 %
Haemorrhages in thoracic cavity	---	19 %	40 %
Haemorrhages in ovary	64 %	9 %	20 %
Inflammation of kidneys	64 %	46 %	60 %
Haemorrhages in kidneys	55 %	46 %	20 %
Enlarged liver with haemorrhages	---	73 %	---
Airsacculitis	64 %	---	---
Haemorrhages in muscle	---	37 %	---
Mortality	46 %	19 %	---

EXAMPLE II

Growth of the bacteria according to the invention, preparation of the vaccine and vaccination of SPF birds.

Strains were grown on Tryptose Broth (TB). Harvest was done at logarithmic growth phase around 6-8 hours post-inoculation depending on the strain. Plate count was made in sheep blood agar for titration. Colony forming units per mililiter (CFU/ml) was determined using 1:10 dilutions of the harvest. Cells were killed by adding formaldehyde to a final concentration of 0.2%. Following a sterility check of this suspension, a minimal titer of 10^8 CFU/ml was added to the final vaccine.

The vaccine was prepared by mixing the two strains (BIV-4895, ATCC No. PTA-3667 and BIV-AVICOR, ATCC No. PTA – 3668) and oil-adjuvant (a water-in-oil emulsion on the basis of a mineral oil with a ratio of 60% oil / 40% water) to a minimal concentration of $10^{7.0}$ CFU/strain/ml.

Specific pathogen free (SPF) chicken were vaccinated at 2, 5 and 9 weeks of age by injection of 0.5 ml of the vaccine subcutaneously halfway down the neck.

EXAMPLE III

Preparation of challenge strains and challenge of vaccinated and control groups.

Bacterial strains BIV-4895, ATCC No. PTA-3667 and BIV-AVICOR, ATCC No. PTA – 3668, were grown on sheep blood agar for 24 hrs. at 37°C. The cells were harvested in Tryptose Broth (TB) until a suspension with an Optical Density of 2.0 was obtained, using a spectrophotometer at wavelength of 540 nm. For challenge, preparations were made that contain the following number of cells in the final challenge-volume :

3×10^9 CFU/ml BIV – AVICOR; ATCC No. PTA-3668
1.45×10^{10} CFU/ml BIV-4895; ATCC No. PTA-3667

At 13 weeks of age, 20 vaccinated and 20 non-vaccinated birds were challenged by intravenous route of 0.2 ml of the inoculum (at least $10^{8.0}$ CFU/bird). Birds were observed for 3 days for morbidity and mortality. After three days of observation all the remaining birds were sacrificed and re-isolation of the bacteria from liver and gonads were made from each bird. Post-mortem lesions of birds that died were also recorded.

RESULTS

Group of birds	Challenge inoculum	Mortality + Reisolation	Protection %
Control Negative	N/A	0	N/A
Control Positive	ATCC No.PTA-3667	77	23
Control Positive	ATCC No.PTA-3668	54	46
Vaccinated	ATCC No.PTA-3667	0	100
Vaccinated	ATCC No.PTA-3668	5	95

EXAMPLE IV

Growth of the bacteria according to the invention, preparation of the vaccine and vaccination of SPF birds.

Strains were grown on Tryptose Broth (TB). Harvest was done at logarithmic growth phase around 6-8 hours post-inoculation depending on the strain. Plate count was made in sheep blood agar for titration. Colony forming units per mililiter (CFU/ml) was determined using 1:10 dilutions of the harvest. Cells were killed by adding formaldehyde to a final concentration of 0.2%. Following a sterility check of this suspension, a minimal titer of 10^8 CFU/ml was added to the final vaccine.

The vaccine was prepared by mixing the three strains (BIV-4895, ATCC No. PTA-3667; BIV-AVICOR, ATCC No. PTA – 3668 and BIV-07990, ATCC No. PTA-3669) and oil-adjuvant (a water-in-oil emulsion on the basis of a mineral oil with a ratio of 60% oil / 40% water) to a minimal concentration of $10^{7.0}$ CFU/strain/ml.

Specific pathogen free (SPF) chicken were vaccinated at 2, 5 and 9 weeks of age by injection of 0.5 ml of the vaccine subcutaneously halfway down the neck.

EXAMPLE V

Preparation of challenge strains and challenge of vaccinated and control groups.

Bacterial strains BIV-4895, ATCC No. PTA-3667; BIV-AVICOR, ATCC No. PTA – 3668 and BIV-07990, ATCC No. PTA-3669, were grown on sheep blood agar for 24 hrs. at 37°C. The cells were harvested in Tryptose Broth (TB) until a suspension with an Optical Density of 2.0 was obtained, using a spectrophotometer at wavelength of 540

nm. For challenge, preparations were made that contain the following number of cells in the final challenge-volume :

8.3×10^9 CFU/ml BIV – AVICOR; ATCC No. PTA-3668
2.2×10^9 CFU/ml BIV-4895; ATCC No. PTA-3667
1.0×10^{10} CFU/ml BIV-07990; ATCC No. PTA-3669

At 13 weeks of age, 20 vaccinated and 20 non-vaccinated birds were challenged by intravenous route of 0.2 ml of the inoculum (at least $10^{8.0}$ CFU/bird). Birds were observed for 3 days for morbidity and mortality. After three days of observation all the remaining birds were sacrificed and re-isolation of the bacteria from liver and gonads were made from each bird. Post-mortem lesions of birds that died were also recorded.

RESULTS

Group of Birds	Challenge Inoculum	Mortality + Reisolation	Protection (%)
Control Negative Vaccinated	N/A	0	N/A
Control Negative Non-vaccinated	N/A	0	N/A
Vaccinated	ATCC No.PTA-3669	27.3	72.7
Vaccinated	ATCC No.PTA-3668	20.9	79.1
Vaccinated	ATCC No.PTA-3667	16.7	83.7
Control Positive	ATCC No.PTA-3669	53.3	46.7
Control Positive	ATCC No.PTA-3668	53.3	46.7
Control Positive	ATCC No.PTA-3667	64.3	35.7

Serological test

Hyperimmune sera were produced in rabbits with isolate representing each biotype, according to the method of Biberstein et. al. (2)

The isolates were grown on blood agar overnight, then harvested in saline containing 0.3% formalin. The cells were washed once and adjusted to 10% transmittance at 575 nm for injection. The injections were by IV route according to the following schedule :

0.5 ml, 1.0, 2.0, 3.0, 3.0, 3.0 at 4 day intervals and all rabbits were bled 4 days after the final injection.

The hyperimmune serum was tested for their specificity using the 3 biotype strains and were reacted with homologous and heterologous rabbit antiserum (2 fold dilutions) by rapid plate agglutination.

Antiserum of each biotype was diluted until the end point was reached to determine the highest dilution that was positive.

Dilution (log^2)
Antigen Biotype 1

Antiserum	1	2	3	4	5	6	7	8	9	10	11
1	+	+	+	+	+	+	+	+	+	+	+
2	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-

Dilution (log^2)
Antigen Biotype 4

Antiserum	1	2	3	4	5	6	7	8	9	10	11
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-
4	+	+	+	+	+	+	+	+	+	+	+

Dilution (log^2)
Antigen Biotype 2

Antiserum	1	2	3	4	5	6	7	8	9	10	11
1	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	-	-	-	-	-	-	-

The biotype specific hyperimmune sera was then used as positive control in micro-plate serum agglutination test.

EXAMPLE VI

Preparation of challenge strains and challenge of vaccinated and control groups.

Bacterial strains BIV-4895, ATCC No. PTA-3667, BIV-AVICOR, ATCC No. PTA – 3668 and BIV – 07990, ATCC No. PTA-3669, were grown on sheep blood agar for 24 hrs. at 37°C. The cells were harvested in Tryptose Broth (TB) until a suspension with an Optical Density of 2.0 was obtained, using a spectrophotometer at wavelength of 540 nm. For challenge, preparations were made that contain the following number of cells in the final challenge-volume :

1.5×10^{10} CFU/ml BIV – AVICOR; ATCC No. PTA-3668
1.7×10^{10} CFU/ml BIV-4895; ATCC No. PTA-3667
1.6×10^{10} CFU/ml BIV-07990; ATCC No. PTA-3669

At 13 weeks of age, 20 vaccinated and 20 non-vaccinated birds were challenged by intravenous route of 0.2 ml of the inoculum (at least $10^{8.0}$ CFU/bird). Birds were observed for 3 days for morbidity and mortality. After three days of observation all the remaining birds were sacrificed, post mortem lesions were recorded and re-isolation of the bacteria from liver, heart and gonads were made from each bird.

RESULTS

Table 1. Evaluation on the effect of vaccine based on mortality and re-isolation.

Group of birds	Challenge inoculum	Mortality + Reisolation	Protection %
Control negative non-vaccinated	N/A	N/A	N/A
Control Positive	BIV - 4895	70	30
Control Positive	BIV - AVICOR	80	20
Control Positive	BIV - 07990	88.8	11.2
Vaccinated	BIV - 4895	10	90
Vaccinated	BIV - AVICOR	10	90
Vaccinated	BIV - 07990	15	85

Table 2. Evaluation on the effect of vaccine based on gross lesions following challenge.

Group of birds	Challenge inoculum	% of Lesions	Protection %
Control negative non-vaccinated	N/A	N/A	N/A
Control Positive	BIV - 4895	74.4	25.6
Control Positive	BIV - AVICOR	27.0	73.0
Control Positive	BIV - 07990	7.0	93.0
Vaccinated	BIV - 4895	4.0	96.0
Vaccinated	BIV - AVICOR	1.1	99.0
Vaccinated	BIV - 07990	2.4	98.0

References

1) Jaworski M.D., D. L. Hunter, A. C. S. Ward. Biovariants of isolates of *Pasteurella* from domestic and wild ruminants. J. Vet. Invest. 1988, **10:** 49-55.

2) Biberstein EL. , Meyer M.E. , and Kenedy P.C. Colonial variation of Pasteurella haemolytica isolated from sheep. J. Bact. 1958, **76:** 445-452.

3) Kearney, J.F., Radbruch A., Liesegang B., Rajewski K. A new mouse myeloma cell line that has lost imunoglobulin expression but permits construction of *anti*body-secreting hybrid cell lines. J. Immunol. 1979, **123:** 1548-1550.

4) Köhler, G., Milstein, C. Continous culture of fused cells secreting antibody of predefined specifity. Nature 1975, **265:** 495-497.

PCT 1-1265-PCT

Original (for SUBMISSION) - printed on 23.10.2002 09:05:55 AM

0-1	Form - PCT/RO/134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	PCT-EASY Version 2.92 (updated 01.10.2002)
0-2	International Application No.	PCT/EP02/11899
0-3	Applicant's or agent's file reference	1-1265-PCT

1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	18
1-2	line	5
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	American Type Culture Collection
1-3-2	Address of depositary institution	10801 University Blvd., Manassas, Virginia 20110-2209United States of America
1-3-3	Date of deposit	22 August 2001 (22.08.2001)
1-3-4	Accession Number	ATCC PTA-3667
1-4	Additional Indications	BIV-4895 Avian Pasteurella trehalosi
1-5	Designated States for Which Indications are Made	all designated States
1-6	Separate Furnishing of Indications	NONE
	These indications will be submitted to the International Bureau later	
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	17
2-2	line	7
2-3	Identification of Deposit	
2-3-1	Name of depositary institution	American Type Culture Collection
2-3-2	Address of depositary institution	10801 University Blvd., Manassas, Virginia 20110-2209United States of America
2-3-3	Date of deposit	22 August 2001 (22.08.2001)
2-3-4	Accession Number	ATCC PTA-3668
2-4	Additional Indications	BIV-AVICOR Avian Pasteurella trehalosi
2-5	Designated States for Which Indications are Made	all designated States
2-6	Separate Furnishing of Indications	NONE
	These indications will be submitted to the International Bureau later	

2/2

PCT 1-1265-PCT

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3	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
3-1	page	16
3-2	line	12
3-3	Identification of Deposit	
3-3-1	Name of depositary institution	American Type Culture Collection
3-3-2	Address of depositary institution	10801 University Blvd., Manassas, Virginia 20110-2209United States of America
3-3-3	Date of deposit	22 August 2001 (22.08.2001)
3-3-4	Accession Number	ATCC PTA-3669
3-4	Additional Indications	BIV-07990 Avian Mannheimia haemolytica
3-5	Designated States for Which Indications are Made	all designated States
3-6	Separate Furnishing of Indications These indications will be submitted to the International Bureau later	NONE

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SEP-13-2001 08:58 ATCC 703 365 2745 P.01

AMERICAN TYPE CULTURE COLLECTION
10801 University Blvd.
Manassas, VA 20110-2209
Telephone: 703-365-2700
Fax: 703-365-2745

FACSIMILE

Date: September 12, 2001

To: Abelardo Aquilar
Fax Number: 52 3 668 80 80

From: ATCC Patent Depository **Number of pages:** **1 (Including this page)**

REFERENCE: **Patent Deposit**

Avian Pasteurella trehalosi: BIV-4895 assigned ATCC No. PTA-3667,
Avian Pasteurella trehalosi: BIV-AVICOR assigned ATCC No. PTA-3668 and
Avian Mannheimia haemolytica: BIV-07990 assigned ATCC No. PTA-3669.

Date of Deposit: August 22, 2001. Paperwork will be forwarded to you in a few days. An invoice will be sent under separate cover:

Standard storage/informing	$ 3,450.00
Viability Test	480.00
Total amount to PTA-3667 to PTA-3669	$ 3,930.00

Marie Harris

Marie Harris, Patent Specialist
ATCC Patent Depository

* **Please note: Patent deposit fees have increased effective January 1, 2001. Deposit forms were revised February 2000. Current deposit forms and fees can be obtained from our web site: www.atcc.org.**

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Budapest Treaty Deposits

American Type Culture Collection

10801 University Blvd., Manassas, VA 20110-2209
Phone (703) 365-2700; fax (703) 365-2745; e-mail applied-sci@atcc.org

TO DEPOSIT OR TO CONVERT A DEPOSIT TO MEET THE REQUIREMENTS OF THE BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

ALL QUESTIONS MUST BE COMPLETED IN ENGLISH. PLEASE USE ONE FORM FOR EACH STRAIN DEPOSITED.

1. Name of deposit. **If microorganism,** give complete scientific name including genus and species and source of material; **if virus,** give name, whether plant or animal, and source including geographic location; **if cell line,** give tissue and species, geographical source of isolation, and any known hazards associated (HIV, EBV, etc.); **if genetic materials,** give name of organism from which vector, clone or library is derived, source of the DNA insert identified by species (e.g. human, mouse) or scientific name, and give name of gene and identity of the host organism; **if consortia or mixed culture,** each component of the mixture must be identified; **if seeds, embryos, insect eggs, etc.,** give common name, scientific name, and geographical source.

   Avian Mannheimia haemolytica formerly known as Pasteurella haemolytica

2. Strain designation (i.e., number, symbols, etc.) BIV - 07990
   The strain designation must correspond with the vial labels.

3. Is this an original deposit under the Budapest Treaty? Yes

4. Is this a request for a conversion of a deposit already at ATCC to meet the requirements of the Budapest Treaty? If so please indicate ATCC designation. Not applicable

5. Is this deposit a mixture of microorganisms or cells? No

6. Provide details necessary to cultivate, test for viability, and store deposit. If mixture, provide description of components and a method to check presence. If plasmid, provide name of host and antibiotic resistance.

   See annex 3

7. Provide sufficient description so that ATCC may confirm deposit properties (e.g., Gram negative rod).

   See annex 3

   a. If deposit is a cell culture, is it being cultured in the presence of antibiotics? If so, please list the antibiotics.
   Not applicable

   b. If deposit is hybridoma, what is the isotype of antibody produced? Not applicable

8. Is this strain hazardous to humans? No Animals? Yes Plants? No. If yes, what is the recommended biosafety level for working with this strain? 1. (Refer to Biosafety in Microbiological and Biomedical Laboratories, 4th ed. U.S. Dept. of Health and Human Services at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm].

9. Availability: Prior to the issuance of a U.S. Patent, ATCC will only make a culture available as instructed by the depositor or relevant patent office. Samples must be provided to a specific investigator if a pertinent patent office under the Budapest Treaty instructs ATCC to do so. The following questions must be answered:

   a) As of date of deposit or conversion to meet the requirements of the Budapest Treaty, do you wish the deposit to be made available to anyone who requests a culture? If yes there are no restrictions on distribution. Answering no will **ensure the deposit is not available until the patent has issued.** Yes ____ No X

   b) As of date of deposit or conversion to meet the requirements of the Budapest Treaty, do you wish the deposit to be made available to requestors which satisfy patent offices in countries not signatory to the Budapest Treaty? Yes ____ No X
   If yes state which countries. ____

Please note that if you are converting your deposit to meet the requirements of the Budapest Treaty and your deposit has already been released for distribution due to the issuance of a U.S. patent, you cannot restrict it from further distribution.

After a U.S. patent issues and we are so notified, ATCC makes the culture available to anyone who requests it, as allowed under USPTO Rules and Regulations (37 CFR 1.808 [a][2]).

10. ATCC will notify you of your ATCC number after confirmation of viability testing is complete.
Name of Individual to notify: Abelardo Aguilar
Fax: (52 3) 668 80 80 Phone: (52 3) 668 8038 E-mail: baguilar@gua.boehringer-ingelheim.com

11. Payment by check, or credit card (Mastercard, VISA or American Express), must accompany the deposit unless prior arrangements for billing have been made and approved. ATCC accepts Purchase Orders in the correct amount:

Purchase Order No. _____ Check No. _____

Credit Card number. Please indicate MasterCard, VISA, or AE. _____

Exp. Date: _____ Name shown on card: _____
(Please type or print clearly.)

_____
Signature of card holder

PAYMENT: ATCC MUST HAVE A BILLING ADDRESS, CONTACT PERSON, PHONE AND FAX NUMBER FOR ALL DEPOSITS:

Contact person : Abelardo Aguilar
Calle 30 No. 2614 Zona Industrial 44940
Guadalajara, Jalisco. México
Phone: (52 3) 668 8038 Fax: (52 3) 668 80 80

12. Name, address, telephone and facsimile number of your attorney of record. _____

_____

_____ (Ref: Docket or Case No. _____)

13. **MUST BE COMPLETED.** Deposited on behalf of: (Verify with your management who owns the deposit. The owner is usually a company or institute and not an individual.)

Boehringer Ingelheim Vetmedica, S.A. de C.V.

I understand and agree that the deposit may not be withdrawn by me for a period specified in Rule 9.1 of the Budapest Treaty (at least 30 years after the date of deposit or 5 years after the date of the most recent request for the deposit, whichever is longer), and that if a culture should die or be destroyed during the life of the patent or the period of time so specified, it is my responsibility to replace it with a living culture of the same organism or cell. In the cases of viruses, cell cultures, plasmids, embryos, and seeds, it is my responsibility to supply a sufficient quantity for distribution for the period of time specified above.

Ing. Jorge Súchil B. [signature] May 24, 200[illegible]
Typed Name Signature Date

Address: Calle 30 No. 2614 Zona Industrial 44940 Guadalajara, Jalisco, México

Phone: (52 3) 668 80 00 Fax: (52 3) 668 80 80 E-mail: jsuchil@gua.boehringer-ingelheim.co

ADDRESS SHIPMENTS AND FORM TO THE ATTENTION OF:

Patent Depository
American Type Culture Collection
10801 University Blvd.
Manassas, VA 20110-2209 U.S.A.

STORAGE: Cultures are stored for 30 years from date of deposit or 5 years after the last request for a sample, whichever is longer, as required under the rules of patent offices in most countries.
FEES: For current fees, check our Web site at www.atcc.org, request a fee sheet by e-mail to applied-sci@atcc.org, or call (703) 365-2700 ext. 320. All fees are subject to change.

ATCC USE ONLY: ATCC DESIGNATION _____ REC'D _____ V.T. RESULT _____

Name of Deposit _____ Strain Designation: _____

Form BP/1 (Rev. 2/00)

Failure to supply all applicable information can delay the processing of this application. PLEASE TYPE OR PRINT CLEARLY

No controlled material, organisms or vectors may be imported or moved interstate unless the data requested on this form is furnished and certified (9 CFR 94, 95, and 122).

According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0579-0015. The time required to complete this information collection is estimated to average between .1.5 and 2 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

FORM APPROVED
OMB NO. 0579-0015

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
National Center for Import-Export, Products Program
4700 River Road, Unit 40
Riverdale, MD 20737-1231

APPLICATION FOR PERMIT TO:

IMPORT OR TRANSPORT CONTROLLED MATERIAL OR ORGANISMS OR VECTORS

1. MODE OF TRANSPORTATION *(Please "X"):*

☐ AIR ☐ SEA ☐ LAND ☐ ANY

2. U.S. Ports of Entry

3. IMPORTER *(Name, organization, complete address, telephone and fax number of individual who will receive and be responsible for the imported material)*

American Type Culture Collection
10801 University Bvd.
Manassas VA 20110 - 2209
Phone (703) 365-2700 Fax 365-2745
Miss Tanya Nunnally (Patent specialist)

4. SHIPPER(s): *(Name and Address of producer/shipper)*

5. DESCRIBE THE MATERIAL TO BE IMPORTED *(Provide the following information, as applicable: Animal species and tissue of origin of animal product, country of origin of the animals from which the raw animal product was sourced, processing country, recombinant system and genetic inserts, antibody immunogens, stabilizers, nutritive factors of animal origin in media.) (COMPLETE VS FORM 16-7 for cell cultures and their products)*

25 glass vials containing freeze dried Avian Mannheimia haemolytica formerly known as Pasteurella haemolytica.
Identified as BIV-07990

6. QUANTITY, FREQUENCY OF IMPORTATION, AND EXPECTED COMPLETION DATE *(estimate)*

7. PROPOSED USE OF MATERIAL AND DERIVATIVES *(Also, for animal pathogens or vectors describe facilities/biosafety procedures)*

8. IF FOR USE IN ANIMALS, SPECIFY THE ANIMAL SPECIES

9. TREATMENT OF MATERIAL PRIOR TO IMPORTATION INTO THE U.S *(Processing/purification methods, including time at specific temperatures, pH, other treatments, disease safeguard etc.)*

See annex 6

10. METHOD OF FINAL DISPOSITION OF IMPORTED MATERIAL AND DERIVATIVES

I CERTIFY AS AUTHORIZED BY THE COMPANY/INSTITUTION THAT I REPRESENT, THAT THIS MATERIAL WILL BE USED IN ACCORDANCE WITH ALL RESTRICTIONS AND PRECAUTIONS AS MAY BE SPECIFIED IN THE PERMIT.

11. SIGNATURE OF APPLICANT

12. TYPED NAME AND TITLE

13. DATE

14. APHIS USER FEE CREDIT ACCOUNT NO. OR METHOD OF USER FEE PAYMENT *(for VISA or Mastercard include number and expiration date.)*

VS FORM 16-3 (NOV 99)

Boehringer Ingelheim

ANNEX 3

Budapest Treaty Deposits

Answer to question No. 6[th].

Culture. This strain could be grown in Brain Heart Infusion broth (Difco) or Tryptose Soy broth. The log phase growth is around six to eight hours of incubation at 37°C.
In Blood Agar peak of growth is observed after twenty four hours at 37°C, an advantage of this method is that hemolysis could be observed.

Preservation by lyophilization. Bacteria is grown in Brain Heart Infusion broth (Difco) for six to eight hours. Use a 10% sterile solution of Bacto Skim Milk (Difco) as stabilizer at a ratio of 1 : 1 (v / v)

Viability test. Take a freeze dried vial and reconstitute with one ml of sterile PBS 1X, pH 7.2. Make serial ten fold dilutions with sterile PBS, pH 7.2. Seed 100 µl of each dilution on Blood Agar plates. Incubate for 24 hours at 37°C . Check viability by colony counting methodology.

Answer to question No. 7[th].

7.3. **BIV – 0799**

Macroscopic morphology
Colonies grown on Sheep Blood Agar for 24 hours, range from 1.0 to 1.5 mm in diameter, bright traslucent, low convex, smooth and creamy, β hemolysis.
Microscopic morphology
Gram negative , non motile, pleomorphic rods, often exhibit bipolar staining.
Biochemical and other tests

TEST	REACTION
Oxidase	+
Catalase	+
Indole	-
Glucose	+
Sacarose	+
MacConkey	+
Urease	-
Nitrate	+
Maltose	+
Mannitol	+
Arabinose	-
Celobiose	-
Sorbitol	+
Xilose	+
Treahalose	-
Salicin	-
Ornithine	-
Esculine	-
β Glucosidase	-
α - Fucosidase	-
β Galactosidase	+

Boehringer Ingelheim

ANNEX 6

Importation Permit

Answer to question No. 6[th] (Treatment of material prior to importation into the U. S.)

BIV – 07990 strain

1. Isolation made from layer oviduct
2. Organ maceration
3. Inoculate in nutrient broth (20 ml of BHI from Difco)
4. Incubation 24 hours at 37°C
5. Seed on sheep blood agar plates
6. Isolate a β haemolytic colony
7. Seed on sheep blood agar plates
8. Identification (Biochemical and other tests)
9. Seed on BHI broth from Difco
10. Grow the bacteria in Brain Heart Infusion broth (Difco) for six to eight hours
11. Add 10% sterile solution of Bacto Skim Milk (Difco) as stabilizer at a ratio of 1 : 1 (v / v)
12. Samples freeze dried
13. Viability, purity and biochemical tests.

Budapest Treaty Deposits

American Type Culture Collection

10801 University Blvd., Manassas, VA 20110-2209
Phone (703) 365-2700; fax (703) 365-2745; e-mail applied-sci@atcc.org

TO DEPOSIT OR TO CONVERT A DEPOSIT TO MEET THE REQUIREMENTS OF THE BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

ALL QUESTIONS MUST BE COMPLETED IN ENGLISH. PLEASE USE ONE FORM FOR EACH STRAIN DEPOSITED.

1. Name of deposit. **If microorganism**, give complete scientific name including genus and species and source of material; **If virus**, give name, whether plant or animal, and source including geographic location; **if cell line**, give tissue and species, geographical source of isolation, and any known hazards associated (HIV, EBV, etc.); **if genetic materials**, give name of organism from which vector, clone or library is derived, source of the DNA insert identified by species (e.g. human, mouse) or scientific name, and give name of gene and identity of the host organism; **If consortia or mixed culture**, each component of the mixture must be identified; **If seeds, embryos, insect eggs, etc.**, give common name, scientific name, and geographical source.
   Avian Pasteurella trehalosi, formerly known as Pasteurella haemolytica

2. Strain designation (i.e., number, symbols, etc.) BIV - AVICOR
   The strain designation must correspond with the vial labels.

3. Is this an original deposit under the Budapest Treaty? Yes

4. Is this a request for a conversion of a deposit already at ATCC to meet the requirements of the Budapest Treaty? If so please indicate ATCC designation. Not applicable

5. Is this deposit a mixture of microorganisms or cells? No

6. Provide details necessary to cultivate, test for viability, and store deposit. If mixture, provide description of components and a method to check presence. If plasmid, provide name of host and antibiotic resistance.
   See annex 2

7. Provide sufficient description so that ATCC may confirm deposit properties (e.g., Gram negative rod).
   See annex 2

   a. If deposit is a cell culture, is it being cultured in the presence of antibiotics? If so, please list the antibiotics.
   Not applicable

   b. If deposit is hybridoma, what is the isotype of antibody produced? Not applicable

8. Is this strain hazardous to humans? No Animals? Yes Plants? No. If yes, what is the recommended biosafety level for working with this strain? 1. (Refer to Biosafety in Microbiological and Biomedical Laboratories, 4th ed. U.S. Dept. of Health and Human Services at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm].

9. Availability: Prior to the issuance of a U.S. Patent, ATCC will only make a culture available as instructed by the depositor or relevant patent office. Samples must be provided to a specific investigator if a pertinent patent office under the Budapest Treaty instructs ATCC to do so. The following questions must be answered:

   a) As of date of deposit or conversion to meet the requirements of the Budapest Treaty, do you wish the deposit to be made available to anyone who requests a culture? If yes there are no restrictions on distribution. Answering no will **ensure the deposit is not available until the patent has issued.** Yes ____ No XX

   b) As of date of deposit or conversion to meet the requirements of the Budapest Treaty, do you wish the deposit to be made available to requestors which satisfy patent offices in countries not signatory to the Budapest Treaty? Yes ____ No X
   If yes state which countries. ____

Please note that if you are converting your deposit to meet the requirements of the Budapest Treaty and your deposit has already been released for distribution due to the issuance of a U.S. patent, you cannot restrict it from further distribution.

After a U.S. patent issues and we are so notified, ATCC makes the culture available to anyone who requests it, as allowed under USPTO Rules and Regulations (37 CFR 1.808 [a][2]).

10. ATCC will notify you of your ATCC number after confirmation of viability testing is complete.
Name of individual to notify: Abelardo Aguilar
Fax: (52 3) 668 80 80 Phone: (52 3) 668 80 38 E-mail: baguilar@gua.boehringer-ingelheim.com

11. Payment by check, or credit card (Mastercard, VISA or American Express), must accompany the deposit unless prior arrangements for billing have been made and approved. ATCC accepts Purchase Orders in the correct amount:

Purchase Order No. _____ Check No. _____

Credit Card number. Please indicate MasterCard, VISA, or AE. _____

Exp. Date: _____ Name shown on card: _____
(Please type or print clearly.)

_____
Signature of card holder

PAYMENT: ATCC MUST HAVE A BILLING ADDRESS, CONTACT PERSON, PHONE AND FAX NUMBER FOR ALL DEPOSITS:
Contact Person : Abelardo Aguilar
Calle 30 No. 2614 Zona Industrial C.P. 44940
Guadalajara, Jalisco. México
Phone: (52 3) 668 80 38 Fax: (52 3) 668 80 80

12. Name, address, telephone and facsimile number of your attorney of record. _____
_____
_____ (Ref: Docket or Case No. _____)

13. **MUST BE COMPLETED.** Deposited on behalf of: (Verify with your management who owns the deposit. The owner is usually a company or institute and not an individual.)
Boehringer Ingelheim Vetmedica, S.A. de C.V.

I understand and agree that the deposit may not be withdrawn by me for a period specified in Rule 9.1 of the Budapest Treaty (at least 30 years after the date of deposit or 5 years after the date of the most recent request for the deposit, whichever is longer), and that if a culture should die or be destroyed during the life of the patent or the period of time so specified, it is my responsibility to replace it with a living culture of the same organism or cell. In the cases of viruses, cell cultures, plasmids, embryos, and seeds, it is my responsibility to supply a sufficient quantity for distribution for the period of time specified above.

Ing. Jorge Súchil B. _____ May 24, 2001
Typed Name Signature Date

Address: Calle 30 No. 2614 Zona Industrial 44940 Guadalajara, Jalisco. México

Phone: (52 3) 668 80 00 Fax: (52 3) 668 80 80 E-mail: jsuchil@gua.boehringer-ingelheim.co

ADDRESS SHIPMENTS AND FORM TO THE ATTENTION OF:

Patent Depository
American Type Culture Collection
10801 University Blvd.
Manassas, VA 20110-2209 U.S.A.

STORAGE: Cultures are stored for 30 years from date of deposit or 5 years after the last request for a sample, whichever is longer, as required under the rules of patent offices in most countries.
FEES: For current fees, check our Web site at www.atcc.org, request a fee sheet by e-mail to applied-sci@atcc.org, or call (703) 365-2700 ext. 320. All fees are subject to change.

ATCC USE ONLY: ATCC DESIGNATION _____ REC'D _____ V.T. RESULT _____

Name of Deposit _____ Strain Designation: _____

Form BP/1 Rev. 2/00

Failure to supply all applicable information can delay the processing of this a pplication. PLEASE TYPE OR PRINT CLEARLY

No controlled material, organisms or vectors may be imported or moved interstate unless the data requested on this form is furnished and certified (9 CFR 84, 95, and 122).

According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0579-0015. The time required to complete this information collection is estimated to average between 1.5 and 3 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

FORM APPROVED
OMB NO. 0579-0015

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
National Center for Import-Export, Products Program
4700 River Road, Unit 40
Riverdale, MD 20737-1231

APPLICATION FOR PERMIT TO:

IMPORT OR TRANSPORT CONTROLLED MATERIAL OR ORGANISMS OR VECTORS

1. MODE OF TRANSPORTATION *(Please "X"):*

☐ AIR ☐ SEA ☐ LAND ☐ ANY

2. U.S. Ports of Entry

3. IMPORTER *(Name, organization, complete address, telephone and fax number of individual who will receive and be responsible for the imported material)*

American Type Culture Collection
10801 University Bvd.
Manassas, VA 20110 - 2209
Phone (703) 365-2700 Fax 365-2745
Miss Tanya Nunnally (Patent specialist)

4. SHIPPER(s): *(Name and Address of producer/shipper)*

5. DESCRIBE THE MATERIAL TO BE IMPORTED *(Provide the following information, as applicable: Animal species and tissue of origin of animal product, country of origin of the animals from which the raw animal product was sourced, processing country, recombinant system and genetic inserts, antibody immunogens, stabilizers, nutritive factors of animal origin in media.)* (COMPLETE VS FORM 16-7 for cell cultures and their products)

25 glass vials containing freeze dried Avian Pasteurella trehalosi , formerly known as Pasteurella haemolytica. Identified as BIV-AVICOR

6. QUANTITY, FREQUENCY OF IMPORTATION, AND EXPECTED COMPLETION DATE *(estimate)*

7. PROPOSED USE OF MATERIAL AND DERIVATIVES *(Also, for animal pathogens or vectors describe facilities/biosafety procedures)*

8. IF FOR USE IN ANIMALS, SPECIFY THE ANIMAL SPECIES

9. TREATMENT OF MATERIAL PRIOR TO IMPORTATION INTO THE U.S *(Processing/purification methods, including time at specific temperatures, pH, other treatments, disease safeguard etc.)*

See annex 5

10. METHOD OF FINAL DISPOSITION OF IMPORTED MATERIAL AND DERIVATIVES

I CERTIFY AS AUTHORIZED BY THE COMPANY/INSTITUTION THAT I REPRESENT, THAT THIS MATERIAL WILL BE USED IN ACCORDANCE WITH ALL RESTRICTIONS AND PRECAUTIONS AS MAY BE SPECIFIED IN THE PERMIT.

11. SIGNATURE OF APPLICANT

12. TYPED NAME AND TITLE

13. DATE

14. APHIS USER FEE CREDIT ACCOUNT NO. OR METHOD OF USER FEE PAYMENT *(for VISA or Mastercard include number and expiration date.)*

VS FORM 16-3 (NOV 99)

Boehringer Ingelheim

ANNEX 2

Budapest Treaty Deposits

Answer to question No. 6th.

Culture. This strain could be grown in Brain Heart Infusion broth (Difco) or Tryptose Soy broth. The log phase growth is around six to eight hours of incubation at 37°C.
In Blood Agar peak of growth is observed after twenty four hours at 37°C, an advantage of this method is that hemolysis could be observed.

Preservation by lyophilization. Bacteria is grown in Brain Heart Infusion broth (Difco) for six to eight hours. Use a 10% sterile solution of Bacto Skim Milk (Difco) as stabilizer at a ratio of 1 : 1 (v / v)

Viability test. Take a freeze dried vial and reconstitute with one ml of sterile PBS 1X, pH 7.2.
Make serial ten fold dilutions with sterile PBS, pH 7.2. Seed 100 µl of each dilution on Blood Agar plates. Incubate for 24 hours at 37°C. Check viability by colony counting methodology.

Answer to question No. 7th.

7.2. BIV – AVICOR

Macroscopic morphology
Colonies grown on Sheep Blood Agar for 24 hours, range from 1.0 to 1.5 mm in diameter, bright traslucent, low convex, smooth and creamy, β hemolysis.
Microscopic morphology
Gram negative, non motile, pleomorphic rods, often exhibit bipolar staining.
Biochemical and other tests

TEST	REACTION
Oxidase	+
Catalase	+
Indole	-
Glucose	+
Sacarose	+
MacConkey	+
Urease	-
Nitrate	+
Maltose	+
Mannitol	+
Arabinose	-
Celobiose	-
Sorbitol	+
Xilose	+
Treahalose	+
Salicin	-
Ornithine	-
Esculine	-
β Glucosidase	+[a]
α - Fucosidase	-
β Galactosidase	+

[a] 80% are positive

Boehringer Ingelheim

ANNEX 5

Importation Permit

Answer to question No. 6[th] (Treatment of material prior to importation into the U. S.)

BIV – AVICOR strain

1. Isolation made from heart of layer hen
2. Organ maceration
3. Inoculate in nutrient broth (20 ml of BHI from Difco)
4. Incubation 24 hours at 37°C
5. Seed on sheep blood agar plates
6. Isolate a β haemolytic colony
7. Seed on sheep blood agar plates
8. Identification (Biochemical and other tests)
9. Seed on BHI broth from Difco
10. Grow the bacteria in Brain Heart Infusion broth (Difco) for six to eight hours
11. Add 10% sterile solution of Bacto Skim Milk (Difco) as stabilizer at a ratio of 1 : 1 (v / v)
12. Samples freeze dried
13. Viability, purity and biochemical tests.

Budapest Treaty Deposits

American Type Culture Collection

10801 University Blvd., Manassas, VA 20110-2209
Phone (703) 365-2700; fax (703) 365-2745; e-mail applied-sci@atcc.org

TO DEPOSIT OR TO CONVERT A DEPOSIT TO MEET THE REQUIREMENTS OF THE BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

ALL QUESTIONS MUST BE COMPLETED IN ENGLISH. PLEASE USE ONE FORM FOR EACH STRAIN DEPOSITED.

1. Name of deposit. **If microorganism**, give complete scientific name including genus and species and source of material; **if virus**, give name, whether plant or animal, and source including geographic location; **if cell line**, give tissue and species, geographical source of isolation, and any known hazards associated (HIV, EBV, etc.); **if genetic materials**, give name of organism from which vector, clone or library is derived, source of the DNA insert identified by species (e.g. human, mouse) or scientific name, and give name of gene and identity of the host organism; **if consortia or mixed culture**, each component of the mixture must be identified; **if seeds, embryos, insect eggs, etc.**, give common name, scientific name, and geographical source.

Avian Pasteurella trehalosi, formerly known as Pasteurella haemolytica

2. Strain designation (i.e., number, symbols, etc.) BIV - 4895
The strain designation must correspond with the vial labels.

3. Is this an original deposit under the Budapest Treaty? Yes

4. Is this a request for a conversion of a deposit already at ATCC to meet the requirements of the Budapest Treaty? If so please indicate ATCC designation. Not applicable

5. Is this deposit a mixture of microorganisms or cells? No

6. Provide details necessary to cultivate, test for viability, and store deposit. If mixture, provide description of components and a method to check presence. If plasmid, provide name of host and antibiotic resistance.
See annex 1

7. Provide sufficient description so that ATCC may confirm deposit properties (e.g., Gram negative rod).
See annex 1

a. If deposit is a cell culture, is it being cultured in the presence of antibiotics? If so, please list the antibiotics.
Not applicable

b. If deposit is hybridoma, what is the isotype of antibody produced? Not applicable

8. Is this strain hazardous to humans? No Animals? Yes Plants? No. If yes, what is the recommended biosafety level for working with this strain? 1. (Refer to Biosafety in Microbiological and Biomedical Laboratories, 4th ed. U.S. Dept. of Health and Human Services at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm].

9. Availability: Prior to the issuance of a U.S. Patent, ATCC will only make a culture available as instructed by the depositor or relevant patent office. Samples must be provided to a specific investigator if a pertinent patent office under the Budapest Treaty instructs ATCC to do so. The following questions must be answered:

a) As of date of deposit or conversion to meet the requirements of the Budapest Treaty, do you wish the deposit to be made available to anyone who requests a culture? If yes there are no restrictions on distribution. **Answering no will ensure the deposit is not available until the patent has issued.** Yes ____ No X

b) As of date of deposit or conversion to meet the requirements of the Budapest Treaty, do you wish the deposit to be made available to requestors which satisfy patent offices in countries not signatory to the Budapest Treaty? Yes ____ No X
If yes state which countries. ____

Please note that if you are converting your deposit to meet the requirements of the Budapest Treaty and your deposit has already been released for distribution due to the issuance of a U.S. patent, you cannot restrict it from further distribution.

After a U.S. patent issues and we are so notified, ATCC makes the culture available to anyone who requests it, as allowed under USPTO Rules and Regulations (37 CFR 1.808 [a][2]).

10. ATCC will notify you of your ATCC number after confirmation of viability testing is complete.
Name of Individual to notify: Abelardo Aguilar
Fax: (52 3) 668 80 80 Phone: (52 3) 668 80 38 E-mail: baguilar@gua.boehringer-ingelheim.com

11. Payment by check, or credit card (Mastercard, VISA, or American Express), must accompany the deposit unless prior arrangements for billing have been made and approved. ATCC accepts Purchase Orders in the correct amount.

Purchase Order No. _____ Check No. _____

Credit Card number. Please indicate MasterCard, VISA, or AE. _____

Exp. Date: _____ Name shown on card: _____
(Please type or print clearly.)

_____
Signature of card holder

PAYMENT: ATCC MUST HAVE A BILLING ADDRESS, CONTACT PERSON, PHONE AND FAX NUMBER FOR ALL DEPOSITS:
Contact person : Abelardo Aguilar
Calle 30 No. 2614 Zona Industrial C.P. 44940
Guadalajara, Jalisco. México
Phone: (52 3) 668 8038 Fax: (52 3) 668 80 80

12. Name, address, telephone and facsimile number of your attorney of record. _____

_____ (Ref: Docket or Case No. _____)

13. **MUST BE COMPLETED.** Deposited on behalf of: (Verify with your management who owns the deposit. The owner is usually a company or institute and not an individual.)
Boehringer Ingelheim Vetmedica, S.A. de C.V.

I understand and agree that the deposit may not be withdrawn by me for a period specified in Rule 9.1 of the Budapest Treaty (at least 30 years after the date of deposit or 5 years after the date of the most recent request for the deposit, whichever is longer), and that if a culture should die or be destroyed during the life of the patent or the period of time so specified, it is my responsibility to replace it with a living culture of the same organism or cell. In the cases of viruses, cell cultures, plasmids, embryos, and seeds, it is my responsibility to supply a sufficient quantity for distribution for the period of time specified above.

Ing. Jorge Súchil B. _____ May 24, 2001
Typed Name Signature Date

Address: Calle 30 No. 2614 Zona Industrial 44940 Guadalajara, Jalisco, México
Phone: (52 3) 668 80 00 Fax: (52 3) 668 80 80 E-mail: jsuchil@gua.boehringer-ingelheim.co

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10801 University Blvd.
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STORAGE: Cultures are stored for 30 years from date of deposit or 5 years after the last request for a sample, whichever is longer, as required under the rules of patent offices in most countries.
FEES: For current fees, check our Web site at www.atcc.org, request a fee sheet by e-mail to applied-sci@atcc.org, or call (703) 365-2700 ext. 320. All fees are subject to change.

ATCC USE ONLY: ATCC DESIGNATION_____ REC'D _____ V.T. RESULT_____

Name of Deposit _____ Strain Designation: _____

Form BP/1 Rev. 2/00

Failure to supply all applicable information can delay the processing of this application. PLEASE TYPE OR PRINT CLEARLY

No controlled material, organisms or vectors may be imported or moved interstate unless the date requested on this form is furnished and verified (9 CFR 94, 95, and 122).

According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0579-0015. The time required to complete this information collection is estimated to average between 1.6 and 3 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information.

FORM APPROVED
OMB NO. 0579-0015

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES
National Center for Import-Export, Products Program
4700 River Road, Unit 40
Riverdale, MD 20737-1231

APPLICATION FOR PERMIT TO:

IMPORT OR TRANSPORT CONTROLLED MATERIAL OR ORGANISMS OR VECTORS

1. MODE OF TRANSPORTATION *(Please "X")*:

☐ AIR ☐ SEA ☐ LAND ☐ ANY

2. U.S. Ports of Entry

3. IMPORTER *(Name, organization, complete address, telephone and fax number of individual who will receive and be responsible for the imported material)*

American Type Culture Collection
10801 University Bvd.
Manassas VA20110 - 2209
Phone (703)365-2700 Fax 365-2745
Miss Tanya Nunnally (Patent specialist)

4. SHIPPER(s): *(Name and Address of producer/shipper)*

5. DESCRIBE THE MATERIAL TO BE IMPORTED *(Provide the following information, as applicable: Animal species and tissue of origin of animal product, country of origin of the animals from which the raw animal product was sourced, processing country, recombinant system and genetic inserts, antibody immunogens, stabilizers, nutritive factors of animal origin in media.) (COMPLETE VS FORM 16-7 for cell cultures and their products)*

25 glass vials containing freeze dried Avian Pasteurella trehalosi, formerly known as Pasteurella haemolytica. Identified as BIV-4895

6. QUANTITY, FREQUENCY OF IMPORTATION, AND EXPECTED COMPLETION DATE *(estimate)*

7. PROPOSED USE OF MATERIAL AND DERIVATIVES *(Also, for animal pathogens or vectors describe facilities/biosafety procedures)*

8. IF FOR USE IN ANIMALS, SPECIFY THE ANIMAL SPECIES

9. TREATMENT OF MATERIAL PRIOR TO IMPORTATION INTO THE U.S *(Processing/purification methods, including time at specific temperatures, pH, other treatments, disease safeguard etc.)*

See annex 4

10. METHOD OF FINAL DISPOSITION OF IMPORTED MATERIAL AND DERIVATIVES

I CERTIFY AS AUTHORIZED BY THE COMPANY/INSTITUTION THAT I REPRESENT, THAT THIS MATERIAL WILL BE USED IN ACCORDANCE WITH ALL RESTRICTIONS AND PRECAUTIONS AS MAY BE SPECIFIED IN THE PERMIT.

11. SIGNATURE OF APPLICANT

12. TYPED NAME AND TITLE

13. DATE

14. APHIS USER FEE CREDIT ACCOUNT NO. OR METHOD OF USER FEE PAYMENT *(for VISA or Mastercard include number and expiration date.)*

VS FORM 16-3 (NOV 99)

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ANNEX I

Budapest Treaty Deposits

Answer to question No. 6th.

Culture. This strain could be grown in Brain Heart Infusion broth (Difco) or Tryptose Soy broth. The log phase growth is around six to eight hours of incubation at 37°C.
In Blood Agar peak of growth is observed after twenty four hours at 37°C, an advantage of this method is that hemolysis could be observed.

Preservation by lyophilization. Bacteria is grown in Brain Heart Infusion broth (Difco) for six to eight hours. Use a 10% sterile solution of Bacto Skim Milk (Difco) as stabilizer at a ratio of 1 : 1 (v / v)

Viability test. Take a freeze dried vial and reconstitute with one ml of sterile PBS 1X, pH 7.2. Make serial ten fold dilutions with sterile PBS, pH 7.2. Seed 100 µl of each dilution on Blood Agar plates. Incubate for 24 hours at 37°C. Check viability by colony counting methodology.

Answer to question No. 7th.

7.1. **BIV – 4895**

Macroscopic morphology
Colonies grown on Sheep Blood Agar for 24 hours, range from 1.0 to 1.5 mm in diameter, bright traslucent, low convex, low solubility and dry, β hemolysis.
Microscopic morphology
Gram negative, non motile, pleomorphic rods, often exhibit bipolar staining.
Biochemical and other tests

TEST	REACTION
Oxidase	+
Catalase	+
Indole	-
Glucose	+
Sacarose	+
MacConkey	+
Urease	-
Nitrate	+
Maltose	-
Mannitol	+
Arabinose	-
Celobiose	-
Sorbitol	+
Xilose	+
Treahalose	+
Salicin	-
Ornithine	-
Esculine	-
β Glucosidase	-
α - Fucosidase	-
β Galactosidase	+

Boehringer Ingelheim

ANNEX 4

Importation Permit

Answer to question No. 6th (Treatment of material prior to importation into the U. S.)

BIV – 4895 strain

1. Isolation made from palatine cleft of layer hen
2. Inoculation on nutrient broth (20 ml of BHI from Difco)
3. Incubation 24 hours at 37°C
4. Seed on sheep blood agar plates
5. Isolate a β haemolytic colony
6. Seed on sheep blood agar plates
7. Identification (Biochemical and other tests)
8. Seed on BHI broth from Difco
9. Grow the bacteria in Brain Heart Infusion broth (Difco) for six to eight hours
10. Add 10% sterile solution of Bacto Skim Milk (Difco) as stabilizer at a ratio of 1 : 1 (v / v)
11. Samples freeze dried
12. Viability, purity and biochemical tests.

Claims

1. Gram-negative, facultative anaerobic,pleomorphic rod-shaped bacteria causing a novel disease of the upper respiratory and of the reproductive tract of poultry, wherein said bacteria are selected from the group of *Pasteurella trehalosi and/or Mannheimia haemolytica* and wherein said Pasteurella and/or Mannheimia are beta haemolysis-positive, Gram-negative, oxidase- positive, catalase-positive, urease-negative, nitrate-positive and indole-negative.
2. *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claim 1, wherein said Pasteurella and/or Mannheimia are MacConkey positive.
3. *Pasteurella trehalosi and/or Mannheimia haemolytica* according to any one of claims 1 or 2, wherein said Pasteurella and/or Mannheimia are Glucose-positive, Sucrose-positive, Mannitol-positive, Arabinose-negative, Celobiose-negative, Xylose-positive, Salicin-negative, Ornithine-negative, Esculin-negative, alpha-Fucosidase-negative, beta-Galactosidase-positive.
4. *Pasteurella trehalosi* according to any one of claims 1 to 3, wherein said Pasteurella are Arabinose-negative and Trehalose-positive.
5. *Mannheimia haemolytica* according to any one of claims 1 to 3, wherein said Mannheimia are Arabinose-negative and Trehalose-negative.
6. *Pasteurella trehalosi andlor Mannheimia haemolytica* according to any one of claims 1 to 5, wherein said poultry is selected from the group of chicken, turkey, duck, goose, dove, pigeon and quail.
7. *Pasteurella trehalosi* as deposited at the under accession number ATCC No. PTA-3667.
8. *Pasteurella trehalosi* as deposited at the under accession number ATCC No. PTA-3668.
9. *Mannheimia haemolytica* as deposited at the under accession number ATCC No. PTA-3669.
10. Method for inactivation of a *Pasteurella trehalosi and/or Mannheimia haemolytica* according to any one of claims 1 to 9 comprising the use of formaldehyde to a final concentration of 0.2%.
11. Inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* obtainable by a method according to claim 10 or by a method known in the art.

12. Inactivated *Pasteurella trehalosi andlor Mannheimia haemolytica* according to claim 11, wherein said *Pasteurella trehalosi andlor Mannheimia haemolytica* are selected from the group of *Pasteurella trehalosi* ATCC No. PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA-3669.
13. Live attenuated *Pasteurella trehalosi and/or Mannheimia haemolytica* wherein the *Pasteurella trehalosi and/or Mannheimia haemolytica* according to any one of claims 1 to 9 are attenuated.
14. Live attenuated *Pasteurella trehalosi andlor Mannheimia haemolytica* according to claim 13, wherein said *Pasteurella trehalosi and/or Mannheimia haemolytica* are selected from the group of *Pasteurella trehalosi* ATCC No. PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No.PTA-3669.
15. Fragments or fractions containing at least one antigen of *Pasteurella trehalosi* and/or *Mannheimia haemolytica* according to any one of claims 1 to 9.
16. Fragments or fractions according to claim 15, wherein said fragments are containing at least one antigen of bacteria selected from the group of *Pasteurella trehalosi* ATCC No.PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No.PTA- 3669.
17. Vaccine composition comprising an inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claim 11 or 12 and/or live *Pasteurella trehalosi and/or Mannheimia haemolytica* according to any one of claims 1 to 9 and/or live attenuated *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claim 13 or 14 and/or fragments or fractions of said *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claim 15 to 16.
18. Vaccine composition according to claim 17, further comprising one or more suitable adjuvant(s) and/or excipient(s) and/or carrier(s).
19. Vaccine composition according to claim 17 or 18, comprising inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica*, wherein said *Pasteurella trehalosi and/or Mannheimia haemolytica* are selected from the group of *Pasteurella trehalosi* ATCC No.PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA- 3669.
20. Vaccine composition according to any one of claims 17 to 19, wherein said vaccine comprises two or more strains of live, and/or live attenuated, and/or

inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claims 1 to 9 and 11 to 14 and/or fractions of two or more strains of said *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claims 15 or 16.

21. Vaccine composition according to any one of claims 17 to 20, wherein said live, and/or live attenuated, and/or inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* according to the invention and/or fractions of said *Pasteurella trehalosi and/or Mannheimia haemolytica* in the vaccine are selected from the group of *Pasteurella trehalosi* ATCC No. PTA-3667, *Pasteurella trehalosi* ATCC No. PTA-3668 and/or *Mannheimia haemolytica* ATCC No. PTA-3669.

22. Vaccine composition according to any one of claims 17 to 21, further comprising at least one other antigen from a virus or microorganism pathogenic to poultry.

23. Vaccine composition according to any one of claims 17 to 22, wherein said virus or microorganism is selected from the group consisting of Infectious Bronchitis Virus, Newcastle Disease Virus, Infectious Bursal Disease Virus, Chicken Anaemia agent, Avian Reovirus, *Mycoplasma gallisepticum*, Avian Pneumovirus, *Haemophilus paragallinarum*, Chicken Poxvirus, Avian Encephalomyelitis virus, *Pasteurella multocida* and *E. coli*.

24. Use of an inactivated *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claim 11 or 12 and/or live *Pasteurella trehalosi and/or Mannheimia haemolytica* according to any one of claims 1 to 9 and/or live attenuated *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claim 13 or 14 and/or fragments or fractions of said *Pasteurella trehalosi and/or Mannheimia haemolytica* according to claim 15 to 16 in the manufacture of a vaccine for the prophylaxis of *Pasteurella trehalosi and/or Mannheimia haemolytica* infections.

25. Method of diagnosis of a disease caused by comprising the steps of obtaining a sample from poultry, wherein said sample is selected from the group of blood, serum, plasma, tissue scrapings, washings, swabs, tissue, analysing said sample for the presence of *Pasteurella trehalosi and/or Mannheimia haemolytica* according to any one of claims 1 to 9.

26. Method of diagnosis according to claim 25, wherein said presence of *Pasteurella trehalosi and/or Mannheimia haemolytica* is determined by an immune test.

27. Method of diagnosis according to claim 25, wherein said presence of *Pasteurella trehalosi and/or Mannheimia haemolytica* is determined by an molecular biology methods.

28. Diagnostic test kit comprising all the components necessary for carrying out a method according to any one of claims 25 to 27.