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- (71) **Applicants:** AQUILON CYL, S.L [ES/ES]; Aquilon CYL, S.l, Campus vegazana s/n, E-24004 León (ES). UNIVERSITAT AUTONOMA DE BARCELONA [ES/ES]; Universitat Autònoma de Barcelona, Edifi. A - Campus Universitari s/n, E-08193 Bellaterra (Barcelona) (ES).
- (72) **Inventors:** LLAGOSTERA CASAS, Montserrat; C/ La Balsa, no. 1, casa 14, E-08290 Cerdanyola del Vallès (ES). BARBÉ GARCÍA, Jordi; C/ La Balsa, no. 1, casa 14, E-08290 Cerdanyola del Vallès (ES). CAMPOY SÁNCHEZ, Susana; C/ Joan Baptista Galí, no. 9 lero 2da, E-08221 Terrassa (ES). MARCA PUIG, Joan; Urb Fournier 19, E-08530 La Garriga (Barcelona) (ES).
- (74) **Agent:** DE CARLOS HERNANDO, Borja; Garrigues IP, S.L.P, Hermosilla, 3, E-28001 Madrid (ES).
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(54) **Title:** NOVEL VETERINARY VACCINE COMPOSITIONS FOR USE IN THE TREATMENT OF INFECTIOUS DISEASES CAUSED BY OR ASSOCIATED WITH PASTEURELLACEAE FAMILY BACTERIA

(57) **Abstract:** The present invention provides vaccine compositions comprising as active ingredient *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract thereof for the therapeutic and prophylactic treatment in animals of respiratory infections which are caused by or associated with bacteria belonging to the family *Pasteurellaceae*, to improve animal health and productivity.



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**NOVEL VETERINARY VACCINE COMPOSITIONS FOR USE IN THE TREATMENT
OF INFECTIOUS DISEASES CAUSED BY OR ASSOCIATED WITH
PASTEURELLACEAE FAMILY BACTERIA**

5 FIELD OF THE INVENTION

The present invention relates to the field of vaccines, and their application as therapeutic and prophylactic agents in the veterinary industry, for the control of pathogen infections in livestock, caused by, or associated with bacteria belonging to the family *Pasteurellaceae*.

- 10 In particular, the present invention provides new vaccine compositions for preventing the onset of, and therapeutically treating, respiratory infections in animals which are caused by, or are associated with bacterial pathogens belonging to the family *Pasteurellaceae*, to improve animal health and productivity. The present invention thus provides new compositions which are therapeutically effective in reducing the clinical
- 15 signs of respiratory diseases caused by bacterial pathogens belonging to the family *Pasteurellaceae*, in particular, those infections caused by the gram-negative bacterium *Actinobacillus pleuropneumoniae*.

BACKGROUND PRIOR ART

- Respiratory diseases in livestock in general cause major welfare and economic
- 20 problems for animal farms despite the widespread use of vaccines and antibiotics. Estimates of the financial cost run into millions per year, due to a combination of production losses, deaths, and veterinary bills.

- Infectious respiratory disease in livestock is a major welfare and economic problem on many farms. Carrier animals that may show no sign of disease, but are able to infect
- 25 others, are difficult to identify and treat. Even with modern drugs, bacteria and viruses in the upper respiratory tract are difficult to remove, making sick animals difficult to cure and susceptible to secondary infections.

Respiratory diseases suffered by farm animals are the results of a combination of primary and opportunistic infectious agents. In addition, adverse environmental and

management conditions of the farms play an important role in the multifactorial nature of infectious respiratory diseases.

Primary pathogens are capable of subverting the animal own defense mechanisms and establish infection on their own, whereas, opportunistic pathogens, take advantage of the virulence mechanisms of the primary pathogens to establish infections. Bacterial agents may act as both primary and opportunistic pathogens, depending on the situation. Although primary respiratory infectious agents can cause serious disease on their own, more often uncomplicated infections with these agents are mild and transient. It is when these primary infections become complicated with opportunistic bacteria that more serious and chronic respiratory diseases result.

Most commonly isolated or studied bacterial pathogens, being the cause or contributing to the onset of a respiratory infectious disease in livestock animals are pathogens belonging to the family of *Pasteurellaceae* bacteria, such as for example, *Pasteurella* sp., *Mannheimia* sp., *Haemophilus* sp., *Histophilus* sp., and *Actinobacillus* sp.

Porcine pleuropneumonia is one of the most important respiratory diseases of intensively raised swine throughout the world. It is caused by the gram-negative bacterium *Actinobacillus pleuropneumoniae*. The economic consequences of porcine pleuropneumoniae can be severe and are principally due to death, decrease average daily gain in weight and feed conversion ratios, slaughter trim losses and intervention costs. Pigs of all ages may be affected, but those 3 to 4 month of age are most frequently found to have this infection. The clinical course of porcine pleuropneumonia can be peracute and acute (characterized by dyspnea, cough, apathy, anorexia and sudden death) or subacute and chronic (characterized by intermittent cough and decrease growth parameters).

Haemophilus parasuis is a member of the family *Pasteurellaceae* and a common inhabitant of the upper respiratory tract of healthy pigs. It is also known as the etiological agent of Glässer's disease in swine, a systemic disease characterized by fibrinous polyserositis, which causes high morbidity and mortality in piglets. *H. parasuis* can also produce pneumonia and sudden death. Glässer's disease has

gained considerable importance in recent years and it is recognized as one of the main causes of economic loss in the pig industry.

Pneumonic pasteurellosis is one of the most economically important infectious diseases of ruminants with a wide prevalence throughout the continents. The disease is characterized by an acute febrile course with severe fibrinous or fibrinopurulent bronchopneumonia, fibrinous pleurisy and septicaemia. Infected animals may die within a few days of the onset of clinical signs, but those which survive the acute attack may become chronically infected. *Mannheimia haemolytica* is well established to be the major aetiological agent of the disease although *Pasteurella multocida* has also been incriminated in many acute outbreaks. Both *Mannheimia* and *Pasteurella* species are commensally resident in the respiratory tract of healthy ruminants and are capable of causing infection in animals with compromised pulmonary defense system. Hence, the disease is essentially triggered by physical or physiological stress created by adverse environmental and climatic conditions such as extremely bad weather, poor management, overcrowding, transportation or previous infection with respiratory viruses, mycoplasma or some other pathogenic organisms.

It is worth mentioning that *Mannheimia haemolytica*, *Pasteurella multocida* and *Pasteurella trehalosi* constitute the most important members of the family *Pasteurellaceae* that pose serious hazards in livestock industry. These species are commensally resident in the animal body as normal constituents of the nasopharyngeal microflora and are all capable of causing infection when the body defense mechanisms are impaired. Their presence is mainly confined to ruminants with most adequately characterized strains originating from cattle, sheep and goats (Biberstein & Hirsh, 1999). Examples of the most commonly recognized diseases associated with *Mannheimia haemolytica* include shipping fever in cattle, primary and secondary pneumonia in cattle sheep and goats, septicaemia and mastitis in sheep and a number of non-specific inflammatory lesions in various species of domestic animals (Quinn et al., 2002). *Pasteurella multocida* is, on the other hand, associated with haemorrhagic septicaemia in cattle and buffaloes and enzootic pneumonia complex in young ruminants (Jones et al., 1997). Other diseases such as fowl cholera and snuffles (an upper respiratory tract infection occasionally accompanied by pleurisy, pneumonia or fatal septicaemia in rabbits) are also caused by *Pasteurella multocida*. With regard to

Pasteurella trehalosi this organism is frequently associated with acute systemic disease or septicaemia in young sheep (Dyson et al., 1981; Jones et al., 1997).

In particular, *Mannheimia haemolytica* is the principal bacterium isolated from respiratory disease in feedlot cattle and is a significant component of enzootic pneumonia in all neonatal calves. For example, in the United States, *Mannheimia*
5 *haemolytica*, serotype 1 is the main bacteria responsible for the clinical signs and lesions of severe bovine pneumonia (shipping fever), particularly disease that is seen within the first week to 10 days after stress such as shipping.

The bacterium is a normal inhabitant of nasal passages and tonsils of ruminants.
10 Following stress or viral infections, the bacterium proliferates and is inhaled into the lungs where it stimulates acute signs of severe respiratory distress: coughing, nasal discharge, high fever, loss of appetite and, maybe, death.

The typical lesion is inflammation of the lung (pneumonia) and chest cavity (pleuritis). These lesions if inadequately treated, can become co-infected with other bacteria,
15 including *Mycoplasma*, resulting in chronic pneumonia with scarring and abscess formation.

Histophilus somni (formerly *Haemophilus somnus*) is a Gram-negative bacterium that is a member of the *Pasteurellaceae* family. It appears microscopically as a coccobacillus, is a facultative anaerobe, non-motile, and a non-spore-forming bacteria.
20 *Histophilus somni* is a commensal organism of cattle that may inhabit certain mucosal surfaces, including the upper airway and reproductive tract. *Histophilus somni* was first recognized as a pathogen in cattle in 1956. *Histophilus somni* is a pathogen of cattle worldwide, but the greatest prevalence of disease is focused in the large beef producing countries of the world, such as the United States and Canada. Infection with
25 *Histophilus somni* is most commonly a feedlot disease, but may be seen in dairy and grazing operations. Young growing cattle age 6-12 months are most commonly infected and show clinical signs. The actual prevalence of the bacteria is very high, and almost all cattle will be exposed at some point in their life. This can be noted in certain herds where 100% of animals have circulating antibodies to *Histophilus somni*. Actual
30 clinical disease, however, is uncommon in susceptible groups, with an incidence rate of

1-2% lower. Clinical disease can be devastating when it occurs. *Histophilus somni* is capable of causing a variety of disease syndromes, including thrombotic meningoencephalitis (TME), respiratory disease (*Histophilus somni* is a component of the Bovine Respiratory Disease Complex, BRDC), myocarditis, polysynovitis, otitis media, mastitis, and reproductive tract diseases.

Several different approaches have been used so far in the treatment of respiratory diseases caused by bacterial pathogens in livestock, however there is still a need for further technical improvement in the present field, to increase efficiency of therapeutic treatments and prevention of the diseases.

10 The state of the art describes treatment methods with antibiotics and vaccines. However, providing an effective agent or treatment method is complicated, as isolating and detecting the primary and/or relevant opportunistic pathogens in an infectious respiratory disease is not always possible.

Moreover, existing treatment methods are based on therapeutic agents or vaccines directed to a specific pathogen, which although effective against that specific pathogen, do not provide the desired preventive or therapeutic effect when treating an infectious respiratory disease, caused or associated with different pathogens.

The present invention is therefore directed to provide vaccine compositions with improved effectiveness for the prevention and therapeutic treatment of infectious respiratory disease in livestock animals caused by, or being associated with bacterial pathogens belonging to the family of *Pasteurellaceae* bacteria.

SUMMARY OF THE INVENTION

The term “infectious respiratory diseases caused by, or being associated with bacterial pathogens” in the context of the present invention refers to any respiratory disease suffered by livestock animals, which etiology is the result of primary infection or opportunistic infection by at least a bacterial pathogen belonging to the family of *Pasteurellaceae* bacteria. Non limiting examples thereof include: acute, subacute or chronic pneumonia, shipping fever, pleuropneumonia, porcine respiratory disease

complex, bovine respiratory disease complex, upper respiratory tract infections, diphtheria, lower respiratory tract infections, Glässer's disease and pasteurellosis.

The terms "livestock animals or animals" in the context of the present invention means animals raised in an agricultural setting to produce commodities such as food, fiber and
5 labor and include: cattle, horses, pigs, birds, poultry, sheep, goat and rabbit.

The term, "bacterial pathogen" in the context of the present invention refers to bacteria which cause a clinically evident illness (i.e., characteristic medical signs and/or symptoms of disease) resulting from a primary or secondary infection, presence and growth in a host animal.

10 The term "family of *Pasteurellaceae* bacteria" in the context of the present invention is meant to include a large family of gram-negative bacteria from the genera *Haemophilus*, *Pasteurella*, *Actinobacillus* and *Mannheimia*, but excluding any virulent strain and serotype of *Pasteurella multocida*. In particular, bacteria belonging to the family of *Pasteurellaceae* bacteria according to the present invention comprise any
15 virulent strain and/or serotype of *Mannheimia* sp., *Haemophilus* sp., *Histophilus* sp., *Pasteurella* sp. (other than *Pasteurella multocida*), and *Actinobacillus* sp. In particular, bacteria species belonging to the *Pasteurellaceae* family according to the present invention are selected from the group consisting of: *Manheimia haemolytica*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Histophilus somni*,
20 *Pasteurella trehalosi* and *Haemophilus paragallinarum*.

The term "therapeutic treatment" in the context of the present invention encompasses preventing or reducing the severity of/or associated with the symptoms of an infectious respiratory disease in an animal (livestock) caused by a bacterial pathogen belonging to the *Pasteurellaceae* family. Reducing the severity of the symptoms of an infectious
25 respiratory disease, according to the present invention include, reducing the clinical signs of an animal after challenged with a virulent bacterial pathogen belonging to the *Pasteurellaceae* family, in particular, *Actinobacillus pleuropneumoniae*. These clinical signs are amongst others, lung lesions, cough, fever, dyspnea, etc.

The term "prophylactic treatment" in the context of the present invention encompasses
30 the treatment of an animal (livestock) not suffering from an infectious respiratory disease caused by a bacterial pathogen belonging to the *Pasteurellaceae* family, to

prevent or at least reduce the likelihood of that animal suffering from that infectious respiratory disease.

The term "acceptable carrier, vehicles or coadjuvants" in the context of the present invention are meant to include any carrier, whether pharmaceutically acceptable or not, which does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound/composition. These vehicles include those acceptable and suitable for administering the compound/composition for an intended prophylactic and/or therapeutic treatment. Examples thereof include: lipopolysaccharides, the Freund's complete or incomplete adjuvant, monophospholipids, such as, monophospholipid A, sulfates, phosphates such as aluminium phosphate, and hydroxides, such as hydrated aluminum hydroxyphosphate and aluminium hydroxide, etc.

According to a first aspect, the invention relates to *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use alone or in combination with other active agents, in the prophylactic and therapeutic treatment of respiratory infections and/or reducing their clinical signs, in livestock animals caused by at least one bacterial pathogen belonging to the *Pasteurellaceae* family other than any virulent strain and serotype of the bacterial species *Pasteurella multocida*.

According to a second aspect, the present invention relates to *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use as defined above, wherein the bacterial pathogen belonging to the *Pasteurellaceae* family is selected from the group consisting of: any virulent strain and/or serotype of *Mannheimia* sp., *Haemophilus* sp., *Histophilus* sp., *Pasteurella* sp. (other than *Pasteurella multocida*), and *Actinobacillus* sp..

According to a third aspect, the present invention relates to *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use as defined above, wherein the bacterial pathogen belonging to the *Pasteurellaceae* family is selected from the group consisting of: any virulent strain and/or serotype of *Manheimia* haemolytica, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Histophilus somni*, *Pasteurella trehalosi* and *Haemophilus paragallinarum*.

According to a further aspect the invention relates to *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use as defined previously, wherein the respiratory infections caused by at least one pathogen belonging to the Pasteurellaceae family are selected from the group consisting of:

- 5 acute, subacute or chronic pneumonia, shipping fever, pleuropneumonia, porcine respiratory disease complex, bovine respiratory disease complex, upper respiratory tract infections, diphtheria, lower respiratory tract infections, Grässer's disease and pasteurellosis.

- A still further aspect of the invention relates to *Pasteurella multocida* strain PM1094 or
10 an outer-membrane protein extract prepared therefrom for use as previously defined in the prophylactic and therapeutic treatment and/or reducing the clinical signs of respiratory infections in livestock animals selected from the group consisting of: cattle, horses, pigs, birds, poultry, sheep, goat and rabbit.

- Additional objects of the present invention are *Pasteurella multocida* strain PM1094 or
15 an outer-membrane protein extract prepared therefrom for use as previously defined in the prophylactic and therapeutic treatment and/or reducing the clinical signs of respiratory infections in pigs caused by *Actinobacillus pleuropneumoniae*, in the prophylactic and therapeutic treatment and/or reducing the clinical signs of respiratory infections in pig, cattle or sheep caused by or associated with *Manheimia haemolytica*,
20 in the prophylactic and therapeutic treatment and/or reducing the clinical signs of respiratory infections in pigs caused by or associated with *Haemophilus parasuis*, in the prophylactic and therapeutic treatment and/or reducing the clinical signs of respiratory infections in cattle caused by or associated with *Histophilus somni*, in the prophylactic and therapeutic treatment and/or reducing the clinical signs of respiratory
25 infections in sheep caused by or associated with *Pasteurella trehalosi*, and in the prophylactic and therapeutic treatment and/or reducing the clinical signs of respiratory infections in poultry caused by or associated with *Haemophilus paragallinarum*.

- It is also an object of the present invention a vaccine compositions against respiratory infections and/or reducing the clinical signs in livestock animals caused by at least one
30 bacterial pathogen belonging to the Pasteurellaceae family other than any virulent strain and serotype of the bacterial species *Pasteurella multocida*, as defined previously, comprising an immunogenically effective amount of *Pasteurella multocida*

strain PM1094 or an outer-membrane protein extract prepared therefrom together with an acceptable carrier, vehicle, optionally an additional active agent and/or an adjuvant. In a further embodiment the invention provides a vaccine for inducing an immune response and/or reducing the clinical signs in an animal suffering from, or susceptible to, a condition associated with respiratory infections caused by at least one bacterial pathogen belonging to the *Pasteurellaceae* family, other than any virulent strain and serotype of *Pasteurella multocida*, in particular, *Actinobacillus pleuropneumoniae*, which comprises an effective amount of *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom, alone or in combination with another active agent.

In a still further embodiment the invention provides a method of increasing survival of an animal suffering from a respiratory disease caused by at least one bacterial pathogen belonging to the *Pasteurellaceae* family, other than any virulent strain and serotype of *Pasteurella multocida*, said method comprising administering to the infected animal an effective amount of *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom together with suitable carriers and vehicles and optionally an additional active agent.

FIGURES

Figure 1: Specific immunoglobulin against OMPs of several strains of *Haemophilus parasuis*, *Haemophilus influenza* and *Manhemia haemolytica*.

Figure 2: Specific immunoglobulin against OMPs of several strains of *Actinobacillus pleuropneumoniae*.

Figure 3: Specific immunoglobulin against OMPs of several strains of *Haemophilus parasuis*, *Haemophilus influenza* and *Manhemia haemolytica*.after subcutaneous vaccination.

Figure 4: Specific immunoglobulin against OMPs of several strains of *Haemophilus parasuis*, *Haemophilus influenza* and *Manhemia haemolytica*.after intraperitoneal vaccination.

Figure 5: Specific immunoglobulin against OMPs of several strains of *Actinobacillus pleuropneumoniae* after subcutaneous vaccination

Figure 6: Specific immunoglobulin against OMPs of several strains of *Actinobacillus pleuropneumoniae* after intraperitoneal vaccination.

- 5 **Figure 7:** Quantification of specific IgG against OMP of *Pasteurella multocida* PM1094 (the vaccine strain) (A), and *Actinobacillus pleuropneumoniae* (B) strains used for the challenge. Day 0, corresponds to the data of sera collected on day 0 of all animal groups; Day 35 are the data of sera collected on day 35 for all the vaccinated (T3 group) and non-vaccinated (T4 group) animals. No differences are detected between
- 10 Day 0 and Day 35 non-vaccinated. The statistical significances are $p < 0.01$ (***) in one-way ANOVA analysis.

Figure 8: Lung scores (average $\pm$ standard deviation) from all the animals from groups T03 (vaccinated) and T04 (non-vaccinated), after infection with *A. pleuropneumoniae*.

- 15 **Figure 9:** Lung scores (average $\pm$ standard deviation) from animals with lesions from groups T03 (vaccinated) and T04 (non-vaccinated), after infection with *A. pleuropneumoniae*.

Figure 10: Dyspnea observed in the animals from group T03 (vaccinated) and T04 (non-vaccinated) after infection with *A. pleuropneumoniae*. Upper panel: percentage of animals affected; lower panel: mean dyspnea scores $\pm$ standard deviation.

- 20 **Figure 11:** Coughing observed in the animals from group T03 (vaccinated) and T04 (non-vaccinated) after infection with *A. pleuropneumoniae*. Upper panel: percentage of animals affected; lower panel: mean cough scores $\pm$ standard deviation.

- Figure 12:** Sneezing observed in the animals from group T03 (vaccinated) and T04 (non-vaccinated) after infection with *A. pleuropneumoniae*. Upper panel: percentage of
- 25 animals affected; lower panel: mean sneezing scores $\pm$ standard deviation.

Figure 13: Body temperature of the pigs infected with *Actinobacillus pleuropneumoniae*. Data are from vaccinated animals (group T03) and non-vaccinated animals (group T04).

Figure 14: Survival of vaccinated (T03) and non-vaccinated (T04) animals after *A. pleuropneumoniae* infection.

Figure 15. ELISA results with outer membrane proteins from *A. pleuropneumoniae* with sera from D0 and D34 (at challenge) in the vaccinated and non-vaccinated control
5 animals.

Figure 16. Percentage of pigs with rectal temperature $>40.5^{\circ}\text{C}$ from the vaccinated group (black circles) and the non-vaccinated group (white circles) after inoculation with *A. pleuropneumoniae*.

Figure 17. Average temperature in pigs from vaccinated (black circles) and non-
10 vaccinated (white squares) groups after challenge with *A. pleuropneumoniae*.

Figure 18. Percentage of animals with severe dyspnea in vaccinated (black circles) and non-vaccinated (white circles) groups after challenge with *A. pleuropneumoniae*.

Figure 19. Representative images of the type of lesions (acute and chronic lesions) considered in our analysis.

15 **Figure 20.** Frequency of animals with different type of lesions (acute, chronic or mixed lesions) in the vaccinated and non-vaccinated groups.

Figure 21. Distribution of animals with acute lesions in vaccinated and non-vaccinated groups. The distribution was significantly different in both groups, with more acute lesions in non-vaccinated pigs (1 tail Fisher test, $P=0.0076$)

20

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel vaccine formulations for the therapeutic and prophylactic treatment of respiratory infectious diseases in livestock animals which are caused by, or are associated with bacterial pathogens belonging to the
25 *Pasteurellaceae* family, excluding any virulent strain and serotype of the species *Pasteurella multocida*. The present invention thus provides new compositions which are therapeutically effective in reducing the clinical signs of respiratory diseases

caused by bacterial pathogens belonging to the family *Pasteurellaceae*, in particular, those infections caused by the gram-negative bacterium *Actinobacillus pleuropneumoniae*.

The inventors of the present application have surprisingly found that the already known
5 mutant *Pasteurella multocida* subsp. *multocida* strain CECT 8081 (PM1094), described in international patent application WO 2009/095518, unexpectedly confers protection in animals against bacterial pathogens belonging to the *Pasteurellaceae* family, and are therefore to be used according to the present invention in vaccine formulations, alone or in combination with other effective agents or as adjuvants for use in the treatment
10 and prevention, and/or for reducing the clinical signs of respiratory infectious diseases caused in animals (livestock) by bacterial pathogens belonging to the *Pasteurellaceae* family, in particular, those infections caused by the gram-negative bacterium *Actinobacillus pleuropneumoniae*.

As will be shown in the present invention, vaccination with *Pasteurella multocida* strain
15 PM1094 is effective in eliciting antibodies against the outer membrane proteins (OMP) of bacterial pathogens belonging to the *Pasteurellaceae* family, is also effective in reducing the clinical signs associated to respiratory infections, such as lung scores, dyspnea, coughing, sneezing and body temperature and also reduces the mortality rate of infected animals.

20 Iron uptake is essential for *in vivo* survival of bacterial pathogens, since free iron inside the host is very limited. This function is regulated by the repressor Fur, which is active under iron rich conditions (these are the conditions commonly found in laboratory culture media). Therefore, in the laboratory, bacteria do not need special systems for iron uptake and these systems remain repressed under the action of Fur. However,
25 during infection, these iron-uptake systems are expressed to guarantee the survival of the pathogen inside the host. For the purpose of the present invention, the *in vivo* situation was mimic by limiting the quantity of free iron in the culture medium (under these conditions bacterial growth is reduced) or by elimination of Fur by mutation. Mutations of the *fur* gene in bacteria produce a derepression of genes involved in iron
30 acquisition. Thus, the elimination of the repression action of Fur translates in an increased transcription and subsequent translation of iron-uptake proteins, some of which are located on the bacterial surface. This is the case for *Pasteurella multocida*

and a bacterine produced with a *fur* mutant has enhanced protective capacity. As has been surprisingly found in the present invention, the mutant *Pasteurella multocida* strain PM1094 and outer membrane proteins (OMPs) thereof showed cross-reaction with OMPs from other bacterial species from the *Pasteurellaceae* family, in particular

5 *Actinobacillus pleuropneumoniae*, where it is well known that currently available vaccines are serotype specific and do not show cross protection. The unexpectedly founded cross-reactivity supports the possibility of providing protection with the *Pasteurella multocida* strain 1094 against pathogens belonging to the *Pasteurellaceae* family as defined above.

10 The strain PM1094 is deposited in the Colección Española de Cultivos Tipo (Spanish Collection of Type Cultures), Parc Científic Universitat de Valencia, Catedrático Agustín Escardino, 9, 46980 Paterna (Valencia), Spain, with the registration number CECT 8180 deposited on October 1, 2012, according to the Treaty of Budapest of 28th April 1977.

15 The deposited mutant *P. multocida* strain CECT 8081 (PM1094) is a double mutant, defective in the Fur and OmpH proteins.

The method for obtaining strain PM1094 is disclosed in WO 2009/095518 and contemplates mutation of the *fur* gene which consists in disruption of the gene by introducing in the bacteria a plasmid containing a fragment of 394 base pairs of the

20 internal region of this gene comprised between nucleotides 18 and 412, and carrying out the nonsense mutations in the *ompH* genes (*ompH1* and *ompH2* genes). The mutation in *ompH1* is a nonsense mutation in position 76 which gives rise to a stop codon instead of a glutamine codon, making it express a truncated protein with 24 amino acids. The mutation in *ompH2* involves several nucleotide changes, including a

25 nonsense mutation in position 670, which gives rise to a truncated protein with 223 amino acids instead of the 350 which the native protein has. The effect caused by the nonsense mutation in *ompH1* and *ompH2* is the absence of the 36KDa outer-membrane protein.

30 Strain PM1094 is known from WO 2009/095518 to provide heterologous protection against virulent *Pasteurella multocida*. This patent application describes compositions

and vaccines containing the said strain (inactivated) or an outer-membrane protein extract prepared therefrom to be used as immunogenic agents for the protection against virulent *Pasteurella multocida* in pigs, cattle and small mammals.

The vaccine formulations according to the present invention comprise, alone or in
5 combination with other active agents, the mutant *Pasteurella multocida* strain PM1094 containing the previously described mutations, which are inactivated thermally or by sonication, or an outer-membrane protein extract prepared therefrom, together with suitable carrier and vehicles.

Inactivation of the strain PM1094 is carried out by thermal treatment at 45-55°C, more
10 preferably at 50°C, during at least 16 h.

Outer-membrane separation techniques used in accordance with the present invention involved the suspension of cells at a concentration from 10^{10} to 10^{12} CFU/ml, preferably 10^{11} CFU/ml, in acetate buffer (0.2 M sodium acetate and 0.2 M lithium chloride), cell
15 disruption with a syringe of 19 gauges and centrifugation at 10 000 rpm during 15 min at 4°C. Afterwards, the supernatant was collected and centrifuged at 23 000 rpm during 2 h 30 min at 4°C. Finally, the pellet obtained was re-suspended in ultrapure water.

To formulate the vaccine of the present invention, the inactivated strain PM1094 or an outer-membrane protein extract prepared therefrom, is combined according to standard practices with any of the typical carrier, vehicles or adjuvants in veterinary vaccine
20 practice, such as, lipopolysaccharides, the Freund's complete or incomplete adjuvant, monophospholipids, such as, monophospholipid A, sulfates, phosphates such as aluminium phosphate, and hydroxides, such as hydrated aluminum hydroxyphosphate and aluminium hydroxide.

The composition of the present invention may optionally include additional active
25 agents which contribute to the therapeutic or preventive effects provided by the present invention.

The dose of the vaccine will vary depending on the concentration of the antigenic material, ranging from 10^6 to 10^{10} CFU/animal, more preferably, from 10^7 to 10^9 CFU/animal. Possible vaccination routes are: intramuscular, oral, intraperitoneal,
30 intratranasal, intratracheal, subcutaneous, etc. Preferred route is intramuscular or intraperitoneal.

Experimental evidence of the invention's industrial application as well as the advantages provided by the invention are detailed in the examples of the present specification.

Throughout the description and claims the word "comprise" and variations of the word, are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples are intended for purposes of illustration only and are not intended to limit the scope of protection. Furthermore, the present invention covers all possible combinations of particular and preferred embodiments described herein.

EXAMPLES

Example 1: Evaluation in mice of the protection against a heterologous challenge (240xLD₅₀), with or without adjuvant

To determine the protective effect of the *Pasteurella multocida* PM1094 strain, four groups of six three-weeks old female Hsd:ICR(CD-1) mice were used. One group was treated with PM1094 inactivated cells at a dose of 10⁸ CFU/animal without adjuvant. Likewise, the second one was also vaccinated but aluminum hydroxide (33% v/v) adjuvant was added to the vaccine. The third and fourth groups were the non-vaccinated controls with and without adjuvant. The vaccine was administered at days 1 and 15 of the experiment. Challenge of all the groups was performed at a dose of 240 x LD₅₀ by intraperitoneal inoculation with the *Pasteurella multocida* PM25 strain which is of a different serovar than that of the vaccine strain. Animals were daily supervised, death was recorded and the percentage of survival was calculated. Results found indicated that vaccine protects the 100% of animals, because all vaccinated animals survived to the challenge.

Table 1. Protection conferred by the vaccine against a heterologous challenge

Group	Adjuvant	Number of animals	Number of dead animals	% Protection
Negative control	-	6	6	0
Adjuvant control	+	6	6	0
Group 1	-	6	0	100
Group 2	+	6	0	100

Example 2: Evaluation in mice of the immune response against OMPS of, *Haemophilus parasuis*, *Haemophilus influenzae*, *Manhemia haemolytica*, and *Actinobacillus pleuropneumoniae*.

- 5 To evaluate the mice immune response after vaccination, ELISA assays were performed to determine the presence, in mouse sera, of specific immunoglobulin (Ig) against OMPs of several bacterial strains (*Haemophilus parasuis* C196 and Nagasaki strains, *Haemophilus influenzae*, *Manhemia haemolytica* PH219, and PH214 strains, *Actinobacillus pleuropneumoniae* C140, CV600, CV424, CV601 and CV-411 strains and *Salmonella enterica* LT2 strain).
- 10

To obtain the serum samples, four groups of six three-weeks old female Hsd:ICR(CD-1) mice were used. One group was treated intraperitoneally with PM1094 inactivated cells at a dose of 10^8 CFU/animal without adjuvant. Likewise, the second one was also vaccinated but adjuvant aluminum hydroxide (33% v/v) adjuvant was added to the

vaccine. The third and fourth groups were the non-vaccinated controls with and without adjuvant. The vaccine was administrated at days 1 and 15 of the experiment. At days 1 (before the first inoculation) and 30, three animals of each group were euthanized and sera were obtained.

- 5 For the ELISA assay, OMPs of the different pathogens were obtained. To do this, cells at a concentration of 10^{11} cfu/ml were resuspended in acetate buffer (0.2 M sodium acetate and 0.2 M lithium chloride), cell disruption was performed by using a syringe of 19 gauges. Then, the suspension was centrifuged at 10 000 rpm during 15 min at 4°C, the supernatant was collected and centrifuged at 23 000 rpm during 2 h 30 min at 4°C.
- 10 Finally, the pellet obtained was resuspended in ultrapure water.

- For each bacterial strain, the corresponding OMPs (0.5 µg per well) were immobilized in microtiter plates. ELISA assays were performed using suitable mice sera dilution and an anti-mice Ig HRP-conjugated antibody as secondary antibody. The obtained results indicate that all animals treated with the inactivated PM1094 vaccine (with or without
- 15 adjuvant) present a significant increase of the specific Ig against OMPs of all bacteria assayed except for those of *Salmonella enterica* strain, which was used as a negative control due to its phylogenetic distance with the *Pasteurellaceae* family. See Figures 1 and 2.

Example 3: Evaluation in rabbit of the immune response against OMP from strains of *M. haemolytica*, *H. parasuis* and *H. influenza*

20

- To evaluate the rabbit immune response after vaccination, ELISA assays were performed to determine the presence, in rabbit sera, of specific immunoglobulin (Ig) against OMPs of several bacterial strains (*Haemophilus parasuis* C196 and Nagasaki strains, *Haemophilus influenzae*, *Manhemia haemolytica* PH219, and PH214 strains
- 25 and *Salmonella enterica* LT2 strain).

- To obtain the serum samples, four groups of six rabbits each one were used. One group was treated with PM1094 inactivated cells at a dose of 10^{10} CFU/animal by subcutaneous innoculation. Likewise, the second one was also vaccinated but by intraperitoneal administration. The third and fourth groups were the non-vaccinated
- 30 controls treated with saline a suspension by subcutaneous or intraperitoneal injection.

The vaccine was administrated at days 1 and 15 of the experiment. At days 1 (before the first inoculation) and 30, serum samples were collected for all the animals of each group.

For the ELISA assay, OMPs of the different pathogens were obtained. To do this, cells
5 at a concentration of 10^{11} cfu/ml were resuspended in acetate buffer (0.2 M sodium acetate and 0.2 M lithium chloride), cell disruption was performed by using a syringe of 19 gauges. Then, the suspension was centrifuged at 10 000 rpm during 15 min at 4°C, the supernatant was collected and centrifuged at 23 000 rpm during 2 h 30 min at 4°C. Finally, the pellet obtained was re suspended in ultrapure water.

10 For each bacterial strain, the corresponding OMPs (0.5 µg per well) were immobilized in microtiter plates. ELISA assays were performed using suitable mice sera dilution and an anti-rabbit Ig HRP-conjugated antibody as secondary antibody.

The obtained results indicate that all animals treated with the inactivated PM1094 vaccine (either by subcutaneous or intraperitoneal administration) present a significant
15 increase of the specific Ig against OMPs of all bacteria assayed except for those of *Salmonella enterica* strain which was used as a negative control due to its phylogenetic distance with the *Pasteurellaceae* family. See Figures 3 and 4.

Subcutaneous and Intraperitoneal vaccination induce an increase in serum total Ig against OMP from strains of *M. haemolytica*, *H. parasuis* and *H. influenza*.

20 **Example 4: Evaluation in rabbit of the immune response against OMP from strains of *Actinobacillus pleuropneumoniae***

To evaluate the rabbit immune response after PM1094 vaccination against OMPs of *Actinobacillus pleuropneumoniae*, ELISA assays were performed using OMPs of *A. pleuropneumoniae* C140, CV600, CV424, CV601 and CV-411 strains and *S. enterica*
25 LT2 strain.

For serum samples obtaining, four groups of six rabbits each one were used. One group was treated with PM1094 inactivated cells at a dose of 10^{10} CFU/animal by subcutaneous inoculation. Likewise, the second one was also vaccinated but by

intraperitoneal administration. The third and fourth groups were the non-vaccinated controls treated with saline a suspension by subcutaneous or intraperitoneal injection. The vaccine was administrated at days 1 and 15 of the experiment. At days 1 (before the first inoculation) and 30, serum samples were collected for all the animals of each group.

For the ELISA assay, OMPs of *S. enterica* LT2 and of the different *A. pleuropneumoniae* strains (C140, CV600, CV424, CV601 and CV-411) were obtained. To do this, cells at a concentration of 10^{11} cfu/ml were re-suspended in acetate buffer (0.2 M sodium acetate and 0.2 M lithium chloride), cell disruption was performed by using a syringe of 19 gauges. Then, the suspension was centrifuged at 10 000 rpm during 15 min at 4°C, the supernatant was collected and centrifuged at 23 000 rpm during 2 h 30 min at 4°C. Finally, the pellet obtained was re-suspended in ultrapure water.

For each bacterial strain, the corresponding OMPs (0.5 µg per well) were immobilized in microtiter plates. ELISA assays were performed using suitable mice sera dilution and an anti-rabbit Ig HRP-conjugated antibody as secondary antibody.

The obtained results indicate that all animals treated with the inactivated PM1094 vaccine (either by subcutaneous or intraperitoneal administration) present a significant increase of the specific Ig against OMPs of all *A. pleuropneumoniae* strains assayed. As expected, no cross reaction exist when OMPs of *S. enterica* LT2 strain were used due to its phylogenetic distance with *Pasteurellaceae* family. See Figures 5 and 6.

Subcutaneous and Intraperitoneal vaccination induce an increase in serum total Ig against OMP from strains of *Actinobacillus pleuropneumoniae*.

Example 5: Obtaining a vaccine preparation based in PM1094

Strain PM1094 was grown overnight at 37°C on several sheep-blood agar plates (COS, Biomérieux, Madrid, Spain). Once grown, the bacterial biomass was harvest and re-suspended in buffered peptone water (BPW, Merck Darmstadt, Germany) generating a concentrated cell suspension stock at about 10^{11} CFU/ml. The exact bacterial concentration was determined by serial dilution plating on sheep-blood agar plates.

The suspension stock was then inactivated by heating at 50°C for 18h. The complete cell inactivation was confirmed by plating 200µl of the cell suspension. The inactivated vaccine was prepared by diluting the inactivated cell suspension stock to a final concentration of 1×10^{10} inactivated CFU/ml and by adding timerosal [1/1000 (v/v)] and
5 aluminum hydroxide [1/3 (v/v)]. Again 1ml of the vaccine was plated onto sheep-blood agar plates to ensure that no contamination has occurred. The vaccine was stored at 4°C until used.

Titration. The concentration of the concentrated stock was 7.1×10^{10} CFU/ml. After inactivation, 15 ml of this concentrated stock was then mixed with 33 ml of aluminum
10 hydroxide, 52 ml of BPW and 0.1 ml of timerosal. Then, the final concentration of the inactivated and adjuvanted vaccine was 1.06×10^{10} CFU/ml.

Example 6: *Actinobacillus pleuropneumoniae* (App) challenge study in pigs vaccinated with *Pasteurella multocida* strain PM1094 vaccine

6.1 Summary of the study

15 The protective capacity of strain PM1094 *Pasteurella multocida* bacterine against infection by *Actinobacillus pleuropneumoniae* (App) was evaluated in the present study. Groups of 12 pigs were either vaccinated (group T03) or not (group T04) and later challenged. Vaccination was performed by intramuscular injection, with 2 doses separated by a 2 weeks interval (D0 and D13) of 1.1×10^{10} CFU of bacteria.
20 Challenge with App was performed by intranasal inoculation of 3.4×10^8 CFU of strain SHOPE 4074 per animal, at D34. Animals were evaluated daily for clinical signs after challenge, with special emphasis on respiratory signs. Vaccinated animals presented a significant increase of antibodies against outer membrane proteins from App. App infected animals (groups T03 and T04) showed signs of acute disease and some
25 animals needed to be euthanatized before the end of the experiment. Although no huge statistical difference in survival was detected, higher percentage of animals survived in the vaccinated group T03. Lesions associated to App infection were found in both groups T03 and T04. The proportion of affected lung was higher in T04 than in T03, but the difference was not statistically significative. Similarly, re-isolation of App in
30 high quantities was more frequent in the animals from the non-vaccinated group T04

than in the vaccinated group T03. Clinical signs, including fever, were observed in both groups with no statistical differences.

6.2 Test product

Pasteurella multocida strain PM1094 vaccine

- 5 Pharmaceutical form: heat inactivated bacterine, *Pasteurella multocida* strain PM1094 vaccine

Storage conditions: timerosal, 4°C

Supplier: Dept. de Genetica i Microbiologia-UAB

Adjuvant: Aluminium hydroxide (Reheis)

- 10 Mock vaccination was performed with PBS and the same adjuvant.

6.3 Test System

Animal species: Pigs

Source: A Farm without respiratory clinical signs and historically free of App 1, 9, 11 serotypes.

- 15 Number: 24

Sex: The gender of the animals is irrelevant for the objective of the experiment

Age: 3 weeks at the start of the study

- 20 Treatments: During the first week, animals were fed with lactofeed containing colistin and zinc. During the following 10 days, a progressive change to starter feed was performed by increasing the proportion of starter feed. Then, only Starter was used to feed the animals until the end of the study. Starter feed contains zinc.

Acclimatization period: After transportation to experimental farm (UAB facilities) and prior to vaccination, the piglets had 17 days of acclimatization

6.4 Housing and grouping

Pigs were housed in 2 rooms at UAB farm facilities. Assignment of the piglets to the treatment group was random. Animals were randomly distributed into 2 groups using List Randomizer at www.random.org.

5 6.5 Animal feeding

At the UAB farm, tap water was available ad libitum and feeding was done in accordance to standard UAB procedures. During the first week, animals were fed with lactofeed containing colistin and zinc. During the following 10 days, a progressive change to starter feed was performed by increasing the proportion of starter feed.
10 Then, only Starter was used to feed the animals until the end of the study. Starter feed contains zinc.

6.6 Inclusion and exclusion criteria

Animals free of respiratory diseases and free of maternal antibodies against App were selected. Only healthy piglets were included in the study. If the piglets showed any
15 clinical signs not attributable to the vaccination, treatment was to be done. If the treatment did not influence the outcome of the experiment, results were to be included in the evaluation. If during the experiment pigs showed pain or distress, which was considered to be non-transient in nature or likely to become more severe as judged by a veterinarian (in consultation with the study director), they were to be euthanized for
20 animal welfare reasons. If treatment would have influenced the outcome of the results, the animals were to be excluded from the study. Any prophylactic or therapeutic treatments would have been administered only after consultation with the study director.

Twenty four animals arrived to the UAB farm and were distributed in 2 groups of 12
25 (T03 and T04).

6.7 Experimental Design

Twenty four pigs were selected from a farm free of respiratory disease and seronegative to *A. pleuropneumoniae*. Animals were transferred to the farm facilities of

the Servei de Granges i Camps Experimentals (SGiCE) from the Universitat Autònoma de Barcelona (UAB) at 3 weeks of age and were allocated in 2 groups of 12 pigs each, which were housed in 2 different rooms. Table 2 summarizes the experimental design.

Table 2: Summary of experiment

Treatment Group	Transport to UAB (3 weeks of age)	Test Product	Day of Admin	Challenge App	Day of euthanasia and necropsy	Number of Animals
T03	Day -17	<i>Pasteurella</i> vaccine PM1094	Day 0 and 13	Day 34	Day 41	12
T04		control				12

5

Vaccination: At 5 (Day 0; D0) and 7 weeks of age (D13), animals in group T03 were vaccinated with the inactivated *P. multocida* strain PM1094 vaccine, while animals in group T04 were mock vaccinated with adjuvant (aluminum hydroxide). Serum samples were taken from all the pigs at both days.

Challenge: At 10 weeks of age (D34), animals in groups T03 and T04 were intranasally infected with *A. pleuropneumoniae* strain 4074. Serum samples were collected from all animals at final challenge (D34).

Clinical observation and necropsies: After challenge, animals were observed daily for clinical signs. If signs of suffering were observed, animals were euthanized following protocol SOP PE ML 517. Rectal temperatures were recorded one day before challenge, at challenge and daily after that. Clinical condition observations were

15

performed daily from challenge onwards. Clinical observation included respiratory signs.

One week after *A. pleuropneumoniae* infection all the remaining animals were euthanized and necropsy was performed: Lung lesions were recorded and scored: A lung scoring system (Hannan et al., 1982) and macroscopic examination of pericarditis, pleuritis and lung abscesses presence were carried out. Lung swabs were taken for bacterial culture. PCR was used for confirmation of the results and serum samples were also collected.

6.8 Procedures

10 Vaccination: To test efficacy of vaccines, treatment should be done using the recommended dose and route of vaccination. In this case, vaccinations were performed intramuscularly with 2 ml of the vaccine, equivalent to 2×10^{10} CFU per animal.

When the piglets were approx. 5 weeks of age (D0), animals from group T03 were vaccinated intramuscularly with 2 ml of the *P. multocida* strain PM 1094 vaccine. Animals in group T04 were mock vaccinated with PBS and adjuvant, aluminum hydroxide. Vaccination was repeated 2 weeks later (D13). Vaccinations were recorded on appropriate forms.

20 Inoculate for challenge: *Actinobacillus pleuropneumonia* (App), strain SHOPE 4074 (Serotype 1), via: intranasal, at a dose of 3.4×10^8 CFU/animal (in 1.5 ml)

Transport conditions: Bacterial challenge suspensions were kept on ice during transport and were applied at room temperature.

Challenge and preparation of challenge material: Challenge will be done so as to mimic the likely natural route of infection. Groups T03 and T04 will be challenged with App at D35 by intranasal inoculation.

Preparation of App inoculum: App inoculum was prepared from freshly streaked chocolate plates (Chocolate polyvitex agar, Biomerieux). After 5 hours at 37°C and 5% CO₂, the bacterial growth was resuspended in commercial PBS (Lonza, B-4800

Verviers, Belgium) to give an optical density at 660 nm of approximately 0.1 (aprox. 108 CFU/ml). Immediately after the inoculum preparation, the real bacterial concentration was confirmed by dilution and plating on chocolate agar plates and resulted to be 2.3×10^8 CFU/ml. Inoculation was performed intranasally with 1.5 ml of the bacterial suspension (3.4×10^8 CFU per animal) and recorded in the corresponding forms.

Serology: Blood samples were collected individually from all piglets at 1st vaccination (D0) and revaccination (D13), at challenge with App (D34) and at necropsy day (D41/42). Samples were transported to the test laboratory at room temperature. Sera were prepared from the clotted blood samples and were stored at -20°C until used. Blood collection was recorded on appropriate forms. Blood samples taken from the animals included in the study were tested by commercial ELISA kit to detect App ApxIV (REF 99.441188). An ELISA to detect antibodies against OMPs from the vaccine strain, PM1094, and the challenge strain App SHOPE 4074 was also performed by the department of Genetics and Microbiology of the Universitat Autònoma de Barcelona, with serum samples from the animals in groups T03 collected at D0 and D34. In addition, serum samples from 6 animals from T04 collected at D34 were included as control. OMPs were obtained from Pm PM1094 (the vaccine strain), and from App strain used in the challenge. OMPs ($0.5 \mu\text{g}$ per well) were immobilized in microtiter plates and 1/600 serum dilution was used. Polyclonal anti-swine IgG HRP-conjugated antibody (Acris R1384HRP) was employed as secondary antibody. For ELISA assays, the sera of 12 and 7 animals of T3 and T4, respectively, were analyzed.

Clinical condition after challenge: After challenge, all pigs were observed daily for clinical signs of disease. Clinical observation included: nasal discharge, coughing, sneezing, and dyspnea. All observations were recorded in appropriate forms and scored as follows: score: 0 (absence of respiratory signs), score: 1 (mild respiratory signs), score: 2 (moderate respiratory signs), score: 3 (severe respiratory signs).

Other specific signs, if found, were also recorded: Body temperatures of the animals were measured 1 day before, the day of challenge and until the end of the study. For determination of the rectal temperature, a digital thermometer was used. The sensor was placed in the rectum until the number on the display was constant. Evaluation of

fever was established by means of a numerical score: 0 (less than 39.5), 1 (between 39.5 and 40.5) and 2 (higher than 40.5). This classification is a modification from that described by Moore et al. (1996). Animals scored 2 were considered to have fever.

Euthanasia, post-mortem examination and sampling: At the end of the experiment, after final bleeding, all animals were euthanized in accordance with standard procedures, using an intravenous overdose of sodium pentobarbital (PE ML 517).

At post-mortem, the animals were examined for lung gross lesions. Particularly, presence of pericarditis, pleuritis and lung consolidation and abscesses were evaluated. Moreover, a lung scoring system according to Hannan et al. (1982) was done. Briefly, each of the 7 lobes of the lungs is allotted a maximum possible lesion score of 5. The area showing pneumonia and /or pleuritis of each lobe is assessed and expressed on a scale of 0-5 to give the pneumonic score per lobe (the maximum total score possible for each complete lung is 35). A tonsillar swab and a lung swab of each lung side were collected from each animal. These samples were transferred to the laboratory where they were immediately processed by means of culture (lung swabs) and PCR (tonsillar swab). Once sampling was completed, all carcasses were destroyed by incineration.

Bacterial culture: Lung swabs were streaked on chocolate agar plates in 2 sections in order to get isolated colonies in the second section. After overnight incubation at 37°C (5% CO₂), bacterial growth was recorded and semiquantified in section 1 as: 0, no growth; 1, 1-20 colonies; 2, isolated colonies in a number higher than 20; 3, lawn.

PCR detection: Tonsillar swabs taken at necropsy were tested by PCR to detect App (PT PCT 049), in the animals infected with this bacterium.

6.9 Evaluation of results

Primary criteria: presence of lung lesions and percentage of lung area affected.
Secondary criteria: clinical respiratory signs, body temperature and mortality associated to App.

Response to vaccination: All animals treated with the inactivated *Pasteurella multocida* strain PM 1094 vaccine presented a significant increase of the specific IgG against OMP of the App bacteria assayed (Fig 7A and B). In all cases the relative specific IgG amount of the Day 35 of the vaccinated group (T03) was significantly higher than that of the non-vaccinated group (T04) at the same time or the level of antibodies of all the animals at day 0 (Fig. 7A and B).

Presence of lung lesions: Table 3 shows the presence of lesions in animals in groups T03 (vaccinated) and T04 (non-vaccinated). Analysis by χ^2 detected no differences between the two groups in total lesions or in pleuritis and pericarditis.

Table 3. Number of animals from groups T03 (vaccinated) and T04 (non-vaccinated) with lesions compatible with *A. pleuropneumoniae* infection.

	Presence of lesions		Presence of pleuritis		Presence of pericarditis	
	number	%	number	%	number	%
T03	9/12	75	9/12	75	3/12	25
T04	11/12	92	9/12	75	5/12	42

The number of animals with lung lesions in the vaccinated group was lower than in the non-vaccinated group. The origin of the lesions was confirmed by App re-isolation from all cases but from animal 200 from group T04, which had lesions but no positive bacterial isolation. Re-isolation of App was possible from 9 out of 9 animals with lesions in vaccinated animals (T03) and in 10 out of 11 animals with lesions in non-vaccinated animals (T04). The quantity of bacteria found in the lesions was semi-quantified and no differences were found between the isolation from both groups of animals. When the bacterial scores from both samples (left and right lung samples) were added, some differences in the highest score were observed between vaccinated and non-vaccinated animals (Table 3). More animals from the non-vaccinated group showed a score of 6. However, this difference was not statistically significant.

Table 4. Number of animals from groups T03 (vaccinated) and T04 (non-vaccinated) with re-isolation of *A. pleuropneumoniae* (App). Quantity of bacteria was semi-quantified in the first section of the agar plate and scored as 0 (no bacteria), 1 (1 to 20

colonies), 2 (isolated colonies in a number higher than 20) and 3 (lawn). The sum of the score from the right and left lung sample was considered in this table.

	App re-isolation (score left lung + score right lung)											
	score 0		score 1		score 2		score 3		score 4		score 5	
T03	3/12	(25%)	0/12	(0%)	0/12	(0%)	2/12	(17%)	1/12	(8%)	2/12	(17%)
T04	2/12	(17%)	1/12	(8%)	0/12	(0%)	1/12	(8%)	1/12	(8%)	0/12	(0%)

- 5 Proportion of lung affected: Lung affected was quantified following a lung scoring system according to Hannan et al. (1982). No statistical difference in lung score was detected when the lung scores from all the animals in groups T03 and T04 were compared (Fig. 8). Although no significant differences were seen, a higher average score was observed in the non-vaccinated animals challenge with App than in the vaccinated ones.

Similar results were obtained when the lung scores from animals with lesions from group T03 and T04 were compared (Fig. 9).

- 15 Clinical respiratory signs: Dyspnea, nasal discharge, coughing and sneezing were evaluated after challenge with App. No differences between groups T03 (vaccinated) and T04 (non-vaccinated) were observed in the proportion of animals showing dyspnea (Fig. 10, upper panel) and the severity of this clinical sign (Fig. 10, lower panel) after infection with App. When coughing was analyzed, no significant differences between groups T03 and T04 were detected (Fig. 11). When sneezing was analyzed, a slight delay in the apparition of this respiratory sign was observed in the vaccinated animals, but the differences were not statistically significant (Fisher exact test at 1 and 2 dpi). (Fig.12)

Body temperature: No significant differences were observed in the body temperature of animals from groups T03 (vaccinated) and T04 (non-vaccinated) after infection with App. (Fig.13).

- 25 Mortality associated to App infection: As expected, animals infected with App showed mortality associated to the infection (Fig. 14). Mortality started 2 days after inoculation,

with 1 animal affected from each group. In group T03 (vaccinated), no more animals needed to be euthanized and 11 animals reached the end of the study. However, in group T04 (control, non-vaccinated), a second animal was euthanized at day 3 and a third animal at day 4 post-inoculation. Nine animals from group T04 reached the end of the study. Survival analysis by Log-Rank or Wilcoxon tests did not detect differences between the two groups. When the χ^2 test was used in the analysis with the animals surviving till the end of the study no differences were detected either between both groups.

6.10 Discussion

Vaccination with a heat-inactivated *Pasteurella multocida* (strain PM1094) was effective in eliciting antibodies against the outer membrane proteins (OMP) from the App challenge strain (strain 4074). To improve the cross-reaction of the induced antibodies different means of inactivation of the vaccine, to better maintain the antigenicity of the vaccine strain, or different adjuvants for the vaccine could be explored.

The challenge with App was successful in reproducing disease. App infection produced acute disease and mortality due to the high severity of the disease. More animals from the vaccinated group survived to the end of the study, but survival analysis did not detect significant differences. Lesions due to App infection were detected in both groups of animals, vaccinated or not, with a higher proportion of pericarditis seen in non-vaccinated animals. Re-isolation of App from infected animals was also slightly higher in non-vaccinated animals, with more animals with the maximum score from both lung samples. Accordingly, lung lesion scores were also slightly higher in non-vaccinated animals. Vaccination produced a mild delay in respiratory signs, with significant differences in dyspnea signs detected in the statistical analysis. It is probable that the detection of statistical differences was low in some of the variables studied due to the low number of animals included in each group.

Example 7: Efficacy study of *Pasteurella multocida* strain PM1094 against *Actinobacillus pleuropneumoniae* (App)

7.1. Objective

Following the results obtained in Example 6, and for the purpose of validating the efficacy of *Pasteurella multocida* strain PM1094 against *Actinobacillus pleuropneumoniae* (App) the present study was designed as a masked (blind), parallel group, randomised and negative controlled study with the objective of testing the efficacy of a *Pasteurella multocida* strain PM1094 vaccine in preventing or reducing the clinical signs of the disease produced by *A. pleuropneumoniae*. Pigs were immunized with the vaccine twice and then infected with a virulent *A. pleuropneumoniae*. Efficacy was monitored by clinical and pathological observations after infection, and by body weight measurement. All pigs were examined during one week after infection and at the end of this period all pigs were euthanized and lesions were evaluated.

7.2 Experimental period schedule

Starting date of the *in vivo* phase: September 29th, 2014.

End of the *in vivo* phase: November 20th, 2014.

7.3 Justification of the selected schedule

Piglets were obtained at 3 weeks of age and were housed for approximately 1 week before starting the protocol. Vaccination was performed after this acclimatization period (D0) and D14, and challenge with a virulent *A. pleuropneumoniae* was performed at D34 and D37. After the challenge, pigs were examined for clinical signs and 10 days after challenge were euthanized and lesions evaluated. The weight of the animals was also measured at the challenge and at necropsy.

7.4 Test product

<u><i>Pasteurella</i> vaccine:</u>	<i>Pasteurella multocida</i> PM1094
	Heat inactivated bacterin
	Adjuvanted with aluminum hydroxide
	10 ¹⁰ CFU / ml

7.5 Test system

7.5.1 Challenge

A virulent *A. pleuropneumoniae* strain was used to challenge the pigs and evaluate the efficacy of the vaccine.

Name: *Actinobacillus pleuropneumoniae* serotype 1

5 Strain: SHOPE 4074 (serotype 1) ATCC Ref 27088

Dose: Approximately 10^8 CFU/animal, in 1.5 ml. Twice.

Inoculation route: Intranasal

7.5.2 Description and justification of the animals used

Forty eight pigs were enrolled in the study. Pigs were purchased from a farm without
10 respiratory clinical signs and historically free of *A. pleuropneumoniae* 1, 9 and 11 serotypes.

7.5.3 Identification method

Each pig was identified individually with a numbered ear tag. Pig identification numbers was recorded and saved as raw data in the Identification sheet.

15 7.5.4 Management and housing of the animals

Animals were housed in two rooms at the corresponding facilities. Animals in one room were vaccinated with the Pasteurella vaccine while the animals in the second room were mock vaccinated and serve as control. Unit conditions (temperature and humidity) were recorded daily. At the time of challenge, all the pigs were infected with *A.*
20 *pleuropneumoniae*.

7.5.5 Animal feeding

During the first week, animals were fed with lactofeed. During the following 3-4 days, a progressive change to prestarter feed was performed by increasing the proportion of prestarter feed. Two weeks later a progressive change to starter feed was performed
25 during 3-4 days by increasing the proportion of starter feed. Then, starter was used to feed the animals until the end of the study. Feed contained colistin and zinc oxide until D36. After D36, only zinc oxide was included in the feed.

7.6 Inclusion and exclusion criteria

Animals from a farm free of clinical respiratory diseases and free of antibodies against *A. pleuropneumoniae* were selected (a serological screening of the farm was performed). Piglets free of maternal antibodies against *A. pleuropneumoniae* were
5 selected. In order to assess this, blood samples taken from the purchased animals for the study were tested using a commercial ELISA kit to detect *A. pleuropneumoniae* ApxIV. All the piglets tested negative in this ELISA test. Only healthy piglets were included in the study.

If the piglets showed any clinical signs not attributable to the vaccination, treatment
10 was performed. This was the case with piglet #47, which showed signs of arthritis and was treated with enrofloxacin. Treatment did not solve the problem and it was considered to be non-transient in nature, therefore it was euthanized for animal welfare reasons and it was excluded from the study.

7.8 Facilities

15 The experimental animal phase was performed at A.M.ANIMALIA BIANYA S.L (Vall de Bianya, Spain). The facilities have an ethical committee and are supervised by a certified veterinarian.

7.9 Experimental design

7.9.1 Description of the study outline

20 Forty eight pigs were selected from a farm free of clinical respiratory disease and seronegative to *A. pleuropneumoniae*. Animals were transferred to the farm facilities of Animalia at 3-4 weeks of age and were allocated in 2 groups of 24 pigs each, which were housed in different rooms.

Vaccination: After about 1 week of adaptation to the facilities (D0), animals in group 2
25 were vaccinated with the inactivated *P. multocida* vaccine, while animals in group 1 were mock vaccinated with adjuvant (aluminum hydroxide). Serum samples were taken from all the pigs. A second vaccination was performed at D14.

Challenge: Three weeks after the second vaccination, all animals were intranasally inoculated with *A. pleuropneumoniae*. Serum samples were taken from all the animals.

Serum samples: Plain blood samples were collected from all animals at D0 and D34. Serum samples were stored at -20°C.

The experimental design is summarized in Table 5.

Table 5: Experimental design

Treatment Group	Vaccine	Dose Volume	Days of Admin	Route of Vac Administration	Day of challenge	Route of challenge	Day of euthanasia and post-mortem examination	Target Number of Animals
T01	Placebo (PW-adjuvant)	2 ml	D0 D14	IM	D34 & D37	IN	D43	24
T02	Pasteurella vaccine	2 ml	D0 D14	IM	D34 & D37	IN		24

5

IM: intramuscular

IN: Intranasal

7.9.2 Randomization and blinding procedure

Pigs were tagged at arrival to the experimental farm. Then, animals were weighed and randomly allocated to experimental treatments forming two weight balanced groups.

10

To avoid any bias in the clinical evaluation, personnel involved in the assessment were blinded. Random list and vaccination record were not available for the personnel involved in the clinical and lung lesions evaluation and were stored by the dispenser of the vaccine.

15

7.10 Procedures

7.10.1 Vaccine preparation

The Δ fur *Pasteurella multocida* strain PM1094 was prepared at the Department of Genetica I Microbiología from the Universitat Autònoma de Barcelona. The *Pasteurella* strain was grown overnight at 37°C on several sheep-blood agar plates. Once grown,

20

the bacterial biomass was harvested and re-suspended in buffered peptone water (BPW) generating a concentrated cell suspension stock of about 10^{11} CFU/ml. The exact bacterial concentration was determined by serial dilution plating on sheep-blood agar plates. The suspension stock was then inactivated by heating at 50°C for 18h.

5 Complete cell inactivation was confirmed by plating 200 µl of the cell suspension. The inactivated vaccine was prepared by diluting the inactivated cell suspension stock to a final concentration of 1×10^{10} inactivated CFU/ml and by adding timerosal [1/1000 (v/v)] and aluminum hydroxide [1/3 (v/v)]. Again 1 ml of the vaccine was plated onto sheep-blood agar plates to ensure that no contamination occurred. The vaccine was stored

10 refrigerated until used.

7.10.2 Vaccination

The group of vaccinated pigs (randomly selected), housed in their particular room, were vaccinated by intramuscular injection with 2 ml of the vaccine per animal. Control group, non-vaccinated, were mock vaccinated with 2 ml of placebo (BPW and

15 adjuvant). This was repeated 2 weeks later (D14).

Vaccinations were recorded on appropriate forms.

7.10.3 Vaccine titration

Vaccine titration was carried out at UAB laboratories during production of the vaccine (see above).

20 7.10.4 Analysis of the response to vaccination by ELISA

An ELISA to detect antibodies against OMPs from *Pasteurella* and *A. pleurpneumoniae* was also performed by the department of Genetics and Microbiology of the Universitat Autònoma de Barcelona, with serum samples from the animals in both groups collected at D0 and D34. OMPs (0.5 µg per well) were immobilized in microtiter plates and 1/600

25 serum dilution was used. Polyclonal anti-swine IgG HRP-conjugated antibody (Acris R1384HRP) was employed as secondary antibody.

7.10.5 Preparation of the inoculum for challenge (infection)

Preparation of the inoculum for challenge was performed at the laboratories. The day before the challenge, *A. pleuropneumoniae* SHOPE 4074 was thawed and streaked for isolation on chocolate agar plates. The plates were incubated overnight at 37°C. The day of the challenge freshly streaked chocolate plates were prepared from the
5 overnight growth. After 5 hours at 37°C and 5% CO₂, the bacterial growth was resuspended in commercial PBS to give an optical density at 660 nm of 0.1 (aprox. 10⁸ CFU/ml). The real bacterial concentration was confirmed by dilution and plating.

7.10.6 Challenge (Infection)

10 Challenge was performed with a target dose of 1 x 10⁸ CFU per animal in a volume of 1.5 ml per animal. Infection was performed by intranasal inoculation using a syringe with a nebulizer. Challenge was performed again at 3 days after the initial inoculation and in the same manner. A challenge record sheet was filled.

7.10.7 Clinical signs

15 After infection, a blinded technician/trained person observed the pigs for clinical signs every day. After challenge, all pigs were observed daily for clinical signs of disease. Clinical observation included: dyspnea, body temperature and lung lesions. All observations were recorded in appropriate forms and scored as follows score 0: absence of respiratory signs, score 1: mild respiratory signs, score 2: moderate
20 respiratory signs and score 3: severe respiratory signs. Other clinical signs if seen were also recorded.

Body temperatures of the animals were measured 1 day before, the day of challenge and until the end of the study. For determination of the rectal temperature, a digital thermometer was used. The sensor was placed in the rectum until the number on the
25 display was constant.

Evaluation of fever was established by means of a numerical score: 0 (less than 39.5), 1 (between 39.5 and 40.5) and 2 (higher than 40.5). This classification is a modification from that described by Moore et al. (1996). Animals with score of 2 were considered to have fever.

7.10.8 Weight and feed consumption

Animals were weighed at arrival (to perform the randomization while maintaining a weight balance between the two groups), at challenges and at necropsy. Weight of each animal was recorded.

- 5 To assess feed consumption, the feed bags for each room were weighed at the days of challenge (D34 and D37) and the day of necropsy (D41). Weight of feed bags was recorded.

7.10.9 Euthanasia and post-mortem examination.

- At the end of the experiment, after final bleeding, all animals were euthanized in
10 accordance with standard procedures, using an intravenous overdose of sodium pentobarbital (PE ML 517).

- At post-mortem, the animals were examined for lung gross lesions. Particularly, presence of pericarditis, pleuritis and lung consolidation and abscesses were evaluated and recorded. Moreover, a lung scoring system according to Hannan et al. (1982) was
15 done. Briefly, each of the 7 lobes of the lungs is allotted a maximum possible lesion score of 5. The area showing pneumonia and /or pleuritis of each lobe is assessed and expressed on a scale of 0-5 to give the pneumonic score per lobe (the maximum total score possible for each complete lung is 35).

- Lung swabs from each lung side were collected from each animal. These samples
20 were transferred under refrigeration to the laboratory where they were processed for bacterial culture and isolation.

Once sampling was completed, all carcasses were destroyed by incineration.

7.10.10 Bacterial culture

- Lung swabs were streaked on chocolate agar plates in 2 sections in order to get
25 isolated colonies in the second section. After overnight incubation at 37°C (5% CO₂), bacterial growth was recorded and semiquantified in section 1 as: 0, no growth; 1, 1-20 colonies; 2, isolated colonies in a number higher than 20; 3, lawn.

7.10.11 Antibody response to vaccination

When the response to vaccination was studied by ELISA with OMPs with *A. pleuropneumoniae*, a mild but significant increase in antibodies was detected in the vaccinated group after vaccination with respect to D0 (Student T test, $P=0.00001$; Fig. 15).

5

7.11 Clinical signs after challenge After the first inoculation, animals showed clinical signs but recover at 2 dpi. The clinical signs were more clearly visible in the vaccinated group than in the non-vaccinated group. The vaccinated group was inoculated first, and the inoculum for the non-vaccinated was maintained under refrigeration while vaccination was performed in the first group. The recovery of the clinical signs at 2 dpi motivated to perform a second inoculation, in which the inoculum was transported at room temperature.

10

15 Animals in both groups showed elevated temperature, but the number of pigs with fever ($T>40.5^{\circ}\text{C}$) at the end of the study (9 dpi) was significantly higher in the non-vaccinated group than in the vaccinated one (Fig. 15). In addition, temperature was more elevated in the non-vaccinated group than in the vaccinated group at 6, 8 and 9 dpi (Fig. 16 and 17; 1 tail Mann-Whitney U, $P=0.0002$; 0.006 ; 0.004 ; for 6 dpi, 8 dpi and
20 9 dpi respectively).

The inoculation caused clinical signs in both groups, especially dyspnea, but to a lesser extent in the vaccinated animals (Table 6).

Table 6. Distribution of scores for dyspnea observed in the pigs from the vaccinated and non-vaccinated groups after challenge with *A. pleuropneumoniae*.

VAC	Dyspnea				Total N
	score 0	score 1	score 2	score 3	
D0	23	0	0	0	23
D1	20	3	0	0	23
D2	15	7	0	0	23
D3	20	3	0	0	23
D4	11	11	1	0	23
D5	10	12	0	1	23
D6	16	6	0	0	22
D7	15	7	0	0	22
D8	13	9	0	0	22
D9	13	9	0	0	22

D0: 1st inoculation
D3: 2nd inoculation

NON-VAC	Dyspnea				Total N
	score 0	score 1	score 2	score 3	
D0	24	0	0	0	24
D1	18	6	0	0	24
D2	20	4	0	0	24
D3	23	1	0	0	24
D4	14	9	1	0	24
D5	7	12	5	0	24
D6	13	9	2	0	24
D7	16	7	0	1	24
D8	12	7	4	0	23
D9	12	10	1	0	23

When severe dyspnea (score > 1) was studied clear differences were observed between the two groups (Fig. 18, Table 7).

Table 7. Distribution of severe dyspnea in vaccinated and non-vaccinated pigs after challenge with *A. pleuropneumoniae*.

VAC	Severe dyspnea (score >1)		Total N
	No	Yes	
D0	23	0	23
D1	23	0	23
D2	23	0	23
D3	23	0	23
D4	22	1	23
D5	22	1	23
D6	22	0	22
D7	22	0	22
D8	22	0	22
D9	22	0	22

NON-VAC	Severe dyspnea (score >1)		Total N
	No	Yes	
D0	24	0	24
D1	24	0	24
D2	24	0	24
D3	24	0	24
D4	23	1	24
D5	19	5	24
D6	22	2	24
D7	23	1	24
D8	19	4	23
D9	22	1	23

Statistical analysis of the occurrence of severe dyspnea in both groups detected differences between the two groups; severe dyspnea was more frequent in non-vaccinated pigs (Fisher test, one tail; $P=0.01$; Table 8)

Table 8. Frequency of animals showing severe dyspnea at any time after challenge.

	Ever Severe dysnea (score >1)		Total N
	No	Yes	
VAC	22	1	23
NON-VAC	16	8	24

All the animals were studied at necropsy and lung lesions were scored. The number of animals showing lesions at necropsy was similar from both groups. In addition, no significant differences in lung scores were observed between both groups of animals.

5

However, the type of lesions observed in both groups was different. Vaccinated animals tended to have more chronic lesions and non-vaccinated pigs showed more acute lesions. When the chronicity of the lesions was evaluated, differences were observed between the two groups. Lesions were categorized as follows (see also

10 Figure 19):

- Acute: lungs with subacute lesions (hemorrhagic or/and fibrinous lesions), which may present some minor fibrous adhesences in pleura, but not abscesses.
- Chronic: lungs with lesions that may have abscesses and fibrosis, but not acute hemorrhagic lesions neither fibrinous pleuritis.
- Mixed: lungs with lesions in both phases of lesion progression (hemorrhagic lesions, abscesses, fibrinous and fibrous lesions

15

Non-vaccinated animals showed more frequently acute lesions than vaccinated ones, which showed more chronic lesions (Chi-Square, $P=0.033$) (Table 9 and Fig. 20).

20

Table 9. Presence of acute lesions in the lungs of pigs from vaccinated and non-vaccinated groups.

	Type of lesion			no lesions	Total pigs
	acute	chronic	mixed		
VAC	0	11	7	4	22
NO VAC	6	5	6	6	23

If the animals with only acute lesions are analysed, these are more frequently seen in the non-vaccinated group (1 tail Fisher test, $P=0.0076$; Figure 21)

7.11 General comments

- 5 The results on temperature, severe dyspnea and chronicity of lesions support an effect of the vaccine in a faster resolution of the infection. The non-vaccinated group showed higher body temperature the last days of the study, with more animals with fever, which is in agreement with the presence of more animals with acute lesions in this group. Vaccinated animals seemed to solve the infection faster, showing more chronic lesions
- 10 at necropsy and lower body temperatures. In addition, the severity of dyspnea was significantly lower in the vaccinated animals than the non-vaccinated ones.

CLAIMS

- 1.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use in the prophylactic and therapeutic treatment and/or for reducing the clinical signs of respiratory infections in livestock animals caused by at least one bacterial pathogen belonging to the *Pasteurellaceae* family other than any virulent strain and serotype of the bacterial species *Pasteurella multocida*.
- 2.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to claim 1, wherein the bacterial pathogen belonging to the *Pasteurellaceae* family is selected from the group consisting of: any virulent strain and/or serotype of *Mannheimia* sp., *Haemophilus* sp., *Histophilus* sp., *Pasteurella* sp. (other than *Pasteurella multocida*), and *Actinobacillus* sp..
- 3.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 and 2, wherein the bacterial pathogen belonging to the *Pasteurellaceae* family is selected from the group consisting of: any virulent strain and/or serotype of *Manheimia haemolytica*, *Haemophilus parasuis*, *Actinobacillus pleuropneumoniae*, *Histophilus somni*, *Pasteurella trehalosi* and *Haemophilus paragallinarum*.
- 4.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 3, wherein the respiratory infections caused by at least one pathogen belonging to the *Pasteurellaceae* family are selected from the group consisting of: acute, subacute or chronic pneumonia, shipping fever, pleuropneumonia, porcine respiratory disease complex, bovine respiratory disease complex, upper respiratory tract infections, diphteria, lower respiratory tract infections, Grässer's disease, pasteurellosis.
- 5.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 4, in the prophylactic and therapeutic treatment of respiratory infections in livestock animals selected from the group consisting of: cattle, horses, pigs, birds, poultry, sheep, goat and rabbit.
- 6.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 5 in the prophylactic and

therapeutic treatment of respiratory infections in pigs caused by *Actinobacillus pleuropneumoniae*.

7.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 5 in the prophylactic and
5 therapeutic treatment of respiratory infections in pig, cattle or sheep caused by or associated with *Manheimia haemolytica*.

8.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 5 in the prophylactic and therapeutic treatment of respiratory infections in pigs caused by *Haemophilus parasuis*.

10 9.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 5 in the prophylactic and therapeutic treatment of respiratory infections in cattle caused by *Histophilus somni*.

10.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 5 in the prophylactic and
15 therapeutic treatment of respiratory infections in sheep caused by *Pasteurella trehalosi*.

11.- *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom for use according to any of claims 1 to 5 in the prophylactic and therapeutic treatment of respiratory infections in poultry caused by *Haemophilus paragallinarum*.

20

12.- A vaccine composition against respiratory infections and/or for reducing the clinical signs of respiratory infections in livestock animals caused by at least one bacterial pathogen belonging to the *Pasteurellaceae* family other than any virulent strain and serotype of the bacterial species *Pasteurella multocida* comprising an immunogenically
25 effective amount of *Pasteurella multocida* strain PM1094 or an outer-membrane protein extract prepared therefrom together with an acceptable carrier, vehicle and/or adjuvant.

13.- A vaccine composition according to claim 12, wherein the respiratory infection is caused by *Actinobacillus pleuropneumoniae*.

30

14.- A vaccine composition according to claim 13, comprising an additional active agent.

FIGURES

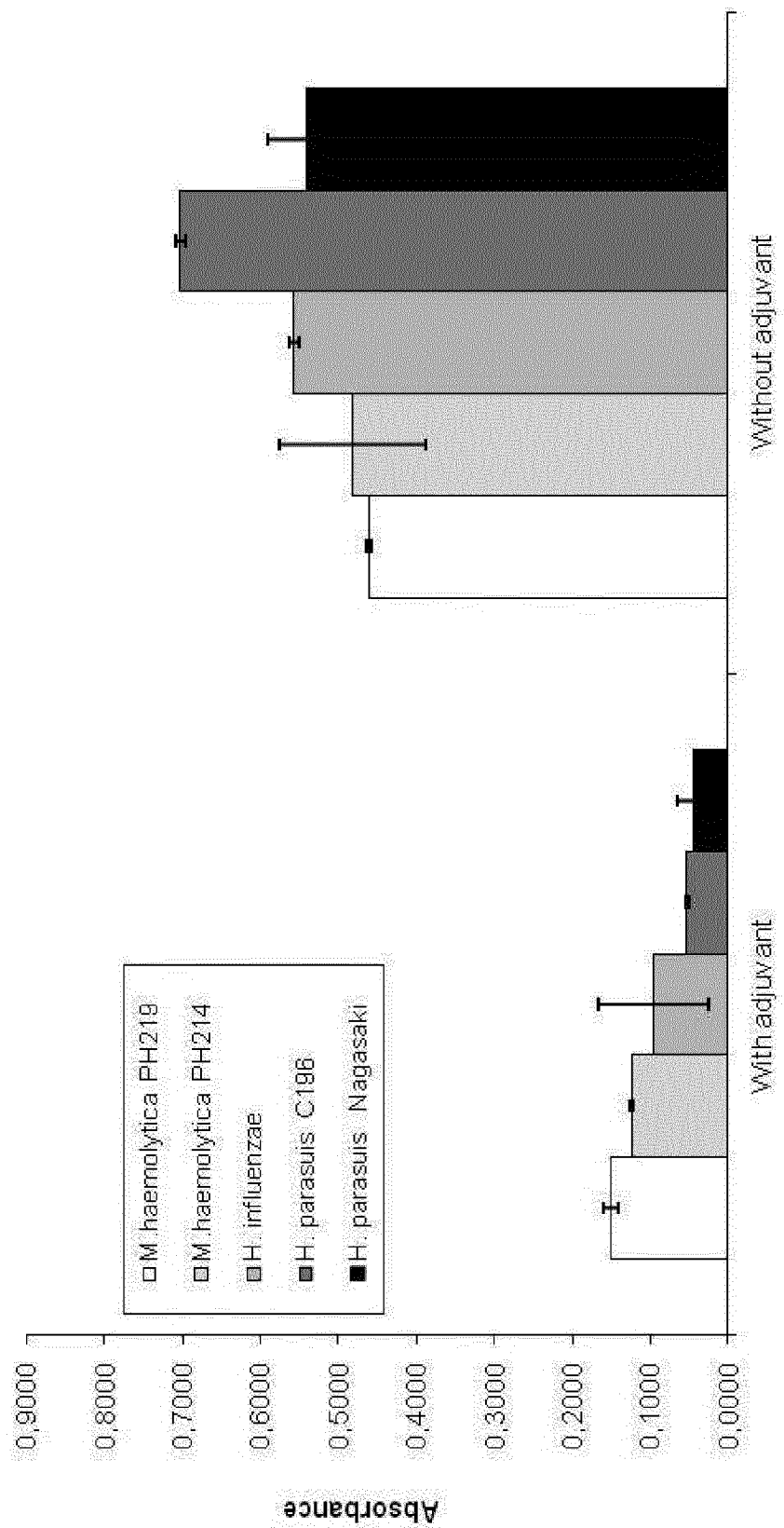


FIGURE 1

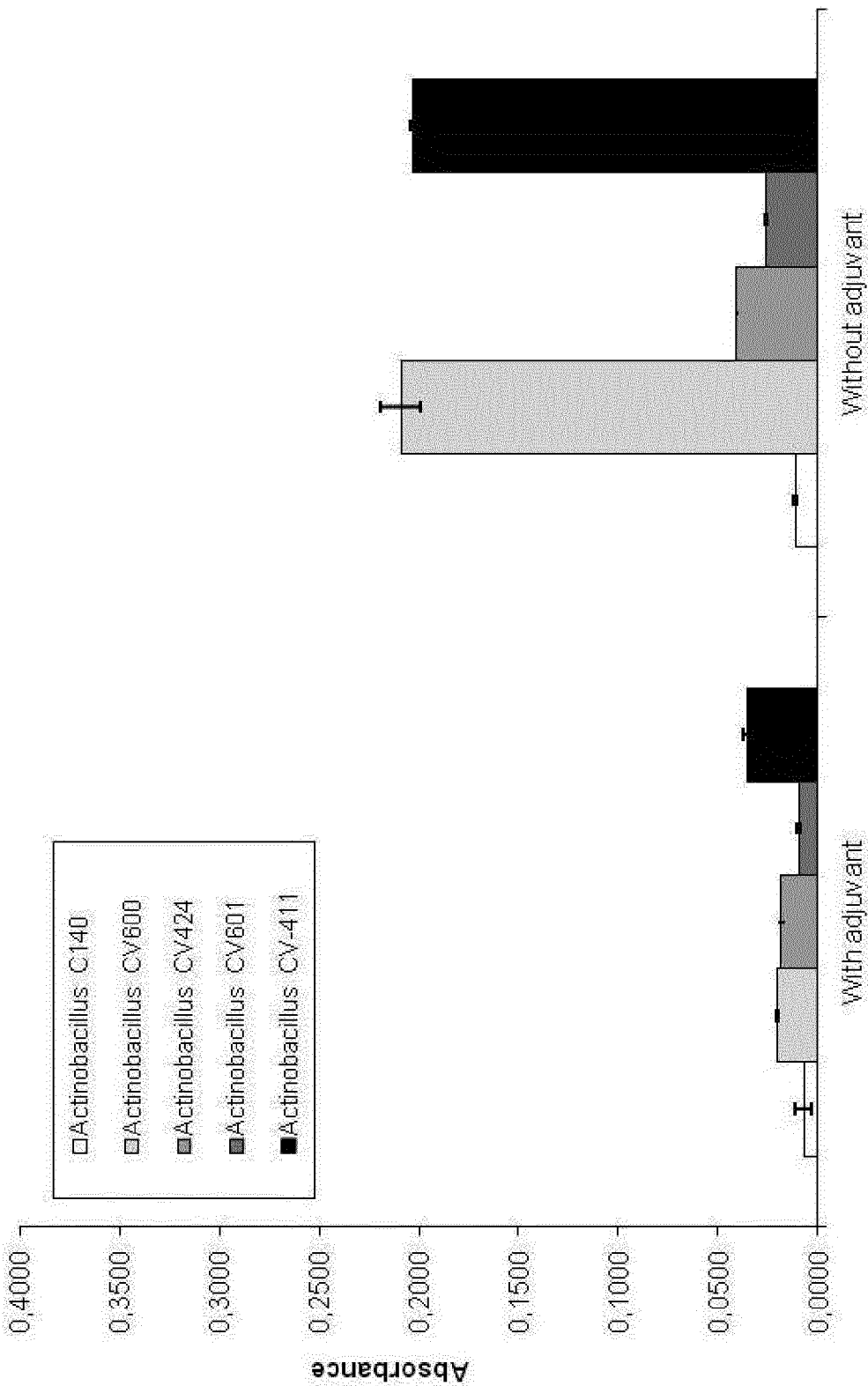


FIGURE 2

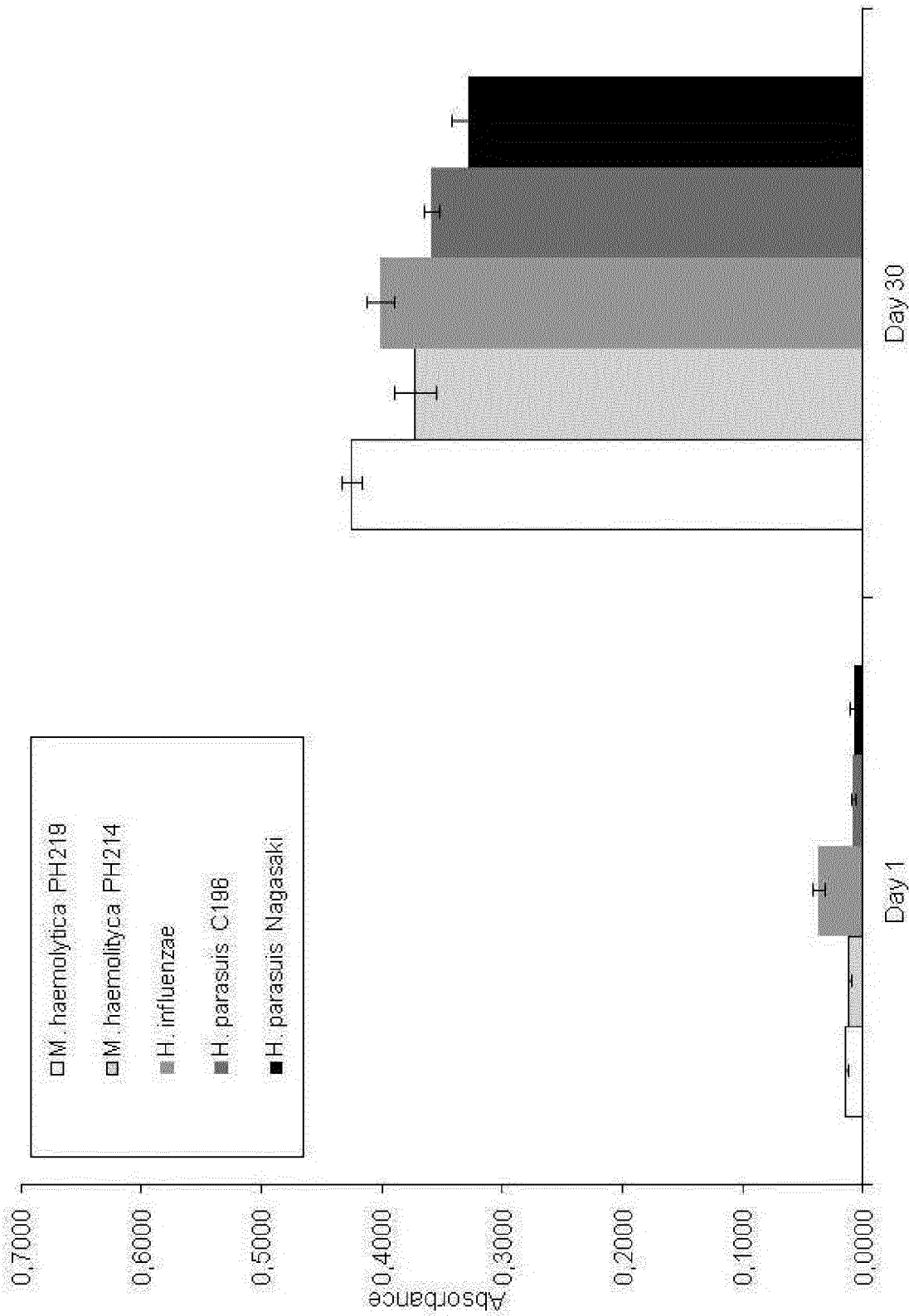


FIGURE 3

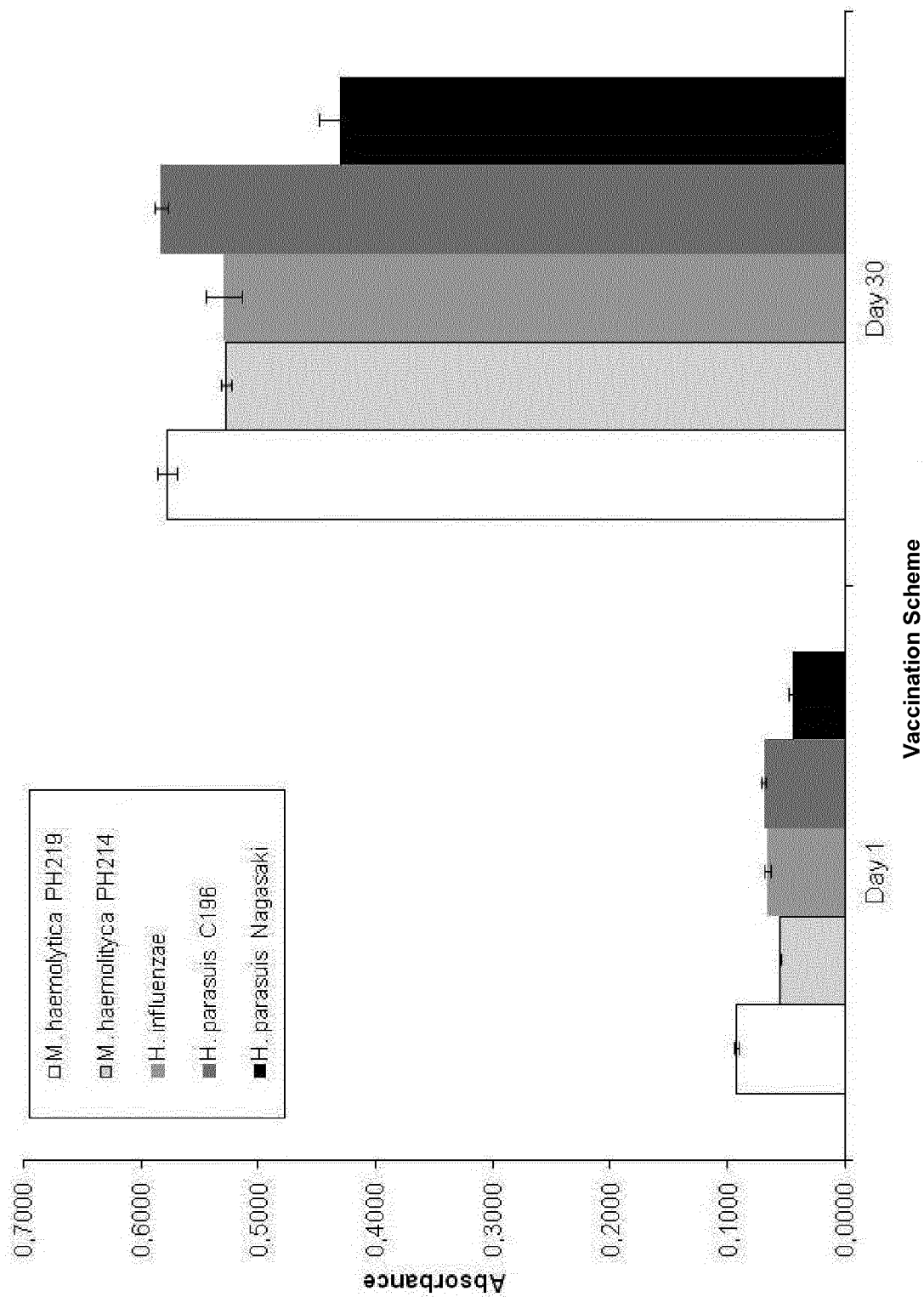


FIGURE 4

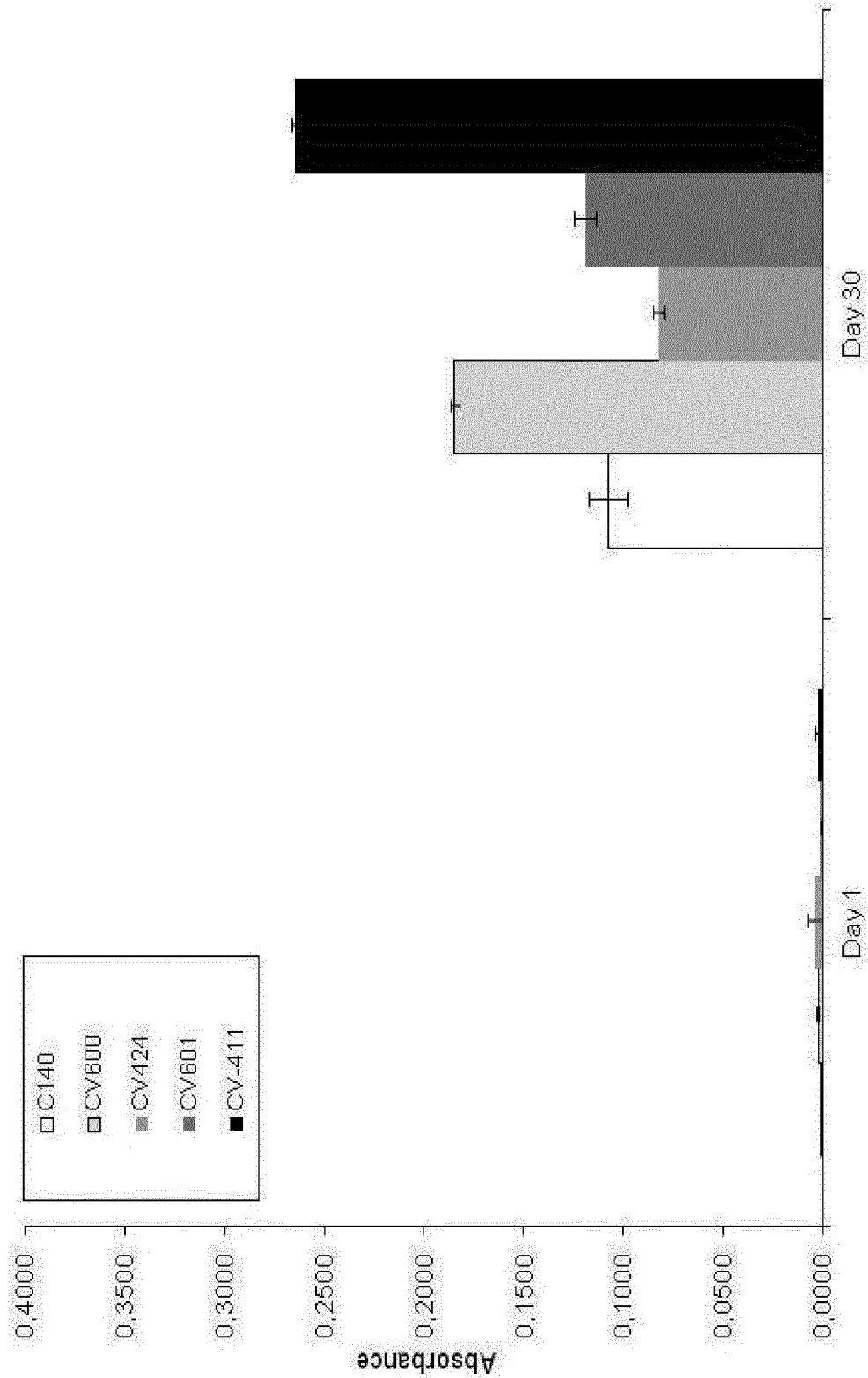


FIGURE 5

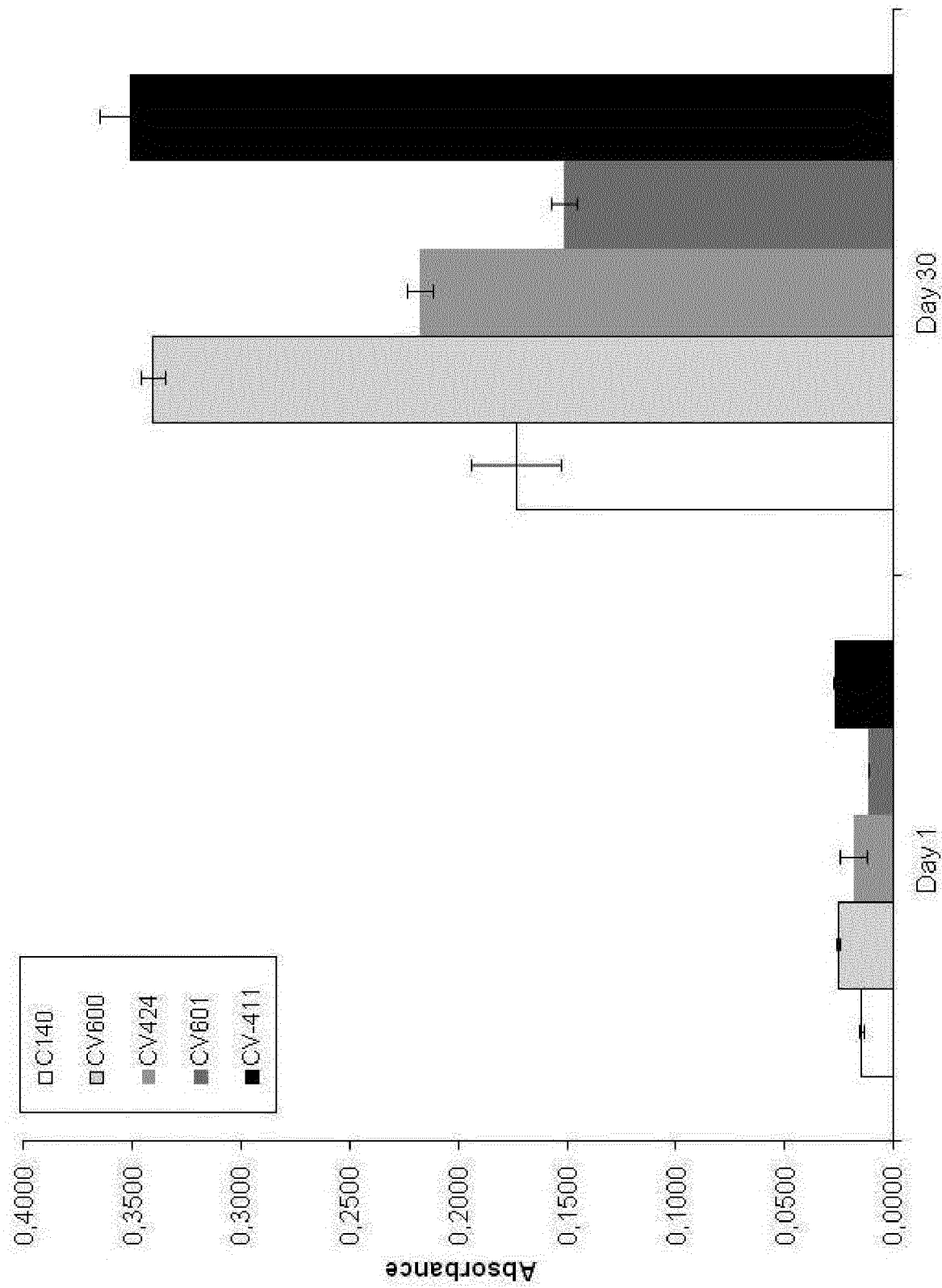


FIGURE 6

FIGURE 7A

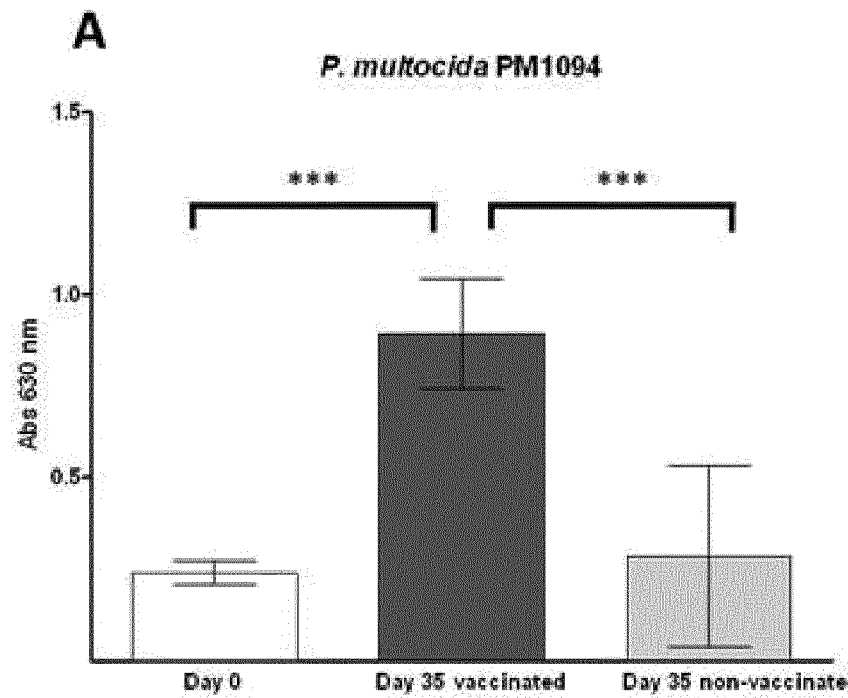


FIGURE 7B

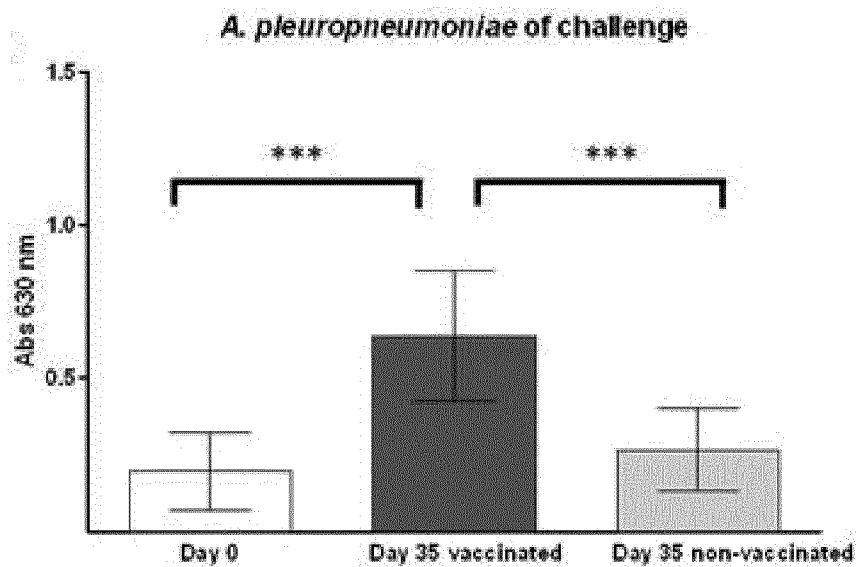


FIGURE 8

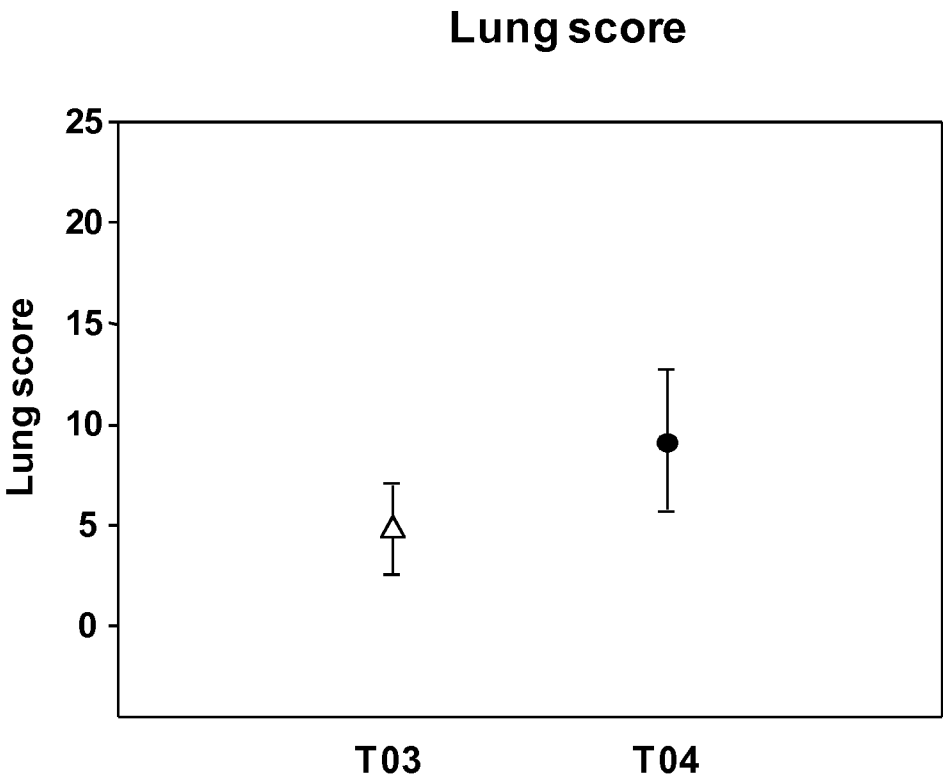
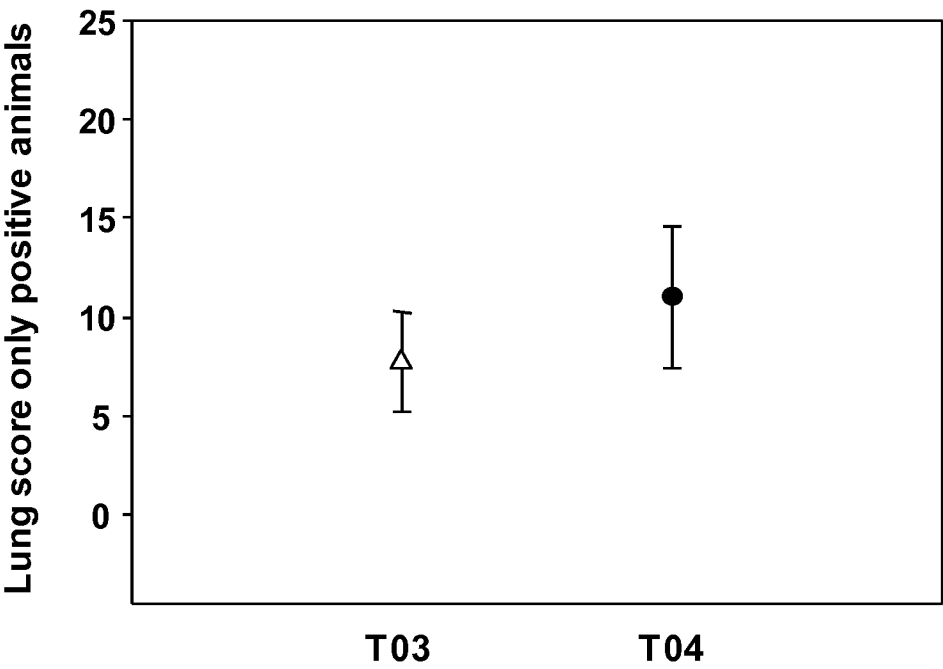
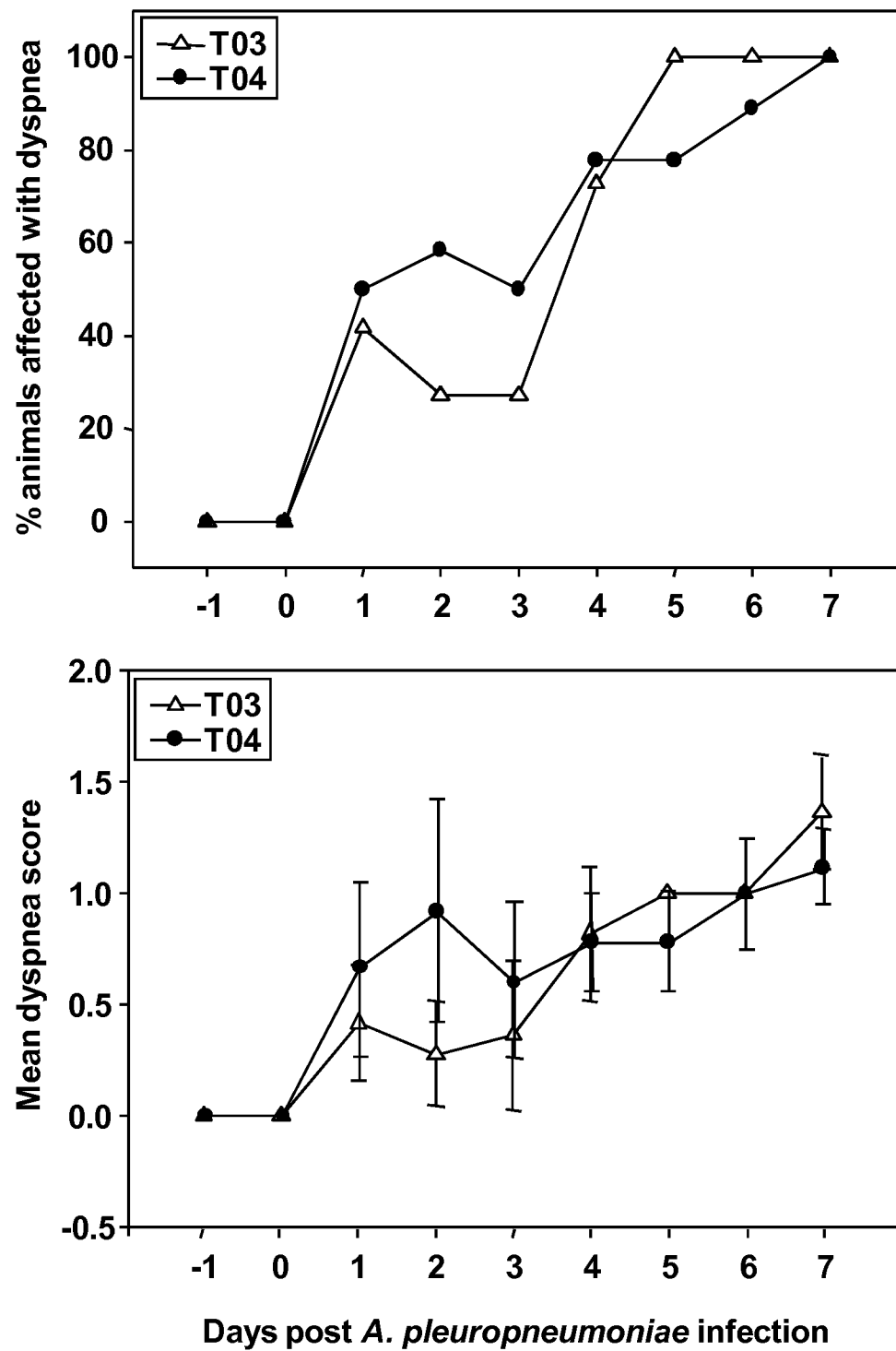


FIGURE 9



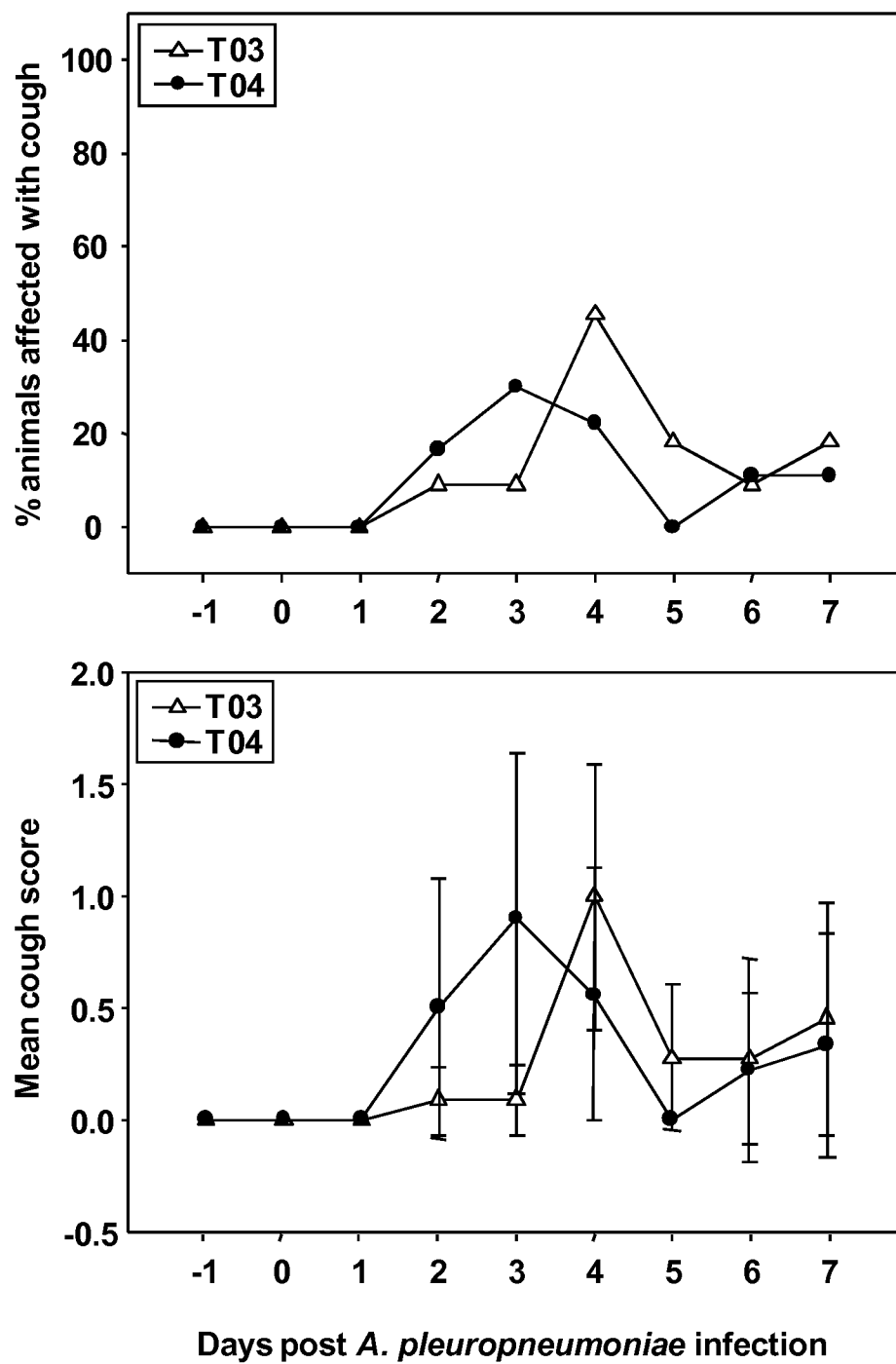
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FIGURE 10



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FIGURE 11



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FIGURE 12

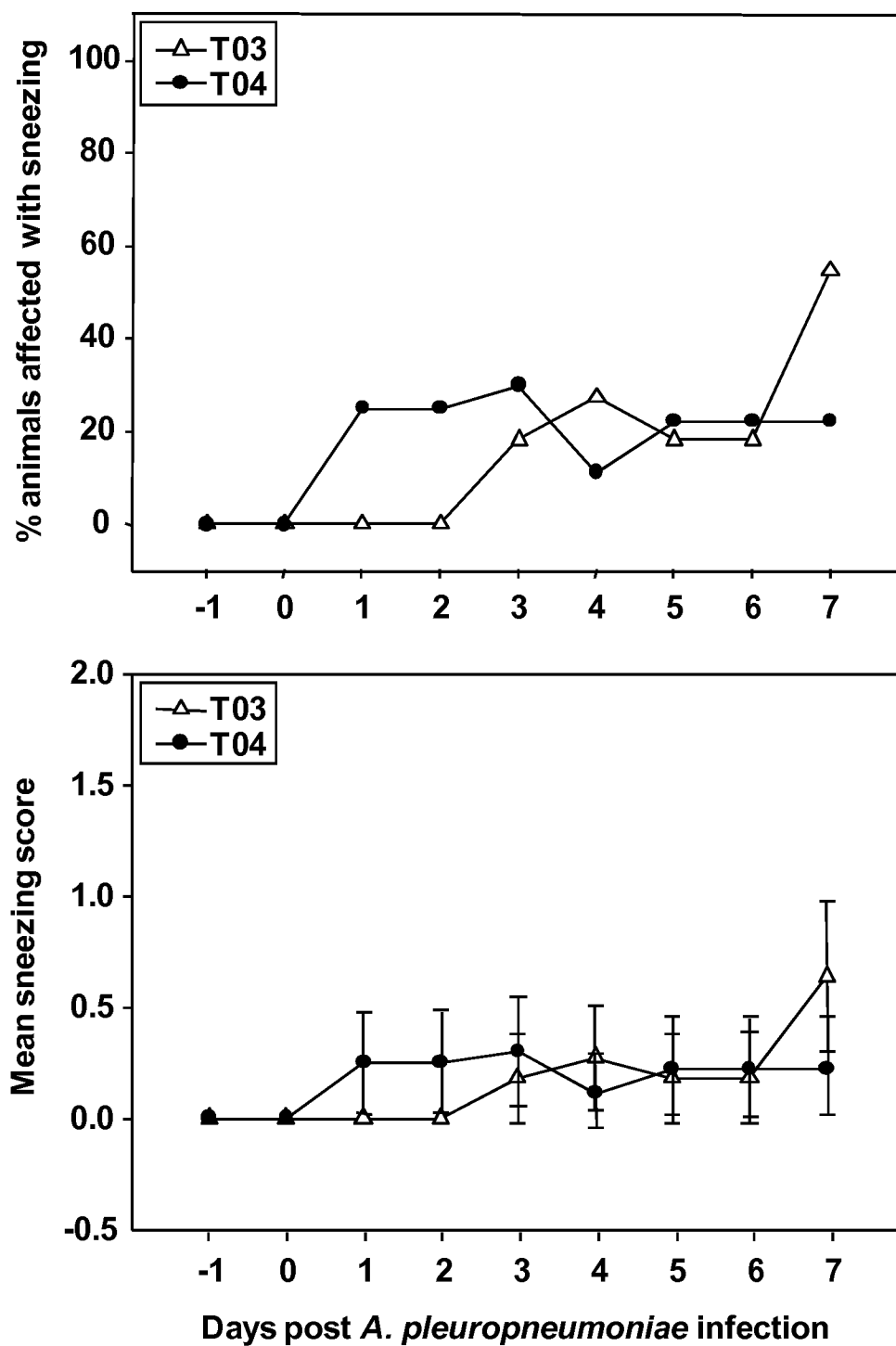


FIGURE 13

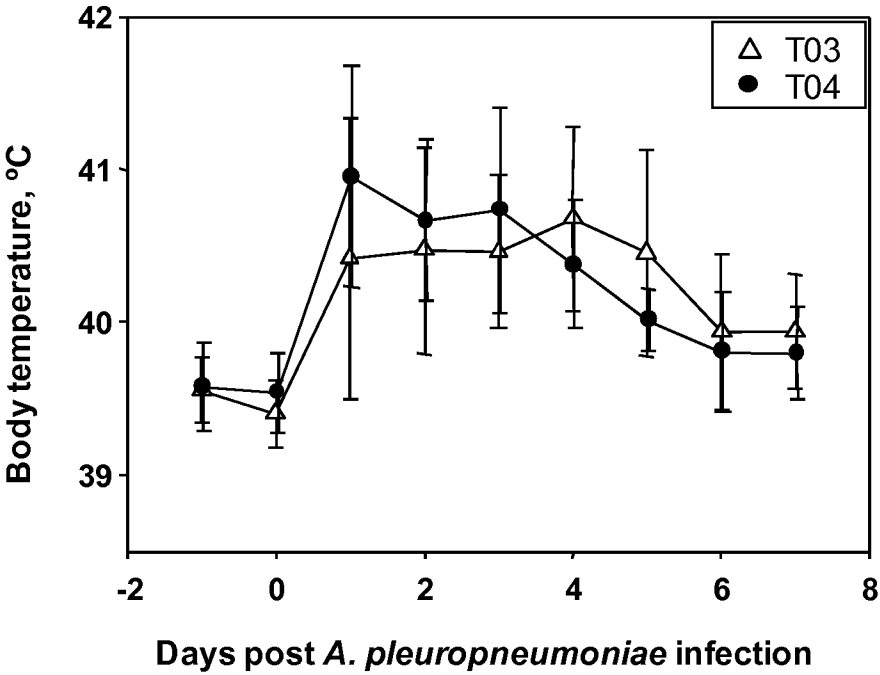


FIGURE 14

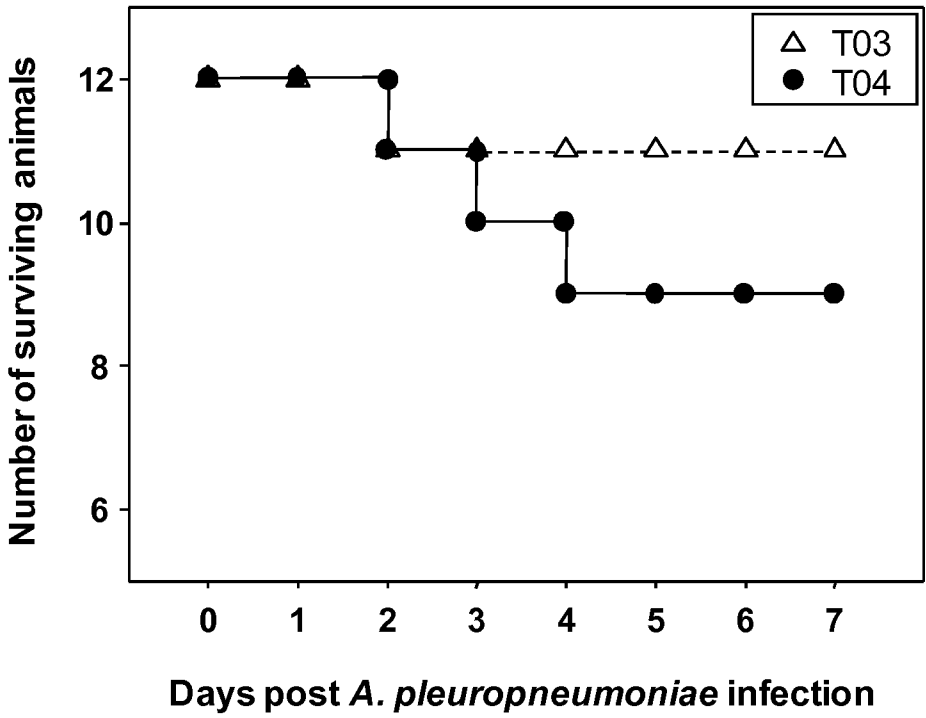


FIGURE 15

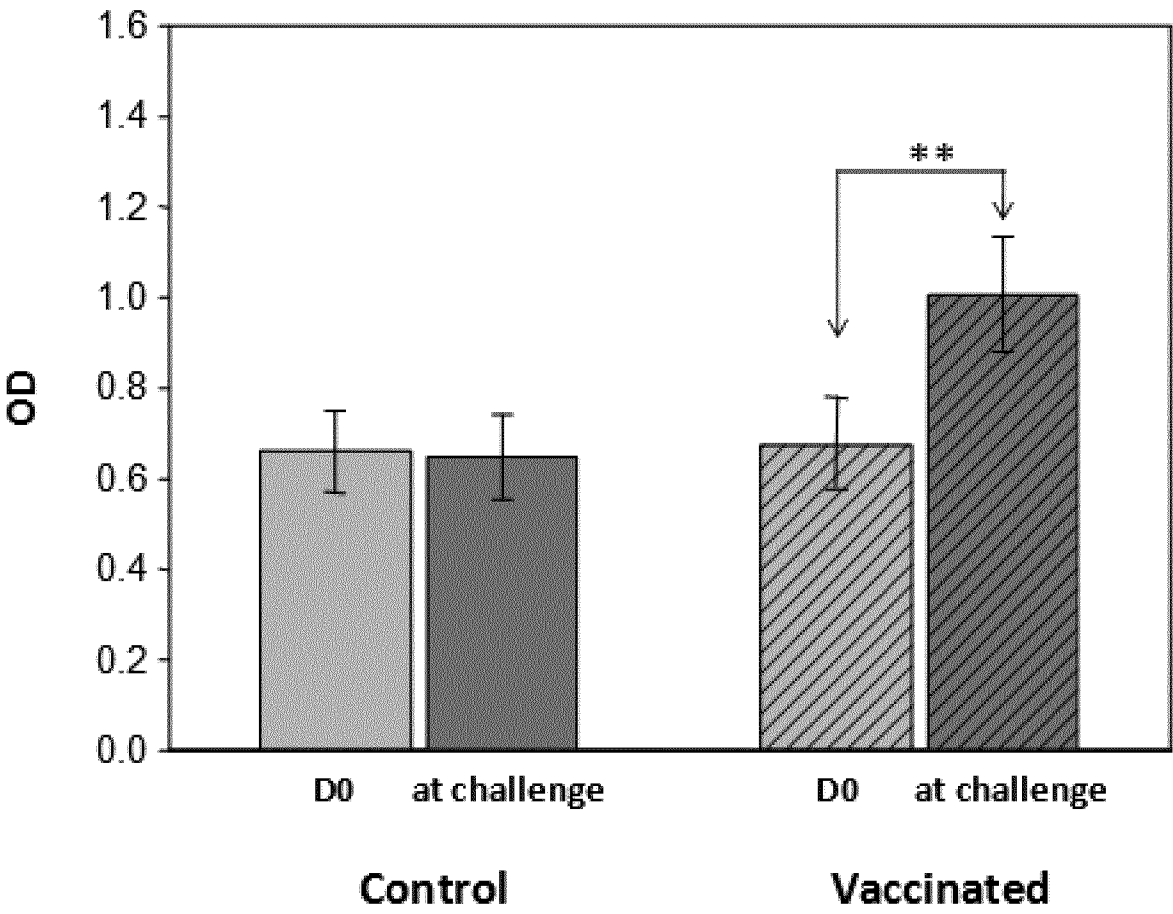


FIGURE 16

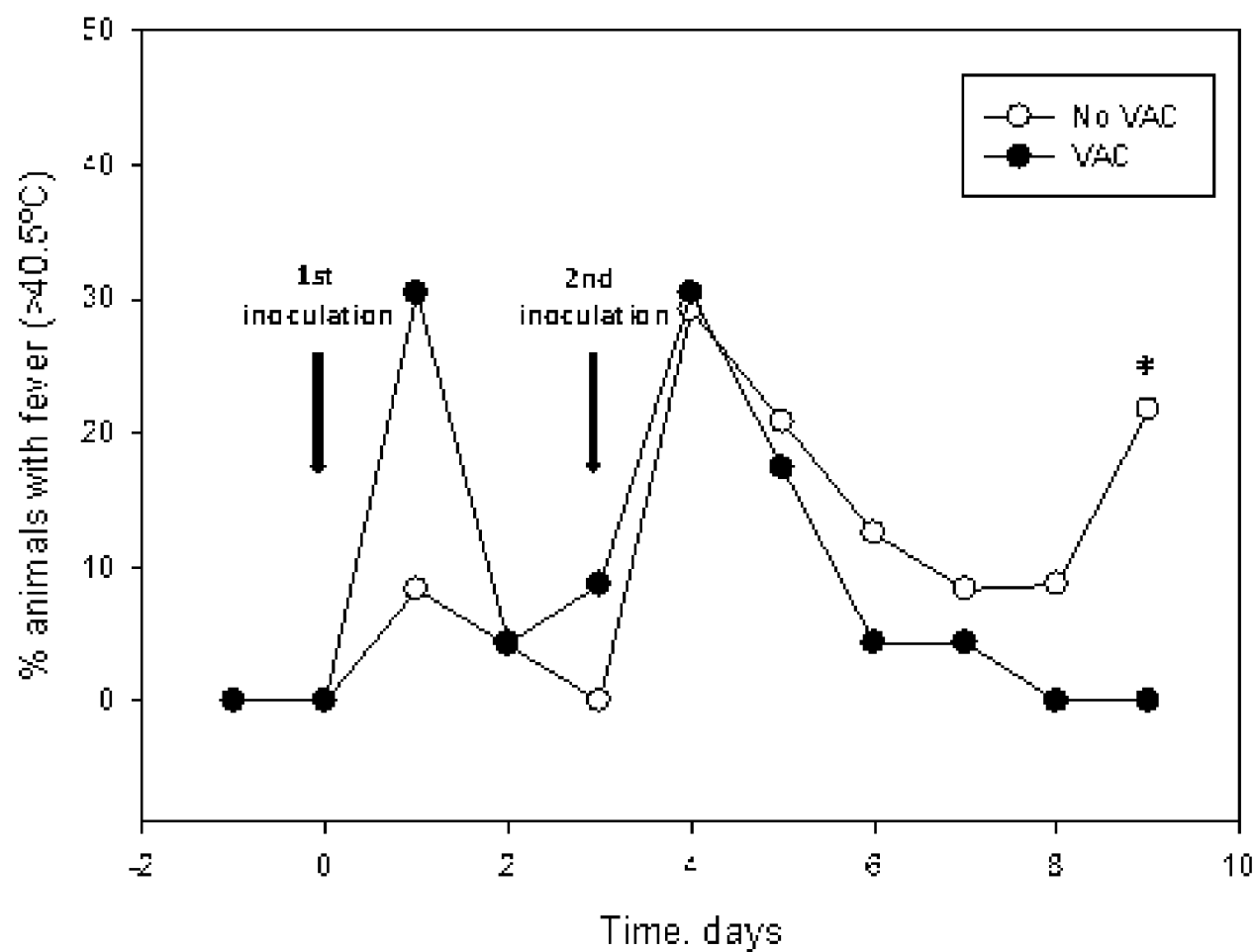


FIGURE 17

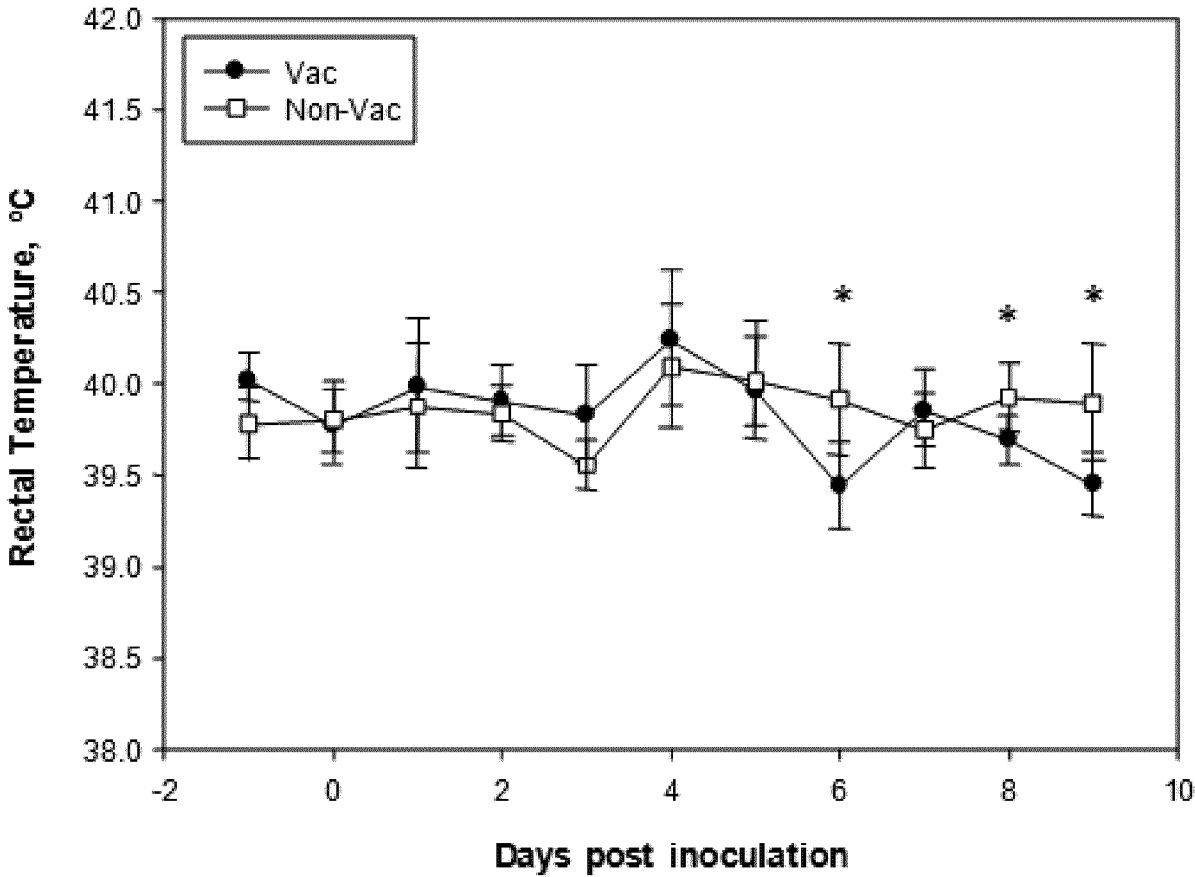


FIGURE 18

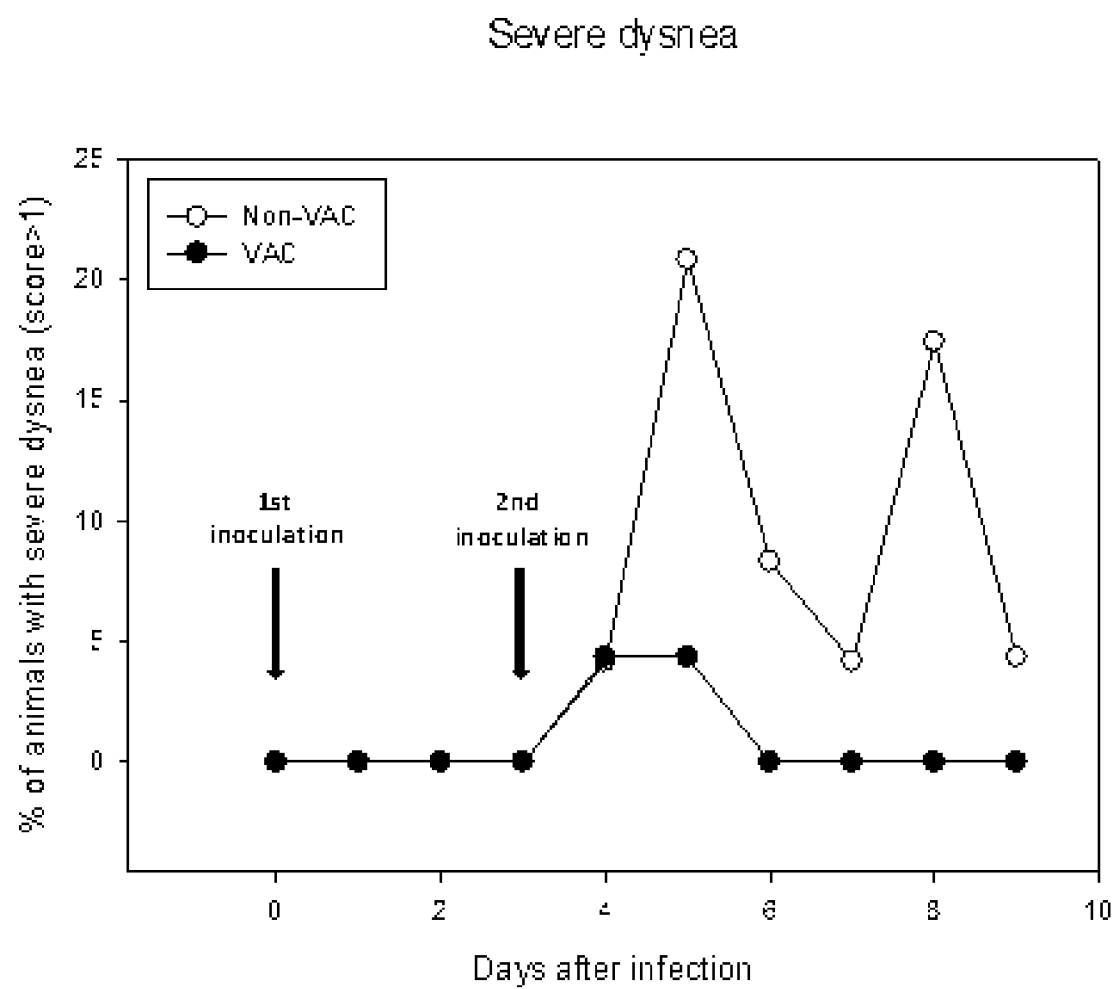


FIGURE 19

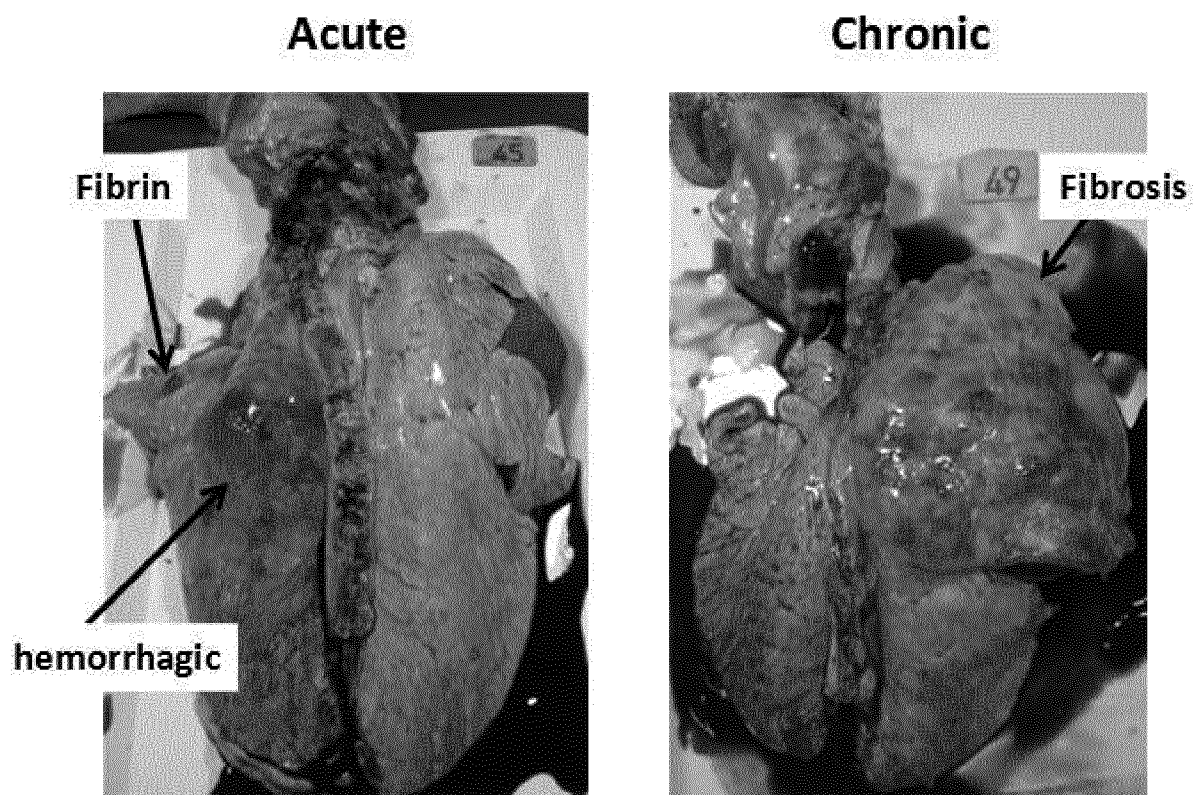


FIGURE 20

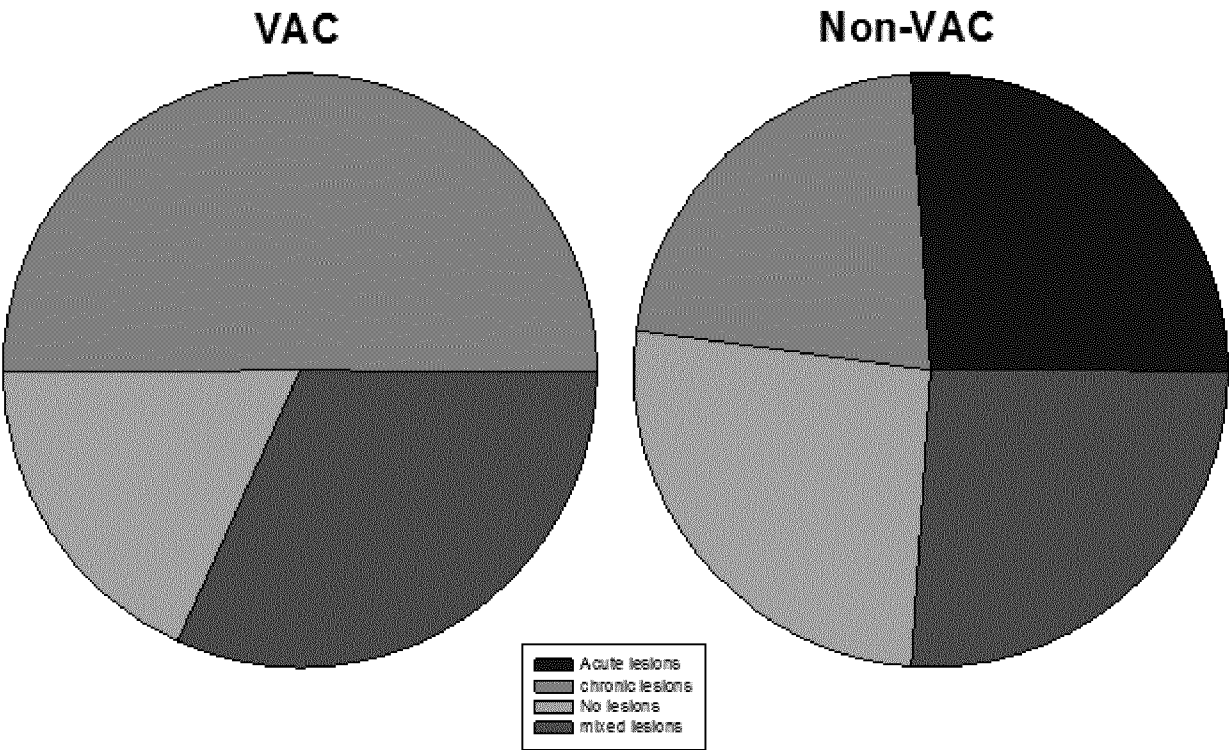
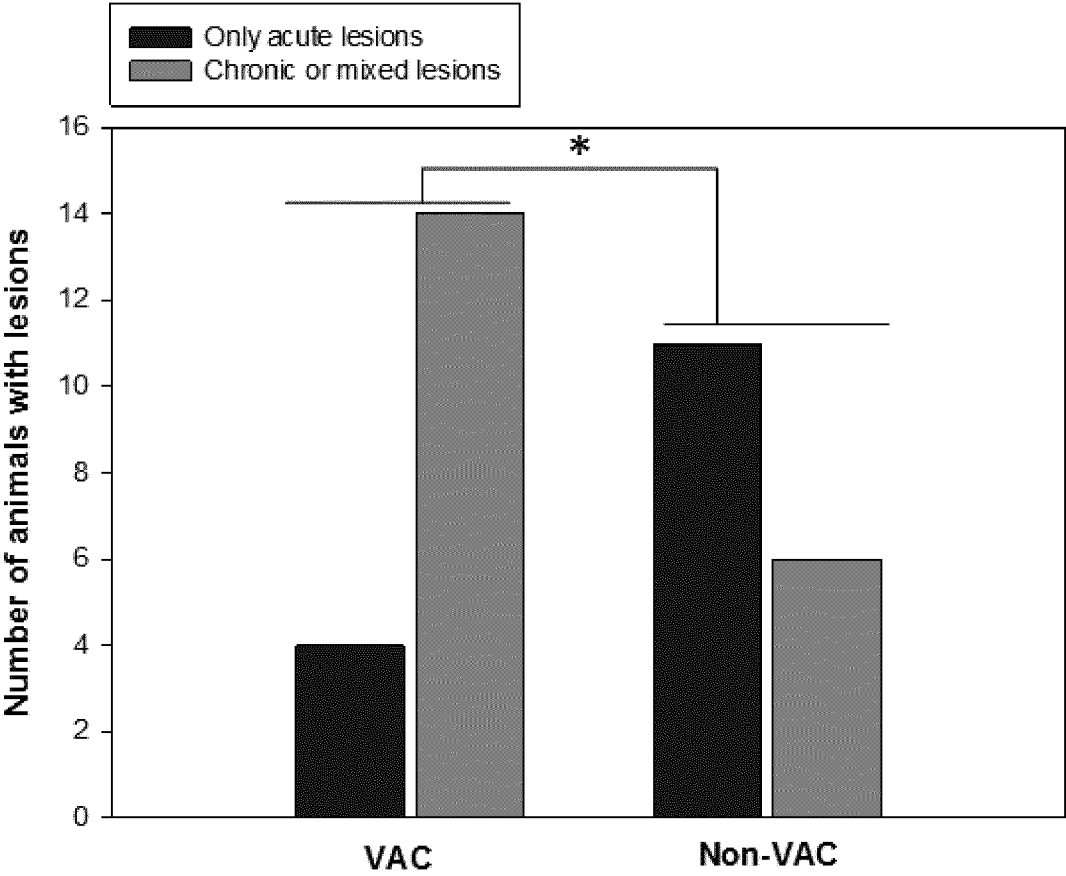


FIGURE 21



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/064763

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/102 C07K14/285 C12N1/36
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	EP 2 256 185 A1 (UNIV BARCELONA AUTONOMA [ES] UNI AUT NOMA DE BARCELONA [ES]) 1 December 2010 (2010-12-01) Abstract * Tab. 3 -/-	1-14



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

27 July 2015

Date of mailing of the international search report

06/08/2015

Name and mailing address of the ISA/

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NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Heder, Andreas

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/064763

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2015/064763

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