VACCINE ADJUVANT, PREPARATION METHOD AND USE THEREOF

Field of the Invention

[0001] The present application relates to the technical field of veterinary vaccine adjuvants, and in particular to a vaccine adjuvant, a preparation method and a use thereof.

Background to the Invention

[0002] Immune adjuvants, referred to as adjuvants for short, are nonspecific immunopotentiators. When injected into an animal prior to an antigen or simultaneously with the antigen, the adjuvant can non-specifically alter or enhance the body's specific immune response to the antigen, playing an important role in an immune response. Common adjuvants used in veterinary vaccines include: aluminum salt adjuvants (aluminum hydroxide, aluminum phosphate, calcium phosphate, etc.), oil adjuvants (mineral oil adjuvants and non-mineral oil adjuvants), aqueous adjuvants (cytokines, liposomes and immunostimulating complexes), drug adjuvants (traditional Chinese medicines and extracts, levamisole), and pathogenic microorganism adjuvants (cholera toxin, monophospholipid, oligodeoxynucleotide, etc.), etc. The adjuvants are important content in immunology and biotechnology, and play a crucial role in improving the body's immune response and immune status, as well as enhancing immune efficacy.

[0003] Currently, commonly used adjuvants in animal experiments include the aluminum salt adjuvants and the oil adjuvants. Experimental research has found that the use of aluminum hydroxide or aluminum phosphate adjuvants can enhance the immunogenicity of the antigen, reduce the dosage of antigens used, and simultaneously achieve an immune response of equivalent efficacy. Therefore, the adjuvants are widely used. However, aluminum adjuvants have defects such as failing to fully activate the body's immune system, interfering with cellular immunity, and having a short duration of immune protection. Oil emulsion adjuvants use mineral oil plus emulsifiers and stabilizers (aluminum stearate) as an oil phase, and a dispersed phase is an aqueous antigen

solution. The two are mixed and emulsified to form oil emulsion adjuvants, including Freund's adjuvant, white oil, and Span80 adjuvant. Their mechanism of action is similar to that of salt adjuvants, which can activate the body's immune cells to produce antibodies. However, some oil adjuvants also have certain defects due to unsatisfactory selection and ratio of mineral oil and emulsifiers, such as: high viscosity of finished vaccines, severe side effects of individual oil adjuvants. The continuous rise in oil prices has led to increased costs, etc. With the deepening of practice, how to develop more advantageous adjuvants and prepare veterinary vaccines with low viscosity, minimal toxic and side effects, and ease of use has become the focus of scientists' attention.

[0004] At present, the selection and use of veterinary vaccine adjuvants remain a key research focus. With the development of molecular biology, the development of new vaccines such as genetic engineering vaccines, synthetic peptide vaccines, and virus-like particle vaccines has put forward higher requirements for vaccine adjuvants. For example, in terms of properties, it is necessary to meet the requirements of good stability, difficult stratification, and easy injection. In terms of safety, it is to meet the requirements of no side effects. In terms of immune efficacy, it is to be able to induce the body to produce long-term and efficient specific immune responses, improve the body's protective ability, and at the same time reduce the dosage of immune substances and lower the production cost of vaccines.

Statement of Invention

[0005] In view of the technical problems existing in the background art, the present application provides a vaccine adjuvant, which has good water solubility, small particle size, good uniformity, high storage stability, no stratification after long-term storage, high safety, and can assist in inducing the body to produce a long-lasting and efficient specific immune response. Meanwhile, the present application further provides a preparation method for a vaccine adjuvant, and the preparation method has the advantages of being simply, convenient, and efficient.

[0006] The present application provides a vaccine adjuvant, including the following

components in parts by mass: 25-60 parts of liposomes and 20-50 parts of pine pollen.

[0007] Preferably, the liposomes include phospholipids, cholesterol, and tocopherol; a mass ratio of the phospholipids to the cholesterol is (10-30):(10-20); and a mass ratio of the phospholipids to the tocopherol is (10-30):(5-10).

[0008] Preferably, saponins are further included, and a mass ratio of the pine pollen to the saponins is (20-50):(10-30).

[0009] The present application provides a vaccine adjuvant dispersion, including the vaccine adjuvant and further including an injectable dispersion.

[0010] Preferably, based on 100 parts by mass of the vaccine adjuvant, mass parts of the injectable dispersion are not more than 200,000 parts.

[0011] The present application provides a preparation method for the vaccine adjuvant, including the following steps:

[0012] S1: dissolving and mixing raw materials of the vaccine adjuvant to obtain a mixed solution; and

[0013] S2: removing a solvent from the mixed solution to obtain a solid substance.

[0014] Preferably, step S1 includes: dissolving the phospholipids and the cholesterol in chloroform to obtain a solution A; dissolving the tocopherol in ethanol to obtain a solution B; dissolving the pine pollen and the saponins in water to obtain a solution C; and mixing the solution A, the solution B, and the solution C to obtain the mixed solution.

[0015] The present application provides a preparation method for the vaccine adjuvant dispersion, including the preparation method for the vaccine adjuvant, and after step S2, further including the following step:

[0016] S3: dissolving the solid substance in the injectable dispersion, and performing ultrasonic treatment and filtering.

[0017] Preferably, a temperature of the ultrasonic treatment in step S3 is 20-30°C; a power of the ultrasonic treatment is 50-70 W.

[0018] Preferably, a total duration of the ultrasonic treatment is 20-30 min; and a cycle

period of the ultrasonic treatment is: ultrasonication for 8-12 s, with an interval of 8-12 s.

[0019] Preferably, a filter pore size specified in step S3 for filtration is 0.10-0.22 μm.

[0020] The present application provides a use of the vaccine adjuvant, or the vaccine adjuvant dispersion, or the vaccine adjuvant prepared by the preparation method, or the vaccine adjuvant dispersion prepared by the preparation method in the preparation of vaccines.

[0021] The present application also provides a vaccine, which includes an inactivated antigen, and further includes the vaccine adjuvant, or the vaccine adjuvant dispersion, or the vaccine adjuvant prepared by the preparation method, or the vaccine adjuvant dispersion prepared by the preparation method.

[0022] Preferably, the inactivated antigen includes one or more of bovine epizootic fever inactivated antigen, porcine Seneca Valley virus inactivated antigen, porcine pseudorabies inactivated antigen, porcine foot-and-mouth disease type O inactivated antigen, porcine foot-and-mouth disease type A inactivated antigen, bovine rotavirus inactivated antigen, and bovine parainfluenza inactivated antigen.

[0023] Beneficial Effects

[0024] The present application provides a vaccine adjuvant, including the following components in parts by mass: 25-60 parts of liposomes and 20-50 parts of pine pollen. The liposomes have a strong ability to carry drugs, and the pine pollen acts as a natural immunostimulant. The combination of liposomes and pine pollen can significantly increase the speed and intensity of the specific immune response of pine pollen, making the immune response faster and stronger, accurately recognize and initiate non-specific defense mechanisms, improve the body's own resistance, and strengthen disease resistance. In addition, the vaccine adjuvant provided by the present application has good water solubility, small particle size, good uniformity, and high storage stability; and the veterinary vaccine further prepared therefrom has strong stability, high safety, no stratification after long-term storage and easy injection, as well as can induce the body to produce a long-lasting and efficient immune response.

Brief Description of the Drawings

[0025] To more clearly illustrate the present application or the technical solutions in the prior art, the drawings required for the embodiments or the description of the prior art are described below.

[0026] FIG. 1 is a particle size distribution diagram of a bovine epizootic fever inactivated vaccine obtained in Example 1 of the present application, and particle size distributions of the bovine epizootic fever inactivated vaccines obtained in other embodiments are similar to that in FIG. 1;

[0027] FIG. 2 is a particle size distribution diagram of a bovine epizootic fever inactivated vaccine obtained in Comparative Example 1;

[0028] FIG. 3 is a particle size distribution diagram of a bovine epizootic fever inactivated vaccine obtained in Comparative Example 2;

[0029] FIG. 4 is a comparison diagram of appearance characteristics of the vaccines prepared in Example 1 and Comparative Examples 1-2, among them, No. 1 shows the appearance of the vaccine of Comparative Example 1, No. 2 shows the appearance of the vaccine of Example 1, and No. 3 shows the appearance of the vaccine of Comparative Example 2;

[0030] FIG. 5 shows curves of neutralizing antibody levels induced by the vaccines prepared in Examples 1-3 and Comparative Examples 1-2 over time and significance difference analysis; and

[0031] FIG. 6 shows curves of neutralizing antibody levels induced by the vaccines prepared in Example 3 and Comparative Examples 3-4 over time and significance difference analysis.

Detailed Description

[0032] The present application provides a vaccine adjuvant, and the vaccine adjuvant components include liposomes and pine pollen.

[0033] In the present application, by weight, a dosage of the liposomes is 25-60 parts,

preferably 40-50 parts, more preferably 45 parts. A dosage of the pine pollen is 20-50 parts, preferably 25-35 parts, more preferably 30 parts.

[0034] The liposomes are similar to biological membranes, generally consisting of an ultramicrospherical preparation formed by a bilayer coated with antigens composed of phospholipids and cholesterol, which can not only transport antigens but also serve as vaccine adjuvants. The source of the liposomes in the present application is not particularly limited, and conventional commercially available liposome products can be used as raw materials. In a preferred example of the present application, the liposomes include phospholipids, cholesterol, and tocopherol. A mass ratio of the phospholipids to the cholesterol is preferably (10-30):(10-20), more preferably (15-20):(12-20), and even more preferably 15:20; and a mass ratio of the phospholipids to the tocopherol is preferably (10-30):(5-10), more preferably (15-20):10, and even more preferably 15:10.

[0035] Phospholipids are indispensable substances for the normal metabolism and healthy survival of organisms, playing a very important role in cell activation, survival and maintenance of organ functions in the body, the activation of muscles and joints, fat metabolism, and body immunity. Phospholipids have emulsifying and dispersing effects, helping in the transport and absorption of drugs. Phospholipids can be used to construct cell membranes, facilitating the presentation of antigens to antigen-presenting cells. Phospholipids can form a phospholipid bilayer, which can not only transport antigens but also serve as the vaccine adjuvants.

[0036] Cholesterol has the functions of forming bile acids, constituting cell membranes, and synthesizing hormones. Cholesterol is an important component of the cell membrane of liposomes, maintaining its normal structure and function. The formed hormones are chemical messengers that coordinate the metabolic processes of different cells in multicellular organisms, participate in the metabolism of various substances in the body, and are very important for maintaining the normal physiological functions of the body.

[0037] Tocopherol has strong antioxidant properties, can maintain and promote various functions of the human body, exert antioxidant effects, improve fat metabolism, and enhance immunity. Among them, tocopherol and its derivatives improve the body's

immune function mainly by enhancing the humoral immunity of B lymphocytes, enhancing the phagocytic function of macrophages, improving the cellular immunity of T lymphocytes, and enhancing the antigen-presenting function of related cells.

[0038] The sources of phospholipids, cholesterol, and tocopherol in the present application are not particularly limited, and conventional commercially available products in the field can be used. In a specific embodiment of the present application, the phospholipids are egg yolk lecithin, purchased from Hebei Siwei Biotechnology Co., Ltd., with a product number of E-30; the cholesterol is purchased from Shanghai Bihe Biochemical Technology Co., Ltd., with a product number of RWE20220112; and the tocopherol is α-tocopherol, purchased from Shanghai Huicheng Biotechnology Co., Ltd., with a product number of CAS: 59-02-9. The present application uses liposomes as the main carrier of vaccine viruses, which helps reduce antigen toxicity and promotes the degradation of antigens in a host body. Moreover, the liposomes can enhance the humoral and cellular immune responses of the body, and the structure is conducive to presenting antigens to antigen-presenting cells.

[0039] The pine pollen is the dry pollen of *Pinus massoniana Lamb*., *Pinus tabuliformis Carr*., or several other plants of the same genus. The pine pollen is warm in nature and has the effects of drying dampness and enhancing immunity. The pine pollen contains a variety of amino acids, vitamins, trace elements, nucleic acids, etc., and is a substance with broad-spectrum biological activity and immunomodulatory effects. As a natural immunostimulant, it has many advantages such as safety, no residue, easy solubility, and easy absorption. As a component of water adjuvants, it has good thermal stability and biocompatibility, and is easily soluble in water. The pine pollen polysaccharides in pine pollen can improve the activity of natural killer cells and the phagocytic capacity of macrophages. When introduced into the body as a component of adjuvants, it can not only increase specific immune responses but also initiate non-specific defense mechanisms, improve the body's own resistance, and strengthen disease resistance. Even when used alone, it can exert the immunostimulatory effect, but embedding it into the bilayer of liposomes can increase its accumulation at the target site, thereby releasing it at specific target sites, and ultimately significantly improving the immune and therapeutic

effects of pine pollen.

[0040] An inner core of liposomes is an aqueous phase, which can encapsulate hydrophilic drugs, and the space between the bilayers is an oil phase, which can encapsulate lipophilic drugs. In addition, an inner aqueous phase of liposomes allows them to be used for the delivery of many biological macromolecules, such as deoxyribonucleic acid (DNA), proteins, or pine pollen. In the present application, liposomes, as drug delivery carriers, help increase the solubility of encapsulated drugs (saponins and pine pollen), improve the therapeutic effect of drugs, reduce drug loss, reduce the adverse reactions of drugs, extend the in vivo circulation time of drugs, improve the targeting of drug delivery, and have good biocompatibility.

[0041] The source of the pine pollen in the present application is not particularly limited, and conventional commercially available pine pollen products in the field can be used. In a specific embodiment of the present application, the pine pollen is purchased from New Era Health (Group) Co., Ltd.

[0042] In a preferred example of the present application, the vaccine adjuvant further includes saponins. The mass ratio of the pine pollen to the saponins is preferably (20-50):(10-30), more preferably (25-35):(15-30), and even more preferably 30:25. In the present application, the saponins are preferably saponin Quil-A, which is a heterogeneous mixture of water-soluble saponins extracted from *Quillaja saponaria* (a tree native to South America).

[0043] Saponins (also known as glycosides) are a class of glycoside compounds, which are glycosides with triterpenoid or spirostane compounds as aglycones. Saponins are mainly distributed in higher terrestrial plants and also exist in small amounts in marine organisms such as starfish and sea cucumbers. The chemical structure of saponins consists of a hydrophilic carbohydrate framework and a lipophilic (hydrophobic) triterpenoid or steroid structure. The hydrophilic and hydrophobic parts interact with the surface tension of water, producing foam in aqueous solutions. Saponin compounds are diverse in types and complex in composition, and have a variety of pharmacological activities. The regulation of immune responses by saponins is controlled by helper T cells

(Th), which can be roughly divided into cell-mediated responses (cell-mediated immunity) mediated by Th1 cells and macrophages, and antibody-mediated immune responses (humoral-mediated immunity) mediated by Th2 cells. The activity of helper T cell antigen responses is enhanced, and the maturation of dendritic cells is promoted. Mature dendritic cells can promote the differentiation of naive T cells into Th1 type through the secretion of interleukin-12, thereby increasing humoral and cell-mediated immune responses.

[0044] The source of the saponins in the present application is not particularly limited, and conventional commercially available products in the field can be used. In a specific embodiment of the present application, the saponins are purchased from Saideao Biotechnology (Beijing) Co., Ltd., with a product number of VSP70.

[0045] Further, in some preferred examples of the present application, the vaccine adjuvant includes the following components in parts by mass: 15 parts of phospholipids, 20 parts of cholesterol, 10 parts of tocopherol, 40 parts of pine pollen, and 15 parts of saponins;

[0046] or, 20 parts of phospholipids, 12 parts of cholesterol, 8 parts of tocopherol, 30 parts of pine pollen, and 30 parts of saponins;

[0047] or, 20 parts of phospholipids, 20 parts of cholesterol, 5 parts of tocopherol, 30 parts of pine pollen, and 25 parts of saponins;

[0048] or, 10 parts of phospholipids, 20 parts of cholesterol, 10 parts of tocopherol, 35 parts of pine pollen, and 25 parts of saponins;

[0049] or, 23 parts of phospholipids, 10 parts of cholesterol, 6 parts of tocopherol, 33 parts of pine pollen, and 28 parts of saponins.

[0050] The vaccine adjuvant provided by the present application is a liposome complex water adjuvant, which can be in a solid dosage form (such as lyophilized powder), or a dispersion (such as phosphate-buffered saline (PBS) solution or normal saline) can be added to prepare a vaccine adjuvant dispersion.

[0051] The present application provides a vaccine adjuvant dispersion, including the aforementioned vaccine adjuvant components and further including an injectable

dispersion. Based on 100 parts by mass of the vaccine adjuvant, mass parts of the injectable dispersion is preferably not more than 200,000 parts, and more preferably not more than 100,000 parts. In a more preferred specific embodiment of the present application, mass parts of the injectable dispersion are 50,000-150,000 parts, preferably 90,000-100,000 parts; more preferably 99,800-100,000 parts. The source of the injectable dispersion in the present application is not particularly limited, and conventional commercially available products in the field can be used. In a specific embodiment of the present application, the injectable dispersion includes PBS solution, DMEM solution, MEM solution, or normal saline.

[0052] Liposomes, as one of the new drug delivery systems successfully applied in clinical practice, possess promising application prospects and numerous advantages. Compared with traditional dosage forms, the present application adds saponins and pine pollen to the liposome bilayer structure, and its advantages include increasing the delivery and absorption capacity of drugs (saponins and pine pollen), extending the in vivo half-life, having the ability to target immune organs and immune cells, improving the pharmacokinetic properties of drugs, enhancing the efficacy of drugs, and reducing adverse reactions. At the same time, strong humoral and cellular immune responses are induced. The raw materials of the vaccine adjuvant provided by the present application, such as phospholipids, pine pollen, tocopherol, saponins, and cholesterol, are all natural substances, and the safety is relatively reliable. Among them, small-molecule hydrophilic components are embedded in lipophilic components, with stable properties. The vaccine adjuvant presents a lipid bilayer structure in the dispersion and can be stored for a long time at about 2-8°C.

[0053] The present application provides a preparation method for the vaccine adjuvant, including the following steps:

[0054] S1: dissolving and mixing raw materials of the vaccine adjuvant to obtain a mixed solution; and

[0055] S2: removing a solvent from the mixed solution to obtain a solid substance.

[0056] In a preferred example of the present application, the liposomes include

phospholipids, cholesterol, and tocopherol. Among them, a solvent for dissolving the phospholipids and the cholesterol preferably includes chloroform; a solvent for dissolving the tocopherol preferably includes ethanol; and a solvent for dissolving the pine pollen and the saponins preferably includes water for injection. The present application preferably dissolves the phospholipids and the cholesterol in chloroform to obtain a solution A; the tocopherol is dissolved in ethanol to obtain a solution B; the pine pollen and the saponins are dissolved in water for injection, shaken and mixed uniformly, filtered after standing, and the supernatant is collected to obtain a solution C; then the solution A, solution B, and solution C are mixed to obtain the mixed solution. The preferred dissolution steps of the present application facilitate the full dissolution of each component to form a liposome network structure. Other methods that can fully dissolve the components to form a liposomal network structure can also be used. For example, each component is dissolved separately to obtain five different solutions, and the mixed solution can also be obtained by mixing.

[0057] In the present application, the method for removing the solvent in step S2 preferably includes rotary evaporation; the temperature of the rotary evaporation is preferably 40-50°C, and the rotation speed is preferably 100-200 r/min.

[0058] In the present application, when the vaccine adjuvant is a vaccine adjuvant dispersion, the preparation method preferably further includes step S3: dissolving the solid substance in the injectable dispersion, performing ultrasonic treatment, and filtering.

[0059] A temperature of the ultrasonic treatment in step S3 is preferably 20-30°C; a power of the ultrasonic treatment is preferably 50-70 W; a total duration of the ultrasonic treatment is preferably 20-30 min; a cycle period of the ultrasonic treatment is preferably: ultrasonication for 8-12 s, with an interval of 8-12 s, more preferably ultrasonication for 10 s, with an interval of 10 s. A filter pore size specified in step S3 for filtration is preferably 0.10-0.22 μm, more preferably 0.22 μm. This filtration range can achieve a good sterilization effect. After ultrasonic treatment and filtration, the obtained vaccine adjuvant has good safety, and is more uniform and stable.

[0060] The present application provides an optional embodiment, and specifically, a

preparation method and use of a water adjuvant includes the following steps:

[0061] T1: dissolving phospholipids and cholesterol in 100 mL of chloroform, and mixing to obtain a solution A after complete dissolution;

[0062] T2: dissolving tocopherol in 100 mL of 95% anhydrous ethanol, and obtaining a solution B after complete dissolution;

[0063] T3: dissolving pine pollen and saponins in 800 mL of purified water, shaking and mixing uniformly, standing still, filtering, and collecting the supernatant to obtain a solution C;

[0064] T4: uniformly shaking and mixing the solution A obtained in step T1, the solution B obtained in step T2, and the solution C obtained in step T3 to obtain a mixed solution D;

[0065] T5: transferring the mixed solution D obtained in step T4 to a rotary evaporator, performing rotary evaporation to remove organic solvents, and forming a solid substance on the inner wall of the flask of the rotary evaporator;

[0066] T6: dissolving the solid substance obtained in step T5 with 1 L of PBS solution to obtain a first mixed solution;

[0067] T7: performing ultrasonic treatment on the first mixed solution obtained in step T6 to obtain a second mixed solution;

[0068] T8: filtering the second mixed solution obtained in step T7 with a 0.22 μm filter membrane to obtain a third mixed solution; and

[0069] T9: diluting the third mixed solution obtained in step T8 100 times with PBS solution to obtain the water adjuvant, and using for preparing veterinary vaccines.

[0070] In a preferred example, the preparation of the PBS solution in steps T6 and T9 is as follows: dissolving 8 g of NaCl, 0.2 g of KCl, 2.68 g of $Na_2HPO_4 \cdot 7H_2O$, and 0.24 g of KH_2PO_4 in 800 mL of water for injection; adjusting the pH value to 7.4 with HCl solution; adding water for injection to make up to 1 L, and sterilizing by filtration through a 0.22 μm filter membrane or autoclaving for later use.

[0071] In a preferred example, the third mixed solution in step T8 is a 100-fold

concentrated water adjuvant. The concentrated solution is convenient for subsequent ultrasonic treatment and storage for later use, and can be diluted 100 times with PBS solution before use.

[0072] In a preferred example, the ultrasonic treatment in step T7 uniformly disperses the particles in the solution to form a liposome water adjuvant with uniform stability and a small particle size.

[0073] Compared with the prior art, the vaccine adjuvant prepared by the present application has the following beneficial effects: forming a uniform and stable phospholipid bilayer water adjuvant with small particle size, easy absorption, good thermal stability, and dissolution activity. The prepared vaccine has low viscosity, good needle penetration, high uniformity, and strong stability, and is easily soluble or dispersible in water. The bilayer formed by the prepared water adjuvant is non-toxic and can reduce antigen toxicity. At the same time, various high-purity active ingredients such as egg yolk lecithin, saponins, pine pollen, and α-tocopherol can stimulate the body's immune defense system, comprehensively enhance the body's humoral and cellular immune responses, and are safe, effective, and reliable to use. It can bind to a variety of antigens, assist in the transport of antigens, and induce strong antibody titers and T-cell responses.

[0074] Based on the water adjuvant provided by the above technical solution, high-purity egg yolk lecithin, saponins, pine pollen, and α-tocopherol are used in its raw materials, so that the vaccine products prepared by using the water adjuvant provided by the present application are uniform, stable in quality, and high in safety. The adjuvant can enhance the humoral immune response, induce a longer immune duration (for example, the immune protection period of the bovine epizootic fever inactivated vaccine (ZJ strain) prepared by using it in calves is similar to that of the ISA206 adjuvant, which can be as long as more than 150 days) and more efficient (for example, the antibody titer of the bovine epizootic fever inactivated vaccine (ZJ strain) prepared by using it in calves can reach more than 1:5079). Therefore, the water adjuvant provided by the present application can help develop veterinary vaccines with strong stability, good needle penetration, and higher safety, and the immune effect is not lower than that of vaccines prepared with commercial oil adjuvants ISA206.

[0075] The present application provides a use of the vaccine adjuvant, or the vaccine adjuvant prepared by the preparation method in the preparation of vaccines.

[0076] The present application further provides a vaccine, the vaccine includes an inactivated antigen, and further includes the vaccine adjuvant, or the vaccine adjuvant prepared by the preparation method.

[0077] Preferably, the inactivated antigen includes one or more of bovine epizootic fever inactivated antigen, porcine Seneca Valley virus inactivated antigen, porcine pseudorabies inactivated antigen, porcine foot-and-mouth disease type O inactivated antigen, porcine foot-and-mouth disease type A inactivated antigen, bovine rotavirus inactivated antigen, and bovine parainfluenza inactivated antigen.

[0078] The technical solutions provided by the present application are described in detail below with reference to examples. The methods used in the following examples are conventional methods unless otherwise specified. The materials, reagents, etc., used can be obtained from commercial channels. The acquisition channels of various biological materials described in the examples are only to provide an experimental acquisition channel for the purpose of specific disclosure, and should not be considered as limiting the sources of biological materials of the present application. In fact, the sources of the biological materials used are extensive, and any biological materials that can be obtained without violating laws and ethical ethics can be used as a substitute according to the prompts in the examples.

[0079] The examples are implemented on the premise of the technical solution of the present application, and detailed implementation modes and specific operation processes are given. The examples will help understand the present application, but should not be considered as limiting the content of the present application.

[0080] Example 1

[0081] In the Example 1, a water adjuvant was prepared as a vaccine adjuvant, and the vaccine adjuvant was mixed with the whole virus inactivated antigen of bovine epizootic fever (ZJ strain) at a mass ratio of 1:1 to prepare a bovine epizootic fever inactivated vaccine, specifically including:

[0082] (1) a titer of the virus liquid for vaccine preparation was $10^{7.5}TCID_{50}$/ml, which was prepared and preserved by Jinyu Baoling Biotechnology Co., Ltd. The specific preparation method was as follows:

[0083] T1: taking 1-2 vials of working seed cells BHK-21, reviving 1-2 generations in adherent cultured, transferring the cells to a shaker flask for suspension culture after growing to form a monolayer, a cell concentration reaching more than 2×10^6 cells/ml, performing stepwise expansion culture of suspension cells, and observing regularly cells;

[0084] T2: inoculating the secondary seed of bovine epizootic fever (ZJ strain) cell virus into the suspension-cultured BHK-21 cells at an MOI value (PFU: cell number = 1:10-1:100) when the number of suspension cells reached 2×10^6 cells/ml, setting culture parameters (pH value, DO value, and stirring speed), and performing suspension culture at 36-37°C, sampling after the dissolved oxygen dropped to zero, observing under a microscope, and harvesting culture when the cell viability was less than 5%;

[0085] T3: determining the $TCID_{50}$ of the bovine epizootic fever (ZJ strain) harvested in step T2 on BHK-21 cells, with a virus content of the virus liquid of ≥$107.5TCID_{50}$/ml;

[0086] T4: adding the inactivator BEI to the virus solution of bovine epizootic fever (ZJ strain) harvested in step T3 in proportion (a final concentration of BEA was 0.003 mol/L) after cyclization, heating to 26°C after fully stirring, starting timing, inactivating for 24 hours, stirring every 200 minutes during the period, adding 50% sodium thiosulfate solution filter-sterilized to the inactivated virus solution after the timing was completed, making a final concentration of sodium thiosulfate 2%, mixing fully, and sampling for inactivation inspection at the same time; and

[0087] T5: performing inactivation inspection on the inactivated virus solution harvested in step T4, using as the virus solution for seedling preparation and storing for later use after passing the inspection; and

[0088] (2) The preparation method for the vaccine adjuvant includes the following steps:

[0089] T1: dissolving 15 g of egg yolk lecithin and 20 g of cholesterol in 100 ml of chloroform and mixing, and fully dissolving to obtain a solution A;

[0090] T2: dissolving 10 g of tocopherol in 100 ml of 95% anhydrous ethanol, and fully dissolving to obtain a solution B;

[0091] T3: dissolving 40 g of pine pollen and 15 g of saponins in 800 ml of purified water, performing shaking and mixed uniformly, standing still, and filtering, and collecting a supernatant to obtain a solution C;

[0092] T4: uniformly shaking and mixing the solution A obtained in step T1, the solution B obtained in step T2, and the solution C obtained in step T3 to obtain a mixed solution D;

[0093] T5: transferring the mixed solution D obtained in step T4 to a rotary evaporator, performing rotary evaporation to remove organic solvents, and forming a solid substance on an inner wall of the flask of the rotary evaporator;

[0094] T6: dissolving the solid substance obtained in step T5 with 1 L of PBS solution to obtain a first mixed solution;

[0095] T7: performing ultrasonic treatment on the first mixed solution obtained in step T6 to obtain a second mixed solution;

[0096] T8: filtering the second mixed solution obtained in step T7 with a 0.22 μm filter membrane to obtain a third mixed solution, namely a 100-fold concentrated water adjuvant; and

[0097] T9: diluting a third mixed solution obtained in step T8 100 times with PBS solution to obtain the water adjuvant, and using for preparing veterinary vaccines.

[0098] A rotary evaporation temperature in step T5 was 40-50°C, and the rotation speed was 100-200 r/min.

[0099] The conditions of the ultrasonic treatment in step T7 included: temperature of 20-30°C, power of 50-70 W, total ultrasonic treatment time of 20-30 min, and ultrasonic mode: ultrasonication for 10 s, pause for 10 s.

[00100] The preparation of the PBS solution in steps T6 and T9 was as follows: dissolving 8 g of NaCl, 0.2 g of KCl, 2.68 g of $Na_2HPO_4 \cdot 7H2O$, and 0.24 g of KH_2PO_4 in 800 mL of water for injection; adjusting a pH value to 7.4 with HCl solution; adding water

for injection to make up to 1 L, and sterilizing by filtration through a 0.22 μm filter membrane or autoclaving for later use.

[00101] (3) The preparation method for the bovine epizootic fever inactivated vaccine (ZJ strain) included the following steps:

[00102] mixing the vaccine adjuvant with the bovine epizootic fever inactivated antigen (ZJ strain) at a mass ratio of 1:1, and emulsifying at 25-30°C and 500 rpm/min for 10 minutes to prepare the bovine epizootic fever inactivated vaccine.

[00103] The particle size distribution of the bovine epizootic fever inactivated vaccine obtained in Example 1 is shown in FIG. 1 (the smaller the particle size, the higher the safety and the better the absorbability). As shown in FIG. 1, a particle size range of the bovine epizootic fever inactivated vaccine obtained in this Example 1 was about 0.086-0.187 μm, and an average particle size was 0.102 μm. The vaccine had a small particle size, and significant advantages were good absorption, painless injection, no side effects, high safety, and strong stability after long-term storage.

[00104] Example 2

[00105] In the Example 2, the vaccine adjuvant was prepared according to the operation steps of Example 1, and the bovine epizootic fever inactivated vaccine was prepared using the bovine epizootic fever inactivated antigen with the same virus content and the adjuvant prepared in Example 2. The only difference was that the contents of each component in the raw materials for preparing the vaccine adjuvant were different.

[00106] The raw materials for preparing the vaccine adjuvant in Example 2 were as follows:

[00107] the dosages of phospholipids and cholesterol were 20 g and 12 g respectively;

[00108] the dosage of tocopherol was 8 g; and

[00109] the dosages of pine pollen and saponins were 30 g and 30 g respectively.

[00110] Example 3

[00111] In the Example 3, the vaccine adjuvant was prepared according to the operation

steps of Example 1, and the bovine epizootic fever inactivated vaccine was prepared by using the bovine epizootic fever inactivated antigen with the same virus content and the adjuvant prepared in Example 3. The only difference was that the contents of each component in the raw materials for preparing the vaccine adjuvant were different.

[00112] The raw materials for preparing the vaccine adjuvant in Example 3 were as follows:

[00113] the dosages of phospholipids and cholesterol were 20 g and 20 g respectively;

[00114] the dosage of tocopherol was 5 g; and

[00115] the dosages of pine pollen and saponins were 30 g and 25 g respectively.

[00116] Comparative Example 1

[00117] The difference between Comparative Example 1 and Example 1 was:

[00118] in the Comparative Example 1, SEPPIC ISA206 vaccine adjuvant (purchased from SEPPIC Company, batch number 200528023520) was mixed with bovine epizootic fever inactivated antigen (ZJ strain) at a mass ratio of 1:1, emulsified at 25-30°C and 500 rpm/min for 15 minutes to prepare the bovine epizootic fever inactivated vaccine.

[00119] A particle size distribution of the bovine epizootic fever inactivated vaccine obtained in this Comparative Example 1 was shown in FIG. 2. It can be seen from FIG. 2 that a particle size range of the bovine epizootic fever inactivated vaccine obtained in this Comparative Example 1 was about 0.0977-0.728 μm, and an average particle size was 0.224 μm. A particle size of the vaccine was larger than that in Example 1, and slight fever and other reactions may occur after injection.

[00120] Comparative Example 2

[00121] The difference between Comparative Example 2 and Example 1 was:

[00122] in the Comparative Example 2, SEPPIC IMS1313VG vaccine adjuvant (purchased from SEPPIC Company, batch number 210126014994) was mixed with bovine epizootic fever inactivated antigen (ZJ strain) at a mass ratio of 1:4, where 1 parts of the 4 parts of the aqueous phase was bovine epizootic fever inactivated antigen (ZJ

strain), and 3 parts were PBS solution. After mixing, the mixture was added as the aqueous phase to the adjuvant, and emulsified at 25-30°C and 500 rpm/min for 15 minutes to prepare the bovine epizootic fever inactivated vaccine.

[00123] The amount of bovine epizootic fever virus contained in Comparative Example 2 was the same as that in Examples 1-3 and Comparative Example 1. The particle size distribution of the bovine epizootic fever inactivated vaccine obtained in the Comparative Example 2 was shown in FIG. 3. It can be seen from FIG. 3 that the particle size range of the bovine epizootic fever inactivated vaccine obtained in this Comparative Example 2 was about 0.091-0.657 μm, and an average particle size was 0.184 μm. A particle size of the vaccine was larger than that in Example 1, but the average particle size was not much different, which was beneficial to injection, good absorption, no side effects, high safety, and strong stability after long-term storage.

[00124] Example 4

[00125] (1) Inspection of Physical and Chemical Properties of Vaccines

[00126] The physical and chemical properties of the vaccines prepared in Examples 1-3 and Comparative Examples 1-2 were inspected, including observing the appearance and dosage form of the vaccines, detecting the pH value of the vaccines, and inspecting the viscosity, stability, and particle size distribution of the vaccines by the following methods.

[00127] Viscosity inspection: 1 mL from each of the bovine epizootic fever inactivated vaccines (ZJ strain) prepared in Examples 1-3 and Comparative Examples 1-2 was aspirated and slowly added into sample cups. The vaccine viscosity was measured with a viscometer under the conditions of torque 40%-60% and rotor speed 6-16 r/min.

[00128] Particle size distribution inspection: 1 mL from each of the bovine epizootic fever inactivated vaccines (ZJ strain) prepared in Examples 1-3 and Comparative Examples 1-2 was aspirated, the scattered light intensity was detected with a Beckman particle size analyzer, and the particle size distribution was calculated.

[00129] Stability inspection: the bovine epizootic fever inactivated vaccines (ZJ strain) prepared in Examples 1-3 and Comparative Examples 1-2 were treated under the

conditions of 8000 rpm and 15 min centrifugation.

[00130] The inspection results of the physical and chemical properties of the vaccines are shown in Table 1 below. As shown in Table 1, except that the appearance of the vaccine in Comparative Example 1 was a milky white slightly viscous emulsion and a dosage form was W/O/W type, the appearances of the vaccines in Examples 1-3 and Comparative Example 2 were all translucent liquids, and the pH values were all in the range of 7-8. Compared with the vaccine in Comparative Example 1, the viscosities of the bovine epizootic fever inactivated vaccines (ZJ strain) prepared in Examples 1-3 were all lower, which was beneficial to the injection of the vaccine and reduced stress reactions; and the vaccines in Examples 1-3 had no precipitation after centrifugation, were uniform and translucent, showing the advantages of high stability and easy injection, which was beneficial to the long-term storage of the vaccine and could provide convenience for the needle-free injection of the vaccine.

[00131] Table 1: Inspection Results of Physical and Chemical Properties of Vaccines

[00132]

Inspection item	Appearance	Dosage form	Viscosity (cp)	8,000 rpm, centrifugation for 15 min	Particle size distribution	pH value
Example 1	Translucent liquid	-	3.01	No change	Single peak	7.86
Example 2	Translucent liquid	-	2.55	No change	Single peak	7.88
Example 3	Translucent liquid	-	2.67	No change	Single peak	7.86
Comparative Example 1	Milky white slightly viscous emulsion	W/O/W	28.77	No aqueous phase	Single peak	7.82
Comparative Example 2	Translucent liquid	-	5.66	No change	Single peak	7.82

[00133] The appearances of the bovine epizootic fever inactivated vaccines obtained in Example 1 and Comparative Examples 1-2 are shown in FIG. 4.

[00134] (2) Safety Inspection of Vaccines

[00135] The safety of the vaccines prepared in Examples 1-3 and Comparative Examples 1-2 was inspected: 60 clean-grade mice (purchased from the Animal Experiment Department of Inner Mongolia University, Hohhot) with a weight of 18-22 g were selected, 5 mice in each group, and randomly divided into 12 groups. Mice were immunized subcutaneously with the vaccine of Example 1 at a single point on the abdomen, 1 ml/mouse, and 5 mice were immunized; mice were intraperitoneally immunized with the vaccine of Example 1 at a single point, 0.5 ml/mouse, 5 mice were immunized; and the vaccines of Examples 2-3 and Comparative Examples 1-2 were immunized and grouped in the same way and with the same immunization dosage, and a PBS control group was set, and mice were immunized with the same immunization dosage and way. Each group was observed continuously for 14 days after immunization. The results are shown in Table 2 below.

[00136] Table 2: Results of Immunizing Mice with Vaccines of Each Group

[00137]

Group	Number of mice	Subcutaneous immunization		Results of subcutaneous immunization	Intraperitoneal immunization		Results of intraperitoneal immunization
		Number of mice (count)	Immunization dosage (mL)		Number of mice (count)	Immunization dosage (mL)	
Example 1	10	5	1	5/5 healthy and alive, no adverse reactions	5	0.5	5/5 healthy and alive, no adverse reactions
Example 2	10	5	1	5/5 healthy and alive, no adverse reactions	5	0.5	5/5 healthy and alive, no adverse reactions

Example 3	10	5	1	5/5 healthy and alive, no adverse reactions	5	0.5	5/5 healthy and alive, no adverse reactions
Comparative Example 1	10	5	1	5/5 healthy and alive, no adverse reactions	5	0.5	5/5 healthy and alive, no adverse reactions
Comparative Example 2	10	5	1	5/5 healthy and alive, no adverse reactions	5	0.5	5/5 healthy and alive, no adverse reactions
PBS control group	10	5	1	5/5 healthy and alive, no adverse reactions	5	0.5	5/5 healthy and alive, no adverse reactions

[00138] It can be seen from Table 2 that under the condition of the same antigen content ($10^{7.5}TCID_{50}$/ml), the mice of same weight were immunized with the vaccines in each group, the PBS control group had a 100% survival rate and no adverse reactions after immunization. Within 14 days after immunization with the bovine epizootic fever inactivated vaccines of Examples 1-3 and Comparative Examples 1-2, no local or systemic adverse reactions caused by the vaccines were observed, and the injection site was normal. The bovine epizootic fever inactivated vaccine provided by the present application had good safety.

[00139] (3) Immunogenicity Inspection

[00140] The immunogenicity of the vaccines prepared in Examples 1-3 and Comparative Examples 1-2 was inspected: 5 healthy susceptible cattle aged 5-6 months with negative bovine epizootic fever (ZJ strain) antigen and antibody (serum neutralizing antibody titer not higher than 1:2) were selected in each group, 2.0 ml of the bovine epizootic fever (ZJ strain) inactivated vaccines prepared in the above Examples 1-3 and Comparative Examples 1-2 were intramuscularly injected into each cattle, and 3 susceptible cattle aged 5-6 months with negative antibody were selected as a blank control group without

vaccination. A booster immunization (i.e., second immunization) was performed in the same vaccination way and with the same immunization dosage 21 days after the first immunization, and the blank control group was not vaccinated. Venous blood was collected from cattle on the 21st day after the first immunization, the 28th day, 90th day, 150th day, and 180th day after the second immunization, serum was separated, and serum neutralizing antibody was detected by a cell neutralization test method. At least 4 of the 5 vaccinated cattle had neutralizing antibodies ≥1:2^6. The body temperature of the test cattle in each test group and the blank control group was monitored in the morning and afternoon on the 3rd day before injection, the 1st day, 2nd day and 3rd day after injection, and up to the 14th day after injection. The feeding activity of the test cattle in each group and whether there were swelling, nodules, ulceration, etc. at an injection site were observed and recorded. The determination results of the antibody titer of the test cattle in each group are shown in Table 3 below; and the average body temperature monitoring results of the test cattle in each group in the morning and afternoon are shown in Table 4 below.

[00141] Table 3: Determination Results of Neutralizing Antibody Titer of Test Cattle in Each Group (log_2)

[00142]

Group	Cattle number	Before immunization	21st day after first immunization	28th day after second immunization	90th day after second immunization	150th day after second immunization	180th day after second immunization
Example 1	1	< 1:2	8	11	12.5	11	9
	2	< 1:2	8.5	12	12.5	11.5	8.5
	3	< 1:2	8.5	12	12	11	9
	4	< 1:2	8	12	12	11	8.5
	5	< 1:2	8.5	12	12	11	9
Example 2	6	< 1:2	8.5	11	12.5	11.5	8.5
	7	< 1:2	8	11	12	11.5	8.5

	8	< 1:2	8.5	12	12	10.5	9
	9	< 1:2	8.5	12	12	11.5	9
	10	< 1:2	8	11	12.5	10	9
Example 3	11	< 1:2	8	12	12	11	9
	12	< 1:2	8	12.5	13	11.5	9
	13	< 1:2	9	12	12	11	9
	14	< 1:2	9	11	13	12	8
	15	< 1:2	9	11	12	11.5	9
Comparative Example 1	16	< 1:2	8	10	11	10	9
	17	< 1:2	9	11	12.5	11	7.5
	18	< 1:2	8.5	10	12	11	8
	19	< 1:2	7.5	10	11	12	8
	20	< 1:2	8	11	12	11	8
Comparative Example 2	21	< 1:2	7.5	9	10	9	7
	22	< 1:2	7	8	9.5	7.5	5.5
	23	< 1:2	6.5	8.5	10	9	6
	24	< 1:2	7	9	10	9	6
	25	< 1:2	7.5	8.5	9.5	7	5
Control group	26	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2
	27	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2
	28	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2

[00143] Table 4: Body Temperature Monitoring Results of Test Cattle in Each Group (unit: °C)

[00144]

Vaccine batch	Cattle number	Body temperature before immunization (°C)				Body temperature after immunization (°C)													
		2 days before	1 day before	On the day	Basal body temperature	1st day	2nd days	3rd days	4th day	5th day	6th day	7th day	8th day	9th day	10th day	11th day	12th day	13th day	14th day
Example 1:	1	38.7	38.5	39.4	38.9	38.9	38.5	38.7	38.7	38.7	39.0	39.4	38.8	39.3	38.6	38.7	39.2	38.8	38.7
	2	38.9	38.7	38.7	38.8	38.9	39.0	38.5	39.3	38.5	38.9	38.7	38.5	38.7	39.3	39.4	38.6	38.5	38.5
	3	38.9	39.1	38.9	39.0	38.9	38.8	39.3	38.7	38.7	39.0	38.7	38.9	38.8	39.3	38.5	38.9	39.3	39.2
	4	38.7	39.0	38.9	38.9	38.8	39.1	39.2	38.8	38.6	39.0	39.2	38.7	39.2	39.4	39.3	39.2	39.1	38.5
	5	38.7	38.6	38.7	38.7	39.0	39.3	39.1	38.8	39.1	38.8	39.4	39.3	38.7	39.1	38.9	39.1	39.4	38.8
Example 2:	6	38.7	38.5	38.8	38.7	38.6	38.7	39.0	38.6	38.9	38.9	39.3	38.8	39.4	38.6	39.3	39.1	38.7	38.7
	7	38.8	38.7	38.6	38.7	38.6	39.4	38.9	38.7	39.0	38.5	38.9	38.8	39.4	38.8	39.1	39.3	38.8	39.2
	8	39.4	39.4	38.9	39.2	39.0	39.2	39.0	38.6	38.8	39.1	38.7	38.6	39.1	38.6	39.4	39.3	38.6	38.8
	9	39.1	38.8	39.0	39.0	38.5	39.3	39.3	39.0	38.7	38.9	39.2	38.6	39.2	38.9	38.8	39.0	38.8	39.4
	10	38.7	38.7	39.0	38.8	38.6	39.0	39.0	39.1	38.6	39.4	38.9	38.6	39.3	38.5	39.4	38.7	38.6	38.5
Example 3:	11	39.2	39.2	39.4	39.3	39.0	39.0	38.7	39.1	39.4	39.0	38.9	38.8	38.8	39.1	38.6	39.0	38.8	38.7
	12	38.5	39.4	38.7	38.9	38.6	38.8	39.1	38.9	39.3	39.0	38.5	39.1	39.2	38.9	39.1	38.6	38.5	39.0
	13	39.1	38.9	38.9	39.0	38.8	38.6	38.8	38.8	38.8	39.4	39.2	39.3	39.0	38.9	39.3	39.2	39.1	39.4
	14	38.5	38.6	38.7	38.6	38.8	38.5	38.6	38.8	38.8	38.8	38.9	39.1	38.9	39.1	39.3	38.5	38.5	38.6
	15	38.6	38.6	38.5	38.6	38.5	38.7	38.8	38.7	38.5	39.3	38.6	38.5	38.5	39.3	39.4	38.9	38.9	38.6
Comparative Example 1	16	38.5	38.6	38.5	38.5	40.0	39.3	38.5	39.4	39.1	39.1	39.4	39.4	38.6	38.8	39.3	38.9	39.0	38.5
	17	38.7	38.6	39.2	38.8	40.3	39.5	38.6	39.2	39.0	39.0	38.9	38.9	39.4	38.6	38.6	38.7	39.0	39.4
	18	39.2	39.2	38.7	39.0	40.1	39.8	39.4	38.8	38.7	38.5	38.8	39.0	39.3	39.1	38.8	39.1	38.7	38.7
	19	39.0	38.8	38.6	38.8	39.8	39.6	38.8	38.6	39.1	39.2	39.0	38.7	39.4	39.2	38.9	38.6	38.9	38.5
	20	39.1	38.5	39.3	39.0	39.9	39.2	38.6	39.0	39.3	39.1	39.2	38.9	38.7	39.4	39.4	38.5	39.3	39.1
Comparative Example 2	21	38.5	38.6	38.6	38.6	38.6	38.7	39.0	39.4	38.7	39.3	39.0	38.7	38.6	38.8	38.9	38.9	39.1	39.1
	22	39.1	38.8	39.3	39.1	38.9	38.6	38.6	38.7	39.1	38.5	39.2	39.2	39.2	39.1	39.2	39.4	39.4	39.2
	23	38.5	39.4	38.8	38.9	39.3	38.6	38.9	38.9	38.8	38.9	39.1	38.5	39.0	38.7	38.5	39.4	39.1	39.0
	24	38.7	38.5	38.9	38.7	39.2	38.9	39.0	38.5	39.1	39.0	38.5	38.9	39.4	38.8	38.9	39.0	38.8	39.2
	25	39.3	39.2	39.2	39.2	38.7	39.1	39.4	38.8	39.2	39.3	39.2	38.8	39.1	39.1	38.6	38.9	38.7	38.5
Blank control group	26	38.6	38.5	38.9	38.7	38.8	38.9	39.2	38.9	39.1	39.1	38.6	38.6	38.6	38.9	39.4	38.7	39.4	38.7
	27	38.8	39.3	38.8	39.0	38.9	39.0	38.9	39.3	39.4	38.6	39.1	39.3	39.1	39.3	39.4	38.9	38.5	38.7
	28	39.4	39.3	39.0	39.2	39.1	38.9	38.6	39.2	38.6	39.3	39.4	38.9	38.9	38.6	39.0	38.6	39.2	38.8
	29	39.4	39.3	38.5	39.1	39.4	39.2	38.7	39.3	39.0	39.4	38.8	38.8	38.7	38.7	39.2	38.5	38.9	38.5
	30	39.4	39.3	38.8	39.2	39.0	39.1	38.9	39.4	38.5	39.1	39.1	38.7	38.8	39.2	38.6	38.9	38.8	38.8

[00145] As can be seen from the results recorded in Table 3 above, under the condition of the same antigen content ($10^{7.5}TCID_{50}$/ml), the antibody dynamics of the 5 immunized groups were similar. Compared with Comparative Example 1, the vaccines of Examples

1-3 had similar immune effects with no significant difference ($P > 0.05$); compared with Comparative Example 2, the vaccines of Examples 1-3 showed a faster increase in antibody titer, and the antibody values at each time point after immunization were higher than those of Comparative Example 2 with significant differences ($P < 0.05$, $P < 0.001$). On the 21st day after the first immunization, the vaccines of Examples 1-3 and Comparative Example 1 all met the efficacy requirement (≥$1:2^8$), and the vaccine of Comparative Example 2 also met the efficacy requirement but with lower antibody values, ranging from $1:2^{6.5}$ to $1:2^{7.5}$. After the second immunization, the antibodies of each immunized group showed a rapid upward trend, reaching the peak on the 90th day after the second immunization. The neutralizing antibody titers of Examples 1-3 were similar to those of Comparative Example 1, with the highest antibody titer of $1:2^{12.5}$ for Examples 1-3 and $1:2^{10}$ for Comparative Example 2. On the 150th day after the second immunization, the antibodies of each immunized group began to decrease slowly, but the neutralizing antibody titers and the rate of decline showed differences. The vaccines of Examples 1-3 still maintained a high antibody level of more than $1:2^{10}$, the antibody value of the vaccine of Comparative Example 1 was more than $1:2^{10}$, and the highest antibody value of the vaccine of Comparative Example 2 was $1:2^9$. On the 180th day after the second immunization, the antibody level of the vaccines of Examples 1-3 ranged from $1:2^8$ to $1:2^9$, that of Comparative Example 1 ranged from $1:2^{7.5}$ to $1:2^9$, and that of Comparative Example 2 ranged from $1:2^{5.5}$ to $1:2^7$. The vaccines of Examples 1-3 exhibited the advantages of long immune duration, good uniformity, and small individual differences (indicating stable vaccine quality); moreover, the antibody levels detected at the same time points were significantly higher than those induced by the vaccine of Comparative Example 2 (each increased by more than 1 titer). Comparative Example 2 was a vaccine prepared using Seppic IMS1313VG immunoadjuvant, which showed a slow increase in antibody titer and induced low antibody levels. The highest efficacy level after 21 days of the first immunization was $1:2^{7.5}$, and the antibody levels of some immunized cattle could no longer meet the efficacy requirement (≥$1:2^6$) on the 180th day after the second immunization, with a short immune duration. The curves of the neutralizing antibody levels induced by the vaccines prepared in Examples 1-3 and Comparative Examples 1-2 over

time and the results of significance difference analysis are shown in FIG. 5. The results proved that the water adjuvant vaccines of Examples 1-3 had similar immune effects to the oil adjuvant vaccine of Comparative Example 1 but with smaller individual differences. Their neutralizing antibody values and immune duration were significantly higher than those of the vaccine prepared in Comparative Example 2. The vaccine products prepared using multiple immunostimulants in Examples 1-3 of the present application could enhance the specific immune response level of antigens in the vaccine, and had long immune duration and good uniformity, thus being able to replace oil adjuvant vaccines.

[00146] As can be seen from the results recorded in Table 4 above, the body temperature of the calves immunized with the bovine epizootic fever inactivated vaccines of Examples 1-3 and Comparative Example 2 basically returned to normal after immunization, with no significant increase, and no local or systemic adverse reactions caused by the vaccines were observed, and the injection site was normal. The body temperature of the calves immunized with the bovine epizootic fever inactivated vaccine of Comparative Example 1 rose to 40.3°C on the first day after immunization and returned to normal on the second day, with no local or systemic adverse reactions caused by the vaccines and a normal injection site. Therefore, the bovine epizootic fever inactivated vaccine provided by the present application has good safety.

[00147] Comparative Example 3

[00148] In Comparative Example 3, the vaccine adjuvant was prepared according to the operation steps of Example 1, and the bovine epizootic fever inactivated vaccine was prepared using the bovine epizootic fever inactivated antigen with the same virus content and the adjuvant prepared in Comparative Example 3. The only difference was that the components in the raw materials for preparing the vaccine adjuvant were different.

[00149] The raw materials for preparing the vaccine adjuvant in Comparative Example 3 were as follows:

[00150] the dosages of phospholipids and cholesterol were 20 g and 20 g respectively;

[00151] the dosage of tocopherol was 5 g; and

[00152] the dosage of saponins was 25 g.

[00153] Comparative Example 4

[00154] In Comparative Example 4, 30 g of pine pollen was weighed and dissolved in 1 L of PBS solution, vortexed and mixed uniformly, filtered with a 0.22 μm filter membrane to obtain a mixed solution, namely a 100-fold concentrated water adjuvant. The mixed solution was diluted 100 times with PBS solution to obtain the water adjuvant of Comparative Example 4, which was used for preparing veterinary vaccines.

[00155] Preparation of PBS solution was as follows: dissolving 8 g of NaCl, 0.2 g of KCl, 2.68 g of $Na_2HPO_4 \cdot 7H_2O$, and 0.24 g of KH_2PO_4 in 800 ml of water for injection, adjusting the pH value to 7.4 with HCl solution, adding water for injection to make up to 1 L, and sterilizing by filtration through a 0.22 μm filter membrane or autoclaving for later use.

[00156] Example 5

[00157] (1) Immunogenicity Test

[00158] The immunogenicity of the vaccines prepared in Example 3 and Comparative Examples 3-4 was tested: 5 healthy susceptible cattle aged 5-6 months with negative bovine epizootic fever (ZJ strain) antigen and antibody (serum neutralizing antibody titer not higher than 1:2) were selected in each group, and 2.0 ml of the bovine epizootic fever (ZJ strain) inactivated vaccines prepared in Example 2 and Comparative Examples 3-4 above were intramuscularly injected into each cattle. In addition, 3 susceptible cattle aged 5-6 months with negative antibody were selected as a blank control group without vaccination. A booster immunization (i.e., the second immunization) was performed in the same vaccination way and with the same immunization dosage 21 days after the first immunization, and the blank control group was not vaccinated. Venous blood was collected from cattle on the 21st day after the first immunization, the 28th day, 90th day, 150th day, and 180th day after the second immunization, serum was separated, and serum neutralizing antibody was detected by the cell neutralization test method. At least 4 of the 5 vaccinated cattle should have a neutralizing antibody $\geq 1:2^6$. The antibody titer determination results of the test cattle in each group are shown in Table 5 below.

[00159] Table 5: Determination results of neutralizing antibody titer of test cattle in each group (log_2)

[00160]

Group	Cattle number	Before immunization	21st day after first immunization	28th day after second immunization	90th day after second immunization	150th day after second immunization	180th day after second immunization
Example 3	20	< 1:2	8	11.5	12.5	12	9
	21	< 1:2	8	11	12	10	8
	22	< 1:2	8.5	11.5	12.5	11.5	9.5
	23	< 1:2	8	12	12	11	9
	24	< 1:2	8	11	12	11	8.5
Comparative Example 3	25	< 1:2	7	8	7.5	5.5	4
	26	< 1:2	7.5	9	8	6.5	4.5
	27	< 1:2	7	8	8.5	6.5	5.5
	28	< 1:2	6.5	7.5	8	6	4
	29	< 1:2	7.5	8	7.5	5.5	4
Comparative Example 4	30	< 1:2	6.5	8	7.5	6	4.5
	31	< 1:2	6	7.5	7.5	6	4
	32	< 1:2	6.5	8	7.5	6	4.5
	33	< 1:2	6.5	7.5	7	6	4

	34	< 1:2	6	7.5	7	5.5	4
Control Group	35	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2
	36	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2
	37	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2	< 1:2

[00161] As can be seen from the results recorded in Table 5 above, for the bovine epizootic fever inactivated vaccines prepared with the same antigen content ($10^{7.5}TCID_{50}$/ml), the difference between Comparative Example 3 and Example 3 was that there was no pine pollen in the adjuvant components, and the difference between Comparative Example 4 and Example 3 was that there was only pine pollen in the adjuvant components. The vaccines in the control group were negative in all immunization periods. Example 3 showed a high neutralizing antibody level in all periods of the first and second immunizations, which was significantly higher than that of Comparative Examples 3 and 4 ($P < 0.001$). The antibody reached the peak of $1:2^{12}$ on the 90th day after the second immunization, and the antibody value showed a slight downward trend on the 150th day after the second immunization, with the antibody value $\geq 1:2^{10}$. By the 180th day after the second immunization, the antibody was still $\geq 1:2^{8}$. The immunogenicity of Comparative Examples 3 and 4 was similar, and the antibody of the former was slightly higher than that of the latter, but the difference was not significant ($P > 0.05$). The antibody titer after the first immunization was between $1:2^{6}$ and $1:2^{7.5}$, and the antibody reached the highest on the 28th day after the second immunization. The average antibody titer of Comparative Example 3 was $1:2^{8}$, and the average antibody titer of Comparative Example 4 was $1:2^{7.7}$. Both groups showed a downward trend on the 90th day after the second immunization, and both were $\leq 1:2^{6.5}$ on the 150th day after the second immunization, and both were lower than $1:2^{6}$ on the 180th day after the second immunization. Comparative Examples 3 and 4 had the common characteristics of low antibody titer, short duration, and large individual differences. Compared with the two, Comparative Example 4 had better uniformity and smaller individual differences. The curve of the neutralizing antibody level induced by the vaccines prepared in Example 3 and Comparative Examples 3-4 with

time and the results of significant difference analysis are shown in FIG. 6.

[00162] Example 6

[00163] In Example 6, the vaccine adjuvant and the bovine epizootic fever inactivated vaccine (ZJ strain) were prepared according to the operation steps of Example 1. The only difference was that the contents of each component in the raw materials for preparing the vaccine adjuvant were different.

[00164] The raw materials for preparing the vaccine adjuvant in Example 6 were as follows:

[00165] the dosages of phospholipids and cholesterol were 10 g and 20 g respectively;

[00166] the dosage of tocopherol was 10 g; and

[00167] the dosages of pine pollen and saponins were 35 g and 25 g respectively.

[00168] Example 7

[00169] In Example 7, the vaccine adjuvant and the bovine epizootic fever inactivated vaccine (ZJ strain) were prepared according to the operation steps of Example 1. The only difference was that the contents of each component in the raw materials for preparing the vaccine adjuvant were different.

[00170] The raw materials for preparing the vaccine adjuvant in Example 7 were as follows:

[00171] the dosages of phospholipids and cholesterol were 23 g and 10 g respectively;

[00172] the dosage of tocopherol was 6 g; and

[00173] the dosages of pine pollen and saponins were 33 g and 28 g respectively.

[00174] Example 8

[00175] 3 healthy susceptible cattle aged 5-6 months with negative bovine epizootic fever (ZJ strain) antigen and antibody (serum neutralizing antibody titer not higher than 1:2) were selected in each group, and 2.0 ml of the bovine epizootic fever (ZJ strain) inactivated vaccines prepared in Examples 4-5 above were intramuscularly injected into

each cattle. In addition, 3 susceptible cattle aged 5-6 months with negative antibody were selected as a blank control group without vaccination. A booster immunization was performed in the same vaccination way and with the same immunization dosage 21 days after immunization, and the blank control group was not vaccinated. Venous blood was collected from cattle on the 21st day after the first immunization, the 28th day, 90th day, 150th day, and 180th day after the second immunization respectively, serum was separated, and serum neutralizing antibody was detected by the cell neutralization test method. At least 2 of the 3 vaccinated cattle should have a neutralizing antibody ≥1:2^6. The results are shown in Table 6 below.

[00176] Table 6: Determination Results of Neutralizing Antibody Titer of Immunized Cattle in Each Group (log_2)

[00177]

Group	Cattle number	Before immunization	21st day after first immunization	28th day after second immunization	90th day after second immunization	150th day after second immunization	180th day after second immunization
Example 6	1	<1：2	8.5	11	12	10	8.5
	2	<1：2	8.5	11.5	12.5	11	9
	3	<1：2	8	11	12	11	8.5
Example 7	4	<1：2	9	12.5	12.5	11.5	9.5
	5	<1：2	9	11	12	11	9
	6	<1：2	9.5	11.5	12.5	11.5	9
Control Group	7	<1：2	<1：2	<1：2	<1：2	<1：2	<1：2
	8	<1：2	<1：2	<1：2	<1：2	<1：2	<1：2
	9	<1：2	<1：2	<1：2	<1：2	<1：2	<1：2

[00178] As can be seen from the results recorded in Table 6 above, under the condition of the same antigen content ($10^{7.5}TCID_{50}$/ml), the bovine epizootic fever inactivated vaccines of Examples 6-7 all produced high specific neutralizing antibodies on the 21st day after the first immunization, which met the efficacy requirement (≥1:2^6). On the 28th day after the second immunization, the antibodies showed a rapid upward trend, and the

antibodies reached the peak on the 90th day after the second immunization, with an average antibody titer of $1:2^{12}$. On the 180th day after the second immunization, the antibody titer was still as high as above $1:2^{8}$, showing the advantages of good immunogenicity, long immune duration, good uniformity, and small individual differences.

[00179] The above examples only express several embodiments of the present application, to facilitate a concentrated and detailed understanding of the technical solution of the present application. However, this can be understood as a limitation on the scope of protection for the patent of the present application. It is to be pointed out that for those of ordinary skill in the art, several modifications and improvements can be made without departing from the concept of the present application, and these all belong to the protection scope of the present application.