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(57) Abstract:



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COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS

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FIELD OF INVENTION

The present invention is related to the fields of microbiology and vaccinology and more specifically concerns the development of a vaccine capable of conferring immunity to infection by *Streptococcus*.

BACKGROUND OF THE INVENTION

The bacterial genus *Streptococcus* is a of a large group of Gram-positive cocci that form long chains of cells. The genus is large and diverse with many important human and agricultural pathogens that cause a wide range of different diseases. The worldwide mortality, morbidity, and economic impacts of streptococcal infections are significant. While effective vaccines have been developed against *S. pneumoniae* (Pneumococcus), there is still great need for affordable and effective vaccines against other species of streptococci that infect humans and animals such as *S. suis* (swine and zoonotic pathogen), *S. agalactiae* (animal, human, and fish pathogen).

As with many genera, classification of the *Streptococcus* genus has undergone many revisions. There are several methods of classification for streptococci (Lancefield, hemolysis, metabolism), but the leading and most comprehensive method to measure genetic relatedness is the use of 16s rDNA sequencing, with taxonomists classifying the genus into 6 major groups containing at least 75 distinct species. Further complicating classification, and ultimately successful immunity, is genome plasticity within the genus which may allow individual strains to develop new traits that allow it to evade an immune response or become more pathogenic. Strains within a species are often referred to as a sequence type (ST) or serotype to indicate their shared genetic or serological traits, respectively.

The capsular polysaccharide (CPS) is an extracellular cellular matrix that surrounds the bacterial cell made up of repeating linear or branched oligosaccharide units connected by amino acids linked to the hydroxyl groups of the sugar rings. The CPS limits the ability of a host's immune system to engulf, destroy, and eradicate the bacterial cells. In most streptococci, the *cps* locus consists of eight genes that encode the biosynthetic pathway for CPS production. The CPS of streptococci and other pathogenic bacteria have been the target of vaccine research for decades as it is highly immunogenic, with success limited to *S. pneumoniae*. Unfortunately, the CPS is also highly variable owing to the fact that streptococci have a highly plastic genome, with high levels of genome recombination and gene switching. Recombination within the *cps* locus gives rise to many serotypes within each streptococcus species and makes developing a broadly effective vaccine against a particular species extremely difficult.

In addition to the CPS, pathogenic streptococci encode an incredibly vast array of surface-associated or secreted components, regulatory genes, or metabolic pathways that all promote the ability to colonize at host and evade the immune system. For example, there are at least 100 known or putative factors within the *S. suis* pan-genome that confer pathogenic capabilities, but because of genome plasticity and recombination, they are not homogeneously distributed within the species leading to ineffective vaccination strategies. Targeting individual traits, serogroups, or sequence types is an ineffective vaccination strategy.

The advent of high-throughput genome sequencing has revolutionized scientists' ability to classify and organize species. More importantly, it has allowed scientists to define core- and pan-genomes of bacteria; genes that are essential and found in all species of genera, and genes that are mobile and are found in certain lineages of a species, respectively. By analyzing and comparing core-genomes of streptococci it is possible to identify highly conserved essential genes that contain potentially antigenic and immunologically protective epitopes across species. These antigens have the potential to provide far greater efficacy because they train a host's immune system to target protein(s) that are essential to the growth and replication of the bacterial cell and are conserved throughout streptococci, providing for broad protection across species.

Thus, there is clearly a need of a broadly protective streptococci vaccine which is antigenic and immunogenic in domestic crossbred commercial swine and provides Nile tilapia protection from infection with *S. agalactiae*.

SUMMARY OF THE INVENTION

The instant invention includes a broadly protective streptococci vaccine which is antigenic and immunogenic.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates Enzyme-linked immunosorbent assays (ELISAs) detecting anti-Strep epitope antibodies in A) secretory IgA in saliva on study day 34, B) secretory IgA in intestinal mucosa on study day 35, C) IgG in serum on study day 34. Data is grouped by vaccination route. All data is relative to the non-treated saline control animals. Ratios greater than 1 indicate antibody levels greater than the saline control animals.

Figure 2 illustrates a Kaplan-Meier survival analysis of Nile tilapia challenged with virulence *S. agalactiae*. The IP-AV (positive control) and SV1 10% immersion and 1% oral fed groups had significantly better survival than the unvaccinated controls ($p < 0.05$).

Figure 3 illustrates the Scheme of Assessment.

Figure 4 illustrates the sIgA S/P ratio from saliva of pigs vaccinated with SV1.

Figure 5 illustrates the IgG S/P ratio from serum of pigs vaccinated with SV1.

DETAILED DESCRIPTION

The present invention now will be described more fully hereinafter in the following detailed description of the invention, in which some, but not all embodiments of the invention are described. Indeed, this invention may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements.

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well as the singular forms, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, steps, operations, elements, components, and/or groups thereof.

Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one having ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and the

present disclosure and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

In describing the invention, it will be understood that a number of techniques and steps are disclosed. Each of these has individual benefit and each can also be used in conjunction with one or more, or in some cases all, of the other disclosed techniques. Accordingly, for the sake of clarity, this description will refrain from repeating every possible combination of the individual steps in an unnecessary fashion. Nevertheless, the specification and claims should be read with the understanding that such combinations are entirely within the scope of the invention and the claims.

Streptococcus is a genus of gram-positive coccus or spherical bacteria that belongs to the family Streptococcaceae, within the order Lactobacillales (lactic acid bacteria), in the phylum Firmicutes. In addition to streptococcal pharyngitis (strep throat), certain *Streptococcus* species are responsible for many cases of pink eye, meningitis, bacterial pneumonia, endocarditis, erysipelas, and necrotizing fasciitis (the 'flesh-eating' bacterial infections). However, many streptococcal species are not pathogenic, and form part of the commensal human microbiota of the mouth, skin, intestine, and upper respiratory tract.

The instant invention identified a *Streptococcus* epitope which was part of a protective antigen. An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or

T cells. The part of an antibody that binds to the epitope is called a paratope. Although epitopes are usually non-self proteins, sequences derived from the host that can be recognized (as in the case of autoimmune diseases) are also epitopes.

The epitopes of protein antigens are divided into two categories, conformational epitopes and linear epitopes, based on their structure and interaction with the paratope. Conformational and linear epitopes interact with the paratope based on the 3-D conformation adopted by the epitope, which is determined by the surface features of the involved epitope residues and the shape or tertiary structure of other segments of the antigen.

- A conformational epitope is formed by the 3-D conformation adopted by the interaction of discontinuous amino acid residues.
- In contrast, a linear epitope is formed by the 3-D conformation adopted by the interaction of contiguous amino acid residues.
 - A linear epitope is not determined solely by the primary structure of the involved amino acids. Residues that flank such amino acid residues, as well as more distant amino acid residues of the antigen affect the ability of the primary structure residues to adopt the epitope's 3-D conformation.
 - 90% of epitopes are conformational.

A T-cell epitope is a small fragment of an antigen that is recognized by a T cell receptor (TCR). T cells are a type of white blood cell that play an important role in the adaptive immune response. They recognize and respond to foreign invaders, such as

bacteria and viruses. T-cell epitopes are typically 8 to 11 amino acids long and are derived from proteins. They are presented to T cells on the surface of antigen-presenting cells (APCs). APCs are cells that have taken up foreign antigens and processed them into peptides. The peptides are then bound to MHC molecules, which are displayed on the surface of the APC.

When a TCR binds to a T-cell epitope, it triggers a series of events that lead to the activation of the T cell. Activated T cells can then release cytokines, which help to recruit other immune cells to the site of infection. They can also kill infected cells directly. The ability of a T cell to recognize a particular T-cell epitope is determined by the structure of the TCR. Each TCR has a unique binding site that is specific for a particular set of T-cell epitopes. The binding site is formed by a combination of amino acids in the TCR molecule.

A B-cell epitope is a small part of an antigen that is recognized by a B cell receptor (BCR). B cells are a type of white blood cell that produce antibodies. Antibodies are proteins that bind to antigens and help to neutralize them. B-cell epitopes are typically 7 to 11 amino acids long and are derived from proteins. They can be linear or conformational. Linear epitopes are short sequences of amino acids that are recognized by the BCR regardless of the three-dimensional structure of the antigen. Conformational epitopes are three-dimensional structures that are recognized by the BCR. The ability of a B cell to recognize a particular B-cell epitope is determined by the structure of the BCR. Each BCR has a unique binding site that is specific for a particular

set of B-cell epitopes. The binding site is formed by a combination of amino acids in the BCR molecule.

T-cell epitopes and B-cell epitopes are each small fragments of an antigen that are recognized by T cells and B cells respectively. Each are important for understanding the immune response and for developing new vaccines and therapies. Epitope-based vaccines stimulate humoral and cellular immune responses using isolated B-cell or T-cell epitopes. These vaccines can use multiple epitopes to increase their efficacy. To find epitopes to use for the vaccine, *in silico* mapping is often used. Once candidate epitopes are found, the constructs are engineered and tested for vaccine efficiency.

A nucleotide codon optimization for *Bacillus subtilis* was utilized to derive the necessary nucleotide sequence for the DNA sequence and a recombinant synthetic DNA sequence was manufactured (Genscript Biotech Corporation - Piscataway NJ, USA). *Bacillus subtilis* is a safe, non-pathogenic bacterium that can be easily genetically modified and produced on a large scale. *Bacillus subtilis* has been used to develop vaccines against a variety of diseases, including anthrax, cholera, and tuberculosis. One advantage of using *Bacillus subtilis* as a vaccine vector is that it can be administered orally, intranasally, and parenterally. This is a major advantage over traditional vaccines, which are typically administered only by injection. Another advantage of using *Bacillus subtilis* as a vaccine vector is that it can be easily produced on a large scale. This is important for the development of vaccines against emerging

diseases, which may require the production of large quantities of vaccine in a short period of time.

The recombinant synthetic DNA sequence, complete with the homologous restriction sites, was amplified using traditional PCR with sequence specific primers resulting in an amplification product. The amplification product was purified by gel extraction techniques, concentrated, digested with BamHI and XbaI overnight, and re-purified. BamHI is a restriction enzyme isolated from the bacterium *Bacillus amyloliquefaciens*. It is a type II restriction endonuclease, which recognizes a specific sequence of DNA and cuts the DNA within that sequence. BamHI recognizes the sequence GGATCC and cuts the DNA between the G and the A. This results in two DNA fragments, each with a sticky end. Sticky ends are complementary to each other, so they can be ligated (joined) together. BamHI is a very versatile restriction enzyme as it can be used to cut DNA from a variety of sources, including bacteria, viruses, and plants. It is also easy to use and it's inexpensive, making it a cost-effective choice. XbaI is a restriction enzyme isolated from the bacterium *Xanthomonas badrii*. It is a type II restriction endonuclease, which recognizes a specific sequence of DNA and cuts the DNA within that sequence. XbaI recognizes the sequence T^ACTAGA and cuts the DNA between the T and the A. XbaI is a very versatile restriction enzyme as it can be used to cut DNA from a variety of sources, including bacteria, viruses, and plants. Like BamHI, it is also easy to use and it's inexpensive, making it a cost-effective choice.

The *Bacillus* expression plasmid pHT10 was digested with BamHI and XbaI, purified, concentrated, and treated with rSAP. The digested synthetic insert and plasmid were then mixed into a T4 DNA ligase reaction overnight at room temperature. T4 DNA ligase is an enzyme that joins together two pieces of DNA by catalyzing the formation of a phosphodiester bond between the 5'-phosphate group of one DNA strand and the 3'-hydroxyl group of the other DNA strand. This reaction is essential for DNA replication, repair, and recombination.

The T4 DNA ligase reaction takes place in the following steps:

1. The T4 DNA ligase enzyme binds to the two DNA strands to be joined.
2. The enzyme breaks the phosphodiester bonds between the 5'-phosphate group of one DNA strand and the 3'-hydroxyl group of the other DNA strand.
3. The enzyme then reforms the phosphodiester bonds between the two DNA strands, joining them together.
4. The T4 DNA ligase enzyme releases the two DNA strands, which are now joined together.

The ligation reaction was transformed into *E. coli* DH5 α (New England Biolabs - Ipswich, Massachusetts, USA) and transformants were screened for the gene insert on LB agar with ampicillin (100 μ g/ml, LB^{Amp}). The ligation reaction is a process that joins together two pieces of DNA. The resulting DNA molecule is then transformed into *E. coli* DH5 α cells. The cells are then grown on a plate containing an antibiotic that kills any

cells that do not contain the cloned gene. The cells that survive are then cultured and the cloned gene is harvested.

The new plasmid, pStrep, was purified from *E. coli* and transformed into *B. subtilis*. Transformants were selected on tryptic soy agar with chloramphenicol (5 µg/ml, TSA^{Cm}), creating the recombinant *Bacillus subtilis* strain, Strep Vaccine 1, (SV1).

A protective subunit is then produced during fermentation of SV1 at between 28 and 37 °C and induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentration ranging from 0.1 to 0.5 mM. The culture is inactivated with formalin. To produce the Test Vaccine and iterations thereof, the inactivated SV1 culture was mixed with a naturally occurring polysaccharide, methyl cellulose (encapsulation media) which acts as the vehicle and protectant for the antigen.

EXAMPLES

Example 1: Immunogenicity study of streptococcus epitope in weanling pigs:

Title: Pilot study determining the humoral immune response in three-week-old piglets after vaccination by various routes with an experimental *Streptococcus* vaccine.

Justification: *Streptococcus suis* and streptococcal-associated diseases are a significant disease and economic burden on intensive, indoor swine operations. Diseases associated with streptococcal infections include meningitis, septicemia, arthritis, polyserositis, valvular endocarditis, myocarditis, pericarditis, and abortion. *Streptococcus suis* is now the most common cause of meningitis in pigs submitted to veterinary diagnostic laboratories. The vaccines currently available fail to induce adequate cross-protection between the many serotypes of *S. suis* thus, a vaccine that induces a strong protective and cross-serotype reactive immune response is needed.

Materials and Methods: Thirty pigs at 3 weeks of age will be weaned from a high health farm with no previous *S. suis* concerns, or other disease confounders (PRRS, *Mycoplasma hyopneumoniae*, PEDv, APP, etc). The pigs will be tagged, weighed, and randomized into one of 10 groups (2 replicates x 5 groups (3 pigs/group)) and allowed to acclimate for 2 to 3 days. On Day 0 of the study, serum samples from each pig will be collected and the first dose of vaccine or saline will be administered as indicated in Table 1. The second dose is administered 14 days later as indicated in Table 1. Treatment groups are separated.

Vaccination: The vaccine will be administered in 2 doses at 2 mL per dose either through an intramuscular injection (IM) behind the ear in the neck, oral gavage, or nasal spray (1 mL/nostril). Saline administration will be by oral gavage.

The study timeline and samples to be collected are outlined in Table 2.

Table 1: Route of administration for vaccine

# animals (Reps)	Group	Dose (Study day)	IM	Oral	Nasal	Saline, Oral
3 (2)	1 & 6	1 st (D0)	X			
		2 nd (D14)	X			
3 (2)	2 & 7	1 st (D0)		X		
		2 nd (D14)		X		
3 (2)	3 & 8	1 st (D0)			X	
		2 nd (D14)			X	
3 (2)	4 & 9	1 st (D0)	X			
		2 nd (D14)		X		
3 (2)	5 & 10	1 st (D0)				X
		2 nd (D14)				X

Table 2: Study timeline and sample collection

Study Day	Activity
Day -3	Pigs are weaned, tagged, weighed, randomized, into groups and vaccinated for PCV2,
Day 0	Serum samples are collected from all pigs before 1 st treatment dose. Pigs are given their first dose of respective treatment according to Table 1. Clinical observations are noted.
Day 14	Serum samples are collected from all pigs before 2 nd treatment dose. Pigs are given their second dose of respective treatment according to Table 1. Clinical observations are noted.
Day 34	Oral fluids collection from animals - individual if possible, or per pen rope collection - Serum samples taken from each pig
Day 35	Clinical observations are noted. Pigs are weighed. Samples taken in necropsy: - Small intestine wash for mucosal sIgA

The results are illustrated in Figure 1 in which Enzyme-linked immunosorbent assays (ELISAs) detecting anti-Strep epitope antibodies in A) secretory IgA in saliva on study day 34, B) secretory IgA in intestinal mucosa on study day 35, C) IgG in serum on study day 34. Data is grouped by vaccination route. All data is relative to the non-treated saline control animals. Ratios greater than 1 indicate antibody levels greater than the saline control animals.

Example 2: Survival of Nile tilapia after vaccination with SV1 and challenge with virulent *Streptococcus agalactiae*.

Experimental Design, Sampling and Vaccination: A total of 180 fingerlings were acclimatized for two days and distributed in four treatments, all in triplicate (3 tanks/treatment, 15 fish/tank). The juveniles were distributed in 12 experimental units of 80 L, totaling 15 fish per tank. Fish were administered treatments as described (Table 1) below and grown for 54 days.

Fish were vaccinated with the SV1 via immersion (imer) and orally (oral) via coating the food pellets. Currently, there are only interparental injectable vaccines commercially available to prevent streptococcosis, thus a commercial *S. agalactiae* vaccine (AquaVac) was used as a positive control treatment (Table 3).

Table 3: Treatment groups, routes of administration and number of replicates per treatment.

Treatments	Challenged	Route of Administration	Replicates (15 fish/tank)
T1. IP-AP	Yes	AquaVac intraperitoneal (IP) injection	3
T2. Immersion 10%	Yes	2 baths (study day 0 and day 45)	3
T3. Oral 10%	Yes	Oral (fed days 30/31 and 45/46)	3
T4. Control	Yes	No treatment	3

Intraperitoneal

The application of the vaccines intraperitoneally was carried out on the 41st day of the experiment, 14 days before the infection challenge. A commercial vaccine, AQUAVAC® Strep SA, MSD, was administered according to the label instructions in the positive control group. The commercial vaccine (AV) was administered at 50 µL per fish.

Immersion

In the immersion application, the baths occurred at two distinct days of the experiment. The first bath was performed at the beginning of the experiment (day zero) and the second bath at 45 days (d 45) of experimentation. A dose of 500 mL of the SV1 in 4.5 L (10% v/v) in which the fish remained immersed for 20 minutes.

Oral

In the oral route, the SV1 was mixed directly on the feed. The fish received the feed containing the SV1 for two consecutive days, at two different times during the period. The first feeding containing SV1 was offered on days 30 and 31 and the second on

days 45 and 46 of the experiment. The inclusion of SV1 in the diet was performed 30 minutes before food management. The dose was defined as 100 μL of the vaccine per gram of feed (10% v/w).

Experimental challenge: On the 55th day of the experiment, fish of all groups were challenged with the bacterial pathogen *S. agalactiae* strain S13 (ST-552, type 1b) isolated from a streptococcosis outbreak in Nile tilapia in Brazilian state of Paraná (Facimoto et al., 2017).

A 50% lethal dose experiment was initially conducted to determine the concentration of the bacteria capable of killing 50% of the unvaccinated and challenged population (LD_{50}). For this dosage trial, 40 Nile tilapia were distributed in 8 experimental units of 30 L, with aeration and controlled temperature, totaling five animals per experimental unit. The LD_{50} was performed in duplicate, and the concentration of bacteria tested were: 1×10^5 ; 1×10^6 ; 1×10^7 ; 1×10^8 CFU mL^{-1} (data not shown). The LD_{50} of *S. agalactiae* S13 by intraperitoneal injection was determined to be $\times 10^7$ CFU mL^{-1} and this concentration was used in the subsequent vaccination and challenge period described below.

The fish were fed for 54 days until they reached the approximate weight of 50 g, a weight in which the animals are more susceptible to diseases (Mian et al., 2009). After this fattening period, each fish was challenged with 1×10^7 CFU mL^{-1} of *S. agalactiae* S13 by intraperitoneal injection. The bacteria were pelleted and washed 3x in cold,

sterile, PBS prior to injection. The fish underwent an anesthetic procedure with eugenol to perform the procedure.

Clinical signs were observed daily for 7 days post-challenge, and all dead fish were removed daily from the tanks and recorded in the trial file. Any fish that was unable to stay submerged or maintain proper orientation was humanely euthanized.

Results:

Table 4: Growth parameters (mean $\pm$ standard deviation) of Nile tilapia after 54 days of experiment. Different letters indicate significant difference among the treatments ($p < 0.05$).

	<i>Immersion 10%</i>	<i>IP-AV</i>	<i>Oral 10%</i>	<i>Control</i>
<i>Weight (g)</i>	58.62 $\pm$ 12.48ab	59.31 $\pm$ 9.78ab	62.17 $\pm$ 11.08a	43.10 $\pm$ 0.10c
<i>Length (cm)</i>	14.39 $\pm$ 1.13b	14.41 $\pm$ 0.41b	15.13 $\pm$ 0.84a	12.94 $\pm$ 0.16

Survival analysis of Nile tilapia challenged with virulent S. agalactiae:

The results are illustrated in Figure 2 which shows a Kaplan-Meier survival analysis of Nile tilapia challenged with virulent *S. agalactiae*. The IP-AV (positive control) and SV1 10% immersion and 10% oral fed groups had significantly better survival than the unvaccinated controls ($p < 0.05$).

Example 3: Determination of the dose volume, route of administration, and kinetics of the humoral immune response in pigs vaccinated with candidate Strep Vaccine 1 (SV1).

Objective

Evaluate the humoral immune response in pigs vaccinated with the candidate streptococcal vaccine formulation using different dose volumes and administration routes in order to determine the optimal vaccine presentation based on the level of specific antibodies determined by antigen-specific immunoassay tests.

Material and method**Animals and Accommodation**

“Las Chirus” is a full-cycle confined pig farm. It has 450 breeders, generating 20 deliveries per week. It is located in French, district of 9 de Julio in the province of Buenos Aires, Republic of Argentina.

In farrowing and post-weaning, work was done with an all-in-all-out system by rooms and the finishing fattening of the animals is done in a continuous flow system.

A total of 105 animals (5 animals/group) were used. They were genetic Agroceres Pig Improvement Company (PIC) females and males obtained from the crossing of females Camborough 1050 with male Terminal 337.

They were housed in rooms with a full slat floor, with a floodable pit. The density was 0.33 animals per m² at termination of fattening phase.

Ventilation by means of curtains (natural ventilation).

Food, water and usual handling of animals.**Food**

Birth-15 days of life: Colostrum and milk

15 days to weaning (21d): milk and pre-starter food

21-30 days of life: food F1 (starter)

30-40 days of life: food F2

40-55 days of life: food F3 (with Amoxicillin 400ppm in the food)

55-70 days of life: food F4 (with Tiamulin 100ppm + Chlortetracycline 300ppm in the food)

Water

Water was provided ad libitum.

Number of pacifiers: 1 for every 10 animals housed.

Other handling during the experimentation period.

Upon admission to weaning boxes (approx. 21 days of age) piglets received a vaccination against PCV2 and *Mycoplasma hyopneumoniae*.

42 days of life: Pigs received *Mycoplasma* revaccination.

Application of injectable Ceftiofur or injectable amoxicillin in animals showing nervous system signs as a result of potential infection by *Streptococcus suis* or *Haemophilus parasuis*.

Treatments

Groups

Vaccine candidate evaluated:

- SV1: Candidate vaccine against *Streptococcus* spp (Ss)

Vaccine candidate	n	Volume	Route of Administration	
			1st Dose	2nd Dose
SV1				
Group 1	5	1 ml	PO	PO
Group 2	5	1 ml	IM	PO
Group 3	5	2 ml ND	PO	----
Group 4	5	2 ml ND	IM	----
Group 5	5	2 ml ND	PO	PO
Group 6	5	2 ml ND	IM	PO
Control (saline)				
Group 7	5	2 ml	IM	PO

Table 5: PO: (oral administration); IM (intramuscular)

Administration date

First dose: Within the first 3 days of life

Second dose: 14-18 days after the first vaccination.

Vaccination Management

Oral administration: the above indicated volume was administered using a dispenser or an automatic syringe.

Intramuscular administration: The above indicated volume was administered with an automatic syringe on the neck region just behind and below the ear, but in front of the shoulder.

Preparation of vaccines in the laboratory

10 microliters were taken from a cryovial from the SV1 working seed, which was plated on TSA plates with chloramphenicol (CP5: 5 mg/ml). The plates were incubated at 37 °C ON. The following day, a colony of the culture was cultured in TSB with CP5. The pre-culture (PC) was incubated at 37 °C overnight. The following day, a certain volume of TSB + CP5 was seeded into a certain large volume of sterile TSB. The culture was incubated for 2 hours at 37 °C, then a certain volume of 0.5 M IPTG (1 ml/L) was added to induce antigen production, and incubation was continued for another 6 hours.

After a total of 8 hours, samples were taken for colony forming unit calculations. Subsequently the culture was inactivated by the addition of a certain volume of formaldehyde and the culture was incubated at room temperature overnight.

The Excipient was prepared following the usual preparation process.

The formulations for the 1 ml and 2 ml dose groups were mixed in equal proportions of inactivated culture (SV1) to excipient (1:1).

Formulations were packed in 10 ml vial, labeled and stored between 2 – 8 °C until use.

Samples and parameters evaluated.**Sampling Date**

Samples were taken on the following days:

- 1st sampling: Primary vaccination day (Week 1)
- 2nd sampling: Booster Day (Week 3)
- 3rd sampling: Week 4
- 4th sampling: Week 6
- 5th sampling: Week 8

Sampling and processing:

Serum Samples

Samples of blood were taken from the jugular vein with 18G x 1.5" needle for pigs and 20G x 1" or 1.5" for piglets. Around 5 ml of blood were taken without anticoagulant in a 15 ml conical tube. To extract serum from blood, the serum is allowed to coagulate in a tube at an angle of at least 60 degrees. Once the clot is formed, it is detached from the glass with the help of a needle. To obtain maximum serum, tubes were heated to 37 °C for a minimum of 30 minutes to a maximum of 1 hour and then placed at 4 °C overnight. The next morning, the tubes were centrifuged at 3000 rpm for 30 minutes and the serum (supernatant) was directly extracted with a pipette. The amount of serum collected was between 500 µl to 1 ml. Serum were stored at -20°C until analysis.

Saliva Samples

IgA determination from Saliva samples were taken as follows:

Oral swabs: Sterile cotton swabs were used to collect saliva from the pigs, the swabs were placed in the pig's mouth until soaked in saliva. The saliva-soaked swabs were

then submerged in 1 ml of sterile PBS, pH 7.4 in a 15 ml conical tube and stored at -20°C until analysis.

Analysis

ELISA for antibody determination

ELISA S/P ratios were used to determine IgA and IgG antibody response to SV1 with the following formula:

Sample – negative control/positive control – negative control

ELISA protocol:

- 1) Coating: BioGreiner plates were coated with 5 µg/ml/well (100 µl/well) of the synthetic Streptococcus peptide antigen covalently linked to BSA and then incubated at room temperature overnight.
- 2) Plates were washed 3X with TBS-T (Tris-buffer saline with 0.1% Tween-20) (200 µl/well)
- 3) The plates were then blocked with 200 µl/well of blocking buffer (Bethyl lab, Cat. E104) and incubated 1 hour at room temperature.
- 4) Plates were washed 3X with TBS-T (200 µl/well)
- 5) Test Samples were diluted as follows:
 - Serum: 1:100 dilution in sample buffer (50 mM Tris buffered saline, pH 8, 1% bovine serum albumin (BSA), 0.05% Tween-20), 100 µl/well
 - Saliva: undiluted, 100 µl/well

The plate was incubated for 1 hour at room temperature.

6) Plates were washed 3X with TBS-T (200 µl/well). Secondary antibodies were added to each well, 100 µl/well, at the following concentrations.

- Anti-Pig IgA HRP 1 µg/ml (A100-102P, Bethyl laboratories)
- Anti-Pig IgG HRP 0.25 µg/ml (A100-205P, Bethyl laboratories)

Incubated for 1 hour at room temperature.

8) Plates were washed 3X with TBS-T (200 µl/well).

9) TMB One component Substrate (E102, Bethyl laboratories) was added (100 µl/well) and incubated 3 minutes at room temperature.

10) The reaction was stopped with 100 µl/well of 0.1 M sulfuric acid.

10) The optical density of each well was read at $\lambda = 450$ nm using a visible light spectrophotometer.

Statistical analysis

An analysis of variance was applied for two factors measured over time using the IBM SPSS Statistic 25 software after determining homoscedastic of variances, sphericity, and normality of the groups.

Experimental Design

The schematic diagram outlining experimental design is illustrated in Figure 3.

RESULTS

Two doses of candidate SV1 vaccine induces antigen-specific secretory IgA and serum IgG in piglets as young as three days of age.

Saliva secretory IgA (sIgA) S/P ratio determination.

Results are shown in Table 6 and in Figure 4. An increase in the antigen-specific salivary sIgA S/P ratios in pigs is observed throughout the weeks in some of the groups evaluated, when the comparison between groups is made within each week, no significant differences are observed (weeks 1, 3, 4 and 6). At week 8, the pigs that received two 2 mL doses of SV1 orally or IM followed by an oral booster, had significantly higher S/P ratio relative to the other groups, with no significant difference between the 2 mL oral/oral and 2 mL IM/oral group. These data indicate that the SV1 candidate vaccine is immunogenic and capable of inducing antigen-specific secretory IgA in pigs as early as 3 days of age, in particular a 2 mL/dose regimen given orally or IM/orally provides a robust sIgA response.

Group	Age of animal at time of sampling									
	Week 1		Week 3		Week 4		Week 6		Week 8	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2 ml Oral	-0.336 ^a	0.422	-0.438 ^a	1.267	0.966 ^a	0.862	0.630 ^a	0.554	0.867 ^{abc}	0.467
2 ml IM	0.251 ^a	0.810	-0.328 ^a	1.571	0.148 ^a	0.105	0.960 ^a	0.839	0.184 ^{ab}	0.900
1 ml Oral/Oral	0.231 ^a	0.803	0.487 ^a	0.718	0.372 ^a	0.546	0.503 ^a	0.174	0.449 ^{ab}	0.311
1 ml IM/Oral	-0.014 ^a	1.112	0.271 ^a	1.594	0.568 ^a	1.261	1.014 ^a	0.448	0.662 ^{abc}	0.483
2 ml Oral/Oral	0.279 ^a	0.479	0.036 ^a	1.314	1.223 ^a	0.836	1.648 ^a	0.902	2.054 ^{cd}	0.176
2 ml IM/Oral	-0.069 ^a	0.269	-0.375 ^a	0.786	0.648 ^a	0.276	1.212 ^a	0.782	2.183 ^d	0.541

Table 6

Serum IgG S/P ratio determination.

Results are shown in Table 7 and in Figure 5. An increase in the antigen-specific serum IgG S/P ratios is observed throughout the weeks in some of the groups evaluated, when the comparison between groups is made within each week, no significant differences

are observed (weeks 1, 3, and 4.). At weeks 6 and 8, the pigs that received two 2 mL doses of SV1 orally or IM followed by an oral booster, had significantly higher S/P ratio relative to the other groups. At 6 weeks of age, the 2 mL IM/Oral group had significantly higher mean S/P ratio than the 2 mL Oral/Oral group, but by 8 weeks of age this difference was no longer present. These data indicate that the SV1 candidate vaccine is immunogenic and capable of inducing antigen-specific serum IgG in pigs as early as 3 days of age, in particular a 2 mL/dose regimen given orally or IM/orally provides a robust serum IgG response.

Group	Age of animal at time of sampling									
	week 1		Week 3		Week 4		Week 6		Week 8	
	Mean	SD	Mean	SD	Mean	Group	Mean	SD	Mean	SD
2 mL Oral	-0.018 ^{ab}	0.038	-0.056 ^a	0.151	0.143 ^a	0.142	0.532 ^a	0.084	0.233 ^{ab}	0.172
2 mL IM	-0.015 ^{ab}	0.05	0.176 ^a	0.453	0.099 ^a	0.1	0.529 ^a	0.719	0.230 ^{ab}	0.174
1 mL Oral/Oral	-0.084 ^a	0.035	-0.107 ^a	0.065	0.123 ^a	0.56	0.609 ^{ab}	0.228	0.341	0.103
1 mL IM/Oral	-0.056 ^a	0.021	0.025 ^a	0.138	0.061 ^a	0.113	1.131 ^{abc}	0.526	0.519	0.339
2 mL Oral/Oral	-0.050 ^{ab}	0.031	-0.126 ^a	0.12	0.046 ^a	0.093	1.404 ^{abc}	0.61	1.668 ^d	0.496
2 mL IM/Oral	-0.048 ^{ab}	0.037	0.018 ^a	0.133	1.181 ^a	1.318	3.052 ^c	0.479	1.878 ^d	0.795

Table 7

Conclusion

These data indicate that candidate vaccine SV1 is immunogenic and antigenic and importantly, the vaccine can protect fish, in this example Nile tilapia, from a challenge with virulent *Streptococcus agalactiae*. Further, the SV1 vaccine induces a robust antigen-specific salivary sIgA and serum IgG response in piglets with the first dose of vaccine administration as early as 3 days of age. The route of administration of either two doses orally or one dose intramuscularly and the second booster dose given orally

provide similar responses for sIgA while the IM/oral induces a higher and quicker serum IgG response as would be expected based on scientists understanding of the immune response and activation, but the response at 8 weeks of age was similar suggesting the new antigenic specific baseline antibody response may not differ. While the volume providing the best antibody response in pigs was 2 mL, it is ultimately irrelevant as the concentration of antigen/mL can be adjusted so that one mL of vaccine could have the same concentration of antigen as a 2 mL dose.

Any method described herein may incorporate any design element contained within this application and any other document/application incorporated by reference herein.

The present invention may be embodied in other forms without departing from the spirit and the essential attributes thereof, and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention. The invention illustratively disclosed herein suitably may be practiced in the absence of any element which is not specifically disclosed herein.

Sequence Listing Information:

DTD Version: V1_3
 File Name: SEQUENCE LISTING 3113.005.xml
 Software Name: WIPO Sequence
 Software Version: 2.3.0
 Production Date: 2023-05-12

General Information:

Current application / IP Office: US
 Current application / Applicant file reference: 3113.005
 Applicant name: Ventanco SA
 Applicant name / Language: en
 Inventor name: Sherry Layton
 Inventor name / Language: en
 Invention title: COMPOSITIONS AND METHODS OF ENHANCING IMMUNE
 RESPONSES TO STREPTOCOCCUS (en)
 Sequence Total Quantity: 3

Sequences:

Sequence Number (ID): 1
 Length: 49
 Molecule Type: AA
 Features Location/Qualifiers:
 - source, 1..49
 > mol_type, protein
 > organism, synthetic construct

Residues:

DPVLVGQEAV AIGELLAESD PKKADYYCQN AATLEGKGPK LADKYSPIF

49

Sequence Number (ID): 2

Length: 878
 Molecule Type: AA
 Features Location/Qualifiers:
 - source, 1..878
 > mol_type, protein
 > organism, synthetic construct

Residues:

MLKATSKTFV TQHTAFSYTA QRFGLKQLGI AGVSEEEPPSP PLTKVPGLED
 MEAGQGIDEA 60
 SLYDPHTWDP VLVGQEAVAI GELLAESDPK KADYYCQNAA TLEGKGPKLA
 DKYSPIFSSS 120
 SSMSGKGPAP GIDLGTITYSC VGVFQHGKVE IANDQGNRT TPSYVAFTDT
 ERLIGDAAKN 180
 QVAMNPTNTI FDAKRLIGRK YDDPTVQSDM KHWPFRRVNE
 GGKPKVQVEY KGEMKTFPE 240
 EISSMVLTKM KEIAEAYLGK KVETAVITVP AYFNDSQRQA TKDAGTITGL
 NVMRIINEPT 300

AAAIAYGLDK KGTRAGEKNV LIFDLGGGTF DVSILTIEDG IFEVKSTAGD
THLGGEDFDN 360
RMVNRVVEEF KGKHKRDNAG NKRAVRRLRT ACERARRTLS SSTQASIEID
SLFEGIDFYT 420
SITARFEEL NADLFRGTLE PVEKALRDAK LDKGQIQEIV LVGGSTRIPK
IQKLLQDFFN 480
GKELNKSINP DEAVAYGAAV QAAILMGDKS ENVQDLLLLD VTPLSLGIET
AGGVMTALIK 540
RNTTIPTKQT QTFTTYS DNQ SSVLVQVYEG ERAMTKDNNL LGKFDLTGIP
PAPRGVPQIE 600
VTFDIDANGI LNVSAVDKST GKENKITITN DKGRLSKDDI DRMVQEAKEY
KAEDEANRDR 660
VGAKNSLESY TYNMKQTVED EKLKGKISDQ DKQKVLDKCQ EVISSLDRNQ
MAEKEEYEHK 720
QKELEKLCNP IVTKLYQGAG GAGAGGSGGP TIEEVDSSSS SSDPVLVGQE
AVAIGELLAE 780
SDPKKADYYC QNAATLEGKG PKLADKYSPI FPLTKVPGLE DMEAGQGIDE
ASLYDPHTWL 840
KATSKTFVTQ HTAFSYTAQR FGLKQLGIAG VSEEEPPSP
878

Sequence Number (ID): 3

Length: 2643

Molecule Type: AA

Features Location/Qualifiers:

- source, 1..2643

> mol_type, protein

> organism, synthetic construct

Residues:

ATGTTAAAGG CTACATCCAA GACTTTTGTG ACACAGCACA
CGGCTTTCAG TTATACAGCG 60
CAACGGTTTG GCTTGAAGCA GTTAGGGATT GCAGGTGTGA
GTGAAGAGGA ACCGTCCCCT 120
CCATTAACAA AAGTCCCTGG GTTGGAAGAC ATGGAGGCCG
GGCAAGGGAT TGATGAAGCC 180
TCACTCTATG ATCCTCATAC TTGGTTGGAC CCGGTGCTTG
TCGGTCAGGA GCGGTAGCC 240
ATCGGAGAGC TTTTGGCTGA ATCAGATCCG AAGAAAGCTG
ATTATTACTG CAAAACGCG 300
GCTACTTTAG AGGGTAAAGG GCCAAAGCTC GCTGACAAGT
ATTCACCGAT ATTTTCATCG 360
AGTTCATCAA TGAGCGGAAA GGGCCCTGCC ATTGGAATCG
ATCTGGGCAC AACATACAGC 420
TGTGTGGGAG TATTTTCAGCA CGGCAAGGTT GAAATCATTG
CAAATGACCA AGGCAACAGA 480

ACAACGCCGT CATATGTCGC TTTTACAGAT ACTGAACGCC
TCATCGGCGA CGCTGCCAAA 540
AACCAAGTGG CGATGAATCC GACAAATACG ATTTTCGACG
CGAAACGTCT CATTGGACGG 600
AAATACGATG ATCCAACAGT ACAATCTGAT ATGAAACATT
GGCCGTTTCG GGTCGTCAAC 660
GAAGGAGGAA AGCCCAAAGT GCAGGTCTGAG TATAAAGGAG
AAATGAAAAC TTTCTTTCCC 720
GAAGAGATAA GTTCCATGGT TTTAACAAAA ATGAAGGAAA
TTGCCGAGGC TTATTTAGGA 780
AAAAAGGTGG AAACGGCGGT GATCACCGTG CCGGCATATT
TTAATGATAG CCAGCGCCAG 840
GCAACCAAGG ATGCGGGAAC CATTACAGGC TTAAATGTTA
TGAGAATTAT TAATGAACCT 900
ACTGCCGCAG CAATCGCTTA CGGACTGGAT AAAAAAGGAA
CAAGAGCAGG CGAAAAGAAT 960
GTACTGATTT TTGATTTAGG GGGAGGTACC TTCGACGTGT
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GGCATCTTTG AAGTGAAATC AACAGCAGGA GATACACACT
TGGGCGGTGA GGATTTTGAC 1080
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AGCATAAACG CGACAATGCC 1140
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TCGAAGGCAT TGACTTCTAC 1260
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AGCTTCTCCA GGATTTTTTT 1440
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TCCAGGACCT TCTGCTTTTA 1560
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GGGTCATGAC GGCTTTGATT 1620
AAACGAAATA CGACCATACC GACGAAGCAG ACACAAACAT
TTACGACGTA CAGCGATAAT 1680
CAGTCATCGG TACTCGTCCA GGTTTATGAA GGAGAACGTG
CAATGACAAA AGATAACAAC 1740
CTTTTGGGGA AATTTGATCT CACAGGGATT CCGCCTGCAC
CAAGGGGTGT GCCTCAAATT 1800
GAAGTAACCT TTGATATCGA CGCCAACGGG ATCTTGAATG
TTAGTGCTGT TGATAAGAGT 1860

ACCGGCAAGG AAAATAAAAT TACAATAACA AACGATAAAG
GCAGACTATC TAAAGACGAT 1920
ATTGACCGCA TGGTGCAAGA GGCTGAAAAG TACAAAGCAG
AGGATGAAGC AAACCGTGAC 1980
CGTGTAGGAG CAAAAAACAG CTTAGAAAGC TATACATATA
ATATGAAACA AACAGTGGAA 2040
GATGAGAAAC TGAAGGGGAA AATAAGCGAT CAAGACAAAC
AGAAAGTTCT TGATAAATGC 2100
CAGGAAGTCA TCTCATCTTT AGATAGAAAT CAAATGGCGG
AGAAAGAAGA ATATGAGCAT 2160
AAGCAAAAAG AGCTGGAAAA ATTATGCAAT CCAATCGTTA
CGAAACTTTA TCAAGGAGCC 2220
GGTGGTGCAG GTGCTGGGGG TTCTGGCGGC CCGACCATTG
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CAGCCACGCT TGAAGGTAAA 2400
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TAACAAAAGT CCCTGGGTTG 2460
GAAGACATGG AGGCCGGGCA AGGGATTGAT GAAGCCTCAC
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CGGCTTTCAG TTATACAGCG 2580
CAACGGTTTG GCTTGAAGCA GTTAGGGATT GCAGGTGTGA
GTGAAGAGGA ACCGTCCCCT 2640
TAA 2643
END

CLAIMS

1. A vaccine composition against *Streptococcus* comprising:
an amino acid sequence selected from the group consisting of Sequence Number (ID): 1, Sequence Number (ID): 2, and Sequence Number (ID): 3; and
a pharmaceutically acceptable carrier.
2. The vaccine composition of claim 1 wherein the vaccine is derived from a protective antigen on which nucleotide optimization was used to optimize for *Bacillus subtilis* to derive the nucleotide sequence for the DNA sequence.
3. The vaccine composition of claim 1 further comprising one or more immunostimulatory polypeptides.
4. The vaccine composition of claim 1 wherein the vaccine composition induces an immune response.
5. The vaccine composition of claim 4 wherein the immune response includes an antibody response.
6. The vaccine composition of claim 1 wherein the vaccine composition is administered by a method selected from the group consisting of oral, intranasal, and parenteral.

7. A method of enhancing the immune response against *Streptococcus* comprising administering to the subject the vaccine composition of claim 1 in an amount effective to enhance the immune response of the subject to one or more strains of *Streptococcus*.
8. The method of claim 7 wherein the vaccine composition induces an immune response.
9. The method of claim 7, wherein the immune response includes an antibody response.
10. The method of claim 7, wherein the vaccine is administered by a method selected from the group consisting of oral, intranasal, and parenteral.

PATENT COOPERATION TREATY

PCT

DECLARATION OF NON-ESTABLISHMENT OF INTERNATIONAL SEARCH REPORT

(PCT Article 17(2)(a), Rules 13ter.1(c) and (d) and 39)

Applicant's or agent's file reference 3113.005-2 (PCT)	IMPORTANT DECLARATION
International application No. PCT/US2024/037808	Date of mailing (day/month/year) 28 October 2024 (28.10.2024)
International filing date (day/month/year) 12 July 2024 (12.07.2024)	(Earliest) Priority Date (day/month/year) 13 July 2023 (13.07.2023)
International Patent Classification (IPC) or both national classification and IPC IPC: A61K 39/108 (2023.01) CPC: A61K 39/0258	
Applicant LAYTON, SHERRYLL	

This International Searching Authority hereby declares, according to Article 17(2)(a), that **no international search report will be established** on the international application for the reasons indicated below.

1. ☐ The subject matter of the international application relates to:
- a. ☐ scientific theories
 - b. ☐ mathematical theories
 - c. ☐ plant varieties
 - d. ☐ animal varieties
 - e. ☐ essentially biological processes for the production of plants and animals, other than microbiological processes and the products of such processes
 - f. ☐ schemes, rules or methods of doing business
 - g. ☐ schemes, rules or methods of performing purely mental acts
 - h. ☐ schemes, rules or methods of playing games
 - i. ☐ methods for treatment of the human body by surgery or therapy
 - j. ☐ methods for treatment of the animal body by surgery or therapy
 - k. ☐ diagnostic methods practised on the human or animal body
 - l. ☐ mere presentations of information
 - m. ☐ computer programs for which this International Searching Authority is not equipped to search prior art
2. ☐ The failure of the following parts of the international application to comply with prescribed requirements prevents a meaningful search from being carried out:
- ☐ the description ☐ the claims ☐ the drawings
3. ☒ A meaningful search could not be carried out without the sequence listing; the applicant did not, within the prescribed time limit:
- ☒ furnish a sequence listing complying with WIPO Standard ST.26, and such listing was not available to the International Searching Authority in a form, language and manner acceptable to it.
 - ☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a).
4. Further comments:

No response was received to the Invitation to Furnish Nucleotide and/or Amino Acid Sequence Listing (Form PCT/ISA/225), issued by the ISA/US on 31 July 2024. As claims 1-10 require a sequence listing for search, an international search was not able to be conducted.

Name and mailing address of the ISA/US COMMISSIONER FOR PATENTS MAIL STOP PCT, ATTN: ISA/US P.O. Box 1450 Alexandria, VA 22313-1450 UNITED STATES OF AMERICA	Authorized officer SHANE THOMAS
Facsimile No. 571-273-8300	Telephone No. (571)272-4188

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference (if desired) (25 characters maximum)
3113.005-2 (PCT)

Box No. I TITLE OF INVENTION	
COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS	
Box No. II APPLICANT ☒ This person is also inventor	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) Layton, Sherryll 141 Nihan Road Quitman, AR	Telephone No. Facsimile No. Applicant's registration No. with the Office
E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send notifications issued in respect of this international application to that e-mail address if those offices are willing to do so. ☐ as advance copies followed by paper notifications; or ☒ exclusively in electronic form (no paper notifications will be sent). E-mail address: bvandegarde@avekip.com; ekdkdocket@kcpatentlaw.com	
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: ☒ all designated States ☐ the States indicated in the Supplemental Box	
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.	
Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) VANDE GARDE, BLAKE E. AVEK IP, LLC 7285 W. 132nd Street, Suite 340 Overland Park, KS 66215 US	Telephone No. 913-549-4700 Facsimile No. Agent's registration No. with the Office 58,264
E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send notifications issued in respect of this international application to that e-mail address if those offices are willing to do so. ☐ as advance copies followed by paper notifications; or ☒ exclusively in electronic form (no paper notifications will be sent). E-mail address: bvandegarde@avekip.com; ekdkdocket@kcpatentlaw.com	
☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Hall, Jeffrey W.
4745 Minnehaha Ave., Unit 101
Minneapolis, MN 55406

This person is:

- ☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

US

State *(that is, country)* of residence:

US

This person is applicant
for the purposes of:

- ☒ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- ☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant
for the purposes of:

- ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- ☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant
for the purposes of:

- ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- ☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant
for the purposes of:

- ☐ all designated States ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No. V DESIGNATIONS

The filing of this request **constitutes under Rule 4.9(a) the designation** of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.

However,

- ☐ DE Germany **is not designated** for any kind of national protection
- ☐ JP Japan **is not designated** for any kind of national protection
- ☐ KR Republic of Korea **is not designated** for any kind of national protection

(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)

Box No. VI PRIORITY CLAIM AND DOCUMENT

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application: regional Office	international application: receiving Office
item (1) 13 July 2023 (13.07.2023)	18/221,713	US		
item (2)				
item (3)				

☐ Further priority claims are indicated in the Supplemental Box.

Furnishing the priority document(s):

☒ The **receiving Office** is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application(s) was filed with the receiving Office which, for the purposes of this international application, is the receiving Office)* identified above as:

☐ all items ☒ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

☐ The **International Bureau** is requested to obtain from a digital library a certified copy of the earlier application(s) identified above, using, where applicable, the access code(s) indicated below *(if the earlier application(s) is available to it from a digital library)*:

☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box
access code _____ access code _____ access code _____

Restore the right of priority: the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (_____). *(See also the Notes to Box No. VI; further information **must** be provided to support a request to restore the right of priority.)*

Incorporation by reference: where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a), or an element or part of the description, claims or drawings referred to in Rule 20.5bis(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if more than one International Searching Authority is competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):*

ISA/ US

Continuation of Box No. VII USE OF EARLIER SEARCH AND CLASSIFICATION RESULTS		
1. Request by the applicant under Rule 4.12		
1.1 ☐	The ISA indicated in Box No. VII is requested to take into account the results of the earlier search(es) indicated below (<i>see also Notes to Continuation of Box No. VII, item 1; use of results of more than one earlier search</i>)	
Filing date (day/month/year)	Application Number	Country (or regional Office)
☐	Statement (Rule 4.12(ii)): this international application is the same, or substantially the same, as the application in respect of which the earlier search was carried out except, where applicable, that it is filed in a different language.	
1.2 Submission of the earlier search results, where necessary*		
☐	Availability of documents (Rules 12bis.1(c) and (d) and 12bis.2(b)): the following documents are available to the ISA in a form and a manner acceptable to it, and therefore DO NOT need to be submitted by the applicant to the receiving Office, or to the ISA.	
☐	a copy of the results of the earlier search,	
☐	a copy of the earlier application,	
☐	a translation of the earlier application into a language which is accepted by the ISA,	
☐	a translation of the results of the earlier search into a language which is accepted by the ISA,	
☐	a copy of any document cited in the earlier search results (<i>if known, please indicate below the documents available to the ISA</i>):	
☐	Request from the applicant to the receiving Office to transmit to the ISA a copy of the earlier search results (Rule 12bis.1(b) and (d)): (<i>where the earlier search was not carried out by the ISA indicated in Box No. VII but by the same Office as that which is acting as receiving Office; or where the earlier search results are otherwise available to the receiving Office</i>): the applicant requests the receiving Office to prepare and transmit to the ISA a copy of the earlier search results.	
* The applicant only needs to furnish a copy of the earlier search results to the receiving Office, or the ISA, if none of the scenarios mentioned under item 1 applies. (<i>See item 10 in the check-list and also Notes to Continuation of Box No. VII, item 1</i>).		
☐	Further earlier searches are indicated on a continuation sheet.	
2. Transmission of the earlier search and classification results to the ISA by the receiving Office where the applicant DID NOT make a request under Rule 4.12		
2.1	Where the international application claims priority of an earlier application, subject to Article 30(2)(a) and (3), the receiving Office:	
	– shall transmit a copy of the earlier search and classification results to the ISA (unless such copy is already available to the ISA), where the earlier application was filed with the same Office as that which is acting as the receiving Office and that Office has carried out the earlier search in respect of the earlier application (Rule 23bis.2(a));	
	– may transmit such a copy if the earlier application was filed with a different Office, but where the results of that earlier search and classification are nevertheless available to the receiving Office (Rule 23bis.2(c)).	
However, where the applicant did not request the receiving Office to transmit to the ISA a copy of the earlier search results under Rule 4.12 (see above item 1), in respect of an earlier search carried out on the following earlier application, the priority of which is subsequently claimed in this international application, the applicant may consider (<i>see also Notes to Continuation of Box No. VII, item 2; use of more than one earlier search</i>):		
	Filing date (day/month/year)	Country (or regional Office)
2.2 Request not to transmit the earlier search results by the receiving Office to the ISA (Rule 23bis.2(b))		
☐	to request that the receiving Office DOES NOT transmit the results of the earlier search to the ISA (Rule 23bis.2(b)) (<i>may only be checked where the international application is filed with the following receiving Offices: DE, FI and SE</i>)	
2.3 Authorization to transmit the earlier search and classification results by the receiving Office to the ISA (Rule 23bis.2(a) and (e))		
☐	to authorize the receiving Office to transmit the results of the earlier search and classification to the ISA (Rule 23bis.2(e)) (<i>may only be checked where the international application is filed with the following receiving Offices: AU, CZ, FI, HU, IL, JP, NO, SE, SG and US</i>)	
☐	to authorize the receiving Office to transmit the results of the earlier international search and classification to the ISA (Rule 23bis.2(a) and Article 30(2)(a) and (3)) (<i>may only be checked where the earlier search concerns an international application, the priority of which is subsequently claimed in this international application and where the earlier international search was carried out by a different ISA than the ISA chosen in Box No. VII</i>)	
☐	Further earlier searches are indicated on a continuation sheet.	
Box No. VIII DECLARATIONS		
The following declarations are contained in Boxes Nos. VIII (i) to (v) (<i>mark the applicable check-boxes below and indicate in the right column the number of each type of declaration</i>):		Number of declarations
☐	Box No. VIII (i) Declaration as to the identity of the inventor :	
☐	Box No. VIII (ii) Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent :	
☐	Box No. VIII (iii) Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application :	
☒	Box No. VIII (iv) Declaration of inventorship (only for the purposes of the designation of the United States of America) :	
☐	Box No. VIII (v) Declaration as to non-prejudicial disclosures or exceptions to lack of novelty :	

Box No. VIII (iv) DECLARATION: INVENTORSHIP (only for the purposes of the designation of the United States of America)

The declaration must conform to the following standardized wording provided for in Section 214; see Notes to Boxes Nos. VIII, VIII (i) to (v) (in general) and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

**Declaration of inventorship (Rules 4.17(iv) and 51bis.1(a)(iv))
for the purposes of the designation of the United States of America:**

I hereby declare that I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

This declaration is directed to the international application of which it forms a part (if filing declaration with application).

This declaration is directed to international application No. PCT/..... (if furnishing declaration pursuant to Rule 26ter).

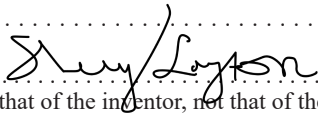
I hereby declare that the above-identified international application was made or authorized to be made by me.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

Name: Sherryll Layton

Residence: Quitman, Arkansas
(city and either US state, if applicable, or country)

Mailing Address: 2 Buck Branch Rd
Quitman, Arkansas 72131, US

Inventor's Signature:  **Date:**
(The signature must be that of the inventor, not that of the agent)

Name: Jeffrey W. Hall

Residence: Minneapolis, Minnesota
(city and either US state, if applicable, or country)

Mailing Address: 4745 Minnehaha Ave., Unit 101
Minneapolis, Minnesota 55406, US

Inventor's Signature: **Date:**
(The signature must be that of the inventor, not that of the agent)

Name:
.....

Residence:
(city and either US state, if applicable, or country)

Mailing Address:
.....
.....

Inventor's Signature: **Date:**
(The signature must be that of the inventor, not that of the agent)



This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

Box No. VIII (iv) DECLARATION: INVENTORSHIP (only for the purposes of the designation of the United States of America)

The declaration must conform to the following standardized wording provided for in Section 214; see Notes to Boxes Nos. VIII, VIII (i) to (v) (in general) and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

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for the purposes of the designation of the United States of America:**

I hereby declare that I believe I am the original inventor or an original joint inventor of a claimed invention in the application.

This declaration is directed to the international application of which it forms a part (if filing declaration with application).

This declaration is directed to international application No. PCT/..... (if furnishing declaration pursuant to Rule 26ter).

I hereby declare that the above-identified international application was made or authorized to be made by me.

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Quitman, Arkansas 72131, US

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Name: Jeffrey W. Hall

Residence: Minneapolis, Minnesota
(city and either US state, if applicable, or country)

Mailing Address: 4745 Minnehaha Ave., Unit 101

Minneapolis, Minnesota 55406, US

Inventor's Signature:  **Date:**
(The signature must be that of the inventor, not that of the agent)

Name:
.....

Residence:
(city and either US state, if applicable, or country)

Mailing Address:
.....
.....

Inventor's Signature: **Date:**
(The signature must be that of the inventor, not that of the agent)

☐ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

Figure of the drawings which should accompany the abstract: 1

/blake e vande garde/
Blake E. Vande Garde
Agent and Attorney for Applicant
Reg. No. 58,264

1. Date of actual receipt of the purported international application:		2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:		
4. Date of timely receipt of the required corrections under PCT Article 11(2):		
5. International Searching Authority (if two or more are competent):	6. ☐ Transmittal of search copy delayed until search fee is paid	

Date of receipt of the record copy
by the International Bureau:

PCT**FEE CALCULATION SHEET****Annex to the Request**

For receiving Office use only

International Application No.

Date stamp of the receiving Office

Applicant's or agent's file reference

3113.005-2 (PCT)

Applicant

CALCULATION OF PRESCRIBED FEES*(Applicants may be entitled to a reduction of certain fees as indicated in the PCT Fee Tables (www.wipo.int/pct/en/fees.pdf))*1. TRANSMITTAL FEE: 104 **T**2. SEARCH FEE: 872 **S**International search to be carried out by: **US**

3. INTERNATIONAL FILING FEE

Enter total number of sheets indicated in Box No IX: **42****i1** Fixed amount for the first 30 sheets 1457 **i1****i2** $\frac{12}{\text{number of sheets in excess of 30}} \times \frac{16}{\text{fee per sheet}} = 192$ **i2**Add amounts entered at i1 and i2 and enter total at I: 1649 **I***(Applicants from certain States are entitled to a reduction of 90% of the international filing fee (see www.wipo.int/pct/en/fees/fee_reduction.pdf). Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 10% of the international filing fee.)*4. FEE FOR PRIORITY DOCUMENT (if applicable): 0 **P**5. FEE FOR RESTORATION OF THE RIGHT OF PRIORITY (if applicable): 0 **RP**6. FEE FOR EARLIER SEARCH DOCUMENTS (if applicable): 0 **ES**

7. TOTAL FEES PAYABLE 2625

Add amounts entered at T, S, I, P, RP and ES, and enter total in the TOTAL box

TOTAL**MODE OF PAYMENT** (Not all modes of payment may be available at all receiving Offices)☒ credit card (details should not be included on this sheet)☒ authorization to charge deposit or current account (see below)☐ bank transfer☐ cash☐ postal money order☐ check☐ revenue stamps☐ other (specify):**AUTHORIZATION TO CHARGE (OR CREDIT) DEPOSIT OR CURRENT ACCOUNT***(This mode of payment may not be available at all receiving Offices)*☐ Authorization to charge the total fees indicated above.☒ (This check-box may be marked only if the conditions for deposit or current accounts of the receiving Office so permit) Authorization to charge any deficiency or credit any overpayment in the total fees indicated above.☐ Authorization to charge the fee for priority document.Receiving Office: RO/ **US**Deposit or Current Account No.: **502790**Date: **July 12, 2024**Name: **Blake E. Vande Garde**Signature: **/blake e vande garde/**

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION,
OBTENTION OR TRANSMITTAL
OF PRIORITY DOCUMENT
(PCT Administrative Instructions, Section 411)

To:

VANDE GARDE, Blake, E.
Avek IP, LLC
7285 W. 132nd Street, Suite 340
Overland Park, KS 66215
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (day/month/year) 06 August 2024 (06.08.2024)	
Applicant's or agent's file reference 3113.005-2 (PCT)	IMPORTANT NOTIFICATION
International application No. PCT/US2024/037808	International filing date (day/month/year) 12 July 2024 (12.07.2024)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 13 July 2023 (13.07.2023)
Applicant LAYTON, Sherryll et al	

The applicant is hereby notified of the date of receipt (or of obtaining by the International Bureau) of the priority document(s) relating to all earlier application(s) whose priority is claimed. Unless otherwise indicated by the letters "NR", in the right-hand column or by an asterisk appearing next to the date of receipt, **the priority document concerned was submitted or transmitted to or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-bis)**. This Form replaces any previously issued notification concerning submission, transmittal or obtaining of priority documents.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
13 July 2023 (13.07.2023)	18/221,713	US	01 August 2024 (01.08.2024)

The letters "NR" denote a priority document which, on the date of mailing of this Form, had not yet been received or obtained by the International Bureau in compliance with Rule 17.1(a), (b) or (b-bis). Where the applicant has failed to either submit, request to prepare and transmit, or to request the International Bureau to obtain the priority document within the applicable time limit under that Rule, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

An asterisk "*" next to a date of receipt, denotes a priority document submitted or transmitted to or obtained by the International Bureau but not in compliance with Rule 17.1(a), (b) or (b-bis) (the priority document was received after the time limit prescribed in Rule 17.1(a); the request to prepare and transmit the priority document was submitted to the receiving Office after the applicable time limit under Rule 17.1(b) or the request to the International Bureau to obtain the priority document was made after the applicable time limit under Rule 17.1(b-bis)). Even though the priority document was not furnished in compliance with Rule 17.1(a), (b) or (b-bis), the International Bureau will nevertheless transmit a copy of the document to the designated Offices, for their consideration. In case such a copy is not accepted by the designated Office as the priority document, Rule 17.1(c) provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Xin Wang e-mail pct.team2@wipo.int Telephone No. +41 22 338 74 02
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DOCUMENT MADE AVAILABLE UNDER THE PATENT COOPERATION TREATY (PCT)

International application number:	PCT/US2024/037808
International filing date:	12 July 2024 (12.07.2024)
Document type:	Certified copy of priority document
Document details:	Country/Office: US
	Number: 18/221,713
	Filing date: 13 July 2023 (13.07.2023)
Date of receipt at the International Bureau:	01 August 2024 (01.08.2024)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a),(b) or (b-bis)

1325020

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

July 31, 2024

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *18/221,713*

FILING DATE: *July 13, 2023*

RELATED PCT APPLICATION NUMBER: *PCT/US24/37808*

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS *US18/221,713*



Certified by

Katherine Kelly Vidal

Performing the Functions and Duties of the
Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

Under the Paperwork Reduction Act of 1995 no persons are required to respond to a collection of information unless it displays a valid OMB control number.

UTILITY PATENT APPLICATION TRANSMITTAL <i>(Only for new nonprovisional applications under 37 CFR 1.53(b))</i>	Attorney Docket No.	3113.005
	First Named Inventor	Sherryll Layton
	Title	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STRE
	Priority Mail Express® Label No.	

APPLICATION ELEMENTS <i>See MPEP chapter 600 concerning utility patent application contents.</i>	Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450
ADDRESS TO:	ACCOMPANYING APPLICATION PAPERS
1. ☐ Fee Transmittal Form (PTO/SB/17 or equivalent) 2. ☒ Applicant asserts small entity status. See 37 CFR 1.27 3. ☐ Applicant certifies micro entity status. See 37 CFR 1.29. Applicant must attach form PTO/SB/15A or B or equivalent. 4. ☒ Specification [Total Pages <u>35</u>] Both the claims and abstract must start on a new page. (See MPEP § 608.01(a) for information on the preferred arrangement) 5. ☒ Drawing(s) (35 U.S.C. 113) [Total Sheets <u>3</u>] 6. Inventor's Oath or Declaration [Total Pages _____] (including substitute statements under 37 CFR 1.64 and assignments serving as an oath or declaration under 37 CFR 1.63(e)) a. ☐ Newly executed (original or copy) b. ☐ A copy from a prior application (37 CFR 1.63(d)) 7. ☒ Application Data Sheet * See note below. See 37 CFR 1.76 (PTO/AIA/14 or equivalent) 8. CD-ROM or CD-R in duplicate, large table, or Computer Program (Appendix) ☐ Landscape Table on CD 9. Nucleotide and/or Amino Acid Sequence Submission (if applicable, items a. – c. are required) a. ☒ Computer Readable Form (CRF) b. ☐ Specification Sequence Listing on: i. ☐ CD-ROM or CD-R (2 copies); or ii. ☐ Paper c. ☐ Statements verifying identity of above copies	10. ☐ Assignment Papers (cover sheet & document(s)) Name of Assignee _____ 11. ☐ 37 CFR 3.73(c) Statement ☒ Power of Attorney (when there is an assignee) 12. ☐ English Translation Document (if applicable) 13. ☐ Information Disclosure Statement (PTO/SB/08 or PTO-1449) ☐ Copies of citations attached 14. ☐ Preliminary Amendment 15. ☐ Return Receipt Postcard (MPEP § 503) (Should be specifically itemized) 16. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed) 17. ☐ Nonpublication Request Under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent. 18. ☐ Other: _____ _____ _____ _____ _____

***Note:** (1) Benefit claims under 37 CFR 1.78 and foreign priority claims under 1.55 **must** be included in an Application Data Sheet (ADS).
(2) For applications filed under 35 U.S.C. 111, the application must contain an ADS specifying the applicant if the applicant is an assignee, person to whom the inventor is under an obligation to assign, or person who otherwise shows sufficient proprietary interest in the matter. See 37 CFR 1.46(b).

19. CORRESPONDENCE ADDRESS					
☒ The address associated with Customer Number: <u>126354</u> OR ☐ Correspondence address below					
Name	Blake E. Vande Garde				
Address	7285 W 132nd St, Ste 340				
City	Overland Park	State	Kansas	Zip Code	66213
Country	USA	Telephone	913-549-4700	Email	ekdkdocket@kcpatentlaw.com
Signature	/Blake E. Vande Garde/			Date	2023-07-13
Name (Print/Type)	Blake E. Vande Garde			Registration No. (Attorney/Agent)	58264

This collection of information is required by 37 CFR 1.53(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (*i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

**COMPOSITIONS AND METHODS OF
ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS**

INVENTOR(S): Sheryll Layton and Jeffrey W. Hall

FIELD OF INVENTION

The present invention is related to the fields of microbiology and vaccinology and more specifically concerns the development of a vaccine capable of conferring immunity to infection by *Streptococcus*.

BACKGROUND OF THE INVENTION

The bacterial genus *Streptococcus* is a of a large group of Gram-positive cocci that form long chains of cells. The genus is large and diverse with many important human and agricultural pathogens that cause a wide range of different diseases. The worldwide mortality, morbidity, and economic impacts of streptococcal infections are significant. While effective vaccines have been developed against *S. pneumoniae* (Pneumococcus), there is still great need for affordable and effective vaccines against other species of streptococci that infect humans and animals such as *S. suis* (swine and zoonotic pathogen), *S. agalactiae* (animal, human, and fish pathogen).

As with many genera, classification of the *Streptococcus* genus has undergone many revisions. There are several methods of classification for streptococci (Lancefield, hemolysis, metabolism), but the leading and most comprehensive method to measure genetic relatedness is the use of 16s rDNA sequencing, with taxonomists classifying the genus into 6 major groups containing at least 75 distinct species. Further complicating classification, and ultimately successful immunity, is genome plasticity within the genus which may allow individual strains to develop new traits that allow it to evade an immune response or become more pathogenic. Strains within a species are often referred to as a sequence type (ST) or serotype to indicate their shared genetic or serological traits, respectively.

The capsular polysaccharide (CPS) is an extracellular cellular matrix that surrounds the bacterial cell made up of repeating linear or branched oligosaccharide units connected by amino acids linked to the hydroxyl groups of the sugar rings. The CPS limits the ability of a host's immune system to engulf, destroy, and eradicate the bacterial cells. In most streptococci, the *cps* locus consists of eight genes that encode the biosynthetic pathway for CPS production. The CPS of streptococci and other pathogenic bacteria have been the target of vaccine research for decades as it is highly immunogenic, with success limited to *S. pneumoniae*. Unfortunately, the CPS is also highly variable owing to the fact that streptococci have a highly plastic genome, with high levels of genome recombination and gene switching. Recombination within the *cps* locus gives rise to many serotypes within each streptococcus species and makes developing a broadly effective vaccine against a particular species extremely difficult.

In addition to the CPS, pathogenic streptococci encode an incredibly vast array of surface-associated or secreted components, regulatory genes, or metabolic pathways that all promote the ability to colonize at host and evade the immune system. For example, there are at least 100 known or putative factors within the *S. suis* pan-genome that confer pathogenic capabilities, but because of genome plasticity and recombination, they are not homogeneously distributed within the species leading to ineffective vaccination strategies. Targeting individual traits, serogroups, or sequence types is an ineffective vaccination strategy.

The advent of high-throughput genome sequencing has revolutionized scientists' ability to classify and organize species. More importantly, it has allowed scientists to define core- and pan-genomes of bacteria; genes that are essential and found in all species of genera, and genes that are mobile and are found in certain lineages of a species, respectively. By analyzing and comparing core-genomes of streptococci it is possible to identify highly conserved essential genes that contain potentially antigenic and immunologically protective epitopes across species. These antigens have the potential to provide far greater efficacy because they train a host's immune system to target protein(s) that are essential to the growth and replication of the bacterial cell and are conserved throughout streptococci, providing for broad protection across species.

Thus, there is clearly a need of a broadly protective streptococci vaccine which is antigenic and immunogenic in domestic crossbred commercial swine and provides Nile tilapia protection from infection with *S. agalactiae*.

SUMMARY OF THE INVENTION

The instant invention includes a broadly protective streptococci vaccine which is antigenic and immunogenic.

DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates Enzyme-linked immunosorbent assays (ELISAs) detecting anti-Strep epitope antibodies in A) secretory IgA in saliva on study day 34, B) secretory IgA in intestinal mucosa on study day 35, C) IgG in serum on study day 34. Data is grouped by vaccination route. All data is relative to the non-treated saline control animals. Ratios greater than 1 indicate antibody levels greater than the saline control animals.

Figure 2 illustrates a Kaplan-Meier survival analysis of Nile tilapia challenged with virulence *S. agalactiae*. The IP-AV (positive control) and SV1 10% immersion and 1% oral fed groups had significantly better survival than the unvaccinated controls ($p < 0.05$).

Figure 3 illustrates the Scheme of Assessment.

Figure 4 illustrates the sIgA S/P ratio from saliva of pigs vaccinated with SV1.

Figure 5 illustrates the IgG S/P ratio from serum of pigs vaccinated with SV1.

DETAILED DESCRIPTION

The present invention now will be described more fully hereinafter in the following detailed description of the invention, in which some, but not all embodiments of the invention are described. Indeed, this invention may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements.

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the term "and/or" includes any and all combinations of one or more of the associated listed items. As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well as the singular forms, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, steps, operations, elements, components, and/or groups thereof.

Unless otherwise defined, all terms (including technical and scientific terms) used herein have the same meaning as commonly understood by one having ordinary skill in the art to which this invention belongs. It will be further understood that terms, such as those defined in commonly used dictionaries, should be interpreted as having a meaning that is consistent with their meaning in the context of the relevant art and the

present disclosure and will not be interpreted in an idealized or overly formal sense unless expressly so defined herein.

In describing the invention, it will be understood that a number of techniques and steps are disclosed. Each of these has individual benefit and each can also be used in conjunction with one or more, or in some cases all, of the other disclosed techniques. Accordingly, for the sake of clarity, this description will refrain from repeating every possible combination of the individual steps in an unnecessary fashion. Nevertheless, the specification and claims should be read with the understanding that such combinations are entirely within the scope of the invention and the claims.

Streptococcus is a genus of gram-positive coccus or spherical bacteria that belongs to the family Streptococcaceae, within the order Lactobacillales (lactic acid bacteria), in the phylum Firmicutes. In addition to streptococcal pharyngitis (strep throat), certain *Streptococcus* species are responsible for many cases of pink eye, meningitis, bacterial pneumonia, endocarditis, erysipelas, and necrotizing fasciitis (the 'flesh-eating' bacterial infections). However, many streptococcal species are not pathogenic, and form part of the commensal human microbiota of the mouth, skin, intestine, and upper respiratory tract.

The instant invention identified a *Streptococcus* epitope which was part of a protective antigen. An epitope, also known as antigenic determinant, is the part of an antigen that is recognized by the immune system, specifically by antibodies, B cells, or

T cells. The part of an antibody that binds to the epitope is called a paratope. Although epitopes are usually non-self proteins, sequences derived from the host that can be recognized (as in the case of autoimmune diseases) are also epitopes.

The epitopes of protein antigens are divided into two categories, conformational epitopes and linear epitopes, based on their structure and interaction with the paratope. Conformational and linear epitopes interact with the paratope based on the 3-D conformation adopted by the epitope, which is determined by the surface features of the involved epitope residues and the shape or tertiary structure of other segments of the antigen.

- A conformational epitope is formed by the 3-D conformation adopted by the interaction of discontinuous amino acid residues.
- In contrast, a linear epitope is formed by the 3-D conformation adopted by the interaction of contiguous amino acid residues.
 - A linear epitope is not determined solely by the primary structure of the involved amino acids. Residues that flank such amino acid residues, as well as more distant amino acid residues of the antigen affect the ability of the primary structure residues to adopt the epitope's 3-D conformation.
 - 90% of epitopes are conformational.

A T-cell epitope is a small fragment of an antigen that is recognized by a T cell receptor (TCR). T cells are a type of white blood cell that play an important role in the adaptive immune response. They recognize and respond to foreign invaders, such as

bacteria and viruses. T-cell epitopes are typically 8 to 11 amino acids long and are derived from proteins. They are presented to T cells on the surface of antigen-presenting cells (APCs). APCs are cells that have taken up foreign antigens and processed them into peptides. The peptides are then bound to MHC molecules, which are displayed on the surface of the APC.

When a TCR binds to a T-cell epitope, it triggers a series of events that lead to the activation of the T cell. Activated T cells can then release cytokines, which help to recruit other immune cells to the site of infection. They can also kill infected cells directly. The ability of a T cell to recognize a particular T-cell epitope is determined by the structure of the TCR. Each TCR has a unique binding site that is specific for a particular set of T-cell epitopes. The binding site is formed by a combination of amino acids in the TCR molecule.

A B-cell epitope is a small part of an antigen that is recognized by a B cell receptor (BCR). B cells are a type of white blood cell that produce antibodies. Antibodies are proteins that bind to antigens and help to neutralize them. B-cell epitopes are typically 7 to 11 amino acids long and are derived from proteins. They can be linear or conformational. Linear epitopes are short sequences of amino acids that are recognized by the BCR regardless of the three-dimensional structure of the antigen. Conformational epitopes are three-dimensional structures that are recognized by the BCR. The ability of a B cell to recognize a particular B-cell epitope is determined by the structure of the BCR. Each BCR has a unique binding site that is specific for a particular

set of B-cell epitopes. The binding site is formed by a combination of amino acids in the BCR molecule.

T-cell epitopes and B-cell epitopes are each small fragments of an antigen that are recognized by T cells and B cells respectively. Each are important for understanding the immune response and for developing new vaccines and therapies. Epitope-based vaccines stimulate humoral and cellular immune responses using isolated B-cell or T-cell epitopes. These vaccines can use multiple epitopes to increase their efficacy. To find epitopes to use for the vaccine, *in silico* mapping is often used. Once candidate epitopes are found, the constructs are engineered and tested for vaccine efficiency.

A nucleotide codon optimization for *Bacillus subtilis* was utilized to derive the necessary nucleotide sequence for the DNA sequence and a recombinant synthetic DNA sequence was manufactured (Genscript Biotech Corporation - Piscataway NJ, USA). *Bacillus subtilis* is a safe, non-pathogenic bacterium that can be easily genetically modified and produced on a large scale. *Bacillus subtilis* has been used to develop vaccines against a variety of diseases, including anthrax, cholera, and tuberculosis. One advantage of using *Bacillus subtilis* as a vaccine vector is that it can be administered orally, intranasally, and parenterally. This is a major advantage over traditional vaccines, which are typically administered only by injection. Another advantage of using *Bacillus subtilis* as a vaccine vector is that it can be easily produced on a large scale. This is important for the development of vaccines against emerging

diseases, which may require the production of large quantities of vaccine in a short period of time.

The recombinant synthetic DNA sequence, complete with the homologous restriction sites, was amplified using traditional PCR with sequence specific primers resulting in an amplification product. The amplification product was purified by gel extraction techniques, concentrated, digested with BamHI and XbaI overnight, and re-purified. BamHI is a restriction enzyme isolated from the bacterium *Bacillus amyloliquefaciens*. It is a type II restriction endonuclease, which recognizes a specific sequence of DNA and cuts the DNA within that sequence. BamHI recognizes the sequence GGATCC and cuts the DNA between the G and the A. This results in two DNA fragments, each with a sticky end. Sticky ends are complementary to each other, so they can be ligated (joined) together. BamHI is a very versatile restriction enzyme as it can be used to cut DNA from a variety of sources, including bacteria, viruses, and plants. It is also easy to use and it's inexpensive, making it a cost-effective choice. XbaI is a restriction enzyme isolated from the bacterium *Xanthomonas badrii*. It is a type II restriction endonuclease, which recognizes a specific sequence of DNA and cuts the DNA within that sequence. XbaI recognizes the sequence T[^]CTAGA and cuts the DNA between the T and the A. XbaI is a very versatile restriction enzyme as it can be used to cut DNA from a variety of sources, including bacteria, viruses, and plants. Like BamHI, it is also easy to use and it's inexpensive, making it a cost-effective choice.

The *Bacillus* expression plasmid pHT10 was digested with BamHI and XbaI, purified, concentrated, and treated with rSAP. The digested synthetic insert and plasmid were then mixed into a T4 DNA ligase reaction overnight at room temperature. T4 DNA ligase is an enzyme that joins together two pieces of DNA by catalyzing the formation of a phosphodiester bond between the 5'-phosphate group of one DNA strand and the 3'-hydroxyl group of the other DNA strand. This reaction is essential for DNA replication, repair, and recombination.

The T4 DNA ligase reaction takes place in the following steps:

1. The T4 DNA ligase enzyme binds to the two DNA strands to be joined.
2. The enzyme breaks the phosphodiester bonds between the 5'-phosphate group of one DNA strand and the 3'-hydroxyl group of the other DNA strand.
3. The enzyme then reforms the phosphodiester bonds between the two DNA strands, joining them together.
4. The T4 DNA ligase enzyme releases the two DNA strands, which are now joined together.

The ligation reaction was transformed into *E. coli* DH5 α (New England Biolabs - Ipswich, Massachusetts, USA) and transformants were screened for the gene insert on LB agar with ampicillin (100 μ g/ml, LB^{Amp}). The ligation reaction is a process that joins together two pieces of DNA. The resulting DNA molecule is then transformed into *E. coli* DH5 α cells. The cells are then grown on a plate containing an antibiotic that kills any

cells that do not contain the cloned gene. The cells that survive are then cultured and the cloned gene is harvested.

The new plasmid, pStrep, was purified from *E. coli* and transformed into *B. subtilis*. Transformants were selected on tryptic soy agar with chloramphenicol (5 µg/ml, TSA^{Cm}), creating the recombinant *Bacillus subtilis* strain, Strep Vaccine 1, (SV1).

A protective subunit is then produced during fermentation of SV1 at between 28 and 37 °C and induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentration ranging from 0.1 to 0.5 mM. The culture is inactivated with formalin. To produce the Test Vaccine and iterations thereof, the inactivated SV1 culture was mixed with a naturally occurring polysaccharide, methyl cellulose (encapsulation media) which acts as the vehicle and protectant for the antigen.

EXAMPLES

Example 1: Immunogenicity study of streptococcus epitope in weanling pigs:

Title: Pilot study determining the humoral immune response in three-week-old piglets after vaccination by various routes with an experimental *Streptococcus* vaccine.

Justification: *Streptococcus suis* and streptococcal-associated diseases are a significant disease and economic burden on intensive, indoor swine operations. Diseases associated with streptococcal infections include meningitis, septicemia, arthritis, polyserositis, valvular endocarditis, myocarditis, pericarditis, and abortion. *Streptococcus suis* is now the most common cause of meningitis in pigs submitted to veterinary diagnostic laboratories. The vaccines currently available fail to induce adequate cross-protection between the many serotypes of *S. suis* thus, a vaccine that induces a strong protective and cross-serotype reactive immune response is needed.

Materials and Methods: Thirty pigs at 3 weeks of age will be weaned from a high health farm with no previous *S. suis* concerns, or other disease confounders (PRRS, *Mycoplasma hyopneumoniae*, PEDv, APP, etc). The pigs will be tagged, weighed, and randomized into one of 10 groups (2 replicates x 5 groups (3 pigs/group)) and allowed to acclimate for 2 to 3 days. On Day 0 of the study, serum samples from each pig will be collected and the first dose of vaccine or saline will be administered as indicated in Table 1. The second dose is administered 14 days later as indicated in Table 1. Treatment groups are separated.

Vaccination: The vaccine will be administered in 2 doses at 2 mL per dose either through an intramuscular injection (IM) behind the ear in the neck, oral gavage, or nasal spray (1 mL/nostril). Saline administration will be by oral gavage.

The study timeline and samples to be collected are outlined in Table 2.

Table 1: Route of administration for vaccine

# animals (Reps)	Group	Dose (Study day)	IM	Oral	Nasal	Saline, Oral
3 (2)	1 & 6	1 st (D0)	X			
		2 nd (D14)	X			
3 (2)	2 & 7	1 st (D0)		X		
		2 nd (D14)		X		
3 (2)	3 & 8	1 st (D0)			X	
		2 nd (D14)			X	
3 (2)	4 & 9	1 st (D0)	X			
		2 nd (D14)		X		
3 (2)	5 & 10	1 st (D0)				X
		2 nd (D14)				X

Table 2: Study timeline and sample collection

Study Day	Activity
Day -3	Pigs are weaned, tagged, weighed, randomized, into groups and vaccinated for PCV2,
Day 0	Serum samples are collected from all pigs before 1 st treatment dose. Pigs are given their first dose of respective treatment according to Table 1. Clinical observations are noted.
Day 14	Serum samples are collected from all pigs before 2 nd treatment dose. Pigs are given their second dose of respective treatment according to Table 1. Clinical observations are noted.
Day 34	Oral fluids collection from animals - individual if possible, or per pen rope collection - Serum samples taken from each pig
Day 35	Clinical observations are noted. Pigs are weighed. Samples taken in necropsy: - Small intestine wash for mucosal sIgA

The results are illustrated in Figure 1 in which Enzyme-linked immunosorbent assays (ELISAs) detecting anti-Strep epitope antibodies in A) secretory IgA in saliva on study day 34, B) secretory IgA in intestinal mucosa on study day 35, C) IgG in serum on study day 34. Data is grouped by vaccination route. All data is relative to the non-treated saline control animals. Ratios greater than 1 indicate antibody levels greater than the saline control animals.

Example 2: Survival of Nile tilapia after vaccination with SV1 and challenge with virulent *Streptococcus agalactiae*.

Experimental Design, Sampling and Vaccination: A total of 180 fingerlings were acclimatized for two days and distributed in four treatments, all in triplicate (3 tanks/treatment, 15 fish/tank). The juveniles were distributed in 12 experimental units of 80 L, totaling 15 fish per tank. Fish were administered treatments as described (Table 1) below and grown for 54 days.

Fish were vaccinated with the SV1 via immersion (imer) and orally (oral) via coating the food pellets. Currently, there are only interparental injectable vaccines commercially available to prevent streptococcosis, thus a commercial *S. agalactiae* vaccine (AquaVac) was used as a positive control treatment (Table 3).

Table 3: Treatment groups, routes of administration and number of replicates per treatment.

Treatments	Challenged	Route of Administration	Replicates (15 fish/tank)
T1. IP-AP	Yes	AquaVac intraperitoneal (IP) injection	3
T2. Immersion 10%	Yes	2 baths (study day 0 and day 45)	3
T3. Oral 10%	Yes	Oral (fed days 30/31 and 45/46)	3
T4. Control	Yes	No treatment	3

Intraperitoneal

The application of the vaccines intraperitoneally was carried out on the 41st day of the experiment, 14 days before the infection challenge. A commercial vaccine, AQUAVAC® Strep SA, MSD, was administered according to the label instructions in the positive control group. The commercial vaccine (AV) was administered at 50 µL per fish.

Immersion

In the immersion application, the baths occurred at two distinct days of the experiment. The first bath was performed at the beginning of the experiment (day zero) and the second bath at 45 days (d 45) of experimentation. A dose of 500 mL of the SV1 in 4.5 L (10% v/v) in which the fish remained immersed for 20 minutes.

Oral

In the oral route, the SV1 was mixed directly on the feed. The fish received the feed containing the SV1 for two consecutive days, at two different times during the period. The first feeding containing SV1 was offered on days 30 and 31 and the second on

days 45 and 46 of the experiment. The inclusion of SV1 in the diet was performed 30 minutes before food management. The dose was defined as 100 μL of the vaccine per gram of feed (10% v/w).

Experimental challenge: On the 55th day of the experiment, fish of all groups were challenged with the bacterial pathogen *S. agalactiae* strain S13 (ST-552, type 1b) isolated from a streptococcosis outbreak in Nile tilapia in Brazilian state of Paraná (Facimoto et al., 2017).

A 50% lethal dose experiment was initially conducted to determine the concentration of the bacteria capable of killing 50% of the unvaccinated and challenged population (LD_{50}). For this dosage trial, 40 Nile tilapia were distributed in 8 experimental units of 30 L, with aeration and controlled temperature, totaling five animals per experimental unit. The LD_{50} was performed in duplicate, and the concentration of bacteria tested were: 1×10^5 ; 1×10^6 ; 1×10^7 ; 1×10^8 CFU mL^{-1} (data not shown). The LD_{50} of *S. agalactiae* S13 by intraperitoneal injection was determined to be $\times 10^7$ CFU mL^{-1} and this concentration was used in the subsequent vaccination and challenge period described below.

The fish were fed for 54 days until they reached the approximate weight of 50 g, a weight in which the animals are more susceptible to diseases (Mian et al., 2009). After this fattening period, each fish was challenged with 1×10^7 CFU mL^{-1} of *S. agalactiae* S13 by intraperitoneal injection. The bacteria were pelleted and washed 3x in cold,

sterile, PBS prior to injection. The fish underwent an anesthetic procedure with eugenol to perform the procedure.

Clinical signs were observed daily for 7 days post-challenge, and all dead fish were removed daily from the tanks and recorded in the trial file. Any fish that was unable to stay submerged or maintain proper orientation was humanely euthanized.

Results:

Table 4: Growth parameters (mean $\pm$ standard deviation) of Nile tilapia after 54 days of experiment. Different letters indicate significant difference among the treatments ($p < 0.05$).

	<i>Immersion 10%</i>	<i>IP-AV</i>	<i>Oral 10%</i>	<i>Control</i>
<i>Weight (g)</i>	58.62 $\pm$ 12.48ab	59.31 $\pm$ 9.78ab	62.17 $\pm$ 11.08a	43.10 $\pm$ 0.10c
<i>Length (cm)</i>	14.39 $\pm$ 1.13b	14.41 $\pm$ 0.41b	15.13 $\pm$ 0.84a	12.94 $\pm$ 0.16

Survival analysis of Nile tilapia challenged with virulent S. agalactiae:

The results are illustrated in Figure 2 which shows a Kaplan-Meier survival analysis of Nile tilapia challenged with virulent *S. agalactiae*. The IP-AV (positive control) and SV1 10% immersion and 10% oral fed groups had significantly better survival than the unvaccinated controls ($p < 0.05$).

Example 3: Determination of the dose volume, route of administration, and kinetics of the humoral immune response in pigs vaccinated with candidate Strep Vaccine 1 (SV1).

Objective

Evaluate the humoral immune response in pigs vaccinated with the candidate streptococcal vaccine formulation using different dose volumes and administration routes in order to determine the optimal vaccine presentation based on the level of specific antibodies determined by antigen-specific immunoassay tests.

Material and method

Animals and Accommodation

“Las Chirus” is a full-cycle confined pig farm. It has 450 breeders, generating 20 deliveries per week. It is located in French, district of 9 de Julio in the province of Buenos Aires, Republic of Argentina.

In farrowing and post-weaning, work was done with an all-in-all-out system by rooms and the finishing fattening of the animals is done in a continuous flow system.

A total of 105 animals (5 animals/group) were used. They were genetic Agroceres Pig Improvement Company (PIC) females and males obtained from the crossing of females Camborough 1050 with male Terminal 337.

They were housed in rooms with a full slat floor, with a floodable pit. The density was 0.33 animals per m² at termination of fattening phase.

Ventilation by means of curtains (natural ventilation).

Food, water and usual handling of animals.

Food

Birth-15 days of life: Colostrum and milk

15 days to weaning (21d): milk and pre-starter food

21-30 days of life: food F1 (starter)

30-40 days of life: food F2

40-55 days of life: food F3 (with Amoxicillin 400ppm in the food)

55-70 days of life: food F4 (with Tiamulin 100ppm + Chlortetracycline 300ppm in the food)

Water

Water was provided ad libitum.

Number of pacifiers: 1 for every 10 animals housed.

Other handling during the experimentation period.

Upon admission to weaning boxes (approx. 21 days of age) piglets received a vaccination against PCV2 and *Mycoplasma hyopneumoniae*.

42 days of life: Pigs received *Mycoplasma* revaccination.

Application of injectable Ceftiofur or injectable amoxicillin in animals showing nervous system signs as a result of potential infection by *Streptococcus suis* or *Haemophilus parasuis*.

Treatments

Groups

Vaccine candidate evaluated:

- SV1: Candidate vaccine against *Streptococcus* spp (Ss)

Vaccine candidate	n	Volume	Route of Administration	
			1st Dose	2nd Dose
SV1				
Group 1	5	1 ml	PO	PO
Group 2	5	1 ml	IM	PO
Group 3	5	2 ml ND	PO	----
Group 4	5	2 ml ND	IM	----
Group 5	5	2 ml ND	PO	PO
Group 6	5	2 ml ND	IM	PO
Control (saline)				
Group 7	5	2 ml	IM	PO

Table 5: PO: (oral administration); IM (intramuscular)

Administration date

First dose: Within the first 3 days of life

Second dose: 14-18 days after the first vaccination.

Vaccination Management

Oral administration: the above indicated volume was administered using a dispenser or an automatic syringe.

Intramuscular administration: The above indicated volume was administered with an automatic syringe on the neck region just behind and below the ear, but in front of the shoulder.

Preparation of vaccines in the laboratory

10 microliters were taken from a cryovial from the SV1 working seed, which was plated on TSA plates with chloramphenicol (CP5: 5 mg/ml). The plates were incubated at 37 °C ON. The following day, a colony of the culture was cultured in TSB with CP5. The pre-culture (PC) was incubated at 37 °C overnight. The following day, a certain volume of TSB + CP5 was seeded into a certain large volume of sterile TSB. The culture was incubated for 2 hours at 37 °C, then a certain volume of 0.5 M IPTG (1 ml/L) was added to induce antigen production, and incubation was continued for another 6 hours.

After a total of 8 hours, samples were taken for colony forming unit calculations. Subsequently the culture was inactivated by the addition of a certain volume of formaldehyde and the culture was incubated at room temperature overnight.

The Excipient was prepared following the usual preparation process.

The formulations for the 1 ml and 2 ml dose groups were mixed in equal proportions of inactivated culture (SV1) to excipient (1:1).

Formulations were packed in 10 ml vial, labeled and stored between 2 – 8 °C until use.

Samples and parameters evaluated.

Sampling Date

Samples were taken on the following days:

- 1st sampling: Primary vaccination day (Week 1)
- 2nd sampling: Booster Day (Week 3)
- 3rd sampling: Week 4
- 4th sampling: Week 6
- 5th sampling: Week 8

Sampling and processing:

Serum Samples

Samples of blood were taken from the jugular vein with 18G x 1.5" needle for pigs and 20G x 1" or 1.5" for piglets. Around 5 ml of blood were taken without anticoagulant in a 15 ml conical tube. To extract serum from blood, the serum is allowed to coagulate in a tube at an angle of at least 60 degrees. Once the clot is formed, it is detached from the glass with the help of a needle. To obtain maximum serum, tubes were heated to 37 °C for a minimum of 30 minutes to a maximum of 1 hour and then placed at 4 °C overnight. The next morning, the tubes were centrifuged at 3000 rpm for 30 minutes and the serum (supernatant) was directly extracted with a pipette. The amount of serum collected was between 500 µl to 1 ml. Serum were stored at -20°C until analysis.

Saliva Samples

IgA determination from Saliva samples were taken as follows:

Oral swabs: Sterile cotton swabs were used to collect saliva from the pigs, the swabs were placed in the pig's mouth until soaked in saliva. The saliva-soaked swabs were

then submerged in 1 ml of sterile PBS, pH 7.4 in a 15 ml conical tube and stored at - 20°C until analysis.

Analysis

ELISA for antibody determination

ELISA S/P ratios were used to determine IgA and IgG antibody response to SV1 with the following formula:

Sample – negative control/positive control – negative control

ELISA protocol:

- 1) Coating: BioGreiner plates were coated with 5 µg/ml/well (100 µl/well) of the synthetic Streptococcus peptide antigen covalently linked to BSA and then incubated at room temperature overnight.
- 2) Plates were washed 3X with TBS-T (Tris-buffer saline with 0.1% Tween-20) (200 µl/well)
- 3) The plates were then blocked with 200 µl/well of blocking buffer (Bethyl lab, Cat. E104) and incubated 1 hour at room temperature.
- 4) Plates were washed 3X with TBS-T (200 µl/well)
- 5) Test Samples were diluted as follows:
 - Serum: 1:100 dilution in sample buffer (50 mM Tris buffered saline, pH 8, 1% bovine serum albumin (BSA), 0.05% Tween-20), 100 µl/well
 - Saliva: undiluted, 100 µl/well

The plate was incubated for 1 hour at room temperature.

6) Plates were washed 3X with TBS-T (200 µl/well). Secondary antibodies were added to each well, 100 µl/well, at the following concentrations.

- Anti-Pig IgA HRP 1 µg/ml (A100-102P, Bethyl laboratories)
- Anti-Pig IgG HRP 0.25 µg/ml (A100-205P, Bethyl laboratories)

Incubated for 1 hour at room temperature.

8) Plates were washed 3X with TBS-T (200 µl/well).

9) TMB One component Substrate (E102, Bethyl laboratories) was added (100 µl/well) and incubated 3 minutes at room temperature.

10) The reaction was stopped with 100 µl/well of 0.1 M sulfuric acid.

10) The optical density of each well was read at $\lambda = 450$ nm using a visible light spectrophotometer.

Statistical analysis

An analysis of variance was applied for two factors measured over time using the IBM SPSS Statistic 25 software after determining homoscedastic of variances, sphericity, and normality of the groups.

Experimental Design

The schematic diagram outlining experimental design is illustrated in Figure 3.

RESULTS

Two doses of candidate SV1 vaccine induces antigen-specific secretory IgA and serum IgG in piglets as young as three days of age.

Saliva secretory IgA (sIgA) S/P ratio determination.

Results are shown in Table 6 and in Figure 4. An increase in the antigen-specific salivary sIgA S/P ratios in pigs is observed throughout the weeks in some of the groups evaluated, when the comparison between groups is made within each week, no significant differences are observed (weeks 1, 3, 4 and 6). At week 8, the pigs that received two 2 mL doses of SV1 orally or IM followed by an oral booster, had significantly higher S/P ratio relative to the other groups, with no significant difference between the 2 mL oral/oral and 2 mL IM/oral group. These data indicate that the SV1 candidate vaccine is immunogenic and capable of inducing antigen-specific secretory IgA in pigs as early as 3 days of age, in particular a 2 mL/dose regimen given orally or IM/orally provides a robust sIgA response.

Group	Age of animal at time of sampling									
	Week 1		Week 3		Week 4		Week 6		Week 8	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2 ml Oral	-0.336 ^a	0.422	-0.438 ^a	1.267	0.966 ^a	0.862	0.630 ^a	0.554	0.867 ^{abc}	0.467
2 ml IM	0.251 ^a	0.810	-0.328 ^a	1.571	0.148 ^a	0.105	0.960 ^a	0.839	0.184 ^{ab}	0.900
1 ml Oral/Oral	0.231 ^a	0.803	0.487 ^a	0.718	0.372 ^a	0.546	0.503 ^a	0.174	0.449 ^{ab}	0.311
1 ml IM/Oral	-0.014 ^a	1.112	0.271 ^a	1.594	0.568 ^a	1.261	1.014 ^a	0.448	0.662 ^{abc}	0.483
2 ml Oral/Oral	0.279 ^a	0.479	0.036 ^a	1.314	1.223 ^a	0.836	1.648 ^a	0.902	2.054 ^{cd}	0.176
2 ml IM/Oral	-0.069 ^a	0.269	-0.375 ^a	0.786	0.648 ^a	0.276	1.212 ^a	0.782	2.183 ^d	0.541

Table 6

Serum IgG S/P ratio determination.

Results are shown in Table 7 and in Figure 5. An increase in the antigen-specific serum IgG S/P ratios is observed throughout the weeks in some of the groups evaluated, when the comparison between groups is made within each week, no significant differences

are observed (weeks 1, 3, and 4.). At weeks 6 and 8, the pigs that received two 2 mL doses of SV1 orally or IM followed by an oral booster, had significantly higher S/P ratio relative to the other groups. At 6 weeks of age, the 2 mL IM/Oral group had significantly higher mean S/P ratio than the 2 mL Oral/Oral group, but by 8 weeks of age this difference was no longer present. These data indicate that the SV1 candidate vaccine is immunogenic and capable of inducing antigen-specific serum IgG in pigs as early as 3 days of age, in particular a 2 mL/dose regimen given orally or IM/orally provides a robust serum IgG response.

Group	Age of animal at time of sampling									
	week 1		Week 3		Week 4		Week 6		Week 8	
	Mean	SD	Mean	SD	Mean	Group	Mean	SD	Mean	SD
2 mL Oral	-0.018 ^{ab}	0.038	-0.056 ^a	0.151	0.143 ^a	0.142	0.532 ^a	0.084	0.233 ^{ab}	0.172
2 mL IM	-0.015 ^{ab}	0.05	0.176 ^a	0.453	0.099 ^a	0.1	0.529 ^a	0.719	0.230 ^{ab}	0.174
1 mL Oral/Oral	-0.084 ^a	0.035	-0.107 ^a	0.065	0.123 ^a	0.56	0.609 ^{ab}	0.228	0.341	0.103
1 mL IM/Oral	-0.056 ^a	0.021	0.025 ^a	0.138	0.061 ^a	0.113	1.131 ^{abc}	0.526	0.519	0.339
2 mL Oral/Oral	-0.050 ^{ab}	0.031	-0.126 ^a	0.12	0.046 ^a	0.093	1.404 ^{abc}	0.61	1.668 ^d	0.496
2 mL IM/Oral	-0.048 ^{ab}	0.037	0.018 ^a	0.133	1.181 ^a	1.318	3.052 ^c	0.479	1.878 ^c	0.795

Table 7

Conclusion

These data indicate that candidate vaccine SV1 is immunogenic and antigenic and importantly, the vaccine can protect fish, in this example Nile tilapia, from a challenge with virulent *Streptococcus agalactiae*. Further, the SV1 vaccine induces a robust antigen-specific salivary sIgA and serum IgG response in piglets with the first dose of vaccine administration as early as 3 days of age. The route of administration of either two doses orally or one dose intramuscularly and the second booster dose given orally

provide similar responses for sIgA while the IM/oral induces a higher and quicker serum IgG response as would be expected based on scientists understanding of the immune response and activation, but the response at 8 weeks of age was similar suggesting the new antigenic specific baseline antibody response may not differ. While the volume providing the best antibody response in pigs was 2 mL, it is ultimately irrelevant as the concentration of antigen/mL can be adjusted so that one mL of vaccine could have the same concentration of antigen as a 2 mL dose.

Any method described herein may incorporate any design element contained within this application and any other document/application incorporated by reference herein.

The present invention may be embodied in other forms without departing from the spirit and the essential attributes thereof, and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention. The invention illustratively disclosed herein suitably may be practiced in the absence of any element which is not specifically disclosed herein.

Sequence Listing Information:

DTD Version: V1_3
File Name: SEQUENCE LISTING 3113.005.xml
Software Name: WIPO Sequence
Software Version: 2.3.0
Production Date: 2023-05-12

General Information:

Current application / IP Office: US
Current application / Applicant file reference: 3113.005
Applicant name: Ventanco SA
Applicant name / Language: en
Inventor name: Sherry Layton
Inventor name / Language: en
Invention title: COMPOSITIONS AND METHODS OF ENHANCING IMMUNE
RESPONSES TO STREPTOCOCCUS (en)

Sequence Total Quantity: 3

Sequences:

Sequence Number (ID): 1
Length: 49
Molecule Type: AA
Features Location/Qualifiers:
- source, 1..49
 > mol_type, protein
 > organism, synthetic construct

Residues:

DPVLVGQEAV AIGELLAESD PKKADYYCQN AATLEGKGPK LADKYSPIF
49

Sequence Number (ID): 2

Length: 878
Molecule Type: AA
Features Location/Qualifiers:
- source, 1..878
 > mol_type, protein
 > organism, synthetic construct

Residues:

MLKATSKTFV TQHTAFSYTA QRFGLKQLGI AGVSEEEESP PLTKVPGLED
MEAGQGIDEA 60
SLYDPHTWDP VLVGQEAVAI GELLAESDPK KADYYCQNAA TLEGKGPKLA
DKYSPIFSSS 120
SSMSGKGPAL GIDLGTITYSC VGVFQHGKVE IANDQGNRT TPSYVAFTDT
ERLIGDAAKN 180
QVAMNPTNTI FDAKRLIGRK YDDPTVQSDM KHWPFRVVNE
GGKPKVQVEY KGEMKTFFPE 240
EISSMVLTKM KEIAEAYLGK KVETAVITVP AYFNDSQRQA TKDAGTITGL
NVMRIINEPT 300

AAAIAYGLDK KGTRAGEKNV LIFDLGGGTF DVSILTIEDG IFEVKSTAGD
 THLGGEDFDN 360
 RMVNRVVEEF KGKHKRDNAG NKRAVRRRLRT ACERARRTLS SSTQASIEID
 SLFEGIDFYT 420
 SITRARFEEL NADLFRGTLE PVEKALRDAK LDKGQIQEIV LVGGSTRIPK
 IQKLLQDFFN 480
 GKELNKSINP DEAVAYGAAV QAAILMGDKS ENVQDLLLLD VTPLSLGIET
 AGGVMTALIK 540
 RNTTIPTKQT QTFTTYSNQN SSVLVQVYEG ERAMTKDNNL LGKFDLTGIP
 PAPRGVPQIE 600
 VTFDIDANGI LNVSAVDKST GKENKITITN DKGRLSKDDI DRMVQEAKEY
 KAEDEANRDR 660
 VGAKNSLESY TYNMKQTVED EKLKGKISDQ DKQKVLDKCQ EVISSLDRNQ
 MAEKEEYEHK 720
 QKELEKLCNP IVTKLYQGAG GAGAGGSGGP TIEEVDSSSS SSDPVLVGQE
 AVAIGELLAE 780
 SDPKKADYYC QNAATLEGKG PKLADKYSPI FPLTKVPGLE DMEAGQGIDE
 ASLYDPHTWL 840
 KATSKTFVTQ HTAFSYTAQR FGLKQLGIAG VSEEEPS
 878

Sequence Number (ID): 3

Length: 2643

Molecule Type: AA

Features Location/Qualifiers:

- source, 1..2643
 - > mol_type, protein
 - > organism, synthetic construct

Residues:

ATGTTAAAGG CTACATCCAA GACTTTTGTG ACACAGCACA
 CGGCTTTCAG TTATACAGCG 60
 CAACGGTTTG GCTTGAAGCA GTTAGGGATT GCAGGTGTGA
 GTGAAGAGGA ACCGTCCCCT 120
 CCATTAACAA AAGTCCCTGG GTTGAAGAC ATGGAGGCCG
 GGCAAGGGAT TGATGAAGCC 180
 TCACTCTATG ATCCTCATAC TTGGTTGGAC CCGGTGCTTG
 TCGGTCAGGA GCGGTAGCC 240
 ATCGGAGAGC TTTTGGCTGA ATCAGATCCG AAGAAAGCTG
 ATTATTACTG CCAAACGCG 300
 GCTACTTTAG AGGGTAAAGG GCCAAAGCTC GCTGACAAGT
 ATTCACCGAT ATTTTCATCG 360
 AGTTCATCAA TGAGCGGAAA GGGCCCTGCC ATTGGAATCG
 ATCTGGGCAC AACATACAGC 420
 TGTGTGGGAG TATTTTCAGCA CGGCAAGGTT GAAATCATTG
 CAAATGACCA AGGCAACAGA 480

ACAACGCCGT CATATGTCGC TTTTACAGAT ACTGAACGCC
 TCATCGGCCGA CGCTGCCAAA 540
 AACCAAGTGG CGATGAATCC GACAAATACG ATTTTCGACG
 CGAAACGTCT CATTGGACGG 600
 AAATACGATG ATCCAACAGT ACAATCTGAT ATGAAACATT
 GGCCGTTTCG GGTCGTCAAC 660
 GAAGGAGGAA AGCCCAAAGT GCAGGTCTGAG TATAAAGGAG
 AAATGAAAAC TTTCTTTCCC 720
 GAAGAGATAA GTTCCATGGT TTTAACAAAA ATGAAGGAAA
 TTGCCGAGGC TTATTTAGGA 780
 AAAAAGGTGG AAACGGCGGT GATCACCGTG CCGGCATATT
 TTAATGATAG CCAGCGCCAG 840
 GCAACCAAGG ATGCGGGAAC CATTACAGGC TTAAATGTTA
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 ACTGCCGCAG CAATCGCTTA CGGACTGGAT AAAAAAGGAA
 CAAGAGCAGG CGAAAAGAAT 960
 GTACTGATTT TTGATTTAGG GGGAGGTACC TTCGACGTGT
 CCATTCTTAC TATAGAAGAC 1020
 GGCATCTTTG AAGTGAAATC AACAGCAGGA GATACACACT
 TGGGCGGTGA GGATTTTGAC 1080
 AACCGGATGG TCAATAGATT CGTCGAAGAA TTAAAGGAA
 AGCATAAACG CGACAATGCC 1140
 GGGAAACAAA GGGCTGTACG ACGGCTGAGG ACTGCCTGTG
 AAAGAGCGCG GCGTACGCTC 1200
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 CGGTTGCATA TGGCGCCGCC 1500
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 GGGTCATGAC GGCTTTGATT 1620
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 TTACGACGTA CAGCGATAAT 1680
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 CAATGACAAA AGATAACAAC 1740
 CTTTTGGGGA AATTTGATCT CACAGGGATT CCGCCTGCAC
 CAAGGGGTGT GCCTCAAATT 1800
 GAAGTAACCT TTGATATCGA CGCCAACGGG ATCTTGAATG
 TTAGTGCTGT TGATAAGAGT 1860

ACCGGCAAGG AAAATAAAAT TACAATAACA AACGATAAAG
 GCAGACTATC TAAAGACGAT 1920
 ATTGACCGCA TGGTGCAAGA GGCTGAAAAG TACAAAGCAG
 AGGATGAAGC AAACCGTGAC 1980
 CGTGTAGGAG CAAAAAACAG CTTAGAAAGC TATACATATA
 ATATGAAACA AACAGTGGAA 2040
 GATGAGAAAC TGAAGGGGAA AATAAGCGAT CAAGACAAAC
 AGAAAGTTCT TGATAAATGC 2100
 CAGGAAGTCA TCTCATCTTT AGATAGAAAT CAAATGGCGG
 AGAAAGAAGA ATATGAGCAT 2160
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 CGAAACTTTA TCAAGGAGCC 2220
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 CAATTGGTGA ATTGCTGGCA 2340
 GAATCTGATC CAAAAAAGC GGAATTAT TGTCAAATG
 CAGCCACGCT TGAAGGTAAA 2400
 GGTCCGAAAC TAGCCGATAA ATACTCTCCT ATCTTCCCAT
 TAACAAAAGT CCCTGGGTTG 2460
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 TCTATGATCC TCATACTTGG 2520
 TTGTTAAAGG CTACATCCAA GACTTTTGTT ACACAGCACA
 CGGCTTTCAG TTATACAGCG 2580
 CAACGGTTTG GCTTGAAGCA GTTAGGGATT GCAGGTGTGA
 GTGAAGAGGA ACCGTCCCCT 2640
 TAA 2643
 END

CLAIMS

1. A vaccine composition against *Streptococcus* comprising:
an amino acid sequence selected from the group consisting of Sequence Number (ID): 1, Sequence Number (ID): 2, and Sequence Number (ID): 3; and
a pharmaceutically acceptable carrier.
2. The vaccine composition of claim 1 wherein the vaccine is derived from a protective antigen on which nucleotide optimization was used to optimize for *Bacillus subtilis* to derive the nucleotide sequence for the DNA sequence.
3. The vaccine composition of claim 1 further comprising one or more immunostimulatory polypeptides.
4. The vaccine composition of claim 1 wherein the vaccine composition induces an immune response.
5. The vaccine composition of claim 4 wherein the immune response includes an antibody response.
6. The vaccine composition of claim 1 wherein the vaccine composition is administered by a method selected from the group consisting of oral, intranasal, and parenteral.

7. A method of enhancing the immune response against *Streptococcus* comprising administering to the subject the vaccine composition of claim 1 in an amount effective to enhance the immune response of the subject to one or more strains of *Streptococcus*.
8. The method of claim 7 wherein the vaccine composition induces an immune response.
9. The method of claim 7, wherein the immune response includes an antibody response.
10. The method of claim 7, wherein the vaccine is administered by a method selected from the group consisting of oral, intranasal, and parenteral.

ABSTRACT

A vaccine composition for mammals/Fish against *Streptococcus* comprising an amino acid sequence selected from the group consisting of Sequence Number (ID): 1, Sequence Number (ID): 2, and Sequence Number (ID): 3, and a pharmaceutically acceptable carrier.

Results:

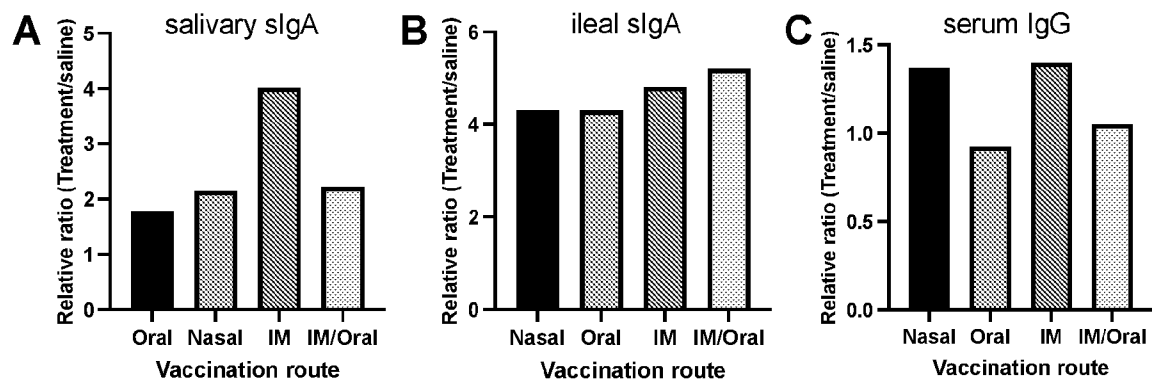


Figure 1

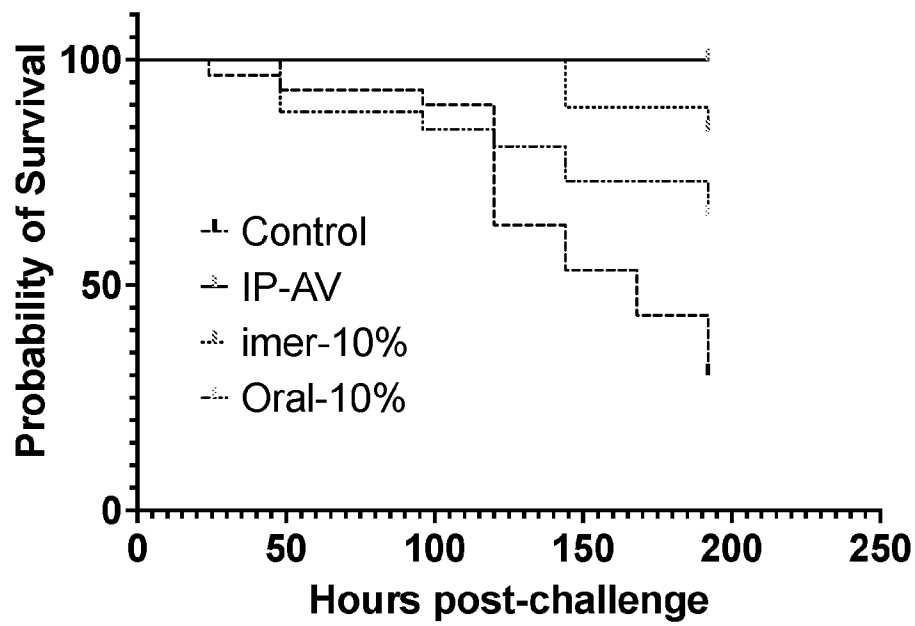


Figure 2

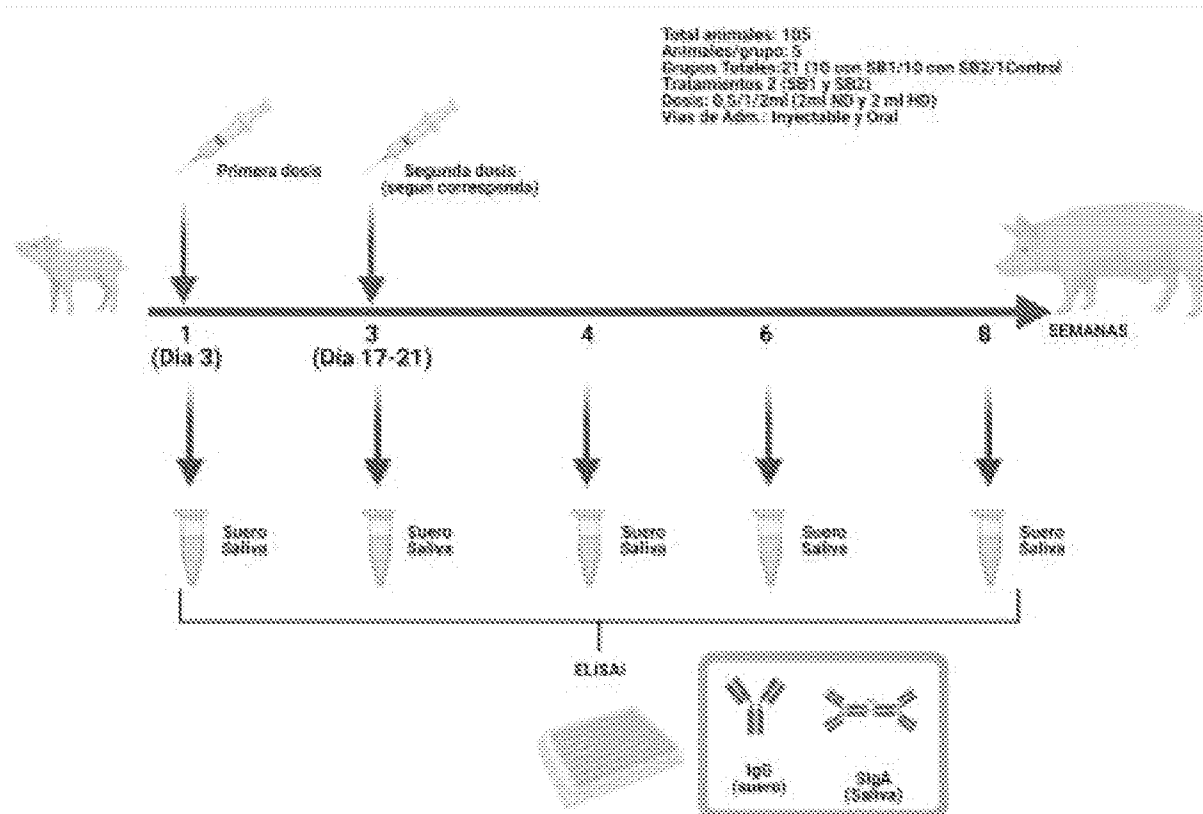


Figure 3

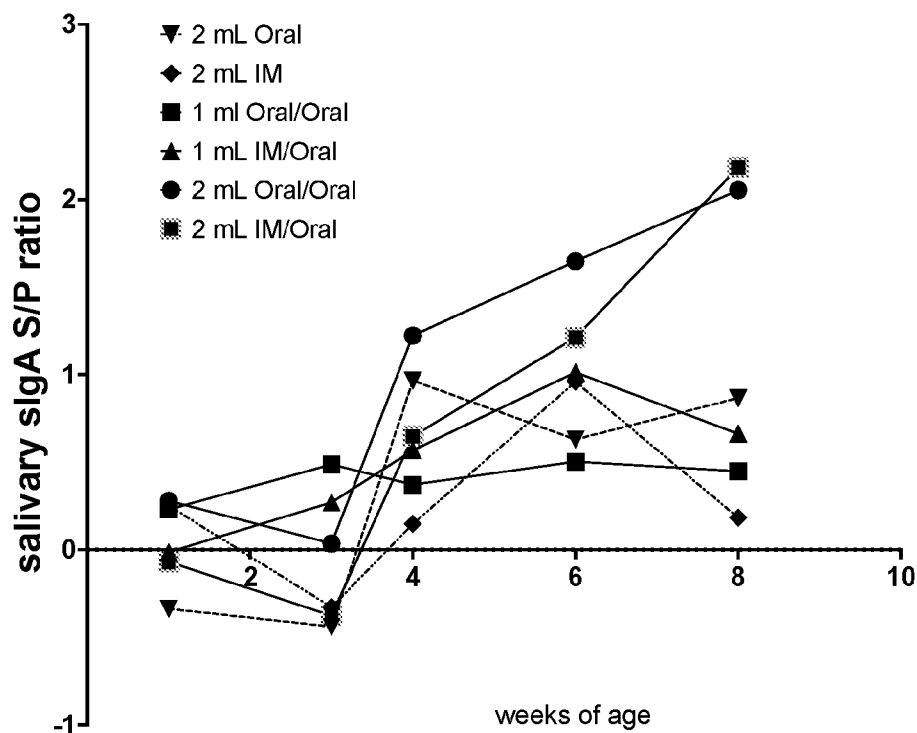


Figure 4

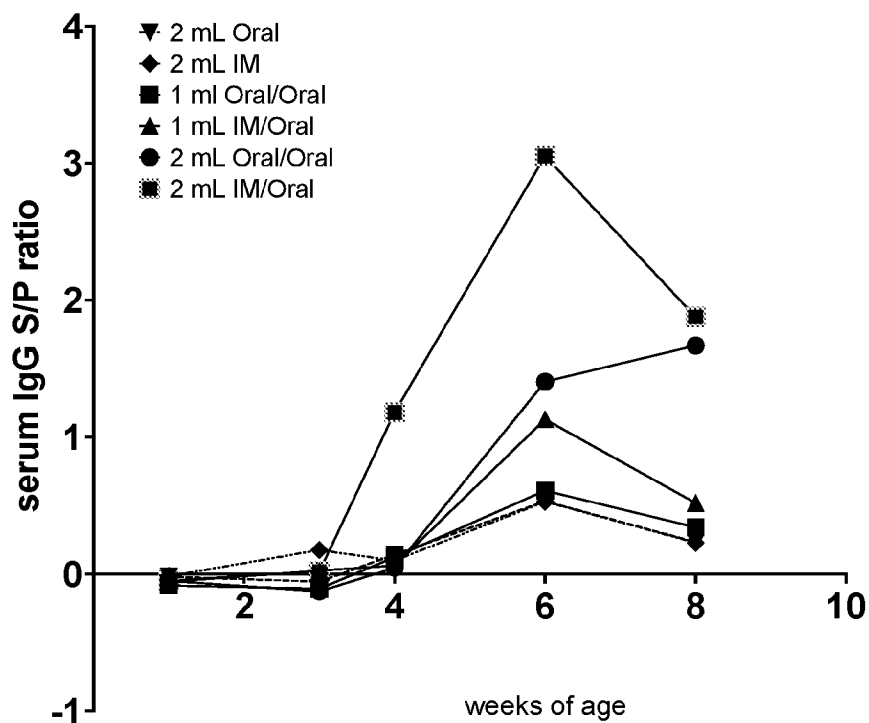


Figure 5

Electronic Acknowledgement Receipt

EFS ID:	48294712
Application Number:	18221713
International Application Number:	
Confirmation Number:	5947
Title of Invention:	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS
First Named Inventor/Applicant Name:	Sheryll Layton
Customer Number:	126354
Filer:	Blake Edward Van Garde/Debbie Denlinger
Filer Authorized By:	Blake Edward Van Garde
Attorney Docket Number:	3113.005
Receipt Date:	13-JUL-2023
Filing Date:	
Time Stamp:	16:52:18
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	CARD
Payment was successfully received in RAM	\$664
RAM confirmation Number	E20237CG54356808
Deposit Account	502790
Authorized User	Debbie Denlinger
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: 37 CFR 1.16 (National application filing, search, and examination fees) 37 CFR 1.17 (Patent application and reexamination processing fees)	

37 CFR 1.19 (Document supply fees)

37 CFR 1.20 (Post Issuance fees)

37 CFR 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Application Data Sheet	aia0014_ADS.pdf	1256063	no	8
			6a719360aa030fefe4c9361845486167ee13c7d6		
Warnings:					
Information:					
2	Transmittal of New Application	aia0015_Utility_Transmittal.pdf	332855	no	2
			233ca7c3234128208d719b0ff776760cff5d8ffa		
Warnings:					
Information:					
3		Specification_Final_20230713.pdf	273401	yes	38
			7b36c80113306313daf4a1b524baf4dc175a06a5		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Specification		1	32	
	Claims		33	34	
	Abstract		35	35	
	Drawings-only black and white line drawings		36	38	
Warnings:					
Information:					
4	Chemical Formulae (Text File)	txt_COMPOSITIONS_AND_METHODS_OF_ENHANCING_IMMUNE_RESPONSES_TO_STREPTOCOCCUS.txt	5907	no	-
Warnings:					
Information:					

5	Fee Worksheet (SB06)	fee-info.pdf	44244	no	2
			93b2454946d922a1e7f312681f9ee2a961f3b60b		

Warnings:

Information:

Total Files Size (in bytes):	1912470
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This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	3113.005
		Application Number	
Title of Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS		
The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76. This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.			

Secrecy Order 37 CFR 5.2:

☐	Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)
--------------------------	---

Inventor Information:

Inventor	1				Remove	
Legal Name						
Prefix	Given Name	Middle Name	Family Name	Suffix		
	Sherryl		ayton			
Residence Information (Select One) • US Residency Non US Residency Active US Military Service						
City	Quitman	State/Province	AR	Country of Residence	US	
Mailing Address of Inventor:						
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Address 2						
City	Quitman	State/Province	AR			
Postal Code	72131	Country i	US			
Inventor	2				Remove	
Legal Name						
Prefix	Given Name	Middle Name	Family Name	Suffix		
	Jeffrey	W.	Hall			
Residence Information (Select One) • US Residency Non US Residency Active US Military Service						
City	Minneapolis	State/Province	MN	Country of Residence	US	
Mailing Address of Inventor:						
Address 1	3608 Boardman Street					
Address 2						
City	Minneapolis	State/Province	MN			
Postal Code	55417	Country i				
All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the Add button.						

Correspondence Information:

Enter either Customer Number or complete the Correspondence Information section below. For further information see 37 CFR 1.33(a).
--

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	3113.005
		Application Number	
Title of Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS		

☐ An Address is being provided for the correspondence information of this application.

Customer Number	126354		
Email Address	ekdkdocket@kcpatentlaw.com	Add Email	Remove Email
Email Address	bvg@kcpatentlaw.com	Add Email	Remove Email

Application Information:

Title of the Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS		
Attorney Docket Number	3113.005	Small Entity Status Claimed	☒
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	3	Suggested Figure for Publication (if any)	1

Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

Application number of the previously filed application	Filing date (YYYY-MM-DD)	Intellectual Property Authority or Country

Publication Information:
☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not be** the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32). Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

Please Select One:	☒ Customer Number	US Patent Practitioner	☐ Limited Recognition (37 CFR 11.9)
Customer Number	126354		

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	3113.005
		Application Number	
Title of Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS		

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78. When referring to the current application, please leave the "Application Number" field blank.

Prior Application Status		Remove
Application Number	Continuity Type	Prior Application Number
Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the Add button.		Add

Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)ⁱ the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Application Number	Country ⁱ	Filing Date (YYYY-MM-DD)	Access Code ^j (if applicable)
Additional Foreign Priority Data may be generated within this form by selecting the Add button.			Add

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013. NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

Application Data Sheet 37 CFR 1.76		Attorney Docket Number	3113.005
		Application Number	
Title of Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS		

Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

NOTE: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)

A. Priority Document Exchange (PDX) - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

B. Search Results from U.S. Application to EPO - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

NOTE: Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	3113.005
		Application Number	
Title of Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS		

Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

Applicant	1	Remove
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If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

[Clear](#)

☒ Assignee Person to whom the inventor is obligated to assign.	☐ Legal Representative under 35 U.S.C. 117 Person who shows sufficient proprietary interest	☐ Joint Inventor
--	--	--------------------------------------

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☐

Prefix	Given Name	Middle Name	Family Name	Suffix

Mailing Address Information For Applicant:

Address 1			
Address 2			
City		State/Province	
Country		Postal Code	
Phone Number		Fax Number	
Email Address			

Additional Applicant Data may be generated within this form by selecting the Add button. [Add](#)

Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	3113.005
	Application Number	
Title of Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS	

Assignee	1
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Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

Prefix	Given Name	Middle Name	Family Name	Suffix

Mailing Address Information For Assignee including Non-Applicant Assignee:

Address 1				
Address 2				
City		State/Province		
Country i		Postal Code		
Phone Number		Fax Number		
Email Address				

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

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Signature:

Remove

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). However, if this Application Data Sheet is submitted with the **INITIAL** filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

Signature	/Blake E. Vande Garde/		Date (YYYY-MM-DD)	2023-07-13
First Name	Blake	Last Name	Vande Garde	Registration Number
				58264

Additional Signature may be generated within this form by selecting the Add button.

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Application Data Sheet 37 CFR 1.76		Attorney Docket Number	3113.005
		Application Number	
Title of Invention	COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO STREPTOCOCCUS		

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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The information provided by you in this form will be subject to the following routine uses:

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6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.
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