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(71) Applicant: UNIVERSITY OF GEORGIA RESEARCH

FOUNDATION, INC. [US/US]; 210 Jackson Street, 110 Terrell Hall, Athens, Georgia 30602 (US).

(72) Inventor: SHARIAT, Nusrat; c/o University of Georgia

Research Foundation, INC., 210 Jackson Street, 110 Terrell Hall, Athens, Georgia 30602 (US).

(74) Agent: JARECKI-BLACK, Judy; Smith, Gambrell &

Russell, LLP, 1105 W. Peachtree Street NE, Suite 1000, Atlanta, Georgia 30309 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR A DIFFERENTIAL DIAGNOSTIC FOR SALMONELLA TYPHIMURIUM INFECTION IN POULTRY

(57) Abstract: A PCR diagnostic assay, methods and a kit for differentiating between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against *Salmonella* Typhimurium.

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COMPOSITIONS AND METHODS FOR A DIFFERENTIAL DIAGNOSTIC FOR SALMONELLA TYPHIMURIUM INFECTION IN POULTRY

REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit to and priority to United States Provisional Application Serial Number 63/506,762, filed on June 7, 2023, and United States Provisional Application Serial Number 63/507,363, filed on June 9, 2023, which are hereby incorporated by this reference in their entireties.

FIELD

[0002] This invention relates generally to compositions, methods and kits for rapidly and differentially identifying field and vaccine strains of *Salmonella* Typhimurium.

BACKGROUND

[0003] *Salmonella*, a genus of rod-shaped gram-negative bacteria of the family Enterobacteriaceae, is further divided into two species are *Salmonella enterica*, comprising six subspecies with over 2600 serotypes, and *Salmonella bongori*. *Salmonella* spp. are facultative intracellular pathogens causing localized or systemic infections, in addition to a chronic asymptomatic carrier state. They are of worldwide economic and public health significance as various *Salmonella* serotypes can infect a variety of animals, poultry and birds, and man. For example, in poultry, fowl typhoid and pullorum disease continue to cause economic losses in those parts of the world where the poultry industries are continuing to intensify and where open sided housing is common. (U.S. Patent No. 8,062,645, the contents of which is hereby incorporated by reference in its entirety). *Salmonella enterica* serovars Typhimurium (*S. typhimurium*) and Enteritidis are known to represent the main risk for public health, with cases of gastroenteritis occurring in humans as a result of infection increasing making *Salmonella* one of the major causes of food-borne illnesses. One study estimates that approximately 1.4 million cases of salmonellosis occur amongst humans in the United States alone (Mead PS, et al., Food-related illness and death in the United States. Emerg Infect Dis. 1999; 607–625).

[0004] The severity of the symptoms associated with *Salmonella* infection depends upon several factors including the type of strain associated with the infection and the age and health status of the host, with children, the elderly, and individuals who are

immunocompromised most severely affected (Acevedo-Villanueva, KY et al, A Novel Approach against Salmonella: A Review of Polymeric Nanoparticle Vaccines for Broilers and Layers. *Vaccines* 2021: 1041). Although the infectious dose varies among *Salmonella* strains and host status, the infective dose for *Salmonella* can be as low as one cell (Food and Drug Administration. *Salmonella* species. In “Bad Bug Book” Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins; Food and Drug Administration: Silver Spring, MD, USA, 2012; Volume 2, pp. 9–13, ISBN 9780323401814).

[0005] More than 70% of human salmonellosis cases have been linked to the consumption of contaminated chicken, chicken products, or eggs (Andino, A, et al, *Salmonella enterica*: Survival, colonization, and virulence differences among serovars. *Sci. World J.* 2015: 520179). One approach to reduce human disease is to vaccinate chickens in broilers and in the hatchery. Although numerous attempts have been made to protect chickens and mammals by immunization with a variety of vaccines, many of the vaccines provide only poor to moderate protection and often require large doses to be completely efficacious. Previously investigated vaccines against salmonellae have generally fallen into four categories: (i) subunit vaccines, including cell fractions or lysates, intact antigens, fragments thereof, or synthetic analogs of naturally occurring antigens or epitopes; (ii) antiidiotypic antibodies; (iii) killed vaccines which corresponds to the whole killed etiologic agent; or (iv) an avirulent or attenuated derivative of the etiologic agent used as a live vaccine. Amongst these, subunit vaccines and whole-cell killed vaccines are in use but give variable results in the prevention of *Salmonella* infections in animals.

[0006] Other vaccine approaches are in research and development. For example, oral administration of live cells from *Salmonella* Typhimurium strains having attenuating deletions in the *cya* and *crp* genes has been shown to provide excellent protection against wild-type *Salmonella* challenge in chickens (Hassan et al., *Res. Microbiol.* 141:839-850, 1990; Hassan et al., *Infect. Immun.* 62:5519-5527, 1994) and systems for stable expression of heterologous antigens in such strains have been developed (Hone et al., *Microb. Pathog.* 5:407-418, 1988; Strugnelli, et al., *Gene* 88:57-63, 1990; Galan et al., *Gene* 94:29-35; 1990; Nakayama et al., *Bio/Technology* 6:693-697, 1995).

[0007] In poultry a number of commercial vaccines, both live and inactivated, are available. For example, Poulvac® ST, from Zoetis, is a live vaccine composed of a

modified-live *Salmonella* Typhimurium, which is claimed as effective for the vaccination of healthy chickens against *Salmonella enteritidis*, *Salmonella* Heidelberg or *Salmonella* Typhimurium colonization of the internal organs, including the intestines and ceca. Live attenuated *Salmonella* vaccines have been found to be superior to inactivated preparations owing to: (i) their ability to induce cell-mediated immunity in addition to antibody responses; (ii) effectiveness after single-dose administration; (iii) induction of immune responses at multiple mucosal sites; (iv) low production cost; and (v) their possible use as carriers for the delivery of recombinant antigens to the immune system. The ability of live attenuated *Salmonella* to colonize the gut-associated lymphoid tissue (GALT; Fever's patches) and the deep tissues following oral administration is the factor that aids stimulation of all arms of the immune response, including mucosal, humoral and cellular immunities.

[0008] While the application of live attenuated *Salmonella* Typhimurium vaccines has significantly helped control *Salmonella* in poultry products, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) scores all *Salmonella* as positive, regardless of origin of the serovar. Attenuated vaccine strains that are identified during poultry processing contribute negatively toward *Salmonella* performance standards. Live attenuated vaccines can remain viable in vaccinated poultry. Therefore, when testing for *Salmonella* in vaccinated poultry, it is unknown if the detected virus is a wild type (WT) strain or a vaccine strain given to the bird to elicit immunity.

[0009] Live attenuated *Salmonella* vaccines are a critical pre-harvest tool for *Salmonella* control and are widely used in industry. With forthcoming regulations that will likely focus on *Salmonella* Typhimurium, along with other serovars, there is a need to distinguish between isolates belonging to the vaccine strain and those that are responsible for causing human illness. The ability to differentiate between WT and vaccine strains would prevent unnecessary decontamination procedures and help processing facilities properly evaluate safety procedures. Therefore, there is a need to provide a diagnostic assay for rapid detection of *Salmonella* Typhimurium and differentiate between WT and vaccine strains.

[0010] Thus, the present invention provides a method to differentiate between wild type (virulent) *Salmonella* Typhimurium and vaccine associated *Salmonella*

Typhimurium. In one embodiment the method contemplates the use of a PCR assay to distinguish between wild-type and vaccine-associated *Salmonella* Typhimurium.

SUMMARY

[0011] In accordance with the purpose(s) of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to a diagnostic assay for differentially identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject comprising primers for detecting *Salmonella* Typhimurium target sequences.

[0012] In one embodiment, the subject is a farm animal selected from poultry, pigs, or cattle. In another embodiment, the subject is a poultry animal selected from a group consisting of chickens, turkeys, geese, ducks, pheasants, bantam, quail, and pigeons. In yet another embodiment, the subject has been vaccinated against a wild-type virulent *Salmonella* serotype.

[0013] In yet another embodiment, the primers detect *Salmonella* Typhimurium cAMP receptor protein (Crp) gene target sequences within a *Salmonella* Typhimurium cAMP receptor protein (Crp) gene, the gene having a nucleic acid sequence of SEQ ID NO:1. In another embodiment, the primers detect *Salmonella* Typhimurium adenylate cyclase (CYA) gene target sequences within a *Salmonella* Typhimurium adenylate cyclase (CYA) gene, the gene having a nucleic acid sequence of SEQ ID NO:2. The *Salmonella* Typhimurium cAMP receptor protein (Crp) gene target sequence are selected from SEQ ID NO: 3, 4 or 5. And the *Salmonella* Typhimurium adenylate cyclase (CYA) gene target sequence are selected from SEQ ID NO: 6, 7 or 8. The primers for detecting the *Salmonella* Typhimurium cAMP receptor protein (Crp) gene target sequence are selected from SEQ ID NO: 9, 10, 11, 12, and 13. The primers for detecting the *Salmonella* Typhimurium adenylate cyclase (CYA) gene target sequence are selected from SEQ ID NO: 14, 15, 16, and 17.

[0014] In one embodiment, the primers detect transposon Tn10 in the *Salmonella* Typhimurium cAMP receptor protein (Crp) gene and *Salmonella* Typhimurium adenylate cyclase (CYA) gene target sequences of the vaccine-associated *Salmonella* serotype. In another embodiment, transposon Tn10 in the Crp gene of *Salmonella* Typhimurium, is detected using the forward primer having a nucleotide sequence as set forth in SEQ ID NO. 13 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 11.

In another embodiment, the transposon Tn10 in the Crp gene of *Salmonella* Typhimurium, is detected using the forward primer having a nucleotide sequence as set forth in SEQ ID NO. 17 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 16.

[0015] In another aspect, the invention relates to a method for differentially diagnosing between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium comprising: (a) extracting sample genomic DNA from the subject; (b) adding samples: adding the sample genomic DNA, a positive control or a negative control into PCR tubes of PCR reaction system respectively to obtain a corresponding sample reaction tube, positive reaction tube or negative reaction tube, wherein the PCR reaction system contains the primers as described above for detecting a Crp or CYA gene; (c) performing PCR reaction: placing the reaction tubes on a PCR instrument, setting circulation parameters, and performing PCR reaction; (d) analyzing results after the PCR reaction is completed; and (e) determining the presence of Tn10 in the sample; wherein the presence of Tn10 indicates the presence vaccine-associated *Salmonella* Typhimurium.

[0016] In yet another aspect, the invention relates to a PCR diagnostic assay kit for differentiating between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium comprising: (a) primers as described for detecting Tn10 in the Crp or CYA genes of *Salmonella* Typhimurium, and the primers for detecting Tn10 comprising a forward primer having a nucleotide sequence as set forth in SEQ ID NO. 13 or 17 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 11 or 16; (b) primers for wild type virulent *Salmonella* Typhimurium, and the primers for detecting wild type virulent *Salmonella* Typhimurium comprising a forward primer having a nucleotide sequence as set forth in SEQ ID NO. 9 or 14 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 10 or 15; (c) control primers for a mutant *Salmonella* Typhimurium, and the primers for detecting mutant *Salmonella* Typhimurium comprising a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 12; (d) DNA polymerase; (e) blocking oligonucleotides; (f) a detectable label; and (g) buffers.

[0017] Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate (one) several embodiment(s) of the invention and together with the description, serve to explain the principles of the invention.

[0019] Figure 1 is a bar graph showing the percentage of *Salmonella* positive samples collected between 2016 to 2022 by the United States Department of Agriculture Food Safety and Inspection Services (USDA-FSIS) from chicken samples.

[0020] Figures 2A-2B are bar graphs showing the amount of Typhimurium due to the vaccine identified by the United States Department of Agriculture Food Safety and Inspection Services (USDA-FSIS) in isolates collected between 2016-2022.

[0021] Figure 2C is a schematic showing the search of whole genome sequencing data (WGS) for isolates of the live attenuated *Salmonella* Typhimurium isolates through a BLAST search for WT and VAC sequences.

[0022] Figure 3A shows an illustration of the creation of unique DNA sequences to *Salmonella* Typhimurium CRP wild type (WT) field strains and vaccine (VAC) strains.

[0023] Figure 3B shows an illustration of the Crp mutant assay.

[0024] Figure 3C shows a PCR assay comparing the primers to the *Salmonella* Typhimurium CRP wild type (WT) field strains and the primers to the vaccine (VAC) strains.

[0025] Figure 4A shows an illustration of the creation of unique DNA sequences to *Salmonella* Typhimurium CYA wild type (WT) field strains and vaccine (VAC) strains.

[0026] Figure 4B show a PCR assay comparing the primers to the *Salmonella* Typhimurium CYA wild type (WT) field strains and the primers to the vaccine (VAC) strains.

[0027] Figure 5A is a PCR assay showing the screening samples that contain Typhimurium for vaccine strain. Some samples consist of mixed serotypes.

[0028] Figure 5B is a multiplex PCR assay using primers for crp: combine primers for PCR-WT and PCR-3.

DETAILED DESCRIPTION

[0029] The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

I. Definitions

[0030] To facilitate an understanding of the principles and features of the various embodiments of the disclosure, various illustrative embodiments are explained herein. Although exemplary embodiments of the disclosure are explained in detail, it is to be understood that other embodiments are contemplated. Accordingly, it is not intended that the disclosure is limited in its scope to the details of construction and arrangement of components set forth in the description or examples. The disclosure is capable of other embodiments and of being practiced or carried out in various ways.

[0031] In describing the exemplary embodiments, specific terminology will be resorted to for the sake of clarity. As used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural references unless the context clearly dictates otherwise. For example, reference to a component is intended also to include composition of a plurality of components. References to a composition containing “a” constituent is intended to include other constituents in addition to the one named.

[0032] Ranges may be expressed herein as from “about” or “approximately” or “substantially” one particular value and/or to “about” or “approximately” or “substantially” another particular value. When such a range is expressed, other exemplary embodiments include from the one particular value and/or to the other particular value.

[0033] Similarly, as used herein, “substantially free” of something, or “substantially pure”, and like characterizations, can include both being “at least substantially free” of something, or “at least substantially pure”, and being “completely free” of something, or “completely pure.”

[0034] By “comprising” or “containing” or “including” is meant that at least the named compound, element, particle, or method step is present in the composition or article or method, but does not exclude the presence of other compounds, materials, particles, method steps, even if the other such compounds, material, particles, method steps have the same function as what is named. The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

[0035] The term "and/or" means one or all of the listed elements or a combination of any two or more of the listed elements.

[0036] The words "preferred" and "preferably" refer to embodiments of the invention that may afford certain benefits, under certain circumstances. However, other embodiments may also be preferred, under the same or other circumstances. Furthermore, the recitation of one or more preferred embodiments does not imply that other embodiments are not useful and is not intended to exclude other embodiments from the scope of the invention.

[0037] Unless otherwise indicated, all numbers expressing quantities of components, molecular weights, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless otherwise indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0038] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. All numerical values, however, inherently contain a range necessarily resulting from the standard deviation found in their respective testing measurements.

[0039] For any method disclosed herein that includes discrete steps, the steps may be conducted in any feasible order. And, as appropriate, any combination of two or more steps may be conducted simultaneously.

[0040] The description exemplifies illustrative embodiments. In several places throughout the application, guidance is provided through lists of examples, which examples can be used in various combinations. In each instance, the recited list serves only as a representative group and should not be interpreted as an exclusive list.

[0041] All headings are for the convenience of the reader and should not be used to limit the meaning of the text that follows the heading, unless so specified.

[0042]

[0043] Unless otherwise specified, the experimental methods, detection methods, and preparation methods disclosed in the present invention all adopt the conventional molecular biology, biochemistry, chromatin structure and analysis, analytical chemistry, cell culture, recombinant DNA technology and related fields in the technical field. These techniques have been well described in the existing literature. For details, please refer to Sambrook et al. MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel et al., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; the series METHODS IN ENZYMOLOGY, Academic Press, San Diego; Wolfe, CHROMATIN STRUCTURE AND FUNCTION, Third edition, Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol.304, Chromatin (PM Wassarman and AP Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, Chromatin Protocols (PB Becker, ed.) Humana Press, Totowa, 1999, et al.

[0044] The terms “oligonucleotide” and “polynucleotide” as used interchangeably herein refer to a polymer of greater than one nucleotide in length of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), hybrid RNA/DNA, modified RNA or DNA, or RNA or DNA mimetics. The polynucleotides may be single- or double-stranded. The terms include polynucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as polynucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted polynucleotides are well-known in the art and for the purposes of the present invention, are referred to as “analogues.”

[0045] The terms “primer” and “polynucleotide primer,” as used herein, refer to a short, single-stranded polynucleotide capable of hybridizing to a complementary sequence in a nucleic acid sample. A primer serves as an initiation point for template-

dependent nucleic acid synthesis. Nucleotides are added to a primer by a nucleic acid polymerase in accordance with the sequence of the template nucleic acid strand. A “primer pair” or “primer set” refers to a set of primers including a 5' upstream primer that hybridizes with the 5' end of the sequence to be amplified and a 3' downstream primer that hybridizes with the complementary 3' end of the sequence to be amplified. The term “forward primer” as used herein, refers to a primer which anneals to the 5' end of the sequence to be amplified. The term “reverse primer”, as used herein, refers to a primer which anneals to the complementary 3' end of the sequence to be amplified.

[0046] The terms “probe” and “polynucleotide probe,” as used herein, refer to a polynucleotide used for detecting the presence of a specific nucleotide sequence in a sample. Probes specifically hybridize to a target nucleotide sequence, or the complementary sequence thereof, and may be single- or double-stranded.

[0047] The terms “annealing” and “hybridization” are used interchangeably and mean the base-pairing interaction of one nucleic acid with another nucleic acid that results in formation of a duplex or other higher-ordered structure. The primary interaction is base specific, i.e. A/T and G/C, by Watson/Crick and Hoogsteen-type hydrogen bonding.

[0048] As used herein, the phrase “hybridization conditions” or “stringent hybridization conditions” refers to hybridization conditions which can take place under a number of pH, salt and temperature conditions. The pH can vary from 6 to 9, preferably 6.8 to 8.5. The salt concentration can vary from 0.15 M sodium to 0.9 M sodium, and other cations can be used as long as the ionic strength is equivalent to that specified for sodium. The temperature of the hybridization reaction can vary from 30° C. to 80° C., preferably from 45° C. to 70° C. Additionally, other compounds can be added to a hybridization reaction to promote specific hybridization at lower temperatures, such as at or approaching room temperature. Among the compounds contemplated for lowering the temperature requirements is formamide. Thus, a polynucleotide is typically “substantially complementary” to a second polynucleotide if hybridization occurs between the polynucleotide and the second polynucleotide. As used herein, “hybridization” or “specific hybridization” refers to hybridization between two polynucleotides under stringent hybridization conditions.

[0049] The term “specifically hybridize,” as used herein, refers to the ability of a polynucleotide to bind detectably and specifically to a target nucleotide sequence. Polynucleotides, oligonucleotides and fragments thereof specifically hybridize to target nucleotide sequences under hybridization and wash conditions that minimize appreciable amounts of detectable binding to non-specific nucleic acids. High stringency conditions can be used to achieve specific hybridization conditions as is known in the art. Typically, hybridization and washing are performed at high stringency according to conventional hybridization procedures and employing one or more washing step in a solution comprising 1-3×SSC, 0.1-1% SDS at 50-70° C. for 5-30 minutes.

[0050] As used herein, the term “hybridizes under stringent conditions” describes conditions for hybridization and washing under which nucleotide sequences having at least 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more base pair matches to each other typically remain hybridized to each other.

[0051] The term “corresponding to” refers to a polynucleotide sequence that is identical to all or a portion of a reference polynucleotide sequence. In contradistinction, the term “complementary to” is used herein to indicate that a polynucleotide sequence is identical to all or a portion of the complementary strand of a reference polynucleotide sequence.

[0052] The terms “target sequence” or “target nucleotide sequence,” as used herein, refer to a particular nucleic acid sequence in a test sample to which a primer and/or probe is intended to specifically hybridize. A “target sequence” is typically longer than the primer or probe sequence and thus can contain multiple “primer target sequences” and “probe target sequences.” A target sequence may be single or double stranded. The term “primer target sequence” as used herein refers to a nucleic acid sequence in a test sample to which a primer is intended to specifically hybridize. The term “probe target sequence” refers to a nucleic acid sequence in a test sample to which a probe is intended to specifically hybridize.

[0053] As used herein an “amplified target polynucleotide sequence product” or “amplified product” refers to the resulting amplicon from an amplification reaction such as a polymerase chain reaction. The resulting amplicon product arises from hybridization of complementary primers to a target polynucleotide sequence under suitable hybridization conditions and the repeating in a cyclic manner the polymerase

chain reaction as catalyzed by DNA polymerase for DNA amplification or RNA polymerase for RNA amplification.

[0054] As used herein, the “polymerase chain reaction” or PCR is a an amplification of nucleic acid consisting of an initial denaturation step which separates the strands of a double stranded nucleic acid sample, followed by repetition of (i) an annealing step, which allows amplification primers to anneal specifically to positions flanking a target sequence; (ii) an extension step which extends the primers in a 5' to 3' direction thereby forming an amplicon polynucleotide complementary to the target sequence, and (iii) a denaturation step which causes the separation of the amplicon from the target sequence (Mullis et al., eds, *The Polymerase Chain Reaction*, BirkHauser, Boston, Mass. (1994). Each of the above steps may be conducted at a different temperature, preferably using an automated thermocycler (Applied Biosystems LLC, a division of Life Technologies Corporation, Foster City, Calif.). If desired, RNA samples can be converted to DNA/RNA heteroduplexes or to duplex cDNA by methods known to one of skill in the art.

[0055] As used herein, “amplifying” and “amplification” refers to a broad range of techniques for increasing polynucleotide sequences, either linearly or exponentially. Exemplary amplification techniques include, but are not limited to, PCR or any other method employing a primer extension step. Other nonlimiting examples of amplification include, but are not limited to, ligase detection reaction (LDR) and ligase chain reaction (LCR). Amplification methods may comprise thermal-cycling or may be performed isothermally. In various embodiments, the term “amplification product” or “amplified product” includes products from any number of cycles of amplification reactions.

[0056] In certain embodiments, amplification methods comprise at least one cycle of amplification, for example, but not limited to, the sequential procedures of: hybridizing primers to primer-specific portions of target sequence or amplification products from any number of cycles of an amplification reaction; synthesizing a strand of nucleotides in a template-dependent manner using a polymerase; and denaturing the newly-formed nucleic acid duplex to separate the strands. The cycle may or may not be repeated.

[0057] Descriptions of certain amplification techniques can be found, among other places, in H. Ehrlich et al., *Science*, 252:1643-50 (1991), M. Innis et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press, New York, N.Y. (1990), R. Favis et al., *Nature Biotechnology* 18:561-64 (2000), and H. F. Rabenau et al., *Infection* 28:97-102 (2000); Sambrook and Russell, *Molecular Cloning*, Third Edition, Cold Spring Harbor Press (2000) (hereinafter “Sambrook and Russell”), Ausubel et al., *Current Protocols in Molecular Biology* (1993) including supplements through September 2005, John Wiley & Sons (hereinafter “Ausubel et al.”).

[0058] The term “end-point analysis” refers to a method where data collection occurs only when a reaction is substantially complete.

[0059] The term “real-time analysis” refers to periodic monitoring during PCR. Certain systems such as the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, Calif.) conduct monitoring during each thermal cycle at a pre-determined or user-defined point. Real-time analysis of PCR with FRET probes measures fluorescent dye signal changes from cycle-to-cycle, preferably minus any internal control signals.

[0060] As used herein, the term “sample” is a portion of a larger source. A sample is optionally a solid, gaseous, or fluidic. A sample is illustratively an environmental or biological sample. An environmental sample is illustratively, but not limited to water, sewage, soil, or air. A “biological sample” is a sample obtained from a biological organism, a tissue, cell, cell culture medium, or any medium suitable for mimicking biological conditions. Non-limiting examples include, saliva, gingival secretions, cerebrospinal fluid, gastrointestinal fluid, mucous, urogenital secretions, synovial fluid, blood, serum, plasma, urine, feces, cystic fluid, lymph fluid, ascites, pleural effusion, interstitial fluid, intracellular fluid, ocular fluids, seminal fluid, mammary secretions, and vitreal fluid, and nasal secretions, throat or nasal materials, eggs, or other food. Methods of obtaining a sample are known in the art. Illustratively, a sample is whole blood, plasma, or serum that is obtained by venipuncture and optionally processed to obtain the final sample.

[0061] As used herein, the term “medium” refers to any liquid or fluid that may or may not contain one or more bacteria. A medium is illustratively a solid sample that has been suspended, solubilized, or otherwise combined with fluid to form a fluidic sample.

Non-limiting examples include buffered saline solution, cell culture medium, acetonitrile, trifluoroacetic acid, combinations thereof, or any other fluid recognized in the art as suitable for combination with bacteria or other cells, or for dilution of a biological sample or amplification product for analysis.

[0062] As used herein, the term "attenuated" refers to a microorganisms and/or bacteria strains and/or viruses that have been genetically modified so as to not cause illness in an animal model. The terms "attenuated" and "avirulent" are used interchangeably herein. By "immunizing amount" as used herein is in fact meant an amount that is able to induce an immune response in the animal that receives the pharmaceutical composition/vaccine. The immune response invoked may be a humoral, mucosal, local and/or a cellular immune response.

[0063] As used herein "subject" refers to avian species including but not limited to chickens, turkeys, geese, ducks, pheasants, bantam, quail, pigeons; however, it is contemplated that a method according to the present invention may also be used as a diagnostic tool in other subjects susceptible to *Salmonella* Typhimurium infection, such as but not limited to reptiles (turtles, lizards, and snakes); amphibians (frogs and toads); rodents (mice, rats, hamsters, and guinea pigs) and other small mammals (hedgehogs); livestock and farm animals (goats, calves, cows, horses sheep, and pigs); and humans.

[0064] The term "multivalent," as used herein may be bivalent, trivalent, quadravalent, and the like and refers to a vaccine which has other antigenic components from related and/or unrelated microorganism and/or viruses, or their components and fragments along with the attenuated *Salmonella* strain. For example, the "multivalent" vaccine could be a combination vaccine having an attenuated *Salmonella* strain in combination with other antigenic components.

II. Compositions

A. *Salmonella* Contamination in Poultry

[0065] *Salmonella* is a genus of the family Enterobacteriaceae, characterized as Gram-negative, facultatively anaerobic, non spore-forming, rod-shaped bacteria, and most strains are motile by flagella. *Salmonella* has an average genome GC content of 50-52%, which is similar to those of *Escherichia coli* and *Shigella*. The genus *Salmonella* is a pathogenic microorganism that causes infections in livestock as well as in human. *Salmonella enterica*, a species of *Salmonella* bacterium, has a variety of serovars

including Gallinarum, Pullorum, Typhimurium, Enteritidis, Typhi, Choleraesuis, and derby (Bopp C A, Brenner F W, Wells J G, Strokebine N A, *Exherichia*, *Shigella*, *Salmonella*. In Murry P R, Baron E J, et al eds Manual of Clinical Microbiology. 7th ed. Washington D.C. American Society for Microbiology 1999; 467-74; Ryan K J. Ray C G (editors) (2004). Sherris Medical Microbiology (4th ed). McGraw Hill. ISBN 0-8385-8529-9.). Of them, *Salmonella* Gallinarum and Pullorum are fowl-adapted pathogens, *Salmonella* Typhi is a human-adapted pathogen, *Salmonella* Choleraesuis and *Salmonella* derby are swine-adapted pathogens, and *Salmonella* Enteritis and *Salmonella* Typhimurium are pathogenic for human and animals. Each serovar causes illness in the respective species, resulting in tremendous damage to farmers or consumers.

[0066] The present invention provides compositions and methods for the quick and reliable detection of *Salmonella*, particularly compositions and methods for detecting *Salmonella* Typhimurium in poultry. A significant source of infection in domestic poultry is from *Salmonella* bacteria, including *Salmonella* Typhimurium (*S. Typhimurium*). Newly hatched birds are particularly susceptible to *Salmonella* infection, and a high mortality rate as a result thereof immediately post-hatching can have serious economic consequences. Live attenuated *Salmonella* vaccines have been shown to protect chickens (Cooper, et al., *Microb. Pathog.*, 9:255-265, 1990; Hassan and Curtis III, *Res. Microbial.*, 141:839-950, 1990). Importantly, the avirulent *S. Typhimurium* aroA deletion mutant microorganism having the identifying characteristics of *S. Typhimurium* STM-1 has been found to be particularly effective in protecting poultry against *Salmonella* infection and disease. The *S. Typhimurium* STM-1 microorganism and the construction thereof is described in U.S. Pat. No. 6,231,871, which is incorporated herein by reference. In addition, the invention further contemplates other live, attenuated *Salmonella* gene mutants, including deletion mutants, in particular aroA deletion mutants, as immunogens as part of a combination vaccine herein described.

[0067] Once an outbreak of salmonellosis by *S. Typhimurium* occurs in poultry, pigs, and cattle, it is difficult to cure by therapeutic agents, including not only because *Salmonella* are intracellular and thus antibiotics are less effective at entering but also because of the increase observed in antibiotic resistance.

[0068] As in livestock, *S. Typhimurium* can cause infections in human via consumption of contaminated, improperly cooked livestock products (e.g., meat

products, poultry products, eggs and by-products). *Salmonella* illness in human usually involves the prompt onset of headache, fever, abdominal pain, diarrhea, nausea, and vomiting. The symptoms commonly appear within 6-72 hours after the ingestion of the organism, and may persist for as long as 4-7 days or even longer (NSW+HEALTH. 2008.01.14.).

[0069] The Centers for Disease Control and Prevention, USA (CDC) estimates *Salmonella* bacteria cause about 1.35 million infections, 26,500 hospitalizations, and 420 deaths in the United States every year. Food is the source for most of these illnesses (Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Foodborne, Waterborne, and Environmental Diseases (DFWED), (2023)). The CDC reports that 16% of human foodborne illness outbreaks between 2005 and 2008 were attributed to *Salmonella* bacteria, and 20% and 18% of that total was caused by *Salmonella Enteritis* and *Salmonella* Typhimurium, respectively. With respect to *Salmonella* foodborne illness in human between 1973 and 1984, the implicated food vehicles of transmission were reportedly chicken (5%), beef (19%), pork (7%), dairy products (6%), and turkey (9%). In 1974-1984, the bacterial contamination test on broilers during the slaughter process showed 35% or more of *Salmonella* incidence. In 1983, *Salmonella* was isolated in 50.6% of chicken, 68.8% of turkey, 60% of goose, 11.6% of pork, and 1.5% of beef. Further, a survey carried out in 2007 reported that *Salmonella* was found in 5.5% of raw poultry meat and 1.1% of raw pork. In particular, it was revealed that *Salmonella Enteritis* commonly originated from contaminated egg or poultry meat, and *Salmonella* Typhimurium from contaminated pork, poultry meat, and beef (Center for Disease Control). A risk assessment conducted by FAO and WHO in 2002 noted that the human incidence of salmonellosis transmitted through eggs and poultry meat appeared to have a linear relationship to the observed *Salmonella* prevalence in poultry. This means that, when reducing the prevalence of *Salmonella* in poultry, the incidence of salmonellosis in humans will fall (*Salmonella* control at the source; World Health Organization. International Food Safety Authorities Network (INFOSAN) Information Note No. 03/2007

B. Live Attenuated *Salmonella* Vaccines

[0070] Live attenuated *Salmonella* vaccines are potentially superior to inactivated preparations owing to: (i) their ability to induce cell-mediated immunity in addition to antibody responses; (ii) oral delivery with no risk of needle contamination; (iii)

effectiveness after single-dose administration; (iv) induction of immune responses at multiple mucosal sites; (v) low production cost; and (vi) their possible use as carriers for the delivery of recombinant antigens to the immune system.

[0071] Few live attenuated *Salmonella* vaccines are actually on the market because results with attenuated mutant strains have not always been good. For example, one study evaluating chickens vaccinated with aro- or cAMP mutants of *Salmonella* resulted in the death of many of the chicks shortly after vaccination. As the infection by *Salmonella* often occurs very early, vaccination of very young chicks is crucial. However, at this age the chicks are very sensitive to *Salmonella*, possibly due to the immaturity of their immune system. In addition to poor protection of the vaccinated chicks, a prolonged excretion of the vaccine strains was often observed with experimental vaccines.

[0072] Other studies have evaluated the vaccination of birds with a live attenuated vaccine that carries deletions in the cya and crp genes (see, for example, US Patents 5,389,368; US 5,855,879; US 5,855,880). Results showed a reduction of the number of birds from which a virulent *Salmonella* challenge strain can be isolated 7 days after challenge.

C. Monitoring and Control of *Salmonella*

[0073] The current level of food safety found in the U.S. is the result of Government regulations combined with industry self-monitoring influenced by market incentives, such as legal liability, brand value, reputation, and the desire to sell more food product. In the U.S., the primary agencies responsible for food safety are the U.S. Department of Agriculture (USDA) Food Safety and Inspection Services (FSIS), which is responsible for the safety of meat, poultry, and processed egg products, and the Food and Drug Administration (FDA), which is responsible for virtually all other foods.

[0074] In 2021, the U.S. Department of Agriculture's Food Safety and Inspection Service (FSIS) announced that it was mobilizing a stronger, and more comprehensive effort to reduce *Salmonella* illnesses associated with poultry products. Despite consistent reductions in the preceding years in the occurrence of *Salmonella* in poultry products, more than 1 million consumer illnesses still occurred due to *Salmonella* annually, and it was estimated that over 23% of those illnesses are due to consumption of chicken and turkey (Foodborne illness source attribution estimates for 2019 for *Salmonella*,

Escherichia coli O157, *Listeria monocytogenes*, and *Campylobacter* using multi-year outbreak surveillance data, United States The Interagency Food Safety Analytics Collaboration (IFSAC) October 2021). USDA proposed specific *Salmonella* control and measurement strategies, including pilot projects, in poultry slaughter and processing establishments. A key component of this approach is encouraging preharvest controls to reduce *Salmonella* contamination coming into the slaughterhouse, including sacrificing *Salmonella* contaminated poultry. Vaccination is an important component in a preharvest program; however, it is well known that a live attenuated *Salmonella* Typhimurium vaccine persists in vaccinates. Therefore, there is a strong possibility that a significant portion of the chickens identified as contaminated with *Salmonella* Typhimurium may actually be harboring the vaccine strain rather than the wild type strain. A quick and reliable method useful to differentially diagnose those birds infected with wild type *Salmonella* from those harboring a vaccine strain, and thus not of concern in a control program, would be a major improvement over current practices.

[0075] Thus, the present invention provides compositions and methods for the differentiation of live *S. Typhimurium* vaccine isolates from wild type or field isolates of *S. Typhimurium* in poultry.

D. Selection of *Salmonella* Target Sequence

[0076] A *Salmonella* nucleic acid sequence targeted for DNA amplification is first selected from *Salmonella* nucleic sequences known in the art. As used herein, the term “*Salmonella* target sequence” refers to a DNA or RNA sequence comprising the nucleic acid sequence of a bacterium of the genus *Salmonella*. It includes but is not limited to, *S. Typhimurium*.

[0077] Exemplary *Salmonella* nucleic acid sequences that may be targeted for amplification according to the present invention are taught by the following publications: Richards AK, et al., Lett Appl Microbiol. 71(2):138-145 (2020); Liu W Q et al., PLoS One, 4(2):e4510 (2009); Thomson N R et al., Genome Res, 18(10):1624-37 (2008); Encheva V et al., BMC Microbiol, 18 ;5:42 (2005); McClelland M et al., Nat Genet, 36(12):1268-74 (2004); Chiu C H et al., Clin Microbiol Rev, 17(2):311-22(2004); Deng W et al., J Bacteriol, 185(7):2330-7 (2003); Parkhill J et al., Nature, 413(6858):848-52 (2001); McClelland M et al., Nature, 413(6858):852-6 (2001), of which contents are incorporated herein by reference.

[0078] An exemplary nucleotide sequence of the complete 4,857,432 bp genome of *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2 is available under Genbank Accession No. NC_003197 and incorporated herein by reference.

1. *Salmonella* Typhimurium Cya and Crp genes

[0079] Provided herein is a PCR detection method using primers for the cAMP receptor protein (Crp) and adenylate cyclase (CYA) genes.

[0080] The nucleic acid sequence for mutant *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ST04-MSC-p1-Delta-CRP is:

```
atgggtgcttg gcaaaccgca aacagacccg actcttgaat ggttcttgtc tcattgccac
attcataagt acccgctcaaa gagcacgctg attcaccagg gtgaaaaagc agaaacgctg
tactacatcg ttaaaggctc cgtggcagtg ctgatcaaag atgaagaagg gaaagaaatg
atcctttctt atctgaatca ggggtgatttt attgggtgaac tgggcctgtt tgaagaaggc
caggaacgca ggcgcctgat gaatccccta atgatttttg taaaaatcat taagttaagg
tggatacaca tcttgtcata tgatcaaata gtttcgcgaa aaatcaataa tcagacaaca
agatgtgcga actcgatatt ttacacgact ctctttacca attctgcccc gaattacact
taaaacgact caacagctta acgttggtt gccacgcatt acttgactgt aaaactctca
ctcttaccga acttgggcgt aacctgccaa ccaaagcgag aacaaaacat aacatcaaac
gaatcgaccg attgttaggt aatcgtcacc tccacaaaga gcgactcgct gtataccgtt
ggcatgctag ctttatctgt tcgggcaata cgatgcccat tgtacttgtt gactggctctg
atattcgtga gcaaaaacga cttatggtat tgcgagcttc agtcgcacta cacggctcgtt
ctgttactct ttatgagaaa gcgttcccg cttcagagca atgttcaaaag aaagctcatg
accaatttct agccgacctt gcgagcattc taccgagtaa caccacaccg ctcatgttca
gtgatgctgg ctttaaagtg ccatggtata aatccgttga gaagctgggt tggtagctgt
taagtcgagt aagaggaaaa gtacaatatg cagacctagg agcggaaaac tggaaacctg
tcagcaactt acatgatatg tcatctagtc actcaaagac tttaggctat aagaggctga
ctaaaagcaa tccaatctca tgccaaattc tattgtataa atctcgctct aaaggccgaa
aaaatcagcg ctcgacacgg actcattgtc accaccgctc acctaataatc tactcagcgt
cggcaaagga gccatgggtt ctagcaacta acttacctgt tgaaattcga acacccaaac
aacttggtta tatctattcg aagcgaatgc agattgaaga aaccttccga gacttgaaaa
gtcctgccta cggactaggc ctacgccata gccgaacgag cagctcagag cgttttgata
tcatgctgct aatcgccctg atgcttcaac taacatgttg gcttgcgggc gttcatgctc
agaaacaagg ttgggacaag cacttccagg ctaacacagt cagaaatcga aacgtactct
caacagttcg cttaggcatg gaagttttgc ggcattcttg ctacacaata acaagggaag
acttactcgt ggctgcaacc ctactagctc aaaatttatt cacacatggt tacgcttttg
ggaaattatg aggggatctc tcagggcaca gcaccagcgt ttgccgtagt gcgtaattaa
tctctgggtg ataaatcagt cttcgccaca tcgatgcaaa caaaaatggc gcatgataaa
acgcgccatt ctgacggaat taacgggtgc cgtagacgac gatggtcttg ccatgcgcgg
agatcagggt ttgatcttcc agcattttca aaatacgacc aacggtttcg cgggagcagc
cgacgatctg gccgatttcc tgacgagtga ttttgatctg catcccatcc ggggtgcgtca
tggcatcggg ctgttttcgcc agattcagca gcgtctgagc gatacgcccc gtgacgtcaa
ggaaggcgag gttacctact ttttcagagg tgacttgtaa gcgacgagcc atctgggaag
agaggcgcat cagaatatcc gggttgacct ggattaattg gcgaaatttt ttgtaggaaa
tttcagcgac ctccatgctg gttttcgcac gtacccaggc gctgcgctga
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(SEQ ID NO:1 available under Genbank Assession No. MT900624).

[0081] The nucleic acid sequence for mutant *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain ST04-MSC-p1-Delta-CYA::Tn10 transposon Tn10 IS4 family transposase ISVsa5 gene is:

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ttgtacctct atattgagac tctgaaacag agactggatg ccataaatca actgcgtgtg
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gatcgcgcgc ttgctgccat gggacccgct tttcagcagg tttacagtct tctgccgaca
ttattgcact atcaccatcc actgatgccg gggttaccttg atggtaacgt tcccagcggg
atttgcttct acacgcctga tgaaccccaa cgccactatc tgaacgaact tgagctgtac
cgcggtatga cgccgcagga cccgccgaag ggcgagctgc cgattaccgg cgtttacacc
atgggcagca cctcctcggt cgggcagagc tgctcgccg acctggatat ctgggtgtgc
catcagtcct ggctcgacgg cgaagagcgt cagttgctgc aacgtaagt tagcctgtg
gaaagctggg ccgcctcgct tggcgttgag gtgagcttct tcctgatcga cgagaaccgt
ttccgccata acgaaagcgg cagtctgggc ggggaagact gtggttctac gcagcatatc
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agtatggtgc cgtgcgacga agaagagcat tacgacgact atgtcatgac gctctatgcg
cagggcgtat taacgcaaaa cgaatggctg gatctggggg gcttaagctc gctctccgcc
gaagagtact ttggcgccag cctgtggcag ctatacaaga gcattgactc gccgtacaaa
gcggtgctga aaacgctgct gctggaagcc tattcatggg aatatcctaa cccacgtctg
ctggcgaaag atattaaaca acgtctgcat gacggtgaaa tcgtatcggt tggactcgat
ccctactgca tgatgctgga acgggtcact gaatacctga cggcgattga agatccgacg
cggctggatt tagtccgccg ctgcttttac ctgaaaagtgt gcgagaaatt aagtcgcgag
cgtgcctgcg taggctggcg tcgggaagta ttaagccagt tagtcagcga gtggggatgg
gacgacgcgc gtctgaccat gctcgataat cgcgcaaaact ggaaaatcga tcaggtgcgc
gaagcccaca acgaattgct cgacgccatg atgcaaagct ctgatgaatc ccctaattgat
ttttatcaaa atcattaagt taaggtagat acacatcttg tcatatgatc aaatggtttc
gccaaaaatc aataatcaga caacaaaatg tgcgaactcg atattttaca cgactctctt
taccaattct gccccgaatt acacttaaaa cgactcaaca gcttaacgtt ggcttgccac
gccttacttg actgtaaaac tctcactctt accgaacttg gccgtaacct gccaaccaaa
gcgagaacaa aacataacat caaacgaatc gaccgattgt taggtaatcg tcacctccac
aaagagcgac tcgctgtata ccgttggcat gctagcttta tctgttcggg caatacgatg
cccattgtac ttgttgactg gtctgatata cgtgagcaaa aacggcttat ggtattgcga
gcttcagtcg cactacacgg tcgttctggt actctttatg agaaagcgtt cccgctttca
gagcaatggt caaagaaagc tcatgaccaa tttctagccg accttgcgag cattctaccg
agtaacacca caccgctcat tgtcagtgat cgtggcttta aagtgccatg gtataaatcc
gttgagaagc tgggttggtg ctggttaagt cgagtaagag gaaaagtaca atatgcagac
ctaggagcgg aaaactggaa acctatcagc aaacttacct atatgtcatc tagtactca
aagactttag gctataagag gctgactaaa agcaatccaa tctcatgcca aattctattg
tataaatctc gctctaaagg ccgaaaaaat cagcgctcga cacggactca ttgtcaccac
ccgtcaccta aaatctactc agcgtcggca aaggagccat ggattctagc aactaactta
cctgttgaaa ttcgaacacc caaacaactt gttaatatct attcgaagcg aatgcagatt
gaagaaacct tccgagactt gaaaagtcct gcctacggac taggcctacg ccatagccga
acgagcagct cagagcgttt tgatatcatg ctgctaactc ccctgatgct tcaactaaca
tggttgcttg cgggcgttca tgctcagaaa caagggtggg acaagcactt ccaggctaac
acagtcagaa atcgaaacgt actctcaaca gttcgcttag gcatggaagt tttgcggcgt
tctgggtaca caataacaag ggaagactca ctcgtggctg caaccctgct tactcaaaat
ctattcacac atgggttacgt tttggggaaa ttatgagggg atctctcagg gctcatcata
agagctatag ctaccactga ataaaacaga gctaatttta tttgtcgttt cttattcacg
caaataagaa tagggttgtt tattgacata taatactgag agatcccctc ataatttccc
caaaacgtaa ccatgtgtga atagattttg agtaagcagg gttgcagcca cgagtgaatc
ttcccttggt attgtgtagc cagaatgccg caaaacttcc atgcctaagc gaactgttga
gagtacgttt cgatttctga ctgtgttagc ctggaagtgc ttgtcccaac cttgtttctg
agcatgaacg cccgcaagcc aacatgttag ttggaagcat agggcgatta gcagcatgat
atcaaaacgc tctgagctgc tcgttcggct atggcgtagg cctagtccgt aggcaggact
tttcaagtct cggaagggtt cttcaatctg cattcgcttc gaatagatat taacaagttg
tttgggtgtt cgaatttcaa caggtaagtt agttgctaga acccatggct cctttgccga
cgctgagtag attttaggtg acgggtgggt acaatgagtc cgtgtcgagc gctgattttt
tcggccttta gagcgagatt tatacaatag aatttggcat gagattggat tgcttttagt
cagcctctta tagcctaaag tctttgagtg actagatgac atatcatgta agttgctgat
aggtttccag ttttccgctc ctaggtctgc atattgtact tttcctctta ctcgacttaa
ccagtaccaa cccagcttct caacggattt ataccatggc acttttaaagc cagcatcact
gacaatgagc ggtgtggtgt tactcggtag aatgctcgca aggtcggcta gaaattgggtc
atgagctttc tttgaacatt gctctgaaag cggaacgct ttctcataaa gagtaacaga
acgaccgtgt agtgcgactg aagctcgcaa taccataagt cgtttttgct cacgaatatc
agaccagtca acaagtacaa tgggcatcgt attgcccga cagataaagc tagcatgcc
acggtataca gcgagtcgct ctttgtggag gtgacgatta cctaacaatc ggtcgattcg
tttgatgtta tgttttgttc tcgctttggt tggcagggtta cggccaagtt cggtaagagt

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gagagtttta cagtcaagta atgctgtggca agccaacggt aagctgttga gtcgttttaa
gtgtaattcg gggcagaatt ggtaaagaga gtcgtgtaaa atatcgagtt cgcacatctt
gttgtctgat tattgatttt tcgcgaaacc atttgatcat atgacaagat gtgtatccac
cttaacttaa tgattttttac caaaatcatt aggggattca tcagtgc aaa gctatcgtaa
tctgattcgc tttgcgcggc gcaacaacct cagcgtgagt gccagccgc aggatatcgg
cgtactgacg cgtaagctgt acgcggcttt tgaagcgttg ccgggtaaaag tcacgctggt
gaacccgcag atatcgccgg atctgtccga gccgaattta acctttatcc atgtgccgcc
gggacgcgcc aaccgttcag gctggtatct ctacaaccgc gcgccgaaca tggattccat
catcagccat cagccgctgg aatataaccg ttatcttaat aagctggtcg cgtgggctg
gttcaacggc ctgctgacgt cgcgaacgca tctgtttatt aagggaacg gtattgtcga
cctgcctaag ttacaggaga tggtcgccga tgtttcgcac catttccgc tgcgcttgcc
tgctccgacg ccgaaagcgc tctacagccc ctgtgaaatt cgccatctgg cgattatcgt
taacctcgaa tatgaccgga cggcggcgtt tcgcaataaa gtggtccatt ttgacttccg
taagctggac gttttcagct ttggcgaaga gcaaaaactgt ctgataggca gtatcgactt
gttatatcgc aactcgtgga acgaagtgcg tactctgcac tttaacggcg agcaggcgat
gatcgaagcg ctgaaaacga ttctggggaa aatgcaccag gatgccgcgc gccgggatag
cgtggagggtg ttctgctaca gtcagcatct tcgcggcctg attcgacccc gtgtgcagca
actggtctcc gaatgtattg agctacgtct ttccagcacc cgtcaggaga ccggtcgctt
caaggcgctg cgggtttccg ggcagacgtg ggggctattc ttcgaacgct tgaatgtctc
ggtgcagaag ctggagaacg ctatcgaatt ctacggcgcg atttcgcata acaagctgca
cgggctgtcg gtacagggtg aaaccaacca ggtgaaattg ccgtcagtgg tggatggctt
cgccagcgaa gggattatcc agttcttctt tgaagaaaca ggcgatgaga aaggctttaa
catttatatt ctggatgaaa gtaaccgggc ggaagtatat caccactgcg aaggtagcaa
ggaagaactg gtgcgcgacg tcagtcgctt ctattcgtca tcgcacgatc gcttcacgta
tggctccagt tttatcaact ttaacctgcc gcagttctac cagatagtga aaaccgatgg
ccgcgcgacg gtgatcccat tccgtacgca gcctatcaac accgtgccgc cagcaaacca
ggatcatgac gcgccgctat tgcagcagta tttttcgtaa

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(SEQ ID NO:2 available under Genbank Assession No. MT900625).

[0082] Cyclic-3'5'-AMP (cAMP) and the cyclic AMP receptor protein are necessary for the transcription of a large number of genes and operons concerned with the transport and breakdown of a large number of catabolites. Evidence has been provided that shows that systems used for transporting fuel/carbon sources are all under positive control by cAMP, as are several amino acid permeases. In addition to its very important role in catabolism, the cAMP concentration in cells also influences lysogenization by temperate phages, synthesis of fimbriae, synthesis of flagella and synthesis of at least one outer membrane protein. Although cAMP is present in mammalian cells, the concentrations present in macrophages and other cells in which *Salmonella* can invade and multiply are below the concentration of 0.1 to 1.0 mM cAMP necessary to allow Δ cya mutants to exhibit a wild-type phenotype in vitro. Furthermore, the inclusion of the Δ crp mutation would essentially abolish any benefit that could accrue from uptake of cAMP in vitro or in vivo by such Δ cya mutants.

[0083] Introduction of the mutations into cya and crp of *S. Typhimurium* can be accomplished by use of transposons, to transfer the mutations from other *Salmonella* strains into *S. Typhimurium*. Transposons can be added to a bacterial chromosome at many points. The characteristics of transposon insertion and deletion have been reviewed

in Kleckner et al. (1977), J. Mol. Biol. 116:125. For example, the transposon Tn10, which confers resistance to tetracycline (and sensitivity to fusaric acid) can be used to create Δcya and Δcrp mutations in a variety of bacterial species, including, for example, *E. coli* and *S. Typhimurium*. Methods for the creation and detection of these mutants in *S. Typhimurium* are described in EPO Pub. No. 315,682 and incorporated herein by reference. Utilizing Tn10, these mutations can be transposed into various isolates of *S. Typhimurium*, preferably those which are highly pathogenic.

[0084] Once rendered avirulent by the introduction of the Δcya and/or Δcrp mutations, the microbes can serve as the immunogenic component of a vaccine to induce immunity against the microbe.

a. Cya and Crp primers

[0085] Oligonucleotides of the instant invention are set forth in SEQ ID NOs: 3-14. CRP and CYA regions as shown in Table 1 were chosen to search for the vaccine strain from whole genome sequencing (WGS) sequences. They were designed to be short ~120-130 nucleotides, so they can be captured from short read sequences..

[0086] Table 1: Sequences used to search for the vaccine strain from WGS sequences

SEQ ID NO:	Mutation	CRP Sequences
3	Jxn 5' and 3' end in field strain	ttattggtgaactgggcctgttgaagaaggccaggaacgcagcgctgggtacgtgcg aaaaccgcatgtgaggtcgctgaaatttcctacaaaaatttcgccaattaatccaggtcaaccgg
4	Jxn Mut 5' end of Tn10	ggtgattttattggtgaactgggcctgttgaagaaggccaggaacgcagcgccctgat gaatcccctaataatgattttggtaaaaatcattaagttaagggtggatacacatcttgcataatgatc
5	Jxn Mut 3' end of Tn10	gggaagacttactcgctggctgcaaccctactagctcaaaattattcacacatgggtacg ctttggggaaattatgaggggatctctcagggcacagcaccagcgttgccgtagtgcgtaatta
		CYA Sequences
6	Jxn 5' and 3' end in WT	gtacgccgatatcctgcgggctggcactcacgctgaggttgttgcgccgcgcaaagcga atcagattacgatagctttgcatcatggcgctcgagcaattcgttgtgggcttcgcgcacctgat
7	Jxn Mut 5' end of Tn10	ggctggcactcacgctgaggttgttgcgccgcgcaaagcgaatcagattacgatagctttg cactgatgaatcccctaataatgattttggtaaaaatcattaagttaagggtggatacacatcttgt
8	Jxn Mut 3' end of Tn10	gtttggttggcaggttacggccaagtgcgtaagagtgagagtttacagtcaagtaaggcgtg gcaagccaacgttaagctgttgagtcgttttaagtgaattcggggcagaattgg

[0087] Oligonucleotides of the instant invention may be used as primers for PCR amplification. Preferred primer pairs and their corresponding targets, blocking oligonucleotides, and probes are shown in Table 2.

[0088] Table 2: PCR primers

SEQ ID NO:	Gene	Primer target/direction	PCR	Primer sequence 5'-3'
9	CRP	WT-fwd	PCR1 and PCR2	ctccgtggcagtgctgatcaaag
10		WT-rev	PCR1	cgtaaggaaggcgagggttacctac
11		Tn-Rev	PCR2	gttctcgcttggtggcaggttac
12		Mut_rev	PCR3	attacgcactacggcaaacgctg
13	CYA	Tn-fwd	PCR3	cgtcacctaaatctactcagcgctcgg
14		WT-fwd	PCR4 and PCR5	gcgatatctgcgggttcaccag
15		WT-rev	PCR4 and PCR6	cgataatcgcgcaaactggaaaatcgatc
16		Tn-Rev	PCR5	tcaagtaatgcgtggcaagccaac
17		Tn-fwd	PCR6	gctcacggatatcagaccagtcaacaag

[0089] These oligonucleotide primers may also be useful for other nucleic acid amplification methods such as the ligase chain reaction (LCR) (EP 0 320 308; Carrino et al., J. Microbiol. Methods 23:3-20 (1995)); nucleic acid sequence-based amplification (NASBA) (Carrino et al., 1995, supra); and self-sustained sequence replication (3SR) and “Q replicase amplification” (Pfeffer et al., Vet. Res. Commun. 19:375-407 (1995)).

[0090] In addition, oligonucleotides of the present invention also may be used as hybridization probes. Hybridization using DNA probes has been frequently used for the detection of pathogens in food, clinical and environmental samples, and the methodologies are generally known to one skilled in the art. It is generally recognized that the degree of sensitivity and specificity of probe hybridization is lower than that achieved through the previously described amplification techniques. The nucleic acid probes of the present invention can also possess a detectable label, such as a reporter-quencher combination as are employed in Scorpion probe assays or in 5'-exonuclease detection assays, such as the Taqman® assay.

[0091] The 3' terminal nucleotide of the nucleic acid probe may be rendered incapable of extension by a nucleic acid polymerase in one embodiment of the invention. Such blocking may be carried out, for example by the attachment of a replication inhibitor moiety, such as a reporter or quencher, to the terminal 3' carbon of the nucleic acid probe by a linking moiety, or by making the 3'-terminal nucleotide a dideoxynucleotide. Alternatively, the 3' end of the nucleic acid probe may be rendered impervious to the 3' to 5' extension activity of a polymerase by incorporating one or more modified internucleotide linkages onto the 3' end of the oligonucleotide. Minimally, the 3' terminal internucleotide linkage must be modified, however, additional internucleotide linkages may be modified. Internucleotide modifications which prevent elongation from the 3' end of the nucleic acid probe and/or which block the 3' to 5' exonuclease activity of the DNA polymerase during PCR may include phosphorothioate linkages, methylphosphonate linkages, boranophosphate linkages, and other similar polymerase-resistant internucleotide linkages. An alternative method to block 3' extension of the probe is to form an adduct at the 3' end of the probe using mitomycin C or other like antitumor antibiotics such as described in Basu et al., *Biochemistry* 32:4708-18 (1993). Thus, the precise mechanism by which the 3' end of the nucleic acid probe is protected from cleavage is not essential so long as the quencher is not cleaved from the nucleic acid probe.

[0092] Some oligonucleotides of the present invention contain both primer and probe regions, and thus can be employed as a primer-probe complex in an appropriate assay, such as a Scorpion probe assay. These primer probe complexes of the instant invention contain a non-amplifiable linker that connects the 3' terminus of the probe region to the 5' terminus of the primer region. This non-amplifiable linker stops extension of a complementary strand from proceeding into the probe region of the primer-probe complex. Examples of such non-amplifiable linkages include 6-carbon linkers and preferably hexethylene glycol (HEG) linkers. Primer-probe complexes of the present invention can also contain a self-complementary region that allows the primer-probe complex to form a stem-loop structure when the probe is unbound from its target DNA, which may be useful, for example, in bringing the reporter and quencher into sufficiently close proximity to one another to cause the reporter signal to be quenched.

III. Methods of Detecting

[0093] Today, most food pathogen testing conducted entails a culture step to enrich the potentially low levels of microorganisms contained in a sample. Following culture of the sample, a portion is removed and tested for the presence of pathogens. Pathogen testing after culture can be done by immunoassays (e.g., bioMerieux's Vidas® automated ELISA platform or SDIX's RapidChek® lateral flow assays) or by PCR-based tests (e.g., DuPont Qualicon's BAX® system, Bio-Rad's iQ-Check™ system). If a pathogen is present in the starting sample, the culture step can increase the concentration of the pathogen as high as $1.0E8$ - $1.0E9$ cfu/mL, so that opening the sample after culture exposes both the user and the environment to a risk of contamination. This exposure inhibits many food producers from conducting pathogen testing on-site, instead choosing to send samples to external laboratories for testing. In addition, since it is unknown which samples contain pathogens and at what levels, food safety test protocols use lengthy culture times to ensure that the worst case scenario of one damaged pathogen is given sufficient time to grow to a detectable concentration. As a consequence, samples with higher pathogen loadings are cultured longer than may be strictly necessary, leading to a delay in time to results. There is thus a need in the field for pathogen test methods that minimize time to results and reduce the risk of exposure of the facility and personnel to cultured pathogens.

[0094] Traditional detection methods, such as non-selective and selective enrichment of bacteria, biochemical characteristics and serological identification, are laborious and time-consuming, which takes 4-7 days to complete detection. Other methods such as antibody detection are fast, but their high false positives make them unsuitable for routine detection. In addition, the low level of pathogen contamination, the “injury” of *Salmonella* caused by food processing and the interference of other food ingredients have limited the detection of *Salmonella*. Therefore, in order to detect pathogenic bacteria in time, control pollution and harm to human health, rapid, specific and sensitive detection methods are urgently needed. Provided herein is a method for rapidly identifying *Salmonella* of specific serotypes.

A. Genome Detection Regions

[0095] Provided herein are compositions and methods that uses a *S. Typhimurium* detection assay developed based on identification of the *Salmonella*

Typhimurium primers. In some embodiments, the assay incorporates primers for detection and differentiation of wild type and vaccine strains of *S. Typhimurium*.

[0096] The present disclosure therefore relates to detection and identification of WT and vaccine *S. Typhimurium* through the use of uses primers for two genes: CRP (cAMP receptor protein) and CYA (adenylate cyclase). These two genes are disrupted by transposons (Tn10) in the vaccine strain. The present detection method finds utility in detection of Tn10 in isolated samples to differentiate between contamination with WT field strain of *S. Typhimurium* or the vaccine stain interrupted by the transposon. The present detection method finds utility in detection of *S. Typhimurium* in any type of sample, for example in appropriate samples for food testing, environmental testing, or human or animal diagnostic testing. While examples of suitable methods for detecting these regions are included herein, it is to be understood that the invention is not limited to the methods described. Rather any suitable method can be employed to detect these DNA regions and subsequently *S. Typhimurium* in a sample.

B. Determining Percent Identity of Nucleic Acid Sequences

[0097] Provided herein are methods of analyzing nucleic acid sequences to determine percent identity. In some embodiments, *Salmonella Typhimurium* short-read sequences from chicken samples uploaded to NCBI Pathogen Detection by FSIS from 2016-2022 were downloaded and assembled. These were analyzed using BLAST with a sequence unique to field strains, followed by a sequence unique to the vaccine strain. To determine the percent identity of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity=number of identical overlapping positions/total number of positions .times.100%). In some embodiments, the two sequences are the same length.

[0098] The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and

Altschul, 1990, PNAS 87:2264 2268, modified as in Karlin and Altschul, 1993, PNAS. 90:5873 5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990, J. Mol. Biol. 215:403. BLAST nucleotide searches are performed with the NBLAST nucleotide program parameters set, e.g., for score=100, wordlength=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present invention. BLAST protein searches are performed with the XBLAST program parameters set, e.g., to score 50, wordlength=3 to obtain amino acid sequences homologous to a protein molecule of the present invention. To obtain gapped alignments for comparison purposes, Gapped BLAST are utilized as described in Altschul et al., 1997, Nucleic Acids Res. 25:3389 3402. Alternatively, PSI BLAST is used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI Blast programs, the default parameters of the respective programs (e.g., of XBLAST and NBLAST) are used (see, e.g., the NCBI website). Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4:11 17. Such an algorithm is incorporated in the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM 120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 is used.

[0099] The percent identity between two sequences is determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

C. Assay Methods

[00100] Detection of the presence of *S. Typhimurium*, may be accomplished in any suitable manner. Preferred methods are primer-directed amplification methods and nucleic acid hybridization methods. These methods may be used to detect *S. Typhimurium* in a sample that is either a complex matrix or a purified culture, e.g., from an animal, environmental, or food source suspected of contamination.

1. Primer-Directed Amplification Assay Methods

[00101] A variety of primer-directed nucleic acid amplification methods are known in the art which can be employed in the present invention, including thermal cycling methods (e.g., PCR, qPCR and LCR), as well as isothermal methods and strand displacement amplification (SDA). The preferred method is PCR.

[00102] PCR is an extremely powerful technique for amplifying specific polynucleotide sequences, including genomic DNA, single-stranded cDNA, and mRNA among others. Various methods of conducting PCR amplification and primer design and construction for PCR amplification will be known to those of skill in the art. Generally, in PCR a double-stranded DNA to be amplified is denatured by heating the sample. New DNA synthesis is then primed by hybridizing primers to the target sequence in the presence of DNA polymerase and excess dNTPs. In subsequent cycles, the primers hybridize to the newly synthesized DNA to produce discrete products with the primer sequences at either end. The products accumulate exponentially with each successive round of amplification.

[00103] The DNA polymerase used in PCR is a thermostable polymerase. This allows the enzyme to continue functioning after repeated cycles of heating necessary to denature the double-stranded DNA. Polymerases that are useful for PCR include, for example, Taq DNA polymerase, Tth DNA polymerase, Tfi DNA polymerase, Tma DNA polymerase, Tli DNA polymerase, and Pfu DNA polymerase. There are many commercially available modified forms of these enzymes including: AmpliTaq® and AmpliTaq Gold® both available from Applied Biosystems. Many are available with or without a 3- to 5' proofreading exonuclease activity. See for example, Vent® and Vent®. (exo-) available from New England Biolabs.

[00104] Other suitable amplification methods include the ligase chain reaction (LCR) (e.g., Wu and Wallace, *Genomics* 4, 560 (1989) and Landegren et al., *Science* 241, 1077 (1988)), transcription amplification (Kwoh et al., *Proc. Natl. Acad. Sci. USA* 86, 1173 (1989)), and self-sustained sequence replication (Guatelli et al., *Proc. Nat. Acad. Sci. USA*, 87, 1874 (1990)) and nucleic acid based sequence amplification (NABSA). (See, U.S. Pat. Nos. 5,409,818, 5,554,517, and 6,063,603). The latter two amplification methods include isothermal reactions based on isothermal transcription, which produce both single-stranded RNA (ssRNA) and double-stranded DNA (dsDNA) as the amplification products in a ratio of about 30 or 100 to 1, respectively.

a. Sample Preparation

[00105] The oligonucleotides and methods according to the instant invention may be used directly with any suitable clinical or environmental samples, without any need for sample preparation. In order to achieve higher sensitivity, and in situations where time is

not a limiting factor, it is preferred that the samples be pre-treated and that pre-amplification enrichment is performed.

[00106] The minimum industry standard for the detection of food-borne bacterial pathogens is a method that will reliably detect the presence of one pathogen cell in 25 g of food matrix as described in Andrews et al., 1984, "Food Sample and Preparation of Sample Homogenate", Chapter 1 in Bacteriological Analytical Manual, 8th Edition, Revision A, U.S. Food and Drug Administration. In order to satisfy this stringent criterion, enrichment methods and media have been developed to enhance the growth of the target pathogen cell in order to facilitate its detection by biochemical, immunological or nucleic acid hybridization means. Typical enrichment procedures employ media that will enhance the growth and health of the target bacteria and also inhibit the growth of any background or non-target microorganisms present.

[00107] Selective media have been developed for a variety of bacterial pathogens and one of skill in the art will know to select a medium appropriate for the particular organism to be enriched, e.g. *S. Typhimurium*. A general discussion and recipes of non-selective media are described in the USDA-FSIS publication "MLG 4.13: Isolation and Identification of *Salmonella* from Meat, Poultry, Pasteurized Egg, Carcass, and Environmental Sponges."

[00108] After selective growth, a sample of the complex mixtures is removed for further analysis. This sampling procedure may be accomplished by a variety of means well known to those skilled in the art. In one embodiment, 5 µl of the enrichment culture is removed and added to 200 µl of lysis solution containing protease. The lysis solution is heated at 37° C. for 20 min followed by protease inactivation at 95° C. for 10 min, and cooled to 4° C. as described in the BAX® System User's Guide, DuPont Nutrition and Health, Wilmington, Del. In another embodiment, selective enrichment broth culture is streaked on to a selective a selective, differential agar (e.g. XLT-4, BGS).

b. PCR Assay Methods

[00109] A preferred method for detecting the presence of the vaccine strain of *S. Typhimurium* in a sample comprises (a) performing PCR amplification using primer pairs listed in Table 2 to produce a PCR amplification result; and (b) detecting the amplification, whereby a positive detection of the amplification indicates the presence of the vaccine strain of *S. Typhimurium* in the sample.

[00110] In another preferred embodiment, prior to performing PCR amplification, a step of preparing the sample may be carried out. The preparing step may comprise at least one of the following processes: (1) bacterial enrichment, (2) separation of bacterial cells from the sample, (3) cell lysis, and (4) total DNA extraction.

c. Amplification Conditions:

[00111] A skilled person will understand that any generally acceptable PCR conditions may be used for successfully detecting *S. Typhimurium* using the oligonucleotides of the instant invention, and depending on the sample to be tested and other laboratory conditions, routine optimization for the PCR conditions may be necessary to achieve optimal sensitivity and specificity. Optimally, they achieve PCR amplification results from all of the intended specific targets while giving no PCR results for other, non-target species.

d. Detection/Examination/Analysis:

[00112] Primer-directed amplification products can be analyzed using various methods. Homogenous detection refers to a preferred method for the detection of amplification products where no separation (such as by gel electrophoresis) of amplification products from template or primers is necessary. Homogeneous detection is typically accomplished by measuring the level of fluorescence of the reaction mixture during or immediately following amplification. In addition, heterogeneous detection methods, which involve separation of amplification products during or prior to detection, can be employed in the present invention.

[00113] Homogenous detection may be employed to carry out “real-time” primer-directed nucleic acid amplification and detection, using primer pairs of the instant invention (e.g., “real-time” PCR and “real-time” RT-PCR). Preferred “real-time” methods are set forth in U.S. Pat. Nos. 6,171,785, 5,994,056, 6,326,145, 5,804,375, 5,538,848, 5,487,972, and 5,210,015, each of which is hereby incorporated by reference in its entirety.

[00114] One “real-time” detection method is the Scorpion probe assay as set forth in U.S. Pat. No. 6,326,145, which is hereby incorporated by reference in its entirety. In the Scorpion probe assay, PCR amplification is performed using a Scorpion probe (either unimolecular or bimolecular) as a primer-probe complex, the Scorpion probe possessing an appropriate reporter-quencher pair to allow the detectable signal of the reporter to be

quenched prior to elongation of the primer. Post-elongation, the quenching effect is eliminated and the amount of signal present is quantitated. As the amount of amplification product increases, an equivalent increase in detectable signal will be observed, thus allowing the amount of amplification product present to be determined as a function of the amount of detectable signal measured. When more than one Scorpion probe is employed in a Scorpion probe assay each probe can have a different detectable label (e.g., reporter-quencher pair) attached, thus allowing each probe to be detected independently of the other probes.

[00115] Another preferred “real-time” detection method is the 5'-exonuclease detection method, as set forth in U.S. Pat. Nos. 5,804,375, 5,538,848, 5,487,972, and 5,210,015, each of which is hereby incorporated by reference in its entirety. In the 5'-exonuclease detection assay a modified probe is employed during PCR which binds intermediate to or between the two members of the amplification primer pair. The modified probe possesses a reporter and a quencher and is designed to generate a detectable signal to indicate that it has hybridized with the target nucleic acid sequence during PCR. As long as both the reporter and the quencher are on the probe, the quencher stops the reporter from emitting a detectable signal. However, as the polymerase extends the primer during amplification, the intrinsic 5' to 3' nuclease activity of the polymerase degrades the probe, separating the reporter from the quencher, and enabling the detectable signal to be emitted. Generally, the amount of detectable signal generated during the amplification cycle is proportional to the amount of product generated in each cycle.

[00116] It is well known that the efficiency of quenching is a strong function of the proximity of the reporter and the quencher, i.e., as the two molecules get closer, the quenching efficiency increases. As quenching is strongly dependent on the physical proximity of the reporter and quencher, the reporter and the quencher are preferably attached to the probe within a few nucleotides of one another, usually within 30 nucleotides of one another, more preferably with a separation of from about 6 to 16 nucleotides. Typically, this separation is achieved by attaching one member of a reporter-quencher pair to the 5' end of the probe and the other member to a nucleotide about 6 to 16 nucleotides away.

[00117] Again, when more than one Taqman® probe is employed in a 5'-exonuclease detection assay, each probe can have a different detectable label (e.g., reporter-quencher pair) attached, thus allowing each probe to be detected independently of the other probes.

[00118] Another preferred method of homogenous detection involves the use of DNA melting curve analysis, particularly with the BAX® System hardware and reagent tablets from DuPont Nutrition and Health. The details of the system are given in U.S. Pat. No. 6,312,930 and PCT Publication Nos. WO 97/11197 and WO 00/66777, each of which is hereby incorporated by reference in its entirety.

[00119] Melting curve analysis detects and quantifies double stranded nucleic acid molecule (“dsDNA” or “target”) by monitoring the fluorescence of the target amplification product (“target amplicon”) during each amplification cycle at selected time points.

[00120] As is well known to the skilled artisan, the two strands of a dsDNA separate or melt, when the temperature is higher than its melting temperature. Melting of a dsDNA molecule is a process, and under a given solution condition, melting starts at a temperature (designated T_{ms} hereinafter), and completes at another temperature (designated T_{me} hereinafter). The familiar term, T_m , designates the temperature at which melting is 50% complete.

[00121] A typical PCR cycle involves a denaturing phase where the target dsDNA is melted, a primer annealing phase where the temperature optimal for the primers to bind to the now-single-stranded target, and a chain elongation phase (at a temperature T_e) where the temperature is optimal for DNA polymerase to function.

[00122] According to the present invention, T_{ms} should be higher than T_e , and T_{me} should be lower (often substantially lower) than the temperature at which the DNA polymerase is heat-inactivated. Melting characteristics are affected by the intrinsic properties of a given dsDNA molecule, such as deoxynucleotide composition and the length of the dsDNA.

[00123] Intercalating dyes will bind to double stranded DNA. The dye/dsDNA complex will fluoresce when exposed to the appropriate excitation wavelength of light, which is dye dependent, and the intensity of the fluorescence may be proportionate to concentration of the dsDNA. Methods taking advantage of the use of DNA intercalating dyes to detect and quantify dsDNA are known in the art. Many dyes are known and used in the art for these purposes. The instant methods also take advantage of such relationship.

[00124] Examples of such intercalating dyes include, but are not limited to, SYBR Green-I®, ethidium bromide, propidium iodide, TOTO®-1 {Quinolinium, 1-1'-[1,3-propanediylbis [(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzothiazolylidene) methyl]]-, tetraiodide}, and YoPro® {Quinolinium, 4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)-propyl]-, diiodide}. Most preferred for the instant invention is a non-asymmetrical cyanide dye such as SYBR Green-I®, manufactured by Molecular Probes, Inc. (Eugene, Oreg.).

[00125] Melting curve analysis is achieved by monitoring the change in fluorescence while the temperature is increased. When the temperature reaches the T_{MS} specific for the target amplicon, the dsDNA begins to denature. When the dsDNA denatures, the intercalating dye dissociates from the DNA and fluorescence decreases. Mathematical analysis of the negative of the change of the log of fluorescence divided by the change in temperature plotted against the temperature results in the graphical peak known as a melting curve.

[00126] It should be understood that the present invention could be operated using a combination of these techniques, such as by having a Scorpion probe directed to one target region and a Taqman® probe directed to a second target region. It should also be understood that the invention is not limited to the above described techniques. Rather, one skilled in the art would recognize that other techniques for detecting amplification as known in the art may also be used. For example, techniques such as PCR-based quantitative sequence detection (QSD) may be performed using nucleic acid probes which, when present in the single-stranded state in solution, are configured such that the reporter and quencher are sufficiently close to substantially quench the reporter's emission. However, upon hybridization of the intact reporter-quencher nucleic acid probe with the amplified target nucleic acid sequence, the reporter and quenchers become sufficiently distant from each other. As a result, the quenching is substantially abated causing an increase in the fluorescence emission detected.

[00127] In addition to homogenous detection methods, a variety of other heterogeneous detection methods are known in the art which can be employed in the present invention, including standard non-denaturing gel electrophoresis (e.g., acrylamide or agarose), denaturing gradient gel electrophoresis, and temperature gradient gel electrophoresis. Standard non-denaturing gel electrophoresis is a simple and quick method of PCR detection, but may not be suitable for all applications.

[00128] Denaturing Gradient Gel Electrophoresis (DGGE) is a separation method that detects differences in the denaturing behavior of small DNA fragments (200-700 bp). The principle of the separation is based on both fragment length and nucleotide sequence. In fragments that are the same length, a difference as little as one base pair can be detected. This is in contrast to non-denaturing gel electrophoresis, where DNA fragments are separated only by size. This limitation of non-denaturing gel electrophoresis results because the difference in charge density between DNA molecules is near neutral and plays little role in their separation. As the size of the DNA fragment increases, its velocity through the gel decreases.

e. Reagents and Kits:

[00129] Any suitable nucleic acid replication composition (“replication composition”) in any format can be used. A typical replication composition for PCR amplification may comprise, for example, dATP, dCTP, dGTP, dTTP, target specific primers and a suitable polymerase.

[00130] If the replication composition is in liquid form, suitable buffers known in the art may be used (Sambrook, J. et al., *supra*).

[00131] Alternatively, if the replication composition is contained in a tablet form, then typical tabletization reagents may be included such as stabilizers and binding agents. Preferred tabletization technology is set forth in U.S. Pat. Nos. 4,762,857 and 4,678,812, each of which is hereby incorporated by reference in its entirety.

[00132] A preferred replication composition of the instant invention comprises (a) the primer pair from Table 2 and (b) thermostable DNA polymerase.

[00133] A more preferred replication composition of the present invention comprises (a) the primer pairs and any corresponding probe or blocking oligonucleotide selected from Table 2, wherein each nucleic acid probe or primer-probe complex employed comprises a detectable label; and (b) thermostable DNA polymerase. Preferably the detectable label comprises a reporter capable of emitting a detectable signal and a quencher capable of substantially quenching the reporter and preventing the emission of the detectable signal when the reporter and quencher are in sufficiently close proximity to one another.

[00134] A preferred kit of the instant invention comprises any one of the above replication compositions. A preferred tablet of the instant invention comprises any one of

the above replication compositions. More preferably, a kit of the instant invention comprises the foregoing preferred tablet.

[00135] In some instances, an internal positive control can be included in the reaction. The internal positive control can include control template nucleic acids (e.g. DNA or RNA), control primers, and control nucleic acid probe. The advantages of an internal positive control contained within a PCR reaction have been previously described (U.S. Pat. No. 6,312,930 and PCT Application No. WO 97/11197, each of which is hereby incorporated by reference in its entirety), and include: (i) the control may be amplified using a single primer; (ii) the amount of the control amplification product is independent of any target DNA or RNA contained in the sample; (iii) the control DNA can be tableted with other amplification reagents for ease of use and high degree of reproducibility in both manual and automated test procedures; (iv) the control can be used with homogeneous detection, i.e., without separation of product DNA from reactants; and (v) the internal control has a melting profile that is distinct from other potential amplification products in the reaction and/or a detectable label on the control nucleic acid that is distinct from the detectable label on the nucleic acid probe directed to the target.

[00136] Control DNA will be of appropriate size and base composition to permit amplification in a primer-directed amplification reaction. The control template DNA sequence may be obtained from the *S. enteritidis* or *S. Typhimurium* genome, or from another source, but must be reproducibly amplified under the same conditions that permit the amplification of the target amplification product.

[00137] The control reaction is useful to validate the amplification reaction. Amplification of the control DNA occurs within the same reaction tube as the sample that is being tested, and therefore indicates a successful amplification reaction when samples are target negative, i.e. no target amplification product is produced. In order to achieve significant validation of the amplification reaction, a suitable number of copies of the control DNA template must be included in each amplification reaction.

[00138] In some instances it may be useful to include an additional negative control replication composition. The negative control replication composition will contain the same reagents as the replication composition but without the polymerase. The primary function of such a control is to monitor spurious background fluorescence in a homogeneous format when the method employs a fluorescent means of detection.

[00139] Replication compositions may be modified depending on whether they are designed to be used to amplify target DNA or the control DNA. Replication compositions that will amplify the target DNA (test replication compositions) may include (i) a polymerase (generally thermostable), (ii) a primer pair capable of hybridizing to the target DNA and (iii) necessary buffers for the amplification reaction to proceed. Replication compositions that will amplify the control DNA (positive control, or positive replication composition) may include (i) a polymerase (generally thermostable) (ii) the control DNA; (iii) at least one primer capable of hybridizing to the control DNA; and (iv) necessary buffers for the amplification reaction to proceed. In addition, the replication composition for either target DNA or control DNA amplification can contain a nucleic acid probe, preferably possessing a detectable label.

g. Nucleic Acid Hybridization Methods

[00140] In addition to primer-directed amplification assay methods, nucleic acid hybridization assay methods can be employed in the present invention for detection of *S. Typhimurium*. The basic components of a nucleic acid hybridization test include probe(s), a sample suspected of containing *S. Typhimurium*, and a specific hybridization method. Typically the probe(s) length can vary from as few as five bases to the full length of the *S. Typhimurium* diagnostic sequence and will depend upon the specific test to be done. Only part of the probe molecule need be complementary to the nucleic acid sequence to be detected. In addition, the complementarity between the probe(s) and the target sequence(s) need not be perfect. Hybridization does occur between imperfectly complementary molecules with the result that a certain fraction of the bases in the hybridized region(s) are not paired with the proper complementary base.

[00141] The sample may or may not contain *S. Typhimurium*. The sample may take a variety of forms, however will generally be extracted from an animal, environmental or food source suspected of contamination. In a preferred embodiment, the sample will be extracted from a chicken. The DNA may be detected directly but most preferably, the sample nucleic acid must be made available to contact the probe before any hybridization of probe(s) and target molecule(s) can occur. Thus the organism's DNA is preferably free from the cell and placed under the proper conditions before hybridization can occur. Methods of in-solution hybridization necessitate the purification of the DNA in order to be able to obtain hybridization of the sample DNA with the probe(s). This has meant that utilization of the in-solution method for detection of target sequences in a sample requires

that the nucleic acids of the sample must first be purified to eliminate protein, lipids, and other cell components, and then contacted with the probe(s) under hybridization conditions. Methods for the purification of the sample nucleic acid are common and well known in the art (Sambrook et al., *supra*).

[00142] Hybridization assays may be conducted directly on cell lysates, without the need to extract the nucleic acids. This eliminates several steps from the sample-handling process and speeds up the assay. To perform such assays on crude cell lysates, a chaotropic agent is typically added to the cell lysates prepared as described above. The chaotropic agent stabilizes nucleic acids by inhibiting nuclease activity. Furthermore, the chaotropic agent allows sensitive and stringent hybridization of short oligonucleotide probes to DNA at room temperature (Van Ness & Chen, *Nucleic Acids Res.* 19:5143-51 (1991)). Suitable chaotropic agents include guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium iodide, and cesium trifluoroacetate, among others. Typically, the chaotropic agent will be present at a final concentration of about 3 M. If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v).

[00143] Alternatively, one can purify the sample nucleic acids prior to probe hybridization. A variety of methods are known to one of skill in the art (e.g., phenol-chloroform extraction, IsoQuick extraction (MicroProbe Corp., Bothell, Wash.), and others). Pre-hybridization purification is particularly useful for standard filter hybridization assays. Furthermore, purification facilitates measures to increase the assay sensitivity by incorporating in vitro RNA amplification methods such as self-sustained sequence replication (see for example Fahy et al., In *PCR Methods and Applications*, Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y. (1991), pp. 25-33) or reverse transcriptase PCR (Kawasaki, In *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis et al., Eds., (1990), pp. 21-27).

[00144] Once the DNA is released, it can be detected by any of a variety of methods. However, the most useful embodiments have at least some characteristics of speed, convenience, sensitivity, and specificity.

[00145] Hybridization methods are well known in the art. Typically the probe and sample must be mixed under conditions which will permit nucleic acid hybridization. This involves contacting the probe and sample in the presence of an inorganic or organic

salt under the proper concentration and temperature conditions. The probe and sample nucleic acids must be in contact for a long enough time that any possible hybridization between the probe and sample nucleic acid may occur. The concentration of probe or target in the mixture will determine the time necessary for hybridization to occur. The higher the probe or target concentration, the shorter the hybridization incubation time needed.

[00146] Various hybridization solutions can be employed. Typically, these comprise from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs about 30-50% v/v formamide, about 0.15 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9), about 0.05 to 0.2% detergent, such as sodium dodecylsulfate, or between 0.5-20 mM EDTA, FICOLL (Pharmacia Inc.) (about 300-500 kilodaltons), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/mL, fragmented nucleic DNA (e.g., calf thymus or salmon sperm DNA, or yeast RNA), and optionally from about 0.5 to 2% wt/vol glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents (e.g., polyethylene glycol), anionic polymers (e.g., polyacrylate or polymethylacrylate), and anionic saccharidic polymers (e.g., dextran sulfate).

[00147] Nucleic acid hybridization is adaptable to a variety of assay formats. One of the most suitable is the sandwich assay format. The sandwich assay is particularly adaptable to hybridization under non-denaturing conditions. A primary component of a sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the DNA sequence.

[00148] The sandwich assay may be encompassed in an assay kit. This kit would include a first component for the collection of samples suspected of contamination and buffers for the disbursement and lysis of the sample. A second component would include media in either dry or liquid form for the hybridization of target and probe polynucleotides, as well as for the removal of undesirable and nonduplexed forms by washing. A third component includes a solid support (dipstick) upon which is fixed (or to which is conjugated) unlabeled nucleic acid probe(s) that is (are) complementary to one or more of the sequences disclosed herein. A fourth component would contain labeled

probe that is complementary to a second and different region of the same DNA strand to which the immobilized, unlabeled nucleic acid probe of the third component is hybridized.

[00149] In a preferred embodiment, polynucleotide sequences disclosed herein or derivations thereof may be used as 3' blocked detection probes in either a homogeneous or heterogeneous assay format. For example, a probe generated from these sequences may be 3' blocked or non-participatory and will not be extended by, or participate in, a nucleic acid amplification reaction. Additionally, the probe incorporates a label that can serve as a reactive ligand that acts as a point of attachment for the immobilization of the probe/analyte hybrid or as a reporter to produce detectable signal. Accordingly, genomic or cDNA isolated from a sample suspected of *S. Typhimurium* contamination is amplified by standard primer-directed amplification protocols in the presence of an excess of the 3' blocked detection probe(s) to produce amplification products. Because the probe(s) is 3' blocked, it does not participate or interfere with the amplification of the target. After the final amplification cycle, the detection probe(s) anneals to the relevant portion of the amplified DNA and the annealed complex is then captured on a support through the reactive ligand.

[00150] In some instances it is desirable to incorporate a ligand labeled dNTP, with the label probe in the replication composition to facilitate immobilization of the PCR reaction product on a support and then detection of the immobilized product by means of the labeled probe reagent. For example a biotin, digoxigenin, or digoxin labeled dNTP could be added to PCR reaction composition. The biotin, digoxigenin, or digoxin incorporated in the PCR product could then be immobilized respectively on to a streptavidin, anti-digoxin or antidigoxigenin antibody support. The immobilized PCR product could then be detected by the presence of the probe label.

[00151] The present invention is illustrated by the following examples. It is to be understood that the particular examples, materials, amounts, and procedures are to be interpreted broadly in accordance with the scope and spirit of the invention as set forth herein.

EXAMPLES

Example 1: Assay for *crp* and *cya* mutants

[00152] Materials and Methods

[00153] Genomic DNA was isolated from vaccine isolates and wild type isolates. 2ul of each was used as template in individual PCRs with the following conditions: 95°C – 3 minutes; 95°C – 30 seconds; 65°C – 30 seconds; 68°C – 30 seconds; cycles: 30.

[00154] PCR was performed on the Analytik Jena T-ONE thermacycler machine. Five (5) ul of each reaction was run on a gel for confirmation (100V, 1 hour). This was tested for Crp using PCR1 (wild type), and PCRs 2 and 3 (vaccine), add for Cya using PCR4 (wild type), and PCRs 5 and 6 (vaccine). Gel images are provided herein PCR assays were developed against field and vaccine strains and validated by screening isolates in our collection. PCR primers according to Table 2 were developed for detecting *Salmonella* Typhimurium cya and crp genes.

[00155] Results

[00156] The application of live attenuated *Salmonella* Typhimurium vaccines has significantly helped control *Salmonella* in poultry products. Because USDA-FSIS scores all *Salmonella* as positive, regardless of serovar, attenuated vaccine strains that are identified at processing contribute negatively toward *Salmonella* performance standards. This study was designed to determine the incidence of a live attenuated *Salmonella* Typhimurium vaccine identified in broiler products by FSIS and to develop a PCR assay for screening of isolates.

[00157] A search of whole genome sequencing (WGS) data was performed to identity the amount of *Salmonella* Typhimurium identified by the USDA-FSIS was due to vaccination. *Salmonella* Typhimurium short-read sequences from chicken samples uploaded to NCBI Pathogen Detection by FSIS from 2016-2022 were downloaded and assembled (FIG. 1). These were analyzed using BLAST with a sequence unique to field strains, followed by a sequence unique to the vaccine strain (FIGs. 2A-2C). PCR assays were developed against field and vaccine strains and validated by screening isolates in our collection. Between 2016-2022, 1,682 *Salmonella* Typhimurium isolates were found on NCBI Pathogen Detection., corresponding to 7.9% of all *Salmonella* identified (FIG. 1).

[00158] Using the PCR assay of this invention, it was found that a total of 104 (6.1%) isolates were identified as the vaccine strain (FIG. 2A-2B). The PCR assay differentiated field strains from the vaccine strain when applied to isolates as well as to overnight mixed *Salmonella* enrichments.

[00159] PCR assays were performed using the unique primers developed for Crp (FIGs. 3A-3B). Both primers for PCR vaccine 1 and PCR vaccine 2 successfully detected the presence of the vaccine *Salmonella* Typhimurium CRP isolate (FIG. 3C). Primers for developed for Cya (FIGs. 4A) also successfully detected the presence of the vaccine *Salmonella* Typhimurium CYA isolate (FIG. 4B).

[00160]

Example 2: Screening of samples that contain *S. Typhimurium* from the vaccine strain

[00161] Results

[00162] Genomic DNA was isolated from mixed serotype cultures known to contain Typhimurium. Two (2) ul was used as a template with the above cycling parameters. Vaccine strain was detected in some mixtures. In cases where wild type was detected, it is not known whether this is Typhimurium or another serotype.

[00163] A PCR assay screening for samples that contain the vaccine strain of *S. Typhimurium* was performed with some samples consisting of mixed serotypes. Figure 5 shows that lanes U2, U3, U6 are positive for vaccine strain. Lanes U2 and U3 also contain other *Salmonella* serotypes. Lanes U1, U4, and U5 did not contain vaccine strain.

Example 3: Testing multiplex PCR using primers for crp

[00164] Results

[00165] PCR primers were mixed to run a multiplex PCR for crp and tested on individual colonies belonging to vaccine strain or to wild type Typhimurium. PCR parameters were as shown above. FIG. 6 shows that the primers for the vaccine strain were only positive for the vaccine strain, while the WT primers were only positive for the WT strain.

[00166] Live attenuated *Salmonella* vaccines are a critical pre-harvest tool for *Salmonella* control and are widely used in industry. With forthcoming regulations that will likely focus on *Salmonella* Typhimurium, along with other serovars, there is a need to distinguish between isolates belonging to the vaccine strain and those that are responsible for causing human illness.

[00167] The complete disclosure of all patents, patent applications, and publications, and electronically available material (including, for instance, nucleotide sequence

submissions in, e.g., GenBank and RefSeq, and amino acid sequence submissions in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq) cited herein are incorporated by reference. In the event that any inconsistency exists between the disclosure of the present application and the disclosure(s) of any document incorporated herein by reference, the disclosure of the present application shall govern. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

What is claimed is:

1. A PCR diagnostic assay for differentially identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject comprising primers for detecting *Salmonella* Typhimurium target sequences.
2. The PCR diagnostic assay of claim 1, wherein the subject is an animal selected from poultry, pigs, cattle, chickens, turkeys, geese, ducks, pheasants, bantam, quail, and pigeons and the like.
3. The PCR diagnostic assay of claim 1, wherein the subject has been vaccinated against wild-type virulent *Salmonella* Typhimurium.
4. The PCR diagnostic assay of claim 1, wherein the primers detect *Salmonella* Typhimurium cAMP receptor protein (Crp) gene target sequences within a *Salmonella* Typhimurium cAMP receptor protein (Crp) gene, or *Salmonella* Typhimurium adenylate cyclase (CYA) gene target sequences within a *Salmonella* Typhimurium adenylate cyclase (CYA) gene, the Crp or CYA target sequences having a nucleic acid sequence of SEQ ID NO:1, 2 or combinations thereof.
5. The PCR diagnostic assay of claim 1, wherein a whole genome search is performed to identify isolates of a vaccine-associated *Salmonella* Typhimurium Crp or CYA gene, the gene having a nucleotide sequences with 95%, 96%, 97%, 98%, 99% or 100% sequence identity SEQ ID NO: 3, 4, 5, 6, 7, 8 or combinations thereof.
6. The PCR diagnostic assay of claim 4, wherein the primers for detecting the *Salmonella* Typhimurium Crp and CYA gene target sequences are selected from SEQ ID NO: 9, 10, 11, 12, 13, 14, 15, 16, 17, or combinations thereof
7. The PCR diagnostic assay of claim 4, wherein the primers detect transposon Tn10 in the *Salmonella* Typhimurium Crp or CYA gene target sequences of the vaccine-associated *Salmonella* Typhimurium.

8. The PCR diagnostic assay of claim 7, wherein transposon Tn10 in the Crp gene of *Salmonella* Typhimurium, is detected using the forward primer having a nucleotide sequence as set forth in SEQ ID NO. 13 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 11.

9. The PCR diagnostic assay of claim 7, wherein transposon Tn10 in the Crp gene of *Salmonella* Typhimurium, is detected using the forward primer having a nucleotide sequence as set forth in SEQ ID NO. 17 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 16.

10. A method for differentially diagnosing between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium comprising:

- (a) extracting sample genomic DNA from the subject;
- (b) adding samples: adding the sample genomic DNA, a positive control or a negative control into PCR tubes of PCR reaction system respectively to obtain a corresponding sample reaction tube, positive reaction tube or negative reaction tube, wherein the PCR reaction system contains the primers for detecting a *Salmonella* Typhimurium Crp or CYA gene;
- (c) performing PCR reaction: placing the reaction tubes on a PCR instrument, setting circulation parameters, and performing PCR reaction;
- (d) analyzing results after the PCR reaction is completed; and
- (e) determining the presence of Tn10 in the sample;

wherein the presence of Tn10 indicates the presence vaccine-associated *Salmonella* Typhimurium.

11. The method of claim 10, wherein the primers detect *Salmonella* Typhimurium Crp gene target sequences within a *Salmonella* Typhimurium Crp gene, the gene having a nucleic acid sequence of SEQ ID NO:1.

12. The method of claim 10, wherein the primers detect *Salmonella* Typhimurium CYA gene target sequences within a *Salmonella* Typhimurium CYA gene, the gene having a nucleic acid sequence of SEQ ID NO:2.

13. The method of claim 11, wherein the primers for detecting the *Salmonella* Typhimurium Crp gene target sequence are selected from SEQ ID NO: 9, 10, 11, 12, and 13.
14. The method of claim 12, wherein the primers for detecting the *Salmonella* Typhimurium CYA gene target sequence are selected from SEQ ID NO: 14, 15, 16, and 17.
15. The method of claim 10, wherein the primers detect transposon Tn10 in the *Salmonella* Typhimurium Crp gene and *Salmonella* Typhimurium CYA gene target sequences of the vaccine-associated *Salmonella* Typhimurium.
16. The method of claim 15, wherein transposon Tn10 in the Crp gene of *Salmonella* Typhimurium, is detected using the forward primer having a nucleotide sequence as set forth in SEQ ID NO. 13 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 11, or the transposon Tn10 in the CYA gene of *Salmonella* Typhimurium, is detected using the forward primer having a nucleotide sequence as set forth in SEQ ID NO. 17 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 16.
17. The method of claim 10, wherein the subject is a farm animal selected from poultry, pigs, or cattle.
18. The method of claim 11, wherein the subject is a poultry animal selected from a group consisting of chickens, turkeys, geese, ducks, pheasants, bantam, quail, and pigeons.
19. The method of claim 10, wherein the subject has been vaccinated against wild-type virulent *Salmonella* Typhimurium.

20. A PCR diagnostic assay kit for differentiating between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium comprising:

- (a) primers for detecting Tn10 in the Crp or CYA genes of *Salmonella* Typhimurium, and the primers for detecting Tn10 comprising a forward primer having a nucleotide sequence as set forth in SEQ ID NO. 13 or 17 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 11 or 16;
- (b) primers for wild type virulent *Salmonella* Typhimurium, and the primers for detecting wild type virulent *Salmonella* Typhimurium comprising a forward primer having a nucleotide sequence as set forth in SEQ ID NO. 9 or 14 and a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 10 or 15;
- (c) control primers for a mutant *Salmonella* Typhimurium, and the primers for detecting mutant *Salmonella* Typhimurium comprising a reverse primer having a nucleotide sequence as set forth in SEQ ID NO. 12;
- (c) DNA polymerase;
- (c) blocking oligonucleotides;
- (d) a detectable label; and
- (e) buffers.

1/8

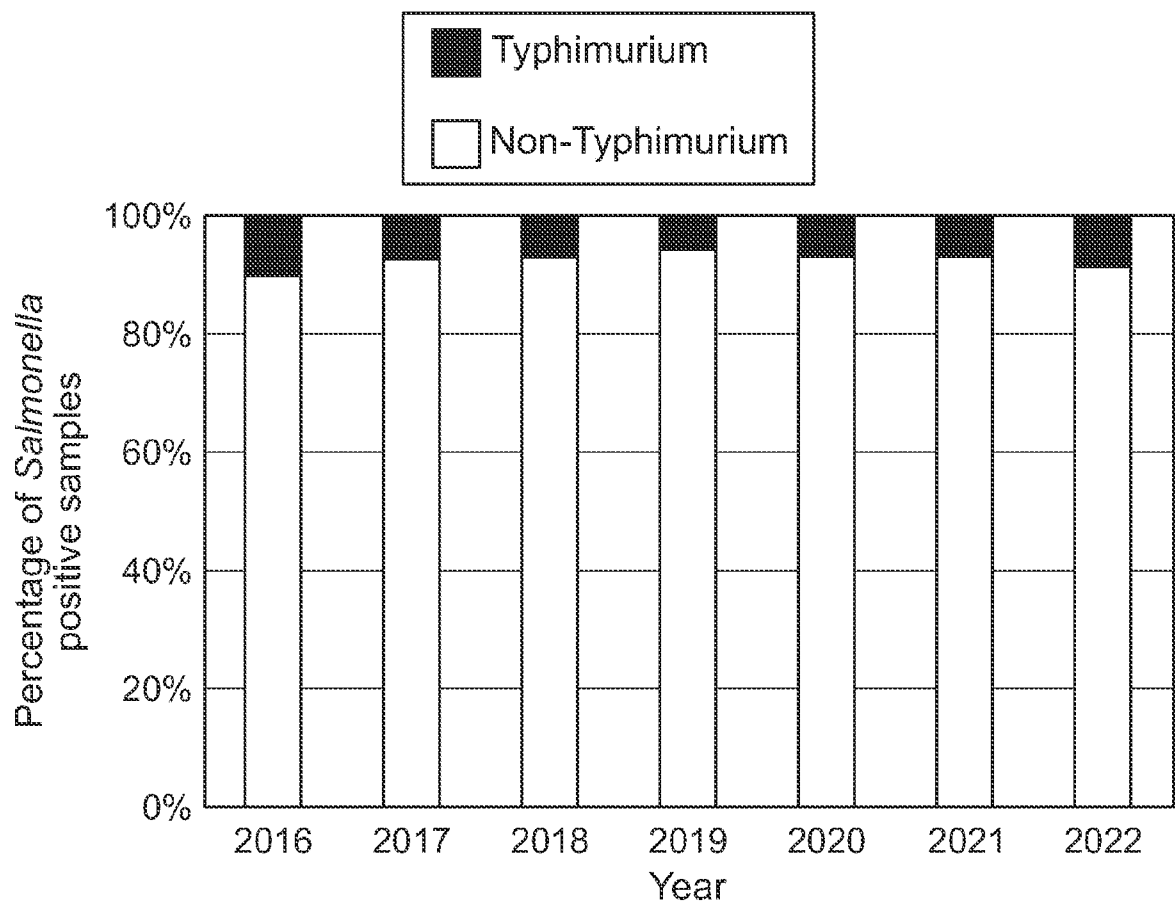


FIG. 1

2/8

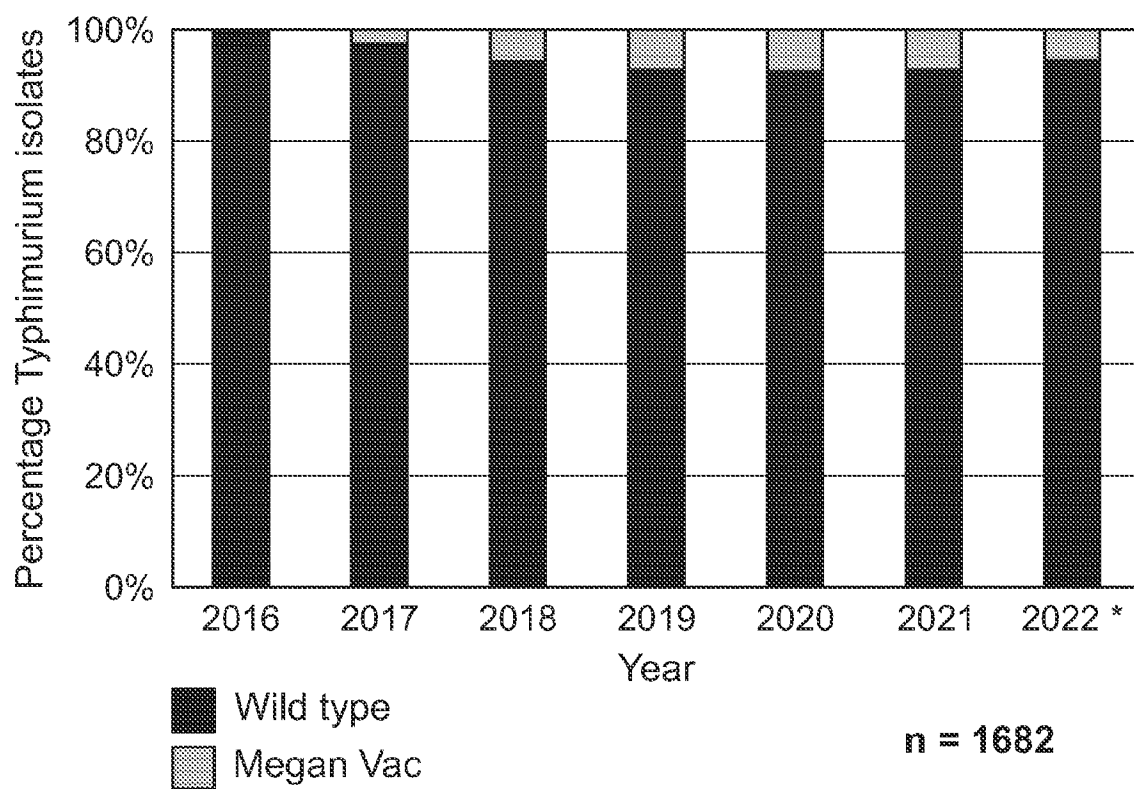


FIG. 2A

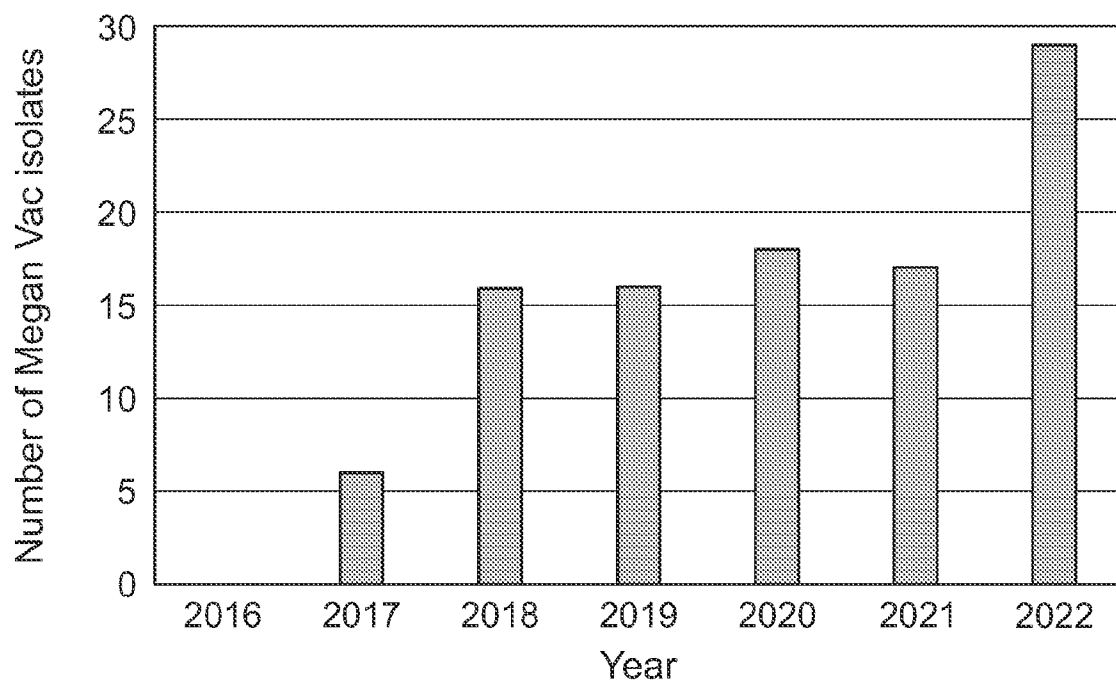


FIG. 2B

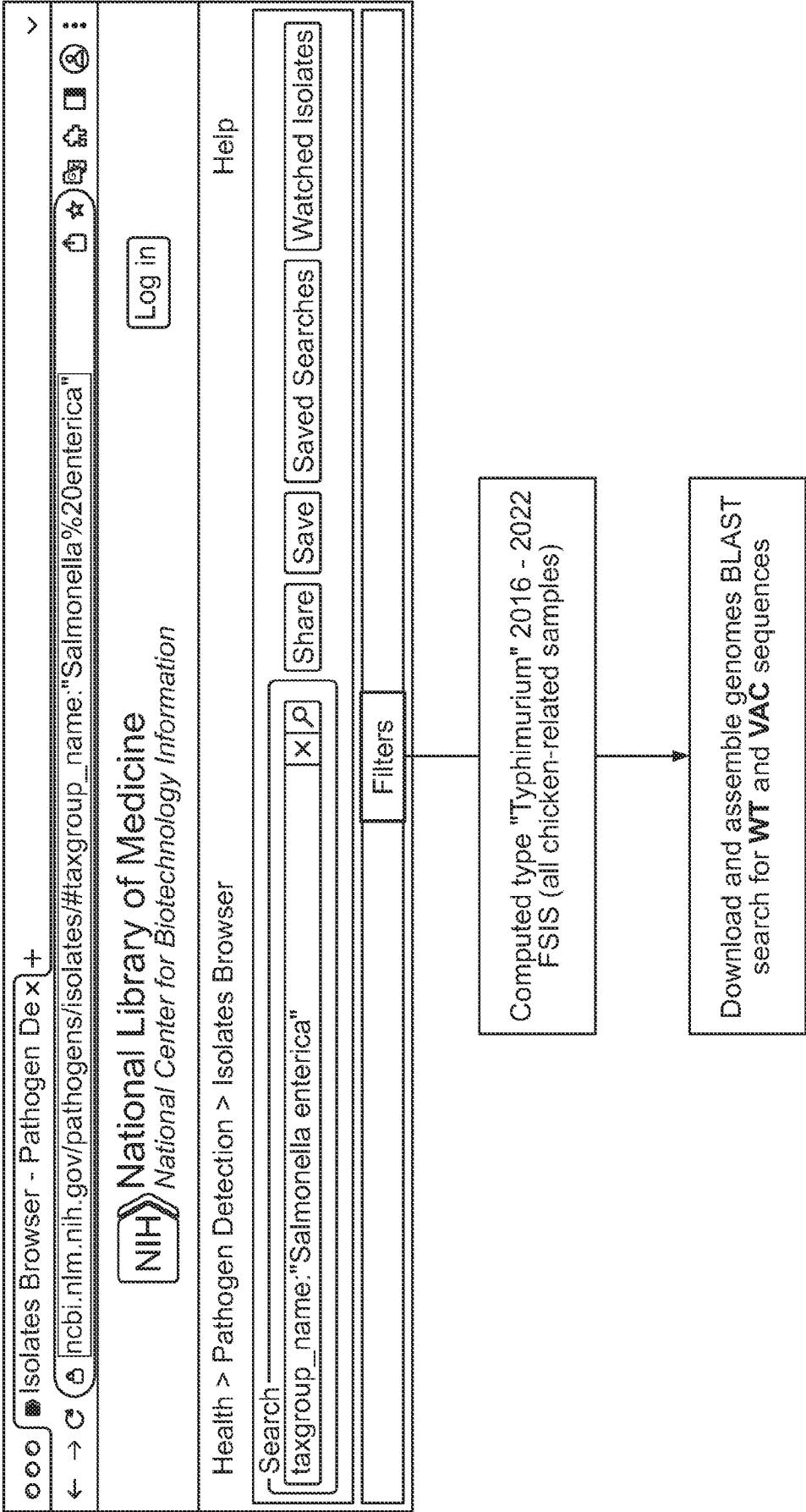


FIG. 2C

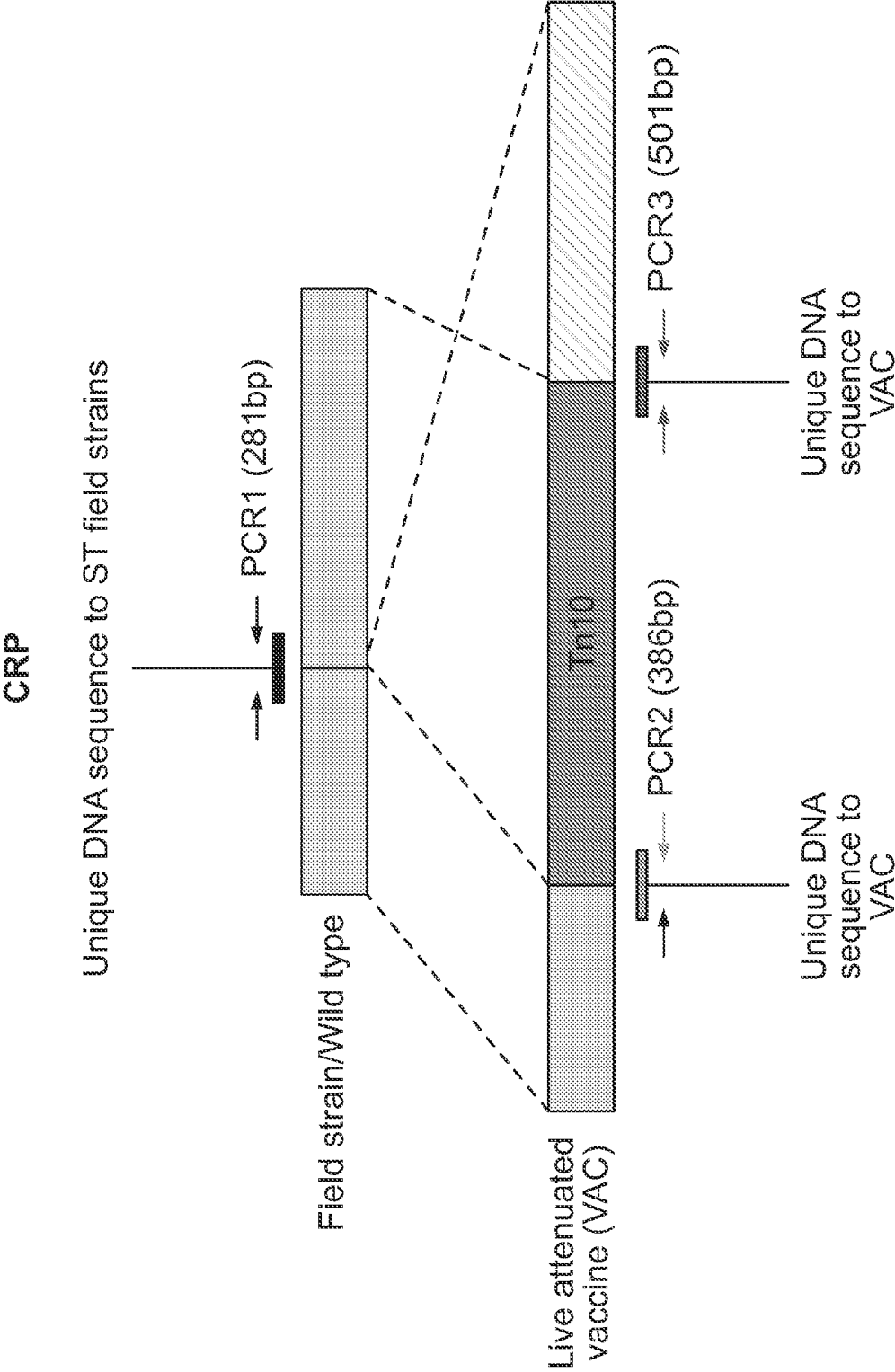


FIG. 3A

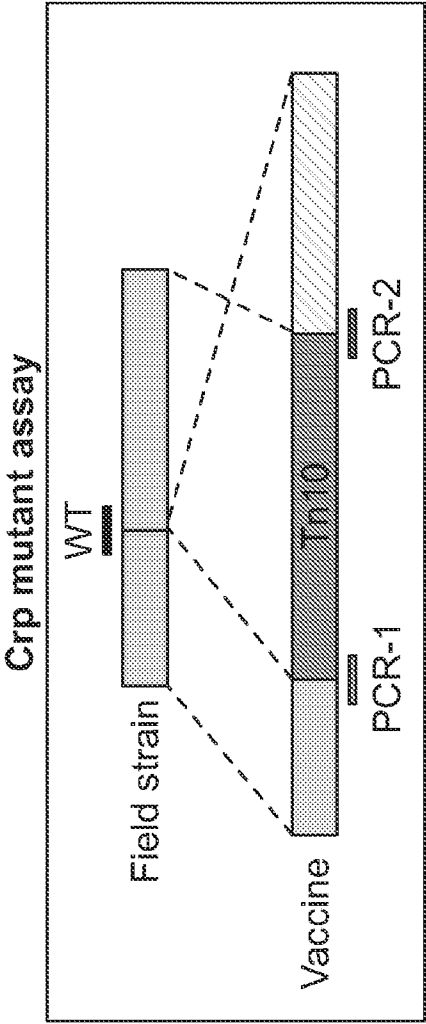


FIG. 3B

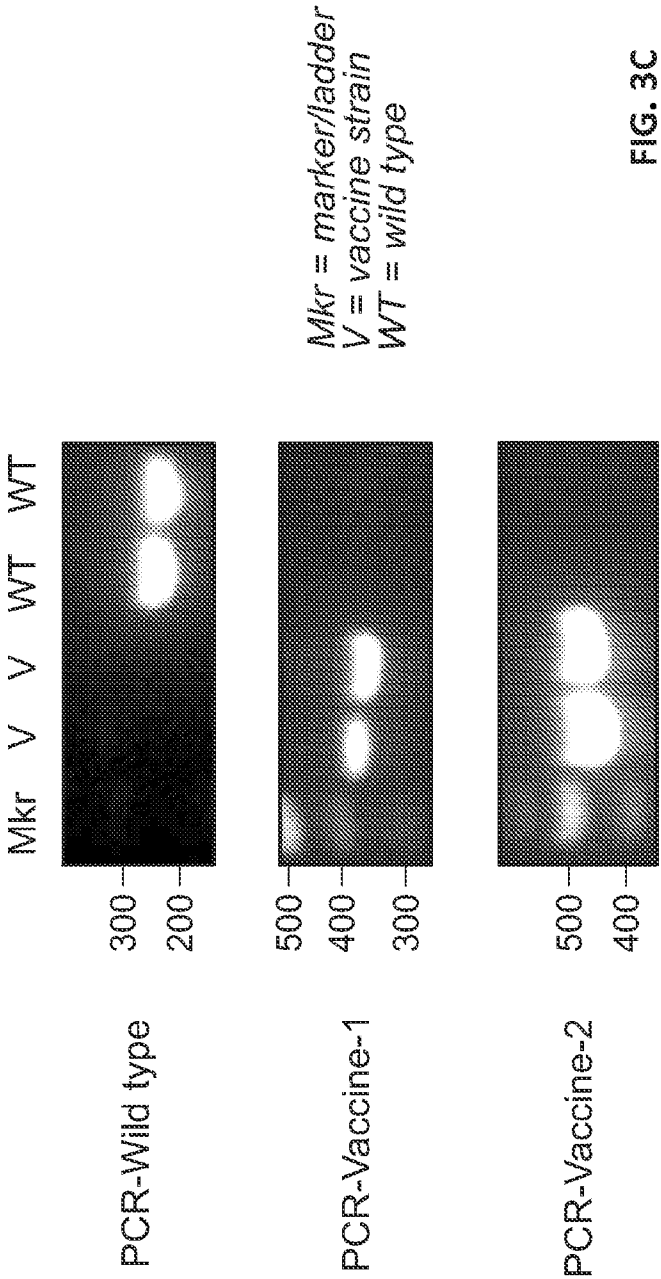


FIG. 3C

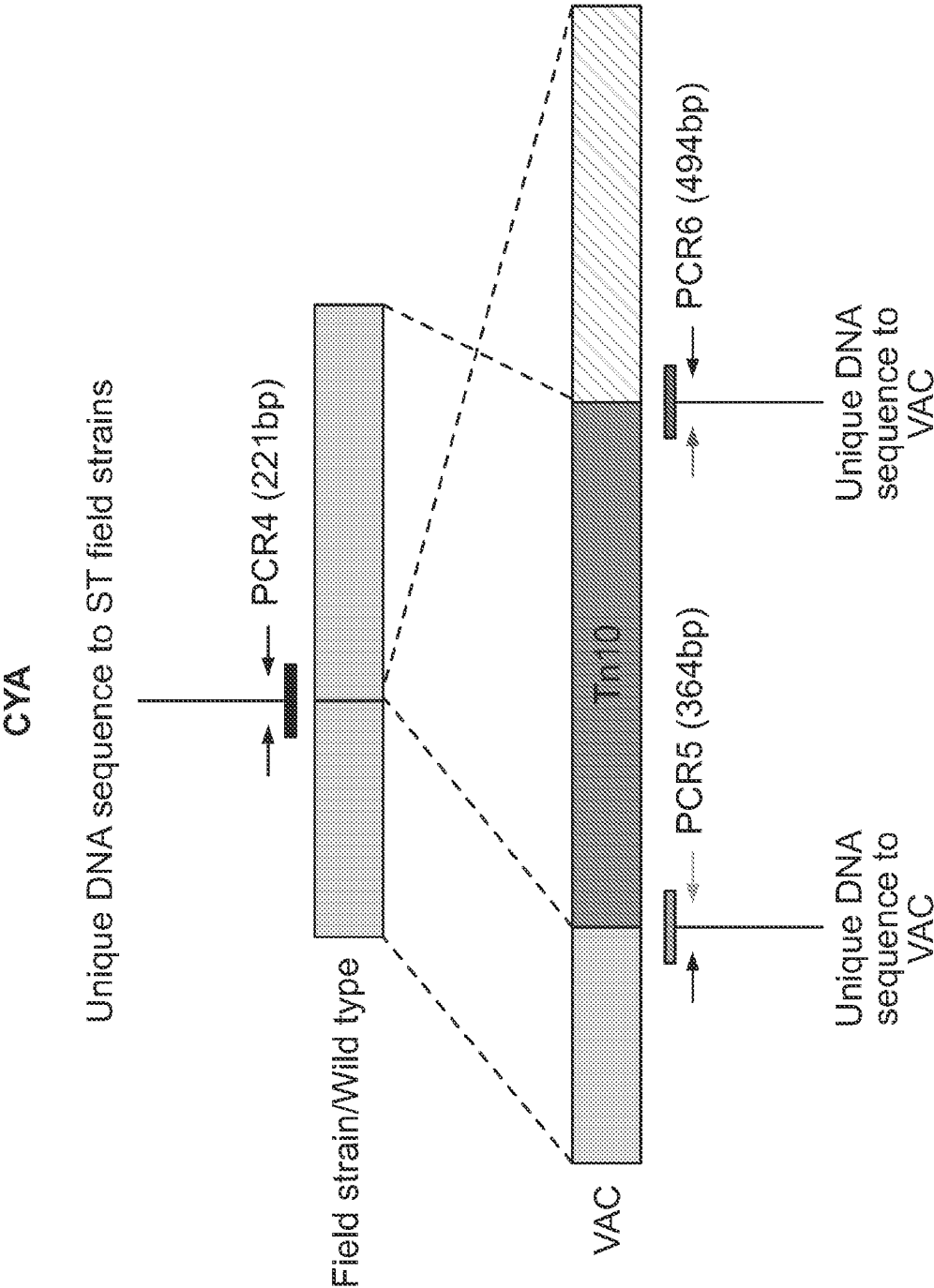
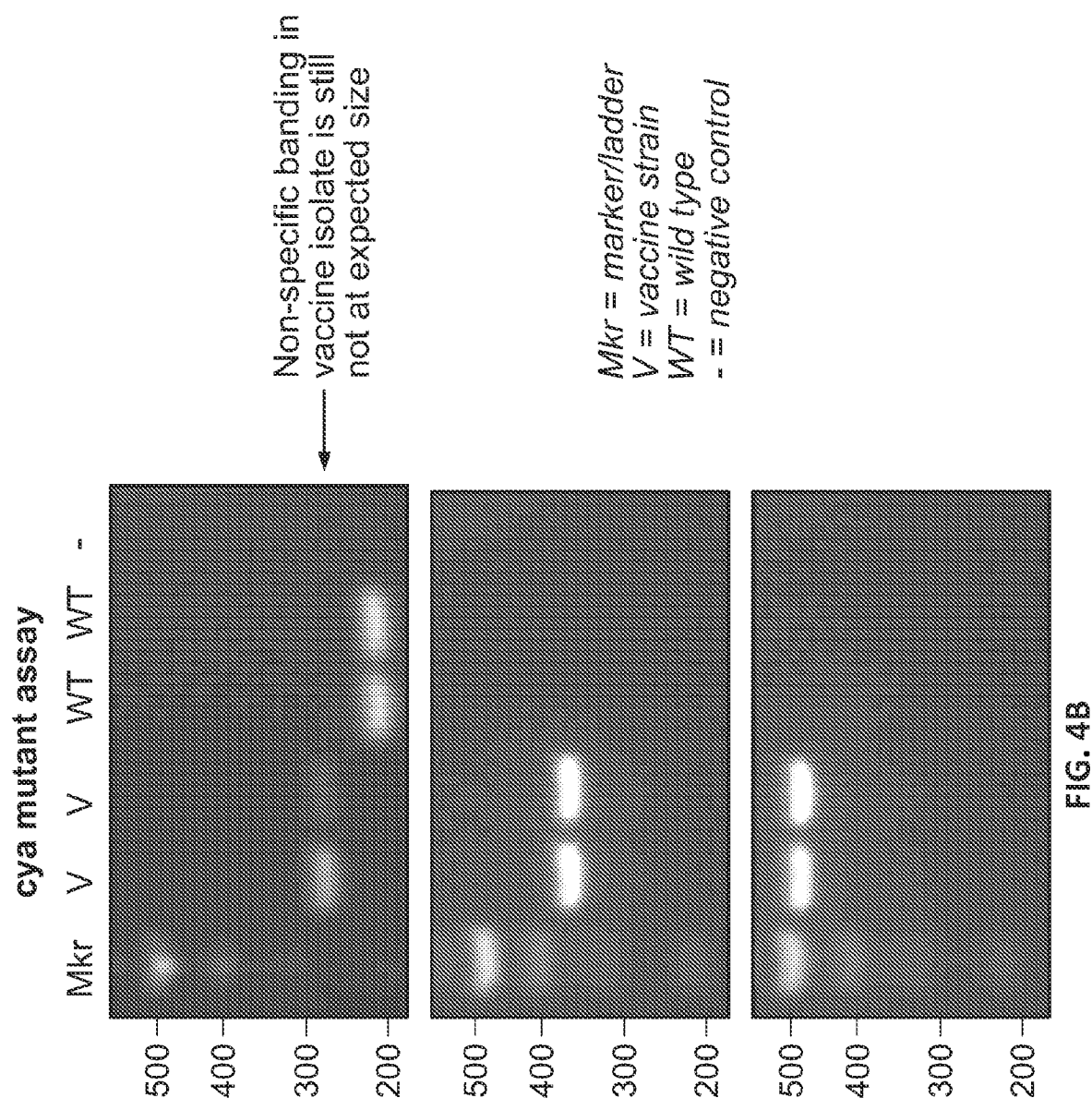
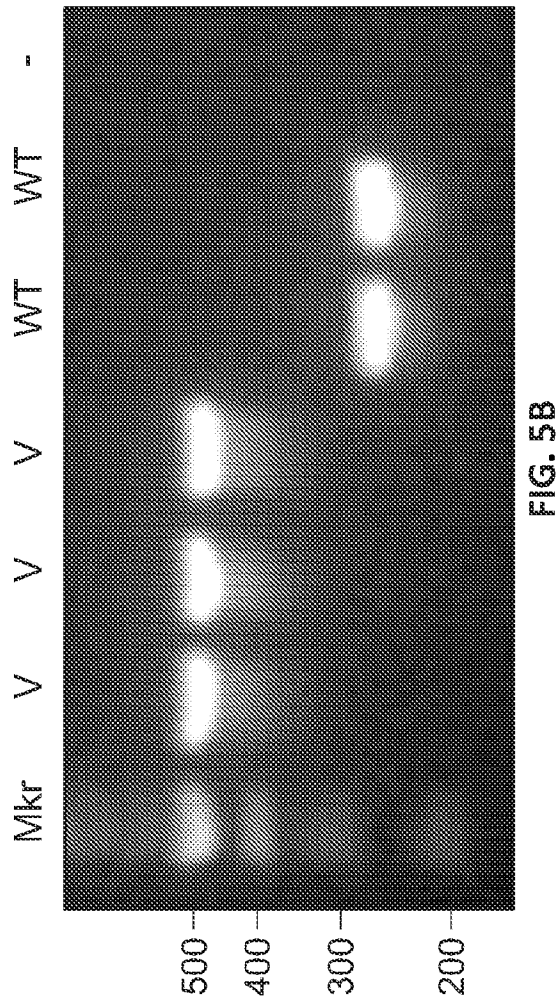
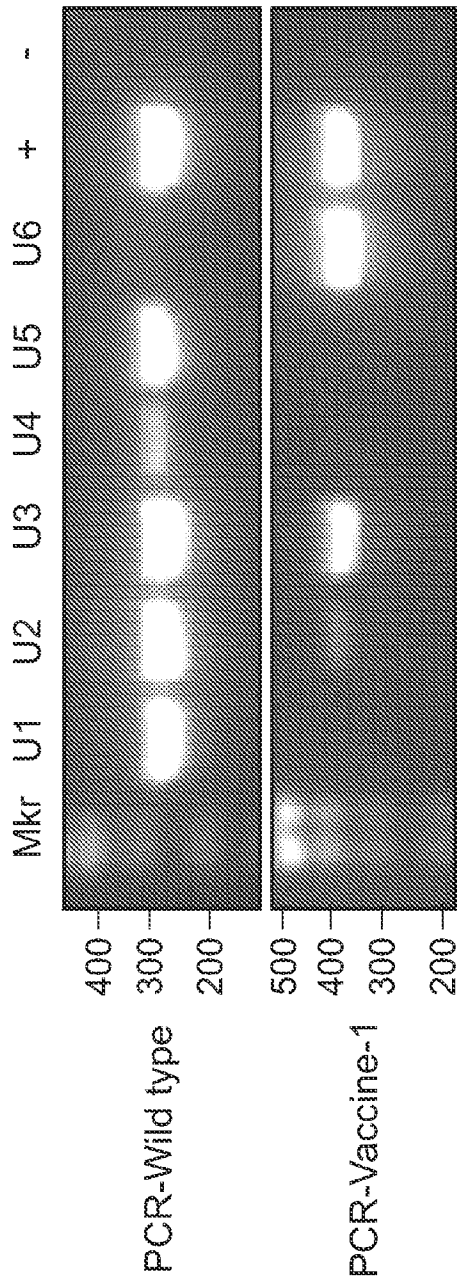


FIG. 4A





INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033003

A. CLASSIFICATION OF SUBJECT MATTERIPC: *C12Q 1/10* (2024.01); *C12Q 1/6888* (2024.01); *G01N 33/569* (2024.01)CPC: *C12Q 1/10*; *C12Q 1/6888*; *G01N 33/56916*; *G01N 2333/255*

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

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

See Search History Document

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

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 101130076 A (BEIJING GREAT GENIUS SCIENCE & TECHNOLOGY DEVELOPMENT) 27 February 2008 (27.02.2008) entire document	1-7
A	WO 1992/008486 A1 (WASHINGTON UNIVERSITY) 29 May 1992 (29.05.1992) entire document	1-7
A	CN 102676419 A (ZHAO et al.) 19 September 2012 (19.09.2012) entire document	1-7
A	CEYSSENS et al., A molecular assay for rapidly distinguishing the AviPro SALMONELLA VAC T vaccine strain from wild-type field isolates, Journal of Microbiological Methods, Vol. 184, No. 106190, 22 March 2021, Pgs. 1-4	1-7
A	MCWHORTER et al., A Long-Term Efficacy Trial of a Live, Attenuated Salmonella Typhimurium Vaccine in Layer Hens, Frontiers in Microbiology, Vol. 9, No. 1380, 26 June 2018, Pgs. 1-13	1-7



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

16 August 2024 (16.08.2024)

Date of mailing of the international search report

11 October 2024 (11.10.2024)

Name and mailing address of the ISA/US

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Commissioner for Patents
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Facsimile No. 571-273-8300

Authorized officer

MATOS
TAINA

Telephone No. 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033003

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	RACCOURSIER et al., In silico and PCR Screening for a Live Attenuated Salmonella Typhimurium Vaccine Strain, Avian Diseases, Vol. 68, No. 1, 09 April 2024, Pgs. 18-24	1-7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033003

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed.
 - b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-9 and 20 are drawn to PCR diagnostic assays for differentially identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject and a PCR diagnostic assay kit for differentiating between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium.

Group II+: claims 10-19 are drawn to methods for differentially diagnosing between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium.

The first invention of Group I+ is restricted to a Crp target sequence selected to be SEQ ID NO: 1, a vaccine-associated *Salmonella* Typhimurium Crp sequence selected to be SEQ ID NO: 3, a forward primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 9, a reverse primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 10, PCR diagnostic assays for differentially identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject comprising the same, and a PCR diagnostic assay kit for differentiating between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium comprising the same. The first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. Specifically, the first named invention was selected based on the first listed Crp target sequence, the first listed vaccine-associated *Salmonella* Typhimurium Crp, and the first listed primers for detecting *Salmonella* Typhimurium Crp presented in the claims (see claims 4-6). It is believed that claims 1-7 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the Crp target sequence selected to be SEQ ID NO: 1, the vaccine-associated *Salmonella* Typhimurium Crp sequence selected to be SEQ ID NO: 3, the forward primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 9, and the reverse primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 10.

An exemplary election of Group II+ is a Crp target sequence selected to be SEQ ID NO: 1, a forward primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 9, a reverse primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 10 and methods for differentially diagnosing between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium comprising the same.

Applicant is invited to elect additional CRP target sequences, vaccine-associated *Salmonella* Typhimurium Crp sequences, forward primer sequences, reverse primer sequences, and their respective, corresponding, SEQ ID NOs to be searched in a specific combination by paying an additional fee for each set of election. An exemplary election would be a Crp target sequence selected to be SEQ ID NO: 1, a vaccine-associated *Salmonella* Typhimurium Crp sequence selected to be SEQ ID NO: 3, a forward primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 9, a reverse primer for detecting the *Salmonella* Typhimurium Crp selected to be SEQ ID NO: 11, PCR diagnostic assays for differentially identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject comprising the same, and a PCR diagnostic assay kit for differentiating between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium comprising the same. Additional CRP target sequences, vaccine-associated *Salmonella* Typhimurium Crp sequences, forward primer sequences, reverse primer sequences and their respective, corresponding, SEQ ID NOs will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

The inventions listed in Groups I+ and II+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ and II+ formulas do not share a significant structural element requiring the selection of CRP target sequences, vaccine-associated *Salmonella* Typhimurium Crp sequences, forward primer sequences, reverse primer sequences where “the primers detect *Salmonella* Typhimurium cAMP receptor protein (Crp) gene target sequences within a *Salmonella* Typhimurium cAMP receptor protein (Crp) gene, or *Salmonella* Typhimurium adenylate cyclase (CYA) gene target sequences within a *Salmonella* Typhimurium adenylate cyclase (CYA) gene, the Crp or CYA target sequences having a nucleic acid sequence of SEQ ID NO:1, 2 or combinations thereof,” where “a whole genome search is performed to identify isolates of a vaccine-associated *Salmonella* Typhimurium Crp or CYA gene, the gene having a nucleotide sequences with 95%, 96%, 97%, 98%, 99% or 100% sequence identity SEQ ID NO: 3, 4, 5, 6, 7, 8 or combinations thereof” and where “the primers for detecting the *Salmonella* Typhimurium Crp and CYA gene target sequences are selected from SEQ ID NO: 9, 10, 11, 12, 13, 14, 15, 16, 17, or combinations thereof.”

The special technical features of Groups I+, PCR diagnostic assays for differentially identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject and a PCR diagnostic assay kit for differentiating between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium, are not present in Groups II+; the special technical features of Groups II+, methods for differentially diagnosing between wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject vaccinated against a *Salmonella* Typhimurium, are not present in Groups I+.

Additionally, even if Groups I+ and II+ were considered to share the technical features of differentially identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject; PCR; primers for detecting a *Salmonella* Typhimurium Crp or CYA gene; and detecting Tn10, these shared technical features do not represent a contribution over the prior art as disclosed by the publication entitled “A molecular assay for rapidly distinguishing the AviPro SALMONELLA VAC T vaccine strain from wild-type field isolates” by Ceyssens et al. (hereinafter, “Ceyssens”), CN 101130076 A to Beijing Great Genius Science & Technology Development (hereinafter, “Beijing”), and WO 1992/008486 A1 to Washington University (hereinafter, “Washington”).

Ceyssens teaches identifying wild-type virulent *Salmonella* Typhimurium and vaccine-associated *Salmonella* Typhimurium in a subject (monitoring of veterinary isolates...we used whole-genome sequencing to identify conserved and specific mutations in resistance and virulence genes which enable to distinguish field and vaccine strains... Based on this information, we developed and validated a Luminex-based assay targeting seven specific single-nucleotide polymorphisms. This molecular test is able to distinguish both *Salmonella* ser. Typhimurium types with 100% sensitivity and specificity within one working day; abstract) and PCR (page 3, second column, first paragraph).

Beijing teaches primers for detecting a *Salmonella* Typhimurium Crp or CYA gene (based on the sequence of the Cya gene of the *Salmonella* typhimurium, the following primers are designed; paragraphs [0459]-[0463]).

Washington teaches detecting Tn10 (Tetracycline to a concentration of 12.5 µg/ml was used to select for transduction of Tn10 insertions and Tn10-induced mutations; page 27, lines 11-14).

The inventions listed in Groups I+ and II+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/033003

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-7**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.