

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
27 May 2010 (27.05.2010)

PCT

(10) International Publication Number
WO 2010/059899 A2

(51) International Patent Classification:
C07K 14/465 (2006.01)

(21) International Application Number:
PCT/US2009/065259

(22) International Filing Date:
20 November 2009 (20.11.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/116,396 20 November 2008 (20.11.2008) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

— with sequence listing part of description (Rule 5.2(a))

(54) Title: VACCINE FOR RUNTING-STUNTING SYNDROME

(57) Abstract: The present invention includes polypeptides, polynucleotides, antibodies, and vaccines associated with Runtling Stunting Syndrome (RSS) in poultry. The present invention also includes methods of protecting poultry, including chickens, against RSS by immunization with such polypeptides and vaccines.



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VACCINE FOR RUNTING-STUNTING SYNDROME

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CONTINUING APPLICATION DATA

This application claims the benefit of U.S. Provisional Application Serial No. 61/116,396, filed November 20, 2008, which is incorporated by reference herein.

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BACKGROUND

The runting stunting syndrome (RSS) in chickens is an economically devastating disease with an unknown causative agent. The disease, also known as MAS, infectious stunting syndrome, broiler runting syndrome, pale bird syndrome, and helicopter syndrome, is characterized by a stunted growth of chickens, an increased feed conversion rate, and poor flock uniformity in the size of the chickens. With RSS, chickens develop diarrhea and show a higher susceptibility to other diseases. Furthermore, cystic enteropathic lesions have been described as one of the hallmarks of the disease. See, for example, Zalvala and Sellers, 2005, "Runting-stunting syndrome," *The Informed Poultry Professional* Issue 85:1-4; Zalvala, 2006, "Runting stunting syndrome (RSS) in broilers: In vivo studies," available on the worldwide web at poultry-health.com/fora/inthelth/zalvala_wpdc_06.pdf; and Zalvala and Barbosa, May 2006, "Runting and stunting in broiler chickens," *Apinco-Facta*, May 2006, available on the worldwide web at poultry-health.com/fora/inthelth/zalvala_apinco_06.pdf; Clark and Jones "Runting-Stunting Syndrome in Broilers," University of Arkansas, Division of Agriculture and published in *Avian Advice*, Spring 2008, available on the worldwide web at thepoultrysite.com/articles/1110/runtingstunting-syndrome-in-broilers; and Rebel et al., 2006, *World's Poultry Sci. J.* 62:17-29.

RSS is an economically devastating disease for the poultry industry. The causative agent is unknown, available diagnostic tests are very limited, and there is no vaccine to prevent or mitigate the disease. There is a need for improved diagnostic and therapeutic reagents and methods for the detection, treatment, and prevention of RSS.

SUMMARY OF THE INVENTION

The present invention includes a polypeptide including an amino acid sequence with at least about 75% sequence identity to the amino acid sequence of SEQ ID NO:2, a truncation, or fragment thereof.

- 5 The present invention includes a polypeptide including an amino acid sequence of SEQ ID NO:2, a truncation, or fragment thereof.

The present invention includes a polypeptide including at least ten consecutive amino acids of SEQ ID NO:2 or at least ten consecutive amino acids of residues 1 to 743 of SEQ ID NO:2.

- 10 The present invention includes a polypeptide having at least fifteen consecutive amino acids of SEQ ID NO:2 or at least ten consecutive amino acids of residues 1 to 743 of SEQ ID NO:2.

The present invention includes a polynucleotide sequence encoding a polypeptide of the present invention.

- 15 The present invention includes a polynucleotide sequence having at least about 75% sequence identity to the nucleotide sequence of SEQ ID NO:1, or a fragment thereof.

The present invention includes a polynucleotide sequence including the nucleotide sequence SEQ ID NO:1.

- 20 The present invention includes a polynucleotide sequence that hybridizes to the polynucleotide sequence of SEQ ID NO:1 at high stringency.

The present invention includes a polypeptide encoded by the polynucleotide sequence of the present invention.

- 25 The present invention includes a vector including a polynucleotide sequence of the present invention. In some embodiments, the vector may be an expression vector.

The present invention includes a host cell including a vector of the present invention.

- 30 The present invention includes an oligonucleotide primer selected from SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16.

The present invention includes a primer pair, wherein the primer pair is SEQ ID NO:3/SEQ ID NO:4; SEQ ID NO:9/SEQ ID NO:10; SEQ ID NO:5/SEQ ID NO:7;

SEQ ID NO:5/SEQ ID NO:12; SEQ ID NO:5/SEQ ID NO:13; SEQ ID NO:8/SEQ ID NO:12; SEQ ID NO:8/SEQ ID NO:13; or SEQ ID NO:11/SEQ ID NO:13.

The present invention includes a composition including one or more polypeptides of the present invention. In some embodiments, the composition further includes an adjuvant. In some embodiments, the composition further includes an antigenic determinant from one or more additional pathogens infectious to poultry.

The present invention includes an immunological composition for raising antibodies in poultry, the composition including one or more polypeptides of the present invention. In some embodiments, the composition further includes an adjuvant. In some embodiments, the composition further includes an antigenic determinant from one or more additional pathogens infectious to poultry.

The present invention includes a vaccine including one or more polypeptides of the present invention. In some embodiments, the vaccine further includes an adjuvant. In some embodiments, the vaccine further includes an antigenic determinant from one or more additional pathogens infectious to poultry.

The present invention includes an antibody that binds to a polypeptide of the present invention. In some embodiments, the antibody may be a monoclonal antibody. The present invention includes a host cell producing a monoclonal antibody of the present invention.

The present invention includes a diagnostic kit including one or more polypeptides of the present invention, one or more polynucleotides of the present invention, one or more primers or primer pairs of the present invention, and/or one or more antibodies of the present invention.

The present invention includes a recombinant virus including one or more polypeptides of the present invention.

The present invention includes a recombinant virus including one or more of the polynucleotides of the present invention.

The present invention includes a method of detecting exposure to runting-stunting syndrome (RSS) in a bird, the method including determining that an antisera sample obtained from the bird specifically binds to a polypeptide of the present invention.

The present invention includes a method of mitigating the symptoms of runting-stunting syndrome (RSS) in poultry, the method including administering one

or more of the polypeptides of the present invention, a composition of the present invention, a vaccine of the present invention, one or more antibodies of the present invention, and/or one or more polynucleotide sequences of the present invention.

The present invention includes a method of producing anti-RSS antibodies in
5 poultry, the method including administering one or more of the polypeptides of the present invention, a composition of the present invention, a vaccine of the present invention, and/or one or more polynucleotide sequences of the present invention.

The present invention includes a method of preventing runting-stunting syndrome (RSS) in poultry, the method including administering one or more of the
10 polypeptides of the present invention, a composition of the present invention, a vaccine of the present invention, one or more antibodies of the present invention, and/or one or more polynucleotide sequences of the present invention.

The present invention includes a method of producing immunity to runting-stunting syndrome (RSS) in poultry, the method including administering one or more
15 of the polypeptides of the present invention, a composition of the present invention, a vaccine of the present invention, and/or one or more polynucleotide sequences of the present invention.

The present invention includes poultry produce by a method of the present invention.

20 In some embodiments of the methods of the present invention, the polypeptide, composition, vaccine, or polynucleotide sequence is administered to a breeder hen. The present invention includes a breeder hen produced by such a method. The present invention includes the offspring of the breeder hen. In some embodiments, the offspring of the breeder hen may demonstrate serum antibodies that bind to a
25 polypeptide having SEQ ID NO:2, a truncation, or fragment thereof, and/or reduced symptoms of RSS.

The present invention includes a method of making the polypeptide, composition, vaccine, polynucleotide sequence, vector, primer, antibody, host cell, diagnostic kit, or recombinant virus of the present invention.

30 The present invention includes a method of detecting a runting stunting syndrome (RSS) infectious agent in a sample, the method including detecting the hybridization of a polynucleotide of the present invention to the sample.

The present invention includes a method of detecting a runting stunting

syndrome (RSS) infectious agent in a sample, the method including producing a polymerase chain reaction (PCR) amplification product with at least one primer of the present invention or a primer pair of the present invention.

The terms "comprises" and variations thereof do not have a limiting meaning where these terms appear in the description and claims.

Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1B. RSS caused a severe weight depression and cystic enteropathy in young broiler chickens. In Fig. 1A, 150 one-day-old commercial broiler chickens were placed on chicken litter from a RSS affected farm (RSS+) or on fresh shavings (RSS-). Twelve days after placement chicken were weight and average of the body weights in gram (g) is shown. The standard deviation is depicted by bars. In Fig. 1B, the presence of cystic enteropathy was histological examined by the analysis of two cross sections of the duodenal loop of each bird. The number of sections which showed lesion out of the total number of analyzed sections is shown in parenthesis. The number of lesions per affected cross section is indicated at the left axis.

Figures 2A-2B. Gut content of RSS negative chickens did not induce lesion or weight depression. One-day old chickens (15 in each group) were inoculated either with PBS (Con/2) or gut content of RSS negative chickens (RSS-/2). As positive control the same numbers of chickens were inoculated with gut content of chickens showing retarded growth and typical lesions in the gut (RSS+/3). In Fig. 2A, the body weight of the chickens was determined before (grey columns) or after (black columns) inoculation. The standard deviation is depicted by bars. In Fig. 2B, the duodenal loop was evaluated for the presence of enteropathic lesions. The number of lesions per section is shown at the left axis. The total number of affected section is showed below the horizontal axis.

Figures 3A-3B. RSS is caused by a non-enveloped virus. In Fig. 3A, 15 one-day-old commercial broiler chickens were inoculated either with PBS (Con), with either gut content of RSS+ affected chicken (RSS+/3) or filtered gut content (RSS+/3filt) or filtered and chloroform treated gut content (RSS+/3filtCF). Before (grey box) and after the experiment (black box) the chicken were weight and the

average of the weight in gram is shown. The standard deviation is shown by error bars. In Fig. 3B, two cross sections of the duodenal loop of each bird was histologically examined for the presence of signs of cystic enteropathy. The number of sections which showed enteropathic lesions out of the total number of analyzed sections is shown in parenthesis. The number of enteropathic lesions per affected cross section is indicated at the right axis.

Figure 4. Cloning of the open reading frame of the astrovirus capsid protein. The obtained RT-PCR fragments and the determined astrovirus sequence are shown. The determined astrovirus sequence is shown as a striped box. The initially amplified RT-PCR fragments are shown as black boxes. RT-PCR fragments amplified with different primer pair combinations (in parenthesis) are depicted as open box. The amplified RT-PCR fragment encompassing the open reading frame of the astrovirus capsid protein with a 6x His at its C-terminus are indicated as dotted box.

Figure 5. Purification of the recombinant astrovirus capsid protein. Samples of lysed cells (1), of a lysate after centrifugation (2), of the centrifugation supernatant incubation with Talon (3), the flow through after the wash (4) and the eluate (5) were separated in 12% polyacrylamide gel by SDS-PAGE. The gels were either stained or a Western blot analysis was performed using an anti His mAb. The binding of the mAbs was visualised by chemoluminescence using a peroxidase-labeled anti-mouse goat serum. A molecular mass marker (M) is shown and the position of the recombinant protein is marked by an arrow.

Figure 6. Vaccination of broiler breeders with the recombinant astrovirus capsid protein induced an immune response in broiler breeder. A group of fifteen 22-week old commercial broiler breeder hens were vaccinated with an oil-emulsion vaccine containing the recombinant protein intramuscularly. One group of 15 hens and three roosters in both groups served as non-vaccinated control. All chickens were bled before the first (0-B) and 14 days after each of the three vaccinations (1st B, 2nd B, and 3rd B). The serum samples were analyzed in the astrovirus capsid protein ELISA along with the positive and negative (SPF) control serum. The extinction at a wave length of 405 nm is shown at the left axis.

Figure 7. Maternal derived antibodies raised against the astrovirus protein are transferred to the progeny of vaccinated hens. The progeny from vaccinated and non-vaccinated broiler hen groups were hatched two times (Gr 1, Gr 2) after each

vaccination. The collection of eggs started 14 days after the 2nd and 3rd vaccination. Blood samples were taken at day of hatch and serum samples were investigated using the astrovirus capsid protein ELISA. Positive and negative (SPF) control serum was analyzed in parallel. The extinction at a wave length of 405 nm is shown at the left
 5 axis.

Figures 8A-8B. Two vaccinations with the recombinant astrovirus capsid protein decreases enteropathic lesions in the gut of the offspring. In Fig. 8A, one-day-old progeny from the vaccinated (VACC) and non-vaccinated (NON-VAC) broiler breeders were divided randomly in groups 12 chickens each. The body weight
 10 was determined before (grey column) and after challenge (black column). The body weight is shown in gram (g) and the scale is shown at the left axis. The standard deviations are indicated by error bars. In Fig. 8B, 12 days after challenge the duodenal loop was taken during necropsy from chickens of all groups and a cross section was histological examined. The presence of enteropathic lesions in the
 15 non-vaccinated/challenged and vaccinated/challenged groups are shown by columns. The number of enteropathic lesions per section is indicated at the left axis.

Figures 9A-9B. Three vaccinations with the recombinant astrovirus capsid protein decreases enteropathic lesions in the gut and mitigates weight depression in the offspring. In Fig. 9A, one-day-old progeny from vaccinated (VACC) and
 20 non-vaccinated (NON-VAC) broiler breeders were divided randomly into two groups of 15 chickens each. The body weight was determined before (grey column) and after challenge (black column) and is shown in gram (g) as indicated at the left axis. Calculated standard deviations are indicated by error bars. The weight difference was calculated between the control group and the challenged group within the
 25 non-vaccinated and vaccinated hatch mates. The weight gain was calculated between day of hatch and day 12 within each group. In Fig. 9B, 12 days after challenge the duodenal loop was taken during necropsy from chickens of all groups and a cross section was histological examined. The presences of histological lesions in the non-vaccinated/challenged and vaccinated/challenged groups are shown by columns.
 30 The number of enteropathic lesions per section is indicated at the left axis.

Figure 10. Representative enteropathic lesions of cross section of the duodenal loop of experimentally RSS affected chicken. Haematoxylin and eosin stained section of the duodenal loop of progeny of vaccinated (VACC) and non-vaccinated

(NON-VACC) which has been RSS challenged (challenge) or not-challenged (control) are shown. The chickens were euthanized at day 12 after challenge. The presence of enteropathic cysts are indicated by an arrow (magnification 16x).

Figures 11A-11B. Three vaccinations with the recombinant astrovirus capsid protein decreases enteropathic lesions in the gut and mitigates weight depression in the offspring. In Fig. 11A, one-day-old progeny from vaccinated (VACC) and non-vaccinated (NON-VAC) broiler breeders were divided randomly into two groups of 12 chickens each. The body weight was determined before (grey column) and after challenge (black column) and is shown in gram (g) as indicated at the left axis. Calculated standard deviations are indicated by error bars. The weight difference was calculated between the control group and the challenged group within the non-vaccinated and vaccinated hatch mates. The weight gain was calculated between day of hatch and day 12 within each group. In Fig. 11B, 12 days after challenge the duodenal loop was taken during necropsy from chickens of all groups and a cross section was histological examined. The presences of histological enteropathic lesions in the non-vaccinated/challenged and vaccinated/challenged groups are shown by columns. The number of lesions per section is indicated at the left axis.

Figure 12. The nucleotide (SEQ ID NO:1) and deduced amino acid (SEQ ID NO:2) sequences of the astrovirus capsid protein ORF.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS OF THE INVENTION

With the present invention, a new viral sequence likely belonging to a virus of the family Astroviridae has been identified using the gut content of chickens affected with the runting stunting syndrome and the open reading frame of the viral capsid protein was cloned and a recombinant baculovirus generated. A purified protein was used as a vaccine in broiler breeders to provide maternal derived antibodies for the protection of the offspring. The presence of specific 32 antibodies was monitored by an ELISA. The offspring of vaccinated breeder hens were partially protected in a RSS challenge model.

The present invention includes diagnostic and therapeutic reagents and methods for the detection, treatment, and prevention of RSS. Molecular methods were

employed to identify novel polynucleotide sequences and deduced polypeptide sequences associated with Runting Stunting Syndrome (RSS) which are capable of protecting poultry, including chickens, against RSS. As used herein, poultry includes domesticated birds that are kept for the purpose of collecting their eggs, or killing for their meat and/or feathers. These most typically are members of the superorder *Galloanserae* (fowl), especially the order *Galliformes* (which includes, for example, chickens, quail, turkeys, and grouse) and the family *Anatidae* (in order *Anseriformes*), commonly known as "waterfowl" (including, for example, ducks, geese, and swans). Poultry may also include other birds which are killed for their meat, such as pigeons or doves or birds considered to be game, like pheasants.

With the present invention, a cloning approach targeting small round viruses (*Picornaviridae*) was used. The nucleotide sequences of the RNA dependent RNA polymerases (RdRp) are the most conserved sequence within RNA virus families. Using degenerate primers based on RdRp sequences of several members of the *Picornaviridae* family, RT-PCR fragments were amplified and sequenced. Using several approaches, a 1.8 kilobase pair (kbp) fragment was amplified. A blastn search in the NCBI database did not identify any similar sequences. The blastn search of the *in silico* translated amino acid sequence showed a 34% similarity to a turkey astrovirus capsid amino acid sequence and a 37% similarity to a turkey astrovirus 3 amino acid sequence. The sequence likely encodes a capsid protein of an astrovirus.

The coding sequences of the open reading frame (ORF) of the capsid protein were amplified, cloned and sequenced using molecular techniques. The open reading frame of the encoding sequence was amplified again to clone the ORF encoding a C-terminal located 6x His tag sequence into the plasmid vector pFastBacDual (Invitrogen). A recombinant baculovirus was generated using the Bac-To-Bac technology (Invitrogen). The recombinant baculovirus was used to infect insect cells (Sf9). The His-tagged protein representing the astrovirus capsid protein was purified using affinity-chromatography. The purified recombinant protein was used to vaccinate broiler breeder hens along with non-vaccinated controls. The experimental vaccine contained the recombinant protein in an oil emulsion vaccine based on Freund's incomplete adjuvant. In addition, an indirect ELISA was established to detect antibodies in serum following vaccination and in serum samples from the field. The broiler breeder hens were vaccinated three times, and antibody titers monitored

using the ELISA system as described above. The offspring of the vaccinated and non-vaccinated broiler breeders were challenged using our previously described RSS challenge model. Twelve days after challenge it was observed that the offspring of vaccinated breeder hens showed a 27% decreased weight gain in comparison to their unchallenged hatch mates.

In contrast, the challenged offspring of the non-vaccinated hens showed about 50% lower weight gain in comparison to their unchallenged hatch mates. Histological examination of the duodenal loop of the challenged and non-challenged offspring showed that in the non-challenged controls of both groups (vaccinated as well as non-vaccinated) no cystic enteropathy was observed. In the duodenum of the challenged offspring from vaccinated breeder hens, a significantly lower number of cystic enteropathic lesions was observed compared to the challenged offspring of the non-vaccinated controls.

After two vaccinations, the vaccinated broiler breeders showed a significant increase in antibody titers against the recombinant protein compared to the non-vaccinated control birds as measured by ELISA. Similarly, elevated antibody titers against the recombinant protein were observed in the offspring the vaccinated breeders. In challenge experiments using the offspring from hens receiving three vaccinations, a level of protection was observed against RSS. Progeny from vaccinated/challenged hens gained significantly more weight compared to offspring from the non-vaccinated/challenged controls. In addition, the number and size of enteropathic cystic lesions in the small intestine was significantly reduced in the offspring from vaccinated hens. Thus, the vaccine of the present invention will be useful in efforts to control RSS.

The present invention includes polypeptides having the amino acid sequence shown in SEQ ID NO:2, truncations and fragments thereof. Truncations include, but are not limited to, amino acid sequences in which one, two, three, four, five, six, or more amino acids are removed from the amino terminus of the amino acid sequence SEQ ID NO:2 and/or one, two, three, four, five, six, or more amino acids are removed from the carboxy terminus of the amino acid sequence SEQ ID NO:2. Truncations may include the amino acid sequence of SEQ ID NO:2 in which the one, two, three, four, five, or six of the C terminal His residues have been removed, including, but not limited to the fragment of amino acid 1 to 743 of SEQ ID NO:2. Truncations of the

present invention may include further C- and N-terminal truncations of the polypeptide including amino acids residues 1 to 743 of SEQ ID NO:2.

Fragments include, but are not limited to, for example, fragments having about 5, about 10, about 15, about 20, about 25, about 50, about 75, about 100, about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, and about 700 consecutive amino acid residues of the sequence of SEQ ID NO:2 or of residues 1 to 743 of SEQ ID NO:2. Fragments also include, for example, fragments of a size range of any combination of the above fragment sizes.

10 Fragments include, but are not limited to, for example, fragments having at least 5, at least 10, at least 15, at least 20, at least 25, at least 50, at least 75, at least 100, at least 150, at least 200, at least 250, at least 300, at least 350, at least 400, at least 450, at least 500, at least 550, at least 600, at least 650, and at least 700 consecutive amino acid residues of SEQ ID NO:2 or a polypeptide including residues 1 to 743 of SEQ ID NO:2.

15 The present invention includes polypeptides having an amino acid sequence with at least about 75% sequence identity with the amino acid sequence of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 75% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 80% sequence identity with the amino acid sequence of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 80% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 85% sequence identity with the amino acid sequence of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 85% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 90% sequence identity with the amino acid sequence of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 90% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequences with at least about 95% sequence identity with the amino acid sequence of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 95% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequences with at least about 96% sequence identity with the amino acid

sequence of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 96% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequences with at least about 97% sequence identity with the amino acid sequence of SEQ ID NO:2, polypeptides having an amino acid sequence
5 with at least about 97% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequences with at least about 98% sequence identity with the amino acid sequence of SEQ ID NO:2, polypeptides having an amino acid sequence with at least about 98% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, polypeptides having an amino acid sequences with at least about 99%
10 sequence identity to the amino acid sequence of SEQ ID NO:2, and polypeptides having an amino acid sequence with at least about 99% sequence identity with amino acids 1 to 743 of SEQ ID NO:2, truncations, or fragments thereof.

The present invention includes polypeptides having an amino acid sequence with one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen,
15 fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, or more amino acid changes from the amino acid sequence of SEQ ID NO:2, truncations, or fragments thereof. Such amino acid changes include, but are not limited to, conservative amino acid changes.

The present invention includes polypeptides encoded by a polynucleotide that
20 hybridizes to the nucleotide sequence of SEQ ID NO:1 under stringent hybridization conditions, and fragments thereof. Stringent hybridization conditions may be, for example, 6X SSC, 5X Denhardt, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/ml fragmented and denatured salmon sperm DNA hybridized overnight at 65°C and washed in 2X SSC, 0.1% SDS at least one time at room temperature for about 10
25 minutes followed by at least one wash at 65°C for about 15 minutes followed by at least one wash in 0.2X SSC, 0.1% SDS at room temperature for at least 3 to 5 minutes. Such polypeptides may be bound by an antibody that specifically binds to a polypeptide including amino acid residues 1 to 743 of SEQ ID NO:2.

Also included in the present invention are compositions including one or more
30 of the polypeptides described herein. Such a composition may include pharmaceutically acceptable carriers or diluents. Carriers include, for example, stabilizers, preservatives and buffers. Suitable stabilizers include, for example, SPGA,

carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers include, for example, alkali metal phosphates. Suitable preservatives include, for example, thimerosal, merthiolate and gentamicin.

- 5 Diluents, include, but are not limited to, water, aqueous buffer (such as buffered saline), alcohols, and polyols (such as glycerol).

The present invention includes immunogenic compositions and vaccines including one or more of the polypeptides described herein. Such a compositions and vaccine may be administered as the active component to immunize a bird to elicit an antibody response to RSS and/or induce immunity against RSS. Immunity may include the induction of a significant higher level of protection in a population of birds after vaccination compared to an unvaccinated group.

- 15 An immunogenic composition or vaccine of the present invention may also include one or more compounds with adjuvant activity. Suitable compounds or compositions for this purpose include aluminum hydroxide, aluminum phosphate, aluminum oxide, plant oils, animal oils, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol F™ or Marcol 52™, Complete Freund's adjuvant, incomplete Freund's adjuvant, or a vegetable oil such as vitamin E acetate, and saponins.

- 20 An immunogenic composition or vaccine of the present invention may also contain one or more stabilizers. Any suitable stabilizer can be used including carbohydrates such as sorbitol, mannitol, starch, sucrose, dextrin, or glucose; proteins such as albumin or casein; and buffers such as alkaline metal phosphate and the like. A stabilizer is particularly advantageous when a dry vaccine preparation is prepared by lyophilization.

- 25 An immunogenic composition or vaccine of the present invention may further include one or more immunogens derived from other pathogens infectious to poultry. Such immunogens may be derived from, for example, Marek's disease virus (MDV), infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg drop syndrome (EDS) virus, turkey rhinotracheitis virus (TRTV), poxvirus, or reovirus.

An immunogenic composition or vaccine of the present invention may be administered by any suitable known method of inoculating poultry including nasally,

ophthalmically, by injection, in drinking water, in the feed, by exposure, *in ovo*, maternally, and the like. The immunogenic composition or vaccine may be administered by mass administration techniques such as by placing the vaccine in drinking water or by spraying the animals' environment. When administered by
5 injection, the immunogenic composition or vaccine may be administered parenterally. Parenteral administration includes, for example, administration by intravenous, subcutaneous, intramuscular, or intraperitoneal injection.

The immunogenic compositions or vaccines of the present invention may be administered to poultry, and although vaccines according to the present invention may
10 be used effectively in chickens, other poultry, such as, for example, turkeys, guinea fowl, ducks, and partridges may be successfully inoculated. Chickens include, but are not limited to, hens, roosters, broilers, roasters, breeder, the offspring of breeder hens, and layers. The vaccine of the present invention may be administered to poultry before or after hatching. Poultry may receive a vaccine at a variety of ages. For
15 example, broilers may be vaccinated *in ovo*, at one-day-old, *in ovo*, or at 2-3 weeks of age. Laying stock or reproduction stock may be vaccinated, for example, at about 6-12 weeks of age and boosted at about 16-20 weeks of age. Such laying stock or reproduction stock may be vaccinated at about 6, at about 7, at about 8, at about 9, at about 10, at about 11, or at about 12 weeks of age. Such laying stock or reproduction
20 stock may be boosted at about 16, at about 17, at about 18, at about 19, or at about 20 weeks of age. The offspring of such laying stock or reproduction stock may demonstrate an antibody titer to a polypeptide including amino acid residues 1 to 743 of SEQ ID NO:2, which may prevent or mitigate the symptoms of an RSS infection in the offspring.

25 The present invention includes polynucleotide sequences that encode the various polypeptides described herein, truncations, or fragments thereof. For example, the present invention includes polynucleotide sequences that encode the amino acid sequence of SEQ ID NO:2, a polypeptide including amino acid residues 1 to 743 of SEQ ID NO:2, truncations, or fragments thereof. The present invention includes the
30 polynucleotide sequence of SEQ ID NO:1, truncation, or fragments thereof.

The present invention includes polynucleotide sequences with at least about 60% sequence identity, polynucleotide sequences with at least about 65% sequence

identity, polynucleotide sequences with at least about 70% sequence identity, polynucleotide sequences with at least about 75% sequence identity, polynucleotide sequences with at least about 80% sequence identity, polynucleotide sequences with at least about 85% sequence identity, polynucleotide sequences with at least about 90% sequence identity, polynucleotide sequences with at least about 95% sequence identity, polynucleotide sequences with at least about 96% sequence identity, polynucleotide sequences with at least about 97% sequence identity, polynucleotide sequences with at least about 98% sequence identity, and polynucleotide sequences with at least about 99% sequence identity to the polynucleotide sequence of SEQ ID NO:1, truncations, or fragments thereof. Such a polynucleotide may encode a polypeptide that is bound by an antibody that specifically binds to a polypeptide including amino acid residues 1 to 743 of SEQ ID NO:2.

The present invention includes polynucleotide sequences that hybridize to the nucleotide sequence of SEQ ID NO:1 under various stringency conditions, and fragments thereof. Stringency conditions include, but are not limited to, moderate and high stringency. High stringency hybridization conditions may be, for example, 6X SSC, 5X Denhardt, 0.5% sodium dodecyl sulfate (SDS), and 100 µg/ml fragmented and denatured salmon sperm DNA hybridized overnight at 65°C and washed in 2X SSC, 0.1% SDS at least one time at room temperature for about 10 minutes followed by at least one wash at 65°C for about 15 minutes followed by at least one wash in 0.2X SSC, 0.1% SDS at room temperature for at least 3 to 5 minutes. Such a polynucleotide may encode a polypeptide that binds to an antibody that specifically binds to a polypeptide including amino acid residues 1 to 743 of SEQ ID NO:2.

The present invention includes polynucleotide sequences having a substitution of one, two, three, four, five, six, seven, eight, nine, ten, or more nucleotides from that of SEQ ID NO:1. The present invention also includes the polynucleotide sequences described herein in which codon usage has been adapted to optimize expression in a given host cell. For example, codon usage may be adapted to optimize for expression in host cells including, but not limited to, baculovirus, yeast, *E. coli*, poultry, or human cells. Such adaptation can be carried out by techniques known in the art. Such a polynucleotide may encode a polypeptide that binds to an antibody that binds to a polypeptide including residues 1 to 743 of SEQ ID NO:2.

The present invention includes primers, including, but not limited to, any of the primers described herein and primers that can be used to generate the sequence of SEQ ID NO:1, or a fragment thereof, in a PCR reaction. In some embodiments, a primer may include at least 5, at least 10, at least 15, at least 20, at least 25, at least 30, at least 35, or at least 40 nucleotide residues. In some embodiments, a primer may include no more than 10, no more than 15, no more than 20, no more than 25, no more than 30, no more than 35, no more than 40, no more than 45, no more than 50, no more than 55, or no more than 60. Such residues may be consecutive sequences of SEQ ID NO:1 or its complement, or may hybridize to SEQ ID NO:1 or its complement.

10 The present invention provides a recombinant vector containing one or more of the nucleotide sequences described herein. Such a recombinant vector may also include other sequences such as expression control sequences, markers, amplifying genes, signal sequences, promoters, and the like, as is known in the art. Useful vectors for this purpose are plasmids, and viruses such as baculoviruses, paramyxovirus, 15 coronavirus, herpes virus (for example, herpes virus of turkeys (HVT)) and pox viruses, for example, fowl pox virus, and the like.

The present invention also includes host cells transformed with a polynucleotide sequence described herein and host cells transformed with a recombinant vector described herein. The host cell may be, for example, a eukaryotic 20 or a prokaryotic host cell. Suitable examples are *E. coli*, insect cell lines such as Sf-9, chicken embryo fibroblast (CEF) cells, chicken embryo kidney (CEK) cells, African green monkey Vero cells and the like.

The present invention includes antibodies that bind to the polypeptides described herein, and various antibody fragments, also referred to as antigen binding 25 fragments, which include only a portion of an intact antibody, generally including an antigen binding site of the intact antibody and thus retaining the ability to bind antigen. Such an antibody, or antigen binding fragment thereof, may, for example, bind to the polypeptide of SEQ ID NO:2. Such an antibody, or antigen binding fragment thereof, may bind to a polypeptide including residues 1 to 743 of SEQ ID 30 NO:2. Such an antibody, or antigen binding fragment thereof, may bind to a polypeptide including at least five, at least ten, at least eleven, at least twelve, at least thirteen, at least fourteen, at least fifteen, at least twenty, at least twenty five, at least

thirty, at least forty, at least fifty, at least seventy-five, at least one hundred, at least two hundred, at least three hundred, at least four hundred, at least five hundred, at least six hundred, or at least seven hundred consecutive amino acid residues of SEQ ID NO:2 and/or residues 1 to 743 of SEQ ID NO:2. In some embodiments, an antibody of the present invention does not bind to a 6X His peptide.

Examples of antibody fragments include, for example, Fab, Fab', Fd, Fd', Fv, dAB, and F(ab')₂ fragments produced by proteolytic digestion and/or reducing disulfide bridges and fragments produced from an Fab expression library. Antibodies include, but are not limited to, polyclonal antibodies and monoclonal antibodies. The antibodies of the present invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Immunoglobulins can have both heavy and light chains. An array of IgG, IgE, IgM, IgD, IgA, and IgY heavy chains can be paired with a light chain of the kappa or lambda form.

The antibodies of the invention can be from any animal origin, including birds and mammals. In some embodiments, the antibodies are human, murine, rat, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins.

Monoclonal antibodies of the present invention can be obtained by various techniques familiar to those skilled in the art. For example, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell. Monoclonal antibodies can be isolated and purified from hybridoma cultures by techniques well known in the art. Other known methods of producing transformed B cell lines that produce monoclonal antibodies may also be used. In some embodiments, the antibody can be recombinantly produced, for example, produced by phage display or by combinatorial methods. Such methods can be used to generate human monoclonal antibodies.

The present invention includes antibodies and binding proteins that include one or more of the complementarity determining regions (CDR) of a monoclonal antibody of the present invention. Such an antibody or binding protein may bind to a

polypeptide including amino acid residues 1 to 743 of SEQ ID NO:2, or a fragment thereof.

Also included in the present invention are hybridoma cell lines, transformed B cell lines, and host cells that produce the monoclonal antibodies of the present invention; the progeny or derivatives of these hybridomas, transformed B cell lines, and host cells; and equivalent or similar hybridomas, transformed B cell lines, and host cells.

The present invention includes isolated polypeptides, polynucleotides, and antibodies. As used herein, "isolated" refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring), and thus is altered "by the hand of man" from its natural state.

The present invention includes kits employing one or more of the polypeptides, polynucleotides, and/or antibodies described herein. Such kits may provide for the administration of a polypeptide of the present invention to an animal in order to elicit an immune response. Such kits may provide for the detection of a polypeptide, antibody or polynucleotide, for example, for the detection of RSS infection or exposure to an RSS agent in an animal. Kits of the present invention may include other reagents such as buffers and solutions needed to practice the invention are also included. Optionally associated with such container(s) can be a notice or printed instructions. As used herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit. The packaging material is constructed by well known methods, preferably to provide a sterile, contaminant-free environment. As used herein, the term "package" refers to a solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding within fixed limits a polypeptide. Kits of the present invention may also include instructions for use. Instructions for use typically include a tangible expression describing the reagent concentration or at least one assay method parameter, such as the relative amounts of reagent and sample to be admixed, maintenance time periods for reagent/sample admixtures, temperature, buffer conditions, and the like.

The present invention includes a variety of methods employing one or more of the polypeptides, polynucleotides, compositions, vaccines, vectors, host cells, recombinant viruses, and/or antibodies described herein.

For example, the polypeptides, polynucleotides, vectors, host cells, recombinant viruses, vaccines, and compositions thereof, may be administered to elicit an immune response in poultry or other animals. The immune response may or may not confer protective immunity. An immune response may include, for example, a humoral response and/or a cell mediated response. Such an immune response may result in a reduction or mitigation of the symptoms of future RSS infection. Such an immune response may prevent a future RSS infection in poultry. Such an immune response may be a humoral immune response, a cellular immune response, and/or a mucosal immune response. A humoral immune response may include an IgG, IgM, IgA, IgD, and/or IgE response. The determination of a humoral, cellular, or mucosal immune response may be determined by any of a variety of methods, including, but not limited to, any of those described herein.

The induction of an immune response may include the priming and/or the stimulation of the immune system of poultry to respond to a future challenge with a RSS infectious agent, providing immunity to future RSS infections. The induction of such an immune response may serve as a protective response, generally resulting in a reduction of the symptoms of RSS in poultry, receiving a challenge with an RSS infectious agent. Preferably, the poultry will display either a therapeutic or protective immunological response such that resistance to new infection will be enhanced and/or the clinical severity of the disease reduced. Such protection may be demonstrated by either a reduction or lack of the symptoms associated with RSS, including, but not limited to, any of those described herein. In some embodiments, a method of the present invention may be used as a vaccination method, vaccinating poultry for the treatment and/or prophylaxis of infection by an RSS infectious agent or a related organism. Any of a wide variety of available assays may be used to determine the effectiveness of the vaccination method of the present invention, including, but not limited to, any of those described herein. For example, clinical scores (including, but not limited to, fecal color, diarrhea, abdominal gut fill, and attitude), histopathology, lesion index (including, but not limited, to size and/or number of cystic enteropathic lesions in the small intestine), percent mortality, or weight gain measurement may be used. Such determinations may be in comparison to non-immunized/RSS challenged, non-immunized/non-RSS challenged, and/or immunized/non-RSS challenged control

animals.

The polypeptides, polynucleotides, vectors, host cells, recombinant viruses, vaccines, and compositions thereof, may be administered to poultry to prevent RSS. The polypeptides, polynucleotides, vectors, host cells, recombinant viruses, vaccines, and compositions thereof, may be administered to poultry at any of a variety of life stages and/or ages; for example, to a breeder hen. The breeder hen may demonstrate serum antibodies that bind to a polypeptide including amino acid residues 1 to 743 of SEQ ID NO:2, or a fragment thereof, and/or reduced symptoms of RSS. The offspring of the breeder hen may demonstrate such antibodies and/or reduced symptoms of RSS.

The polypeptides, polynucleotides, vectors, host cells, recombinant viruses, vaccines, and compositions thereof may be administered to poultry or other animals, to produce antibodies. Other animals include, but are not limited to, mice, rat, donkey, sheep, rabbit, goat, guinea pig, camel, and horse.

The present invention also includes methods for the detection of RSS agents and antibodies to RSS, including the detection of an RSS infection or the detection of previous exposure of an animal to an RSS agent. Such a method may employ determining that an antisera sample includes antibodies that specifically bind to a polypeptide of the present invention. Such a method may employ detecting the hybridization of a polynucleotide of the present invention to a sample. Such a method may employ producing a polymerase chain reaction (PCR) amplification utilizing at one or more of the oligonucleotide primers described herein. Such primers include, for example, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, or SEQ ID NO:16. Such a method may employ producing a polymerase chain reaction (PCR) amplification utilizing a primer pair described herein. Such primer pairs include, for example, SEQ ID NO:3/SEQ ID NO:4; SEQ ID NO:9/SEQ ID NO:10; SEQ ID NO:5/SEQ ID NO:7; SEQ ID NO:5/SEQ ID NO:12; SEQ ID NO:5/SEQ ID NO:13; SEQ ID NO:8/SEQ ID NO:12; SEQ ID NO:8/SEQ ID NO:13; or SEQ ID NO:11/SEQ ID NO:13. The polypeptides, polynucleotides, and/or antibodies may be labeled with one or more of the detectable markers known to the skilled artisan. In some aspects, the polypeptides,

polynucleotides, and/or antibodies may be bound to a solid substrate.

Antibodies may be detected by any of a variety of methods, including, but not limited to, the methods described herein and any suitable method available to the skilled artisan. Immunoassays that can be used include, but are not limited to, competitive and non-competitive assay systems using techniques such as BIAcore analysis, FACS (Fluorescence activated cell sorter) analysis, immunofluorescence, immunocytochemistry, Western blots, radio-immunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art. With any of the methods of the present invention, the intensity of a signal from an anti-RSS antibody may be indicative of the relative amount of the anti-RSS antibody in a sample when compared to a positive and negative control reading.

Any of the diagnostic methods of the present invention may include the additional step of providing a report or print out of the results. The sample may be any sample in which RSS antibodies, antigens, or nucleotides are present, for example, a blood, serum or tissue sample. Such methods and kits may also provide for the detection of infectious RSS agents in environmental samples.

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.

25

Example 1
Development of a recombinant vaccine against
Runting and Stunting Syndrome in chickens

Runting and stunting syndrome (RSS) has been recognized since the late 1970s. RSS has also been referred to as mal adsorption syndrome (MAS), infectious stunting syndrome, broiler runting syndrome, pale bird syndrome and helicopter syndrome. All associations to this disease manifestation have been based on clinical

signs and microscopical lesions in organs of the small intestine. Clinical signs are growth retardation (stunted chickens), ruffled feathers, and slight diarrhea.

Histopathologic changes of the stunted chicks include villous atrophy of the small intestine and a distention of crypts of Lieberkühn (Nili et al., 2007, *Comp Clin Pathol*;

- 5 16:161-6; and Otto et al., 2006, *Avian Dis*; 50:411-8). Although environmental, nutritional and management issues play an important role in the manifestation of this syndrome, infectious agents, namely viruses have been implicated as etiologic agent(s). Specifically, reoviruses, rotaviruses, enteroviruses, astroviruses and other small round viruses have been observed in the intestines and contents of clinically
- 10 affected birds by virus isolation and/or electron microscopy. See, for example, McNulty et al., 1984, *Avian Path*; 13:429-439; Reece and Frazier, 1990, *Avian Path*; 19:723-758; Frazier and Reece, 1990, *Avian Path*; 19:759-777; Goodwin Davis, and Player, 1993, *Avian Dis*; 37:229-233; and Goodwin et al., 1993, *Avian Dis*; 37:451-458. A similar enteric syndrome has been described for young turkey poults,
- 15 Poult Enteritis and Mortality Syndrome (PEMS). Viral etiologies associated with this disease are Turkey coronavirus (TCV), Turkey Astrovirus (TAsTV) and turkey reovirus (Guy, 1998, *Poultry Sci*; 77:1166-1175; Heggen-Pey et al., 2002, *Avian Dis*; 46:32-47; Kapczynski et al., 2002, *Virus Genes*; 25:95-100; Sellers et al., 2004, *Avian Dis*; 48:531-539; and Spackman et al., 2005,
- 20 *Avian Pathol*; 34:291-296).

Avian reoviruses (ARV) are commonly isolated from healthy chickens but are also associated with several diseases, namely viral arthritis and MAS. The relationship of reoviruses in cases of MAS is not well understood. Reoviruses isolated from cases of MAS have been shown to cause lesions in the duodenum, jejunum and

- 25 ileum with (Decaesstecker et al., 1986, *Avian Pathol*; 15:769-782; Kouwenhoven et al., 1988, *Avian Pathol*; 17:879-892) or without an associated weight suppression (Songserm et al., 2003, *Vet. Res*; 34:285-295). In addition, reovirus antigen staining was observed in intestinal lesions of experimentally infected broilers with an MAS homogenate. However, the intestinal lesions associated with avian reovirus MAS
- 30 isolates nor antigen staining was present in the crypt of the Lieberkuhn (Songserm et al., 2000, *Avian Dis*; 44:556-567), where numerous cysts have been observed in affected birds. Other studies performed by researchers in Europe (Meulemanns et al.,

1986, "Runting syndrome in broiler chickens. Experimental reproduction studies," In: Acute virus infections of poultry. J.B. McFerran and M.S. McNulty, eds. Martinus Nijhoff, Dordrecht, The Netherlands. pp. 115-124; and McFerran and McNulty, 1986, "Recent advances in enterovirus infections of birds," In: Acute virus infections of poultry. J.B. McFerran and M.S. McNulty, eds. Martinus Nijhoff, Dordrecht, The Netherlands. pp. 195-201) and Australia (Pass et al., 1982, Vet. Rec; 110:386-387) found little evidence that avian reovirus played a significant role in RSS.

Avian nephritis virus (ANV), a recently classified astrovirus has also been implicated in RSS. This virus was initially classified as a picornavirus (entero- and entero-like virus), based on morphological features, until the full length genome was sequenced in 2000 (Imada et al., 2000, J. Virol; 74:8487-8493). At this time, ANV was re-classified as a new genus in the family *Astroviridae*. Early investigations into the etiologies of RSS identified entero-like viruses in young chicks. A crude inoculum containing this virus and a reovirus consistently produced slow feathering, fecal changes and depressed weight gains in broilers inoculated at one day of age (McNulty et al., 1984, Avian Path; 13:429-439). Co-infection of avian reovirus and ANVs isolated from broilers exhibiting a runting syndrome has been examined in two lines of SPF chickens (Shirai et al., 1990, Avian Dis; 34:295-303). In single challenge with the reovirus resulted in weight suppression in both lines of chickens. However, single challenge with the ANV resulted in growth depression and nephritis in only one of the two lines. Challenge of the same line with ANV at day of age and avian reovirus at three days of age resulted in growth depression less severe than the single avian reovirus challenge. These results suggest a breed-related susceptibility for ANV.

Diagnostic tools available for detecting enteric viruses of poultry were previously limited to electron microscopy. However, advancements in molecular technology have made RT-PCR and PCR valuable tools for detecting these viruses which are typically difficult to culture. We previously developed a multiplex RT-PCR for detection of TAsTV, coronavirus and reovirus (Spackman et al., 2004, Avian Dis; 49:86-91) in feces of turkey poultts experiencing PEMS. In TAsTV experimentally inoculated turkey poultts, viral replication was limited to cells along the base and sides of the villi, primarily in the distal small intestine and cecum.

Material and Methods

Establishment of a challenge model for RSS. Chicken litter was collected and transported from a farm with clinical signs of RSS to experimental isolation houses (colony houses). The chicken litter was distributed onto the floor of a colony house with an approximate area of 10 m² (RSS+). In parallel, fresh shavings were distributed onto the floor of another colony house (RSS-). In each house, 150 one-day-old broiler chickens from a commercial farm were raised. Water and feed was provided *ad libitum*. Twelve days after placing the birds in the colony houses, the birds were removed and humanely euthanized with CO₂ and body weights recorded.

- 5 Post mortem necropsy was performed, the small intestine was removed and tissue samples from the duodenal loop were harvested and fixed in neutral buffered formalin for subsequent histological examination. The remaining sections of the small intestine were homogenized in a Waring blender, diluted 1:1 with sterile PBS and aliquots were stored frozen at -80C. In subsequent experiments, 15 one-day-old commercial broiler chickens were gavaged by feeding tube with 1ml of the homogenized gut of either RSS+ or RSS- birds. One group was left untreated to serve as true negative controls. All three groups were held in HEPA filtered Horsfal Bauer units with forced air positive pressure. Water and feed was provided *ad libitum*. Twelve days after inoculation the birds were euthanized with CO₂ and the duodenal loop was harvested, fixed in neutral buffered formalin and examined for histological lesions.

- Based on the assumption that RSS has a viral etiology, commercial broiler chickens were gavaged as described above infected with three different inocula, non-treated gut content, filtered gut content and filtered/chloroform treated gut content. The gut content was obtained from RSS+ chickens. Before filtration, the gut content was centrifuged at 3500 x g for 30 minutes (min) at 4C. The supernatant was obtained and centrifuged at 16000 x g for 10 minutes at 4C. Supernatant was subsequently filtered through a 0.45 µm filter using a 10 ml syringe. The obtained filtrate was filtered again using a 0.22 µm filter. The final filtrate was left untreated or was mixed with 0.5 volume of chloroform, repeatedly vortexed for 15 seconds (s) in a one minute interval over 15 minutes. The suspension was centrifuged at 3500 x g for 30 minutes at 4C and the upper phase was removed and used for further experiments.

In next experiments, one-day-old broiler commercial broiler chickens were held in Horsfal Baur units with positive pressure HEPA filtered air. Water and food was supplied *ad libitum*. The chickens were gavaged with one of the following treatments: 1) 1 ml of the non-treated RSS+ gut content, 2) filtered RSS+-gut content, 3)

- 5 filtered/chloroform treated RSS+-gut content or 4) no treatment. The chickens were monitored daily. At day 12 after infection, the chickens were euthanized with CO₂ and body weights recorded. During necropsy, the duodenal loop was harvested, fixed in neutral buffered formalin and histologically examined. The tissue samples of all experiments were fixed in 10% buffered formalin and routinely processed, embedded,
10 sectioned and stained with hematoxylin and eosin (H&E).

- Delineation of oligonucleotides.* Based on the results from the experiments described above and the hypothesis that the infectious agent is a small round virus, available sequences of RNA-dependent RNA polymerases (RdRp) of the virus family *Picornaviridae* were aligned using the ClustalW (available on the worldwide web at
15 ebi.ac.uk/Tools/clustalw2/index.html), due to a possibly existing higher similarity between the single nucleotide sequences of the RdRp. Based on the similarities, two groups of viruses were formed and the sequences investigated, one, polioviruses, coxsackieviruses including duck picornaviruses (DuckPico), and, two, aphthoviruses (FMDV). The requirement for a possible primer pair was that the resulting RT-PCR
20 fragment should not be larger than 350 bp. The resulting primer pairs (FMDVFP1 (SEQ ID NO:3) /FMDVRP1 (SEQ ID NO:4); DucpicoFP1 (SEQ ID NO:9) / DucpicoRP1 (SEQ ID NO:10)) were used for RT-PCR (see Table 1).

Table 1. Oligonucleotides used for cloning of the Astrovirus sequences

	Name	Sequence	Orientation	Location ^a	Genbank No ^b	Virus
5	FMDVFP1	GGGTTTACAAACCTGTGATG (SEQ ID NO:3)	sense	7786-7806	DQ248888	FMDV
	FMDVRP1	CCGCACACGGCGITCACCC (SEQ ID NO:4)	antisense	7982-7920	DQ248888	FMDV
	FMDVFP2	TAAGGACITTTGTGGTCTATG (SEQ ID NO:5)	sense			
	FMDVFP2Rev	CATAGACCACAAAGTCCTTA (SEQ ID NO:6)	antisense			
	FMDVRP2	GGCCTCGATGCTTGGGAGCC (SEQ ID NO:7)	antisense			
10	FMDVRP2Rev	GGCTCCCAAGCATCGAGGCC (SEQ ID NO:8)	antisense			
	Ducpico FP1	AGATTGATTGAAGCCTCCAGT TTG (SEQ ID NO:9)	sense	6505-6528	AY278552	Human poliovirus 2
				7113-7136	AY563023	Duck picornavirus
	Ducpico RP1	ATGSWDGTNCCHGARCABCC YGADGGCAT (SEQ ID NO:10)	antisense	6841-6869	AY278552	Human poliovirus 2
				7458-7486	AY563023	Duck picornavirus
15	DucPicoFP2	GGAAAGGAAGATGAGGGCAT TG (SEQ ID NO:11)	sense			
	DucPicoFP2rev	CAATGCCCTCATCTTCCTTTCC (SEQ ID NO:12)	antisense			
	DucPicoRP2	GCCAGTTTGGAGAGTATTAC (SEQ ID NO:13)	antisense			
	DucPicoRP2Rev	GTAAATACTCTCCAACTGGC (SEQ ID NO:14)	sense			
	poly-T-GC	GCGCGCGCTTTTTTTTTT (SEQ ID NO:17)	antisense			
20	CapFP	^{EcoRI} ccGAATTCATGGCCGATAAGG CTGGGCCGC (SEQ ID NO:15)	sense			
	CapRP	^{Not I} ggGCGGCCGCTAGTGATGGTG ATGGTGATGCTCGGCGTGGCC GCGGCTGCTAGCAGG (SEQ ID NO:16)	antisense			

^a Location of the oligonucleotides in agreement with the sequences of FMDV (foot and mouth disease virus), duck picornavirus, and human poliovirus 2

^b NCBI genbank

RNA purification and RT-PCR. Ten milliliters (ml) of the homogenized tissue samples were clarified by low speed centrifugation (3500 x g for 20 min) and the supernatant was filtered using a 0.45 µM filter. The resulting fluid was ultracentrifuged at 174899 x g for one hour. The resulting pellet was resuspended in
 5 200 microliter (µl) sterile PBS. The RNA was purified by using the High-Pure-RNA-Isolation-Kit (Roche, Applied-Science). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using SuperScript™ III One-Step RT-PCR System with Platinum® Taq (Invitrogen) following the standard protocol as provided by the manufacturer. For the
 10 amplification of the 3' end of the genomic RNA a RAMP-RT-PCR was performed. To this end the time from the annealing step to the extension step during PCR was set with an increment of 30% of the normal RAMP using the Eppendorf Mastercycler ep (Eppendorf, Hamburg, Germany).

Cloning and sequence analysis. Amplified PCR fragments were cloned into
 15 the vector pCR2.1 using the TopoTA cloning kit (Invitrogen). Purified plasmid DNA was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Lincoln, CA, USA). Resulting sequences were compared using the computer program Gene-Runner Version 3.1 (Hastings Software, Hudson, NY, USA). Subsequent data analysis was performed using online computer programs available on
 20 the worldwide web at expasy.org/tools/dna.html and ebi.ac.uk/Tools/clustalw2.

Generation of recombinant baculovirus expressing the astrovirus capsid protein. For expression of the astrovirus capsid protein a recombinant baculovirus was generated based on the baculovirus transfer vector pFastBac™ Dual and the Bac-to-Bac® Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA). To
 25 construct an appropriate plasmid, a 2267 bp fragment was amplified by RT-PCR using a pair of oligonucleotides (CAP-FP (SEQ ID NO:15); CAP-RP (SEQ ID NO:16), see Table 1). The resulting RT-PCR fragment was eluted from a 1% agarose gel, cloned into pCR2.1 (CAP-pCR2.1) using the TopoTA cloning Kit (Invitrogen). After verification by sequencing, CAP-pCR2.1 was cleaved with EcoR I/Not I and the
 30 eluted DNA fragment encompassing the capsid protein coding region and a 6xHis encoding region at its C-terminus was ligated into EcoR I/Not I cleaved baculovirus transfer vector pFastBac™ Dual to obtain pFAST-CAP. After verification of the

nucleotide sequence a recombinant bacmid containing the ORF of the astrovirus capsid protein was generated using the Bac-to-Bac® Baculovirus Expression System (Invitrogen) following the protocols as provided by the manufacturer. Transfection and subsequent propagation of recombinant baculovirus was performed in Sf9 cells as recommended by the manufacturer using Cellfectin® (Invitrogen). Sf9 cells were cultivated in serum free medium (HyClone SFX-Insect, ThermoFisher) containing ampicillin (100 IU/ml) and streptomycin (100 µg/ml). The baculovirus expressing the astrovirus capsid protein (Cap-Bac) was used for infection of Sf9 cells at a MOI of 1. 72 h p.i. infected Sf9 cells were harvested by centrifugation at 1000 x g for 10 min at 4°C. The sedimented cells were used either immediately for protein purification or stored at -20°C until purification. Protein purification was performed as previously described by Letzel et al. (2007).

Determination of the virus titer. The TCID₅₀ was determined by indirect immunofluorescence. To this end, tenfold dilutions of the viral stock were performed in serum-free medium (HyClone SFX-Insect, ThermoFisher). 100 µl of Sf9 cells (300,000 cells/ml) were seeded into each well of a 96 well cell culture plate and infected with 100 µl of the appropriate virus dilution. Three days after inoculation, the cells were fixed with ice-cold ethanol and indirect immunofluorescence was performed using a monoclonal antibody (mAb) directed against Baculovirus V5 protein (mAb-V5, Sigma-Aldrich, St. Louis, MO) as primary antibody. As a secondary antibody, a FITC labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) was used. The TCID₅₀ was calculated using the formula of Reed and Muench (Reed and Muench, 1938, Am. J. Epidemiol; 27:493-497).

Detection of recombinant protein. For Western blot analysis, samples were separated by sodium dodecylsulphate-12% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The membrane was incubated with blocking solution using 5% non-fat dry milk in TBST (150 mM NaCl, 10 mM Tris, pH 8.0, 0.05% Tween 20) for one hour. After washing in TBST the membranes were incubated with the anti-6xHis mAb (Clone His-1, Sigma-Aldrich, ST. Louis, MO, USA) as primary antibodies and peroxidase-labeled goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) as secondary antibody. The binding

of the antibodies was monitored using a chemiluminescent substrate, Immobilon Western (Millipore, Billerica, MA) and Gel Logic 2200 (Carestream Health, New Haven, CT). For monitoring of the recombinant protein purification, samples were separated by sodium dodecylsulphate-12% polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Imperial Protein Stain (ThermoScientific, Rockford, IL, USA) following the manufacturers instructions.

Development of ELISA for the detection of astrovirus capsid specific antibodies in chicken sera.

For ELISA, the protein detector ELISA kit (KPL, Gaithersburg, MD) was used. The antigen was diluted in coating buffer to a final concentration of 500 ng/ml and 50 µl were added in each well of a 96-well flat bottom plate (FisherBrand, Santa Clara, CA). After incubation over night at 4°C, non-bound antigen was removed, wells were rinsed three times with wash solution (KPL, Gaithersburg, MD) and 100 µl blocking solution were added (KPL, Gaithersburg, MD). The plate was incubated for 45 min at 37°C. Following the washing procedure, 50 µl of diluted sera (1:100) were pipetted into the wells and incubated for one hour at 37°C and rinsed three times with wash solution. Finally 50 µl of 1:1000 diluted peroxidase conjugated goat anti-chicken serum (KPL, Gaithersburg, MD) were added. After incubation for one hour at 37°C, the plate was washed three times, 50 µl of peroxidase substrate solution (KPL, Gaithersburg, MD) were added and the plate incubated for 15 min at room temperature. The enzymatic reaction was stopped by adding 50 µl of stop solution per well (KPL, Gaithersburg, MD). The optical density (OD) values were measured at 405 nanometers (nm) using an ELISA reader (ELX 808, BioTek, Winooski, VT).

To obtain a standard serum from chicken, five 21-day-old SPF leghorn chicken were bleed and vaccinated as described below. Per chicken, 500 µl of the purified protein (250 µg) were mixed with Freund's incomplete adjuvant and a stabile emulsion was obtained by ultrasonic using Branson Sonifier 150 at level 2. Appropriate controls were left unvaccinated. The chickens were vaccinated three times at intervals of four weeks. At each time point prior to vaccination and three weeks following final vaccination, the chickens were bled and the serum was tested for reactivity in the described ELISA system. Four weeks following the final vaccination, a final bleed was obtained from the chickens and euthanized with CO₂.

Vaccination of broiler breeders. One group of fifteen 21-week-old broiler breeder hens was vaccinated with the purified recombinant protein and a second group was left non-vaccinated. In both groups three broiler roosters were present. One group of hens was vaccinated three times (week 22, 26, 30) intramuscularly in the breast muscle with the experimental vaccine produced as described above. The roosters were not vaccinated and held as sentinel controls. No adverse effect in the breast muscle was observed during the duration of the experiments. Before vaccination and two weeks after each vaccination, blood was drawn from the brachial vein to obtain serum samples. The serum samples were monitored by the recombinant protein ELISA.

Challenge experiments in commercial broiler chicken. Starting at fourteen days following the second and third vaccination, eggs were collected from both groups and chickens were hatched separately. From each hatch, two groups of fifteen chickens were weighed and placed into a Horsfal Bauer isolation unit and given water and food *ad libitum*. One group of chickens from each vaccination group was challenged by a gavage of filtered gut content from RSS affected chickens. One group was mock-challenged with PBS. The chickens were monitored daily. From additional progeny not used for the experiments, blood samples were taken and evaluated by the recombinant protein ELISA. Twelve days after infection chickens were euthanized by CO₂ and weighed. At necropsy the duodenal loop was taken for histopathological examination. The tissue sample taken was a cross section of the duodenal loop just above the tip of the pancreas including the ascending and descending portions of the loop.

Results

Runting-Stunting-Syndrome is caused by non-enveloped viruses. In order to establish a challenge model for RSS in commercial broilers, chicken litter from an affected farm was transported to a colony house and 150 one-day-old commercial broiler chickens were placed on that litter (RSS+/1). In parallel 150 commercial broiler chickens were placed on fresh shavings (RSS-/1). During the experiment RSS+/1 chickens developed slight diarrhea, ruffled and dirty feathers, and lassitude. The control chickens (RSS-/1) showed no clinical signs. Twelve days after infection,

chickens were euthanized, weighed and necropsy was performed. The macroscopic inspection of the RSS+/1 chickens revealed a difference in size compared to the RSS-/1 control chickens. The ventral abdomen of the RSS+/1 chicken was swollen in a number of chickens. Macroscopic lesions during necropsy of the RSS+/1 chickens

5 consisted of a thinning of small intestines, gaseous ceacae, swollen and dark liver, massive filled bile, and enlarged spleen. Comparison of body weights showed that RSS+/1 chickens showed an approximate 50 % weight depression in comparison to the RSS- chicken (Fig. 1). For histological examination from each duodenal loop, two sections were cut and following H&E staining, scored for the presence and number of

10 lesions per section. In 28 out of 60 sections, lesions typical for cystic enteropathy in the RSS+/1 group were observed (Fig. 1). The number of lesions per section varied between 1 and 10.

Based on the results obtained, we performed the next experiment where fifteen one-day-old commercial broiler chickens were inoculated with minced gut content of

15 either RSS+/1 chickens (RSS+/2) or RSS-/1 chickens (RSS-/2). One group was mock inoculated with PBS (Con/2). All three groups were held in Horsfal Bauer units with positive pressure. Twelve days after inoculation the chickens were evaluated. The macroscopic inspection of the RSS+/2 chickens showed distention of the abdomen, retarded growth, dirty feathers, and signs of diarrhea. At RSS-/2 chickens as well as

20 Con/2 chickens, no clinical signs were observed. The average weight of the RSS+/2 chickens was 53% below the control chickens (Con/2) whereas the weight of the RSS-/2 chickens showed a not significant weight reduction of 8% in comparison to the controls (Fig. 2A). Macroscopic lesions of the abdominal organs of RSS+/2 chickens were consistent with the signs of RSS+/1 chickens. Abdominal organs of the RSS-/2

25 and the control chickens (Con/2) showed no macroscopic lesions. The histological examination of the duodenal loop showed the presence of cystic enteropathy and shorter gut villi of RSS+/2 chickens in comparison to RSS-/2 and control chickens (Con/2) where no microscopical lesions were observed (Fig. 2B). These results show that using a small group of chickens the macroscopic and microscopic lesions could be

30 observed in RSS+ chickens.

This challenge model was further refined. To this end the gut content of RSS+/1 chicken was centrifuged and filtered, as described in the Material and

Methods section. Fifteen one-day-old broiler chickens were orally inoculated with either the untreated gut content (RSS+/3), the filtered gut content (RSS+/3filt), or the filtered and chloroform treated gut content (RSS+/3filtCF). One group was mock inoculated with PBS (Con). The chickens were weighed before inoculation and 12 days after inoculation (Fig. 3A). The weight depression of the group receiving the non-treated material (RSS+/3) was 64 % in comparison to the non-treated control group. In this group three out of 15 chickens died during the experiment. The group which was inoculated with the filtered material showed a weight depression of 45% whereas the filtered and CHCL3-treated material caused a weight depression of 33%.

The difference in body weight between the RSS+/3filt group and the RSS+/3filtCF group was not statistically significant. Macroscopic lesions were not observed in the control group whereas in the group inoculated with the non-treated material, the filtered, and the filtered/chloroform treated material, macroscopic lesions were observed as described above. The data resulting from the histology showed that microscopic lesions in the duodenal loop were observed even after treatment with chloroform (Fig. 3B). Based on this data it was concluded that the causative agent for RSS is a non-enveloped virus whereas the presence of other agents (such as bacteria, fungi) might have an impact on the severity of the disease.

Cloning of the open reading frame of the astrovirus capsid protein. Based on the above performed experiments we decided to clone nucleotide sequences which might be the genomic information of a virus which might cause the clinical picture of the RSS. Since it has been described that small round viruses were found consistently in the gut content of RSS affected chicken, oligonucleotides focusing on RdRp sequences of picornaviruses were delineated. For RNA purification filtered gut content of RSS+/1 and RSS-/1 was used in standard RT-PCR using two primer sets of primer pairs (FMDVFP1 (SEQ ID NO:3)/ FMDVRP1 (SEQ ID NO:4); and Ducpico FP1 (SEQ ID NO:9)/ Ducpico RP1 (SEQ ID NO:10)). The RT-PCR using the primer pair FMDVFP1/FMDVRP1 revealed a cDNA fragment of approximately 280 bp (FMDV-frag1) whereas by using the second primer pair a cDNA fragment of approximately 500 bp was amplified (DucPic1-frag). The RT-PCR using RNA from the gut content of RSS-/1 resulted in no fragment. In parallel, PCR was performed without an RT step and no fragment was amplified which indicated that the template

for the cDNA fragments was RNA. Both fragments resulting from the RT-PCR were eluted from the gel and cloned using the Topo TA cloning system. Sequence analysis using Blastn search (NCBI database) showed no similarities with any nucleotide sequence present in the database. The next experiments were performed to elucidate if

- 5 both fragment belong to one RNA molecule or to different RNA's. To this end, nested, internal primer were delineated which should amplify a cDNA fragment if both previous RT-PCR fragments belonged to one RNA (see Table 1) and RT-PCR were performed using RNA from RSS+/1. As expected the primer pairs FMDVFP2 (SEQ ID NO:5)/FMDVRP2 (SEQ ID NO:7) and DucpicoFP2 (SEQ ID NO:11)
- 10 /DucpicoRP2 (SEQ ID NO:13) resulted in RT-PCR fragments of the expected size, 232 bp (FMDV-frag2) and 418 bp (DucPic-frag2), respectively. Sequence analysis confirmed the identity of the sequence of the amplified fragments with the previous sequences of the appropriate fragments DucPic1-frag and FMDV-frag1.

- Next, a number of RT-PCR's were performed. Each primer of each fragment
- 15 was used for an RT-PCR using the primer of the other fragment. In addition, the reverse-complementary sequences of each primer (see Table 1) were also used since the location of each RT-PCR fragment to each other was unknown. Only four combinations resulted in amplification of fragments (FMDVFP2 (SEQ ID NO:5)/DucPicoFP2Rev (SEQ ID NO:12), FMDVFP2 (SEQ ID NO:5)/DucPicoRP2
- 20 (SEQ ID NO:13); FMDVRP2rev (SEQ ID NO:8)/DucPicoFP2Rev (SEQ ID NO:12), FMDVRP2rev (SEQ ID NO:8)/DucPicoRP2 (SEQ ID NO:13)). All fragments were cloned and sequenced. The sequence of the longest fragment (FMDVFP2 (SEQ ID NO:5)/DucPicoRP2 (SEQ ID NO:13)) comprised all sequences of the other three fragments.

- 25 Based on this result, it was concluded that both sequence fragments which were amplified during our first RT-PCR were part of a single RNA molecule. The sequence was used for a blastn search in the NCBI database but no similarities were found. The nucleotide sequence was now *in silico* translated in all six open reading frames using a software program available on the worldwide web at expasy.org/tools/dna.html. The highest similarity (53%) at the amino acid level was found with
- 30 the capsid precursor protein of a turkey astrovirus 2 (GENBANK Accession Number AAV37186) and the RNA dependent RNA polymerase of turkey astrovirus 2 (61%,

GENBANK Accession Number ABX46565) using a blastp search using all six proteins sequences resulting from the *in silico* translation. Thus the amplified sequence likely belonged to an astrovirus which has not been described yet. From the probable capsid protein open reading frame (ORF) the start codon was already
 5 determined.

To amplify the complete ORF a RAMP-RT-PCR was performed. Since it is known that the genomic RNA of astroviruses contain an poly-A tail at its 3' end an oligonucleotide was delineated which contained an poly T tail with an GC-clamp sequence (see Table 1, polyT-GC). The Ramp-RT-PCR was performed with a primer
 10 pair (DucPicoRP2Rev (SEQ ID NO:14), polyT-GC) and yielded a faint fragment of 1846 bp. The fragment was cloned and sequenced and a probable ORF was determined by the use of the previously determined nucleotide sequences (Astro-Frag1), as shown in Fig. 12. The location of the single RT-PCR fragments is depicted in Fig. 4.

A blast search using blastn (NCBI database) showed no similarities with any
 15 nucleotide sequence. Only a small portion of the sequence (34 nucleotides) showed a 96% identity with human astroviruses (e.g Human astrovirus type 8 T8/US/KL1553/1998 capsid protein precursor, GENBANK Accession Number AY304469). The similarities with amino acid sequences of turkey astrovirus 2 (TAV
 20 2) (Genbank accession number AAV37186, similarity: 46.2%), TAV 3 (AAV37187, 38.5%), TAV (ABX46578, 37.5%), duck astrovirus (YP_002728003, 36.3%), avian nephritis virus 1 (BAA92849, 33.0%), avian nephritis virus 2 (BAB21617, 30.8%), human astrovirus (BAA33721, 29.8%), bat astrovirus (ACN88712, 29.0%), ovine astrovirus (NP_059946, 28.6%), mink astrovirus (NP_795336, 28.5%), feline
 25 astrovirus (AAC13556, 28.0%), and porcine astrovirus (CAB95000, 27.5%). This showed that this new astrovirus had a limited similarity to other astroviruses and seemed indeed a new member of the family *Astroviridae*. The low similarities to other chicken astroviruses were somewhat surprising, since avian nephritis virus 1 showed a similarity of 70% to avian nephritis virus 2. Also the similarities between turkey
 30 astroviruses were comparable high. The amino acid sequence of TAV showed a similarity to TAV 2 and TAV 3 of 85% and 81%, respectively.

Generation of a recombinant baculovirus expressing the astrovirus capsid

protein. Using the determined nucleotide sequence of 2951 nucleotide (nt), an open reading frame (ORF) of 2250 nucleotides (SEQ ID NO:1) was deduced which encoded a protein of 743 aa (amino acids 1-743 of SEQ ID NO:2). The ORF of the capsid protein was amplified by RT-PCR using two oligonucleotides, CapFP (SEQ ID NO:15) and CapRP (SEQ ID NO:16). CapRP also encoded a 6x His sequence to obtain a His-tag at the C-terminus of the capsid protein for subsequent purification procedures. The obtained RT-PCR fragment (FragAstroCap-ORF) using RNA prepared from purified gut content of RSS+/1 chicken was cloned and sequenced. The fragment was cleaved from the cloning vector pCR2.1 with EcoRI/NotI and ligated into the appropriately cleaved baculovirus transfer vector pFASTDUAL (Invitrogen). The resulting plasmid (pFAST-AstroBac) was used for the generation of a recombinant baculovirus using the Bac-to-Bac system (Invitrogen) as described by the manufacturer. The existence of a recombinant baculovirus was confirmed by PCR using the primer pair which was used for the amplification of the ORF from the RNA (CapFP, CapRP). This PCR fragment was cloned and the identity of the sequence was verified by sequencing. The recombinant virus (AstCap-Bac) was propagated in Sf9 cells and used for infection of Sf9 cells for protein purification with an MOI of 1. Three days after infection the protein was purified as previously published (Letzel et al., 2007, J Gen Virol; 88:2824-2833). The protein purification was monitored by SDS-PAGE followed by staining of the proteins and Western Blot (Fig. 5). The stained protein gel showed no prominent band in the lysate of infected cells. In the eluate, a single band with an approximate molecular weight of 80 kDa was observed. This molecular weight was in accordance with the theoretical molecular weight of 82.91 kDa. By Western blot analysis using a 6xHis specific mAb, a band with a similar molecular weight was observed in the lysate before and after centrifugation and after elution of the protein from the Talon matrix. The identity of the protein was confirmed by MALDI-TOF MS/MS analysis using the single stained protein band after the purification process.

Generation of tools for the detection of antigens and antibodies. In order to detect antibodies raised against the protein, three-week-old SPF birds were immunized with the purified protein. The chicken serum was used to establish an ELISA system. First a checker-board titration was performed to obtain the best ratio between the

positive and negative chicken serum at a certain antigen dilution. The antigen was diluted 1:10 in binding buffer and the chicken serum was diluted 1:100 in dilution buffer prior adding to the plate. Using these parameters we used 100 sera from one-day-old SPF chickens and determined the cut off point as defined by the average of the adsorption plus three-times the standard deviation. The average adsorption was 0.064 with a standard deviation of 0.009 which resulted in a cut off of 0.091. The cut-off point used for the ELISA was set at 0.1.

Vaccination of broiler breeder and challenge of the progeny. For the study, 30 broiler breeder hens and six broiler breeder rooster were obtained at 21 weeks of age. The animals were divided into two groups of 15 breeder hens and three roosters each. After one week of adaptation to the new facility, hens and rooster were bled to obtain a pre-immunserum. One group of hens was vaccinated with the experimental vaccine of Incomplete Freund's adjuvant and the recombinant protein. The roosters were not vaccinated and served as sentinel controls in each group. Development of the serum antibody titer was measured by an indirect ELISA using the recombinant antigen (Fig. 6). Two weeks after each immunization the birds were bled and the serum antibody level specific for this antigen was determined. At 21 weeks of age both groups showed a similar antibody titer, as measured by ELISA. In the non-vaccinated group the antibody titer declined over the duration of the experiment with the lowest titer at the time of the third bleed. Interestingly, a high standard deviation was observed which indicates that the antibody titers varied between the single hens. In contrast, in the group of hens vaccinated with the recombinant antigen the average antibody titer increased and reached a plateau 14 days after the second vaccination. Moreover, the standard deviation within this group was lower than in the non-vaccinated group. The non-vaccinated roosters were bled in parallel with the breeder hens. The ELISA data showed that the titer of the rooster's sera fluctuated over the tested time but stayed below the titer of the vaccinated group independent of whether or not the serum was taken from roosters in the non-vaccinated group or the vaccinated group.

Vaccination with the recombinant antigen protects against RSS. Chickens were hatched after the second and third vaccination and serum samples were obtained at day of hatch. The serum samples were analyzed in the ELISA (Fig. 7). The antibody titer of chickens in the non-vaccinated group did not increase but a certain

level of antibodies raised against the recombinant capsid protein was present. This is not surprising since the non-vaccinated breeder hens also showed reactivity with this particular antigen. It has to be mentioned that single chicken sera in this group showed an ELISA optical density (OD) of greater than 1, but most sera were below an
5 OD of 0.5. In the group of progeny from the vaccinated breeder hens, the titer was higher after the second vaccination which is in agreement with the ELISA data obtained from the sera of the broiler breeder. The titer in broiler chickens elevated further after the third vaccination. ELISA titer in the broiler breeder group increased not significantly after the third vaccination probably because the immune response
10 likely reached a plateau against this particular antigen. Furthermore it was observed that the standard deviation in the offspring of the non-vaccinated group was very high but was comparably low in the vaccinated group indicating a more uniform antibody titer in the latter group.

To test if this antibody response was sufficient to protect from RSS challenge, experiments were performed using 15 chickens hatched from eggs collected beginning
15 at 14 days following the vaccination time point. The offspring from both groups were randomly divided into two groups each and weighed individually before infection by gavage. The chickens were either gavaged with filtered gut content or mock-infected with PBS. The results of the experiments demonstrated that two vaccinations were not
20 sufficient to prevent the differences in weight 12 days after infection (Fig. 8A). Both challenge groups showed macroscopic as well as microscopic lesions. Microscopic lesions were present in fewer gut sections in the vaccinated group in comparison to the offspring of the non-vaccinated group (Fig. 8B). The first infection experiment using the one-day-old progeny after the third vaccination indicated that presence of maternal
25 derived antibodies were able to reduce the difference in weight between the non-inoculated control birds and the challenged birds (Fig. 9A). During the experiment the chickens of the non-vaccinated/challenged group showed slight diarrhea and lassitude. In the chickens of the other three groups no signs of disease were observed. In addition, only the chickens in the non-vaccinated/challenged group
30 showed macroscopic lesions of RSS. The weight difference between non-challenged control birds and their challenged hatch mates was 26% in the vaccinated group whereas a difference of 46% was observed between the non-challenged and challenged

chicken of the non-vaccinated group.

To obtain a more objective picture we calculated the average weight gain. To this end we calculated for each group the average weight at day one and day 12 and subtracted the average weight of day one from the average weight of day 12. The chickens of the vaccinated/challenged group gained on average 94 g more weight during the 12 days period of the experiment in comparison to the progeny of the challenged/non-vaccinated group. Histological examination of the duodenal loop indicated that vaccination of breeder hens with the recombinant protein mitigated the number of affected guts as well as the severity of the lesions (Fig. 9B). In both non-challenged control groups no lesions in the gut typical of RSS were found. In addition, the average size of the lesions was smaller in the vaccinated/ challenged group (Fig. 10) in comparison to the challenged/ non-vaccinated group. To confirm the results a second experiment was performed using the same parameter as in the previous experiment. The average weight difference between challenged and the non-challenged group was 45% of the progeny from the non-vaccinated breeder hens (Fig. 11A). Again, the progeny of the vaccinated group which was challenged showed only 25% weight difference to the non-challenged hatch mates. The calculation of the weight gain showed clearly that vaccinated broiler chickens gained 83% more weight (288 grams (g)) than the non-vaccinated broiler chickens (157g). The evaluation of the histological lesions showed that only three sections out of 24 sections showed cystic enteropathy to a low degree in the group of vaccinated and challenged chicken. In contrast, in the duodenal loop of the non-vaccinated/challenged chickens more (13/24) and to a higher degree cystic lesions in the gut were observed (Fig. 11B).

25 Discussion

RSS in chickens is an important problem in the poultry industry especially in the broiler production. To date, the etiological agent has not been described, likely due to the fact that the agent, or several agents, can not be isolated in cultural systems such as cell culture or embryonated eggs. To develop and subsequently test a vaccine candidate an appropriate challenge model needs to be in place. Since no agent was isolated initially an approach was applied which resembled possible field condition. The approach using non treated and/or filtered gut content to reproduce the disease has

been described before (Nili et al., 2007, *Comp Clin Pathol*; 16:161–6; Montgomery et al., 1997, *Avian Dis*; 41:80-92; and Songserm et al., 2000, *Avian Dis* 2000;44:556-67). The experimental animal model system used in this example resulted in typical signs for RSS such as growth retardation, and cystic lesions in the gut as observed during necropsy and by histopathology. The results obtained during the experiments using gut content of non-clinical diseased chickens versus the gut content of clinical diseased chickens indicate that an infectious agent induced the weight depression. With the present example, a 0.22 μ m filtrate induced a significant reduction in weight and the presence of gut lesions, indicating a viral etiology. But it needed to be mentioned that the body weight in the group inoculated with the unfiltered gut content also showed a difference in weight in comparison to the group inoculated with the filtrate. These results are in agreement with Nili et al. ((Nili et al., 2007, *Comp Clin Pathol*; 16:161–6) and suggest that the presence of infectious agents other than viruses might also play a role in the severity of the disease. The presence of both, gut lesions and reduction in body weight, after treatment of the filtrate with chloroform supported the hypothesis that the agent(s) responsible for RSS are non-enveloped viruses.

Based on the description that a small round virus might play an important role in the genesis of RSS, with this example, the genomic sequence of a picornavirus was amplified. The use of a combination of degenerated primers and primers containing sequences of conserved regions based on nucleotide sequences of picornaviral RdRP resulted in the discovery of a sequence of a new, undescribed astrovirus. The closest relative amino acid sequence was a turkey astrovirus 2 sequence whereas the nucleotide sequence showed no similarities to any sequences present in the NCBI database.

Several attempts in primary chicken liver cells and primary chicken kidney cells failed to isolate a virus which reacted with the anti-capsid protein serum from chickens (ck-Cap+) in immunofluorescence. Thus, the baculovirus-vector approach was chosen to develop a vaccine. The expression of viral proteins using recombinant baculoviruses for the use in vaccines has been shown for several viral antigens, e.g. human paramyxovirus 3 (van Wyke Coelingh et al., 1987, *Virology*; 160:465-72), dengue virus (Zhang et al., 1988, *J Virol*; 62:3027-31), West Nile virus (Bonafé et al.,

2009, Vaccine; 27:213-22), and influenza A virus (Mahmood et al., 2008, Vaccine; 26:5393-9). The presented example showed clearly that the purified protein induces an immune response in broiler breeders. In addition, field sera obtained from 20 breeder flocks (layers and broiler, 10 sera per flock) from different states in the 625
5 USA (GA, DE, AL) were investigated for the presence of antibodies against the recombinant protein using the indirect ELISA. The results also indicated that this virus or an antigenic related virus was present in the field.

Due to the prime/boost scheme the level of antibodies were elevated in the vaccinated birds whereas the level of antibodies declined in the non vaccinated group.
10 After the third vaccination the antibody level in the breeder hens was further elevated but with a lower standard deviation. A similar behavior of the antibody levels was observed in the appropriate serum samples of the progeny. This might explain the results after the challenge experiments. Infected chickens obtained after the second vaccination of the breeder hens showed no difference in weight gain to their
15 nonvaccinated/challenged control chickens. The only observed difference was that a lower number of chickens of the vaccinated hens showed gut lesions in comparison to the chickens of the nonvaccinated controls. These results can be interpreted that the antibody level was not sufficient to provide protection from retarded growth of the chickens. After the third vaccination the challenged offspring showed a further
20 elevated antibody titer and both, the presence of gut lesions and the differences in result was confirmed by a second experiment performed 14 day later. These results showed that a certain level of antibodies needs to be present to induce a partial protection against the differences in weight gain and gut lesions. Cystic lesions in the intestine are an important characteristic of the disease, since the cystic lesions in the
25 small intestine were always present in the infected group but not in the controls. In conclusion the recombinant astrovirus capsid protein provided partial protection to the offspring of breeder hens three times vaccinated with a recombinant astrovirus capsid protein. This is the first report for a vaccine candidate able to induce partial protection against RSS in chickens.

30

Example 2
Preparation of Vaccine under Industrial Conditions

Antigen and vaccine preparation. Sf9 cells were infected in suspension culture with a multiplicity of infection of 1 with the recombinant astrovirus capsid protein encoding baculovirus. Three days after infection the cell will be harvested and centrifuged at 2000 xg for 20 minutes at 4°C. The supernatant will be discarded and the cells will be incubated with lysis buffer as described by Letzel et al. (Letzel et al., 2007, J Gen Virol; 88:2824-2833). The supernatant was obtained after centrifugation (17000xg for 45 minutes at 4°C) and was processed by continuous diafiltration on a Quixstand concentrating unit in a horizontal flow hood using and 30,000 MWCO hollow fiber membrane. The filtration was performed with six liters of PBS. Next the recombinant baculovirus was inactivated applying a treatment with beta-Propiolactone (Sigma) for 72 hours at 4°C at a final concentration of 0.1%. The obtained inactivated lysate was tested for the presence of infectious virus, as described below. To obtain a sufficient antigenic mass for vaccination the proteins in the lysate were 11.7-fold concentrated with Amicon Ultra-15 Centrifugal Filter Units-30 k (Millipore). The lysate was used to formulate water-in-oil emulsion vaccine using an adjuvant provided by Merial. Using this approach 300 ml of an experimental vaccine was produced.

Testing for inactivation of the recombinant virus. The initial sample before any treatment (S0) and the final inactivated sample before vaccine formulation (SF) were tested for the presence and the titer of infectious virus using a 10-fold titration in Sf9 cells. The cells were incubated for 7 days at 28°C. The samples were subsequently passaged five times. Each passage was analyzed for the presence of infectious virus by transfer of 100 ul of cell culture supernatant to 100 ul fresh diluted Sf9 cells (10^5 /ml). The cells were incubated for three days. The cells were fixed with ice-cold (-20°C) ethanol for 10 minutes. The fixed cells were tested for the presence of infectious baculovirus by using an anti-baculovirus specific monoclonal antibody (Mouse Anti-AcV5 Monoclonal Antibody, Clone ACV5 antibody; Sigma Aldrich) and a goat anti-mouse FITC-conjugate (Jackson ImmunoResearch).

Example 3
Vaccine Application Under Semi-Field Conditions

With this example commercial broiler breeders will be vaccinated with a vaccine prepared as in Example 2 above under industrial conditions to determine if sufficient maternal derived antibodies are induced to provide protection to the offspring under challenge conditions similar to the condition which are prevalent in the field.

Vaccination of breeder hens. Six week old commercial broiler breeder hens (BH) and rooster will be housed in floor pens. At seven weeks of age the birds will be bled by puncture of the brachial vein to obtain serum samples. The serum samples will be analyzed for the presence of antibodies which are specific for the recombinant astrovirus capsid protein by an indirect ELISA. At eleven weeks of age the broiler breeder hens will be subcutaneously vaccinated in the neck using one ml of the experimental vaccine (BH-Vac). One group of breeder hens and the rooster will serve as non-vaccinated control (BH-Con). Four weeks after vaccination serum samples will be obtained as described above. The next subcutaneous vaccination will be performed at an age of 18 weeks (booster vaccination). Four weeks, and every subsequent four weeks after booster vaccination serum samples will be obtained as described above and analyzed for the presence of antibodies which are specific for the recombinant astrovirus capsid protein by an indirect ELISA. This will result in data regarding the dynamic level of antibodies in the breeders over time. Starting with 24 weeks the eggs will be collected and stored at 4°C until use. The eggs will be incubated to hatch the offspring of the vaccinated broiler hens and the offspring of the appropriate non-vaccinated controls. The offspring will be challenged in a colony house-floor challenge model. Serum samples from one-day-old broiler chicken from the offspring of BH-Vac and BH-Con will be monitored for the presence of antibodies raised against the recombinant protein.

Colony house-floor challenge model. Chicken litter will be collected and transported from a farm with clinical signs of RSS to experimental isolation houses (colony houses, CH). The chicken litter will distributed onto the floor of a colony house with an approximate area of 10 m² (CH-RSS+). In parallel, fresh wood shavings will distributed onto the floor of another colony house (negative control,

CH-RSS-neg). In each house, 150 one-day-old broiler chickens from a commercial hatchery will be raised. Water and feed will be provided ad libitum. Twelve days after placing the birds in the colony houses, the birds will be removed and humanely euthanized with CO₂. The body weights of all birds will be recorded. Once the difference in weight gain between the RSS+ and RSS control group reached 40% to 50% the hatched offspring of the BH-Vac and BH-Con will be placed either on litter of the CH-RSS+ or on fresh wood shavings of CH-RSS-neg. Twelve days after placing the birds in the colony houses, the birds will be removed and humanely euthanized with CO₂. The body weights of all birds will be recorded and necropsy will be performed, the small intestine removed, tissue samples from the duodenal loop harvested and fixed in neutral buffered formalin for subsequent histological examination.

15. Example 4
Field study

This example will determine if the vaccine prepared under industrial conditions induces sufficient maternal derived antibodies in vaccinated commercial broiler breeders to provide protection to the offspring under field conditions.

Antigen and vaccine preparation. Vaccine will be prepared as described in Example 2. The production of this vaccine will result in a batch of approximately 80,000 vaccine doses for a field experiment. Testing for inactivation of the recombinant virus will be as described in Example 2.

25 *Vaccination of breeder hens under field conditions with an experimental vaccine.* Breeder hens of one house (approximately 39,000 breeder hens) of one company will be subcutaneously vaccinated with the experimental vaccine at 11 weeks and 18 weeks of age under production conditions. The remaining breeder hens of the same complex will serve as control. Serum samples from both groups (20 per
30 flock) will be collected before vaccination, four weeks after each vaccination and every four weeks after the last vaccination. The eggs of the vaccinated hens will be separately hatched and placed in separate broiler production houses on a farm with a history of RSS. The performance of the broiler offspring of the vaccinated hens will

be compared with the performance of the broiler offspring of the non-vaccinated hens in the other houses based on weight gain, feed conversion rate, mortality, and condemnation rate at the processing plant. Serum samples from one-day-old broiler chicken resulting from the offspring of vaccinated and non-vaccinated broiler breeder
5 hens will be monitored for the presence of antibodies raised against the recombinant protein.

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
10 in, e.g., SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GENBANK and RefSeq) cited herein are incorporated by reference. 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
15 in the art will be included within the invention defined by the claims. 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.

Sequence Listing Free Text

- 5 SEQ ID NO:1 Nucleotide sequence of chicken astrovirus capsid (runting stunting) ORF.
- SEQ ID NO:2 Deduced amino acid sequence of the runting stunting ORF.
- 10 SEQ ID NO:3-17 Synthetic oligonucleotide primers.

What is claimed is:

1. A polypeptide comprising an amino acid sequence with at least about 75% sequence identity to the amino acid sequence of SEQ ID NO:2, a truncation, or fragment thereof.
2. A polypeptide comprising an amino acid sequence of SEQ ID NO:2, a truncation, or fragment thereof.
3. A polypeptide comprising at least ten consecutive amino acids of SEQ ID NO:2 or at least ten consecutive amino acids of residues 1 to 743 of SEQ ID NO:2.
4. A polypeptide comprising at least fifteen consecutive amino acids of SEQ ID NO:2 or at least ten consecutive amino acids of residues 1 to 743 of SEQ ID NO:2.
5. A polynucleotide sequence encoding a polypeptide of any one of claims 1-4.
6. A polynucleotide sequence comprising at least about 75% sequence identity to the nucleotide sequence of SEQ ID NO:1, or a fragment thereof.
7. A polynucleotide sequence comprising the nucleotide sequence SEQ ID NO:1.
8. A polynucleotide sequence that hybridizes to the polynucleotide sequence of SEQ ID NO:1 at high stringency.
9. A polypeptide encoded by the polynucleotide sequence of any one of claims 5-8.
10. A vector comprising the polynucleotide sequence of any one of claims 5-8.
11. The vector of claim 10 wherein the vector is an expression vector.
12. A host cell comprising the vector of claim 10 or 11.

13. An oligonucleotide primer selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, and SEQ ID NO:16.
14. A primer pair, wherein the primer pair is SEQ ID NO:3/SEQ ID NO:4; SEQ ID NO:9/SEQ ID NO:10; SEQ ID NO:5/SEQ ID NO:7; SEQ ID NO:5/SEQ ID NO:12; SEQ ID NO:5/SEQ ID NO:13; SEQ ID NO:8/SEQ ID NO:12; SEQ ID NO:8/SEQ ID NO:13; or SEQ ID NO:11/SEQ ID NO:13.
15. A composition comprising the polypeptide of any one of claims 1-4 or 9.
16. An immunological composition for raising antibodies in poultry, the composition comprising the polypeptide of any one of claims 1-4 or 9.
17. A vaccine comprising the polypeptide of any one of claims 1-4 or 9.
18. The composition of claim 15 or 16 or the vaccine of claim 17 further comprising an adjuvant.
19. The composition of claim 15 or 16 or the vaccine of claim 17 further comprising an antigenic determinant from one or more additional pathogens infectious to poultry.
20. An antibody that binds to the polypeptide of any one of claims 1-4 or 9.
21. The antibody of claim 10, wherein the antibody is a monoclonal antibody.
22. A host cell producing the monoclonal antibody of claim 21.
23. A diagnostic kit comprising one or more polypeptides of any one of claims 1-4 or 9, one or more polynucleotide of any one of claims 5-8, one or more primers of claim 13, one or more primer pairs of claim 14 or one or more antibodies of claims 20 or 21.

24. A recombinant virus comprising the polypeptide of any one of claims 1-4 or 9.
25. A recombinant virus comprising the polynucleotide of any one of claims 5-8.
26. A method of detecting exposure to runting-stunting syndrome (RSS) in a bird, the method comprising determining that an antisera sample obtained from the bird specifically binds to a polypeptide of any one of claims 1-4 or 9.
27. A method of mitigating the symptoms of runting-stunting syndrome (RSS) in poultry, the method comprising administering a polypeptide of any one of claims 1-4 or 9, the composition or vaccine of any one of claims 15-19, or the polynucleotide sequence of any one of claims 5-8.
28. A method of producing anti-RSS antibodies in poultry, the method comprising administering a polypeptide of any one of claims 1-4 or 9, the composition or vaccine of any one of claims 15-19, or the polynucleotide sequence of any one of claims 5-8.
29. A method of preventing runting-stunting syndrome (RSS) in poultry, the method comprising administering a polypeptide of any one of claims 1-4 or 9, the composition or vaccine of any one of claims 15-19, or the polynucleotide sequence of any one of claims 5-8.
30. A method of producing immunity to runting-stunting syndrome (RSS) in poultry, the method comprising administering a polypeptide of any one of claims 1-4 or 9, the composition or vaccine of any one of claims 15-19, or the polynucleotide sequence of any one of claims 5-8.
31. Poultry produced by the method of any one of claims 27-30.
32. The method of any one of claims 27-30, wherein the polypeptide, composition, vaccine, or polynucleotide sequence is administered to a breeder hen.

33. A breeder hen produced by the method of claim 32.

34. The method of claim 32 wherein the offspring of the breeder hen demonstrate serum antibodies that bind to a polypeptide having SEQ ID NO:2, a truncation, or fragment thereof, and/or reduced symptoms of RSS.

35. The offspring of the breeder hen produced by the method of claim 32.

36. A method of making the polypeptide, composition, vaccine, polynucleotide sequence, vector, primer, antibody, host cell, diagnostic kit, or recombinant virus of any one of claims 1-25.

35. A method of detecting a runting stunting syndrome (RSS) infectious agent in a sample, the method comprising detecting the hybridization of a polynucleotide of any one of claims 4-7 to the sample.

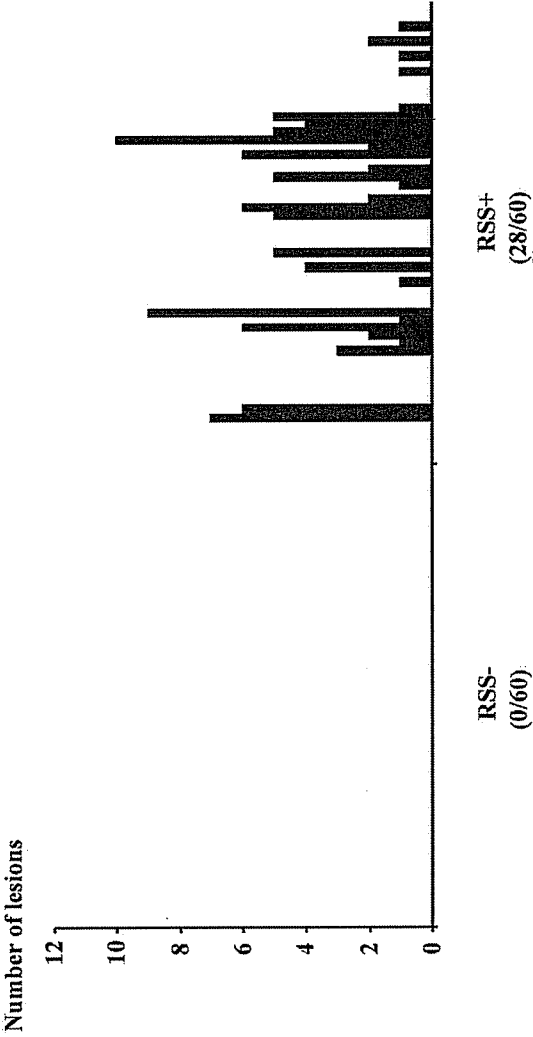
36. A method of detecting a runting stunting syndrome (RSS) infectious agent in a sample, the method comprising producing a polymerase chain reaction (PCR) amplification product with at least one primer of claim 12 or a primer pair of claim 13.

Figure 1

A

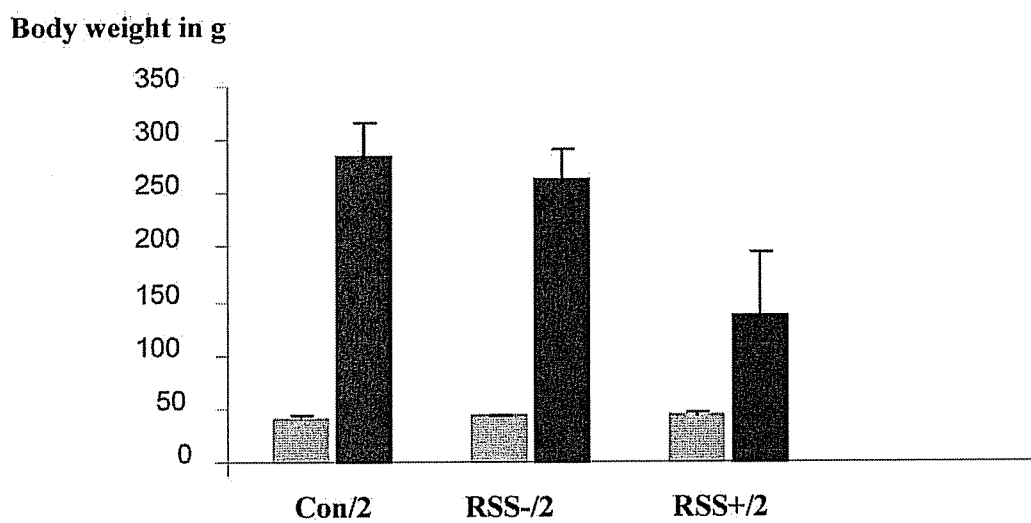


B

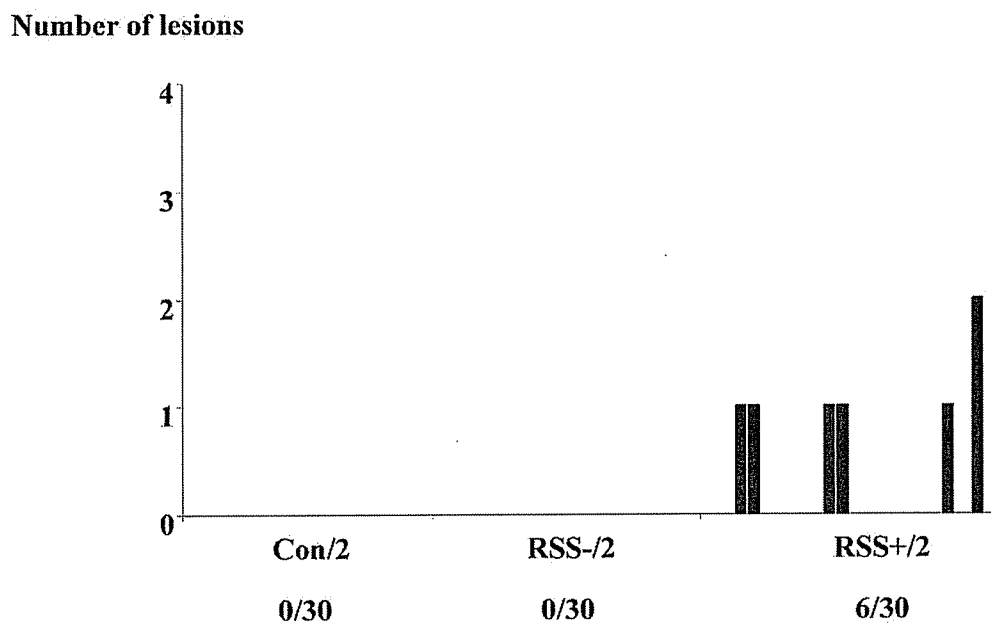


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A



B

*Figure 2*

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Figure 3

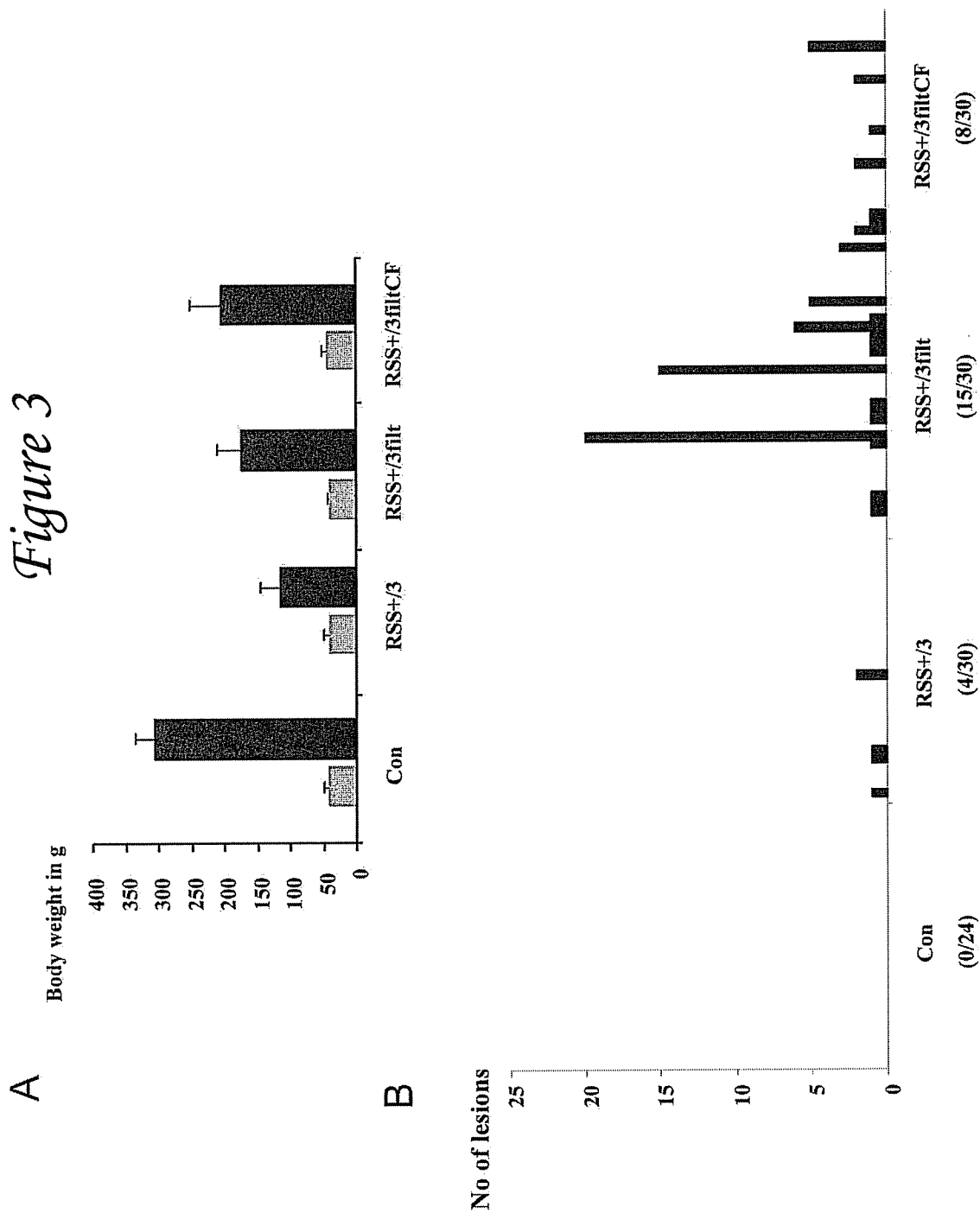
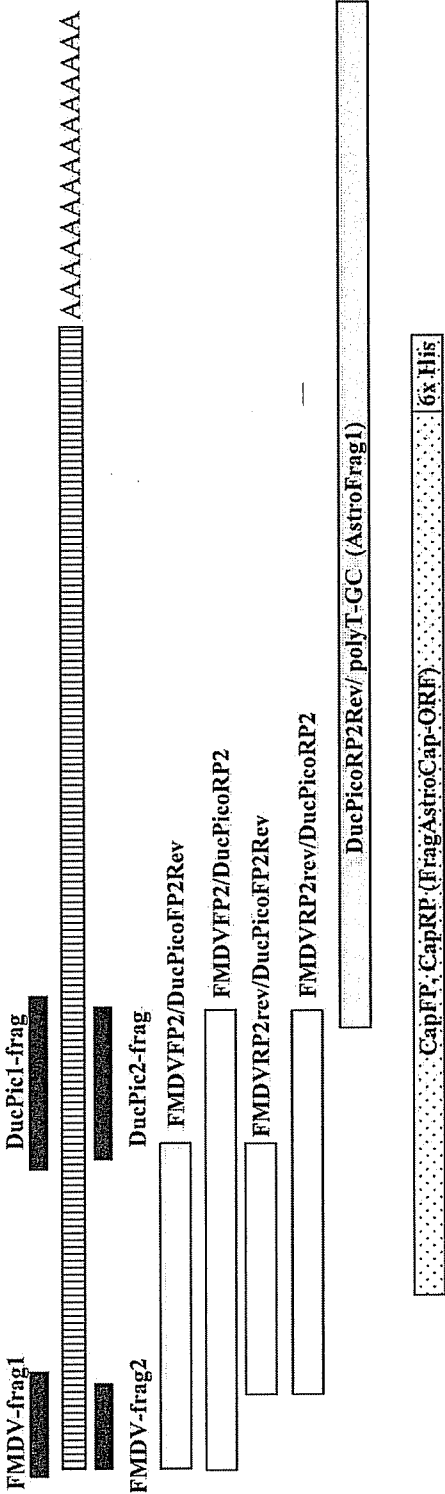


Figure 4



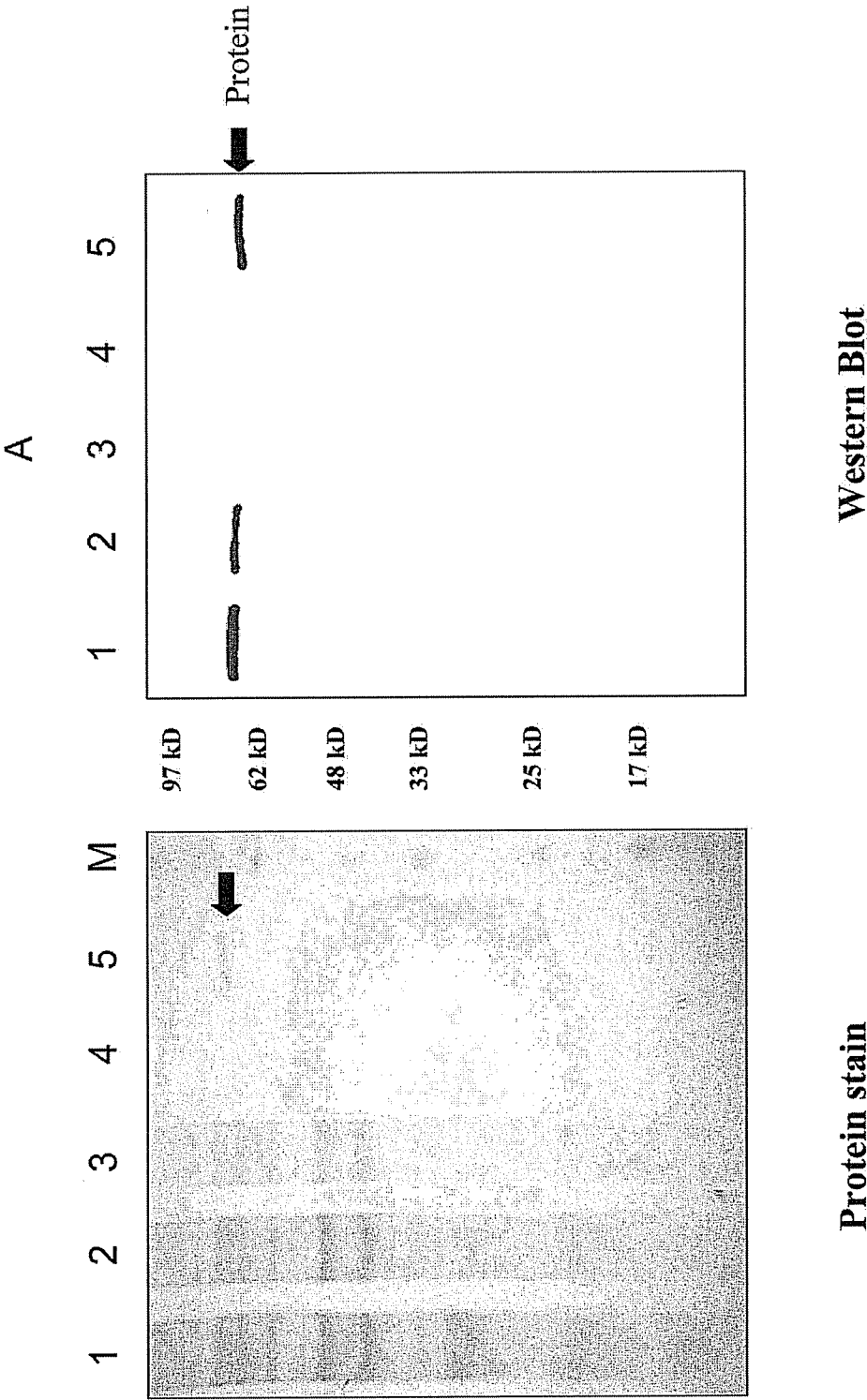


Figure 5

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Figure 6

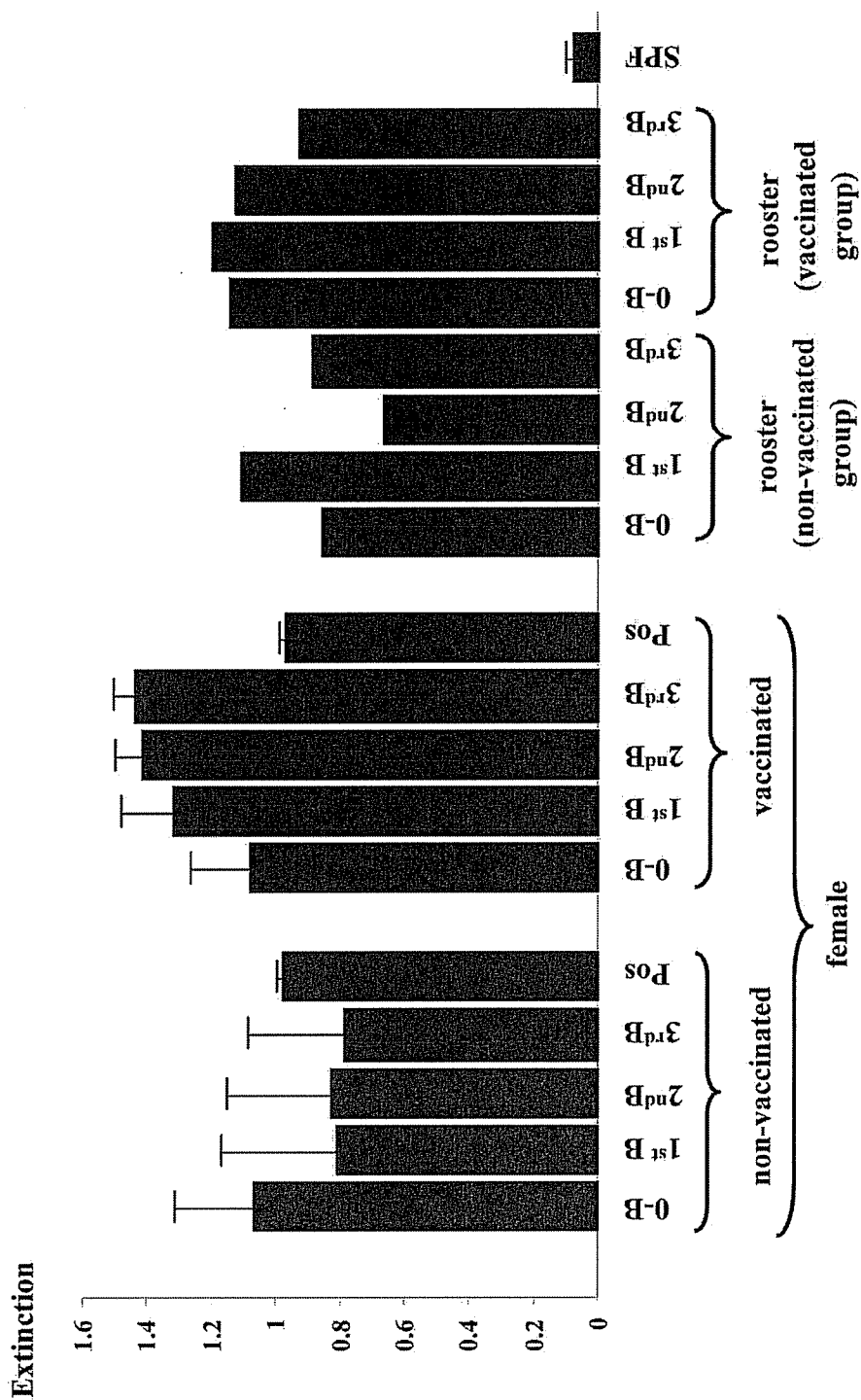
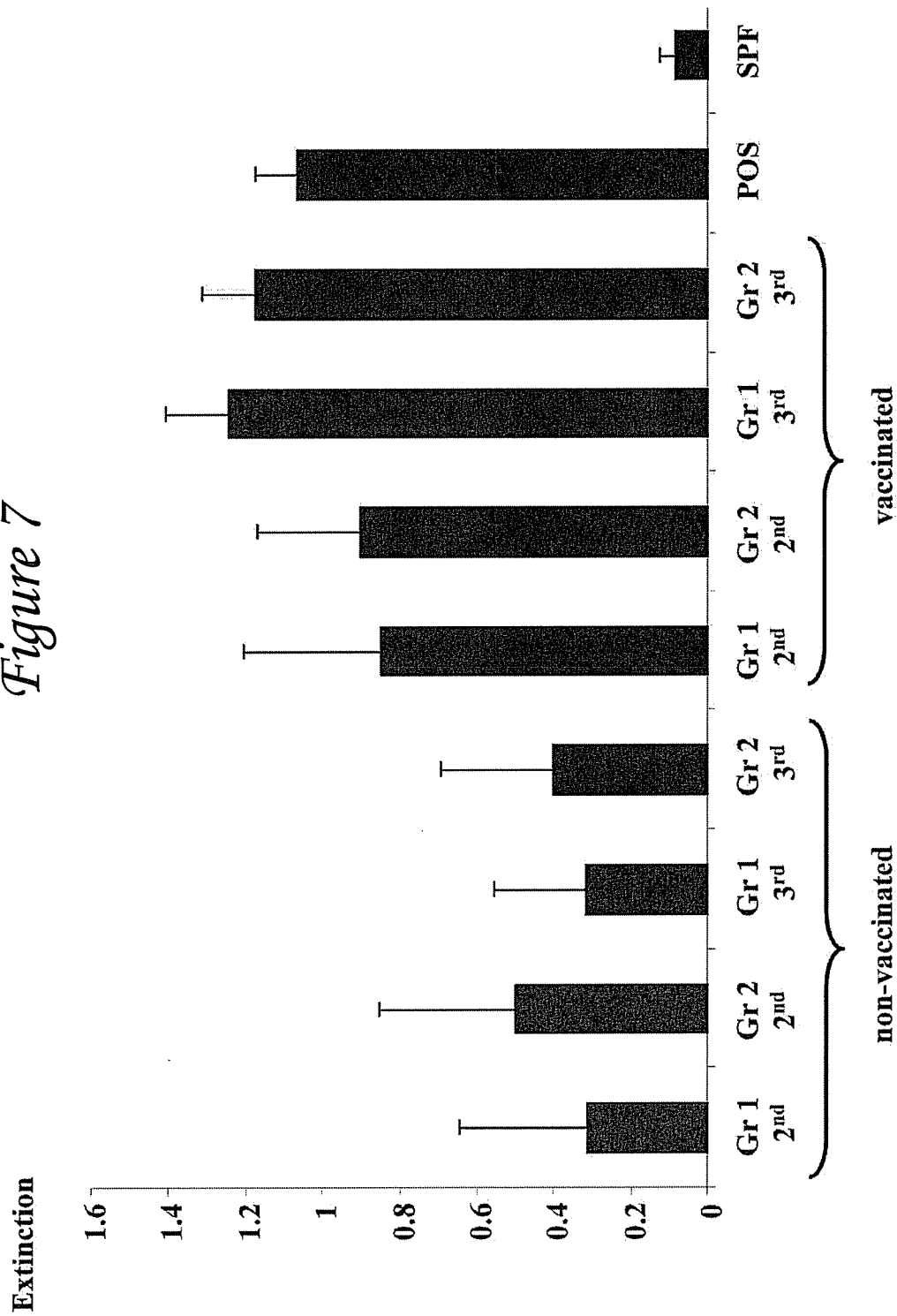
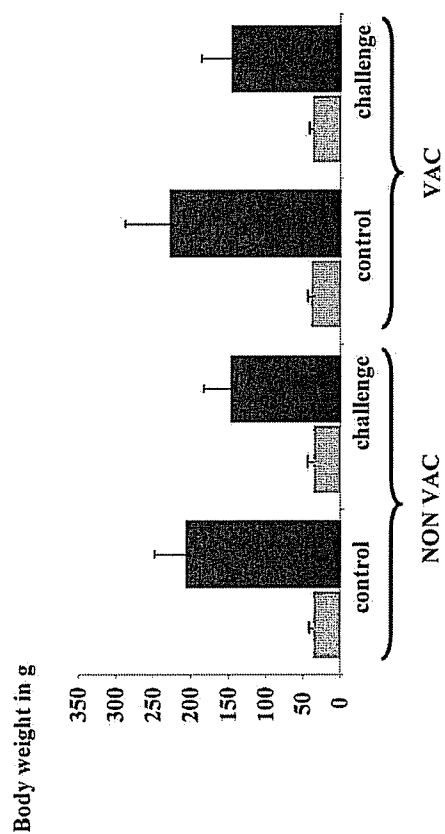


Figure 7

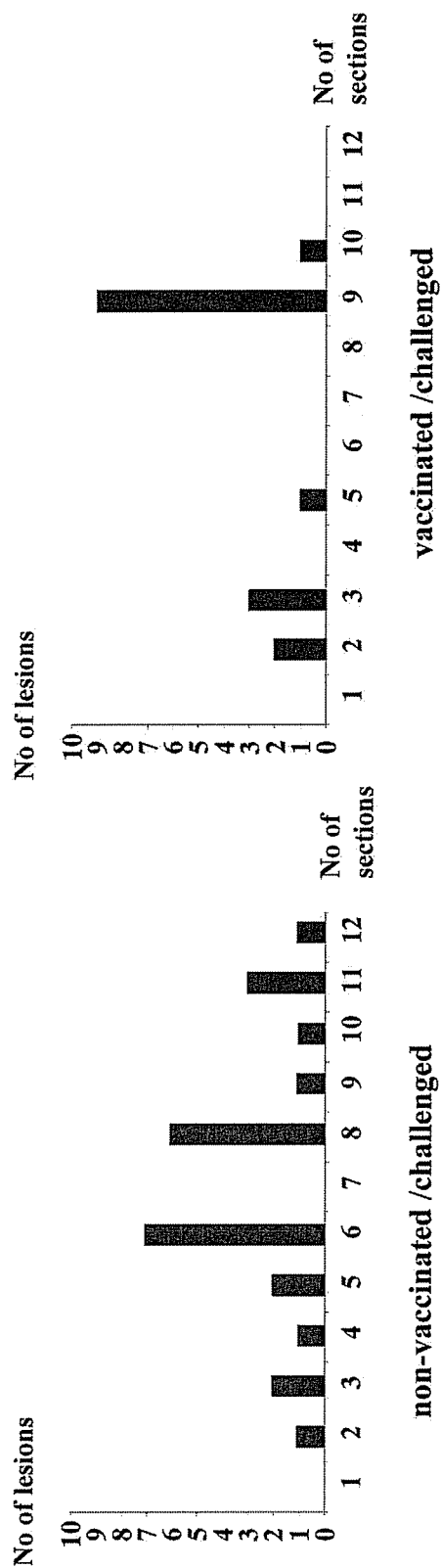


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Figure 8



B



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Figure 9

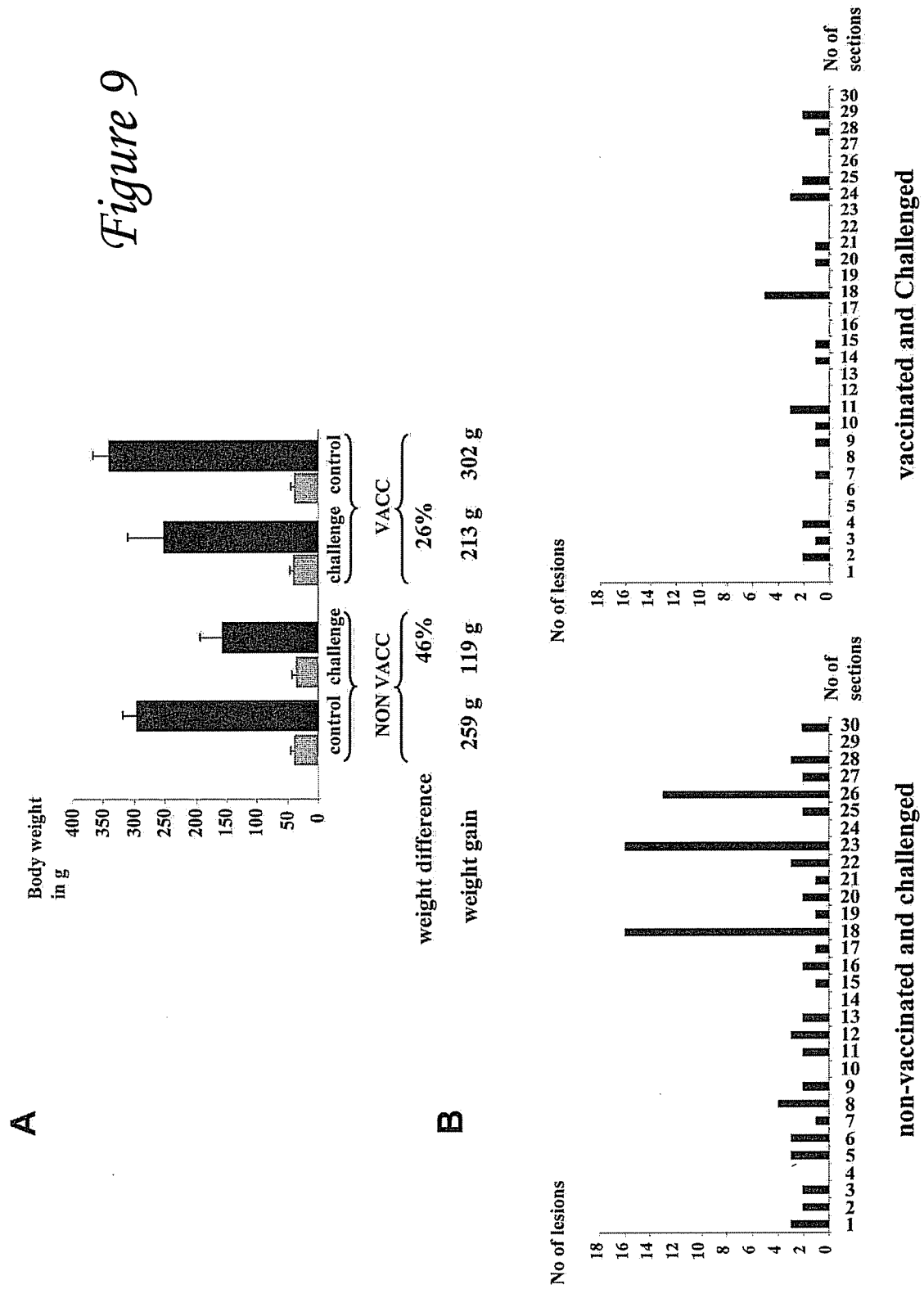


Figure 10

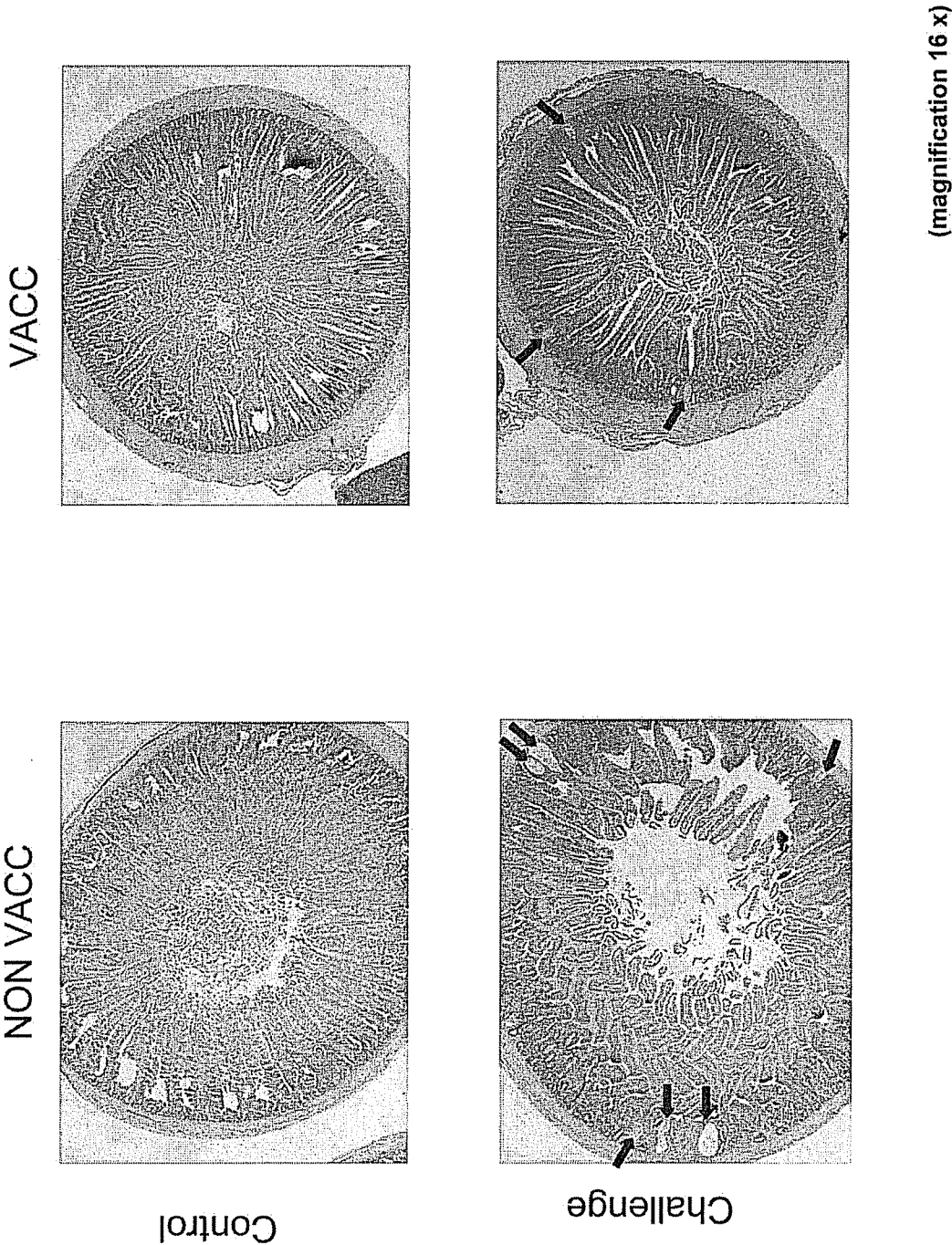
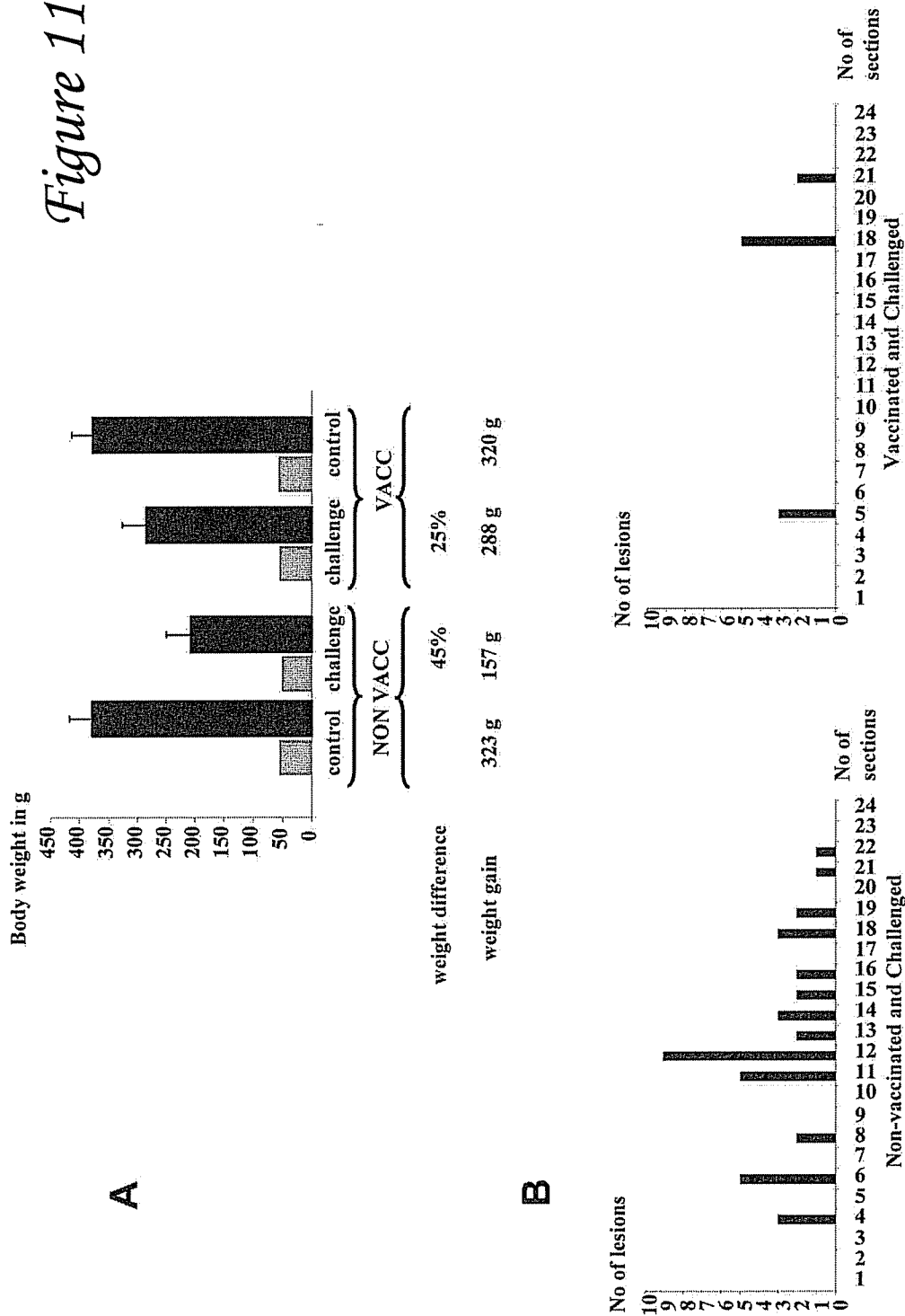


Figure 11



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1	Met	Ala	Asp	Lys	Ala	Gly	Pro	Gln	Lys	Arg	Arg	Val	Ser	Arg	Arg	Gly	16
1	ATG	GCC	GAT	AAG	GCT	GGG	CCG	CAG	AAG	AGG	AGG	GTA	TCT	AGG	CGC	GGT	48
17	Arg	Gly	Arg	Ser	Arg	Ser	Arg	Ser	Arg	Ser	Arg	Ser	Arg	Ser	Arg	Asn	32
49	CGT	GGC	CGC	TCT	CGG	TCA	AGG	TCG	CGC	TCA	CGT	TCT	CGA	TCA	AGA	AAT	96
33	Arg	Val	Lys	Lys	Thr	Val	Thr	Ile	Val	Glu	Thr	Lys	Lys	Thr	Pro	Ser	48
97	CGT	GTA	AAG	AAA	ACA	GTC	ACG	ATC	GTT	GAA	ACA	AAA	AAG	ACC	CCA	AGT	144
49	Lys	Ser	Ile	Leu	Lys	Lys	Glu	Leu	Asp	Asn	His	Glu	Arg	Lys	Asp	Arg	64
145	AAG	TCA	ATC	CTG	AAA	AAG	GAG	TTG	GAC	AAT	CAT	GAG	AGG	AAA	GAT	AGA	192
65	Lys	Arg	Phe	Arg	Lys	Leu	Glu	Lys	Lys	Leu	Asn	Gly	Pro	Lys	Ile	His	80
193	AAA	AGG	TTC	AGA	AAA	TTG	GAA	AAG	AAA	TTA	AAT	GGA	CCA	AAA	ATT	CAC	240
81	Asp	Arg	Met	Ala	Val	Thr	Thr	Thr	Leu	Gly	Val	Leu	Thr	Gly	Asn	Ser	96
241	GAT	CGC	ATG	GCG	GTT	ACA	ACC	ACT	CTT	GGT	GTT	CTC	ACC	GGC	AAT	TCT	288
97	Asp	Asn	Asn	Leu	Glu	Arg	Lys	Met	Arg	Ala	Leu	Leu	Asn	Pro	Leu	Leu	112
289	GAC	AAT	AAT	TTG	GAA	AGG	AAG	ATG	AGG	GCA	TTG	CTT	AAT	CCC	TTA	CTC	336
113	Leu	Lys	Ser	Gln	Asn	Thr	Gly	Ala	Ser	Ala	Ser	Pro	Leu	Ser	Leu	Arg	128
337	TTG	AAA	TCC	CAG	AAC	ACT	GGA	GCA	TCG	GCA	TCA	CCA	CTC	TCC	CTT	AGA	384
129	Ala	Ser	Gln	Tyr	Ser	Met	Trp	Lys	Ile	Gln	Arg	Cys	Val	Val	Lys	Phe	144
385	GCC	TCA	CAG	TAT	TCA	ATG	TGG	AAA	ATA	CAG	CGG	TGT	GTT	GTT	AAA	TTT	432
145	Val	Pro	Leu	Val	Gly	Ala	Ala	Asn	Val	Ala	Gly	Ser	Val	Ser	Phe	Val	160
433	GTA	CCG	CTG	GTG	GGA	GCA	GCA	AAT	GTT	GCT	GGA	AGT	GTG	TCA	TTC	GTG	480
161	Ser	Leu	Asp	Gln	Asp	Ala	Thr	Ser	Ser	Gln	Pro	Glu	Ser	Pro	Asp	Thr	176
481	TCG	CTG	GAC	CAA	GAT	GCA	ACA	TCC	TCT	CAA	CCT	GAA	TCA	CCT	GAT	ACC	528
177	Ile	Lys	Ala	Lys	Val	His	Ala	Glu	Val	Ser	Ile	Gly	Gln	Arg	Phe	Asn	192
529	ATA	AAG	GCG	AAG	GTG	CAT	GCC	GAG	GTG	TCC	ATT	GGC	CAG	AGG	TTC	AAC	576
193	Trp	Asn	Val	Gln	Ser	Arg	Tyr	Leu	Val	Gly	Pro	Arg	Ser	Gly	Trp	Trp	208
577	TGG	AAT	GTG	CAA	TCT	AGA	TAC	CTT	GTT	GGA	CCG	CGG	TCT	GGC	TGG	TGG	624
209	Gly	Met	Asp	Thr	Gly	Glu	Ser	Pro	Thr	Asp	Thr	Val	Gly	Pro	Ala	Leu	224
625	GGT	ATG	GAC	ACC	GGA	GAG	TCA	CCA	ACT	GAC	ACA	GTT	GGT	CCA	GCA	CTT	672
225	Asp	Phe	Trp	Asn	Leu	Tyr	Arg	Thr	Val	Asn	Thr	Leu	Gln	Thr	Gly	Thr	240
673	GAC	TTC	TGG	AAT	TTA	TAT	AGG	ACA	GTA	AAT	ACT	CTC	CAA	ACT	GGC	ACA	720
241	Thr	Ser	Gln	Val	Tyr	Thr	Ala	Pro	Leu	Phe	Ser	Ile	Glu	Val	Phe	Thr	256
721	ACA	TCT	CAG	GTT	TAC	ACC	GCT	CCA	CTA	TTT	TCT	ATA	GAA	GTA	TTC	ACC	768
257	Val	Tyr	Val	Phe	Ser	Gly	Tyr	Glu	Pro	Lys	Pro	Ala	Leu	Ala	Thr	Met	272
769	GTC	TAT	GTA	TTT	TCA	GGG	TAC	GAA	CCC	AAG	CCT	GCC	CTT	GCC	ACA	ATG	816
273	Thr	Asn	Ser	Thr	Phe	Glu	Ser	Gln	Gln	Gly	Val	Thr	Ile	Thr	Asn	Gly	288
817	ACA	AAT	TCA	ACT	TTT	GAA	AGT	CAG	CAG	GGG	GTG	ACT	ATA	ACA	AAT	GGC	864

Figure 12-1

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289	Thr	Asn	Gly	Glu	Leu	Leu	Leu	Asn	Val	Pro	Gln	Arg	Ser	Ala	Leu	Ser	304
865	ACA	AAT	GGT	GAA	CTA	CTT	CTT	AAT	GTC	CCA	CAA	CGT	TCA	GCC	CTT	TCA	912
305	Glu	Arg	Leu	Arg	Glu	Lys	Glu	Val	Pro	His	Arg	Val	Gln	Asn	Gln	Thr	320
913	GAA	AGG	CTT	CGC	GAA	AAA	GAG	GTT	CCA	CAC	CGT	GTA	CAA	AAT	CAA	ACG	960
321	Gly	Gly	Val	Gly	Glu	Val	Leu	Trp	Ala	Val	Ala	Ser	Gly	Ala	Val	Glu	336
961	GGT	GGT	GTT	GGA	GAA	GTA	TTA	TGG	GCA	GTT	GCA	TCA	GGA	GCG	GTG	GAG	1008
337	Gly	Ala	Ala	Glu	Ala	Leu	Gly	Pro	Trp	Gly	Trp	Leu	Leu	Arg	Gly	Gly	352
1009	GGG	GCT	GCA	GAA	GCT	TTA	GGA	CCC	TGG	GGG	TGG	CTA	CTA	AGA	GGT	GGT	1056
353	Trp	Trp	Val	Ile	Lys	Lys	Leu	Phe	Gly	Arg	Thr	Gly	Glu	Asp	Ala	Asn	368
1057	TGG	TGG	GTC	ATT	AAA	AAA	CTT	TTT	GGC	AGA	ACC	GGA	GAA	GAT	GCA	AAT	1104
369	Asp	Val	Tyr	Val	Met	Tyr	Ser	Ser	Ile	Glu	Asp	Ala	Asn	Lys	Asp	Ser	384
1105	GAT	GTG	TAC	GTA	ATG	TAC	TCT	TCA	ATT	GAA	GAT	GCA	AAC	AAG	GAC	AGT	1152
385	Arg	Ile	Tyr	Gln	Thr	Val	Thr	Gly	Ser	Val	Gln	Ile	Gln	Gln	Gly	Pro	400
1153	AGA	ATA	TAT	CAA	ACT	GTC	ACT	GGA	TCG	GTG	CAA	ATA	CAA	CAA	GGC	CCA	1200
401	Leu	Val	Leu	Thr	Gln	Ile	Ser	Ser	Pro	Asn	Val	Asn	Thr	Ser	Gly	Gly	416
1201	CTC	GTT	CTA	ACA	CAA	ATC	TCA	TCG	CCG	AAT	GTG	AAT	ACA	TCC	GGA	GGG	1248
417	Val	Val	Gln	Val	Asn	Ser	Thr	Thr	Pro	Asn	Asp	Tyr	Leu	Pro	Leu	Ser	432
1249	GTT	GTT	CAG	GTA	AAT	TCA	ACC	ACT	CCA	AAT	GAC	TAC	TTG	CCC	CTC	TCT	1296
433	Gln	Glu	Ser	Tyr	Ala	Glu	Thr	Pro	Leu	Lys	Lys	Tyr	Val	Leu	Tyr	Asp	448
1297	CAA	GAA	AGT	TAT	GCA	GAG	ACA	CCA	TTG	AAA	AAA	TAT	GTA	CTT	TAT	GAC	1344
449	Ser	Thr	Gly	Asn	Pro	Val	Asp	Ser	Asn	Met	Ser	His	Thr	Met	Arg	Ile	464
1345	AGC	ACC	GGG	AAC	CCC	GTT	GAT	AGC	AAT	ATG	AGC	CAC	ACC	ATG	AGG	ATA	1392
465	Thr	Gly	Tyr	Pro	Glu	Ser	Lys	Leu	Val	Thr	Ser	Ser	Ser	Val	Trp	Leu	480
1393	ACA	GGG	TAT	CCT	GAG	TCA	AAA	CTA	GTG	ACA	TCA	AGC	TCA	GTC	TGG	CTC	1440
481	Gly	Thr	Thr	Gly	Lys	Ser	Val	Gln	Ser	Thr	Lys	Trp	Leu	Met	Ser	Asp	496
1441	GGT	ACA	ACT	GGT	AAG	AGC	GTA	CAA	TCA	ACT	AAA	TGG	CTG	ATG	TCC	GAT	1488
497	Tyr	Thr	Asn	Thr	Gly	Val	Ile	Phe	Gly	Phe	Pro	Tyr	Thr	Ser	Ala	Pro	512
1489	TAC	ACA	AAT	ACA	GGG	GTC	ATA	TTT	GGT	TTT	CCT	TAT	ACG	AGC	GCA	CCA	1536
513	Pro	Gly	Ala	Thr	Val	Gly	Asn	Ile	Gly	Val	Ile	His	Thr	Ala	Lys	Ser	528
1537	CCG	GGA	GCA	ACT	GTC	GGC	AAC	ATT	GGT	GTC	ATT	CAT	ACT	GCA	AAA	TCA	1584
529	Leu	Ile	Lys	Thr	Ile	Arg	Tyr	Arg	Arg	Gln	Asn	His	Leu	Pro	Thr	Thr	544
1585	TTA	ATT	AAG	ACA	ATC	AGA	TAC	AGA	AGA	CAA	AAC	CAT	CTT	CCA	ACA	ACA	1632
545	Pro	Phe	Glu	Ser	Ser	Leu	Ile	Pro	Ser	Ala	Ser	Lys	Gly	Pro	Ser	Gln	560
1633	CCT	TTT	GAA	TCT	TCC	CTG	ATA	CCG	TCA	GCG	TCA	AAA	GGA	CCC	AGT	CAA	1680
561	Met	Leu	Gly	Cys	Phe	Asp	Thr	Pro	Tyr	Val	Trp	Cys	Arg	Val	Cys	Asp	576
1681	ATG	CTA	GGA	TGT	TTT	GAC	ACC	CCA	TAC	GTA	TGG	TGT	AGA	GTT	TGT	GAT	1728

Figure 12-2

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577	Asn	Thr	Cys	Ser	Thr	Lys	Pro	Thr	Asp	Gly	Ala	Val	Thr	Gln	Arg	Tyr	592
1729	AAT	ACA	TGC	TCC	ACC	AAG	CCT	ACT	GAT	GGT	GCA	GTT	ACA	CAG	AGG	TAC	1776
593	Asn	Ala	Trp	Gly	Leu	Leu	Val	Val	Ser	Leu	Ala	His	Asp	Lys	Val	Tyr	608
1777	AAT	GCA	TGG	GGC	CTG	CTG	GTG	GTT	AGC	CTT	GCC	CAT	GAT	AAG	GTG	TAT	1824
609	Val	Leu	Ala	Gly	Tyr	Pro	Asn	Ser	Gln	Thr	Ala	Val	Pro	Val	Gln	Gln	624
1825	GTA	CTA	GCA	GGC	TAT	CCA	AAT	TCA	CAA	ACA	GCA	GTA	CCA	GTG	CAA	CAA	1872
625	Leu	Val	Trp	Asp	Thr	Phe	Asp	Trp	Asp	Ala	Asn	Phe	Ser	Thr	Gly	Arg	640
1873	TTG	GTT	TGG	GAT	ACT	TTT	GAC	TGG	GAT	GCA	AAT	TTT	TCC	ACT	GGC	AGA	1920
641	Ile	Tyr	Ser	Ala	Val	Trp	Pro	Gly	Glu	Asp	Gly	Ala	Glu	Gln	Glu	Gly	656
1921	ATT	TAT	AGC	GCA	GTA	TGG	CCA	GGT	GAA	GAT	GGT	GCT	GAA	CAG	GAA	GGT	1968
657	Ser	Asp	Thr	Asp	Asp	Ala	Asp	Ser	Asp	Ile	Ser	Ser	Leu	Phe	Asp	Pro	672
1969	TCA	GAC	ACT	GAT	GAT	GCT	GAC	TCT	GAC	ATT	TCC	AGT	CTT	TTT	GAC	CCA	2016
673	Met	Asn	Glu	Val	Glu	Lys	Asp	Phe	His	Phe	Gln	Cys	Ser	Leu	Lys	Thr	688
2017	ATG	AAT	GAG	GTG	GAA	AAG	GAC	TTC	CAT	TTC	CAG	TGT	AGT	CTC	AAA	ACT	2064
689	Ser	Asp	Tyr	Leu	Lys	Glu	Glu	Ala	Asp	Phe	Trp	Lys	Ala	Lys	Ala	Gln	704
2065	TCT	GAC	TAC	TTA	AAA	GAG	GAG	GCT	GAC	TTT	TGG	AAA	GCA	AAG	GCG	CAA	2112
705	Gln	Leu	Leu	Met	Glu	Lys	Ala	Met	Glu	Lys	Pro	Ser	Ala	Asn	Pro	Pro	720
2113	CAG	CTA	CTC	ATG	GAG	AAG	GCA	ATG	GAA	AAA	CCA	AGT	GCT	AAT	CCT	CCT	2160
721	Leu	Val	Arg	Phe	Glu	Lys	Gly	Gly	Pro	Glu	Gln	Gln	Lys	Gln	Pro	Ala	736
2161	CTT	GTC	CGC	TTC	GAG	AAG	GGT	GGA	CCT	GAG	CAG	CAA	AAA	CAA	CCT	GCT	2208
737	Ser	Ser	Arg	Gly	His	Ala	Glu	His	His	His	His	His	His	His	***		750
2209	AGC	AGC	CGC	GGC	CAC	GCC	GAG	CAT	CAC	CAT	CAC	CAT	CAC	CAC	TAG		2250

Figure 12-3