Biochemistry & Physiology: Open Access

Vaillant, et al., Biochem Physiol 2014, 3:4
DOI: 10.4172/2168-9652.1000146

Research Article Open Access

Laying Hen's Primary Immune Response to Staphylococcal Protein A (SpA)

Angel Alberto Justiz Vaillant[1*], Sehlule Vuma[1], Wayne Mohammed[1], and Norma McFarlane-Anderson[2]

[1]*Pathology and Microbiology Unit, Department of Para-Clinical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago, West Indies*

[2]*Department of Basic Medical Sciences, The University of the West Indies, Mona campus, Jamaica, West Indies*

[*]**Corresponding author:** Angel Alberto Justiz Vaillant MD, PhD, Department of Para-Clinical Sciences, The University of the West Indies, St. Augustine, Trinidad and Tobago, Tel: +868-773-5914; E-mail: avail4883@gmail.com

Received date: Nov 05, 2014; **Accepted date:** Nov 19, 2014;**Published date:**Nov 26, 2014

Abstract

Our aim was to provide information about the production of Egg White Immunoglobulin (EWIg) with specificity to Staphylococcal protein-A, a surface antigen of *Staphylococcus aureus* and to study the inhibition of this bacterium growth in pre- and post-immunized hens. A sandwich Enzyme-Immunosorbent Assay (ELISA) showed a large concentration of anti-SpA antibodies in the eggs from hens immunized with protein A. The titer of these antibodies was at least 5 to 6-folds of that of the eggs from pre-immunized hens 10 days post-immunization. Inhibition of the growth of *S. aureus* by anti-SpA antibodies purified by SpA-affinity chromatography (PURE-1A) was observed in laying hens vaccinated. Growth of the bacteria in blood agar plates occurred in antibody samples from pre-immunized laying hens only. Inhibition of the agglutination of SpA-bearing *Staphylococcus aureus* cells by purified anti-SpA antibodies was observed *in vitro*. The authors are not aware of previous studies of the primary immune system response developed in eggs from laying hens, so this research could set a precedent in the field of egg white immunoglobulin technology. The use of hyper-immune eggs as alternative to the use of antibiotics could be advantageous for the large amount of antibodies produced, low cost, the reduction of antigenic variation and very low toxicity.

Keywords: Egg white immunoglobulin; Staphylococcal protein-A (SpA); ELISA; Vaccine

Introduction

Purifying IgY from the yolk of avian egg is of particular interest as a source of specific antibodies for oral immunization to prevent infection. The IgM concentration in the egg white is 0.15 mg/ml [1]. Oral administration of egg yolk antibodies has been successfully used to prevent bacterial infection in animals including *E. coli* infections in piglets [2] and dental caries caused by Streptococci species in rats [3]. IgY antibodies have been used in the immunodiagnosis of several infectious diseases [4].

However, we know very little about Egg White Immunoglobulins (EWIg) and their potential. The avian egg white has several proteins, which can be beneficial to human health. For example Yao et al, 1998 suggested that avidin may be a promising vehicle for the delivery of radioisotopes, drugs, toxins, or therapeutic genes to intraperitoneal tumors [5]. Our aim was to provide information about the production of EWIg with specificity to Staphylococcal protein-A, a surface antigen of *Staphylococcus aureus* and to study the inhibition of the *S. aureus* growth in pre- and immunized laying hens [6,7].

Materials and Methods

Production of anti-protein A antibodies in egg white from chickens

Six healthy layer chickens (brown Leghorn), aged 6 months, wereinjected intramuscularly at multiple sites on the breast with 1 mg of SpA in 0.5 ml Complete Freund's Adjuvant (CFA) on day 0, and 1 mg of the same antigen in 0.5 ml Incomplete Freund's Adjuvant (IFA) on days 8.The eggs were collected pre- and post-immunization from laying hens. The egg white was manually andcarefully separated from the egg yolk to prevent contamination of egg yolk anti-SpA antibodies (immunoglobulin Y, IgY) with those of the egg whites (IgM and IgA) [8].

Sandwich ELISA for detection of anti-SpA antibodies

The 96 well polystyrene microplates (U-shaped bottom) were coated with 500 ng of SpA (Sigma-Aldrich) in coating buffer for 4h at 37°C. The microplates were washed four times with PBS-Tween-20 and blocked with 3% non-fat milk in PBS, 25 µl/well, 1h, at room temperature (RT). The microplates were washed four times again. Samples were added:50 µl of 1:50 dilutions of egg whites. After incubation for 1h at RT the microplates were washed four times and 50 µl of peroxidase-labelled protein-A in dilution of 1:3000 (Sigma-Aldrich) was added. The microplates were then incubated for 1h at RT, washed four times. Tetramethyl benzidine (TMB) solution (50 µl) was added to each well. After a further incubation for 15 min in the dark, the reaction was stopped and read in a microplate reader at 450 nm [9,10].

Purification of anti-SpA antibodies in eggs whites from the eggs of laying hens by affinity chromatography

Laying Hens egg whites immunoglobulins (300 µl) were purified using a protein-A affinity chromatography commercial kit, PURE-1A (Sigma Aldrich Co). In this procedure the manufacturer's instructions were followed. The antibody concentration was adjusted to 0.4 mg/ml. The purified antibodies were stored at -20°C [8,11].

In vitro inhibition of *S. aureus* growth by anti-SpA antibodies from the egg white of hens

In vitro testing for investigating the effect of purified anti-SpA antibodies on *S. aureus* isolates. The investigation of the neutralizing capacity of purified anti-SpA antibodies was carried out as follows: One ml of Brain Heart Infusion (BHI) broths was placed in 11 sterile test tubes. To each of these tubes was added equal volume of 10 µl of purified anti-SpA antibodies in concentration of 2.5 µg/ µl. An inoculum of the ATCC *Staphylococcus aureus* strain (ATCC #33592) was prepared to 0.5 commercially prepared McFarland scale standards (1=300 x 10^6/ml bacteria concentration). Then 10 µl of this inoculum was added serially from Tubes 1 to 10. None was added to Tube 11 and 12 (controls) [7].

Preparations from the tubes were plated out on Blood agar and also incubated overnight at 35°C. The Optical Density (OD) value of the bacterial growth was measured and plotted against the different bacterial concentration at different serial dilutions (1:10, 1:100 or 1:1000).

Inhibition of the agglutination of SpA-bearing *Staphylococcus aureus* cells by purified anti-SpA antibodies *in vitro*

Briefly serial dilutions of 25 µl (60 µg/µl) of purified anti-SpA were added in duplicate to 96 wells micro-titer plates containing 20 µl of SpA-bearing *S. aureus* cells (Sigma-Aldrich Co) and incubated for 1 hour at RT. Inhibition of agglutination were seen in positive samples (containing anti-SpA antibodies) and agglutination at the bottoms of the wells was seen in negatives samples (as in turtle serum, Sigma-Aldrich Co). In this test a commercial human serum (Sigma-Aldrich Co.) was used as a positive control, given the capacity of human IgG to bind to SpA.

Statistical analysis

Statistical data were analyzed using the SPSS version 18. A P value <0.05 was considered significant.

Results and Discussion

The sandwich ELISA was standardized by determination of reaction conditions and optimal concentrations of coating reagent, antigen, conjugate and substrate using checkerboard titration. The basis for the development of the sandwich ELISA was the determination of the presence of antibodies against protein A in the eggs white from immunized chicken with purified protein A. Several photometric measurements were conducted to establish the cut-off point of the test that resulted in a mean absorbance value of at least three fold of that of the negative controls (cutoff point=0.36). The sandwich ELISA showed sensitivity and specificity of 95% and 97% respectively as shown in Figures 1 and 2.

The use of SpA-coating microplates and peroxidase-labeled SpA conjugate guaranteed that only anti-SpA antibodies could test positive in the ELISA, since other chicken immunoglobulins does not bind to protein A in a non-specific manner. ELISA showed a large concentration of anti-SpA antibodies in the egg whites from hens immunized with protein A. The titer of these antibodies was at least 5 to 6-folds of that of the eggs of the pre-immunized hens 10 days post-immunization. Six out of 6 (100%) layer hens tested positive for anti-SpA antibodies as shown in Table 1.

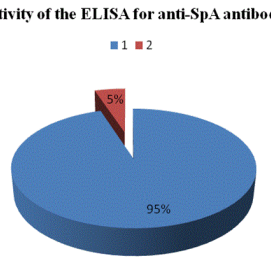

Figure 1: Pie: the representation of the sensitivity of the ELISA for anti-SpA antibodies. 1: True positive samples represented the 95%. 2: False positive samples represented the 5%.

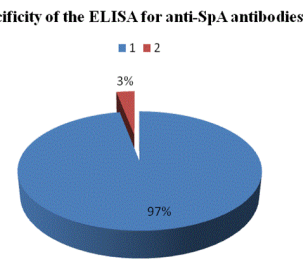

Figure 2: Pie: the representation of the specificity of the ELISA for anti-SpA antibodies. 1: True negative samples represented the 97% and 2: False negative samples represented the 3%.

Variables	Mean OD at 450 nm	Standard Deviation
Laying hen 1	1.412	0.06
Laying hen 2	1.687	0.044
Laying hen 3	1.511	0.057
Laying hen 4	1.283	0.073
Laying hen 5	1.482	0.065
Laying hen 6	0.933	0.047
Positive control 1	1.88	0.05
Positive control 2	1.356	0.078
Negative control 1	0.11	0.008
Negative control 2	0.178	0.011
Blank 1	0.065	0.004
Blank 2	0.048	0.002

Table 1: Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for detection of anti-SpA antibodies in Layer hens

The production of anti-SpA antibodies was shown earlier by affinity chromatography from 10 days post-immunization egg white. As shown in Table 2 when the bacteria concentration was the serial dilution of 1:10 the optical density of the bacteria growth in pooled egg

whites from post-immunized laying hens was 1.562 versus pre-immunized hens that showed an OD values of 0.209. So to speak there was a significant difference in the absorbance values of immunized and pre-immunized animals in the three serial dilutions of bacteria. Similar results were shown in a previous investigation on the inhibition of the growth of *B. burgdorferi* in an *in vitro* assay, testing dogs for specific antibodies to the surface antigen [7].

Bacterial concentration[A]	Immunized/pre-immunized hens[B]	P-value
1:10	0.209/1.562	< 0.001
1:100	0.187/1.347	< 0.001
1:1000	0.155/1.233	< 0.001

Table 2: Showing the laboratory results of bacteria growth observed in immunized and pre-immunized laying hens 13 days pos-immunization. [A] = Bacteria concentration in ml after serial dilutions; [B] = Optical density values of pooled egg white from immunized and pre-immunized laying hens; P value <0.05 is statistically significant.

The preparations that were streaked out on blood agar plate and incubated overnight confirmed the previous results, that there was growth inhibition of the *S. aureus* in the plates incubated with anti-SpA antibody samples. Growth of the bacteria occurred only in negative anti-SpA antibody samples as those from pre-immunized layer hens. As shown in Table 3 the number of inhibited samples from immunized hens was 48 out of 50, which represented a percentage inhibition of96% and it was statistically significant.

Birds	N. of inhibited/Total	% Inhibition	P-value
Immunized laying hens	48/50	96	< 0.001
Pre-immunized laying hens	8/50	16	> 0.1

Table 3: Percentage inhibition of the growth of *S. aureus* in blood agar plates streaked out with egg white preparations. The presence of anti-SpA antibodies in the egg white was responsible for the inhibition of the growth of the bacterium *S. aureus*.

On the other hand in pre-immunized hens the percentage inhibition was not statistically significant, the number of inhibited samples was 8 out of 50 and we presumed that this amount of samples that inhibited the growth of *S. aureus* in a non-specific manner might be in relation with the presence of natural antibodies in the egg white samples.

Another result was the inhibition of the agglutination of SpA-bearing *S. aureus* cells by purified anti-SpA antibodies *in vitro*, which substantiated the previous results. This can be explained by the binding of anti-SpA antibodies to SpA-bearing *S. aureus* cells, preventing those from agglutination at the bottom of the wells. As shown in Figure 3 a pooled positive sample for anti-SpA antibody egg white sample inhibited the agglutination of SpA-bearing *S. aureus* cells in dilutions of 1:2048 as compared with the negative one that agglutinated SpA-bearing *S. aureus* from 1:8 to 1:4096 dilutions. The reciprocal titer was used to describe these results in the Figure 4. The positive samples have a value of one and the negative samples of zero.

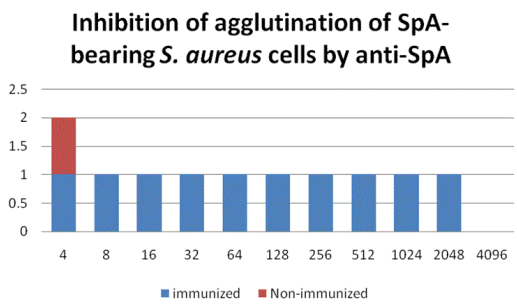

Figure 3: Inhibition of the agglutination of SpA-bearing *Staphylococcus aureus* cells by purified anti-SpA antibodies *in vitro*.

Summary

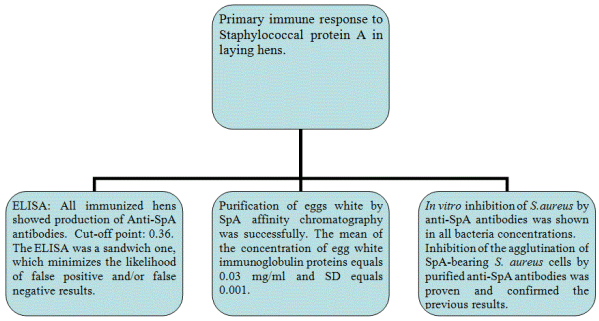

Figure 4: Diagram that summarizes the laying hen's primary immune response to Staphylococcal protein A (SpA). The four techniques have shown that only immunized laying hens produced anti-SpA antibody.

Conclusion

The authors are not aware of previous studies of the primary immune system response developed in the egg white from laying hens, so this research could set a precedent in the field of egg white immunoglobulin technology. Anti-SpA antibodies (IgY) were produced in the egg white of immunized hens. Growth of the bacteria in blood agar plates occurred in samples plated out with egg whites from pre-immunized laying hens only. The SpA-affinity chromatography was useful in the characterization of the anti-SpA antibodies. Inhibition of the agglutination of SpA-bearing *Staphylococcus aureus* cells by purified anti-SpA antibodies was observed *in vitro*. They demonstrated the high titer of anti-SpA antibodies produced by immunized laying hens. The use of hyper-immune eggs as alternative to the use of antibiotics could be advantageous for the immunotherapy of infectious diseases, for the large amount of antibodies produced, low cost, the reduction of antigenic variation and very low toxicity.

Acknowledgements

To Department of Microbiology, University of the West Indies, Mona campus, Jamaica, WI.

Competing interests

Authors have declared no competing interests exist.

References

1. Larsson A, Bålöw RM, Lindahl TL, Forsberg PO (1993) Chicken antibodies: taking advantage of evolution--a review. PoultSci 72: 1807-1812.
2. Wiedemann V, Kühlmann R, Schmidt P, Erhardt W, Lösch U (1990) Chicken egg antibodies for prophylaxis and therapy of infectious intestinal diseases. III. In vivo tenacity test in piglets with artificial jejunal fistula. ZentralblVeterinarmed B 37: 163-172.
3. Hamada S, Horikoshi T, Minami T, Kawabata S, Hiraoka J, et al. (1991) Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of Streptococcus mutans. Infect Immun 59: 4161-4167.
4. Losonczy S, Batke J (1997) Application of specific immune-globulin (IgY) of the egg yolk of birds in the veterinary immuno-diagnosis and immuno-therapy. Magy Aon Lapjan 119: 339-343.
5. Yao Z, Zhang M, Sakahara H, Saga T, Arano Y, et al. (1998) Avidin targeting of intraperitoneal tumor xenografts. J Natl Cancer Inst 90: 25-29.
6. Wang LH, Li XY, Jin LJ, You JS, Zhou Y, et al. (2011) Characterization of chicken egg yolk immunoglobulins (IgYs) specific for the most prevalent capsular serotypes of mastitis-causing Staphylococcus aureus. Vet Microbiol 149: 415-421.
7. Straubinger RK, Chang YF, Jacobson RH, Appel MJ (1995) Sera from OspA-vaccinated dogs, but not those from tick-infected dogs, inhibit in vitro growth of Borreliaburgdorferi. J ClinMicrobiol 33: 2745-2751.
8. JustizVaillant AA, Ramirez N, Cadiz A, Ferrer B, Akpaka P, et al. (2013) Separation and Reactivity of Avian Immunoglobulin Y. J Chromat Separation Techniq 4: 173.
9. JustizVaillant AA, McFarlane-Anderson N, Akpaka PE, Smikle MP, Ramirez N, et al. (2013) Use of Dot Blots Analysis in the Separation of Anti-HIV Antibodies in Animals. J Chromat Separation Techniq 4: 181.
10. Stöbel K, Schönberg A, Staak C (2002) A new non-species dependent ELISA for detection of antibodies to Borreliaburgdorferi s. l. in zoo animals. Int J Med Microbiol 291 Suppl 33: 88-99.
11. JustizVaillant AA, Akpaka PE, McFarlane-Anderson N, Smikle MP, Wisdom B (2012) Purification of Immunoglobulin Y (IgY) from the Ostrich (Struthiocamelus) by Staphylococcal Protein A (SpA) Affinity Chromatography. J Chromat Separation Techniq 3: 127.