Production and characteristics of a novel chicken egg yolk antibody (IgY) against periodontitis-associated pathogens

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ABSTRACT
Periodontitis is a bacterial biofilm-induced oral disease, mostly caused by Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans) and Porphyromonas gingivalis (P. gingivalis). Oral administration of chicken egg yolk antibody (IgY) is a promising nutritional strategy to control pathogen infections. The objective of this study was to produce an A. actinomycetemcomitans- and P. gingivalis-specific IgY and evaluate its effects on bacterial agglutination and biofilm formation. Thirty laying hens were immunized with a complex of lysozyme containing typical molecular weights of membrane proteins of A. actinomycetemcomitans and P. gingivalis. IgY was isolated by polyethylene glycol 6000 and ammonium sulfate and purified by dialysis. The results of enzyme-linked immunosorbent assay showed that the obtained IgY were specific to both A. actinomycetemcomitans and P. gingivalis. In addition, immunoelectron microscopy scanning and crystal violet staining showed that the IgY could bind to cell wall of the pathogens and efficiently accelerate agglutination and inhibit biofilm formation. Furthermore, the activity of the IgY remained stable at different temperature, pH, and storage period. This is the first report that a novel two-in-one IgY was produced to modulate the agglutination and biofilm formation of A. actinomycetemcomitans and P. gingivalis, suggesting the potential of IgY to control periodontitis caused by oral pathogens.

Introduction
Periodontitis is a plaque biofilm-induced inflammatory disease of the supporting tissue of the tooth. Although periodontitis is actually not fatal, it can cause tooth loss and considerable restrictions in quality of life like chewing function, phonetics, and esthetics of individuals [1]. It is well-accepted that periodontitis is initiated by pathogens embedded in subgingival dental plaque (biofilm) [2]. Not all bacteria in the biofilm are equally pathogenic, but Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans) and Porphyromonas gingivalis (P. gingivalis) show particularly strong association with periodontitis [3]. A. actinomycetemcomitans has been implicated in the etiology of aggressive periodontitis but it has also been associated with chronic periodontitis. P. gingivalis is related to chronic periodontitis and periodontal disease progression [4, 5]. Antibiotics, used in conjunction with mechanical therapy like scaling and root planning, are usually applied to the treatment of periodontal disease [6]. However, antibiotics are deficient in specificity and inevitably show strong side effects, such as the emergence of drug-resistant bacteria [7]. It is urgent to explore alternative therapies targeting pathogenic strains to treat periodontal diseases.

Specific antibody has been known to be protective against pathogen infections [8]. Chicken egg yolk antibody (IgY) is the predominant immunoglobulin found in laying hens. Similar to the placental transfer of IgG in mammals, IgY is actively transported from serum to egg yolk in immunizing hens [9]. Importantly, it is easy to achieve high concentration (about 100 mg/egg) and high titer of IgY with appropriate immunization methods [10]. In contrast to other animal-produced antibodies, the production of IgY is much less painful for animals [11]. In addition, comparing with antibiotics, IgY is environmentally friendly without undesirable side effects, disease resistance, or toxic residues. IgY-based therapies have many advantages, such as reducing cost, biosafety, and easy of mass preparation. Currently, specific IgYs have been prepared to neutralize a variety of pathogens, including bacteria, viruses, and parasites [12]. For instance, specific IgY has been used in passive immunization trials against parasitic infection, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, Ebola virus infection, and other infections [13–15]. In the treatment of oral disease, specific IgY targeted against P. gingivalis gingipains, Streptococcus mutans glucan-binding protein
B, or Solobacterium moorei has been developed [16–18]. However, IgY against aggressive periodontitis-related *A. actinomycetemcomitans* and against both *A. actinomycetemcomitans* and chronic periodontitis-related *P. gingivalis* are still to be explored. Therefore, this study was conducted to produce an *A. actinomycetemcomitans* - and *P. gingivalis*-specific IgY and to evaluate its effects on agglutination and biofilm formation.

**Materials and methods**

**Bacterial strains and maintenance**

*A. actinomycetemcomitans* (ATCC 29523, GIM 1.393) and *P. gingivalis* (ATCC 33277, GIM 1.851) were from Guangdong Microbial Culture Collection Center (Guangzhou, China). *A. actinomycetemcomitans* was cultured on blood agar plates at 37°C for 24 h in a 5% CO₂ incubator, while the liquid medium used to culture *A. actinomycetemcomitans* was tryptone soy broth. *P. gingivalis* was streaked onto blood agar plates and cultured at 37°C for 5 d using an anaerobic package.

**Preparation of antigen and immunization of hens**

*A. actinomycetemcomitans* was cultured on blood agar plates for 24 h, while *P. gingivalis* was cultured on blood agar plates for 72 h. Colonies were removed from the agar by washing with sterile phosphate-buffered saline (PBS, pH 7.4), treated with 0.5% formalin, and stored at 4°C for further studies. The colonies were adjusted to 2.1 × 10⁸ CFU/mL with PBS and then lysed on ice by ultrasonic sonication at a frequency of 20 kHz (20 min, cycles of each 10 s on and 10 s off). The lysate of the two bacteria was mixed in an equal volume and emulsified with an equal volume of complete (for the initial injection) or incomplete (for the five booster injections) Freund’s adjuvant (Sigma-Aldrich, St Louis, MO, USA) for antigen preparation.

All animal procedures were performed under the Guidelines for Care and Use of Laboratory Animals of Guangdong University of Technology (Guangzhou, China) and experiments were approved by the Animal Ethics Committee of Guangdong University of Technology (Guangzhou, China). Thirty White Leghorn laying hens (25 weeks old) were immunized with six injections of 1 mL of the antigen solution. Each hen was injected at two different sites (0.5 mL per site) of pectoral muscle. The injections were given at a 9-day interval, and after the second immunization, eggs laid by the hens were collected for 3 months and stored at 4°C. The eggs laid by hens which immunized with sterile PBS were collected as a control.

**Isolation and purification of IgY**

Isolation and purification of IgY were performed as described previously with minor modification [19]. Briefly, egg contents were poured on to Whatman filter paper after making a wide bore without puncturing yolk sac and then the paper was moved sideways to get yolk sac without albumin. The yolk sac was punctured and its contents were collected in a measuring bottle. A volume of yolk was added to 3 volumes of buffer containing 3.5% polyethylene glycol (PEG) 6000 (w/v), followed by stirring at room temperature for 30 min. After the mixture was centrifuged at 10000 g for 20 min at 4°C, the supernatant was filtered through four layers of sterile gauze. Subsequently, PEG6000 was added to the filtrate with gentle stirring to adjust a final concentration of 12% (w/v). Precipitate was collected by centrifugation. A portion of the precipitate was dissolved in the PBS, mixed with an equal volume of saturated ammonium sulfate, and centrifuged to collect pellets. The pellets were desalted for 72 h using dialysis tubes to get purified IgY antibodies, and then lyophilized into powders for further studies.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

Molecular weight of antigens and IgY antibody were determined by SDS-PAGE, with a 5% stacking gel and 10% separating gel, as previously described [20]. The gel was stained with Coomassie brilliant blue R-250 solution (Bio-Rad Laboratories, Hercules, CA, USA), and images and purification of IgY were obtained using Phoretix 1D Pro system (Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK).

**Indirect enzyme-linked immunosorbent assay (iELISA)**

**Antibody titer analysis**

The titer of the specific IgY against *A. actinomycetemcomitans* and *P. gingivalis* was determined by iELISA [21]. Briefly, 96-well polystyrene plates were coated with 1 × 10⁸ CFU of *A. actinomycetemcomitans* or *P. gingivalis* in carbonate-bicarbonate solution (0.05 M, pH 9.6) and incubated overnight at 4°C. The plate was washed three times with PBS (pH 7.4, 0.01 M) containing 0.05% Tween-20 (PBST) and were blocked using 100 μL/well of PBS containing 3% (w/v) skim milk powder at 37°C for 2 h. After three rinses with PBST, 100 μL of gradient diluted samples of the crude extract of IgY (initiated from 1 mg/mL, 1:10 to 1:10240 dilution) were added to corresponding
wells and incubated at 37°C for 1 h. The crude extract of IgY (100 μL, 1:10000 dilution) from the control hens was used as the control. After washing 3 times with PBST, 100 μL/well of HRP- rabbit anti-chicken secondary antibody (EarthOx, LLC, San Francisco, CA, USA, 1:5000 dilution) was added. The plate was incubated at 37°C for 1 h, following washing with PBST. Then, 100 μL of tetramethylbenzidine (TMB) substrate solution was added to each well, and the plate was incubated at room temperature for 15 min to allow chromophore development followed by adding 50 μL of 2 mol/L H2SO4 to stop the reaction. The optical density (OD) values were obtained by a microtiter plate reader (Thermo scientific Multiskan™, Massachusetts, USA) at a wavelength of 450 nm. When ODsample/ONDnegative ≥ 2.1, the maximum dilution multiple of the sample was determined as the IgY titer.

Cross-reactivity of IgY
iELISA was also conducted to assess the cross-reactivity of IgY to other bacteria, including Bacillus subtilis, Listeria monocytogenes, Enterococcus faecium, Vibrio parahaemolyticus, Yersinia enterocolitica, Pseudomonas fluorescens, Solobacterium moorei, Salmonella typhimurium, Escherichia coli, Staphylococcus aureus, Staphylococcus albus, Staphylococcus epidermidis, Streptococcus mutans, and Pseudomonas aeruginosa. The cross-reactivity of the IgY against these bacteria was determined by the maximum dilution, in which ODsample/ONDnegative ≥ 2.1.

Stability of IgY
Briefly, the purified IgY was subjected to different conditions, including temperature (4 to 90°C under pH 7.0 for 2 h), pH (2.0 to 10.0 at 37°C for 2 h), and storage duration (0 to 35 days under 7.0) and performed to measure OD450 values against A. actinomycetemcomitans.

Immunoelectron microscopy scanning
A. actinomycetemcomitans (10⁷ CFU/mL) or P. gingivalis (10⁷ CFU/mL) were incubated with specific IgY (6 mg/mL dissolved in PBSA), nonspecific IgY (6 mg/mL dissolved in PBSA), or PBS. Exactly, 1 mL of the bacterial solution was incubated at 37°C for 2 h and then at 4°C overnight. After two washing steps with PBSA, 300 μL of colloidal gold-conjugated rabbit anti-chicken IgG antibody (Beijing Biosynthesis Biotechnology Company, Beijing, China), diluted 1:14 in PBSA, were added, and the samples were incubated at 37°C for 2 h. After three rinses with PBSA, a 300-mesh copper grid was used to mount the bacteria. Subsequently, the samples were negatively stained with 2% phosphotungstic acid (pH 7.0) and observed under a transmission electron microscope (Hitachi HT7700, Tokyo, Japan).

Microbial agglutination assay
The microbial agglutination assay was performed as previously described with minor modification [22]. Briefly, A. actinomycetemcomitans or P. gingivalis at a final concentration of 10⁶ CFU/mL was incubated with specific IgY (2.0 mg/mL), nonspecific IgY (2.0 mg/mL), or PBS for 1 h at 37°C. The agglutination activity was observed under an inverted fluorescence microscope (Olympus IX71, Tokyo, Japan).

Evaluation of biofilm formation
A. actinomycetemcomitans and P. gingivalis were cultured in tryptone soy broth liquid medium, and supplemented with specific IgY (at a final concentration of 2, 4, or 8 mg/mL), nonspecific IgY (at a final concentration of 8 mg/mL, the negative control), or PBS (the blank control) to produce a final bacterial concentration of 10⁶ CFU/mL. A treatment with ampicillin (0.1 mg/mL) was served as the positive control. Exactly 100 μL of each mixture was added to a 96-well plate and cultured at 37°C for 24 h. The plate was rinsed 3 times with distilled water and stained with 50 μL 0.5% crystal violet (w/v) for 15 min at room temperature. After washing 3 times, 200 μL 95% ethanol was added to extract the bound dye, and biofilm formation was quantified by OD595nm values [23].

Statistical analysis
The data were performed with GraphPad Prism software version 7 (GraphPad, La Jolla, CA, USA) and were expressed as means ± standard deviation (SD). Statistical significance was determined by one-way ANOVA with a Tukey post hoc test. Differences between groups were considered statistically highly significant if P < 0.01.

Results
Preparation of antigens and IgY
The molecular weights of antigens and IgY were determined by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). As showed in Figure 1a, the molecular weight of proteins in lysate of A. actinomycetemcomitans varied below 120 kDa, and the most abundant weight was ~64 kDa which maybe one of the prominent membrane proteins in A. actinomycetemcomitans [24]. Whereas, the molecular weight of most proteins in lysate of P. gingivalis ranged from 38 to 120 kDa, containing particular molecular weights of membrane proteins in P. gingivalis [25].
The results indicated that two protein bands with a 70-kDa heavy chain and a 25-kDa light chain were observed in crude and purified IgY (Figure 1b). The presence of a band of ~38 kDa may be due to the C-terminal fragment of vitellogenin II [26].

**Titer, cross-reactivity, and stability of IgY**

The conditions of indirect ELISA were performed to determine titer, cross-reactivity, and stability of IgY. The results showed that the antibody titer increased slowly in the first 40 days. After the fifth immunization, however, the antibody titer of IgY against *A. actinomycetemcomitans* or *P. gingivalis* showed a sharp increase and reached the peak which was lasted to roughly 15 days (Figure 2a).

Cross-reactivity was performed to assess the specificity of the IgY. The results showed a strong binding activity of the IgY to *A. actinomycetemcomitans* and *P. gingivalis*, while a weak binding activity with other bacterial strains (Figure 2b). These results confirmed that the IgY possesses a high specificity towards *A. actinomycetemcomitans* and *P. gingivalis*.

The stability assessment showed that the IgY was stable at 4 to 60°C and at pH 4.0 to 10.0. Moreover, the IgY kept stable for 35 days under 4, 25 and 37°C (Figure 3). The results indicated that the IgY is stable under variant conditions, which is a vital property of supplementation in therapy products for periodontitis.

**Targeting of IgY against *A. actinomycetemcomitans* and *P. gingivalis***

The binding of IgY was observed under a fluorescence microscope and a transmission electron microscope. The results showed that large amount of immunogold particles were observed in the surface of *A. actinomycetemcomitans* and *P. gingivalis* of the specific IgY group (Figure 4a,d). There were few immunogold particles in the nonspecific IgY group and the control group (Figure 4b,c,e, f). In addition, fluorescence was observed in the specific IgY group, whereas there was no obvious fluorescence in the nonspecific IgY group and the blank control group (data not shown). These results demonstrated specific binding of the IgY to the cell wall of *A. actinomycetemcomitans* and *P. gingivalis*.

**Agglutination of *A. actinomycetemcomitans* and *P. gingivalis***

Agglutination is the reaction between surface antigens of bacteria and specific antibodies. Microbial agglutination assay was conducted to verify whether the IgY could specifically recognize and agglutinate *A. actinomycetemcomitans* and *P. gingivalis*. The results showed that the IgY efficiently agglutinated *A. actinomycetemcomitans* and *P. gingivalis*, while the nonspecific IgY and the control groups showed no bacterial agglutination (Figure 5).
Figure 2. The titer of IgY against A. actinomycetemcomitans and P. gingivalis (a) and cross-reactivity of IgY (b) determined by ELISA. Briefly, 96-well polystyrene plates were coated with A. actinomycetemcomitans or P. gingivalis and blocked with PBS containing skim milk powder. Gradient dilutions of IgY crude extract were added to the corresponding wells, followed by incubation with HRP- rabbit anti-chicken secondary antibody. The optical density of each well was determined by the chromochromic degree responding to tetramethylbenzidine. When OD_{sample}/OD_{negative} ≥ 2.1, the maximum dilution multiple of the sample was determined as the IgY titer. The results are the means of 6 replicates.

**Antibiofilm activity of IgY**

Biofilms are the formation and persistence of surface-associated microbial communities, which are encapsulated in an extracellular matrix of polymeric substance and confers several advantages to resident pathogens. In contrast to the blank control and the negative control groups, the specific IgY group showed lower OD values, demonstrating that the biofilm formation of both A. actinomycetemcomitans (Figure 6a) and P. gingivalis (Figure 6b) was inhibited by the IgY. Moreover, the inhibition effect increased as the concentration of the IgY increased.

**Discussion**

Periodontitis is a complex infection, in which bacteria are responsible for the immune inflammatory process resulting in the loss of support of affected teeth [27]. To prevent and treat this disease, it is key to recognize the pathogenic role of bacteria that accumulate in the periodontal pocket [28]. The aetiology of periodontitis is closely linked to the manifestation of proinflammatory bacterial dysbiosis through the overgrowth of specific, mostly gram-negative, germs in bacterial biofilms. Therefore, the reduction of bacterial biofilm must be the aim of any established periodontal therapy [29]. As mechanical cleaning alone cannot fully eliminate all bacteria involved in periodontal disease, antibiotics are applied to the treatment of periodontal disease. However, along with antibiotic misuse, there are lots of antibiotic-resistant bacteria, leading to a global public health problem. Consequently, alternatives to antibiotics are urgently need.

Previous works demonstrated that IgY can specifically inhibit the growth of periodontitis pathogens and the application of IgY has the potential to substitute antibiotic therapy [30]. However, the antibacterial characteristics of IgY are to be documented. In this study, we established
a novel IgY against two pathogens that are strongly associated with periodontitis. Theoretically, the cost of producing a two-in-one IgY is much lower than that of producing complexes of two kinds of IgY. The two-in-one IgY was high-yield, stability, and specific to *A. actinomycetemcomitans* and *P. gingivalis*. Importantly, the activities of agglutination and antibiofilm of the IgY indicate potential to treat periodontitis.
Design and preparation of an antigen is the basis for a successful production of specific antibody. Therefore, a complex of lysate with typical molecular weights of membrane proteins of of *A. actinomycetemcomitans* and *P. gingivalis* was used as the antigen in this study. The properties of IgY are profoundly affected by methods of isolation and purification. Various IgY isolation methods are reviewed in detail, including water dilution method, ammonium or sodium sulfate precipitation method, PEG precipitation method, dextran sulfate precipitation method, precooled propane and acetone method, and water dilution ultrafiltration method [30]. IgY isolation by means of PEG-precipitation is very cost-effective and results in highly specific antibodies with a stable titer. After isolated by PEG-precipitation and purified by ammonium sulfate precipitation, the purity of IgY in this study was highly increased. Minor impurities, corresponding to molecular weight around 35–40 kDa, probably were the C-terminal fragment of vitellogenin II [26]. The IgY showed high binding ability to *A. actinomycetemcomitans* and *P. gingivalis* and weakly bound ability to other bacterial strains. Meanwhile, the same changing tendency along with the immunization was found in the antibody titer of IgY against *A. actinomycetemcomitans* or *P. gingivalis*. Titer of the IgY against *P. gingivalis* was higher than that of *A. actinomycetemcomitans*. The possible reason is that the immunogenicity of *P. gingivalis* was stronger than that of *A. actinomycetemcomitans*.

Functional receptors of bacteria such as their outer membrane proteins, lipopolysaccharides, fimbriae, and flagella, are crucial factors for bacterial colonization. Specific IgY can recognize distinct antigenic
epitopes, including supernatant containing cytoplasmic proteins and precipitated membrane proteins [31]. Our results of ELISA demonstrated that the IgY is specific to A. actinomycetemcomitans and P. gingivalis. In addition, immunofluorescence staining (data not showed) and transmission electron scanning indicated that the IgY can bind to the cell wall components of A. actinomycetemcomitans and P. gingivalis. The results revealed that ultrasound for crushing A. actinomycetemcomitans and P. gingivalis is suitable to antigen preparation. The binding of IgY to bacteria may block or impair the functions of bacterial components, leading to agglutination and biofilm damage [32,33]. Agglutination is a process to concentrate microbes, resulting in growth inhibition and death promotion. Our results indicated that the IgY largely accelerates the agglutination of A. actinomycetemcomitans and P. gingivalis. This action also might reduce the adhesion of A. actinomycetemcomitans and P. gingivalis to oral epithelial cells. What’s more, in the oral cavity, the development of the biofilm community can help microbial cell escape the flowing of fluid and the movement of the tongue. Inhibiting biofilm formation can reduce the growth of anaerobic bacteria or facultative anaerobic microorganisms without providing an anaerobic environment and location of colonization [34]. Interestingly, biofilm formation of both A. actinomycetemcomitans and P. gingivalis was inhibited by the IgY in a concentration-dependent manner in this study.

In addition, in vivo trials are introduced to investigate the therapeutic effects of IgY. For instance, Oba and colleagues demonstrated that the consumption of specific IgY against P. gingivalis gingipain reduces the accumulation of subgingival dental plaque in cats [35]. Also, treatment with IgY from hens immunized with whole F. nucleatum cells results in a marked decrease in alveolar bone loss after F. nucleatum infection in a mouse model [36]. A randomized controlled clinical trial of 64 patients with periodontitis showed that the adjunctive use of lozenges containing IgY against P. gingivalis gingipains results in reduction of gingival bleeding index and P. gingivalis cell in gingival pockets [37]. Further in vivo studies are needed to directly demonstrate the therapeutic effects of our two-in-one IgY.

Collectively, this is the first report to prepare a novel IgY against both A. actinomycetemcomitans and P. gingivalis with stability under various conditions. The IgY can specifically binds to the cell wall components and then accelerates agglutination and decreases biofilm formation. Those properties of the IgY make it a potential alternative to antibiotics in treatment with periodontitis induced by A. actinomycetemcomitans and P. gingivalis. However, the actual therapeutic effects are under explored.

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Disclosure statement

The authors declare that there is no conflict of interests to publish this paper.

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