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Specific egg-yolk antibodies (IgY) confer protection against *Acinetobacter baumannii* in a murine pneumonia model

Running title: Specific IgYs against *Acinetobacter baumannii*

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Abstract

Aim: *Acinetobacter baumannii*, an increasingly serious health threat, is considered as one of the six most dangerous microbes of high mortality rate. However, treatment of its infections is difficult because of the lack of efficient antibiotic or commercial vaccines. Passive immunization through administration of specific antibodies such as IgY, could be an attractive practical solution.

Methods and Results: In the current study, antigenicity of two recombinant outer membrane proteins (OmpA and Omp34) as well as inactivated whole cell of *A. baumannii* was assessed by ELISA. Moreover, prophylactic effects of specific IgY antibodies (avian antibody) raised against these antigens were evaluated in a murine pneumonia model. The specific IgY antibodies had various prophylactic effect in the pneumonia model. OmpA was the most potent antigen in terms of triggering antibody and conferring protection. While synergic effect was observed in ELISA for antibodies raised against combination of OmpA and Omp34 (which known as Omp33-36 and Omp34kDa), antagonist effect was unexpectedly seen in challenges. The reason of this phenomenon remains to be precisely addressed.

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Conclusion: All the specific IgY antibodies could protect mice against pneumonia caused by *A. baumannii*.

Significance and Impact of Study: The specific IgY antibodies could be employed as a pharmaceutic against pneumonia caused by *A. baumannii*.

Keywords: OmpA; Omp33-36; Omp34kDa; Avian antibody; Passive immunization

1. Introduction

Acinetobacter baumannii, a Gram-negative, non-motile coccobacillus and one of the most successful nosocomial pathogens (Perez and Bonomo 2014), is an increasingly serious health threat assigned as one of the six most dangerous microbes by the Infectious Diseases Society of America (IDSA) (Huang et al. 2016). Mortality rate of *A. baumannii*-associated infections had been reported up to 70% (Vila and Pachón 2012). The clinical management of *A. baumannii* infections is difficult due to the emergence of highly antibiotic-resistant strains including multidrug-resistant (MDR) and pan-drug resistant (PDR) (Wang et al. 2003; Dijkshoorn et al. 2007; Pachón and McConnell 2014). In spite of increasing prevalence of MDR strains and their high mortality, efficient antibiotic is not provided by the pharmaceutical industry for treatment of its infections (Pachón and McConnell 2014). These implications highlight active and passive immunizations as cost-effective approaches to reduce the clinical and economic burden of infections caused by this notorious pathogen (Ahmad et al. 2016). Hence, specific antibodies could be considered one of the most promising strategies to prevent and treat infections caused by the bacterium (Cohen et al.

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1961). In this regard, specific polyclonal antibodies could be isolated from the immunized mammal's serum. IgY, a mammalian IgG counterpart in birds, is the major antibody in serum and egg yolk with several advantages over IgG. For example, IgY does not activate the complement system. It is a cost-effective natural product. High titer of specific IgY could be found in the immunized hen egg yolks for a long time. High amount of IgY could be obtained from an egg yolk. No pain and stressful bleeding is needed for production of IgY; so, its production is a non-invasive method (Li et al. 2015; Müller et al. 2015). This technology has already been successfully applied in clinical trials against *Pseudomonas aeruginosa* (Kollberg et al. 2003). GMP production of Anti-*Pseudomonas* IgY was set up in Sweden. In November 2003, a special permission was obtained from the Swedish Medical Products Agency for treatment of CF patients to be treated with IMMUNSYSTEM's Anti-Pseudomonas IgY. In 2008, the European Medicines Agency (EMEA) granted an orphan drug designation to the IgY for treatment of cystic fibrosis (http://www.immunsystem.com/cf_therapy.html). Recently, it is demonstrated that IgY targeting *P. aeruginosa* modifies bacterial fitness, facilitate formation of immobilized bacteria in aggregates and increase bacterial hydrophobicity, thereby enhance bacterial killing by Polymorphonuclear neutrophils (PMN) mediated phagocytosis and facilitate bacterial clearance in the cystic fibrosis airways (Thomsen et al. 2015; Thomsen et al. 2016b). The effects of anti-*P. aeruginosa* IgY investigated on bacterial eradication in a murine (BALB/c) pneumonia model has shown that administration of anti-*P. aeruginosa* IgY significantly reduced the bacterial burden and inflammatory cytokines in lung (Thomsen et al. 2016a).

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Various immunogens have so far been introduced among which, protein antigens such as OmpA (Luo et al. 2012; Lin et al. 2013; Fajardo Bonin et al. 2014), OmpW (Huang et al. 2015), Omp22 (Huang et al. 2016), NucAb (Garg et al. 2016), FilF (Singh et al. 2016), Ata (Bentancor et al. 2012), Bap (Fattahian et al. 2011) and Omp34 (Fajardo Bonin et al. 2014), are highly appreciated as promising ones. OmpA, a pivotal virulence factor of *A. baumannii* (Smani et al. 2012) and highly immunogenic in mice and human (Luo et al. 2012; Lin et al. 2013; Fajardo Bonin et al. 2014), is the most promising immunogen amongst other protein antigens (Chen 2015). Sub-lethal infection of *A. baumannii* in mice, could trigger antibodies against this antigen as a predominant target of humoral immunity (Luo et al. 2012). Hence, OmpA is nominated as an appropriate vaccine candidate against *A. baumannii*, however, its cytotoxicity needs to be pointed as a drawback. Omp34 is another appropriate immunogen of *A. baumannii* (Islam et al. 2011; Fajardo Bonin et al. 2014). This OMP, known also as Omp34kDa and Omp33-36, is a specific antigen which has no cross-reaction with sera from patients infected by other bacteria (Islam et al. 2011). McConnell et al. (McConnell et al. 2011) used outer membrane vesicles (OMVs) as an immunogen against *A. baumannii* and showed abundance of Omp34 in the OMVs. This OMP had recently been highlighted as a potential vaccine candidate against *A. baumannii* in an immunoproteomic study (Fajardo Bonin et al. 2014). Furthermore, this highly immunogenic protein could bind to fibronectin and thereby play a substantial role in interaction of *A. baumannii* to host cells. It has also been described as a virulence factor associated with metabolic fitness of *A. baumannii,* as well as the pathogen adherence and invasion to human epithelial cells (Smani et al. 2012). This protein induces apoptosis and inhibits autophagy in human cells. Inhibition of autophagy modulated by Omp34 enables the pathogen to persist within autophagosomes (Rumbo et al. 2014). A mutant strain of Omp34 has exhibited higher lethal dose and lesser dissemination in

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a murine sepsis model (Smani et al. 2013). Recently, it is nominated as a 14-stranded barrel by an in silico study on Omp34 3D structure (Jahangiri et al. 2018b).

In the current study, specific IgYs were raised against inactivated whole cells of *A. baumannii* as well as two recombinant protein antigens (OmpA and Omp34). Then the antibodies were purified from egg yolks and their protective efficacy against viable pathogen was assessed in a murine pneumonia model. The results are discussed from a novel candidate perspective for efficient treatment of infections caused by *A. baumannii*.

2. Materials and Methods

2.1. Antigen preparation

Three antigens were used in the current study viz., Inactivated Whole Cell of *A. baumannii,* and its recombinant OmpA (rOmpA) and recombinant Omp34 (rOmp34).

2.1.1. Whole cell inactivation

The *A. baumannii* ATCC 19606 was grown in Mueller-Hinton broth to an OD_{600} of 0.6. The bacterial cells were harvested by centrifugation and were washed in sterile PBS. Inactivation of the bacteria was carried out in 0.05 mol l^{-1} formalin for 18 h and was confirmed by plating on blood agar (McConnell and Pachón 2010).

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2.1.2. Recombinant OMPs

The *ompA* gene, corresponding to amino acids 23-341 of OmpA with accession number WP_000777878 and the *omp34* gene, corresponding to amino acids 20-299 of Omp34 with accession number WP_000733005.1were amplified by polymerase chain reaction (PCR) from genomic DNA of *A. baumannii* ATCC 19606 as template. Following primers were designed and used:

Forward (*ompA*): 5'-ATAAGAATTCATGGCTAATGCTGGCGTAAC-3'

Reverse (*ompA*): 5'-GGATGTCGACTTAAGTACGGCTACCAGTG-3'

Forward (*omp34*): 5'- TGCTGGATCCATGTATCAATTTGAAGTTC-3'

Reverse (*omp34*): 5'- TAAAGTCGACTTAGAAACGGAATTTAGCATT-3'

The restriction sites (*Eco*RI: GAATTC; *Sal*I: GTCGAC; *BamH*I: GGATCC) are underlined. The amplified fragments were cloned into plasmid pET28a (+) vector (Invitrogen). The constructs were transformed into *E. coli* BL21 (DE3) for expression of the recombinant proteins. After confirming the gene fragments by sequencing, the recombinant proteins were expressed and were then purified in denatured condition using Ni-NTA agarose columns (Qiagen).

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2.2. Immunization of hens

The inactivated bacteria were suspended in sterile PBS. The concentration of the inactivated bacteria was adjusted to 4×10^9 cell ml^{-1}. All antigens including inactivated bacteria and recombinant proteins were combined at 1:1 (v:v) ratio with Freund adjuvant (Sigma).

Four groups of Hy-line laying hens were immunized intramuscularly with 1000µl of the vaccines at four sites of the breast muscle (total dose = 2×10^9 Inactivated cells (group 1), 100 µg rOmpA (group 2), 100 µg rOmp34 (group 3) and 100 µg rOmpA + 100 µg rOmp34 (group 4)) on days zero, 14, 28 and 42. For the first injection the antigens were emulsified in the complete Freund's adjuvant while for subsequent boosters were emulsified in the incomplete one.

2.3. IgY preparation

Pre-immune IgY was purified from eggs laid prior to immunization and the anti-*A. baumannii* IgY from eggs collected at least one week after immunization. The IgYs were purified by a modified water dilution method (Hodek et al. 2013). Egg yolks separated from the whites were diluted to 1:7 (v:v) with distilled water. The pH was set at 5.0 with 0.5 mol l^{-1} HCl, and the mixtures were frozen at -20 °C. The frozen mixtures were transferred to a conventional filter paper in room temperature. After spontaneous thawing and filtering of the frozen mixtures, 8.8% NaCl was added to the cleared flow liquids and their pH was set as 4.0 with 0.5 mol l^{-1} HCl. After 2 h stirring at room temperature, the mixtures were centrifuged (3,700 g, 20 min, 4 °C). Supernatants were removed and the pellets were suspended in PBS.

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IgY concentration was estimated via Bradford method. The IgY samples purity was determined by 8% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Hodek et al. 2013).

2.4. ELISA

The immunoreactivity of IgYs was assessed by indirect ELISA. ELISA plate wells were coated with 100 µl of antigen solutions (*A.baumannii* at 3.6×10^8 CFU ml^{-1} concentration, 20 µg/ml OmpA or Omp34 in 50 mmol l^{-1} sodium carbonate buffer, pH 9.6). The plates were then incubated at 4 °C overnight. The supernatants were removed and wells were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and were blocked with 100 µl of 5% skim milk solution in PBST followed by incubating for 1 h at 37 °C. After washing the wells (three times) with PBST, 100 µl of specific IgYs prepared in PBS (1:2 serial dilutions of pre-immune or immune IgY preparations) was added to the wells and the plates were incubated for 1 h at 37 °C. The highest concentration of IgY solutions was 50 µg ml^{-1}. The washing procedure was repeated as above and then 100 µl of 1:1500 PBST dilution of HRP-conjugated rabbit anti-chicken IgG antibody (Sigma) was added to the wells and incubated at 37 °C for 1 h. The wells were washed with PBST and then 100 µl of TMB solution was added to the wells. The plate was kept at room temperature until control wells developed colour. The reaction was stopped by adding 100 µl/well of three mol l^{-1} H_2SO_4. The developed color was measured at 450 nm with an ELISA reader.

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2.5. Hydrophobicity assay

Effect of specific IgYs on bacterial hydrophobicity was assessed by microbial adhesion to hydrocarbons (MATH) test. A water-hexane two-phase system was applied to evaluate the affinity of bacteria to hexane. The bacteria were washed twice with sterile PBS and were then incubated with 10, 40, 200, 1000 and 2000 μg ml^{-1} of the control or specific anti-*A. baumannii* IgY for 1 h at 37 °C with gentle shaking. Three ml of the bacterial suspension were transferred to a sterile tube and 0.5 ml of *n*-hexane was layered on it. Vortex was performed for 60 s, and after occurrence of phase separation, the OD of the aqueous phase was measured. The hydrophobicity index (HI), relative bacterial hydrophobicity, was calculated as follows: HI = (initial absorbance – absorbance after phase separation)/(initial absorbance). Accordingly, higher hydrophobicity shows lower HIs. After achieving appropriate concentration of IgY, the test was repeated by anti-OmpA and anti-Omp34 IgYs.

2.6. Nasal challenge of mice

All animal experiments were conducted in compliance with the Welfare Act and regulations related to experiments involving animals. All experiments have adhered to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications no. 8023, revised 1978). The animal care rule was ethically approved by Shahed University. The animals were maintained in suitable well aerated environment with water and animal feed ad libitum. Male BALB/c mice with 10-12 week age (27-32 g) were used in this study. The minimal number of mice were used in order to meet ethical aspects. Total number of about 100 mice were employed in the current study. All animals were randomly allocated into 22 groups of four or six animals each.

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The animals were housed in standard cages such that their normal physiologic and behavioral needs, adequate ventilation and accessing to food and water were provided. Room temperature was set as 20-26 ºC. The relative humidity was in range 40-60%.

2.6.1. *A. baumannii* ATCC 19606

Pilot challenges were initially carried out with standard strain of *A. baumannii* ATCC 19606. For pilot challenges, only IgY raised against Inactivated Whole Cell (IgY-IWC) and control IgY purified from non-immunized hens (IgY-C) were employed to estimate appropriate amount of effective IgYs. The 50% lethal dose (LD_{50}) of *A. baumannii* ATCC 19606 in the mice pneumonia model was determined by intranasal administration of 40 µl bacterial suspensions in PBS at doses ranging from 1.8×10^7 to 1.8×10^9 CFU. This test was carried out simultaneously in four groups of BALB/c mice with six mice per group. The mice received intraperitoneal injections of 150 µg g^{-1} cyclophosphamide on days 1 and 2. Challenges were performed on day 4. The number of survivals three days after challenge was used to estimate LD_{50} by the Probit method (Lieberman 1983).

Five groups of 27-32 g BALB/c mice received intraperitoneal injections of 150 µg g^{-1} of cyclophosphamide on days 1 and 2. These animals were also challenged intranasally with 10X LD_{50} of *A. baumannii* ATCC 19606 on day 4. The mice were anesthetized by intraperitoneal administration of xylazine (20 mg kg^{-1}) + ketamine (100 mg kg^{-1}). One h before challenge, the mice received 20 µl of PBS or IgYs. The groups were as follow: 1) control received sterile PBS, 2) IgY-C1 received 20 µg of control IgYs purified from non-immunized hens, 3) IgY-C2 received 40 µg of control IgYs purified from non-immunized hens, 4) IgY-IWC1 received 20 µg of IgYs purified from immunized hens by inactivated whole cell of *A. baumannii* and 5) IgY-IWC2 received 40 µg of IgYs purified from

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immunized hens with inactivated whole cell of *A. baumannii*. Mice survival was monitored for 14 days. The survivals were euthanized after 14 days. The spleen and lung were removed, weighted and homogenized aseptically in 2 ml and 4 ml of 0.9% sterile NaCl. The homogenized tissue suspensions were serially diluted. 100 µl of each dilution was plated on LB agar medium. Colony counting was carried out after incubating the plates at 37 °C overnight.

2.6.2. Clinical strain

Six clinical *A. baumannii* strains from bacterial culture collection of Shahed University (under #AbI001, #AbI036, #AbI041, #AbI052, #AbI101 and #AbI104) were evaluated to achieve the most virulent one in the murine pneumonia model. These strains had been isolated from blood, lung secretions, trachea, lung, BAL and lung respectively. Among the evaluated isolates, the most virulent one was selected based on mortality in the murine pneumonia model. Whole ELISA was also performed (as described in "**2.4. ELISA"** section) for the selected clinical isolate to determine and compare the cross reaction of the prepared IgY with this strain.

The mice were divided in seven groups. The groups were as follow: 1. Control (receiving bacteria suspended in PBS), 2. IgY-C-C (receiving only IgY-C suspended in PBS), 3. IgY-C (receiving IgY-C and bacteria suspended in PBS), 4. IgY-A (receiving IgY-A and bacteria suspended in PBS), 5. IgY-34 (receiving IgY-34 and bacteria suspended in PBS), 6. IgY-A34 (receiving IgY-A34 and bacteria suspended in PBS) 7. (receiving IgY-IWC and bacteria suspended in PBS); . Challenges were carried out by intranasal administration of 7.4×10^8 CFU *A. baumannii* AbI101 suspended in 20 µl PBS. Mice survival was monitored for eight days after which bacterial load in spleen and lung of the survived mice were assessed via

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colony counts as described in previous section. The clinical symptoms of the mice were noted in addition to survival monitoring.

2.7. Statistical analyses

Quantitative data were analysed with the One-way ANOVA test. Non-parametric data were analysed by the Kruskal–Wallis in the statistical program SPSS. Those results with p values of ≤ 0.05 were considered as significant.

3. Results

3.1. Antigens and IgYs

Western blotting by Anti-His HRP-conjugated antibodies for rOmpA and rOmp34 is shown in Figure S1. There was a molecular weight discrepancy of about nine kDa in OmpA with the apparent weight on the SDS-PAGE. No colony was seen after plating the inactivated bacteria. The purified IgYs loaded in non-reduced condition showed a band of ~180 kDa. In reduced condition, two bands of the light heavy chain were presented in the SDS-PAGE (Figure S2).

3.2. Immunoassay tests

IgY raised against the combination of rOmpA and rOmp34 showed the highest absorbance while, IgYs raised against Inactivated Whole Cell showed the lowest one (Fig. 1).

3.3. Hydrophobicity assay

One thousand and 2000 mg ml^{-1} of IgYs conferred significant difference in absorbance obtained before and after of mixing with n-hexane. The 1000 mg ml^{-1} concentration was selected for the further hydrophobicity assay (Table 1).

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3.4. Challenges

3.4.1 *A. baumannii* ATCC 19606

LD_{50} of *A. baumannii* ATCC 19606 was determined as 1.75×10^8 CFU. Clinical signs such as weight loss, eye condition (a range between no sign and closed septic eyes), clustering, piloerection, hypothermia and tachypnea were observed in mice challenged with the bacteria. Survival diagram is plotted in Fig. 2. All control mice as well as those treated with 20 µg of IgYs were died before 80 hours. Although 25% of mice treated with 20 µg of IgY-IWC was survived after 72 h, no significant difference was observed for survivability among the groups of mice. However, all mice treated with 40 µg of IgY-IWC were survived for eight days. All control mice and 75% of mice treated with 40 µg of IgY-C were died within four days.

3.4.2. Clinical strain

A. baumannii AbI101 was selected as the most fatal strain among the evaluated isolates. ELISA results showed no significant differences in comparison with ATCC19606 (data not shown). LD_{50} of *A. baumannii* AbI101 was determined as 2.04×10^7 CFU. Based on the observed clinical signs, average eye condition score of various groups on day eight post infection in descending order was as follow: positive control > Y-34 > Y-IWC > Y-A34 > Y-C > Y-A > control, received only IgY. Details of average eye condition in various groups during eight days post infection with *A. baumannii* AbI101 are provided as Figure S3. Average scores of other clinical symptoms in various groups on day eight post infection were as follow: control, received only bacteria > Y-A34 > Y-34 > Y-IWC > Y-C > Y-A > control, received only IgY. Details of other clinical symptoms in various groups during eight days post infection with *A. baumannii* AbI101 are provided as Figure S4. Survival plots are shown in Fig. 3. All control mice were died within eight days; however, all mice treated with Y-A

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were survived. During the monitoring time, 75% of mice treated with Y-C, Y-34 or Y-IWC were survived while 25% of mice treated with Y-A34 were survived.

No colony was seen after plating homogenized spleen of Y-C, Y-A34 or Y-IWC groups. The number of viable bacteria in spleen of Y-A and Y-34 was 66 and 95 CFU respectively. Plating results of homogenized lung from various groups were as follow: Y-C: 3.9×10^4 CFU, Y-A: 1.3×10^3 CFU, Y-34: 2.2×10^3 CFU and Y-A34: 1.6×10^3 CFU, and Y-IWC: 8.3×10^6 CFU.

4. Discussion

Since signal peptides could not be effective in raising protective antibodies (Jahangiri et al. 2017; 2018a), these segments were removed in the amplified genes by considering the issue in primer designs. Moreover, based on the previous in silico analyses, 15 aa at the C-terminus of OmpA has negative effect on its immunogenicity. Removal of this region could enhance antigenicity of OmpA (Jahangiri et al. 2017); Hence, this region was also removed via forward primer design. The observed discrepancy in apparent molecular weight of OmpA could be attributed to native localization (OM) of this protein. Some OMPs including OmpA (McConnell and Pachón 2011) show apparent weight increase following heat treatment. This phenomenon may be due to transition of the barrel structure to an α-helix (Nandi et al. 2005; McClean 2012).

Since the rOmpA and rOmp34 were purified and administered in denatured condition, their structures and consequently their conformational epitopes could be disturbed. So, antibodies which could recognize these OMPs on the surface of *A. baumannii* (native proteins) are those raised against exposed linear B-cell epitopes. Hence, the protection conferred thereby could

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be attributed to the specific IgYs raised against exposed linear B-cell epitopes. Toobak et. al. have investigated immunization of three recombinant OMPs (OmpA, OmpC and OmpF) of *Salmonella enterica* serovar Typhi purified in denaturing condition (Toobak et al. 2013). No protection had been reported where these antigens separately administered (Toobak et al. 2013). Our results show both IgY antibodies raised against denatured rOmpA or rOmp34 are protective. To the best of our knowledge this is the first report about protective immunization of Omp34 against *A. baumannii*. Amount of administered antigens has critical role in IgY production. Very high or very low amount of a given antigen could result in undesirable immunomodulatory effects such as suppression and tolerance (Marcq et al. 2013). Since no reliable study was available about immunization of hens by IWC of *A. baumannii*, data obtained from a mice immunization study with *A. baumanii* (McConnell and Pachón 2010) and a hen immunization study with *Pseudomonas aeruginosa* (Kollberg et al. 2003) were invoked. For protein antigens, administration of 10-1000 µg is recommended (Cook and Trott 2010; Marcq et al. 2013). The used doses of protein antigens (100 µg, in single and 200 µg in combination form) are fall into the recommended rage. Intramuscular route was selected since it could raise specific IgY titer about 10-fold higher than subcutaneous one (Leenaars and Hendriksen 2005). Generally, protein antigens are stronger immunogens than inactivated whole cells (Marcq et al. 2013). Higher absorbance observed in ELISA for the IgY-A, IgY-34 and IgY-A34 in comparison with IgY-IWC could be interpreted via this fact. The lower absorbance observed in ELISA for the IgY against whole cell is in line with other previous findings (Lee et al. 2002; Kollberg et al. 2003). Comparison of whole cell ELISA results with previous studies (Lee et al. 2002; Kollberg et al. 2003) showed that the administered dose of inactivated bacteria was appropriate for generating high titer of IgY. The low absorbance observed for IgY-C in ELISA could be attributed to existed immunity due to source of hens (a commercial poultry facility in Karaj, Iran). Based on the previous in silico studies

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(Jahangiri et al. 2017; 2018a), antigenicity of OmpA is higher than Omp34. The ELISA results confirmed the obtained in silico results. This finding is in accordance with Toobak et. al. study (Toobak et al. 2013). Based on VaxiJen results in their study, antigenicity order of OmpF, OmpC and OmpA was as OmpF > OmpC > OmpA which was confirmed by their ELISA results (Toobak et al. 2013). Interestingly, OmpA and Omp34 showed synergic effect in terms of antibody triggering. This effect could be due to existence of common epitopes in both OmpA and Omp34 antigens. Hydrophobicity assay showed that IgY-IWC could increase bacterial hydrophobicity. It could be probably due to the numbers of target positions recognized by IgY-IWC. Since antibodies triggered against inactivated whole cell were raised against more exposed surface antigenic determinants, these antibodies could cover more positions on bacteria surface rather than Y-A, Y-34 and Y-A34. A strange event was increasing absorbance of IgY-C after mixing with n-hexane. This issue could be explained by hydrophobicity of Fc in IgY representing behavior similar to phospholipids.

In mice challenges, since IgY-C could also recognize the bacteria, the observed protective effect is reasonable. Neutrophils are one of major effectors in mechanism of IgY effect (Thomsen et al. 2015). Until now, four modes of action are known for IgY: agglutination, adherence-blockade, opsonization improving phagocytosis and toxin neutralization (Xu et al. 2011). Based on literature, no toxin is produced by *A. baumannii*. Neutrophils are involved in phagocytosis improved by opsonization. Since neutropenic mice were used for challenges, this mechanism is impaired in these mice. Hence, bacterial adherence blocking could be considered as the main mechanism of IgY mediated anti-bacterial activity in this model. Owing to pivotal roles of OmpA and Omp34 in *A. baumannii* pathogenicity and its adherence to the epithelial cells, higher protection observed in mice groups received anti-OmpA or anti-Omp34 IgYs could be reasonable. Synergic effects of antigens are in contrast with those

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observed in our previous study (Toobak et al. 2013). In our previous study, no synergic effect had been observed in ELISA for combination of OmpA, OmpC and OmpF; while in the present study, this effect was observed for OmpA and Omp34. In contrast, synergic protection had been conferred to the mice immunized with combination of OmpA, OmpC and OmpF; while, antagonist effect was unexpectedly observed in mice received IgY-A34. Although the reason of this result remains to be explored, a hypothetical explanation could be provided. It is probably due to existence of common immunodominant epitopes in both antigens which are topologically inaccessible to antibodies where expressed in parent bacteria (*A. baumannii*).

In conclusion, specific IgY raised against OmpA, Omp34 and inactivated whole cell of *A. baumannii* could cause protection in the murine pneumonia model among which, IgY raised against OmpA has the highest protective effect. So, IgY could be employed as a safe natural product against pneumonia caused by *A. baumannii*.

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Authors contribution

Professor Iraj Rasooli (IR) and Parviz Owlia (PO) conceived and planned the project. Abolfazl Jahangiri (AJ) carried out the experiments. Jafar Salimian (JS) supervised the immunological aspects of the project. Ehsan Derakhshanifar (ED), Aleme Naghipour Erami (ANE), Elham Darzi Eslam (EDE) contributed to sample preparation and laboratory

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instrumentation. Shakiba Darvish Alipour Astaneh (SDAA) kindly helped in trouble shooting. AJ took the lead in writing the manuscript. IR and PO contributed to the interpretation and analysis of the results. All authors provided critical feedback and helped shape the research.

Conflict of interest

All authors attest they meet the ICMJE criteria for authorship. The authors declare that they have no conflict of interest.

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Table 1. Shift percentage of OD_{600} of bacteria treated by various IgYs after mixing with n-hexane.

Sample	Percentage of shift
IgY-C	+58.9%
Bacteria	+2.6%
Bacteria + IgY-A	+24.8%
Bacteria + IgY-34	+22.4%
Bacteria + IgY-A34	+28.6%
Bacteria + IgY-C	+19.0%
Bacteria + IgY-IWC	-13.0%

Supporting Information

Supplementary Figure captions

Figure S1. Western blotting result by Anti-His HRP-conjugated antibodies for the purified recombinant proteins (rOmpA and rOmp34).

Figure S2. Purified IgY lanes are on the right side of the ladder show IgYs treated with 2-Mercaptoethanol

Figure S3. Average eye condition of various groups during eight days post infection with *A. baumannii* AbI101. zero: no sign, four: closed and infected eye.

Figure S4. Average clinical symptoms of challenged mice during eight days post infection with *A. baumannii* AbI101. zero: no sign, six: died

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Figures

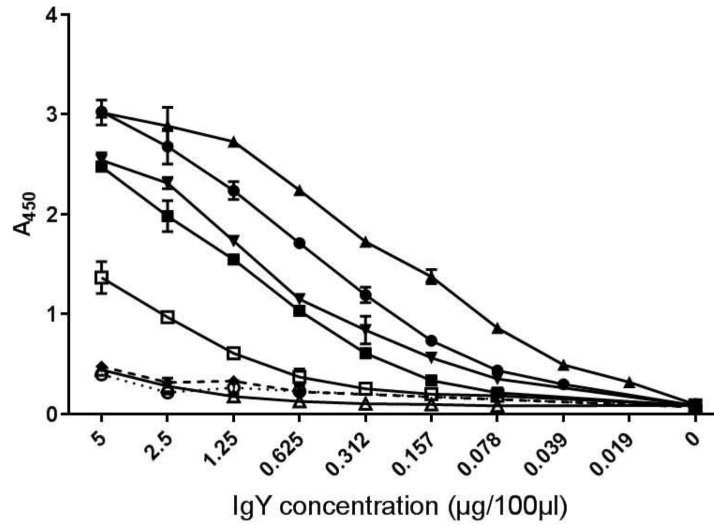

Fig. 1 ELISA results of specific IgYs. (●): specific IgY raised against rOmpA; rOmpA as coated antigen, Y-34 (■): specific IgY raised against rOmp34; rOmp34 as coated antigen, Y-A34 (▲): specific IgY raised against combination of rOmpA and rOmp34; rOmpA as coated antigen, (▼):specific IgY raised against combination of rOmpA and rOmp34; rOmp34 as coated antigen, (♦):control IgYs purified from non-immunized hens; rOmpA as coated antigen, (○):control IgYs purified from non-immunized hens; rOmp34 as coated antigen, (Δ): control IgYs purified from non-immunized hens; whole cell of *A. baumannii* as coated antigen, (□): specific IgY raised against inactivated whole cell of *A. baumannii* ATCC 19606; whole cell of *A. baumannii* as coated antigen.

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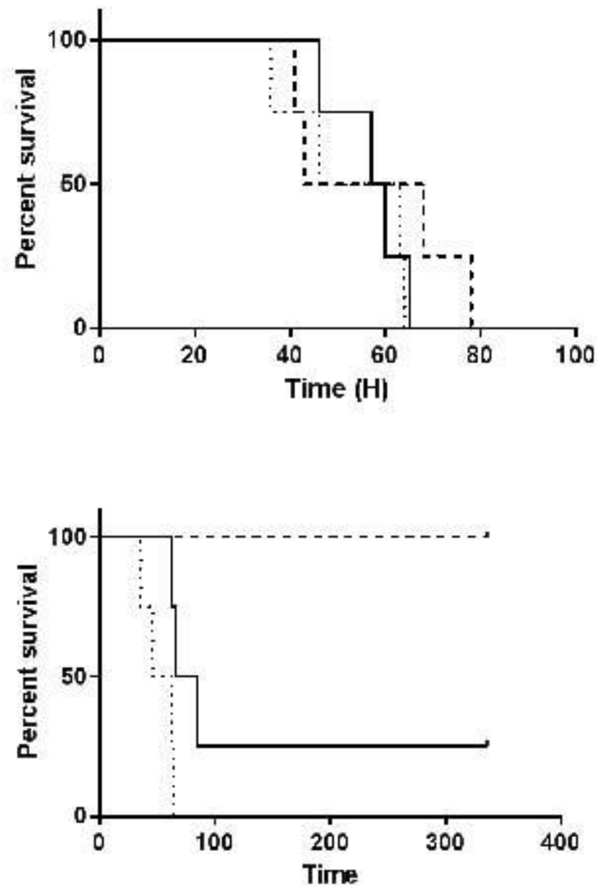

Fig. 2 Survival plots of mice received 20 µg (above) or 40 µg (below) of specific IgYs 1 hour before challenge with *A. baumannii* ATCC 19606. (…): control mice, (—): mice received IgY-C, (---): mice treated by IgY-IWC.

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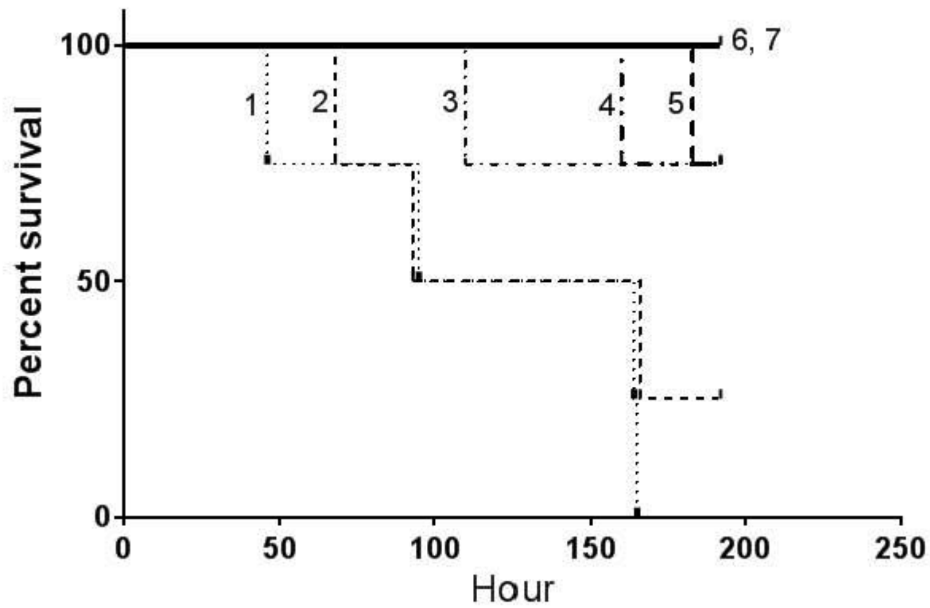

Fig. 3 Survival plots of mice received 40 µg of specific IgYs 1 hour before challenge with *A. baumannii* AbI101. (1): Control (receiving bacteria suspended in PBS), (2): receiving IgY-A34 and bacteria suspended in PBS, (3): receiving IgY-C and bacteria suspended in PBS, (4): receiving IgY-34 and bacteria suspended in PBS, (5): receiving IgY-IWC and bacteria suspended in PBS, (6): receiving only IgY-C suspended in PBS, (7): receiving IgY-A and bacteria suspended in PBS.

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