



RESEARCH ARTICLE

**REVISED** The protective effects of antigen-specific IgY on pyocyanin-treated human lymphoma Raji cells [version 2; peer review: 2 approved]

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**Abstract**

**Background:** Pyocyanin (PCN), a highly pathogenic pigment produced by *Pseudomonas aeruginosa*, induces caspase 3-dependent human B cell (Raji cells) death. The aim of the present study, therefore, was to assess whether antigen-specific IgY antibodies may be protective on PCN-induced Raji cell death.

**Methods:** Chickens were subcutaneously immunized with Freund's complete adjuvant containing PCN, and then given two boosted immunizations. Anti-PCN IgY antibodies were purified from egg yolk and detected using an agar gel precipitation test (AGPT) and ELISA. Protective effects of antigen-specific IgY on Raji cells were tested using a cell viability assay.

**Results:** AGPT results showed the formation of strong immune complex precipitates, whilst ELISA further confirmed the presence of IgY antibodies specific to PCN at significant concentration. Further experiments showed that anti-PCN IgY antibodies significantly increased PCN-treated Raji cell viability in a dose-dependent fashion (p<0.05).

**Conclusions:** The results of the present study suggest that anti-PCN IgY antibodies may be protective on PCN-induced Raji cell death.

**Keywords**

*Pseudomonas aeruginosa*, pyocyanin, IgY, protective effect

**Open Peer Review**

Reviewer Status

	Invited Reviewers	
	1	2
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version 1 published 04 Jul 2019	? report	 report

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Any reports and responses or comments on the article can be found at the end of the article.

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**Author roles:** **Susilowati H:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Validation, Visualization, Writing – Original Draft Preparation; **Artanto S:** Investigation, Methodology, Resources; **Yulianto HDK:** Data Curation, Investigation, Software, Writing – Original Draft Preparation; **Sosroseno W:** Software, Visualization, Writing – Review & Editing; **Hutomo S:** Investigation, Writing – Original Draft Preparation

**Competing interests:** No competing interests were disclosed.

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**REVISED Amendments from Version 1**

1. We replaced [Figure 4A, B, and C](#) as our response to Dr. Madhavan regarding magnification of micrograph.
2. We have already added two new references published in 2019 to the reference list. These are papers published by Li *et al.*, 2019 and Ruffin & Brochiero, 2019.
3. We added new sentences in Discussion section line 21 as response to Dr. Boy Bachtir comment about possible mechanism of PCN-induced cell death inhibition by IgY.
4. We have already added new data of quantitative results of acridine orange/ethidium bromide staining. Data is available in <https://doi.org/10.6084/m9.figshare.8115701.v6> as [Figure 4D](#)
5. We have already added new explanation to accommodate rational reason of the use of pyocyanin. The explanation is added to line 13 in Discussion section, and added new references No.24 and 25
6. According to reviewer comment to the reference no. 16 that PCN is not having a role in the bacterial invasion to the host cells we have revised the manuscript, precisely on the 3rd line of the discussion section.
7. Our suggestion for the next study were listed in the end of Discussion section.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

*Pseudomonas aeruginosa*, an opportunistic Gram-negative bacterium, is found in the environment with a broad spectrum of habitats and is responsible for severe nosocomial infections in the urinary tract<sup>1</sup>, the respiratory tract<sup>2</sup>, the vascular system<sup>3</sup> and the central nervous system<sup>4</sup>. It is known for one of the most common pathogens infecting patients with cystic fibrosis, leading to increase its morbidity and mortality due to the resisting abilities of this pathogen to against antibiotic treatments<sup>5,6</sup>. The presence of *P. aeruginosa* in dental pulp and periapical lesions may cause failure of endodontic treatments<sup>7,8</sup>. In the initial stage of infection, *P. aeruginosa* releases various virulent mediators, such as elastases, proteases, exotoxin A, and pyocyanin (PCN), after which chronic infection and persistent bacterial colonization at the *P. aeruginosa*-infected sites would be established<sup>9</sup>. PCN, a blue redox-active secondary metabolite and a member of tricyclic phenazine family, is known to function as a gene controller during the stationary growth phase<sup>10</sup>, an antibiotic<sup>11</sup>, an electron transfer facilitator<sup>12</sup>, and a potent mammary cell-damaging virulence factor<sup>13</sup>. Reports indicate that PCN inhibits B cell, T cell and macrophage functions<sup>14,15</sup> and induces neutrophil apoptosis<sup>16</sup>, suggesting that PCN suppresses both innate and antigen-specific adaptive immune response.

The existence of multidrug-resistant (MDR) *P. aeruginosa* leads to the development of alternative treatment strategies to eradicate an established chronic *P. aeruginosa* infection. Of these treatments, both active and passive immunotherapies have been reported. Active immunization with *P. aeruginosa*-derived flagella in cystic fibrosis patients resulted in increased serum antigen-specific IgG antibodies and reduced number of *P. aeruginosa* strains, suggesting the reduction of *P. aeruginosa* infection risk in cystic fibrosis patients by active vaccination<sup>17</sup>. Passive immunization with egg yolk immunoglobulin (IgY) specific

to *P. aeruginosa* in patients with cystic fibrosis prevented bacterial colonization and infection, perhaps by acting as an opsonin, which in turn enhanced neutrophil phagocytosis to this pathogen<sup>18–20</sup>. A recent study showed that PCN induces caspase 3-dependent human B cell (Raji Cells) death<sup>21</sup>. The aim of the present study, therefore, was to determine whether antigen-specific IgY antibodies may prevent PCN-induced Raji cell death.

## Methods

### IgY preparation and purification

PCN (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO at a concentration of 1 mg/ml. Five Leghorn chickens aged 3 months were subcutaneously immunized with 500 µl of Freund's complete adjuvant (Sigma-Aldrich) containing 100 µg of PCN in the back of the neck. Two weeks later, a booster was given by injecting 500 µl incomplete Freund adjuvant containing 40 µg PCN as above and the same immunization regime was repeated two weeks later. Eggs were collected one week after the last immunization and IgY was isolated by using Pierce® Chicken IgY Purification Kit (Thermo Fisher Scientific Pierce Biotechnology, Rockford, USA) according to the manufacturer. The presence of anti-PCN IgY antibodies was detected using the agarose gel precipitation test (AGPT) as previously reported<sup>22</sup> and its concentration was assessed using the Chicken IgY ELISA Kit (Elabscience Biotechnology Co., Ltd, USA). The AGPT test was performed three times, each of 4 isolates from the first and second IgY purification results. The ELISA was then performed on two IgY batches.

### Cell cultures

Raji cells, a human B cell line, obtained from central university laboratory LPPT, Universitas Gadjah Mada, Yogyakarta, Indonesia, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 UI/ml of penicillin-streptomycin, and 250 µl/ml of amphotericin B and then incubated in 5% CO<sub>2</sub> humidity. All materials for culture medium were purchased from Sigma-Aldrich. The cells were cultured in 96-well plates and five replicates were carried out for assays.

### Cell viability

PCN (Sigma-Aldrich) was initially dissolved in DMSO (Sigma-Aldrich) at a concentration of 1 mg/ml and then diluted in RPMI to a final concentration of 1 µg/ml, 10 µg/ml, 25 µg/ml, and 50 µg/ml. Raji cells at  $2 \times 10^4$  cells incubated without the presence of PCN were used as a negative control. After exposure to various concentration of PCN then the cultures were incubated at 37°C for 24 hours. In the next experiments, the cells, at a concentration of  $2 \times 10^4$  cells/well, were treated with 50 µg/ml PCN with or without the presence of various concentration (6.71 µg/ml, 13.42 µg/ml, 28.19 µg/ml, 55.49 µg/ml, 111.87 µg/ml, and 223.75 µg/ml) of anti-PCN IgY were cultured in 96-well plates and incubated for 16 hours. Cell survivability was assessed by MTT assay as described previously<sup>21</sup>. Experiments were carried out three times with 8 replicates in each group.

In order to assess cell viability,  $5 \times 10^4$  cells/well were cultured on sterile coverslips in 24-well plates for 24 hours and then treated with PCN in the presence or absence of anti-PCN IgY

(55.49  $\mu\text{g/ml}$ ) for 16 hours. Subsequently, the cells were stained with acrydine orange/ethidium bromide and viewed under Digital Carl Zeiss-Axioscope 40 (Carl Zeiss Vision, Oberkochen, Germany) by which viable and death cells appeared as green and orange/red, respectively.

### Statistical analysis

The results of PCN cytotoxicity assay on Raji cells were analyzed by using one way analysis of variance followed by LSD test. Data obtained from the experiments on the effects of anti-PCN IgY on PCN-treated Raji cells was analysed by using one-way ANOVA followed by Tukey's Test. Statistical analysis was calculated by using IBM SPSS Statistics Version 22 (SPSS Inc., IBM Corp., Chicago, IL).

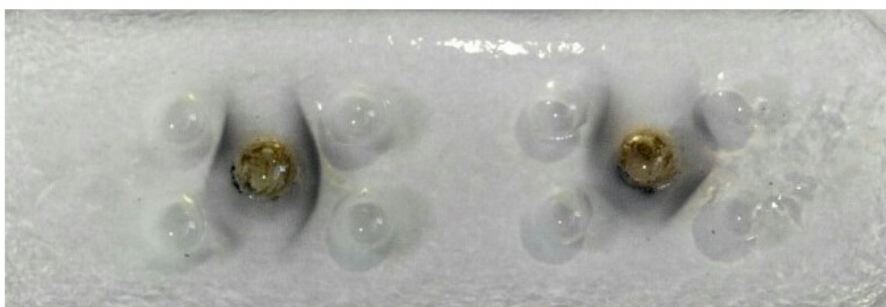
### Results

Following isolation and purification of IgY from the egg immunized chickens, PCN-IgY complexes were detected by using

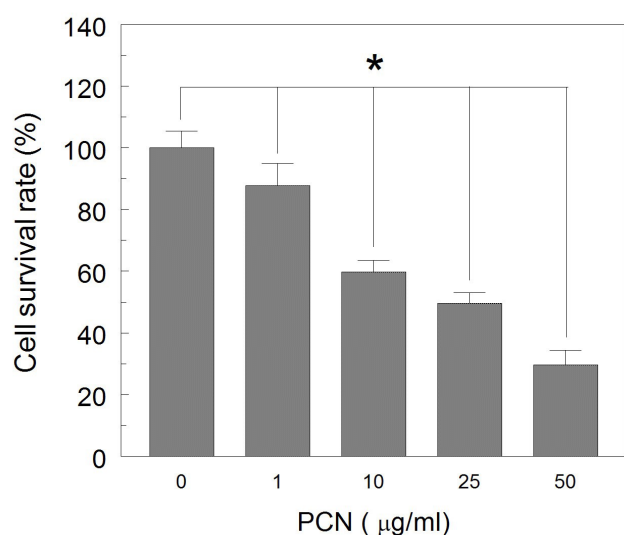
AGPT. As seen in **Figure 1**, clear lines of precipitates from two IgY batches in the agarose matrix indicated the presence of PCN-specific antibodies. A further assessment using ELISA demonstrated that the first batch gives high amount of specific IgY antibodies (8.95  $\mu\text{g}/\mu\text{l}$ ) than that one of the second (3.02  $\mu\text{g}/\mu\text{l}$ ) which were then used for the rest of experiments.

The results of this study showed that PCN at 1 mg/ml was toxic to the Raji cells. This cytotoxic effect of PCN on the cells was steadily increased in a dose dependent fashion ( $p < 0.05$ ) (**Figure 2**).

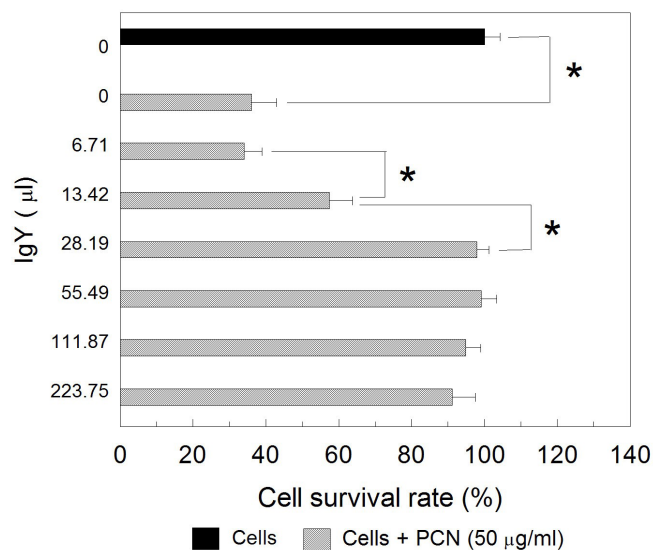
Further experimentation demonstrated that anti-PCN IgY at concentrations of 28.19  $\mu\text{g/mL}$  or higher was able to suppress the cytotoxic effect of PCN on Raji cells as compared with the negative control ( $p < 0.05$ ) (**Figure 3**). No significant differences between the cells treated with PCN and specific anti-PCN IgY antibodies at the concentration above 55  $\mu\text{g/ml}$  were



**Figure 1.** Agarose gel precipitation test of pyocyanin (PCN)-IgY antibody complex batch I and II. The presence of PCN-IgY antibody was detected through the presence of precipitates formed on agarose gel.



**Figure 2.** The effects of pyocyanin (PCN) on Raji cell survivability. After incubation with various concentration of PCN, Raji cell viabilities were assessed by MTT assay. PCN-treated Raji cell survivability was calculated against the control cells. \* $p < 0.05$ .



**Figure 3.** The effects of IgY specific antibodies on pyocyanin (PCN)-treated Raji cell survivability. PCN was incubated with various concentration of IgY antibodies. Raji cells were then incubated with the PCN-IgY mixtures. Viable cells were assessed by MTT and their percentage was calculated as in **Figure 2**. \* $p < 0.05$ .

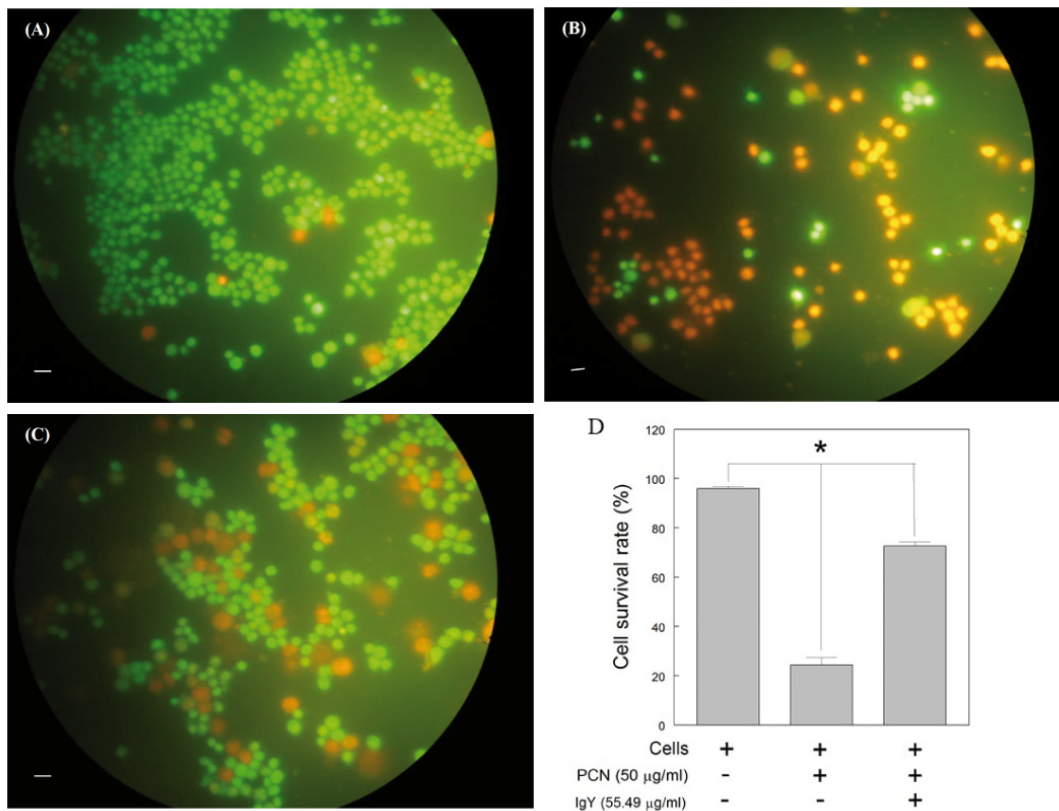
observed, however ( $p > 0.05$ ) (Figure 3). Microscopically, the number of viable cells treated with PCN-IgY complexes was much higher than those treated with PCN only (Figure 4). The results showed that anti-PCN IgY did increase the survivability of PCN-exposed cells from 24.3% up to 72.6% at concentration at 55.49  $\mu\text{g/ml}$  (Figure 4). Statistically, significant differences existed between cell only and PCN-treated or PCN-IgY-treated cells. The survivability of cells treated by PCN was significantly lower than that treated with IgY prior to PCN stimulation ( $p < 0.05$ ). Raw cell viability counts, along with other raw results and images, are available as *Underlying data*<sup>23</sup>.

## Discussion

The results of the present study showed that PCN does induce cell death in Raji cells as also seen in our previous report<sup>21</sup>. Similarly, other also demonstrated that PCN plays an important role in *P. aeruginosa* pulmonary infection through the induction of neutrophil cell death, which involves the release of reactive oxygen species and the activation of mitochondrial acid sphingomyelinase<sup>16</sup>. Therefore, efforts to inhibit excessive host cell damage induced by PCN are imminent.

Further results of the present study demonstrated that anti-PCN IgY antibodies specific to PCN significantly reduce the ability

of this pathogen to induce Raji cell death in a dose-dependent fashion. Whilst no previous studies showing prevention of PCN-induced cell death by antigen-specific IgY have yet been reported to our knowledge, the present results are supported by the fact that antigen-specific IgY antibodies did prevent *P. aeruginosa* infection in humans by both active and passive immunization<sup>17-19</sup>, suggesting that *P. aeruginosa*-specific IgY antibodies may inhibit cellular inflammatory responses induced by this pathogen. Pyocyanin is secreted by *P. aeruginosa* when the nutrition for the bacterium is limited. As important virulent factor, PCN plays a key role in bacterial infection because its ability to cross cell membrane which then causing disturbance in cellular electron transport and innate immune response<sup>24</sup>. Since PCN functions to kill host cells<sup>25</sup> it is possible to inhibit cellular and tissue damage by blocking PCN using the anti PCN-antibody IgY. Antigen-specific IgY antibodies also stimulated *P. aeruginosa* aggregation and increased human neutrophil phagocytic activities<sup>20</sup>. The exact mechanism by which antigen-specific IgY antibodies inhibited PCN-induced Raji cell death seen in the present study remains unclear, however. Zhao *et al.* demonstrated the activation of caspase-3 in hepatoma HepG2 cell death mechanism induced by PCN<sup>26</sup>. Other has shown that PCN stimulates NK cell death via increased intracellular calcium levels and mitochondrial disruption<sup>27</sup>,



**Figure 4. Microscopic features of Raji cells treated with pyocyanin (PCN) or PCN-IgY antibody complexes.** Raji cells were incubated without PCN (A) and with PCN (B) or the mixture of PCN-IgY antibodies (C) and then stained with acridine orange/ethidium bromide. Green or orange fluorescence stained cells are viable and dead cells, respectively. The cell survivability was increased on the cells treated with IgY antibody prior to PCN exposure (D).

suggesting that cell death induced by PCN may utilize different apoptotic signal pathways, perhaps, in a cell type-dependent fashion. Our previous study indicated that PCN induced Raji cell death via a caspase 3-activation pathway<sup>21</sup>. It seems plausible, therefore, that PCN-IgY antibody complexes may fail to activate Raji cell-derived caspase 3 and hence, inhibit cell death. However, more studies are required to delineate this speculation.

The extrapolation of the results of the present study in the therapy of *P. aeruginosa* infection remains to be further investigated. *P. aeruginosa* with its multiple mechanisms for adaptation and survival is well known as one of the main pathogens that lead to increase nosocomial infections<sup>1-4</sup> and morbidity and mortality in cystic fibrosis patients<sup>5</sup>. The difficulty to eradicate *P. aeruginosa* infection has been even more complicated by the presence of MDR *P. aeruginosa*, and hence alternative supplemental treatment approaches have been put forward based upon its bacterial virulent factors, such as exotoxin, lipopolysaccharide, and flagellin<sup>28</sup>. Immunotherapies using IgY specific to *P. aeruginosa* to delay initial infection and reduce both frequency of infection and development of chronic infection seem to be promising. Both passive and active immunization with antigen-specific IgY antibodies in humans resulted in inhibition of *P. aeruginosa* infection. For example, oral immunization with specific IgY antibodies in patients with cystic fibrosis led to the inhibition of *P. aeruginosa* colonization<sup>18,19</sup>, suggesting the usefulness of antigen-specific IgY antibodies an immunotherapy to prevent *P. aeruginosa* infection in patients with cystic fibrosis. Therefore, PCN-specific IgY antibodies used as an immunotherapy alongside with the common antibiotic treatments for *P. aeruginosa* infection are highly possible.

In conclusion, the present study showed that eggs from PCN-immunized chickens contain substantial amount of IgY antibodies that recognize PCN. Furthermore, IgY antibodies derived from PCN-immunized chicken were able to inhibit PCN-induced Raji cell death, suggesting that PCN-specific IgY

antibodies may be protective against PCN-induced Raji cell death. Future studies need to clarify the mechanisms involved in inhibiting the death of Raji cells induced by PCN. Furthermore, it is also necessary to study the effect of IgY antibodies derived from PCN-immunized chicken on the in vivo model of *P. aeruginosa* infection.

### Data availability

Figshare: Cytotoxicity of PCN.xlsx. <https://doi.org/10.6084/m9.figshare.8115701.v6><sup>23</sup>.

This project contains the following underlying data:

- Cytotoxicity of PCN.xlsx (raw cell viability data following treatment with pyocyanin)
- The effect of IgY on cell viability.xlsx (raw cell viability data following treatment with pyocyanin and IgY)
- Fig 4A untreated cells.JPG (raw image used for Figure 4A)
- Fig 4B PCN-treated cells.JPG (raw image used for Figure 4B)
- Fig 4C PCN IgY-treated cells.JPG (raw image used for Figure 4C)
- Fig 4D Cell survivability (raw image used for Figure 4D)
- Acridine orange-ethidium bromide results (raw cell viability data)
- IMG-AGPT.jpg (raw image of agar gel precipitation test)
- Elisa results Sept 1.xls (raw ELISA data)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license \(CC-BY 4.0\)](https://creativecommons.org/licenses/by/4.0/).

### Acknowledgements

We also express our gratitude to Ericko Tedy Hananto, who has helped with full responsibility during antibody preparation.

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# Open Peer Review

Current Peer Review Status:  

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## Version 2

Reviewer Report 20 December 2019

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**Boy M. Bachtiar** 

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**Competing Interests:** No competing interests were disclosed.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Author Response 13 Jan 2020

**Heni Susilowati**, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Indonesia

Dear Dr. Boy M Bachtiar,

We express our high appreciation for the review, critical analysis, and suggestions given for the improvement of our manuscript. We hope this research report will be useful for the development of science in the health field.

**Competing Interests:** We declare that in the past and at present we have never had a conflict of interest with anyone regarding the process in our manuscript peer review.

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## Version 1

Reviewer Report 27 August 2019

<https://doi.org/10.5256/f1000research.21186.r52110>



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**Priya Madhavan** 

School of Medicine, Faculty of Health and Medical Sciences, Taylor's University, Subang Jaya, Malaysia

Overall, the study design is of an acceptable quality. Immunotherapy using IgY specific to *P. aeruginosa* looks promising. Minor English editing and grammar check are required.

- Magnification/Scale on Figure 4 is missing. Best to include this.
- Why isn't there a positive control? I can only see results with negative control. Please provide a justification for not including a positive control.
- Best to also include other articles from year 2018-2019.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Immunogenetics, Antimicrobial resistance, Molecular Microbiology.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 16 July 2019

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**Boy M. Bachtiar**

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### General comments:

In this manuscript, the authors provide data account of an IgY-antibody that was purified from eggs of hens immunized with PCN secreted by *P. aeruginosa*. The specificity of PCN-IgY binding was determined by using AGPT, and it was further examined in vitro experiment to mimic the naturally occurring of bacterium-host cell interaction. In my opinion, it is necessary to explain (in the discussion session), when the toxin (PCN) is secreted and injected into host cells. For example, after cell contact (adherence phase).

### Specific comments:

#### Methods:

1. In the experiment, PCN was added into the cultured cell in the presence or absence of anti-PCN IgY (acted as control). This means, in the absence of the IgY, the toxin can enter the host cell (might be in adherence phase) due to the presence of a receptor, then induced the intracellular pathway leading to human B cell (Raji Cells) death (21). If this is the case, please explain it in the discussion session.

#### Results:

1. In order to exclude chicken serum as the other IgY source, please change “the immunized chickens” to “egg of immunized chickens”.
2. In this study, they used a microscope (fluorescence microscope?) to compare the Raji cells number between groups tested, qualitatively, thus the obtained results was not quantitative.

#### Discussion:

1. “anti-PCN IgY antibodies specific .....to this virulence”. I suggest changing this virulence with this pathogen.
2. “the present results are supported by the fact that antigen-specific IgY antibodies did prevent *P. aeruginosa* infection....., suggesting that *P. aeruginosa*-specific IgY antibodies may inhibit cellular inflammatory responses induced by this pathogen”. Since the IgY used in this study was specifically bind to the secreted PCN, not to bacterium’s whole cell, the rationale behind using this antibody is need to be explored.
3. According to the reference (16), the toxin is not having a role in the bacterial invasion into the host cell, but it involves in the mechanisms by which the bacterium kills the host cell tested (neutrophils).
4. “Furthermore, antigen-specific IgY antibodies were....., suggesting that PCN-specific IgY antibodies may be protective against PCN-induced Raji cell death”. Not clear, what (cell surface)

antigen/s that the authors assumed to bind specifically to the IgY, in addition to the secreted toxin (PCN).

5. Based on this study, please suggest, what should be done in future studies.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Oral microbiology, host agent interaction

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

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