

## ***In Vitro* Evaluation of the Effect of Storage Time on Immunogenicity of the 10-Valent Pneumococcal Conjugate Vaccine (PCV-10) Using Baby Rabbit Complement & HL-60 Cells**

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*Submitted 2<sup>nd</sup> May 2023; Accepted 07<sup>th</sup> March 2024 and; Published 07<sup>th</sup> March 2024*

### **ABSTRACT**

Pneumococcal diseases, caused by *Streptococcus pneumoniae*, pose significant health risks for children, leading to infections such as meningitis, pneumonia, and *otitis media*. Despite the introduction of the 10-valent pneumococcal conjugate vaccine (PCV-10) in Kenya since 2011, the mortality rate among children under five remains high. This study investigates the impact of storage time on the immunogenicity of PCV-10. The study aimed to assess whether different storage durations affect the antibody functionality and immunogenicity of the PCV-10 vaccine in Kenya. An experimental design using White New Zealand rabbits was employed. Three cohorts received the PCV-10 vaccine at intervals of 0, 4, and 8 weeks, while a control group received a placebo. Blood samples were analyzed using the Pneumococcal-Specific IgG-Enzyme Linked Immunosorbent Assay and an opsonophagocytic activity assay. Initial IgG antibody titers ranged from 0.23-0.27 µg/ml across cohorts. Following vaccination, there was a consistent rise in titers, with significant increases observed after Dose II and Dose III, surpassing WHO's recommended sero-protective levels (0.3-0.50 µg/ml). Additionally, opsonophagocytic activity increased with escalating vaccine doses. Cohort 1, stored for 4 weeks, exhibited the highest efficacy, killing 35.1% of bacteria after three doses. The study underscores the robust immunogenicity of PCV-10, particularly after the second and third doses. However, decreased efficacy with prolonged storage underscores the importance of stringent storage protocols. Recommendations include prioritizing doses II and III, establishing policies for careful storage practices not exceeding 8 weeks, and continuous monitoring to optimize vaccine effectiveness.

**Keywords:** *IgG Antibodies, Opsonophagocytic Activity, Pneumococcal Vaccine*

## INTRODUCTION

Pneumococcal diseases caused by the encapsulated bacterium; *Streptococcal pneumoniae*, vary in severity and are the major cause of illness during the childhood period (Center for Disease Control and Prevention, 2022). They include meningitis, otitis media, pneumonia, bronchitis and sinusitis. Such infections are treated using antibiotics and can also be prevented through intramuscular vaccination in infants and children between 6 weeks and 5 years of age. There are two primary types of pneumococcal vaccines: pneumococcal conjugate vaccines (PCVs) and pneumococcal polysaccharide vaccines (PPVs). Pneumococcal conjugate vaccines include *PCV-13 (Pneumovax 13)*, *PCV-10 (Synflorix)* while pneumococcal polysaccharide vaccine includes *PPSV-23 [Pneumovax 23]* (Tereziu & Minter, 2023). Both types are generally stored in a refrigerated environment at temperatures ranging from 2°C to 8°C (36°F to 46°F) and should be protected from freezing (Centers for Disease Control and Prevention, 2023).

In Kenya, the 10-valent pneumococcal conjugate vaccine (PCV-10) has been integrated to be part of the National Expanded Program on Immunization (KEPI) since 2011 to mitigate the high mortality and incidence rates of pneumococcal diseases being witnessed among children less than five years (Hammit *et al.*, 2014). However, 8 years down the line, prevention of pneumococcal infections has not been fully achieved; remaining the uncontested primary killer of children under five years (O'Brien *et al.*, 2009). Additionally, the rate of antimicrobial resistance exhibited by *S. pneumoniae* has been increasing in recent past making treatment a challenge (Cillóniz *et al.*, 2018). Since 2011, the Global Alliance of Vaccines & Immunization (GAVI) has been donating 10v-PCV vaccine to Kenya as part of the sponsorship between the two countries (Ojal *et al.*, 2019). Kenya being a developing country and with the sponsor due for exit, much concerns are raised whether GoK should continue injecting colossal financial input into use of 10v-PCV vaccine yet the mortality rates are still high.

Though the 10v-PCV vaccine has been shown to be very effective, current incidences and mortality rates of pneumococcus in Kenya raises questions on their efficacy (Githii *et al.*, 2013). From an immunological point of view, efficacy of a vaccine is influenced by several factors including host intrinsic, perinatal, nutritional, environmental, administration, extrinsic, vaccine and behavioural factors (Zimmermann & Curtis, 2019). Effective immunogenicity of the 10v-PCV vaccine relies on the activity of adjuvants (Lockhart, 2003). Adjuvants such as aluminum salt are usually added to vaccines to boost their immune responses (Blin, 2019). Storage of the vaccine for long periods of time has been associated with settling down of the adjuvant molecules and consequent failure of the molecules to re-disperse (Zhang *et al.*, 2012). Evidently, since this vaccine is not locally manufactured, storage time during transit and the cold chain process might be the reasons behind its perceived ineffectiveness. Additionally, dysfunctional antibodies not capable of opsonizing Pneumococci antigens and facilitating activation of the complement proteins also may contribute to the vaccine's poor performance in Kenya.

PCV-10 (Synflorix) is manufactured by GlaxoSmithKline (GSK) in Brentford, United Kingdom. It is a turbid white suspension given only as an intramuscular injection. It contains polysaccharides derived from ten *Streptococcal pneumoniae* serotypes: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F and utilize either protein D, tetanus toxoid (18C) or diphtheria (19F) toxoid as carrier protein. Additionally, each 0.5ml dose contains 0.5mg of aluminum phosphate as an adjuvant. Aluminum salts such aluminum phosphate are commonly and widely employed as adjuvants in vaccine

development (Mei et al., 2019). It takes at least 8 weeks for a product to be dispatched and cleared by the Kenya port authorities from the UK. During this time the vaccine adjuvant molecules would have settled and the degree of immunogenicity significantly deteriorated. This study aimed to assess whether different storage durations and antibody functionality affects the immunogenicity of the 10v-PCV vaccine used in Kenya.

## METHODOLOGY

### Study Design and Sample Population

This study employed an experimental research design as outlined by Fehrenbacher (2012). A total of nine rabbits (*White New Zealand*) of the same sex was procured and split into three cohorts of three rabbits each. For each cohort, there were two experimental rabbits and one control rabbit.

### Acquisition, Care and Maintenance of White New Zealand Rabbits

White New Zealand rabbits were obtained from the Institute of Primate Research (IPR) and maintained in accordance with the guidelines stipulated for use of animals in research (Chave, 2003). A comprehensive record, including the procurement details, species, gender, feeding records, and all medical/diagnostic procedures, was also maintained for all the nine rabbits procured. The experimental group for each cohort consisted of two White New Zealand Rabbits, each receiving a complete PCV-10 vaccine dose. The control group for each cohort consisted of a single White New Zealand Rabbit which was given a placebo. Both experimental and control group were housed in separate cages but kept in distinct laboratories, partitioned by well-established walls within the Biomedical Science Laboratories, Kabarak University.

### Administration of PCV-10 (Synflorix, GSK) Vaccine

PCV-10 vaccine vials with the same production date were obtained from the Unit of Vaccines & Immunization Services (UVIS) and stored at temperatures ranging from 4°C to 8°C. The production of T-cell independent Pneumococci-specific IgG was induced by immunizing the White New Zealand Rabbits with PCV-10 {(Synflorix), Diphtheria CRM 197, GSK}. A complete dose of PCV-10 vaccination requires three doses administered at weeks 6, 10 and 14 of an infant's life. In this study, this was replicated as follows:

*Cohort I:* Each of the two experimental rabbits in cohort I received an initial 100 µL (0.34g) dose of PCV-10 vaccine solution on day 0, the same day the vaccines were obtained and brought into the Biomedical Science Laboratories at Kabarak University. Subsequent doses were administered at 4-week intervals, with the second dose given at week 4 and the third at week 8 from day 0. The control rabbit in this cohort received Phosphate Buffered Saline (PBS) for each corresponding vaccine dose to serve as a control.

*Cohort II:* Each of the two experimental rabbits in cohort II received the initial 100 µL (0.34g) dose of PCV-10 vaccine solution at the 4<sup>th</sup> week, aligning with the timing of the second dose for the rabbits in cohort I. The second dose was administered at week 8, corresponding to when the cohort I rabbits received their third dose, and the third dose was given at week 12 from day 0. The control rabbit in this cohort received Phosphate Buffered Saline (PBS) for each corresponding vaccine dose to serve as a control.

*Cohort III:* Each of the two experimental rabbits in cohort III received the initial 100  $\mu$ L (0.34g) dose of PCV-10 vaccine solution at the 8<sup>th</sup> week, corresponding to when the cohort I rabbits received their third dose. Subsequent doses were administered at 4-week intervals, with the second dose given at week 12 and the third at week 16 from day 0. The control rabbit in this cohort received Phosphate Buffered Saline (PBS) for each corresponding vaccine dose to serve as a control.

### **Collection of Blood for Laboratory Analysis**

A quantity of 2.5 mL of whole blood was obtained from each mouse in both experimental and control groups before and after injections with PCV-10 and PBS, respectively. The volume of blood to be collected was determined based on the total body weight of the White New Zealand Rabbits, following the calculation recommended by Bourin et al. (2007), typically at 10% per kilogram of body weight. The whole blood was then gently centrifuged to collect serum, which was used subsequently for evaluating blood antibody levels.

### **Sero-Survey for Pneumococci Specific IgG Titers**

The Pneumococci Specific IgG-Enzyme Linked Immunosorbent Assay (Pneumococci Specific IgG ELISA) was used following the protocol by Nahm & Goldblatt (2002). This assay assessed pneumococci-specific IgG antibody levels for PCV-10 serotypes: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F. Evaluations were done before PCV-10 and PBS interventions, and subsequently at four-week intervals post-interventions. A serum antibody level of 0.35  $\mu$ g/ml at 450nm for each PCV-10 serotype indicated protective immunity.

### **Immunoglobulin G Antibody Opsonophagocytic Activity Assay**

The functionality of Pneumococci Serotype IgG antibodies induced after administering PCV-10 in White New Zealand Rabbits was evaluated through opsonophagocytic activity assay. Functionality refers to the antibodies' ability to opsonize Pneumococci antigens, facilitating their elimination by phagocytes and activating complement system proteins. The test followed the protocol outlined by Martinez et al. (1999).

### **Ethical Considerations**

The study obtained ethics review from both the Kabarak University Research Ethics Committee (KUREC) and the permit from National Commission for Science, Technology and Innovation (NACOSTI) - NACOSTI/P/17/65428/15801. The research strictly adhered to ethical principles, prioritizing the welfare and rights of the animals involved in the experiment. Measures were implemented to minimize discomfort and distress to the baby rabbits used in the study, with strict protocols ensuring their humane treatment and proper handling throughout the experiment. All procedures were conducted in strict accordance with established ethical guidelines and regulations to uphold the integrity and validity of the research findings.

## Data Analysis

The collected data was entered, cleaned, and analyzed using SPSS version 22. Descriptive statistics were employed to profile Pneumococci IgG titers and antibody functionality capacities. The analysis focused on summarizing the data using measures such as means, standard deviations, and frequency distributions. This approach provided a clear understanding of the central tendency and variability of the variables under study. Results were presented in the form of tables and graphs to facilitate easy interpretation.

## RESULTS

### Pneumococcal IgG Antibodies Titer Induced by 10v-PCV Vaccine

Table 1 present the IgG antibody titers (in  $\mu\text{g/ml}$ ) for three cohorts (EXP-I, EXP-II, CTRL-III) across different doses of the PCV-10 vaccine. At baseline, all cohorts show relatively similar IgG antibody titers, ranging from 0.23 to 0.27  $\mu\text{g/ml}$ . Following the first dose, there is a modest increase in titers across all cohorts. Notably, Dose II sees a more substantial elevation, and by Dose III, the titers continue to rise. The CTRL-III cohort, which received a placebo, maintains consistent baseline-level titers throughout. Comparing the results to the recommended seroprotective titers (0.3-0.50  $\mu\text{g/ml}$ ) set by World Health Organization in 2010, all cohorts generally fall within or exceed this range after Dose II and Dose III.

**Table 1:**  
*IgG Ab Titres (ug/ML) for Cohorts 1, 2 & 3*

PCV-10 (0.5ml)	COHORT 1 (GMC- $\mu\text{g/ml}$ )			COHORT 2 (GMC- $\mu\text{g/ml}$ )			COHORT 3 (GMC- $\mu\text{g/ml}$ )		
	EXP-I	EXP-II	CTRL-III	EXP-I	EXP-II	CTRL-III	EXP-I	EXP-II	CTRL-III
Baseline	0.25	0.23	0.26	0.23	0.27	0.25	0.24	0.24	0.26
Dose I	0.29	0.31	0.25	0.28	0.31	0.27	0.28	0.28	0.26
Dose II	0.34	0.34	0.26	0.34	0.31	0.25	0.33	0.32	0.26
Dose III	0.41	0.39	0.25	0.37	0.35	0.25	0.35	0.36	0.26

*Recommended IgG Ab sero-protective titres [0.3-0.50  $\mu\text{g/ml}$ ] (WHO, 2010)*

### Opsonophagocytic Activity of Pneumococcal IgG Antibodies Induced by the 10v-PCV Vaccine

Table 2 below shows the opsonophagocytic assay of activity of IgG antibodies obtained from rabbits that were injected with 10v-PCV vaccines of different storage durations. In general, out of the 97 pneumococci bacteria assayed in each cohort, there was a general increase in number of the bacteria killed with increase in dose administered from 1 to three. Conversely, as the storage duration increased from 4 weeks (cohort 1) to 12 weeks (cohort 3), it was noted that the number of s pneumoniae killed decreases at each dose except for the second dose given to subjects in cohort 2.

**Table 2:**  
*Pneumococci IgG Opsonophagocytic Activity Assay Evaluation Using HL-60 Cells & BRC*

Experiment	Reaction incubation conditions	No. of Pneumococci assayed	No. of <i>Streptococcal pneumoniae</i> killed at completion of each Expt. Dose (n)			
			Baseline	After Dose 1 of PCV-10	After Dose 2 of PCV -10	After Dose 3 of PCV 10
Cohort – 1 (PCV-10 storage time – 4 weeks)	BRC + HL-60 cells, 37°C, 5%CO <sub>2</sub> , 30 minutes	97	8	26 (26.8%)	28 (28.9%)	34 (35.1%)
Cohort– 2 (PCV-10 storage time – 8 weeks)	BRC + HL-60 cells, 37°C, 5%CO <sub>2</sub> , 30 minutes	97	5	26 (26.8%)	29 (29.9%)	30 (30.9%)
Cohort – 3 (PCV-10 storage time – 12 weeks)	BRC + HL-60 cells, 37°C, 5%CO <sub>2</sub> , 30 minutes	97	7	25 (25.8%)	27 (27.8%)	27 (27.8%)

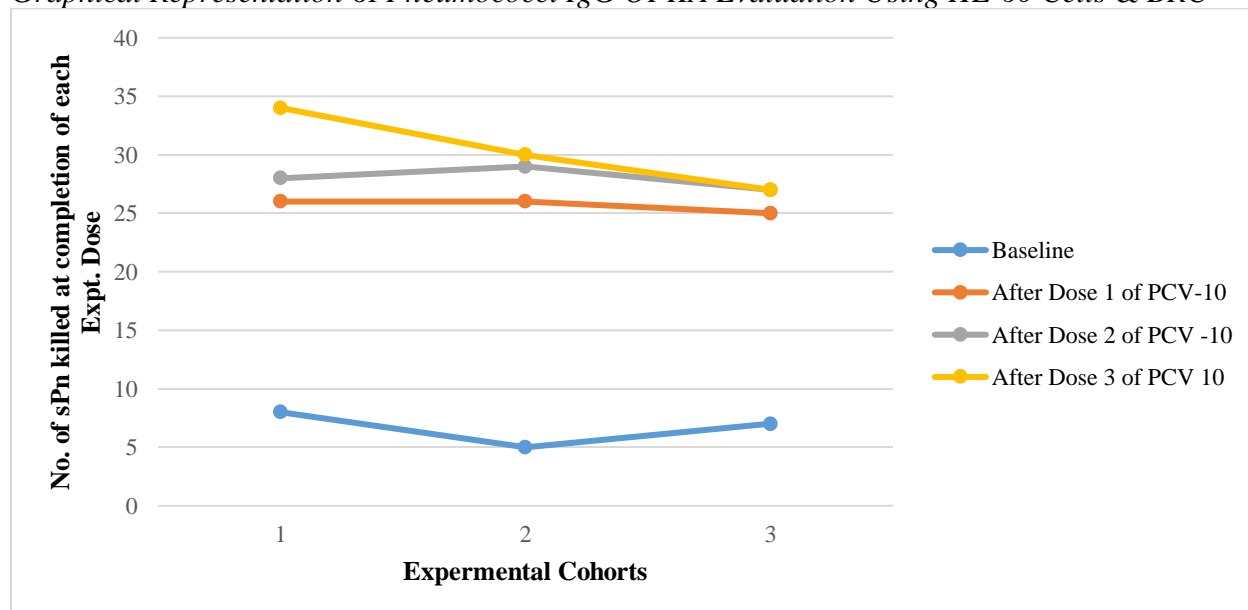
*BRC*=baby rabbit complement; *PCV- 10* =10-valent *Pneumococcal conjugate vaccine*; *Exp* =experiment; *CO<sub>2</sub>* =carbon IV oxide; The No. of pneumococci assayed represents an average for two experimental animals in each cohort

### Dose-Dependent Dynamics in Pneumococci Killing

Figure 1 below is a graphical representation of the number of pneumococci killed in each cohort and after every dose. The number of bacteria killed increased with subsequent dose administration. Additionally, the number of bacteria in cohort 1 that were killed at each subsequent dose is higher compared to those in cohort 3. Notably, higher number of bacteria were killed at the second dose in cohort 2 compared to first dose.

**Figure 1:**

*Graphical Representation of Pneumococci IgG OPKA Evaluation Using HL-60 Cells & BRC*





## DISCUSSION

The storage duration and functionality of antibodies are some of the key factors that directly influence effectiveness and efficacy of vaccines (Pollard & Bijker, 2020). Vaccine formulation consist of several ingredients that have to be kept in specific conditions to optimize the delivery of active compounds (D'Amico et al., 2021). Suspension is a key component in vaccine formulation as it ensures the active component is equally distributed in the formulation (Taraban et al., 2019). However, when left undisturbed in conditions such as long storage duration, suspended particles tend to settle at the bottom of vials and harden, forming a cake. Shaking of such vials prior to administration may not re-disperse the active compound equally and as a result, a patient may only get adjuvants or other excipients (Taraban et al., 2019). This results in ineffective vaccination. Kenya is yet to start producing her own pneumococcal vaccines, and the ones being used are usually imported from other countries. Transportation of such vaccines over long distances and then their storage at depots enables constituent sedimentation. This study looked at effect of storage time on the immunogenicity of 10v-PCV vaccine by assessing the opsonophagocytic activity of antibodies generated.

### **Pneumococcal IgG Antibodies Titer Induced by 10v-PCV Vaccine**

The study investigated Pneumococcal IgG antibody titers induced by the PCV-10 vaccine across three cohorts (EXP-I, EXP-II, CTRL-III). At baseline, all cohorts exhibit similar IgG titers (0.23-0.27  $\mu\text{g/ml}$ ). Following the first dose, a modest increase is evident, suggesting an immediate response to vaccination. Notably, Dose II prompts a significant elevation, and this trend persists through Dose III, signifying a cumulative and sustained immune response. This parallels study done by Van Westen et al. (2018) who found that IgG antibody concentrations were high after the third dose and had reached the WHO set seroprotective range of 0.35  $\mu\text{g/mL}$ . Similarly, Udah et al. (2023) while assessing antibody response to PCV-10 among Nigeria children under 5 years found that the mean antibody concentration was within seroprotective range and concluded that the PCV-10 that was currently used in Nigeria was sufficiently antigenic.

The control rabbits which were administered with a placebo, maintained baseline titers, confirming that the observed changes are vaccine-induced. Importantly, all cohorts surpass the WHO's seroprotective threshold post Dose II and Dose III, underscoring PCV-10's efficacy in eliciting a robust and persistent IgG antibody response. These findings suggest PCV-10 induces a robust and sustained IgG antibody response, crucial for protective immunity against pneumococcal infections. The robust and sustained IgG antibody response induced by the PCV-10 vaccine, as evidenced by the escalating titers post Dose II and Dose III, suggests the vaccine's efficacy in establishing protective immunity against pneumococcal infections. This sustained response is crucial for long-term immunity, potentially reducing the burden of pneumococcal-related diseases. The confirmation that changes in antibody titers are vaccine-induced, demonstrated by the placebo group maintaining baseline levels, reinforces the specificity of the vaccine's impact.

## **Opsonophagocytic Activity of Pneumococcal IgG Antibodies Induced by the 10v-PCV Vaccine**

The study assesses the opsonophagocytic activity of IgG antibodies induced by the 10v-PCV vaccine, considering different storage durations. In cohort 1 (4 weeks), a consistent increase in killed pneumococci accompanies each administered dose, indicating a positive correlation between vaccine dosage and opsonophagocytic efficacy. Cohort 2 (8 weeks) mirrors this trend. Cohort 3 (12 weeks) displays a reduction in killed bacteria, deviating from the expected pattern and emphasizing the potential influence of prolonged storage on vaccine potency. These findings underscore a nuanced relationship between storage time, vaccine doses, and opsonophagocytic activity. The positive correlation between vaccine dosage and opsonophagocytic efficacy in cohorts 1 and 2 underscores the importance of proper dosage in enhancing immune response. However, the reduction in killed bacteria in cohort 3, with prolonged storage, highlights a potential vulnerability in vaccine potency over time. This emphasizes the need for meticulous protocol optimization, considering both storage conditions and dosage strategies, to ensure consistent and effective immune responses.

Generally, these findings underscore the importance of continuous monitoring and optimization of vaccine administration protocols. Insights into the dynamics of immune responses and the impact of storage conditions can inform vaccination strategies, contributing to the development of more effective and durable vaccines. This knowledge is crucial for public health initiatives, guiding policymakers and healthcare professionals in optimizing vaccine delivery, ensuring the sustained protection of populations against pneumococcal infections.

### **Conclusion (s)**

In conclusion:

- IgG antibody titers in three cohorts (EXP-I, EXP-II, CTRL-III) increased progressively with PCV-10 doses, surpassing or meeting WHO-recommended sero-protective titers after Dose II and Dose III.
- CTRL-III (placebo) maintained consistent baseline titers, emphasizing the vaccine's impact on antibody response.
- Opsonophagocytic activity against pneumococci increased with higher vaccine doses, indicating a dose-dependent response.
- Extended storage duration (12 weeks) reduced killing efficiency, except for the second dose in cohort 2, suggesting a nuanced impact of storage on vaccine efficacy.

### **Recommendation (s)**

Based on the results and conclusions, this study recommends the following:

- In terms of optimizing vaccination schedule, doses II and III of PCV-10 should be prioritized to ensure robust antibody responses that meet WHO recommended titers.
- Vigilant vaccine storage practices and policies need to be implemented to avoid extended vaccine storage beyond 8 weeks to ensure efficacy is maintained when administered.
- Continuous monitoring systems should be established to adapt vaccination strategies to the emerging data.



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