Comparative Analysis of Haemolysin Production and Complement Levels in Indigenous Rabbits (Oryclolagus cunicali) and Other Animal Species

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Abstract
The complement system is an essential part of the immune response, defending against infections and contributing to inflammation. This study focuses on haemolysin production and complement levels in local rabbits, using subcutaneous and intravenous inoculation. It also evaluated complement levels in various animal species and healthy humans for comparison. Serum samples were collected, and the complement fixation test was performed using sheep red blood cells as markers. The findings revealed changes in red blood cell lysis with different haemolysin dilutions over time. The study also provides information on complement minimum haemolytic dose concentrations in various animal species. A graph illustrates the antibody levels in response to different inoculation routes. This research enhances our understanding of haemolysin and complement levels, emphasizing the need for standardized protocols and larger population studies to explore clinical implications to benefit immunological studies.

Introduction
The complement system plays a crucial role in host defense against infectious agents and the inflammatory process, contributing to the overall immune response. It is comprised of a complex network of plasma proteins and cell surface receptors that act as enzymatic components or binding proteins, respectively [1]. Genetic deficiencies of certain complement components have demonstrated the significance of the complement system in host defense. These deficiencies...
can lead to life-threatening recurrent bacterial infections or immune complex diseases [1,2]. For instance, deficiencies in components such as C3, C2, C1q, C1r, C1s, or C4 have been associated with increased susceptibility to bacterial infections or autoimmune disorders [2,3].

In addition to its role in host defense, the complement system is involved in inflammation and tissue injury, as evidenced by clinical investigations and experimental studies [2,4]. The complement system can be activated through three different pathways: the classical complement pathway, the alternative complement pathway, and the lectin pathway. Furthermore, complement is closely linked to the adaptive immune response, highlighting its importance in immune regulation and immune-mediated diseases [1,3]. One of the key components for the detection and quantification of complement activity is haemolysin, an antibody specific to sheep red blood cells (SRBC). The complement fixation test (CFT) relies on the detection of haemolysin to assess the presence of antibodies or immune complexes in serum samples [2]. However, the production of haemolysin and the levels of complement in local rabbits (Oryctolagus cunicali), an important animal model for immunological studies, have not been extensively characterized. Additionally, the levels of complement in sera from different animal species and apparently healthy humans need to be evaluated for comparative analysis and potential clinical implications.

Therefore, the present study aims to investigate the production of anti-sheep antibody (haemolysin) in local rabbits using subcutaneous and intravenous routes of inoculation. This investigation will provide valuable insights into the capacity of local rabbits to produce haemolysin, which is essential for the complement fixation test and other immune hemolytic reactions. Additionally, the study aims to determine the levels of haemolysin produced in local rabbits and evaluate its potential application in immunological studies. Furthermore, the evaluation of complement levels in sera from different animal species and apparently healthy humans need to be evaluated for comparative analysis and potential clinical implications.

Materials and Methods

Study Design

Serum Samples

A total of 90 samples were collected from cattle, sheep, rabbits, goats, dogs, humans, donkeys, camels, and chickens, with an equal split of 5 males and 5 females from each species. Sera were separated by centrifugation and stored at -20°C until tested.

Experimental Animals

Six-month-old local rabbits (Oryctolagus cunicali) were obtained from Maiduguri Monday market. The rabbits were fed a diet of green leaves and rabbit pellets (Pfizer plc) and housed in cages. Additionally, four guinea pigs weighing 0.5-1 kg (2 males and 2 females) were obtained from the same source and fed a diet of green leaves and mice pellets. The animals were dewormed and stabilized on a high-energy diet before the start of the experiment.

Sample Collection and Preparation of Antigen

Heterophile antigen was prepared using sheep blood. The blood was collected from healthy ewes and mixed with a cold (4°C) 3.8% sodium citrate solution. After centrifugation, the sedimented cells were washed and resuspended in cold acetate buffer. The sheep red blood cell (SRBC) stroma was then heated, and its protein content was determined. The antigen was adjusted to a concentration of 2 mg/ml and stored at -20°C until required.

Inoculation of Rabbits

The rabbits were randomly divided into three groups: Group 1 (B1, B2, and B3), Group 2 (A1), and Group 3 (A2, control). In Group 1, the three rabbits were intravenously inoculated with 1ml of SRBC antigen on specific days. Blood samples were collected from each rabbit on designated days, and the serum was separated, incubated, and stored at -20°C. In Group 2, the rabbits were subcutaneously inoculated with 2ml of the SRBC antigen, and serum samples were collected and stored as in Group 1. The sera were later incubated and stored at -20°C for the haemolysin assay.

Complement Fixation Test (CFT)

Sheep red blood cells (SRBC) served as markers to assess complement fixation. These cells were sensitized by haemolysin and then diluted to attain a concentration of 4 to 6 mean haemolytic doses (MHD). The haemolysin, which had undergone heat treatment, was combined with Veronal Buffer (VB) in various proportions to achieve the desired dilutions. To obtain a 1:10 dilution, one part of heat-treated haemolysin was mixed with nine parts of VB. Subsequently, for a 1:50 dilution, one part of the 1:10 dilution was combined with four parts of VB. Similarly, a 1:100 dilution was achieved by combining one part of the 1:10 dilution with nine parts of VB.

Further dilutions were prepared based on the 1:100 dilution. For a 1:200 dilution, one part of the 1:100 dilution was mixed with one part of VB. Similarly, a 1:400 dilution was obtained by combining one part of the 1:100 dilution with three parts of VB. To achieve a 1:800 dilution, one part of the 1:100 dilution was mixed with seven parts of VB. Finally, for a 1:1000 dilution, two parts of the 1:100 dilution were combined with eighteen parts of VB.
Determination of Complement Levels in Human and Animal Sera

Fresh guinea pig serum was used as the source of complement. The procedure for conducting the master dilution of complement levels in human and animal sera involved the preparation of eight tubes labeled with specific tube numbers. Starting with Tube No. 1, a 0.1 ml volume of a 1:40 complement was added, followed by the addition of increasing volumes of complement (0.2 ml, 0.3 ml, and so on) in subsequent tubes up to Tube No. 8.

To complete the dilution, Veronal Buffer (VB) was added to each tube. The specific volumes of VB added varied for each tube, ranging from 1.9 ml in Tube No. 1 to 1.4 ml in Tube No. 8. Thorough mixing was performed to ensure proper dilution of the complement and VB. It was then incubated at 37°C. The titration results were read after 30 minutes, and the dilution showing complete or almost complete haemolysis was considered to contain 1 unit of complement.

For the final titration of complement levels in human and different animal species, eight tubes were prepared, each containing specific components. In each tube, a complement master dilution of 0.2 was added, followed by 0.1 volume of Veronal Buffer and 0.1 volume of sensitized cells. The final volume of each tube was adjusted to obtain a 1:25 serum diluted complement. The specific volumes for each component were calculated to achieve the desired dilution. The resulting mixture in each tube was then used for further analysis.

Results

The observations from the table 1, Indicate that lysis rates generally decrease with higher dilutions of haemolysin, signifying a lower level of red blood cell lysis. Specifically, higher lysis rates are observed at lower dilutions, such as 1:10 and 1:50, particularly on day 14. Furthermore, over the course of the experiment from day 14 to day 22, the lysis rates tend to decrease, suggesting a potential decline in haemolysin activity. The control group, serving as a reference, exhibits no lysis as indicated by the 0% values in the “Control %” column. It is worth noting that the titre of haemolysin used in the experiment is specified as 1:25.

<table>
<thead>
<tr>
<th>Day 14(IV)%</th>
<th>Day 22 (IV)%</th>
<th>Day 14(sc) %</th>
<th>Day 22(se)%</th>
<th>Control %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10-100</td>
<td>1:10-75</td>
<td>1:10-75</td>
<td>1:10-25</td>
<td>1:10-0</td>
</tr>
<tr>
<td>1:50-95</td>
<td>1:50-75</td>
<td>1:50-50</td>
<td>1:50-25</td>
<td>1:50-0</td>
</tr>
<tr>
<td>1:100-75</td>
<td>1:100-50</td>
<td>1:100-50</td>
<td>1:100-0</td>
<td>1:100-0</td>
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<td>1:200-50</td>
<td>1:200-25</td>
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<tr>
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<td>1:800-25</td>
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<td>1:800-0</td>
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<td>1:1000-0</td>
<td>1:1000-0</td>
<td>1:1000-0</td>
</tr>
</tbody>
</table>

Note: The titre of Haemolysin used in the experiment was 1:25

Figure 1 Graphical Estimation of 50% Haemolytic Antibody Level in (I/V) and (SC) Inoculation, presents a line graph depicting the changes in the 50% haemolytic antibody level over the course of days of inoculation. The graph demonstrates the trend of the haemolytic antibody level over time, where there is a gradual increase in the antibody level, followed by a peak around day 16 where the level reaches approximately 600. Subsequently, there is a decline, leading to lower antibody levels.

The two lines labeled "N.B = (I/V)" and "(SC)" represent the haemolytic antibody levels for intravenous (I/V) and subcutaneous (SC) inoculation, respectively.

Figure 1: Graphical Representation of 50% Haemolytic Antibody Level in Intravenous and Subcutaneous Inoculation Over Time
Table 2 illustrates the concentrations of complement minimum haemolytic doses for various animal species, including humans, categorized by gender. For male and female donkeys, the complement minimum haemolytic dose concentrations are 0.03 and 0.06, respectively. No specific concentration is provided for male rabbits, except for female rabbits where it is 0.06. Male cattle exhibit a complement minimum haemolytic dose concentration of 0.01, while female cattle have a concentration of 0.04. The concentration for male chickens is unspecified in this table, except for female chickens, where it is 0.06. There is no complement minimum haemolytic dose concentration specified for dogs. Female camels have a complement minimum haemolytic dose concentration of 0.04. For sheep, the concentration is 0.01 for males, whereas it is unspecified for females. The concentration for goats is not specified in this table. The concentration for humans is not specified, except for females, where it is 0.06.

Furthermore, both positive and negative controls were successfully executed at the optimal dilution.

**Table 2: Complement Minimum Haemolytic Dose Concentrations for Different Animal Species and Humans**

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Complement minimum haemolytic dose concentration</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Donkey</td>
<td>0.03</td>
</tr>
<tr>
<td>Rabbit</td>
<td>-</td>
</tr>
<tr>
<td>Cattle</td>
<td>0.01</td>
</tr>
<tr>
<td>Chicken</td>
<td>-</td>
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<tr>
<td>Dog</td>
<td>-</td>
</tr>
<tr>
<td>Camel</td>
<td>-</td>
</tr>
<tr>
<td>Sheep</td>
<td>0.01</td>
</tr>
<tr>
<td>Goat</td>
<td>-</td>
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<tr>
<td>Human</td>
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</table>

**Note:**

N.B

* __________________________ = NIL

* Both positive and negative control had worked at optimum dilution.

**Discussion**

The findings of this study revealed important insights regarding the lysis rates of red blood cells in response to different dilutions of haemolysin and over various days of inoculation. Consistent with previous studies [5], our findings demonstrate that lysis rates generally decrease with higher dilutions of haemolysin, indicating a lower level of red blood cell lysis. This suggests that higher dilutions of haemolysin result in reduced potency and effectiveness in inducing lysis.

Interestingly, our results show that lower dilutions, particularly 1:10 and 1:50, exhibit higher lysis rates, especially on day 14. This finding is in line with the work of Marianne et al., who reported that lower dilutions of haemolysin can more efficiently induce red blood cell lysis [6]. However, as the experiment progresses from day 14 to day 22, the lysis rate decreased, indicating a potential decline in haemolysin activity over time. This decline in activity could be attributed to factors such as degradation or clearance of the haemolysin molecules [7].

The control group in our study, serving as a reference, exhibited no lysis, as evidenced by the 0% values in the "Control %" column. The absence of lysis in the control group validates the reliability of our experimental setup and confirms that the observed lysis in other groups is attributed to the haemolysin.

Furthermore, the titre of haemolysin used in our experiment was specified as 1:25. This information is crucial for comparing our findings with other studies and understanding the potency of the haemolysin utilized. It is noteworthy that the haemolytic activity of haemolysin can vary depending on factors such as the source of haemolysin, preparation method, and experimental conditions.

Moving on, our study provides information on the complement minimum haemolytic dose concentrations for different animal species, including humans. Comparing our findings with other similar studies conducted, the complement minimum haemolytic dose concentrations for donkeys in our study align with the findings reported by Lilyan [8]. However, we did not come across similar studies conducted on cows, rabbits and chickens, indicating complement minimum haemolytic dose concentrations. This highlights the need for further research to explore the complement minimum haemolytic dose concentrations in dogs within the Nigerian context.

In terms of Graphical Estimation of 50% Haemolytic Antibody Level in Intravenous and Subcutaneous Inoculation Over Time, the graph provides a visual representation of the changes in the 50% haemolytic antibody level over the course of days of inoculation. The graph shows a gradual increase in the antibody level, reaching a peak around day 16 at approximately 600. This peak indicates the highest level of haemolytic antibody activity during the experiment. Subsequently, there is a decline in the antibody levels, suggesting a decrease in haemolytic activity. Liriye et al. reported a similar trend of increasing haemolytic antibody levels followed by a decline in a study malaria vaccine [9]. This consistency strengthens the reliability and reproducibility of our results.

These similarities in the trends observed across different studies, suggest that the dynamics of haemolytic antibody levels in response to inoculation...
may be consistent across different populations and geographical locations.

Conclusion
In conclusion, our study provides valuable insights into the lysis rates of red blood cells in response to different dilutions of haemolysin and the dynamics of haemolytic antibody levels over time. The observations align with previous studies conducted within and outside Nigeria, supporting the consistency of these findings across different populations.

Limitations
The sample size for some animal species was small, limiting the generalizability of the results. Additionally, further investigations involving larger sample sizes and a wider range of dilutions would enhance our understanding of the haemolytic activity of the tested haemolysin.

Variations in experimental protocols, such as the source of haemolysin and the specific inoculation methods used, may contribute to slight discrepancies in the exact antibody levels observed. Therefore, future studies should aim to standardize experimental protocols and replicate these findings in larger and more diverse populations to enhance the generalizability of the results.

Conflict of Interest
The authors declare no conflict of interest in the publication of this study.

References