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Optimization of the T-cell proliferation assay in fascioliasis using a non-radioactive method, the Alamar Blue Assay

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Abstract

T-cell proliferation studies are traditionally carried out with radioactive reagents or fluorescent reagents that require measurement with advanced technology instrumentation. We attempted to calibrate the optimal conditions suitable for the use of a non-radioactive assay for the measurement of a T-cell proliferation assay in bovine fascioliasis, but applicable to the study of other infectious diseases in our developing country setting. Crude antigen extract was prepared from 15 adult *Fasciola gigantica* flukes. Cellular responses were detected by the proliferation of peripheral blood mononuclear cells (PBMC) in response to stimulation by serial dilutions of the crude antigen extract. The results showed that the antigen dilution 1:1,600 gave the highest PBMC proliferative response (Stimulation Index, S.I = 1.10± 0.2). Percentage reduced Alamar Blue was 27.3-71.6%. This suggests that the cell-mediated immune response in bovine immunity to *Fasciola* infection may be reliably measured in our setting with the Alamar Blue Assay.

Keywords: T-cell proliferation, bovine, fascioliasis, alamar blue assay.

Introduction

Fasciola hepatica (temperate liver fluke) and *Fasciola gigantica* (tropical liver fluke) are digenetic trematodes of the Phylum Platyhelminthes and class Trematoda that parasitize several mammalian hosts, including cattle, sheep, goats, and humans. In temperate regions, *F. hepatica* is the most common cause of fascioliasis. In contrast to this, *F. gigantica* is the common cause of fascioliasis in Africa and Asia where it is recognized as a major source of loss of production in domestic ruminants [1-5].

The primary pathological changes associated with fascioliasis are extensive hepatic fibrosis due to trauma caused by migrating juveniles and inflammation and edema of the bile ducts [3]. The protective resolution of liver fluke (*F. hepatica* and *F. gigantica*) infection is a dynamic interplay between the host's effector responses and the parasite's defense and immunomodulatory

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ystems [6-9]. Both cellular and humoral immune responses are known to be elicited by infection with *Fasciola* species and it has been reported that protective mmunity can be elicited in cattle, goats and rats by nfection with irradiated metacercariae, somatic fluke extracts and excretory/secretory fluke antigens but there s little evidence of immunological resistance of sheep to challenge infection with *F. hepatica* when judged by worm burdens [6-9]. McCole *et al* (1999) found that lood lymphocytes from infected animals demonstrated transient, but marked elevation in responsiveness to *F. hepatica* antigen and resistance to *F. hepatica* nfection in rats involved both antibodies and lymphoid ells. The importance of T-cells in protective immunity against *F. hepatica* was indicated by studies performed on cattle, which demonstrated successful transfer ymphoid cells to a recipient monozygotic twin calf. This was shown to confer 80% resistance to *F. hepatica* infection. [10 and 11]. In a recent study [7], during the arly phase of *F. gigantica* infection in cattle, the redominant T-cell protective immune response was the Th 2 response, which was later polarized to the Th 0 esponse during the chronic phase of the infection. This uggests the involvement of cellular immune mechanisms in resistance to *Fasciola* infection.

The Alamar Blue Assay was developed as a non-adioactive lymphocyte proliferation assay that indirectly measures cell proliferation [15]. The dye is added in an xidized form (blue colour) and is reduced (red colour) ith cell proliferation. There is great interest in the development of a safe effective colorimetric method to measure cell growth. This assay system has been tried applications and also in parasite systems [15]. In Nigeria, the few studies in the immunology of fascioliasis have focused on the humoral immune response of uminants to *Fasciola* infections [12], so, little is known bout the role and importance of the cellular immune response in acquired resistance to this infection in cattle.

This study calibrated the optimal conditions for isolating ovine peripheral blood mononuclear cells (PBMC) and investigated the role of T cells in the immune response *F. gigantica* in infected cattle in a local abattoir.

Materials and methods

tudy-design

rom the throat of adult cattle being slaughtered in the abattoir, 5 ml of blood was collected into a labeled EDTA ottle, and properly shaken to prevent coagulation of the lood samples. Blood samples collected at the abattoir were taken to the laboratory for analysis.

Parasite collection

Adult *F. gigantica* flukes were obtained from infected bovine livers. The flukes were teased from the bile ducts after transverse slices were made across the entire ventral surface of the liver.

Antigen preparation

Antigen was prepared [1] by homogenizing 15 adult flukes in ice-cold phosphate buffered saline (PBS). This crude homogenate was centrifuged at 10,000 rpm for 20 minutes and dialyzed at 4°C overnight against phosphate buffered saline. It was then filtered and sterilized by passing it through 0.7 im Millipore filters. The fluke extract was stored in aliquots at –80°C.

Peripheral blood mononuclear cells (PBMC) isolation Lymphocytes were isolated using a protocol modified from [13] for the isolation of human PBMC on Ficoll hypaque, and washes were done at low speeds for 5 minutes. Cells were suspended in complete RPMI culture medium.

Lymphocyte proliferation assay

Cells were diluted to a concentration of 3 x 10^5cells/ml and proliferation assays were carried out in triplicate at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hours.

Antigen stimulation of lymphocyte

Serial dilutions (1:100-1:6,400) of the fluke extract and phytohaemaglutinin (PHA) were used to stimulate the lymphocytes. These were then incubated at 37°C in a humid atmosphere of 5% CO_2 in air for 24 hours. After a 24 hr period of incubation, 20il of Alamar Blue dye was added into each well to indirectly measure the proliferation of the cells; the cells were further incubated for another 24 hours. Absorbance was measured at 570 nm and 600 nm with an ELISA plate reader.

Lymphocyte proliferation was determined by calculating the percentage (%) reduced Alamar Blue using this formula:

$$\% \text{ Reduced Alamar Blue} = \frac{(Ó_{ox}\ ë_2)\ (Aë_1) - (Ó_{ox}\ ë_1)\ (Aë_2)}{(Ó_{red}\ ë_1)\ (A'\ ë_2) - (Ó_{red}\ ë_2)\ (A'\ ë_1)} \times 100 \quad \ldots\ [1]$$

Where $Ó_{ox}\ ë_1$, $Ó_{ox}\ ë_2$, $Ó_{red}\ ë_1$, and $Ó_{red}\ ë_2$ are constants, $Aë_1$ and $Aë_2$ are absorbance at 570 nm and 600 nm respectively for stimulated cells while $A'\ ë_1$ and $A'\ ë_2$ are absorbance at 570 nm and 600 nm respectively for

medium alone. The stimulation index was calculated by dividing the mean OD values of antigen-stimulated cells by the mean OD values of unstimulated cells. Cells with Stimulation Index (SI) of =1 were considered positive.

Statistical analysis

The association between antigen stimulation and lymphocyte proliferation was examined by correlation analysis. Paired or unpaired students't-tests and analysis of variance were used to compare test and control data where appropriate.

Results

Lymphocyte proliferation responses

The results from antigen stimulation of PBMCs showed that the percentage reduced Alamar Blue was between 27.3 and 71.6% (Table 1) and that 1:1,600 antigen dilution gave the highest level of percentage reduced Alamar Blue with an average of 45.9% across the entire samples. PBMCs from all but one animal examined proliferated (S.I ≥1) in response to antigen stimulation at 1:1,600 dilution (Table 2). There was low level of proliferation at serial dilutions 1:100-1:800 and a sharp increase at 1:1,600 and then a reduction from 1:3,200 dilution across the entire samples except in two samples that had a low percentage of reduced Alamar Blue at 1:1,600 dilution (Figure 1).

There was a strong positive correlation (r = 0.97) between the average stimulation index and average percentage reduced Alamar Blue across all the samples; that is, the higher the stimulation index the higher the proliferation observed.

Discussion

The present study developed and established the optimal conditions required for harvesting bovine peripheral blood mononuclear cells and setting up a cell culture for proliferation using Alamar Blue assay for investigating cellular immunity to fascioliasis in cattle.

The reduced proliferation observed with very high and very low concentration of antigen dilutions could be explained by the concept of immunogenicity of an antigen. It is known that antigen concentration is important for the stimulation of T-cells. This means that concentrations lower than the optimal concentration will not elicit immune response because it will not be recognized by the immune system while concentrations higher than the optimal could lead to immune paralysis or immune tolerance [13]. This could explain why a low proliferative response was observed at antigen dilutions

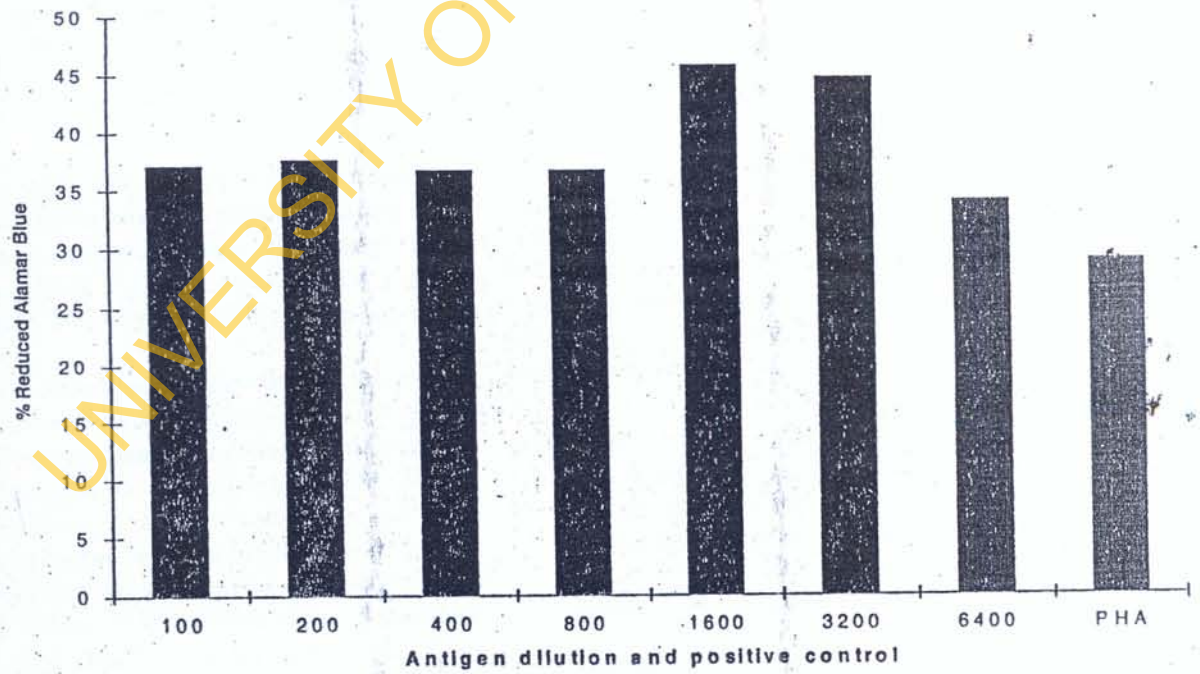

Figure 1: Average level of cell proliferation when stimulated by the different antigen dilutions and phytohaemaglutinin (PHA).

Table 1: Percentage of alamar blue dye reduced by PBMC from cattle in response to stimulation by *Fgigantiea* crude antigens in different concentrations.

Cattle No.	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	PHA
1	30.6	31.3	31.4	28.2	39.0	41.3	30.1	26.9
2	27.3	29.2	31.7	28.6	35.0	37.4	28.8	29.5
3	31.7	31.4	29.2	34.5	34.3	32.3	29.2	30.2
4	48.9	46.2	43.1	51.3	50.3	47.1	43.1	32.1
5	37.6	37.4	34.8	39.4	39.8	38.0	37.1	30.0
6	30.9	30.4	30.6	30.8	30.4	27.9	28.8	25.2
7	47.9	49.1	48.4	49.4	66.4	62.5	62.5	41.4
8	28.4	29.4	30.4	28.2	35.4	39.0	32.3	27.4
9	41.6	46.7	44.5	38.4	56.8	56.2	48.7	37.7
10	46.6	45.9	44.1	39.5	71.6	66.0	59.9	39.7

Table 2: Stimulation Index (SI) of the serial antigen dilutions in all the cattle samples showing the stimulatory effects of the antigen dilutions on the cell proliferation.

Cattle No.	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	PHA
1	0.89	0.89	0.90	0.80	1.11	1.17	0.91	0.69
2	0.72	0.77	0.83	0.75	0.93	0.98	0.94	0.70
3	1.00	0.99	0.92	1.09	1.08	1.02	0.92	0.95
4	1.05	0.99	0.92	1.10	1.08	1.01	0.92	0.70
5	0.99	0.98	0.90	1.04	1.04	0.99	0.96	0.78
6	1.10	1.07	1.08	1.09	1.07	0.98	1.01	0.89
7	0.82	0.84	0.83	0.86	1.16	1.08	0.95	0.70
8	0.85	0.83	0.79	0.71	1.29	1.19	1.02	0.71
9	1.28	1.11	1.09	1.03	1.34	1.47	1.19	0.98
10	0.92	1.02	0.96	0.86	1.22	1.21	1.05	0.80

1:100-1:800 and ≥ 1:3,200. This same reason could also explain the low proliferation observed with the cells stimulated with phytohaemaglutinin. The concentration used in this study was 3 µg/ml, much higher than the 0.05 µg/ml used by McCole *et al* (1999) to give a positive stimulation index.

A low positive cut-off was chosen for the stimulation index because the incubation period lasted for only 48 hours. The low stimulation index observed in this study agrees with several studies that observed low PBMC proliferation (S.I=1.1±0.2) during the first 7 days of culture until day 11 when vigorous proliferative responses to *Fasciola hepatica* were observed reaching its peak at day 21 [1]. Others [14] reported a low stimulation index (S.I≤ 2) until after day 5 of incubation, and after a 7-day incubation. Much work still remains to be done in calibrating the Alamar blue assay system for characterizing the t cell proliferation in response to antigens from the whole worm especially the tegument antigens, although it is generally considered that the excretory/secretory antigens are more relevant to immunological studies of liver fluke infections [9].

This is one of the few reports that have used Alamar Blue Assay to monitor PBMC proliferation. In our hands, this assay was simple to use in monitoring PBMC proliferation. This is the first report of *Fasciola* T-cell work in Nigeria.

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