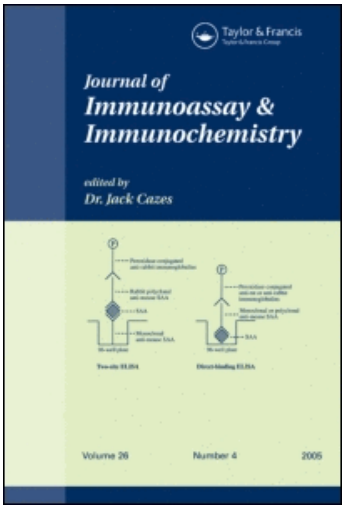

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T-Cell Responses to *Mycobacterium avium* PPD Antigens in Gastro-intestinal Helminth Co-infected Chickens in Central Ethiopia

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ABSTRACT

A cross-sectional study was conducted on extensively reared chickens of three selected agro-climatic zones in Central Ethiopia to examine the predisposing effect of gastro-intestinal helminthes to intestinal *Mycobacterium avium* when it occurs as co-infection. This was done through a Lymphocyte Stimulation Test (LST) using avian PPD on peripheral blood mononuclear cells obtained from the blood of chickens and gross examination of digestive tract for the presence of helminth parasites. Data were analyzed using the statistical softwares SAS (1994) and Intercooled STATA version 6. Fourteen (14.7%) out of the 95 examined chickens were positive in in vitro LST showing stimulation index (SI) ≥ 2. There was a significant ($\chi^2 = 9.93$, $P < 0.01$) difference in prevalence of *M. avium* by altitude: highest in chickens from lowland (27.8%) areas, followed by 13.3% in chickens from mid altitude and none was reacted to LST from highland region. A significant relationship ($\chi^2 = 9.58$, $P < 0.01$) in cestode co-infection with *M. avium* was found. There was no significant ($\chi^2 = 1.66$, $P > 0.05$) relationship in nematode co-infection with *M. avium*.

Key Words: Avian tuberculosis; Stimulation index; Cestode; *M. avium*; Nematode.

INTRODUCTION

Avian tuberculosis (AT)[1] is principally an important chronic infectious disease of free-range domestic poultry caused by *Mycobacterium avium* (*M. avium*), an intracellular, acid-fast bacillus that infects mononuclear cells.[2,3] Its significance has diminished in many richer countries as a result of introduction of more intensive production system; however, it remained to be a problem in developing countries where the production system is still an extensive (traditional) one under which the free-range and backyard systems are undertaken.[2,4] Even though most developed countries have managed to control the disease in their poultry industry, it has remained to be a serious problem in many zoological gardens where some of the breeds of wild birds in these countries are forced into extinction.[2,5,6]

Avian tuberculosis is extrapulmonary, mainly intestinal, and is a common disease of free-range domestic chickens characterized by generalized wasting manifested by noticeable atrophy of the thigh and breast muscles, diarrhoea, anaemia, icterus, and bilateral lameness in

older chickens with caseous nodular formation in affected visceral organs.[2,7,8] The most important factor in AT epidemiology is the tremendous number of tubercle bacilli exuded from ulcerated tuberculous lesion of poultry, which creates a constant source of the virulent bacteria.[1,2] The contaminated environment containing bacilli-laden soil and litter is of greatest importance in transmission of the disease to other uninfected chickens as well as to other domestic animals and possibly man.[3,9] Even though the *M. avium* infection is common in patients with Acquired Immunodeficiency Syndrome (AIDS),[10,11] there are also reports of *M. avium* in normal human hosts patients without predisposing conditions.[12,13] The same external environment that is incriminated as a source for *M. avium* bacilli is the same that is incriminated as a source for gastro-intestinal endoparasite in domestic chickens.[9,14,8,15] AT and gastro-intestinal helminthes share the same predilection site, which is the GI tract, inflicting damage in their own way.[2,4,8,16] Studies have managed to show that intestinal helminthes infection reduces the efficacy of BCG vaccines in cattle.[17] Similarly, other studies on human have shown that in most cases where BCG vaccine confers the least protection are characterized by a high endemic prevalence of chronic infectious diseases, particularly helminthes.[18–20] This sheds a light that hosts exposed to GI helminthosis responds less to the challenge, because of altered host immune response to subsequent infections.[17,19,20] Although several studies have been done on AT in other countries, it is still not proven to date the actual existence of the disease in Ethiopia except a report by OIE/FAO/WHO[21] on the probable existence of the disease in Ethiopia. This study was aimed to: (1) investigate the prevalence of *M. avium* in local breed domestic chickens in three selected agro-climatic zones regarded as major chicken rearing areas in Ethiopia, and (2) discern its association with GI parasitosis.

EXPERIMENTAL

Study Area and Animals

A cross-sectional study on AT in local chickens was carried out in three selected agro-climatic zones of central Ethiopia which were considered to be representative of high (Debre Berhan, located at 2780 m altitude; mean annual temperature range between 6.3 and 18.8°C), mid (Sebeta, located at 2240 m altitude; mean annual temperature range between 15 and 21°C),

and low (Nazareth, located at 1300 m altitude; mean annual temperature range between 15 and 28°C).

Study animals included were randomly selected, 95 local[22] chickens from both sexes (male and female), and age groups (young and adult): 29 from high altitude, 30 from mid altitude, and 36 from low altitude areas.

Questionnaire Survey

A structured questionnaire survey was made in parallel by a house-to-house observational visit in all the three agro-climatic zones to collect additional information which revealed the varying number of chickens which are found in one household, the poor type of management in terms of food and shelter, low amount of output per chicken in terms of eggs, weight gain and hatched chicks, the low income and poor socio-economic status of each household, the long intervals on which chicken droppings are cleaned from their dwellings that implicates the poor management, and husbandry which may attribute to the poor hygiene that will maximize losses from the root causes of the most bacterial and parasite infections, the long duration of the time that chickens are kept in the household for egg laying and breeding purpose in females and males, respectively along with the number of newly introduced chickens to the flock. Moreover, since both the chickens and human share the same dwelling, which is also not hygienic, there is a strong probability for the transfer of *M. avium* infection from the natural host, chicken to human.

Parasitological Examination

During ante-mortem examination, all clinical signs whether indicative of AT or not, was recorded. Live weight (in grams) was taken to assess the status of body condition of study chickens using spring balance before and after sacrifice. Cervical disarticulation was employed as recommended[23] so that post-mortem examination can be done. The digestive tract was separated and detached from the mesentery and from other adjacent organs. A dissection that extended from the oesophagus up to the rectum was made.[23,24] Those worms that were only visible with naked eye were picked by thumb forceps and

counted. In this study, no species identification of the respective worms was done.

Lymphocyte Stimulation Test (LST)

Peripheral Blood Mononuclear Cell Separation

Peripheral blood (20 mL) was collected via the intra-cardiac route in 2% EDTA for Lymphocyte Stimulation Test (LST) and was diluted with RPMI-1640 (Sigma-Aldrich Corp., St. Louis, MO, USA) in 2:1 ratio. Then the peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque solution density gradient (Sigma Diagnostics, St. Louis, MO, USA) centrifugation. The cells that were collected from the interface was washed with cold RPMI-1640, enriched with 100 μg/mL of L-glutamine (Life Technologies, Paisley, UK), heat inactivated with 10% foetal calf serum (FCS) (Serva, D-6900 Heidelberg 1, Germany) to which added was 100 U penicillin/mL, 100 μg/mL Streptomycin (Sigma-Aldrich Corp., St. Louis, MO, USA), 5×10^{-5} M of 2-Mercaptoethanol (Serva Feinbiochemica, Heidelberg, Germany), 1 mM sodium pyruvate, 10 mM HEPES buffer (Sigma Chemicals Co., St Louis, MO, USA) for the maintenance of a suitable pH for longer period handling procedures, and 5 μg/mL of 5-fluorocytosine/mL (Sigma-Aldrich Corp., St. Louis, MO, USA). The viability of the PBMC was assessed and 95% viability per field was accepted using Trypan Blue. Cells not taking the Trypan Blue under a microscope at 40× magnification in haemocytometer chamber were considered as viable cells.

Cell Proliferation

Peripheral blood mononuclear cells (8×10^6 per well) were cultured in a round and flat-bottomed 96-well micro-titre plate. The cultures were set up in triplicate where three wells are stimulated with the mitogen, Concanavalin A (Con A) (Pharmacia, Uppsala, Sweden) with a concentration of 10 μg/mL for 3 days that may serve as positive controls, three wells were stimulated by Avian PPD (Statens Serum Institute, Copenhagen, Norway) with a concentration of 5 μg/mL for 5 days to be used as test antigen, and another three wells were cultured with RPMI media alone to serve as negative controls. The cultures were then kept at 40°C at 95% humidity and 5% CO_2 incubator. Cells

which are stimulated with Con A were pulsed on day 3 whereas cells which are stimulated with PPD were pulsed on day 5 with 25 μL of 40 μCi ^{3}H-Thymidine (Amersham Life Sciences, UK) 20 h before harvesting. Proliferation was assessed in liquid scintillation counter (LKB, β-counter; LKB Wallak 1216, Turku, Finland) and the radioactivity counted in scintillation fluid (β-Plate Scint; LKB Wallak, Turku, Finland) was expressed as a stimulation index (SI) that is mean CPM with antigen or mean CPM without antigen. Stimulation index greater than or equal to two ($SI \geq 2$) are considered to respond positively to the avian PPD.[14,17]

Statistical Analysis

The prevalence rate for *M. avium* is defined as the proportion of the number of LST positive animals (i.e., $SI > 2$) to the total number of animals tested expressed in percent. The prevalence for nematode and cestode is the proportion of nematode or cestodes positive animals to the total number of animals tested expressed in percent. These were generated by FREQ procedures of the Statistical Analysis System.[25] Variation of prevalence by altitude, sex, and age was investigated by the chi-square test. Student *t*-test was used for the assessment of nematode and cestode infection and infection intensity (worm burden) (STATA version 6.0). Odds ratio (OR) was computed by FREQ procedures with the option of Cochran-Mantel-Haenszel statistic of Statistical Analysis System[25] to estimate the level of risk of avian tuberculosis by explanatory variables. Odds ratio (OR) is the ratio of the odds of diseases occurring among animals exposed to a factor and the odds of disease occurring among animals not exposed to a factor.[26] In cases, where the population showed skewed distribution, it was logarithmically transformed and the geometric mean was used as a measure of central tendency. Correlation between live weight and SI was done by altitude.

RESULTS

The status of the negative controls is presented in Table 1. All negative controls were negative for parasites egg counts and had $SI < 1$. This was compared with positive animals of the study subjects in Fig. 1.

Table 1. Basic information on negative control animals.

Areas & subject #	Sex	Age	Stimulation index		Parasite egg counts		Measurements			
			Con A (mitogen)	PPD	Nematode	Cestode	Live weight (g)	Carcass weight (g)	Breast dimension (g)	Thigh circumference (cm)
Debre Berhan (high altitude)										
DC 001	M	Y		0.84	0	0	1500	1100	15	14
DC 002	F	Y		0.50	0	0	800	500	12	10
DC 003	M	A		0.67	0	0	1800	1400	15	14
DC 004	F	A		0.73	0	0	1200	900	12	11
Sebeta (mid altitude)										
SC 005	M	Y		0.48	0	0	800	500	10	8
SC 006	F	Y		0.63	0	0	700	500	11	9
SC 007	M	A		0.81	0	0	1300	1000	13	10
SC 008	F	A		0.55	0	0	1200	1000	13	11
Nazareth (low altitude)										
NC 009	M	Y		0.63	0	0	900	700	10	10
NC 010	F	Y		0.94	0	0	700	400	8	8
NC 011	M	A		0.59	0	0	1300	1000	10	10
NC 012	F	A		0.88	0	0	1000	700	9	9

Sex: M = male; F = female.
Age: Y = young; A = adult.

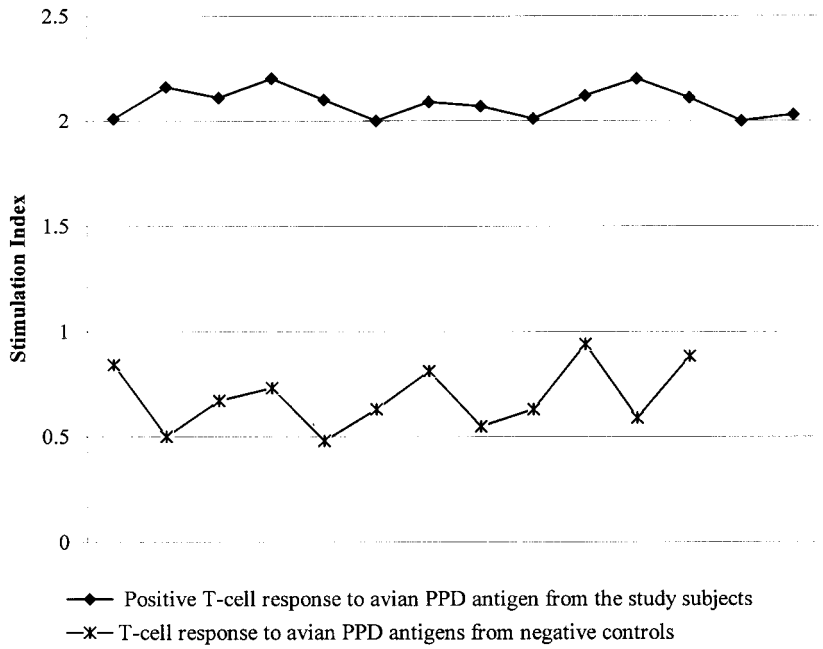

Figure 1. Comparison of stimulation index of the negative controls and positive animals.

Avian PPD Specific In Vitro Cellular Response

Out of a total of 95 chickens studied, 14 (14.7%) were positive to the LST with SI ≥ 2. The prevalence of *M. avium* was significantly ($P < 0.01$) different by altitude: 27.8% (10/30) in chickens from low altitude, 13.3% (4/29) from mid altitude, and none (0%) from high altitude areas (Table 2). The difference in prevalence between males (13.3%) and females (16%) as well as between young (11.3%) and adult (17.6%) was not statistically significant ($P > 0.05$, Table 2).

Prevalence of Gastro-Intestinal Helminthes and Its Association with *M. avium*

Out of the 95 chickens examined, 51 (53.7%) and 59 (62.1%) were found infected with different species of nematodes and cestodes,

Table 2. Prevalence of *M. avium* as judged from in vitro cellular assay with positive T-cell response to avian PPD antigens.

Factors	Number examined	Number positive	Prevalence (%)	Significance
Overall	95	14	14.74	
Altitude[a]				$\chi^2 = 9.93,\ P < 0.01$
High	29	0	0.00	
Mid	30	4	13.33	
Low	36	10	27.78	
Sex				$\chi^2 = 0.13,\ P > 0.05$
Female	50	8	16.00	
Male	45	6	13.33	
Age				$\chi^2 = 0.74,\ P > 0.05$
Young	44	5	11.36	
Adult	51	9	17.65	

[a]Altitude: high altitude, 2780; mid altitude, 2240; low altitude, 1300 m above sea level.

respectively (Table 3). The helminth infection intensity (worm burden) or geometric mean for nematodes and cestodes was 19.5 and 57.4, respectively. A co-infection of nematode and cestode in a single subject was encountered in 12 cases. Area specific analysis showed that the prevalence and infection intensity (geometric mean) of nematodes showed a significant difference ($P < 0.01$) in chickens sampled from mid (76.6%; 24.5), high (51.7%; 16.2), and low (36.1%; 18.5) altitudes. The prevalence of nematode infection between males and females was not significantly different (60% vs. 48%, $P > 0.05$). However, there was significant difference ($P < 0.05$) between young (40.9%) and adult (64.7%) age group for nematode infection. Similar to that of nematodes prevalence, a significant difference ($P < 0.05$) was found in the prevalence and infection intensity of cestodes (geometric mean) in low (77.7%; 94.7), mid (63.3%; 66.6), and high (44.8%; 23.8) altitudes. Furthermore, there were significant differences in prevalence of cestode infections between sex (males, 73.3% vs. females, 52%; $P < 0.05$) and age (young, 47.7% vs. adult, 74.5%; $P < 0.01$). In this study, a reverse relation of nematodes vs. cestodes prevalence was seen in relation to altitude.

The relationship between avian tuberculosis and gastro-intestinal helminthosis is shown in Table 4. Those chickens that were AT positive (50%) and AT negative (54.3%) with nematode infection did not show a significant difference ($P > 0.05$). However, there was a significant

Table 3. Prevalence of gastro-intestinal helminthosis in chickens.

		Nematodes		Cestodes	
Factors	Number examined	Number positive	Prevalence (%)	Number positive	Prevalence (%)
Overall	95	51	53.68	59	62.11
Altitude[a]			$\chi^2 = 10.89,\ P < 0.01$		$\chi^2 = 9.07,\ P < 0.05$
High	29	15	51.72	12	41.38
Mid	30	23	76.67	19	63.33
Low	36	13	36.11	28	77.78
Sex			$\chi^2 = 1.37,\ P > 0.05$		$\chi^2 = 4.58,\ P < 0.05$
Female	50	24	48.00	26	52.00
Male	45	27	60.00	33	73.33
Age			$\chi^2 = 9.89,\ P < 0.01$		$\chi^2 = 7.20,\ P < 0.01$
Young	44	16	36.36	21	47.73
Adult	51	35	68.63	38	74.51

[a]Altitude: high altitude, 2780; mid altitude, 2240; low altitude, 1300 m above sea level.

($P < 0.01$) relationship between AT positive (100%) and AT negative (56.7%) with cestode infection status in chickens.

Correlations Between SI and Live Weight in Chickens by Agro-climatic Zones

There was positive correlation between live weight and SI in chickens sampled from high ($r = 0.74$) where no and mid ($r = 0.62$) altitude areas whereas the correlation that was seen in chickens from low ($r = -0.24$) altitude areas was negative.

DISCUSSION

The highest number of chickens with AT positive result was recorded in low altitude followed by mid altitude areas. This may be related to a relatively higher temperature in low and mid altitude areas than in higher altitude areas favoring the survival of *M. avium* organisms. Schalk and colleagues[27] demonstrated that virulent strains of *M. avium* survive in soil and saw dust for 168 days at 20°C and 244 days at 37°C. It was also

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Table 4. Relationship between avian tuberculosis and gastro-intestinal helminthosis.

Chickens reactivity to *M. avium*	Nematode infection status[a]			Cestode infection status[b]		
	Positive	Negative	Total	Positive	Negative	Total
Positive	7 (50.0)	7 (50.0)	14 (14.7)	14 (100.0)	0 (0.0)	14 (14.7)
Negative	44 (54.3)	37 (45.7)	81 (85.3)	46 (56.8)	35 (43.2)	81 (85.3)
Total	51 (53.7)	44 (46.3)	95 (100.0)	60 (63.2)	35 (36.8)	95 (100.0)

Figures in parentheses are percentages.
[a] $\chi^2 = 1.66$, $P > 0.05$.
[b] $\chi^2 = 9.58$, $P < 0.01$.

reported that tubercle bacilli live in an environment with temperature ranging between 25°C and 45°C.[28]

The absence of significant sex difference in the prevalence of *M. avium* may be related to traditional husbandry practices such as keeping both cocks and hens for breeding and egg laying purposes for long period of time giving enough time for infection to establish itself and spread in the flock.[27] Similarly, no significant age difference for *M. avium* prevalence was found in this study. Different to our observation, higher prevalence in adult than in young chickens was also observed.[27] This may suggest that age could possibly influence manifestation of the clinical disease and not the resistance to infection.

The highest prevalence of nematodes seen at the mid altitude area was in agreement with previous reports from Ethiopia[8,16] and from Tanzania.[15] This may be due to the suitable ambient temperature in mid (15 to 26°C) altitude areas that falls between the optimal temperature ranges required for the development of nematode larvae, which is 18 to 26°C.[29] The high prevalence of nematodes seen in the adult age group may probably be due to the repeated exposure of the adults for the nematode larvae of the intermediate hosts that may have extended throughout their life span. Moreover, the length and size of the adult chicken gut may have a contributory effect on harboring the infections.

In our study, the prevalence and infection intensity of cestodes did not significantly differ in the different altitudes. The insignificant difference that was observed in low and mid altitude areas may be due to the small temperature differences between the two. This was in agreement with the results obtained in Ethiopia[8,16] who considered chickens from different agro-climatic zones. Moreover, the variation of the cestode prevalence at different altitudes was previously reported,[15] where optimal temperature and relative humidity contributes for the growth of the cestode eggs and the intermediate hosts such as beetles, earth worms, and hence resulting higher prevalence in specific altitude areas. Especially regarding cestode infection intensity, the result obtained in this study is similar with the findings by Tigabu[8] where the highest burden was seen also in low altitude area followed by mid and high altitude areas.

The present study indicated that the prevalence of AT was not affected by the prevalence and intensity of the nematode infection. This may probably be due to the fact that nematodes mostly exert their pathogenic effect by their presence causing mild inflammation and petecchial haemorrhage of the mucous membrane of the gut in the process of feeding and migration; the worms can block the lumen of the gut completely. Most of these nematodes compete for digestible

nutrient rather than inflicting direct damage to the gut wall.[5] On the other hand, cestodes, which are mostly acquired from intermediate hosts (beetles, snails, slugs, earthworms, crustaceans, etc.) simultaneously, can attack the gut by thousands of hold fast suckers, which is an attachment apparatus for cestodes. Moreover, multiple nodules and hyperplastic enteritis on the gut wall at a higher degree was encountered[24] that confuses with the nodular tubercles that develop during AT infection.

The significant difference in prevalence of cestodes between mid and low altitude areas may be attributed to the presence of favorable environment for the development of both the cestode egg and intermediate host.[15,29] The high incidence of cestodes infection intensity in low altitude are combined with the highest prevalence of chickens that responded positively to the in vitro avian PPD test may show a relationship between the cestode infection and *M. avium* infection implicating that there can be a possibility for *M. avium* that facilitates its colonization and establishment in the gut as a result of the severe damage inflicted by the cestode.[24,30]

The association seen between results of the in vitro cellular assay and the measurements of live weight indicated that it has influenced negatively the body condition of the respective chickens. The negative correlation coefficient seen at the low altitude area depicted that as the stimulation index increases the live weight has decreased. In other words, it has been shown that there was some degree of muscular wastage[1,2] in the low altitude areas where large number of the chickens with $SI \geq 2$ and cestode infection was encountered.

In conclusion, AT and cestode infection are most prevalent in low altitude. Because of the resulting low live weight, a significant correlation between the two infections may have an ominous economic impact. It is, therefore, imperative for any improvement in chicken breeding could include cestode and AT control measures. The control of AT in chicken is particularly important since some strains of avian strains may cause disease in humans.

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