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Cytokines Levels and Anti-tuberculosis Drug Resistance among HIV Seropositive Patients in Ibadan, Southwest, Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Susceptibility to TB disease rises on mutations in cytokine receptors which ultimately lead to defective signaling and alter the immune surveillance and bacterial killing. Therefore, this study was undertaken to investigate the association between cytokines and anti-TB drug resistances. A total of 217 people living with human immunodeficiency virus at University College Hospital, Ibadan were recruited. Venous blood and sputum samples were collected. Sputum samples were cultured for Mycobacterium tuberculosis complex and characterized using standard methods. Anti-TB drug resistance was detected using phenotypic and genotypic methods. Cytokines levels (IL-10 and IFN-Y) were estimated using ELISA method. Association between cytokines and anti-TB drug resistance was determined using linear regression analysis. There were 105(48.4%) males and 112(51.6%) females with a mean age of 42.95 ± 8.286 years and a range of 28-65 years. The prevalence of TB among PLHIV was found to be 19.1% with male having higher rate than female

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(*P*<0.05), and highest rate among aged ≥40. There was no significant association between cytokines (IL-10 and IFN-y) and sex of the subjects (*P*>0.05). However, females had higher IFN-y level of 231.06±61.81 IU/ml when compared to 227.53±57.92 IU/ml obtained from males. The prevalence of multi-drug resistance TB was 11.9% with Rifampicin resistance having the highest rate of 5.5%. There was no significant association between cytokines (IL-10 and IFN-y) and sex, but age group >40 years had higher IL-10 and IFN-y level of 226.53±52.39 IU/ml and 232.41±66.63 IU/ml respectively. There was an association between high cytokines levels and anti-TB drug resistance (*P*<0.05). Highest cytokines levels; IL-10 (401.00±7.07 IU/ml) and IFN-Y (401.00±7.07 IU/ml) were recorded in Levofloxacin resistance. There was an association between high cytokines levels and genotypic anti- TB drug resistance (*P*<0.05) with highest cytokines levels; IL-10 (400.12±12.37 IU/ml) and IFN-Y (472.87±31.93 IU/ml) recorded in Rifampicin resistance. This entire study revealed that there is high prevalence of MDR-TB among PLHIV, with Rifampicin monoresistance having the highest resistance rate among the drugs tested, and pro and inflammatory cytokines (IL-10 and IFN-y) play significant role in the development of Multi Drug Resistant-Tuberculosis among people living with HIV.

Keywords: Tuberculosis; cytokines levels; anti-tuberculosis drug resistance.

1. INTRODUCTION

The major causes of failure to effective solutions to tuberculosis (TB) are human immunodeficiency virus (HIV) infection, epidemics of TB and delay in diagnosis and proper treatment (Naidoo et al., 2019). In 2022, the estimated number of incident cases of TB were about 10.6 million, deaths from TB among HIV-negative patients were1.13 million and deaths from HIV-positive TB were about 0.17 million (WHO, 2023).

Drug-resistant TB may arise due to the failure in implementing proper TB control programs and properly managing TB cases (WHO, 2022). As a result, more than 206,030 MDR-TB cases are estimated to occur worldwide each year, of which about 6% takes place in Nigeria (Dayyab et al., 2022). World Health Organization (WHO) pointed out in a survey recently that about 20% of MDR-TB isolates were also extensively drug-resistant (XDR) i.e. resistant to INH and RIF and to at least one of the most efficient second-line drugs (WHO, 2022; Elewski et al., 2021). Bacteria may become antibiotic resistant due to two reasons: one mutation in some drug target genes and second through horizontal transfer in which an organism obtain drug resistance genes from other microorganisms (Peraman et al., 2021). Most of the microorganisms acquire resistance in the environment through horizontal transfer because mutations occur at low frequency (Valerga et al., 2015).

Cytokines have a protective and pathological responses with the network of pro-inflammatory, T1 and T2 cytokines through reactions with complex mycobacterial antigens and RD regions (Vilchèze & Jacobs, 2019). Additional role of pro-inflammatory cytokines as biomarker signature for disease severity, bacterial load, culture conversion and treatment outcome have been discovered in previous studies (Sampath et al., 2023). Mycobacterial growth prevention is achieved by the joint role of cytokines and chemokines as they coordinate the initiation, expansion, inflammation, recruitment, differentiation, activation and localization of mononuclear cells. Array of cytokines such as IL-12, IL-18, IFN-γ, TNF-α, and IL-6 play early protection roles against both MDR-TB and DS-TB. Mycobacterium tuberculosis (MTB) clearance in granuloma of non-human primate model rendered through the T cells with balanced secretion of pro and anti-inflammatory cytokines (Giri et al., 2020). However, in the chronic infection setting, the balance between protection and pathology is lost due to altered expression of cytokines that accompany delayed resolution of inflammation and tissue repair. The susceptibility to TB disease rises on mutations in cytokine receptors which ultimately lead to defective signaling and aborts the immune surveillance and bacterial killing (Pavithra et al, 2023; Kumar et al., 2019).

There is limited studies on cytokine signatures for DR-TB and latent TB. Hence, this study aimed to determine the relationship of cytokines levels and drug resistance among TB/HIV co-infected clients in Ibadan.

2. MATERIALS AND METHODS

2.1 Study Area

The study was carried out at the University College Hospital (UCH), Ibadan. The hospital

serves as the central hub for healthcare services, medical education, and research in the southwest region of Nigeria. It is located at longitude 7.3569°N and latitude 3.8743°E.

2.2 Study Population

The target population included individuals attending the University College Hospital in Ibadan, Nigeria, who are living with HIV, as well as people without HIV infection who serve as control group. Informed consent was obtained from each subject before their inclusion study.

2.3 Sample Collection

Collection of sputum sample: Sputum samples were collected from eligible people living with HIV and they were instructed on the proper technique for providing sputum samples. Each subject was instructed to produce and submit 2 sputum specimens within two consecutive days. Each subject completed a structured questionnaire that requested information about age, sex, occupation, bacilli Calmette-Guerin (BCG) vaccination, history of chronic cough or TB. Collection was done through the support of medical house officers/registrars posted to HIV unit; a consultant was responsible for overall supervision.

Collection of samples for biochemical and haematological assays: About 10mls of blood was collected from each eligible and consented client. Out of which 5mls were added to K_3 EDTA bottles for HIV confirmation test, viral load determination, CD4+ cell count, cytokines, and full blood count estimation. The remaining 5mls blood was added to lithium heparin bottles for electrolyte panel analysis, liver function tests, and lipid profile estimation.

2.4 Estimation of Immunological Markers

CD4 $^+$ Cell Count: $CD4^+$ was done using Partec Cyflow machine as described by the manufacturer

Cytokine Estimation: IL10 & IFN-y. IL-10: The estimation of the interleukin 10 was done using the NWLSS Human IL-10 ELISA kit, which is able to recognize native and recombinant human IL-10. IFN-y: The estimation of the IFN-γ, or type II interferon was done using the Cohesion Bioscience Human IFN gamma ELISA kit, which employs an antibody specific for Human IFN-gamma coated on a 96-well plate.

2.5 Analysis of Sputum Specimens

Decontamination: Sputum samples were decontaminated using n-acetyl L-cysteine-sodium hydroxide (NALC/NaOH) method

Ziehl-Neelsen staining: Smears were made from the sediments of decontaminated and stained by Ziehl-Neelsen staining technique.

Inoculation and Culture Methods: The sediment pellet of decontaminated sputum was resuspended in deionized water by vortexing to make homogeneous inoculum; 50 microlitres of this inoculum was inoculated on Lowenstein Jensen (LJ) slants. The inoculated slants were incubated in MSE incubator model 4032 at 37^0C for eight weeks. These were observed after 48 hours and thereafter weekly for appearance of macroscopic growth.

Inoculation and Culture Method: The sediment pellet of decontaminated sputum was resuspended in deionized water by vortexing to make homogeneous inoculum; 50 microlitres of this inoculum was inoculated on Lowenstein Jensen (LJ) slants, Ogawa slants as well as blood agar slants in triplicate. These bottles were sealed with parafilm to prevent contamination and desiccation.

The inoculated slants were incubated in MSE incubator model 4032 at 37^0C for eight weeks. These were observed after 48 hours and thereafter weekly for appearance of macroscopic growth. The growth on LJ, Ogawa as well as blood agar slants were observed after keeping the slants in diffuse sunlight for about one hour for pigmentation (Murli et al., 2009) .The methods for the preparation of the three media used as shown in the appendix.

Estimation of cytokine IL10: The estimation of the interleukin 10 was done using the NWLSS Human IL-10 ELISA kit, which is able to recognize native and recombinant human IL-10.

Estimation of cytokine IFN-γ: The estimation of the IFN-γ, or type II interferon was done using the Cohesion Bioscience Human IFN gamma ELISA kit, which employs an antibody specific for Human IFN-gamma coated on a 96-well plate.

2.6 Data Analysis

Analyses of all obtained data were performed by using STATA/IC version 23.0. The x^2 test was used to calculate p value when appropriate. P values <0.05 was considered statistically significant.

3. RESULTS

Table 1 showed that 105(48.4%) were male, while 112(51.6%) were females. Moreover, 26(52.0%) of the control subjects were male, while 24(48.0%) were females. The mean age with SD was 42.95 ± 8.286 years with a range of 28-65 years, while the control group had a mean age with SD of 42.24 ± 4.745 years with a range of 35-50 years.

Table 2 showed that male subjects had higher *M.tuberculosis* isolation rate of 19.0% compared to 13.4% for females. Similarly, higher M.bovis isolation rate of 4.7% was found in males while females had 2.7%. NTM had more occurrences in males (3.8%), compared to females (1.8%). However, higher *M.tuberculosis* isolation rate of 21.4% was found among people aged ≥40 years compared to 10.5% among people < 40 years at *(P<0.05)*. Age group ≥40 years had higher isolation rate (4.5%) of *M.bovis* compared to those <40 (2.9%), though, not statistically significant at *(P>0.05).* NTM had a non-significant higher isolation rate of 3.6% among age group ≥40 years compared to 1.9% for age group <40 years *(P>0.05).*

Table 3 showed that there was no significant association between cytokines (IL-10 and IFN-y) and sex of the subjects *(P>0.05).* However, females had higher IFN-Y level of 231.06±61.81 IU/ml when compared to 227.53±57.92 IU/ml obtained from males. Moreover, there was no significant association between cytokines (IL-10 and IFN-y) and sex of the subjects *(P>0.05)*. However, age group >40 years had higher IL-10 and IFN-G level of 226.53±52.39 IU/ml and 232.41±66.63 IU/ml respectively when compared to 221.22±51.20 IU/ml and 232.41±66.63 obtained from age group <40 years.

Table 4 showed that there was significant association between culture positivity (TB) and cytokines (IL-10 and IFN-y) *(P<0.05)*

Table 5 showed that GeneXpert positivity (TB) is significantly associated with high cytokines levels *(P<0.05).*

Table 6 showed the relationship between Phenotypic DST and Cytokines. There was an association between high cytokines levels and anti-TB drug resistance *(P<0.05)*. Highest cytokines levels; IL-10 (401.00±7.07 IU/ml) and IFN-Y (401.00±7.07 IU/ml) were recorded in Levofloxacin resistance.

Table 1. Demographic characteristics of the participants

Respondents' Socio-demographic Characteristics	Cases n(%)	Control n(%)	Total n(%)
Sex			
Male	105(48.4)	26(52.0)	131(49.1)
Female	112(51.6)	24(48.0)	136(50.9)
Age Group (years)			
<40	90(41.5)	17(34.0)	107(40.1)
40-49	83(38.2)	30(60.0)	113(42.3)
50-59	34(15.7)	3(6.0)	37(13.9)
≥60	10(4.6)	0(0.0)	10(3.7)
≥40	127(58.5)	33(66.0)	160(59.9)
Mean (±SD)	42.95 ± 8.286 years	42.24 ± 4.745 years	42.82 ± 7.745 years
Range	28 to 65 years	35 to 50 years	28 to 65 years
Total	**217**	**50**	**267**

**(P>0.05)*

Table 2. Relationship between Sex and age of *Mycobacterium* species

Sex	N	*M.tuberculosis* n(%)	*M.bovis* n(%)	NTM n(%)
Male	105	20 (19.0)	5 (4.7)	4(3.8)
Female	112	15 (13.4)	3 (2.7)	2(1.8)
Age group (years)				
< 40	105	11 (10.5)	3 (2.9)	2(1.9)
≥40	112	24 (21.4)	5 (4.5)	4(3.6)

**(P<0.05)*

Table 3. Relationship between gender and age vs Cytokines (IL-10 and IFN-y)

Cytokines	Male (n=105)	Female (n=112)	t-test	p-value
IL_10	224.48±48.30	224.18±55.18	0.042	0.966
IFN_Y	227.53±57.92	231.06±61.81	0.434	0.665
	Age group (years)			
	<40 years (n=90)	**≥40 years (n=127)**	**t-test**	**p-value**
IL_10	221.22±51.20	226.53±52.39	0.742	0.459
IFN_G	225.04±48.73	232.41±66.63	0.893	0.373

**P>0.05*

Table 4. Relationship between Culture and Cytokines

Cytokines	Culture results			
	Positive (n=49)	**Negative (n=168)**	**t-test**	**p-value**
IL_2	285.33±75.31	206.53±20.89	12.112	0.000*
IFN_G	300.76±91.63	208.52±16.94	12.404	0.000*

**P<0.05*

Table 5. Relationship between GeneXpert (DS-TB and DRTB) and Cytokines

		Cytokines (IL-10)			
Variables	**N**	**Mean IL_2**	**Standard deviation**	**F-test**	**p-value**
GeneXpert (DRTB)				**68.729**	**0.000***
Negative (NOT DETECTED)	177	208.95	29.25		
Positive (DSTB)	36	292.87	74.99		
DRTB	4	287.87	45.12		
Total	**217**	**224.33**	**51.85**		
	Cytokines (IFN-y)				
Variables	**N**	**Mean IFN_G**	**Standard deviation**	**F-test**	**p-value**
GeneXpert (DRTB)				**74.616**	**0.000***
Negative (NOT DETECTED)	177	211.16	31.63		
Positive (DSTB)	36	309.23	88.15		
DRTB	4	315.37	50.51		
Total	**217**	**229.35**	**59.84**		

**P<0.05 (i.e. Significant)*

Table 6. Relationship between Phenotypic DST and Cytokines

	Phenotypic DST/Cytokines			
RIF	**Sensitive**	**Resistant**	**t-test**	**p-value**
IL_10	268.59±69.88	336.95±70.12	2.942	0.005*
IFN_Y	276.08±81.32	376.87±81.40	3.730	0.001*
INH				
IL_10	272.58±71.64	350.68±61.10	2.880	0.006*
IFN_Y	277.39±76.01	420.56±70.40	4.926	0.000*
ETB				
IL_10	275.71±70.62	393.62±24.17	3.294	0.002*
IFN_Y	286.26±79.72	463.87±49.92	4.355	0.000*
LEV				
IL_10	280.41±72.88	401.00±7.07	2.316	0.025*
IFN_Y	292.78±84.66	488.25±1.06	3.232	0.002*
ETH				
IL_10	278.90±72.93	384.00±29.86	2.462	0.018*
IFN_Y	292.03±85.43	434.66±92.81	2.791	0.008*

**P<0.05*

Key; Rifampicin, Isoniazid, ETB-Ethambutol, LEV-Levofloxacin, ETH-Ethionamide

Table 7. Relationship between LPA DST and Cytokines

LPA DST/Cytokines				
RIF	**Sensitive**	**Resistant**	**t-test**	**p-value**
IL_10	281.02±63.65	367.07±71.47	3.212	0.003*
IFN_Y	294.38±73.30	416.07±85.33	3.918	0.000*
INH				
IL_10	284.25±66.22	400.12±12.37	3.457	0.001*
IFN_Y	297.92±73.34	472.87±31.93	4.684	0.000*
FLQ				
IL_10	288.13±69.30	387.00±24.75	2.4dd35	0.019*
IFN_Y	303.62±79.72	455.16±57.30	3.214	0.003*

**P<0.05*

Key: RIF-Rifampicin, INH, Isoniazid, FLQ-Flouroquinolone

Table 7 Relationship between LPA and Cytokines as shown in Table 7 revealed that there was an association between high cytokines levels and genotypic anti- drug resistance *(P<0.05).* Highest cytokines levels; IL-10 (400.12±12.37 IU/ml) and IFN-Y (472.87±31.93 IU/ml) were recorded in Rifamicin resistance.

4. DISCUSSION

HIV and TB co-infection has been described as a lethal combination, as each disease speeds up the other's progress. A realization which probably led to updating of the WHO TB/HIV treatment approach, in 2012, to place persons with HIV on TB-Preventive therapy upon confirmation of HIV.

Tuberculosis continues to be the leading cause of mortality among PLHIV in sub-Saharan Africa (Sekayi, 2023). In this study, the high isolation rate(19%) of M.*tuberculosis* among male PLHIV obtained is similar to 17.0% reported by Purushotan et al., (2013) in India, but markedly lower than 37.4% rate reported by Melkamu (2021) in Ethiopia. The higher rate of TB/HIV co-infection cases might be explained by impairing the host's immune response to both diseases, lengthening the time it takes for TB treatments to be effective by increasing the bacilli load, which could make the bacteria resistant to anti-TB drugs, and lead to MDR-TB.

TB/HIV co-infection is a major public health problem in many parts of the world, but the prevalence of co –infection varies among countries (Melkanu, 2021). The risk of developing TB among the millions of people living with HIV is 18 times higher than the rest of the global population. TB remains the overall leading cause of death among people living with HIV, accounting for around one third HIV-related deaths. In this study, occurrence of *M.bovis* in PLHIV was higher among males (4.7%) when compared with (2.7%) recorded in females. However, this is in contrast to the study conducted by Valegra et al. (2021) in Europe. The authors reported higher isolation rate of 9.5% among female subjects.

Human disease caused by M.*bovis* has been confirmed in African countries. HIV infection may be an important risk factor for M.*bovis* disease, and M.*bovis* has been associated with mortality among PLHIV (Rodwell, 2022). Non-tuberculous Mycobacteria (NTM) is a common opportunistic infection in PLHIV. AS obtained in this study, the occurrence of NTM in PLHIV has been reported (Lee et al.,2022). Although the prevalence is low in this study, however, NTM occurred more in males (3.8%) than in females' clients (1.8%). Similarly, NTM occurred more in the age group >40 years (3.9%). These data indicated the persistence of NTM disease even in the modern *cART era*. Lee et al., (2022) reported that the distribution of NTM reflects geographical diversity, wherein species vary according to region and country.

Drug-resistant TB impairs the current treatment strategies and worsens the unfavorable outcomes. The knowledge on host immune responses between drug-sensitive and drug-resistant infection is inadequate to understand the pathophysiological differences and disease severity. Secreted proteins called cytokines display versatile behavior upon infection with M.tuberculosis and their imbalances often tend to assist disease pathology than protection. In this study, high cytokines levels were significantly associated with anti-TB drug resistance (P<0.05). This is in line with the report of Pavithra et al., (2023) that drug-resistant tuberculosis was associated with altered cytokine levels. These TB biomarkers can be considered

crucial to achieve the global TB elimination targets. The effort to understand causal factors like cytokines and their differential expression during different stages of TB is valuable in identifying unique biological signatures (Mensah et al., 2021). Cytokines mount protective and pathological responses with the network of pro-inflammatory, Th1 and Th2 cytokines through interaction with complex mycobacterial antigens and RD regions (Mustafa et al., 2021). Previous studies determined the augmented role of pro-inflammatory cytokines as biomarker signature for disease severity, bacterial burden, culture conversion and treatment outcomes (Mihret et al., 2022; Chowdhury et al.,2023; Domingo et al.,2023). The susceptibility to TB disease rises on mutations in cytokines receptor which ultimately lead to defective signaling and aborts the immune surveillance and bacterial killing (Kumar et al*.,* 2019).

It was found in this study that pro and inflammatory cytokines (IL-10 and IFN-y) were associated with TB (P<0.05). This is in-line with Marina et al (2021) that the imbalance of cytokine secretion in HIV infection affects the function of the immune system and the course of the function of the immune system and the course of the disease, increasing or suppressing viral replication (Jacobs et al., 2019).

5. CONCLUSION

This study revealed that pro and inflammatory cytokines (IL10 and IFN-y) are significantly associated with TB and MDR-TB among People living with HIV. There is high prevalence of MDR-TB among People living with HIV, with Rifampicin monoresistance having the highest resistance rate among the drugs tested. Pro and inflammatory cytokines (IL-10 and IFN-y play significant role in the development of Multi Drug Resistant-Tuberculosis among people living with HIV.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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CONSENT AND ETHICAL APPROVAL

The study proposal was examined, approved, and permission for work was granted by the ethical committee of University College Hospital/University of Ibadan. All study participants were educated on the purpose of conducting the research, after which written consent was obtained from each participants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

REAGENTS

4% NaOH SOLUTION

- 40 g of NaOH pellets was dissolved in 100mls of distilled water. Magnetic stirrer was used to mix well.
- The beaker was kept cool to minimize heat build up
- It was then transferred to a volumetric flask and distilled water added to 1,000mls mark.
- It was transferred to a reagent bottle and labeled.

2% CHLORHEXIDINE (HIBITANE) SOLUTION

- 20 mls of hibitane solution was added to a volumetric flask
- Distilled water was added up to 1,000mls mark.
- It was then transferred to a reagent bottle and labeled.

NaOH – NALC SOLUTION

- **SOLUTION 1**
- **4% NaOH solution –**
- 40 g of NaOH pellets was dissolved in 100 mls of distilled water. Magnetic stirrer was used to mix well.
- The beaker was kept cool to minimize heat build up
- It was then transferred to a volumetric flask and distilled water added to 1,000 mark.

SOLUTION 2

2.94% Na Citrate

* 29 g Tri-sodium citrate-dihydrate was dissolved in 100 mls of distilled water
* The beaker was placed on magnetic stirrer and the stir bar was used to mix well.
* The solution was transferred to a volumetric flask and distilled water added to the 1000 mls mark.

NaOH-Na Citrate working solution

- Equal volumes of solution 1 and solution 2 was combined in screw cap flasks
- It was autoclave at 121^0C for 15 minutes, and allowed to cool down to room temperature
- It was then stored in refrigerator at 2 – 8^0C

WORKING NALC-NaOH solution

- This working solution was made fresh each day of use.
- 2.5 g of NALC reagent was added to 500 mls of NaOH – Na Citrate solution in a volumetric flask.

Phosphate Buffer 0.067M, pH 6.8

Stock Alkaline Buffer

- 9.07 g Na_2HPO_4 was dissolved in 100 ml of distilled water and made up to 1000 ml in a volumetric flask.

Stock Acid Buffer

- 9.07 g KH_2PO_4 in 100 mls of distilled water and made up to 1000 ml in a volumetric flask.

Working Phosphate Buffer, 0.067M, pH 6.8

- Equal volumes of stock alkaline and acid buffer in a 1 Liter screw cap flask
- The pH of the mixture was checked with a pH meter.
- It was titrated with the stock buffer if pH of mixture was not 6.8
- The mixture was autoclave at 121^0C for 15 minutes, and allowed to cool down to room temperature.
- Buffer was stored in refrigerator at 2 – 8^0C until use.

CULTURE MEDIA

LOWENSTEIN – JENSEN (LJ) MEDIUM

Mineral salt base solution:

- Potassium dihydrogen phosphate anhydrous (KH_2PO_4) 2.4 g
- Magnesium sulfate ($MgSO_4.7H_2O$) 0.24 g
- Magnesium citrate 0.6 g
- Asparagine 3.6 g
- Glycerol (reagent grade) 12 ml
- Distilled water 600mls

The ingredients were dissolved in order, in the distilled water by heating. It was autoclaved at 121^oC for 30 minutes to sterilize. It was then cooled to room temperature.

Malachite green solution:

- Malachite green dye 2.0 g
- Sterile distilled water 100 mls

Using aseptic techniques the dye was dissolved in sterile distilled water by placing in the incubator for 1 – 2 hours.

Homogenized whole eggs:

Fresh hen's eggs (not more than 7 days old), from hens that have not been fed antibiotic containing feed, were cleaned by scrubbing thoroughly with a brush in warm water and a plain alkaline soap. The eggs were cracked with sterile knife into a sterile knife into a sterile flask.

Preparation of medium

The following ingredients were aseptically pooled in a large, sterile flask and mixed well:

- Mineral salt solution 600mls
- Malachite green solution 20 ml
- Homogenized eggs (25 eggs) 1000ml

The complete medium is distributed in 6 – 8 mls volumes in sterile 28 mls McCartney bottles and inspissated within 15 minutes of distribution to prevent sedimentation of heavier ingredients.

Coagulation of medium

Before loading, the inspissator to 85^{0}C. The bottles were placed in a slanted position in the inspissator and the medium coagulated for 45 minutes at 85^{0}C.

Sterility check

After inspissation, a representative sample of culture bottles were incubated at 37^{o}C for 24 hours as a sterility check.

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