CHARACTERIZATION AND THERMOSTABILIZATION OF *AVIBACTERIUM PARAGALLINARUM* CANDIDATE STRAINS WITH POTENTIAL USE AS CANDIDATE VACCINE STRAINS IN TANZANIA

ANDREW CLAUD CHOTA

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ABSTRACT

This study was conducted to isolate, characterize, thermostabilize and develop an easier and cheap technique for preparation of inactivated and live vaccines against Infectious coryza (IC). Fifty six (56) samples were collected from chicken. Fourty eight percent (27) isolates had phenotypic characteristics suggestive of both pathogenic and non pathogenic *Avibacterium* species. Fifty nine percent (16) of 27 isolates were catalase negative, oxidase positive and NAD dependent. Moreover, they were positive to fructose, maltose, and sucrose, but negative to lactose and urease suggesting that they were *Avibacterium paragallinarum.* Identification and serotyping by polymerase chain reaction (PCR) confirmed that they were *Av. paragallinarum* serovar B. Chocolate broth (CB), a novel medium was used to propagate *Av. paragallinarum.* The broth's ability to support growth of *Av. paragallinarum* was compared to other three routine media and analysed using t-test. Results showed that null hypothesis was accepted between allantoic fluid (AF) and CB and between CB and modified brain heart infusion (MBHI) and was rejected between CB and brain heart infusion (BHI). The cost of production of 1 mL of CB was twenty six (26) times lower than the cost of producing AF and four (4) times lower than MBHI and BHI, therefore, CB is preferred as it is an easy to prepare and cheap medium. *Av. paragallinarum* isolates were thermostable at room temperature (RT) at the temperature range of 27-29^{0}C. The bacteria were recovered after storage for three months at RT in 2.5% gelatin. The findings from the present study have shown that *Av. paragallinarum* serotype B and other members of the genus *Avibacterium* exist in Tanzania. Propagation of *Av. paragallinarum* CB will possibly result in production of vaccines that are cheap and affordable to farmers.

Furthermore, preservation of *Av. paragallinarum* in 2.5% gelatin for 3 months is a significant finding for development of live vaccines against IC.

DECLARATION

I, Andrew Claud Chota, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been nor concurrently being submitted for a higher degree award in any other university.

…… ……

Andrew Claud Chota **Date**

(Msc. MBB candidate)

The above declaration is confirmed

…… ……

Dr. Kasanga C. J. **Date**

(Supervisor)

…… ……

Prof. Wambura P. N. **Date**

(Supervisor)

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DEDICATION

This work is dedicated to my lovely parents, Rosina Joseph Mponzi and the late Claud Paul Chota for bringing me up and spending their energy and resources to support the family education. Their commitment made it possible for me come this far.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percent
µl	Microlitre
μM	Micromole
^{0}C	Degrees of centigrade
A/R	Reverse primer for serovar A
ABC/F	Forward primer for serovars A, B and C
AF	Allantoic fluid
AP-1F	*Avibacterium paragallinarum* forward primer
AP-1R	*Avibacterium paragallinarum* reverse primer
B/R	Reverse primer for serovar B
BA	Blood agar
BAB	Blood Agar Base
BHI	Brain heart infusion
bp	Base pair
BTC	Belgian Technical Coorperation
C/R	Reverse primer for serovar C
CA	Chocolate agar
CB	Chocolate broth
CFU	Colony forming unit
CMI	Chicken Meat Infusion
CO_2	Carbon dioxide
CVL	Central Veterinary Laboratory
DNA	Deoxyribose nucleic acid

EDTA	Ethly-Dimethyl Triacetate
ERIC	enterobacterial repetitive intergenic consensus
et al	and others
g	Gram
h	Hours
kbp	Kilobase pair
lb	Pound
MBB	Molecular Biology and Biotechnology
MBHI	Modified brain heart infusion
MCA	MacConkey agar
min	Minutes
mls	Mililtres
MS	Master seed
Msc	Masters of science
NAD	Nicotinamide dinucleotide
*n*g	Nanogram
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribose nucleic acid
rpm	Revolutions per minute
RT	Room temperature
SUA	Sokoine University of Agriculture
Taq	Thermo *aquaticus*

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Tanzania has an area of 945 000 km^2 and a population of 35.03 million poultry, of which 94% (32.9 million) are local scavenging chickens, 1.8% (0.6 million) are commercial chickens and 4.2% (1.5 million) are other poultry (Ciamarra and Nsiima, 2011). Diseases are regarded by 95.5% of flock owners as the major constraint in poultry industry especially village chickens (Yongolo, *et al.*, 1997). Among other diseases, Infectious coryza (IC) is reported by farmers and cases recorded reflect the diagnosis derived at postmortem examination. In Tanzania, the disease is associated with overcrowding, low levels of biosecurity, rampant movement of poultry in households and mixing of birds from different flocks (Msami, 2007).

IC is caused by *Avibacterium Paragallinarum (Av. Paragallinarum),* an important avian pathogen occurring worldwide (Blackall *et al.*, 2005) especially in farms keeping multi-aged birds and is influenced by environmental factors (Blackall, 1999). The bacteria have been isolated and characterized in neighboring countries where Byarugaba *et al.* (2006) isolated these bacteria in Uganda and reported that there were more positive samples by ERIC-PCR than cultures.

A report by Mouahid *et al*. (1989) showed that IC causes major economic losses in the poultry industry due to increased culling rates and reduction in egg production (up to 40%). Mortalities can be as high as 48% (Bland *et al.*, 2002). The situation

can be even worse in Tanzania due to overcrowding, keeping multiaged flocks and low level of biosecurity.

Use of antibiotics for treatment against IC does not give good results due to high resistance of *Av. paragallinarum* to these antibiotics (Byarugaba *et al.,* 2011). Studies show that preparation of the vaccine from *Av. paragallinarum* local strain Tan 1-05 provoked production of antibodies and protective immunity in chickens (Wambura, 2010) and that vaccination using international vaccines has not shown good results (Bragg *et al.,* 1996; Terzolo *et al.,* 1997). Therefore , use of autogenous vaccines is highly recommended. This study aimed at isolating, characterizing and selecting a candidate strain and developing an easy and cheaper technique for preparation of attenuated live and inactivated vaccines for control of IC in Tanzania.

1.2. Objectives

1.2.1 Overall objective

To contribute towards the development of live and inactivated vaccines that can be used in control of IC in poultry in Tanzania.

1.2.2 Specific objectives

i. To identify, isolate and characterize *Av. paragallinarum* in order to select candidate strains for use in vaccine development.

ii. To develop an easy and cheap novel broth for propagation of *Av. paragallinarum.*

iii. To determine the thermostability condition(s) of the candidate strains for use in vaccine development.

1.2.3 Hypotheses

Ho Ability of CB and AF to support growth of *Av. paragallinarum* are not different

Hi Ability of CB and AF to support growth of *Av. paragallinarum* are different

Ho Ability of CB and MBHI to support growth of *Av. paragallinarum* are not different

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CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Infectious coryza

Infectious coryza is caused by *Av. paragallinarum*, and affects the upper respiratory tract and parasinuses of chickens (Blackall et al., 2005) causing high economic losses due to loss in condition of broilers and reduced egg production in layers (Mouahid *et al.*, 1989).

2.2. Infectious coryza epidemiology

2.2.1. Etiologic agent

Av. paragallinarum is the causative agent of IC, the bacteria is the member of the family Pasteurellaceae and were re grouped from *Haemophilus paragallinarum* (Blackall et al., 2005). The bacteria are gram - negative, nonmotile, filamentous, and pleomorphic with small grey colonies on chocolate agar (Mendoza-Espinoza *et al.*, 2008). All isolates of the disease causing agents previously showed to require only Nicotinamide Adenine Dinucleotide (NAD) for growth (Rajurkar, *et al.*, 2009). However, recent findings show that A*v. paragallinarum* can grow in absence of NAD (Garcia *et al.*, 2002) and some grow on modified media that are not capable of supporting other typical *Av. paragallinarum* isolates (Blackall *et al*., 2011). Currently there are two different but related serotyping schemes for *Av. paragallinarum* namely Page and Kume schemes (Blackall, 1999). Page agglutination scheme, a serologic classification of *Av. paragallinarum* is the most widely used and in this a total of three serovars namely, serovars A, B and C are recognized (Page, 1962). Using inhibition-hemagglutination technique seven

serovars (HA1 to HA7) were identified. These were distributed into three serogroups (I, II and III) in a scheme known as Kume scheme (Kume et al., 1983).

2.2.2. Transmission

Chronically ill or healthy carrier birds are the reservoir of infection. Chickens of all ages are susceptible, but susceptibility increases with age. The incubation period is 1-3 days, and the clinical disease persist for 2-3 weeks. Under field conditions, the duration may be longer in the presence of concurrent diseases, eg, mycoplasmosis, and infected flocks are a constant threat to uninfected flocks. Transmission is by direct contact, airborne droplets, and contamination of drinking water. Chickens become susceptible at about 4 weeks of age from hatching (Blackall, 2011), a fact that calls for a timely early vaccination.

2.3. Clinical Signs

Mild form of the disease manifests as depression, serous nasal discharge and slight facial swelling, and in severe disease swelling of one or both infraorbital sinuses is marked and oedema of the surrounding tissues may extend to the wattles (Rajurkar, *et al.,* 2009).

2.4. Diagnosis

2.4.1. Culture techniques

The causative agent of IC is a highly fastidious organism; for a long time it was believed that usual culture media were not supporting growth of these bacteria and that their growth was only possible in the presence of NAD (Blackall *et al.,* 2005).

However, there are some isolates of *Av. paragallinarum* that do not need NAD (Garcia *et al.*, 2002). Culturing *Av. paragallinarum* on the other hand, needs careful handling because recently there have been reports of growth of these bacteria in media that are not capable of supporting other typical *Av. paragallinarum* (Blackall *et al*., 2011). The most universally medium used for isolation of *Av. paragallinarum* remains to be 5% sheep blood agar with a cross-streak of a nurse culture of *Staphylococcus epidermidis* to provide the required NAD. This medium aids in the recognition of NAD dependent *Av. paragallinarum* as, the organism will show satellitism (Blackall, 2011).

2.4.2. Biochemical techniques

Following isolation, biochemical tests at minimum, Gram staining, catalase test and dependence on NAD for NAD-dependent *Av. paragallinarum* from birds showing clinical signs (Blackall, 2011) have to be done. NAD-independent *Av. paragallinarum* are catalase negative but does not need NAD factor or growth.

2.4.3. Molecular techniques

In laboratories lacking experience in handling NAD-requiring organisms and in regions where NAD-independent *Av. paragallinarum* are common the use of PCR is the best approach to diagnose the disease (Blackall, 2011). This conventional PCR targeting the hemagglutinin gene was developed specifically for identification of *Av. paragallinarum* (Chen *et al.*, 1996) and was validated for use on isolates and directly on birds (Chen *et al.*, 1998). This technique is more sensitive as compares to other methods of diagnosis (Byarugaba et al., 2006). A new technique for serotyping

of *Av. paragallinarum* isolates has been developed by Sakamoto *et al.* (2012). In this technique a hypervariable region *HMTp210* which encodes for the hemagglutinin (HA) gene is used as a target gene. This technique is more easy and sensitive as compared to the other serotyping schemes.

2.5. Treatment and control

Following treatment with various antibiotics like tetracycline and enrofloxacin, relapses do occur and recovered birds remain as carriers (Yamamoto, 1991; Byarugaba *et al.,* 2011). Due to poor biosecurity (Wambura, 2010) and repeated movement of poultry in the house hold (Msami, 2007), vaccination using autogenous vaccines has remained the most reliable way to prevent the occurrence of the disease (Wambura, 2010). Vaccines developed using local strains are highly advocated (Blackall and Reid, 1987) due to poor results on protection shown by international vaccines (Terzolo *et al.,* 1997). There is now widespread acceptance that the Page serovars (A, B and C) do not cross-protect. It is also known that not all serovar B isolates are cross-protective (Yamaguchi *et al.,* 1991). Due to the inability of different serovar B isolates to cross-protect, commercial vaccines now include multiple B strains (Jacobs *et al.,* 2003). A study by Soriano *et al.* (2004) showed that cross-protection was good within the four serovar A subtypes and poor within the serovar C subtypes. Use of recombinant vaccines have shown to provide good protection (Wang *et al.,* 2007; Noro *et al.,* 2008 and Wu *et al.,* 2011). However, their production and use in developing countries remains difficult.

2.6. Infectious coryza vaccines

2.6.1. Killed vaccines

Inactivated vaccines are made from microorganisms (viruses, bacteria, other) that have been killed through physical or chemical processes. These killed organisms cannot cause disease. Several studies indicate that chickens are protected when autogenous vaccines are used (Chukiatsiri, *et al.,* 2009; Wambura, 2010). While only killed whole cell coryza vaccines remain the only current commercially available vaccines, there has been active research on possible alternative approaches. Vaccines prepared in mineral oil and aluminium hydroxide adjuvants show better results in terms of protection (Chukiatsiri, *et al.,* 2009; Wafaa and El-Ghany, 2011).

2.6.2. Live vaccines

Live attenuated vaccine (LAV), A vaccine prepared from living micro-organisms (viruses, bacteria currently available) that have been weakened under laboratory conditions. LAV vaccines will replicate in a vaccinated individual and produce an immune response but usually cause mild or no disease. Microorganisms used in preparation of live attenuated vaccines grow in a vaccinated individual, but because they are weak, they will cause no or very mild disease. To date efforts are still underway to develop a live vaccine against IC. The difficult challenges to development of the live vaccines are hinged to the fastidious nature of the bacteria which leads to a very short life span, Stokes (1962), reported on a short term preservation of two days for Haemophilus on BA and CA. Trials on vaccinations against IC using live vaccines have, for a long time, been limited to the use of live

cultures from embryonated chicken eggs (Tennison and Siddle, 1961). Scientists have been working on the same principle for over twenty years (Blackall, P. personal communication, 2012). Live vaccine may solve the problem of failures in cross protection among serovars.

2.6.3. Subunit vaccines

Subunit vaccines, like inactivated whole-cell vaccines do not contain live components of the pathogen. They differ from inactivated whole-cell vaccines, by containing only the antigenic parts of the pathogen. These parts are necessary to elicit a protective immune response. Advances on cell biology research have lead to development of recombinant vaccines. Recombinant haemagglutinin antigens can provide protection against *Av. paragallinarum* (Noro *et al.*, 2008; Wu *et al.*, 2011). Additionally, some polypeptides have also shown to be capable of providing protection (Wang *et al.*, 2007). However, on my opinion use of advanced and sophisticated technology in production, may result in development of vaccines that are expensive and farmers in developing countries cannot afford to purchase.

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Study area

Samples were collected from different zones of Tanzania. The selection criterion was representation of the ecological zones including, Southern (SZ) zone, Southern highlands (SHZ) zone, Central (CZ) zone, Eastern (EZ) zone, Western (WZ) and Northern (NZ) zones and some areas of Zanzibar isles (ZI) (Appendix 1). These areas differ in various aspects ranging from rainfall amount, weather topography, culture of inhabiting society, altitude and geographical locations sea.

Laboratory works were carried out at Sokoine University of Agriculture, Department of Veterinary Microbiology and Parasitology and Inqaba Biotechnical Industries, Pretoria SA.

3.2. Sample collection

Samples were collected by taking nasal and tracheal swabs from apparently healthy chicken, clinically sick birds and dead birds with history suggesting that previously they had clinical signs suggestive of IC. The samples from apparently healthy and clinically sick birds were streaked on chocolate agar (CA) immediately and the agar were kept in candle jars and transported to the laboratory. However, in case of dead birds, heads were chopped off and transported to the laboratory and nasal and tracheal swabs were streaked on CA. Samples were sourced from different ecological zones in Tanzania to try to isolate different serovars (Appendix 1). A total of 56 samples were collected (Table 1) and processed to isolate the bacteria. Isolation from different ecological zones that differ in rainfall amount, weather,

topography, culture of inhabiting society and altitude and geographical locations at getting representative samples to try and identify the serovars circulating in the country.

Table 1: Number of samples of tracheal and nasal swabs collected from each ecological zone in Tanzania.

Ecological zone	Representative location	Number of samples
Central	Dodoma	7
Western	Tabora	13
Southern highlands	Iringa	6
Northern	Manyara	3
Eastern	Morogoro	11
Southern	Mtwara	6
Zanzibar Isles	Bungi (Southern Unguja)	8

3.3. Media preparation

Chocolate agar (CA) was prepared from blood agar base (BAB) as per manufacturer's (HiMedia Laboratories Pvt.Ltd) instructions with modification. Briefly, BAB was measured (21.25 g then dissolved in 500 ml of distilled water-), heated and autoclaved at 15lb at 121^0C for 15 min. BAB was cooled to 45^0C and horse blood was added (1:10) and modified by heating in a 80^0C water bath for 15 min to expose the V factor, cooled to 45^0C and then poured on plates to prepare CA. Blood agar (BA) and MacConkey agar (MCA) were prepared as per manufacturer's HiMedia Laboratories Pvt. Ltd and TULIP DIAGNOSTICS (P) LTD respectively) instructions. Briefly, BAB and MCA were measured (21.25 g then dissolved in 500 ml of distilled water) and (55 g then dissolved in 1000 ml of distilled water) respectively, autoclaved, cooled to 45^0C and poured on plates. The media prepared were allowed to solidify on plates and incubated at 37^0C for 24h to check for sterility.

3.4. Characterization of isolates

3.4.1. Biological characterization

3.4.1.1. Bacterial culture

The culture media were incubated in 5% CO_2 in a candle jars at 37^0C for 24h (Dousse *et al*., 2008). Dew drop colonies from the primary culture were picked up using a straight wire loop and were subcultured in new sterile CA to get pure cultures. Pure cultures were tested for dependence on NAD by growing on BA and MCA (Wafaa and El Ghany, 2011) on similar conditions. Colonies were stained by gram and Indian ink staining methods and biochemical and molecular techniques were carried out (Byarugaba *et al*., 2006).

3.4.1.2. Gram staining

Pure colonies from CA were stained using the Gram staining technique (Adderson, 2008). Briefly, smears were prepared on glass slides with bacterial colonies and normal saline, air dried and heat fixed. Smears were then stained with methyl violet and lugol's iodine was used as a mordant, washed with acetone alcohol and counter stained with neutral red then examined under the microscope using 100 x magnifications micromophological characteristics.

3.4.1.3. Capsule staining

Capsule staining was done by using Indian ink (Adderson, 2008). Briefly, normal saline was placed on the glass slide close to an Indian ink drop. Then the bacterial colonies were taken from plates by using a sterile wire loop. Bacterial colonies were mixed with normal saline, spread with a sterile wire loop while slowly mixing with

Indian ink. Then wet preparations were covered with cover slips and examined under the microscope using 100 x magnifications for motility and capsules.

3.4.2. Biochemical characterization

All isolates with phenotypic characteristics suggestive of *Avibacterium* species were characterized biochemically. The bacteria were tested for dependence on NAD, oxidase, urease, catalase positivity and ability to ferment sugars that is, glucose, sucrose, mannose, maltose and fructose (Byarugaba *et al.*, 2006 and Wambura, 2010). Sugars were prepared at 2% concentration where Andrade's peptone water was used as an indicator. Andrade peptone water was prepared as per manufacturer's (HiMedia Laboratories Pvt.Ltd) instructions. After aliquoting in bottles the sugars were steamed for 15 min, cooled at RT then incubated at 37^0C for 24h and sterile sugars were stored for use.

3.4.3. Molecular characterization

3.4.3.1. DNA extraction

DNA was extracted from colonies suspected to be of *Av. paragallinarum* using Sepa gene extraction kit as per manufacture's (*Sepa Gene* Ltd) instructions with modifications (extraction kit protocol is shown in appendix 2). Modification was made by using pure colonies (instead of tissues) as a starting material. Briefly pure colonies were suspended in 200 µl of Nuclease Free Water in 1.5 mls Eppendorf tubes. Seven hundred microlitre of sterile buffered saline (PBS) was added in each tube and centrifuged at low speed of 3000 rpm for 10 minutes at 4^0C. Supernatant was not discarded as per protocol but was transferred into new Eppendorf tubes.

Fifty microlitre of solution I was added to the supernatant and vortex-mixed. Suspension was incubated at room temperature for 10 minutes before adding 50 µl of solution II and 350 µl of solution III, followed by 200 µl of solution IV and vortex-mixed. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4^0C. Supernatant was transferred into new Eppendorf tubes and 30 µl of solution V was added followed by 330 µl of Isopropanol and incubation at -20^0C for 1.30 hours. The mixture was then centrifuged at 13,000 rpm at 4^0C for 30 minutes. The pellets were then washed with 40 µl of 70% chilled alcohol and resuspend in 50 µl of sterile TE buffer. DNA samples were tested for purity by running on agarose gel (Roodt, 2009) then stored at -20^0C until use.

3.4.3.2. Polymerase chain reaction (PCR)

Identification PCR for *Av. paragallinarum* was carried out at Inqaba Biotechnological Industries, Pretoria-South Africa (SA) using a PCR assay based on species-specific DNA primers (Table 1), AP-1F and AP-1R (Chen *et al.*, 1996). The reaction mixture contained 2µM of the primer sets AP-1F and AP-1R (Table 1), 0.5µg of genomic DNA, 5µl of 10 x ThermoPol buffer, 200µM of dNTP's and 2 units of Taq polymerase, filled to 50µl with Nuclease Free Water. The reaction cycle included an initial denaturation cycle of 10 min at 94°C applied to whole cell PCR in order to break cells to enable DNA release after which the 2 units *Taq* polymerase were added. This was followed by 30 cycles of denaturation (94°C for 25 sec); annealing (55°C for 50 sec); elongation (72°C for 45 sec) and a final elongation cycle of 7 min at 72°C, with an expected amplification result of approximately 500 bp.

3.4.3.3. Serotyping of *Av. paragallinarum* using PCR

The technique of amplifying the specific serovars of *Av. paragallinarum* as described by Sakamoto *et al.* (2012) was used with slight modification. Briefly normal PCR was used rather that a Multiplex PCR used by Sakamoto and others. PCR was done by running selected samples using primer sets in table 1. 0.2 μM each of ABC forward primer, A-reverse, B-reverse and C-reverse primers (Table 2), 12.5 μM of the master mix (The Chemo-Sero-Therapeutic Research Institute, Kikuchi Research Center, 1314–1 Kawabe Kyokushi, Kikuchi, Kumamoto, 869–1298, Japan) and 100 *n*g of the template DNA. The reaction cycles were as follows: 98^0C for 1 min; 30 cycles of 98^0C for 10 sec, 56^0C for 10 sec and 72^0C for 2 min; and final step at 72^0C for 7 min. PCR products were analyzed by agarose gel electrophoresis. Each sample was run using all three sets of the primers that is ABC/A, ABC/B, and ABC/C.

Table 2: The set of primer pairs used in this study

Primer set #	Primer	Primer sequence	Fragment length
1	ABC forward	5'-GGCTCACAGCTTTATGCAACGAA-3'	0.8 kbp
	A reverse	5'-CGCGGGATTGTTGATTTTGTT-3'	
2	ABC forward	5'-GGCTCACAGCTTTATGCAACGAA-3'	1.1 kbp
	B reverse	5'-GGTGAATTTCACCACACCAC-3	
3	ABC forward	5'-GGCTCACAGCTTTATGCAACGAA-3'	1.6 kbp
	C reverse	5'-TAATTTTCTTATTCCCAGCATCAATACCAT-3'	
4	AP-1F	TGA GGG TAG TCT TGC ACG CGA AT	500 bp
	AP-1R	CAA GGT ATC GAT CGT CTC TCT ACT	

3.5. Novel technique for bacterial propagation

3.5.1. Preparation of the culture media

3.5.1.1. Chocolate broth (CB)

Chocolate broth was prepared by enriching nutrient broth (NB) with horse blood (Turk., 1964) with modification. NB {with peptic digest of animal tissue (5.0 g/litre), sodium chloride (5.0 g/litre), beef extract (1.5 g/litre) and yeast extract (1.5 g/litre)} were measured and prepared as per manufacture's (HiMedia Laboratories Limited, Mumbai) instruction. After autoclaving (121^0C, 15lb for 15 minutes), NB was cooled to 45^0C and horse blood collected in EDTA was aseptically added {10 milliltre ml)} in every 100 ml of NB to make a 10% enriched medium. This medium was then heated in a water bath set at 80^0C while gently agitating for 15 minutes. Sterile gauze was used to filter the CB to remove unwanted debris. The broth was tested for sterility by streaking on CA, BA and MCA and incubating at 37^0C in 5%CO_2 for 24h and stored in sterile containers at 4^0C until use.

3.5.1.2. Brain heart infusion broth (BHI)

A standard media brain heart infusion broth was prepared as per manufacture's instruction (Oxoid Ltd, Basingstoke, and Hants, England). Briefly, BHI broth Briefly, BHI broth {with Calf Brain Infusion Solids (12.5 g/litre), Beef Heart infusion solids (5.0 g/litre), Proteose peptone (Oxoid L46), (10.0 g/litre), Sodium chloride (5.0 g/litre), Dextrose (2.0 g/litre) and Disodium phosphate anhydrous (2.5 g/litre)} were measured and prepared as per manufacturer's (Oxoid Ltd, Basingstoke, Hants, England) instructions. Autoclaved BHI was aliquoted and stored at 4^0C for use.

3.5.1.3. Modified brain heart infusion broth (MBHI)

MBHI broth was prepared by enriching BHI broth with horse blood (Mendoza-Espinoza *et al*., 2008) with modification. Briefly, BHI broth {with Calf Brain Infusion Solids (12.5 g/litre), Beef Heart infusion solids (5.0 g/litre), Proteose peptone (Oxoid L46), (10.0 g/litre), Sodium chloride (5.0 g/litre), Dextrose (2.0 g/litre) and Disodium phosphate anhydrous (2.5 g/litre)} were measured and prepared as per manufacturer's (Oxoid Ltd, Basingstoke, Hants, England) instructions. Autoclaved BHI was cooled to 45^0C and horse blood collected in EDTA was aseptically added (10 ml) in every 100 ml of NB to make a 10% enriched medium. This medium was then heated in a water bath set at 80^0C while gently agitating for 15 min. sterile gauze was used to filter the MBHI to remove unwanted debris. The broth was tested for sterility and stored in sterile containers at 4^0C until use.

3.5.1.4. Embryonated chicken eggs

Embryonated chicken eggs (ECE) were bought from Sokoine University of Agriculture (SUA) farm, examined, cleaned with alcohol, labelled and incubated for 8 days at 37^0C in a moistured incubator (Tennison and Siddle, 1961; Wambura 2010) with small modification on the age of inoculation. Eggs were candled and all eggs with dead embryos were discarded while fertile eggs were used for propagation of the bacteria.

3.5.1.5. Sterility

All media, CB, MBHI and BHI and ECE swabs were tested for sterility by striking on BA, MCA and CA in duplicate (Wambura, 2010) with modification. One pair was incubated on 5% CO_2 in candle jars and the other pair in aerobic condition at 37^0C for 24h. The culture media were aseptically suspended in sterile universal bottles in duplicate and each pair was tested for sterility by striking on BA, MCA and CA followed by incubation in 5% CO_2 candle jars and in aerobic conditions at 37^0C for 24h.

3.5.2. Bacterial propagation

3.5.2.1. *Av. paragallinarum* inoculation in CB, BHI and MBHI

Five milliliters of each medium suspended in duplicate in universal bottles were inoculated with 1 millilitres (ml) of the *Av. paragallinarum* suspended in aliquoted normal saline.

3.5.2.2. Incubation of the inoculated media

Inoculated media were incubated at 37^0C on 5% CO_2 in candle jars for 24h. Media were examined for turbidity and subcultured in CA under similar conditions after 24h. After 48h of incubation, the culture media was used for ten fold dilution and counting. Inoculation was done only on media that had passed a sterility test.

3.5.2.3. *Av. paragallinarum* inoculation in AF

The ECE were candled after 9 days and all those with viable embryos were cleaned again, labelled and a hole of was made 0.1mm above the air space mark. Then 0.1

millilitre of *Av. paragallinarum* suspended in the sterile normal saline in aliquots was inoculated through allantoic cavity route as described by Reid and Blackall (1983). However Reid and Blackall inoculated 1 ml of the bacterium the hole was then sealed with paraffin wax.

3.5.2.4. Incubation of embryonated chicken eggs

Inoculated embryonated chicken eggs were incubated at 37^0C for 24h. After 24h, the eggs were candled to examine embryo viability and all eggs with dead embryos were removed. Following incubation for 48h, allantoic fluid was harvested from all eggs with viable embryos. AF was then streaked on CA and incubated on 5% CO_2 in candle jars at 37^0C for 24h. Another sample was taken for hemagglutination test and bacterial count.

3.5.3. Bacterial count

Bacterial count was done by ten fold serial dilution of the bacterium as described previously by Cruickshank *et al.* (1968). Briefly, sterile universal bottles were used in making a ten fold serial dilution of the bacterium grown on different growth media. Ten universal bottles were used for each growth medium. One milliliter of each inoculated growth media was diluted in 9 ml of sterile normal saline and then serially transferred and diluted in 9 ml in the next nine universal bottles (Cruickshank *et al.,* 1968).

After dilution 0.1 ml of the diluted broth was inoculated on CA and incubated at 37^0C on 5% CO_2 in candle jars for 24h. The resulting counts were multiplied by 10 to get the number of colonies in 1 ml using the formula:

N = Dx where:-

N = Number of colonies per ml.

D = Dilution factor.

X = Average number of colonies counted.

Four countings were done on each growth medium. The first counting was done by the author and the second, third and fourth countings were blind counting done by other fellow scientist on request. The plates were labelled W for AF, X for CB, Y for BHI and Z for MBHI and counting was done by three fellows who had no information other than plates identification. The average of the four counts was taken. Amount of experimental vaccine each 1 ml of the medium can produce was calculated using the formula $C_1V_1 = C_2V_2$ and costs of producing 1 ml of each culture media were calculated basing on the cost of purchasing 500g of each medium and cost of purchasing an embryonated chicken egg.

3.6. Thermostabilization of the Isolates

3.6.1. Bacteria preparation

Isolates Avp.D3 and Avp.M4 were used in thermostabilization. The isolates were passaged in the CA daily with four to five days of storage at 4^0C in between. After fourteen passages fully grown bacteria were left at room temperature in tightly closed jars for three days and recovered again. Recovered bacteria were stained by Gram and Indian ink staining techniques for microscopic and motility and capsule examination. Catalase positivity test and was done and dependence on NAD was done by streaking the colonies on MCA, BA and CA. After adaptation to the

laboratory environment, a single colony was harvested and inoculated in 5 mls of CB for propagation and storage (Turk, 1964) with small modification that Turk used CA and in case a novel media CB was used. Inoculated medium was incubated at 37^0C under microaerophilic conditions in candle jar for 48h. *Av. paragallinarum* were serially diluted in normal saline and counted (Cruickshank *et al.*, 1968). Colony forming unit (CFU) / ml was established and bacteria were preserved in different gelatin concentrations.

3.6.2. Gelatin preparation as a stabilizer

Gelatin was prepared at 5%, 7.5%, 10%, 15% and 20% in duplicate. Colonies of *Av. paragallinarum* were harvested and suspended on tubes containing CB and incubated at 37^0C at microaerophilic condition for 48h. The resulting propagated *Av. paragallinarum* were suspended in gelatin at 4.56 x 10^{12} CFU/ mL. Final concentration of each gelatin was adjusted to 2.5%, 3.75%, 5%, 7.5% and 10%.

3.6.3. Bacteria preservation

Av. paragallinarum preserved in gelatin at different concentrations were kept in duplicates at room temperature (RT) with a range of 27-29^0C, 4^0C, and -20^0C (Sengiyumva, 2010) with modifications on gelatin concentrations used.

3.6.4. Bacteria recovery

Bacteria were then subcultured in CA daily for 1 week followed by once in a week for one month and after every one month to see the viability (Turk, 1964) with small modification as Turk used CA to store *Haemophilus influenzae*. Recording of

recovered bacteria was inferred as survival and started at the second week. Scores were given as 3+ if the whole plate had the colonies, 2+ if at least 75% of the plate had colonies and 1+ if at least 50% of the plate had colonies. However, even a single colony was counted as a survivor (Sengiyumva, 2010). A second set of the gelatin-preserved bacteria were subcultured after two months to establish the effect of repeated freezing and thawing cycles. The bacteria were preserved for three months.

3.7. Data analysis

Data were analysed in Excel spreadsheet and Epi-info version 3.5 and others were presented as mean values and were analysed statistically by the Student's t – test. . Mean values of CFU/ml of the different media (CB, MBHI, BHI and AF) were compared

CHAPTER FOUR

4.0. RESULTS

4.1. Characterization of *Av. paragallinarum* isolates

4.1.1. Biological characterization

4.1.1.1. Bacterial culture

Figure 2, shows dew drop colonies of Av. paragallinarum grown on agar plates, Figure 2A, a still picture and Figure 2B a stereo microscopic picture of the colonies appearing opaque. The chocolate coloured background is CA. There were no growth on BA and MCA indicating that the growth factor NAD is required for *Av. paragallinarum* to grow.

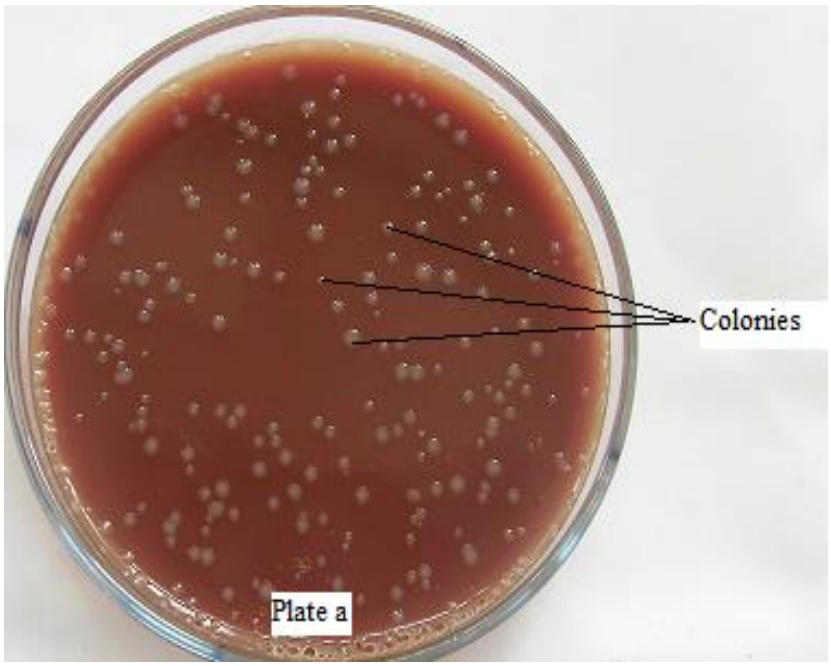
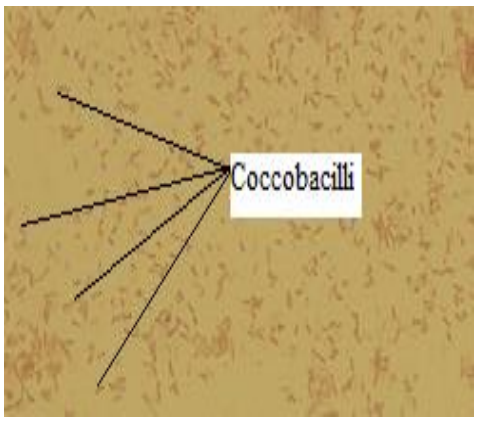


Figure 1: *Av. paragallinarum* colonial morphology in chocolate agar: dew drop colonies are seen as opaque after overnight incubation.

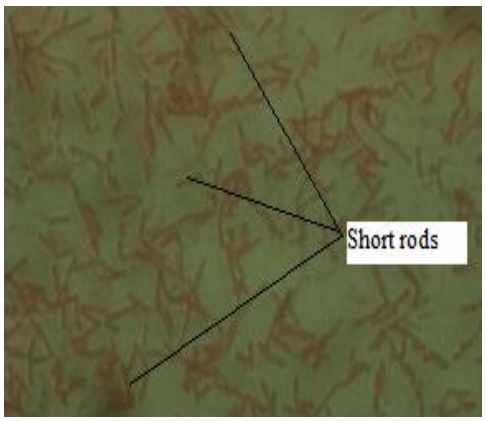
4.1.1.2.. Bacterial gram stain

Isolates were gram negative coccobacilli on gram staining (Figure 2A). Some of them showed pleomorphism while older cultures that stayed for about 72 hours at 4^0C formed short rods as seen in Figure 2B and filamentous form as seen in Figure 2C.

A



B



C

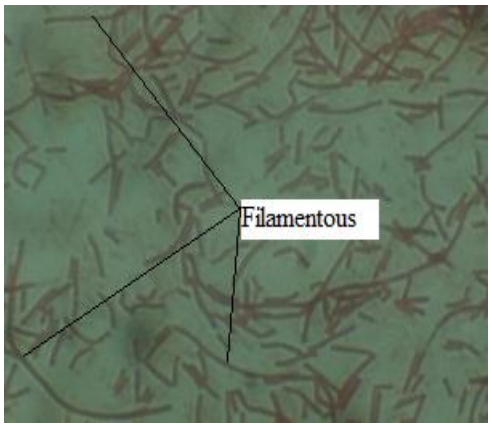


Figure 2: *Av. paragallinarum* Gram staining showing different forms of the bacteria as it change with time, A. gram negative Coccobacilli, B. gram negative short rods and C. gram negative filamentous form

4.1.1.3.. Bacterial capsule stain

Wet smears stained by Indian ink stain revealed that the bacteria are non motile and capsulated. In Figure 3 below capsules are seen as hollow structures because they did not take the stain when background was stained with an Indian ink.

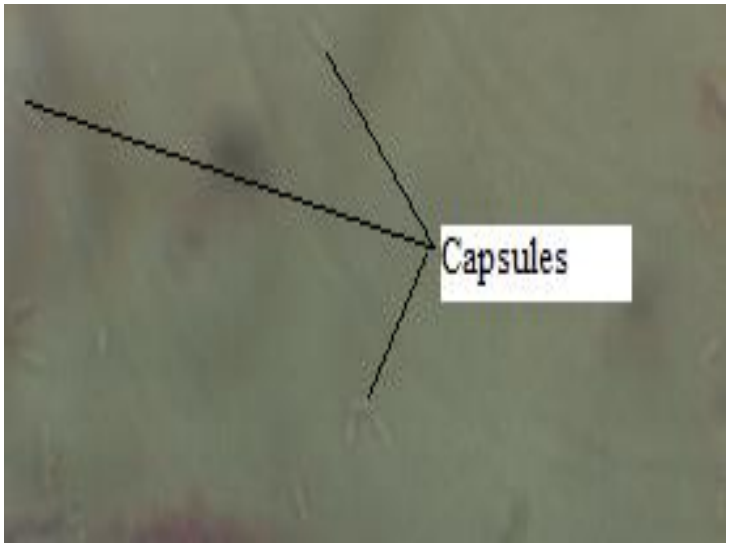


Figure 3: *Av. paragallinarum* Indian ink stain, the bacterial capsules appear as hollow whitish structures and the background is stained bluish.

From the biological characterization tests used above it was shown that 48% (27) of the samples had phenotypical characteristics of *Avibacterium* species including Avibacterium *paragallinarum*, *Avibacterium avium* and *Avibacterium volantium*. Ecological zones from which positive samples were sourced are shown in Figure 4.

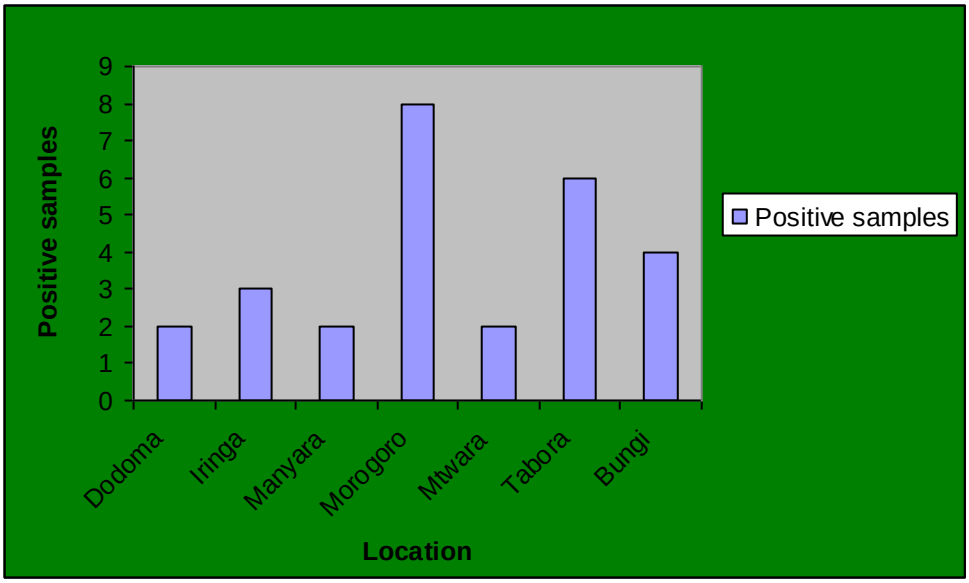


Figure 4: Samples from different ecological zones that tested positive to *Avibacterium* species.

Legend: Dodoma (Central Zone), Iringa (Southern Highland Zone), Manyara (Northern Zone), Morogoro (Eastern Zone), Mtwara (Southern Zone), Tabora (Western Zone) and Zanzibar (Bungi).

4.1.2. Biochemical characterization

All isolates were NAD dependent, oxidase positive and urease negative. However, they did not ferment lactose but fermented fructose, maltose, mannose, glucose and sucrose. Of those isolates, 59% (16) isolates were *Av. paragallinarum* as they were catalase negative.

4.1.3. Molecular characterization

4.1.3.1 Identification PCR

Isolates L2 were amplified by *Av. paragallinarum* specific primers and had a band size of 500 bp (Figure 5) which is the expected band size of *Av. paragallinarum* according to Chen *et al*. (1996).

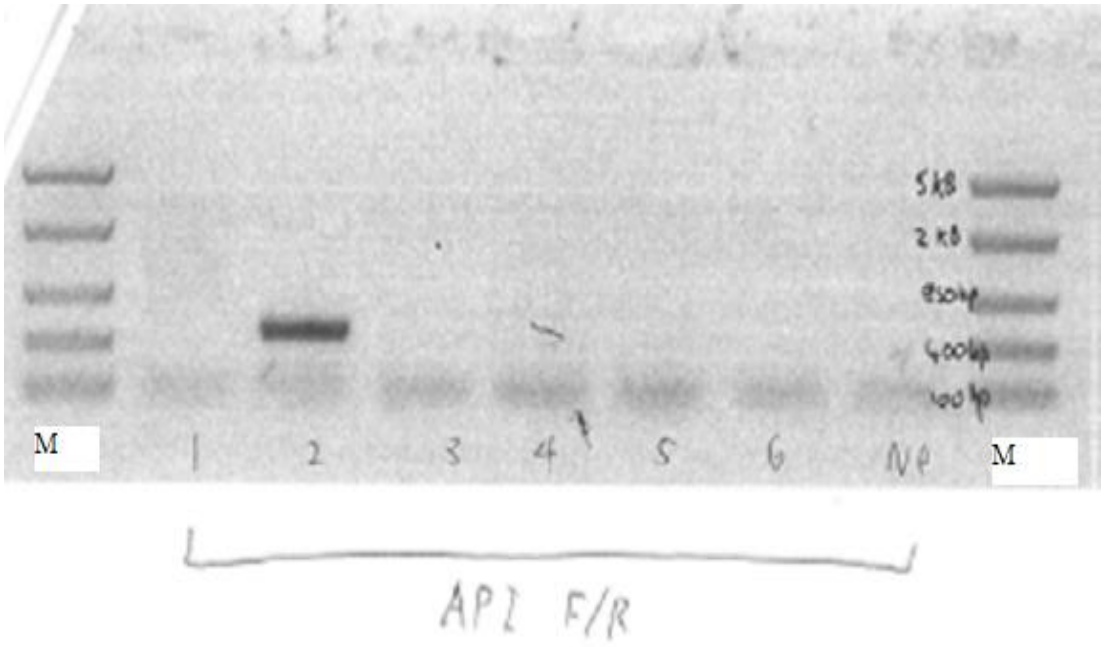


Figure 5: *Av. paragallinarum* identification by PCR using specific primers for identification of the bacteria.

Key: M indicates the molecular marker; lane Ne indicates the negative control and lanes L1, L3, L4, L5 and L6 were not amplified. GeneRuler^{TM} (Fermentas) was used as molecular marker

4.1.3.2. Serotyping of *Av. paragallinarum*

Isolates 3 and 4 were amplified by primers specific for serovar B with a 1.1 kbp band size. These are *Av. paragallinarum* serovar B (Figure 6) according to Sakamoto *et al* (2012). Of all the samples serotyped, none of the other serovars (serovar A and serovar C) was identified.

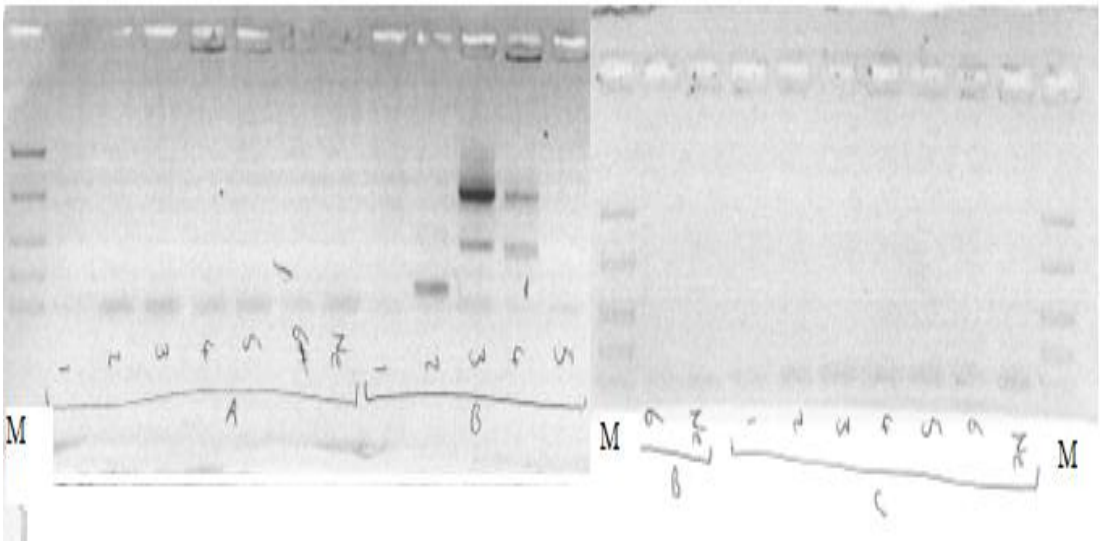


Figure 6: Serotyping of *Av. Paragallinarum* using specific primers for serotypes A, B and C

Key: M indicates the molecular marker, Ne indicates the negative control and lanes L1, L2, L3, L4, L5, L6 (A) were samples processed using ABC/A, F/R primers and were all negative, lanes were L1, L2, L3, L4, L5, L6 (B) samples processed using ABC/B, F/R and L2, L3, L4 were positive where as L1, L5 and L6 were negative and lanes L1, L2, L3, L4, L5, L6 (C) were samples processed using ABC/C, F/R primers and were all negative.

4.2. Novel media for bacterial propagation

4.2.1. Bacterial growth

All media and ECE supported propagation of *Av. paragallinarum*. Dew drop colonies were seen on CA streaked with propagated *Av. paragallinarum* from each medium and allantoic fluid (AF) from ECE. However, there were no growth on MCA and BA streaked with bacteria from the same media. CFU counts (Table 3) showed that *Av. paragallinarum* propagation was good in ECE, followed by CB, MBHI and BHI broth was the least. Since t- calculated is smaller than t-tabulated

between AF and CB and between CB and MBHI [AF and CB at p=0.05 (|T| =1.1405 ≤ t (0.05, 2) = 2.447)) and between CB and MBHI p=0.05 (|T| =0.9691 ≤ t (0.05, 2) = 2.447)], the null hypothesis that the ability of AF and CB and ability of CB and MBHI to support propagation of *Av. paragallinarum* are not different was accepted and the alternative hypothesis that the ability are different was rejected. On the other hand since t-calculated is greater than t-tabulated between CB and BHI, between BHI and MBHI, Between AF and BHI and between AF and MBHI [CB and BHI at p=0.05 (|T| =3.4547 ≥ t (0.05, 2) = 2.447) , BHI and MBHI at p=0.05 (|T| =5.0168 ≥ t (0.05, 2) = 2.447), AF and BHI at p=0.05 (|T|=5.3693 ≥ t(0.05,2) = 2.447) and AF and MBHI p=0.05 (|T| =2.5153 ≥ t(0.05,2) = 2.447)] therefore we reject the null hypothesis that the ability of CB and BHI, BHI and MBHI, AF and BHI and AF and MBHI to support propagation of *Av. paragallinarum* are not different and accept the alternative hypothesis that their abilities are different. *Av. paragallinarum* propagated in CB were preserved in 2.5% gelatin for use in vaccine production.

Table 3: Mean CFU count / mL of each medium (row 2) and also the same mean CFU / mL expressed as a Log of 10 (row 3).

Type of growth media / No of counts	**BHI**	**MBHI**	**CB**	**AF**
Average CFU/mL	2.19×10^{11}	1.94×10^{12}	2.72×10^{12}	3.84×10^{12}
Log 10 of CFU/mL	3.11	3.29	3.44	3.59

Key: AF– Allantoic fluid, BHI - Brain heart infusion, CB – Chocolate broth CFU – Colony forming unit, MBHI – Modified brain heart infusion

4.2.2. Amount of vaccine that can be produced by each 1 ml of medium

Taking into account the mean CFU / mL obtained results shows that 1 ml of AF can possibly produce approximately 38.4 litres of vaccine, followed by CB which can

produce 27.2 litres, MBHI about 19.4 litres and the least was BHI which has shown that 1 mL can produces only 2.2 litres of the vaccine (Figure 7).

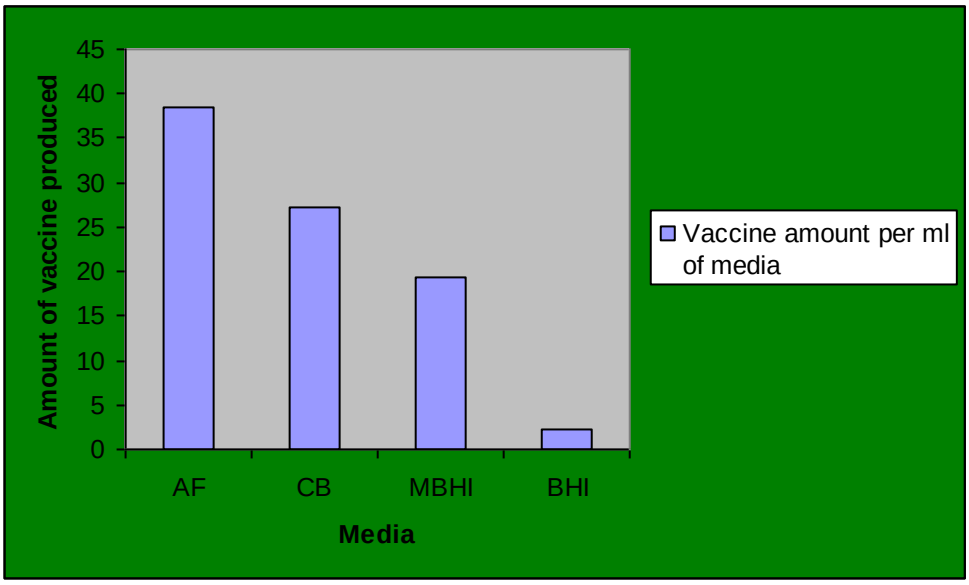


Figure 7: Mean volume in litres of the vaccine that can be produced from each 1 ml of the medium

4.2.3. Cost of producing 1 ml of each media

Cost of producing 1 ml of each media has shown that, AF costing 90 Tsh. was the media that is produced at the highest cost than all others. On the other hand production cost of 1 ml of CB which was 3.5 Tsh. was the lowest (Figure 8). Cost of producing 1 ml of CB was around 26 times lower than producing 1 mL of AF and four (4) times lower than producing 1 ml of MBHI and BHI.

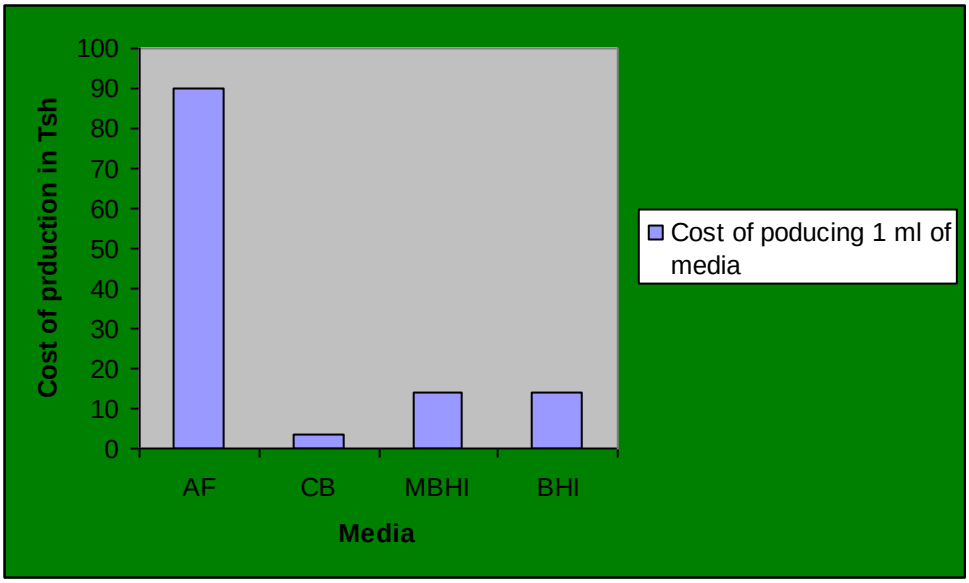


Figure 8: Estimated cost of producing 1 ml of AF, BHI, CB and MBHI. Figures are expressed in Tsh.

4.3. Thermostability

4.3.1. Room temperature records

Room temperature recorded was high at the beginning of the experiment in February and declined in the last month of the experiment which was May. Room temperature was not controlled, however daily records were recorded three times a day. The room temperature range was 27-29^{0}C with and average of 28^{0}C as depicted in Figure 9.

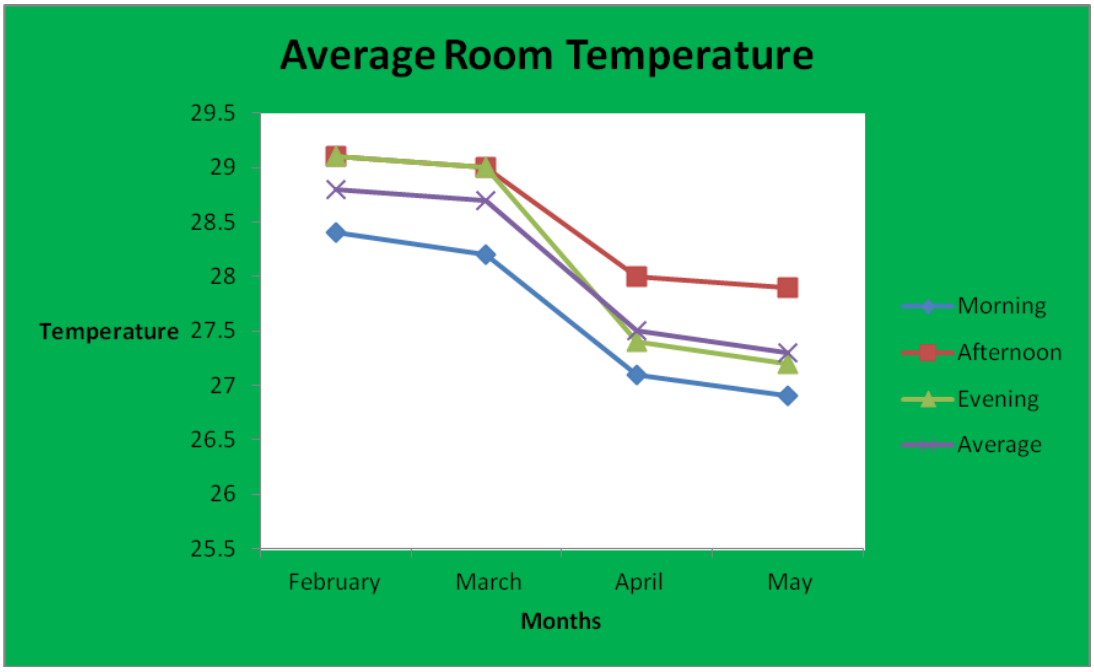


Figure 9: The average room temperature recorded in the laboratory where the bacteria were preserved for a period of 4 months, temperature was recorded three times a day.

4.3.2. Survival of *Av. paragallinarum* at room temperature.

Figure 10 shows the trend of survival of *Av. paragallinarum* stabilized by gelatin and stored at room temperature for the period of three months. Recovery rate was 100% (3^{+}) in the first four weeks; CFU count was high in 2.5% gelatin (4.33×10^{11} CFU/ml) and low in 10% gelatin (4.12×10^{7} CFU/ml). In the eighth week, recovery rate declined to 75% (2^{+}) recording 2.88×10^{10} CFU/ml in 2.5% gelatin and to 2.44×10^{4} CFU/ml in 10% gelatin. In the twelfth week, there were no bacteria recovered from 7.5% gelatin and 10% gelatin. On the other hand recovery rate was 50% (1^{+}) in 2.5% (1.48×10^{7} CFU/ml), 3.75% (1.44×10^{4} CFU/ml) and 5% (1.24×10^{3} CFU/ml). Trends showed that *Av. paragallinarum* stored in different gelatin concentrations were more stable in first two months of storage and that was February to March which recorded an average room temperature of 28.8^{0}C as

compared to the last month os storage which was May which had an average of 27^0C.

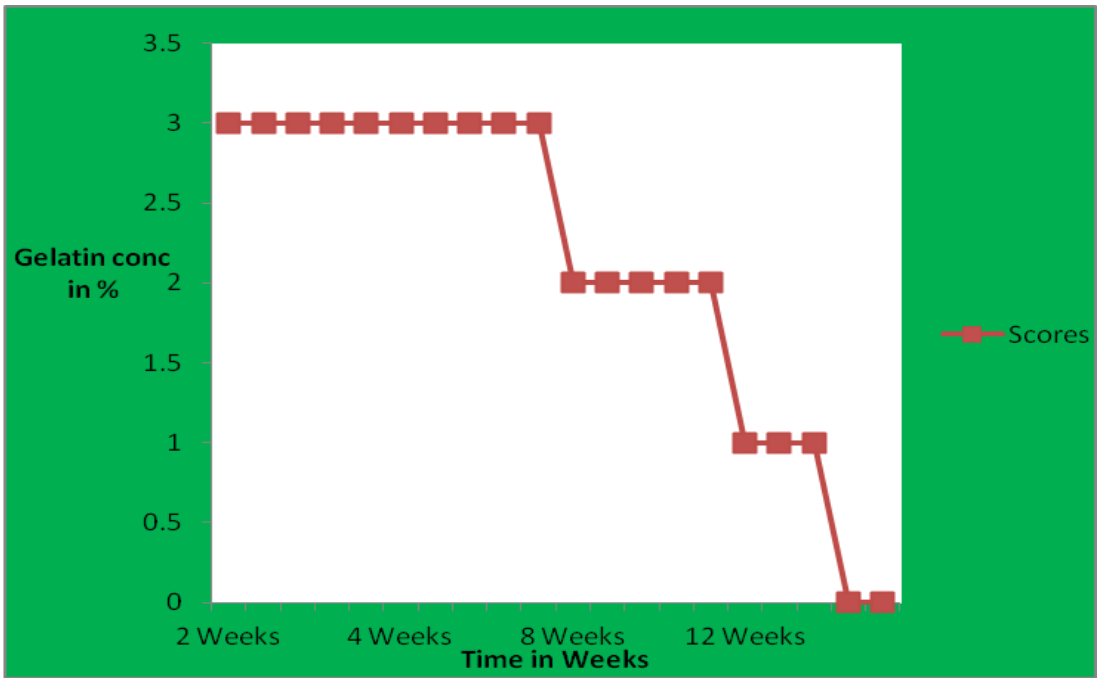


Figure 10: Survival of *Av. paragallinarum* at room temperature after stabilization with gelatin.

4.3.3. Survival of *Av. Paragallinarum* at 4^0C

Recovery of *Av. paragallinarum* stored at 4^0C has shown that in the first four weeks it was 100% in 2.5% gelatin (4.33×10^{10} CFU / ml) and declined to 75% in 10% gelatin (3.16×10^6 CFU / ml). In the 8^{th} week there was 75% recovery in 2.5% gelatin (4.52×10^6 CFU / ml) and also at 3.75% gelatin (3.47×10^6 CFU / ml). However, at the same 8 weeks time, it declined to 50% in 5%, 7.5%, 10% gelatin (3.61×10^4 CFU / ml), (3.73×10^3 CFU / ml) and (2.77×10^2 CFU / ml) respectively. Recovery rate was mantained at 50% in the 12^{th} week generally low and the records were 3.41×10^3 CFU / ml, 3.39×10^3 CFU / ml, 3.22×10^2 CFU / ml

in 2.5%, 3.75% and 5% gelatin, respectively. However, it declined to zero at 7.5% and 10% gelatin concentrations (Figure 11)..

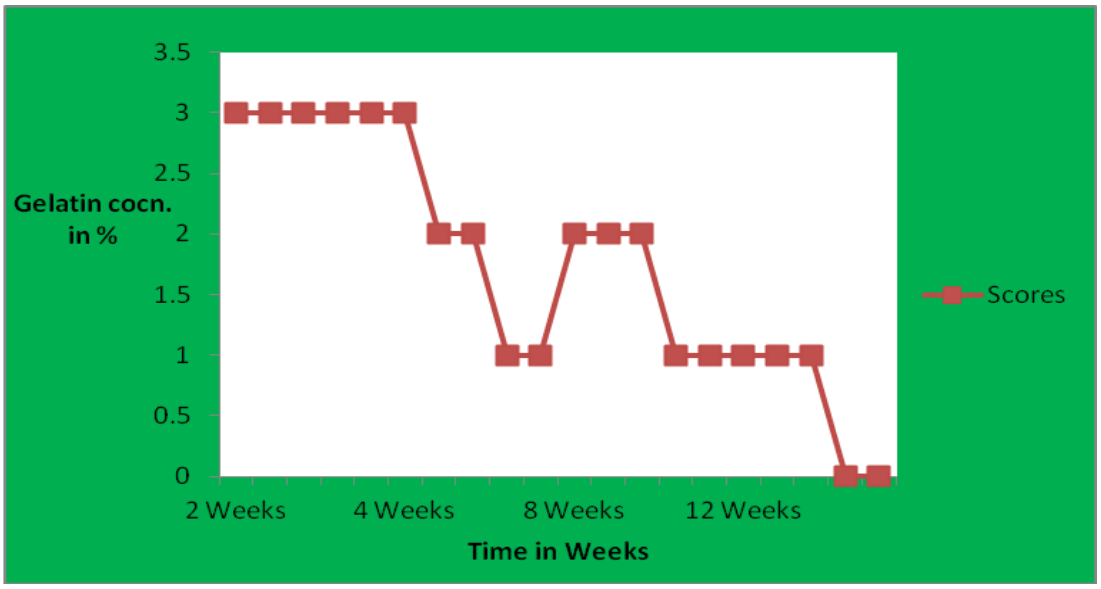


Figure 11: Survival of *Av. paragallinarum* at 4^0C after stabilization with gelatin.

4.3.4. Effect of repeated freezing thawing cycles on *Av. paragallinarum*

Repeated thawing and freezing has shown to affect the survival of *Av. paragallinarum* in gelatin. *Av. paragallinarum* preserved in gelatin and stored at room temperature without being opened for the period of 8 weeks were more stable recording (4.14×10^{10} CFU / ml, 2.33×10^{10} CFU / ml, 4.75×10^{9} CFU / ml, 3.17×10^{9} CFU / ml, 3.89×10^{8} CFU / ml in 2.5% gelatin, 3.75% gelatin, 5% gelatin, 7.5% gelatin and 10% gelatin respectively) as compared to those stored at 4^0C without repeated thawing and freezing cycles which recorded (3.49×10^{10} CFU / ml, 5.29×10^{9} CFU / ml, 6.67×10^{9} CFU / ml, 6.31×10^{7} CFU / ml, 3.22×10^{7} CFU / ml in 2.5% gelatin, 3.75% gelatin, 5% gelatin, 7.5% gelatin and 10% gelatin), respectively (Figure 12a).

Similary *Av. paragallinarum* preserved in gelatin and stored at room temperature without being opened which recorded 4.14 x 10^{10} CFU / ml, 2.33 x 10^{10} CFU / ml, 4.75 x 10^{9} CFU / ml, 3.17 x 10^{9} CFU / ml, 3.89 x 10^{8} CFU / ml in 2.5% gelatin, 3.75% gelatin, 5% gelatin, 7.5% gelatin and 10% gelatin were more stable than both, those stored at 4^{0}C without repeated freezing and thawing cycles which recorded 3.49 x 10^{10} CFU / ml, 5.29 x 10^{9} CFU / ml, 6.67 x 10^{9} CFU / ml, 6.31x 10^{7} CFU / ml, 3.22 x 10^{7} CFU / ml in 2.5% gelatin, 3.75% gelatin, 5% gelatin, 7.5% gelatin and 10% gelatin respectively and those stored at 4^{0}C with repeated thawing-freezing cycles which recorded 4.52 x 10^{6} CFU / ml, 3.47 x 10^{6} CFU / ml, 3.61 x 10^{4} CFU / ml, 3.61 x 10^{4} CFU / ml, 3.73 x 10^{3} CFU / ml and 2.77 x 10^{2} CFU/ml in 2.5% gelatin, 3.75% gelatin, 5% gelatin, 7.5% gelatin and 10% gelatin respectively.

The difference in CFU / ml between *Av. paragallinarum* preserved in gelatin and stored at 4^{0}C without being opened and those stored at 4^{0}C with repeated freezing and thawing cycles shows that they are affected by the repeated freezing and thawing cycles (Figure 12b).

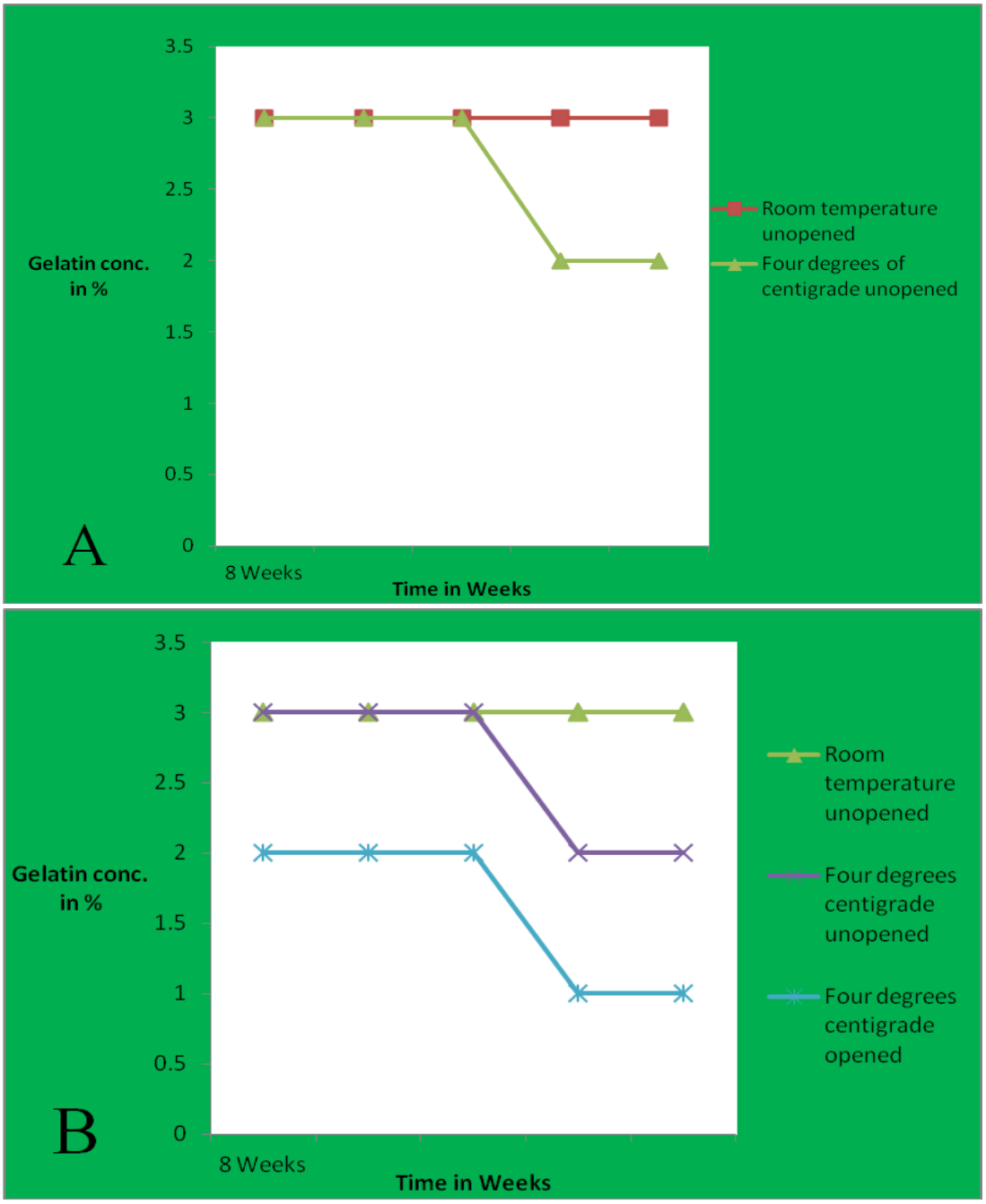


Figure 12: Effect of repeated thawing – freezing cycles on *Av. paragallinarum* after stabilization with gelatin.

Legend: A – Survival rate of *Av. paragallinarum* at Room temperature and at 4^0C after preservation for 8 weeks without opening the vials. B – Survival rate of *Av. paragallinarum* at Room temperature and 4^0C after preservation for 8 weeks without

being opened as compared to survival at 4^0C with repeted thawing and freezing cycles in the period of 8 weeks.

4.3.5. Survival of *Av. paragallinarum* at -20^0C

Av. paragallinarum was poorly recovered at -20^0C from gelatin at all concentrations as shown in Figure 15. There were no recovery in all gelatin concentrtions in the 4^{th} week of storage. The bacteria were well recovered in the first 7 days of storage and no recovery of the bacteria was done in 10% gelatin in the 2^{nd} week of storage. Towards the end of two weeks of storage recovery rate had declined to 3.44 x 10^6 CFU / ml, 3.12 x 10^4 CFU / ml, 2.71 x 10^4 CFU / ml and 2.56 x 10^4 in 2.5%, 3.75%, 5%, and 7.5% gelatin concentrations respectively.

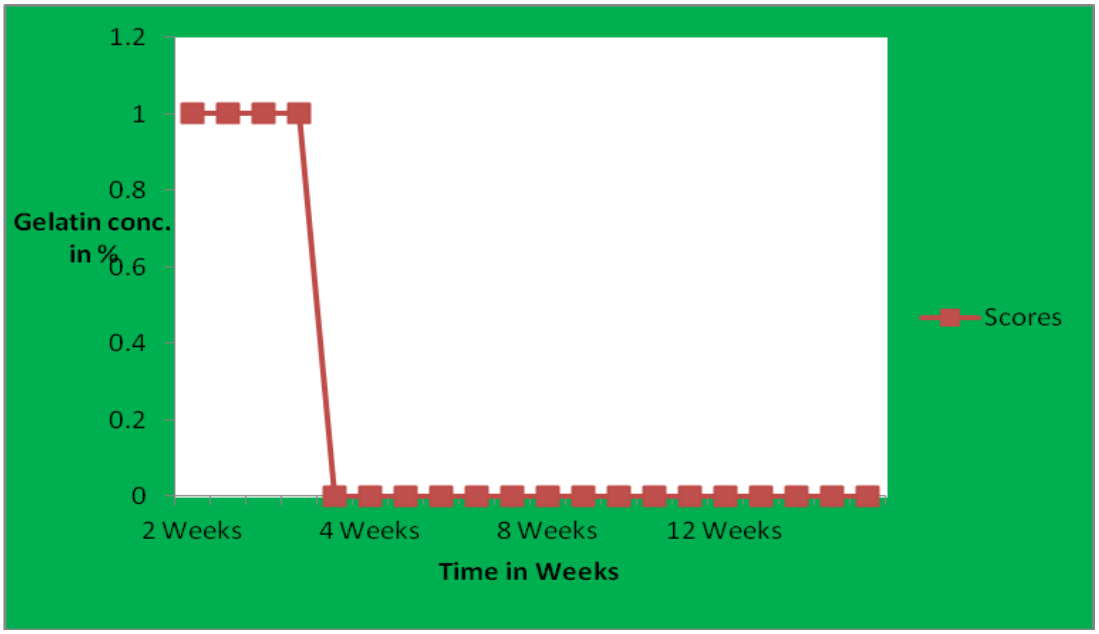


Figure 13: Survival of *Av. paragallinarum* at -20^0C after stabilization with gelatin.

CHAPTER FIVE

5.0. DISCUSSION

5.1. Characterization of Isolates of *Av. paragallinarum*

In this study, Av. paragallinarum serovar B corresponding to Page serovar scheme (Page, 1962: Blackall *et al*., 1990) and other members of the genus Avibacterium (*Av. volantium* and *Av. avium*) have been isolated from apparently health, clinically sick and dead chickens. Isolation of this causative agent in apparently healthy chickens in this study indicated presence of carrier birds. The findings are in agreement with those made by Byarugaba *et al.* (2011).

Bacteria isolated in this study depicted biological characteristics similar to those previously reported by Olsen *et al.* (2004) and Roodt (2009). Yellowish colonies of *Av. volantium* were also seen and this was in agreement with the description by Mutters *et al.* (1985).

Biochemically, all isolates of *Avibacterium* species were oxidase positive, urease negative and required NAD as a growth requirement. However, *Av. paragallinarum* were catalase negative (Blackall *et al.,* 2005; Wambura 2010) where as *Av. avium* and *Av. volantium* were catalase positive, these results are in agreement to those reported by other workers (Biberstein and White, 1969 and Mutters *et al.* 1985). This is the first time *Av. volantium* and *Av. avium* are isolated in Tanzania. Identification by PCR using species-specific DNA primers revealed an amplification of 500bp, which is similar to that reported by Roodt (2009). Isolates 3 and 4 were amplified with serotypic technique as described by Sakamoto *et al.* (2012) and

corresponds to serovar B of the Page scheme (Page, 1962). The isolates were previously not amplified by the ID PCR in the present study indicating that they might be new strains, similar to the observation by Roodt (2009). Identification of *Av. paragallinarum* in apparently healthy birds clinically sick and dead birds indicate the presence of the carrier birds and possibility of them being major sources of infection and poor production in most of the farms visited and reported.

5.2. Novel media for propagation of *Av. paragallinarum*

Propagation of *Av. paragallinarum* for vaccine production has for a long time being done in different media (Wambura, 2010; Wafaa and El Ghany, 2011). However, in all studies done to date, there is no single study that has compared the ability of different growth media to support propagation of the bacteria.

In this study, the ability of a novel media for propagating *Av. paragallinarum* was optimized. These findings about the ability of *Av. paragallinarum*, to grow on CB were similar to the findings by Turk (1964) on the ability of CA to support storage of *Haemophilus influenzae,* the other member of the family *Pasteurellaceae.* Growth of *Av. paragallinarum* in CB which is cheap and easy to prepare will possibly make production of broth autogenous vaccines which have up to now shown ability to provide good protection much easier (Blackall, 1995). As a result of easy and cheap preparation of the novel media, vaccines produced will probably be cheap and easily available to farmers and hence facilitate the control of the disease.

5.3. Thermostability of *Av. paragallinarum*

Poor delivery system in the line of the cold chain is a big problem in the efforts to control various diseases by vaccination. Report by Simba and Msamanga (1994) showed that there are weak points in the cold chain along the way to livestock keepers. Due to this there is a high concern in ensuring proper storage, handling and developing heat stable vaccines as it was also suggested by Cheyne (1989).

Despite the concept that it is difficult to preserve members of the genus *Haemophilus*, from which *Av. paragallinarum* was reclassified (Blackall *et al*, 2005), this study has shown that *Av. paragallinarum* can be preserved better in gelatin at lower concentration at room temperature. Similar findings (not published, based on personal communication) were observed by Sengiyumva (2010). Ability of *Av. paragallinarum* to be stored for a long time is in line with the finding on the storage of *Haemophilus influenzae* as described by Turk (1964). In this study *Av. paragallinarum* was recovered from gelatin at 2.5% concentration after being stored for 3 months.

The survival of *Av. paragallinarum* at -20^0C was poor and the reasons for this might be the effect of the initial concentration as was previously reported by Saab *et al.* (2001) and thawing and repeated freezing cycles as was also reported by Stamp (1947). Despite the fact that *Av. paragallinarum* survived in gelatin for a period of 3 months, other studies shown that preservation by drying supports much better and can make the bacteria survive for years (Stamp, 1947). Further studies have to be done to see whether *Av. paragallinarum* will depict similar results as to other bacteria.

Long term preservation of *Av. paragallinarum* is important for the development of live vaccines. Up to date efforts are done to develop a live vaccine against IC (Blackall, P. personal communication, 2012), the best level so far reached is the use of live cultures from embryonated chicken eggs. The findings of this study are very important in the efforts that are underway to develop the vaccine that can be effectively delivered without much dependence on the cold chain.

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

IC is a disease that causes high economic losses among poultry keepers in Tanzania. Poor growth in meat chicken and reduced egg production in layers is a set back to the efforts shown by poultry keepers to alleviate poverty and improve the protein availability in many families.

Despite the difficulty in isolation of the bacteria due to its fastidious nature, in this study the *Av. paragallinarum* and other members of the same genus *Avibacterium avium and Av. volantium* have been isolated. This shows that *Av. paragallinarum* serotype B exists in Tanzania.

Preparation of CB has been shown to be easy and cheap as compared to other media namely MBHI, BHI and AF. *Av. paragallinarum* titres in CB are high and this makes it a broth of choice in preparation of both live and killed broth vaccines. Since the cost of media preparation is low this will possibly pave way for preparation of vaccines that are cheap and affordable to the farmers.

Preservation of *Av. paragallinarum* in 2.5% gelatin at room temperature for 3 months is a key finding for development of live vaccines against IC. This sets a foundation to overcome the need of repeated isolation and propagation of *Av. paragallinarum* for development of vaccines. Manipulation of the bacteria is made possible and the live vaccines can be produced and easily stored and delivered along the cold chain.

6.2. Recommendations

The author recommends that live and killed vaccines should be developed from *Av. paragallinarum* preserved in gelatin and tested for safety, sterility, potency and efficacy. It is further recommended that different routes of administration should be tested to obtain a safer route that will result in the required stimulation of the antibodies to confer the required protection. Further passaging of the *Av. paragallinarum* should be done to remove the capsule and obtain non capsulated bacteria. Test for the virulence of the non capsulated bacterium should also be performed.

REFERENCES

Adderson, E.E. (2008). *Avibacterium paragallinarum.* In *Practical Handbook of Microbiology.* Edited by (Goldman, E. and Green, L.H.) CRC press. pp. 519 – 531.

Biberstein, E.L and White, D.C. (1969). A proposal for establishment of two new *Haemophilus* species. *Journal of Medical Microbiology* 2:75-78.

Blackall, P.J. (1995). Vaccines against infectious coryza. *World Poultry Science Journal* 15:17-26.

Blackall, P.J. (1999). Infectious coryza: overview of the disease and new diagnostic options. *Clinical Microbiology Review* 12:627-632.

Blackall, P.J. (2011). *An update on the diagnosis and prevention of fowl cholera and infectious coryza. In: XXII Latin America Poultry Congress*.

Blackall, P.J. and Reid, G.G. (1987). Further efficacy studies on inactivated aluminium hydroxide adsorbed vaccines against infectious coryza. *Avian Diseases* 31:527-532.

Blackall, P.J. and Reid, G.G. (1987). Further efficacy studies on inactivated alminium hydroxide adsorbed vaccines against infectious coryza. Avian Diseases 31: 527 – 532.

Blackall, P.J., Christensen, H. and Bisgaard, M. (2011). Unusual growth variants of *Avibacterium paragallinarum*. *Australian Veterinarian Journal* 89:273-275.

Blackall, P.J., Christensen, H., Beckenham, T., Blackall, L.L. and Bisgaard, M. (2005). Reclassification of *Pasteurella gallinarum*, *[Haemophilus] paragallinarum*, *Pasteurella avium* and *Pasteurella volantium* as

Avibacterium gallinarum gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov. and *Avibacterium volantium* comb. nov. *International Journal of Systematic Evolutionary Microbiology* 55:353-62.

Blackall, P.J., Eaves, L.R. and Rogers, D.G. (1990). Proposal of new serovar and altered nomenclature for *Haemophilus paragallinarum* in Kume haemagglutinin scheme. *Journal of Clinical Microbiology* 28:1185-1187.

Bland, M.P., Bickford, A.A., Charlton, B.R., Cooper, G.C., Sommer, F. and Cutler, G. (2002). Case report: a severe infectious coryza infection in a multi-age layer complex in central California. In: 51st Western Poultry Disease Conference/XXVII Convencion Anual ANECA, Peurto Vallajarta, Mexico, pp 56- 57.

Bragg, R.R., Coetzee, L. and Verschoor, J.A. (1996). Changes in the incidences of the different serovars of *Haemophilus paragallinarum* in South Africa: a possible explanation for vaccination failures. *Onderstepoort Journal of Veterinary Research* 63: 217–226.

Byarugaba, D.K., Minga, U.M., Gwakisa, P.S., Katunguka, E.R., Bisgaard, M. and Olsen. J.E. (2006). Occurrence, isolation and characterisation of *Avibacterium paragallinarum* from poultry in Uganda. In: *Proceedings of the 11th International Symposium on Veterinary Epidemiology and Economics*. [http://www.sciquest.org.nz] site visited on 1/5/2012.

Byarugaba, D.K., Minga, U.M., Gwakisa, P.S., Katunguka-Rwakishaya, E., Bisgaard, M., Christensen, H. and Olsen J.E. (2011). Demonstration of

antibiotic resistance genes *str*A, *blaTEM*, *tet*A, *tet*C and *sul*2 in *Avibacterium paragallinarum*. *African Journal of Microbiology Research* 5(22): 3624-3627.

Chen, X., Song, C., Gong, Y. and Blackall, P.J. (1996). Further studies on the use of a polymerase chain reaction test for the diagnosis of infectious coryza. *Avian Pathology* 27: 618-624.

Chen, X., Song, C., Gong, Y., and Blackall, P. J. (1998). Further studies on the use of a polymerase chain reaction test for the diagnosis of infectious coryza. *Avian Pathology* **27:** 618-624.

Cheyne J. (1989). Vaccine delivery management. *Reviews of Infectious Diseases*, 11: 617-622.

Chukiatsiri, K., Sasipreeyajan, J., Neramitmansuk, W. and Chansiripornchai, N. (2009). Efficacy of autogenous killed vaccine of *Avibacterium paragallinarum*. *Avian Diseases* 53:382–386.

Ciamarra, U.P. and Nsiima, L. (2011). Technical Workshop for Improving Agricultural Statistics: Challenges and Opportunities. In: *Livestock data in Tanzania: Status and Prospects,* 24 October, 2011. Dar es Salaam, Tanzania. pp. 1-13.

Cruickshank, R., Duguid, J.P., Swain, R.H.A. (1968). Counting bacteria and measuring bacterial growth. In *Medical Microbiology*; The English Language Book Society and Churchill Livingstone, Teviot Place, Edinburgh. pp 870 -875.

Dousse, F., Thomann, A., Brodard, I., Korczak, B.M. Schlatter, Y., Kuhnert, P., Miserez, R. and Frey, J. (2008). Routine phenotypic identification of

bacterial species of the family *Pasteurellaceae* isolated from animals. *Journal of Veterinary Diagnostic Investigations* 20:716–724.

Garcı´a1, F. Romo, A., Ortiz, M. and Blackall, P.J. (2008). The vaccination-challenge trial: the gold standard test to evaluate the protective efficacy of infectious coryza vaccines. *Avian Pathology* 37(2):183-186.

Garcia, A., Angulo, E. and Blackall, P.J. (2002). The presence of NAD independent *Haemophilus paragallinarum* in Mexico. *Avian Diseases* 48:425-429.

Jacobs, A.A., van den Berg, K. and Malo, A. (2003) Efficacy of a new tetravalent coryza vaccine against emerging variant type B strains. *Avian Pathology* 32:265-269.

Kume, K., A. Sawata, Nakai, T. and Matsumoto, M. (1983). Serological classification of *Haemophilus paragallinarum* with a hemagglutinin system. *Journal of Clinical Microbiology* 17:958–964.

Matsumoto, M. and Yamamoto, R. (1975). Protective quality of aluminium hydroxide adsorbed broth bacterins against Infectious coryza. *American Journal of Veterinary Research* 36:579 – 582.

Mendoza-Espinoza, A., Koga, Y. and Zavaleta, A. I. (2008). Amplified 16S ribosomal DNA restriction analysis for identification of *Avibacterium paragallinarum*. *Avian Diseases* 52:54–58.

Mouahid, M., Bouzoubaa, K. and Zouagui, Z. (1989). Chicken infectious coryza in Morocco: Epidemiological study and pathogenicity trials. *Actes de l'Institut Agronomique et Veterinaire Hassan II* 9: 11-16.

Msami, H. (2007). Veterinary health, public health, biosecurity measures. In: Poultry sector country review: Review based on the structure, marketing

and importance of the commercial and village poultry industry: an analysis of the poultry sector in Tanzania. Central Veterinary Laboratory, Dar es Salaam Tanzania. pp 31 – 34.

Mutters, R., Piechulla, K., Hinz, K.H and Mannheim, W. (1985). *Pasteurella avium* and *Pasteurella volantium* sp. nov. *International Journal of Systematic Bacteriology* 35:5-9.

Noro, T., Oishi, E., Kaneshige, T., Yaguchi, K., Amimoto, K. and Shimizu, M. (2008) Identification and characterization of haemagglutinin epitopes of *Avibacterium paragallinarum* Serovar C. *Veterinary Microbiology* 131:406 - 413.

Olsen, I., Dewhirst, F.E., Paster, B.J. and Busse, H.J. (2004). Family *Pasteurellaceae*. In Bergey's Manual of Systemic Bacteriology. 2: 36 - 49.

Page, L.A. (1962). Haemophilus infections in chicken. 1. Characteristics of 12 *Haemophilus* isolates recovered from diseased chickens. *American Journal of Veterinary Research* 23: 85-95.

Page, L.A., Rosenwald, A.S. and Price, F.C. (1963). *Haemophilus* infections in chicken. IV Results of laboratory and field trials of formalized bacterins for prevention of the disease caused by *Haemophilus gallinarum*. *Avian Diseases* 7: 239 – 256.

Project Report, submitted at Sokoine University of Agriculture, Morogoro – Tanzania. pp 11-12.

Rajurkar, G., Roy, A. and Yadav, M.M. (2009). An overview on epidemiologic investigations of infectious coryza. *Veterinary World* 2(10):401-403.

Roodt, Y. (2009). Towards unraveling the genome of *Avibacterium paragallinarum*. A PhD thesis at Free State University, Bloemfontein - South Africa. pp 89 – 116.

Saab, O.C.A, Castillo, M.C, Ruiz Holgado, A.P. and Nader O.M. (2001). A comparative study of preservation and storage of *Haemophilus influenzae*. *Memorial Institute Oswaldo Cruz, Rio de Janeiro* 96: 583-586.

Sakamoto, R., Kino, Y. and Sakaguchi, M. (2012). Development of a multiplex PCR and PCR-RFLP Method for serotyping of *Avibacterium paragallinarum*. *Journal of Veterinary Medical Science*74: 271–273.

Sengiyumva, E.K. (2010). Development and evaluation of a live vaccine against *Avibacterium paragallinarum* infection in chickens: *A Special*

Simba, D.O. Msamanga, G.I. (1994). Use of cold chain to assess vaccine exposure to adverse temperatures in rural Tanzania. *East African Medical Journal* 71: 445-446.

Soriano, V.E., Longinos, G.M., Téllez, G., Fernández, R.P., Suárez-Güemes, F. and Blackall, P.J. (2004) Cross-protection study of the nine serovars of *Haemophilus paragallinarum* in the Kume haemagglutinin scheme. *Avian Pathology* 33:506-511.

Stamp, L. (1947). The preservation of bacteria by drying. Journal General Microbiology, 1:251-265.

Tennison, L.B. and Siddle, P.J. (1961). Limited Study with *Haemophilus* Cultures. *Avian Diseases* 5 (3): 352-354.

Terzolo, H.R., Sandoval, V.E. and Gonzalez Pondal, F. (1997). Evaluation of inactivated infectious coryza vaccines in chickens challenged by serovar B strains of *Haemophilus paragallinarum*. *Avian Pathology* 26: 365–376.

Turk, D.C. (1964). Short – term storage of *Haemophilus influenzae*. *Journal of Clinical Pathology* 17:297-300.

Wafaa, A. and El-Ghany, A. (2011). Evaluation of autogenous *Avibacterium paragallinarum* bacterins in chickens. *International Journal of Poultry Science* 10 (1): 56-61.

Wambura, P.N. (2010). Preparation and use of autogenous vaccine from *Avibacterium paragallinarum* (strain Tan 1-05) in layer chickens. *Tropical Animal Health and Production* 42: 483–486.

Wang, H., Gao, Y., Gong, Y., Chen, X., Liu, C., Zhou, X., Blackall, P.J., Zhang, P. and Yang, H. (2007) Identification and immunogenicity of an immunodominant mimotope of *Avibacterium paragallinarum* from a phage display peptide library. *Veterinary Microbiology* 119:231-239.

Wu, J.R., Chen, P.Y., Shien, J.H., Shyu, C.L., Shieh, H.K., Chang, F. and Chang, P.C. (2010). Analysis of the biosynthesis genes and chemical components of the capsule of *Avibacterium paragallinarum*. *Veterinary Microbiology* 145:90–99.

Wu, J.R., Wu, Y.-R., Shien, J.H., Hsu, Y.M., Chen, C.F., Shieh, H.K. and Chang, P.C. (2011) Recombinant proteins containing the hypervariable region of the haemagglutinin protect chickens against challenge with *Avibacterium paragallinarum*. *Vaccine* 29:660-667.

Yamaguchi, T., Blackall, P.J., Takigami, S., Iritani, Y. and Hayashi, Y. (1991) Immunogenicity of *Haemophilus paragallinarum* serovar B strains. *Avian Diseases* 35:965-968.

Yamamoto, R. (1991). Infectious coryza. In: *Diseases of Poultry*. Edited by (M. S. Hofstad, H. J. Barnes, B. W. Calnek, W. M. Reid, and H. E. Yonder, Jr.) Iowa State University Press, Ames. pp 186-195.

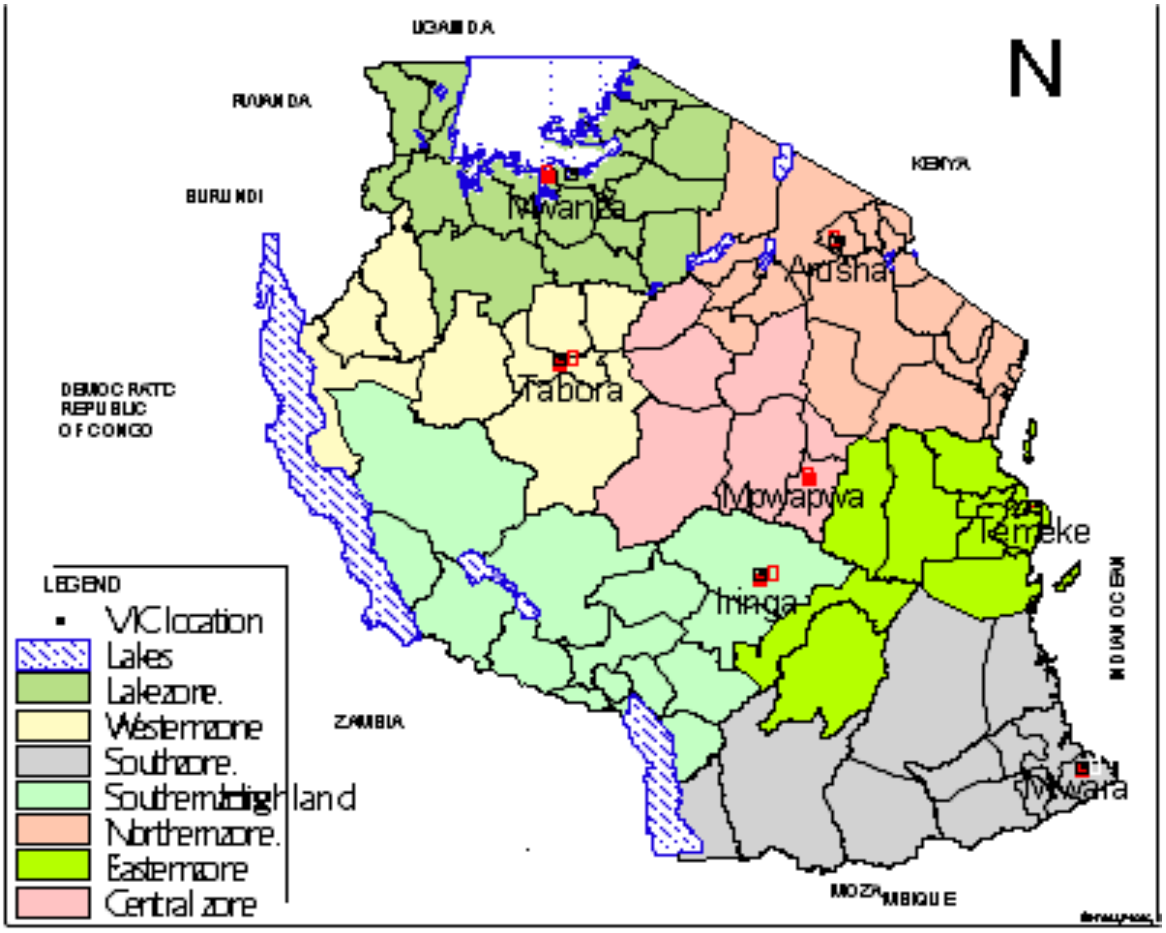
Yongolo, M.G.S., Mtambo, M.M., Minga, U.M., Boki, K. (1997). Strategies for enhancement of poultry productivity in Tanzania. In: Proceedings of the 20th Anniversary of the Faculty of Veterinary Medicine, SUA, June 1997, Morogoro, Tanzania, pp. 85-94.

APPENDICES

Appendix 1: Table showing details on locations and sample collection

Zone	Location	Samples collected from	Samples collected by	Number of samples	Number of birds sampled
Central	Dodoma	live birds	Tracheal swab	2	7
		Clinically sick birds	Nasal swab	5	
			Tracheal swab	5	
Western	Tabora	live birds	Nasal swab	6	13
			Tracheal swab	6	
		Dead birds	Nasal swab	2	
			Tracheal swab	2	
		Clinically sick birds	Nasal swab	5	
			Tracheal swab	5	
Southern highlands	Iringa	live birds	Nasal swab	2	6
			Tracheal swab	2	
		Dead birds	Nasal swab		
			Tracheal swab		
		Clinically sick birds	Nasal swab	4	
			Tracheal swab	4	
Northern	Manyara	live birds	Nasal swab		3
			Tracheal swab		
		Dead birds	Nasal swab	3	
			Tracheal swab	3	
		Clinically sick birds	Nasal swab		
			Tracheal swab		
Eastern	Morogoro	live birds	Nasal swab	4	11
			Tracheal swab	4	
		Dead birds	Nasal swab	1	
			Tracheal swab	1	
		Clinically sick birds	Nasal swab	5	
			Tracheal swab	5	
Southern	Mtwara	live birds	Nasal swab	2	6
			Tracheal swab	2	
		Dead birds	Nasal swab	2	
			Tracheal swab	2	
		Clinically sick birds	Nasal swab	2	
			Tracheal swab	2	
Zanzibar	Bungi	live birds	Nasal swab	3	8
			Tracheal swab	3	
		Dead birds	Nasal swab	2	
			Tracheal swab	2	
		Clinically sick birds	Nasal swab	3	
			Tracheal swab	3	

Appendix 2; Map of Tanzania showing ecological zones



Map adopted from the National Livestock policy, Ministry of Agriculture and Food Security, 2006.

Appendix 3: Sepa gene extraction protocol.

DNA EXTRACTION (*Sepa* Gene extraction protocol)

1. 1-2g of tissue (or 200mg of feaces) in 700µl sterilized PBS and homogenize
2. Centrifuge 3,000 rpm x10 min x 4^0C
3. Transfer the supernatant into new eppendorf tubes
4. Centrifuge 13,000 – 15,000 rpm x 30 – 60 min x 4^0C
5. Discard the supernatant and take the pellet
6. Add 50µl of Solution I to the pellet and homogenate and/or resuspend by pipetting
7. Incubate at room temperature (20-25^0C) for 10 min
8. Then add 50µl of Solution II and mix by pipetting without making bubbles
9. Then add 350µl of Solution III and mix a bit
10. Then add 200µl of Solution IV
11. Mix the contents (vortex) until the mixture is uniformly turbid (milky in colour)
12. Centrifuge at 12,000 rpm x 15 min x 4^0C
13. Take the supernatant and transfer into new tubes and add 30µl of Solution V
14. Then add 330µl of Isopropanol and mix uniformly by tilting
15. Incubate at either -80^0C for 5 min, -30^0C for 60 min or 4^0C overnight
16. Centrifuge at 13,000 – 15,000 rpm x 30 min x 4^0C
17. Discard the supernatant
18. Wash the pellet with 70% chilled ethanol
19. Air-dry the pellet on clean bench
20. Add 30-50µl of Tris-EDTA (TE) buffer or sterilized distilled water (DW) and store at -30^0C