ISOLATION AND IDENTIFICATION OF CELLULAR STRESS PROTEINS ASSOCIATED WITH BOID INCLUSION BODY DISEASE

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By

YUSUF MAINA ILYASU

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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DEDICATION

To my mother: Fatima Mohammed Maina Yusuf of blessed memory

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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July 2016

Chairman : Professor Noordin Mohamed Mustapha, PhD
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Boid inclusion body disease is one of the fatal and most important diseases of captive boid snakes worldwide. Till today, cases were diagnosed by the demonstration of eosinophilic intracytoplasmic inclusion bodies from tissue sections under light microscopy. However, inclusion bodies are also found in many other viral infections. Understanding the specific etiologic agent and the disease pathogenesis has eluded researchers for over three decades since the disease was first discovered in the 1970s. Recently however, highly divergent and novel arenaviruses were isolated from tissues of snakes with the disease. Even though the arenaviruses isolated were novel and highly divergent in each case, researchers were able to establish causal linkage with the disease in vitro. Research has now focused on understanding the formation and nature of the inclusion protein commonly found in tissues of affected snakes. It is believed that understanding the nature and the chemical composition of this protein may lead to a better understanding of the cause, progression and diagnosis of the disease. Various cellular stress proteins have frequently been found as common component of cellular response to viral infections associated with protein aggregation. A proteomic profile of such proteins can be used to understand the disease pathogenesis leading to a better understanding of the disease diagnosis and consequently its treatment. The present study therefore attempts to shed some light towards further understanding the pathogenesis of BIBD in snakes through a comparative study of the protein profiles from BIBD infected and healthy specimens by means of electrophoresis and peptide mass spectrometry. Tissue samples obtained at necropsy from snakes that died naturally of the disease as well as those obtained from experimentally infected chicken embryos were subjected to total protein isolation using PRO-PREP^{TM} protein isolation solution according to the manufacturer's protocol and quantified by the Bradford method. Protein separation was accomplished through SDS-PAGE, and the protein bands of various sizes were purified, trypsin-digested and identified by mass spectrometry. The peptide sequences obtained were analysed using the Mascot sequence matching software [Matrix Science] with Ludwig NR database. The peptide sequences were compared against known protein sequences on the data base. Fourteen proteins were identified from the infected specimens using peptide mass finger printing with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry, twelve out of which were heat shock proteins. These were heat shock protein 5 (hsp5), heat shock cognate protein 71 (hsc71) and glucose regulatory protein 78 (grp78) with protein hit score values greater than 32 significantly different at

($p<0.05$). Specimens from the BIBD negative snake did not show these proteins in their profile. Specimens from the chicken embryo showed inclusion bodies at histopathology in their tissues, but did not yield any protein band on the electrophoretogram. Heat shock protein 70 family have frequently been associated with protein aggregation diseases and because of the known role they play in the progression of such diseases, the study therefore added some knowledge that may help in understanding the pathogenesis of BIBD in snakes. The study also confirms that BIBD-associated pathogen can be propagated in embryonated chicken egg, a finding that might be of immense benefit to laboratories and diagnostic facilities that have interest in the study of this virus but lack cell culture capabilities for its propagation.

Abstrak thesis yang dikemukan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Master Sains

PENGASINGAN DAN PENGENALPASTIAN PROTEIN SELULAR STRES BERKAIT DENGAN PENYAKIT JASAD RANGKUMAN BOID

Oleh

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Penyakit jasad rangkuman boid (BIBD) merupakan satu penyakit maut serta amat penting pada ular kurungan seluruh dunia. Hingga kini, ia didiagnosis secara mikroskopi dengan kehadiran jasad rangkuman intrasitoplasma bereosinofil pada tisu. Bagaimanapun, jasad rangkuman juga dilihat pada pelbagai jangkitan virus yang lain. Kefahaman mengenai agen etiologi khusus serta patogenesis penyakit ini telah membingungkan penyelidi semenjak ia ditemui pada tahun tujuh puluhan. Kini terdapat penemuan arenavirus yang amat berbeza dan novel telah diasing kan dari tisu ular terjangkit. Walaupun arenaviruses yang diasingkan adalah amat novel dan berbeza pada setiap kes, penyelidik masih berupaya mengukuhkan hubungan penyebab dengan penyakit secara in vitro. Penyelidik sekarang menumpukan kepada kefahaman pembentukan dan bentuk asli protein rangkuman yang terdapat pada tisu ular terjangkit bagi merungkai patogenesis, peluasan dan diagnosis penyakit ini. Pelbagai protein tegasan ditemui sebagai unsur greakbalas sel kepada jangkitan virus berkait dengan protein gumpalan. Profil proteomik protein seperti ini boleh diguna bagi memahami patogenesis penyakit yang membawa kepada pemahaman diagnosis penyakit dan akhirnya rawatan yang lebih kukuh. Kajian ini merintis kepada kefahaman mendalam BIBD pada ular melalui kajian perbandingan profil protein daripada spesimen ular terjangkit IBD dan ular normal menggunakan elektroforesis dan spektrometri jisim protein. Sampel tisu yang diperolehi dari nekropsi ular yang mati secara semulajadi akibat BIBD dan telur ayam berembrio yang dijangkiti dengan virus ini diuji pengasingan protein penuh total protein menggunakan pengasingan protein PRO-PREP^{TM} mengikut syor pengeluar yang dihitung dengan kaedah Bradford. Pemisahan protein dilakukan melalui SDS-PAGE, dan garisan pelbagai saiz protein ditulinkan, cerna-tripsin dan dikenalpasti melalui spektrometri jisim. Jujukan peptid yang diperolehi di analisis menggunakan perisian jujukan padanan Mascot [Matrix Science] dengan pengkalan data NR Ludwig. Jujukan peptid dibanding dengan jujukan protein yang diketahui dalam pengkalan data. Daripada 14 protein telah dikenalpasti daripada specimen menggunakan cetakan peptid jisim jari dengan spektrometri penyahserapan/pengionana-masa penerbangan bantuan-matriks laser, 12 adalah protein tegasan. Protein ini adalah hsp5, hsp70, hsc71 dan grp78 dengan nilai skor melanda protein melebihi 32 ($p<0.05$). Spesimen daripada ular negatif untuk BIBD tidak menunjukan protein berkenaan pada profilnya. Secara histopatologi, spesimen daripada telur ayam berembrio menunjukan jasad rangkuman tetapi tidak menghasilkan garisan protein pada elektroforetogram. Keluarga protein renjatan 70 kerapkali dikaitkan dengan

penyakit pengumpulan protein serta memandangkan peranan yang dimainkannya dalam pembentukan penyakit sedemikian, kajian ini telah menambah ilmu yang boleh membantu kepada kefahaman pathogenesis BIBD pada ular. Kajian ini juga telah mengesahkan bahawa pathogen terkait-BIBD boleh dicambah dalam telur ayam berembrio. Ini adalah penemuan yang sungguh bermanfaat kepada makmal dan fasiliti diagnosis yang berminat mengkaji virus ini tetapi mempunayi kekangan dengan kemudahan kultur sel.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science**.** The members of the Supervisory committee were as follows:

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LIST OF ABBREVIATIONS

AC	Allantoic cavity
BIBD	Boid Inclusion Body Disease
BIBDP	Boid Inclusion Body Disease Protein
BIBDAV	Boid Inclusion Body Disease-Arenaviruses
CASV	California Academy of Science Virus
CAM	Chorioallantoic membrane
CNS	Central Nervous System
CO	Carbon mono oxide
CPE	Cytopathic Effect
ELISA	Enzyme-linked immunosorbent assay
GP	Glycoprotein
GPC	Glycoprotein complex
GGV	Golden Gate Virus
Grp	Glucose regulated protein
HSP	Heat shock protein
Hsps	Heat shock proteins
Hsc	Heat shock cognate
H&E	Haematoxylin and Eosin
IB	Inclusion Body
IBDP	Inclusion Body Disease Protein
KDa	Kilo Dalton
MAB	Monoclonal antibody
MALDI	Matrix-assisted laser desorption/ionization
MHC	Major histocompatibility complex
NWA	New world arenavirus
OWA	Old world arenavirus
PCR	Polymerase chain reaction
PTAH	Phosphotungstic acid-haematoxylin
PAS	Periodic acid- Schiff
RPM	Revolution per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel

	electrophoresis
SSP	Stable signal protein
SSRNA	Single stranded RNA
SOP	Standard operating procedure
TEM	Transmission electron microscopy
TOF/TOF	Time of flight/Time of flight
UHV	University of Helsinki Virus
UPM	Universiti Putra Malaysia

CHAPTER ONE

INTRODUCTION

Inclusion Body Disease (IBD) otherwise known as Boid Inclusion Body Disease (BIBD) is the most important infectious disease that is commonly fatal in captive snakes of the family Boidae (boas and pythons), and sometimes among vipers and cobras (Jacobson et al., 2001; Raymond et al., 2001; Wozniak et al., 2000; Schumacher et al., 1994). The disease was first recognized in 1970s among captive snakes and has frequently been associated with the eradication of entire infected boid collections (Schumacher et al., 1994).

Nineteen percent of all reptiles maintained as pets in the United States today are snakes (Schumacher, 2006), majority of these snakes are members of the Boidae family (Boidae and Pythonidae) and millions of which are kept and bred in breeding facilities for the pet trade. There is currently some concern in countries where BIBD has been found among captive snakes that the disease may become popular among the local populations (Banajee et al., 2012).

The disease has been discovered in several boid snakes including boa constrictor (*Boa constrictor*), green anaconda (*Eunectes murinus*), Haitian boa (*Epicrates striatus*), ringed tree boa (*Corallus annulatus*), garden tree boa (*Corallus hortulanus*), Burmese python (*Python molurus*), reticulated python (*Python reticulatus*), ball python (*Python regius*), and Australian python (*Morelia spilota variegate and Morelia spilota spilota*) (Orós et al., 1998).

Until recently, retroviruses and paramyxoviruses have widely been suspected as the causative agents for a very long time as they were frequently isolated from tissue samples of positive snakes, (Jacobson et al., 2001; Schumacher et al., 1994), a claim that was later discountenanced, as these family of viruses were later recognized as endogenous to these snakes and were also frequently isolated from genome sequences of negative snakes as well. Most recently however, two novel arenavirus genomes were detected while another two were isolated from tissues of snakes histologically diagnosed as positive of the disease (Bodewes et al., 2013; Stenglein et al., 2012) and are confirmed to be the causative agents (Hetzel et al., 2013).

Clinically in boas, the signs are often variable, with regurgitation commonly observed as the first indication of the disease, followed by anorexia. The affected snakes may show central nervous disorder, such as head tremor and opisthotonus. Dysecdysis or abnormal skin shedding frequently occurs as a result of the partial paralysis of the posterior half of the snake (Schumacher et al., 1994). Death may occur from secondary bacterial, fungal and protozoan infections. Other signs include encephalitis, pneumonia, hepatitis, enteritis and osteomyelitis. Neoplastic processes including lymphomas may also occur (Schilliger et al., 2011). This may result from a direct

consequence of immunosuppression that may occur from the impairment of cellular function due to inclusion body (IB) formation in red and white blood cells as well as myelopoietic cells (Chang & Jacobson, 2010; Wozniak et al., 2000). Both the clinical manifestations and the disease progression differ in boas and pythons (Chang & Jacobson, 2010; Schumacher et al., 1994). While the disease can run an acute or chronic course in some affected species, boas die within weeks or months with less nervous signs, or become asymptomatic carriers (Chang & Jacobson, 2010; Vancraeynest et al., 2006). Pythons on the other hand were shown to suffer mostly an acute form of the disease, with clinical manifestation of a severe fatal nervous involvement (Chang & Jacobson, 2010; Vancraeynest et al., 2006; Raymond et al., 2001; Carlisle et al., 1998; Schumacher et al., 1994;).

Until recently, the most rapid diagnostic technique for BIBD was by the detection of intracytoplasmic inclusion bodies in peripheral leucocytes (Bodewes et al., 2013) which was considered the gold standard for the diagnosis of BIBD. Biopsy samples from organs such as the liver, kidney and spleen often proved valuable for an early and successful detection of inclusion bodies (Chang & Jacobson, 2010). Because the pathogenesis was unclear to scientist, the disease remains a mystery, and therefore there is still no drug for its treatment and no vaccine is available for its prevention.

Evidence available suggests that the disease can be transmitted among snakes, but the exact modes of transmission are still not understood (Schumacher et al., 1994). However, the blood sucking snake mite *Ophionyssus natricis* have frequently been associated with snake collections during outbreaks and may therefore act as a vector, even though, there is no experimental evidence supporting this hypothesis (Schumacher, 1996).

A histologically distinctive attribute of the disease is the frequent deposition of typical intracytoplasmic inclusion bodies that are eosinophilic and often described as electron dense and consisting of a protein with a molecular weight of 68kDa (Wozniak et al., 2000) commonly found in peripheral blood cells as well as tissues and organs under light microscopy (Chang & Jacobson, 2010).

The inclusion bodies were sometimes described as granular and non-membrane bound aggregates material (Schumacher et al., 1994), with morphology most closely resembling proteinaceous non-viral inclusions (Del Rosario et al., 1994; Jensen & Gluud, 1994; Manetto et al., 1989; French, 1983; Denk et al., 1979; Norkin et al., 1960). These intracytoplasmic, eosinophilic, non-viral inclusions have also been described in human and animal tissues affected by other disease conditions as well (Del Rosario et al., 1994; Jensen & Gluud, 1994; Scroggs et al., 1989; French, 1983; Denk et al., 1979).

Very little is known regarding the nature, chemical composition and the origin of the 68 kDa Boid Inclusion Body Disease Protein (BIBDP) found within the inclusion bodies (Wozniak et al., 2000), however, several stressors such as infection,

inflammation, exposure to toxins, and heat induce the production of stress related proteins (Boston et al., 1996; Waters et al., 1996; Vierling, 1991). This group of proteins including heat shock proteins, are frequently encountered in protein aggregation diseases and therefore suggest that they may have a role in their formation or in the maintenance of the native conformation of these inclusions, hence their presence as common components of the cellular stress response (Waters et al., 1996).

To test this hypothesis, the present study therefore aims to confirm that BIBD is another protein aggregation disease through the proteomic profiling of heat shock proteins (Hsps) present as common component of the inclusion body in BIBD infected boid snakes through the following objectives:

1. Isolate and identify heat shock proteins from Embryonated Chicken Egg experimentally infected with BIBD pathogen.

2. Isolate and identify heat shock proteins from tissues of boid snakes that died of confirmed Boid Inclusion Body Disease.

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APPENDICES

Appendix 1

Proteomics International Pty Ltd

Box 3008, Broadway, Nedlands, Western Australia 6009
Tel: +61 8 9389 1992 Fax: +61 8 9389 1981
Email: info@proteomics.com.au ABN 78 096 013 455
www.proteomics.com.au ISO 17025:2005

IN CONFIDENCE
Yusut Ilyasu
UPM

Purchase order Number: BASEM-21396

31st July 2014

Protein Identification by MS
Report S140728YIALv2_3374

SAMPLES
PI-reference: 3374
Date received: 21/07/2014
Number of samples: 3
Source of sample: Coomassie stained gel bands
Service required: Protein identification by MS

METHODS
The protein samples were trypsin digested and peptides extracted according to standard techniques (Bringans et al. Proteomics 2008). Peptides were analysed by MALDI-TOF/TOF mass spectrometer using a 5800 Proteomics Analyzer [AB Sciex]. Spectra were analysed to identify protein of interest using Mascot sequence matching software [Matrix Science] with Ludwig NR Database.
Database: Ludwig NR
Taxonomy: Serpentes (July 2014, 40536 sequences)

Accredited for compliance with ISO/IEC 17025, NATA accredited list of SOP's:
0_14 In-gel destain, reduction/alkylation, digestion & extraction of peptides
1_04 4800/5800 MALDI Matrices and Sample Spotting
1_01 Operation of 4800 5800 TOF/TOF Mass Spectrometer
1_06 Generation of Mascot MS Results
2_07 Silver Destaining MS Compatible

SOPs carried out at Proteomics International Facility, located at Harry Perkins Institute of Medical Research, QEII Medical Centre, Nedlands, Perth, Australia.

RESULTS
The results provided relate only to the samples as listed below.

Results can be viewed by going to the web address:
https://sysbio-mascot.wehi.edu.au/mascot

Login
Username: PI-Client226
Password: Laundress

After logging in to the web page, click HOME (top right side of page). Under the Mascot Utilities go to SEARCH LOG. To see all searches, increase the number under the HOW MANY section (e.g from 50 to 100). Click on JOB NUMBER to view the results. Under the FORMAT AS button select **STANDARD SCORING** to display results.

The table below shows how the samples were labeled. The labels correspond to the numbers under SEARCH TITLE in the Mascot Search results page.

The PI ref is 140723 PI-3374 Plate# 7542

Sample name	MS job run and PI-number	Mascot job#
L	140723 – 3374A, A12	249853
H	140723 – 3374B, A13	249854
SP	140723 – 3374C, A14	249852
QC BSA	140723 – QC, A11	249765

All results are stored on a secure server and password protected; data will be available until 2015.

NOTES ON INTERPRETING THE RESULTS

Database: Ludwig NR
Ludwig NR is a comprehensive, audited database designed specifically for mass spectrometry applications. It contains non-identical protein sequence information based on all major publicly available datasets. For further information see:

http://www.matrixscience.com/help/seq_db_setup_nr.html

Viewing the results via the weblink
To view the results in an interface similar to the old version of MASCOT, select the peptide summary option [the peptide summary].

Each peptide is fragmented within the mass spectrometer to produce ions that give amino acid sequence information. In each case the peptide ion data is matched to possible amino acid sequences in the database. This data frequently lends itself to *more than one sequence interpretation.*

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. This means protein scores are the sum of a series of peptide scores and this determines the ranking of protein hits. The probability is a measure of how unlikely it is that the hit is a random event.

The help page [[help]] in the results file provides detailed explanations on most of the features used by the MASCOT software. This can be accessed at http://www.matrixscience.com/help/msms_summaries_help.html#PEPSUM

1. Data in **red** indicates that the protein hit ranked number one in the list of possible sequences (move the cursor over the number in the QUERY column to see the list).

2. **Black** indicates the protein hit ranked lower down the list of possible sequences. Clicking on the number in the QUERY column shows the MS/MS peptide spectra that matched the sequence.

3. When data appears in **bold** this is the first time a peptide found in the dataset has been matched to a protein.

4. Peptides not in bold are seen further down the list of hits, and show the peptide has already been matched to a protein at a higher level of significance.

5. The lists of peptides not assigned to protein hits at the end of the report are sequences of low significance also contained within the sample.

In all cases the best results are achieved where two or more peptides map to the same protein. One matched peptide at high confidence is indicative**. Search results are not absolute and matches near the significance threshold should be closely examined. Evaluate hits carefully if the molecular mass data does not support the Mascot hit. A hit lower down the list but of the correct size may indicate a better match.**

6. The results shown are generated by automatic database searching. Where no significant hit is obtained this may indicate that there is insufficient protein concentration or the protein is not in the database. Analysis against an alternative database or further de novo peptide sequencing may be beneficial.

Search parameters: The search parameters for MALDI analysis on the 5800 MALDI-TOF/TOF mass spectrometer [Applied Biosystems] are as follows:

Peptide tolerance (Peptide tol): ± 0.4
MS/MS tol: ± 0.4
Peptide charge: +1
Mass: monoisotopic
Enzyme: Trypsin
Miss cleavage: 1

7. **Re-searching a database**:
This feature on the new Mascot server can be used to search the spectra data against other available databases listed on the server.

Follow the instructions below to re-search the spectra data against the database of interest.

a. In the Mascot search result page select RE-SEARCH. (If you cannot locate the RE-SEARCH button on the result page go to instruction step e).

b. To re-search the spectra data against any available database, select the respective database and set the taxonomy to the corresponding taxonomic group of the target organism.

c. Click START SEARCH and wait for the results to be displayed.

d. The above search parameters are recommended and will usually appear as the default parameters when the RE-SEARCH option is selected. Note that, search results might differ if the search parameters are altered.

e. If you cannot readily locate the RE-SEARCH button in the search result page look for the FORMAT AS button and change the option to PROTEIN FAMILY SUMMARY.

f. Click on FORMAT AS to update the page. The RE-SEARCH button will be seen at the updated page.

g. Click on the RE-SEARCH button to display all available databases and follow step A to C as detailed above.

APPENDIX 2

Histopathological procedure

Tissue processing

The following procedures are performed during tissue processing**.**

1-Dehydration

2- Clearing

3-Impregnating

4-Embedding

5-Sectioning

6-Staining

Dehydration

Alcohol was used for the removal of all extractable water by dehydrant diffusing through the tissues. Dehydration is done so that the paraffin wax used for impregnation will be easily compatible. An automated tissue processor was used for the dehydration; the duration for the procedure is as follows:

70% alcohol-1hour

70% alcohol -1 hour

95% alcohol -1 hour

Absolute alcohol -1hour

Absolute alcohol – 1 hour

Absolute alcohol -1hour

Clearing

Xylene was used to clear all the excess alcohol and water. Dehydrant is removed and the tissue becomes clear and translucent**.**

Impregnation

The entire clearing agents are been removed in order to allow the paraffin to penetrate the tissue. The tissues are kept in a wax bath containing a molten paraffin wax and the temperature for melting point for the paraffin wax is maintained at $56\text{-}58^{0}$. Impregnating help the tissues to become harden which makes sectioning easier.

Embedding

After the tissue is cleared with alcohol, it is then transferred into a melted paraffin wax, each piece of tissue is placed in a position with its appropriate identifying name beside the tissue pan. The tissue is place down gently with forceps and making all the tissue to flattening, and then it is filled with the melted liquid paraffin wax, after that the pan is placed at the cooler part of the machine containing ice, it makes it harder and it is removed gently.

Sectioning

This is the process where the blocks are sectioned in to a thin ribbon. 3 microtome thickness is used to cut the block gently and the ribbon like is allowed to flow on the water bath and is pick up gently with cover slide and allowed to dry overnight.

Staining

Harris haematoxylin and eosin

Slide were submerged in xylene for 5min

↓

Slide were submerged in 100% alcohol for 5min

↓

Slide were submerged in 70% alcohol for 5min

↓

Rinse

↓

Slide were submerged in haematoxylin for 5min

↓

Rise 3-5 times

↓

Slide is dip in 1% alcohol for 3 seconds

↓

Slides were put under running water for 5 min

↓

95% alcohol was sprayed on the slides, cleared and were allowed to dry.

↓

Clear and dry

↓

DPX is applied together with the cover slip

BIODATA OF STUDENT

Yusuf Ilyasu Maina was born into the family of Mohammed Maina Yusuf and Fatima MM Yusuf 50 years ago at Potiskum town of Yobe State of Nigeria. He started his primary education in 1971 at Central Primary School in Potiskum town where upon his successful completion in 1977, he proceeded to Government Secondary School Damboa in Borno state also in Nigeria. He sat for his General certificate of education (GCE) examination and passed in 1982. He was offered admission by University of Maiduguri same year where he studied Veterinary Medicine. He graduated in 1989 and joined the Nigerian Customs Service in 1990 as an Assistant Superintendent of Customs (ASC). He resigned in 1992 and took up an appointment as Veterinary Officer with Yobe State Veterinary Service where he rose to the rank of Director Veterinary Service. He held several positions in the service and coordinated several projects including the World Bank funded Avian Influenza Control Project, an initiative by the World Bank that helped in the containment of the 2006 outbreak of the Highly Pathogenic Avian Influenza (HPAI, H5N1) that swept across most of Asia and Africa. He also coordinated the European Union sponsored Pan- African programme for the control of Epizootics (PACE), an initiative that helped some African countries implement the OIE- pathway for the eradication of Trans boundary Animal Diseases (TADs) including Rinderpest with a view to help those countries participate in global trade in livestock and livestock products. He is currently coordinating the containment of the resurgence of Avian Influenza in his home state in Nigeria. Yusuf is blissfully married to Zara Aliyu.

LIST OF PUBLICATIONS

Diagnosis of Boid Inclusion Body Disease: Challenges and Future Prospects
Y.Ilyasu , Y. Abba, Z. Zunita, M. L. Mohd-Azmi and M. M. Noordin*
*Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400, UPM Serdang, Selangor, Malaysia.**Corresponding author: E-mail:noordinmm@upm.edu.myMobile: +0133626972Journal of Agriculture and Veterinary Science (IOSR-JAVS) Volume 8, Issue 1 Ver, 1 (Jan. 2015), PP 20-25

Submitted for Publication to Pakistan Veterinary Journal PVJ

Propagation of Boid Inclusion Body Disease Pathogen in Embryonated Chicken Egg
Y. Ilyasu, Y. Abba, Z. Zunita, M. L. Mohd-Azmi and M. M. Noordin*
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