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This seminar series is a publication of seminar papers presented by staff and visiting scientists to the National Veterinary Research Institute, Vom during 2013

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INTRODUCTION

Information on results of research findings and or trials of innovations or fabrications in Research Institutes needs to be presented and publicized at a formal setting. The Seminar and Publications Committee, NVRI, Vom ensured the coordination, organization and presentation of seminars in the Institute regularly in 2013. This provided a platform for researchers, scientists, visiting lecturers on Sabbatical, students and at times other interested stakeholders to brainstorm on such presentations with the aim of fine-tuning these findings for enhanced agricultural production. Undoubtedly, when properly harnessed, such findings will translate into better agricultural output and poverty alleviation in line with the agricultural transformation agenda of the federal government of Nigeria.

In this series we presented highlights of seminar presentations in 2013 covering topical issues of veterinary and public health interest such as: The use of molecular detection method to detect and characterize pigeon variant of Newcastle disease virus (NCDV) from a local chicken in Nigeria, which underscores the role of this avian species in the epidemiology of NCD; The first isolation of pandemic 2009 influenza A/ H1N1 from pigs in Nigeria was presented highlighting a phenomenon known as "reverse zonoosis". Other topics on detection, seroprevalence and binomial regression analysis of zoonotic agents such as *Camphylobacter* spp. in fecal matrices, *Leptospira* spp in rodents, *Brucella* spp. in livestock and Human papilloma virus using hospital records were aptly presented and discussed. A review of rabies diagnosis, which chronicles the activities of the Rabies Unit of the Central Diagnostic Laboratory (CDL) from 2003- 2012, was presented. This report showed that rabies is endemic in Nigeria and concerted efforts from Veterinarians, policy makers and the public should be made to curtail the avoidable deaths from this preventable disease.

An attempt by a researcher to surmount the hurdle of viral DNA extraction from formalin fixed tissues for African Swine Fever (ASF) virus detection

using conventional DNA extraction methods was presented in this compendium. This will certainly paved way for research on ASFV in museum and archeological specimens as well as in forensic veterinary science. High level of lead, above the minimum allowable levels by international health safety organizations was detected in several visceral organs of cattle slaughtered for human consumption, buttressing the fact that the environment (pasture and water) may be highly polluted by this element. This calls for caution when ordering for the popular offal pepper soup to avoid accumulation of lead in our tissues and its attendant health implications.

It is hoped that this publication will serve as a reference material and a guide for researchers in the area of veterinary science and public health.

Dr P. A. Okewole

Chairman

Seminar and Publications Committee

ON BINOMIAL REGRESSION ANALYSIS OF HUMAN PAPILLOMAVIRUS (HPV) INFECTION DATA

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INTRODUCTION

In this article we describe a methodology for the problem of estimating response probabilities where the response variable is categorical, with two or more categories, and where the predictors are also categorical. We are presently concerned with the case where the response variable is reducible to a dichotomous form (Binary).Our aim is to use Human papillomavirus infection data obtained from ABUTH, Zaria to estimate the probabilities for each response category taking factor (predictors) effect into account. To examine model adequacy using goodness of fit statistics. To use Wald statistics to test the hypotheses that there is no difference in responses for the factors and examine whether the fishers exact test will produce the same result. We achieved our aim by using a combination of logistic regression model for categorical data and fishers exact probability test. Following Agresti (2002), we identified an approach amongst the Binomial Logit models for binary data. We apply the methodology to a problem from genital Human papillomavirus infection data.

Cancer is still a major cause of death globally, with the developing countries contributing the highest quota of about 64%, (Jemal et al, 2011). Cancer is not limited by geography or gender. In Nigeria, 250,000 new cases of cancer related deaths are recorded annually, breast and cervical cancer being the most common in Nigeria. The prevalence of cervical infection with Human papillomavirus (HPV), particularly the high risk types that cause cervical cancer, varies greatly worldwide. An estimated global HPV prevalence of 11.7% has been reported, (Bruni *et al,* 2010). It has also been documented that there is a strong positive relationship between the prevalence of high risk types of

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HPV in middle aged women and high incidence of cervical cancer,(Ana et al,2010). Recent studies in Nigeria have shown that about 32 different HPV types cause cervical infection, with the high risk types HPV 16, 31, 35 and 58 dominating. Nigeria has documented an overall HPV prevalence of 26.3% (Clement *et al*, 2010). Most infections are caused by more than one type of HPV, (Paola *et al,* 2009). Since any test for HPV infection is limited to one subtype or a group of subtypes, it will miss a certain number of infections and, therefore the response variable is bound to be affected by misclassification, producing some false negative results, (Carlos *et al,* 2003). Misclassification has implications for proper estimation of prevalence level, proper diagnosis, proper treatment of the infection, and the design of effective control strategies. In the Nigerian situation, high prevalence of HPV is a distinctive feature, probably because the HPV transmission continues into middle and old age without proper detection and intervention, thereby resulting in high prevalence of cervical cancer.

MATERIALS AND METHOD

For this study 131, women among the many being screened at the ante-natal clinic of ABUTH aged 13-45+ were selected to participate in this study. For each woman her infection status is recorded by testing for HPV DNA in cervical samples, whether she was married and the type of marriage arrangement she lives under, whether she has a history of sexually transmitted infection, whether she has history of contraceptive use, and whether she experiences post coital bleeding. The test of independence between the factors and response category was carried out using the Fishers exact probability test. Binomial regression analysis was also carried out in which the multinomial response is subjected to the effect of four factors (Marital status of the subject, whether subject experiences Post coital bleeding, whether subject use contraceptives, and whether subject has a history of sexually transmitted infection). The approach described by Agresti (2002) provides binomial parameters, prediction equations, estimates of the probabilities for each response category taking factors effect into account. The Binomial regression model is a member of the Logistic regression models and is useful in problems where the dependent

variables take on only a few discrete values. Major Fields of application include econometric, time series, biostatistics, medical applications and educational testing, (Annette, 2002).

RESULT

The Fishers exact probability test indicate that Marital status, Post coital bleeding (PCB), use of contraceptives, and having a history of sexually transmitted infection is independent of Human papillomavirus(HPV) infection status of subjects at the ABUTH, Zaria, Nigeria. The Wald statistics on the other hand affirms that all the factors contribute significantly to the predictive ability of the model.

Logit Phi 5/Phi 1 appears to fit the data well. The likelihood ratio chi-square statistics is significant compared with the Pearson chi-square distribution. This suggests that the explanatory variables are important as 93% of the "variation is explained by these factors" in the model. The Wald statistics at alpha= 0.05 also confirms that all the factors in the model contributes significantly to the predictive ability of the model.

CONCLUSION

Motivated by a study of Human papillomavirus (HPV) infection in women, binomial regression analysis is presented in which the multinomial response is subjected to the effect of four factors (Marital status, Post coital bleeding, use of Contraceptives and sexually Transmitted Infections). The approach described by Agresti, (2002) provides binomial parameters, prediction equations, estimates of the probabilities for each response category taking factors effects into accounts. Goodness of fit statistics indicated optimal logit and together with Wald statistics confirms that the factors effects are very significant. It also shows that the logit approach is preferred above the Fishers exact probability test in determining the contributions of the factors to the responses.

Table 1: Goodness of fit statistic for Base Category logit model for HPV infection data based on dummy for the response category and the factors

Logit	$\log\frac{\pi_6}{\pi_1}$	$\log\frac{\pi_5}{\pi_1}$	$\log\frac{\pi_4}{\pi_1}$	$\log\frac{\pi_3}{\pi_1}$	$\log\frac{\pi_2}{\pi_1}$
Deviance	2.836	2.147	0.000	0.000	5.657
Scaled deviance	2.836	2.147	0.000	0.000	5.657
Pearson chi-square	1.426	1.078	0.000	0.000	3.985
Scaled Pearson chi-square	1.426	1.078	0.000	0.000	3.985
Log likelihood (fitted model,11 parameters)	−1.418	−1.074	−9.778	−9.778	−6.569
Log likelihood (minimal model, 1 parameter)	−17.894	−14.295	−17.894	−50.560	−10.349
Likelihood ratio chi-square	$32.953(10)df$	$26.443(10)df$	$16.231(10)df$	$81.562(10)df$	$7.559(10)df$
Pseudo R^2	0.92	0.93	0.453	0.806	0.365
Akaike information criterion (AIC)	24.836	24.147	41.557	41.557	35.138
Finite sample corrected (AICC)	27.054	26.366	43.775	43.775	37.357
Bayesian information criterion (BIC)	56.463	55.774	73.184	73.184	66.765
Consistent AIC (CAIC)	67.463	66.774	84.184	84.184	77.765

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ISOLATION AND MOLECULAR CHARACTERIZATION OF PIGEON VARIANT OF NEWCASTLE DISEASE VIRUS FROM A LOCAL CHICKEN

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INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease of poultry that may result in 100% morbidity and mortality in susceptible flocks. Newcastle disease virus (NDV), the aetiologic agent of Newcastle disease belongs to the genus Avulavirus within the family *paramyxoviridae* in the order *mononegavirales.* It is designated Avian paramyxovirus 1 (Alexander, 1997; Mayo, 2002; Lamb and Kolakofsky, 2002; and Pedersen *et al.*, 2004). It is an envelope, single stranded non-segmented, negative sense RNA virus (Seal *et al.*, 2000).

ND has been established in at least 241 species of birds (Kaleta and Baldauf, 1988). Most species of birds; Chickens, Turkeys, Pheasants, Quails and Guinea-fowl are highly susceptible to virulent APMV-1. A pigeon-adapted variant of avian paramyxovirus serotype 1 also known as Pigeon paramyxovirus serotype 1 (PPMV-1) classified as Lineage 4 (Aldous *et al.,* 2003) primarily affects pigeons and doves (Columbiformes) it is however known to infect chickens and other poultry species. (Alexander *et al.,* 1985)
So far, four ND panzootics have been reported in the world. The third panzootic was caused by PPMV-1 (lineage 4) which started during the early 1980s involving racing pigeons and doves kept by fanciers. The outbreak spread through contact between birds at pigeon races, bird shows and through international trade in these bird species (Alexander, 2001).
Outbreaks of PPMV-1 in pigeons have been recorded in Japan since 1984 and still continued. Characterization of the Pigeon paramyxovirus (PPMV-1) isolated from chickens in South Africa indicated close similarity to the Japanese strains (Abolnik *et al.,* 2004).

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Whereas PPMV-1 have been recovered from a parrot in Nigeria (accession number FM200798) in 2007 (Snoeck *et al.*, 2009), there is no report of similar isolation and characterization in chickens. This paper to our knowledge reports for the first time the isolation and molecular characterization of PPMV-1 virus recovered from a local chicken in Nigeria.

MATERIALS AND METHODS

Virus

Since the first report of the highly pathogenic avian influenza (H5N1) in Nigeria in 2006, there has been continued investigation of cloacal and tracheal swabs taken from apparently healthy and sick birds in addition to tissues from dead birds, for the purpose of early detection of H5N1 virus and the monitoring of Newcastle disease viruses.

Virus isolate NGCK1208 - HQ456844 was recovered from a chicken that died in a live bird market in Yenogoa, Bayelsa state during targeted live bird surveillance for avian influenza and Newcastle disease in 2008.Virus isolation was done at the Avian influenza laboratory of National Veterinary Research Institute, Vom. By inoculation of tissue suspension in 9 day old embryonated chicken eggs, the Newcastle disease virus isolate was identified by Haemagglutination(HA) and Haemagglutination inhibition (HI) tests, using standard procedures (OIE, 2009).

Virus RNA extraction and RT-PCR

Viral RNA was extracted from fresh allantoic fluid using Trizol(R) LS Reagent (Invitrogen) according to the manufacturer's instructions.

RT-PCR

One-step RT-PCR was performed for the amplification of a fragment of the fusion gene including the Fo cleavage site, in a 30µl reaction mixture containing; oligonucleotide pair described by Abolnik *et al.,* (2008).

Reaction mixture was cycled in an Applied Bio systems Verity ™ 96-well thermal cycler (model #: 9902, S/N 299020808, Singapore) under the following cycling condition; 42^{o}C for 20 min, Reverse transcription 94^{o}C for 5 minutes, initial denaturation. 35 cycles of: 94^{o}C for 30 sec denaturation 53^{o}C for 30 sec. annealing72^{o}C for 1 min Elongation

DNA sequencing and editing

To determine the exact order of the nucleotide bases and amino acid sequences within the F_o cleavage site, the purified PCR product was sequenced using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit V4.1 (Applied Bio systems) followed by analysis with an

AB1377™ automated sequencer according to the manufacturer's instructions. Nucleotide sequence editing was conducted with Bio Edit.

Phylogenetic analysis

A blast homology search of the sequence was done to identify closely related sequences, which were included in a multiple sequence alignment, using Muscle. Phylogenetic analysis of the partial F gene nucleotide sequence (nt 61 to 374) was done using Mega 5.2(Tamura *et al.,* 2011)

RESULTS AND DISCUSSION

A haemagglutinating agent was detected using HA test which was further identified as Newcastle disease virus using HI test. The HI test was also used to exclude the presence of avian influenza viruses. Following the trizol extraction of genomic RNA from fresh allantoic fluid, a 1180bp fragment was amplified by RT-PCR.

Sequence and phylogenetic analysis of this strain NGCK1208 - HQ456844 revealed a clustering with lineage 4 viruses designated as PPMV-1(fig. 1)

The mean distance similarity of sequence of NGCK1208- HQ456844 with sequences of reference strains was 96.9% with mean difference of 0.031%. Differing by 0.040% from strain FM200798 recovered from a parrot in Nigeria in 2007 and 96% similar with two substitutions; a $N \rightarrow S^{30}$ and $K \rightarrow Q^{114}$ at the cleavage site. It also shared a 95% similarity with strain AY288996 from a pigeon isolated in Italy in 2000. Genetic distance of 0.043% between NGCK1208- HQ456844 and two pigeon isolates from New York City; FJ410145 and EF520716 isolated in 1984. A difference of 0.061% was recorded between the Nigerian strain and the strain (AY445669 ZA469/PPMV1/02) recovered from chickens in South Africa. The deduced amino acids of Nigerian and South African stain revealed multiple substitutions; $L \rightarrow P^{28}$, $S \rightarrow N^{30}$, $V \rightarrow A^{49}$, $V \rightarrow G^{111}$ and $K \rightarrow Q^{114}$ at the cleavage site.

Outbreaks of ND in domestic poultry due to infection with PPMV-1 have been reported. This was believed to be as a result of contamination of poultry feed with faeces from infected feral pigeons (Alexander *et al.,* 1984). Similarly, there are reports of outbreaks of ND in unvaccinated poultry associated with the pigeon variant of APMV-1 infection mainly due to contact between infected pigeon and poultry (Alexander 2001; Dilaveris et al, 2007). Newcastle disease due to infection with PPMV-1 in grey Partridges (*Perdix perdix*) has been reported. Feral pigeons living above the affected pens of partridges were incriminated as the source of infections (Irvine *et al.*, 2009). Several outbreaks of ND in Pheasants due to PPMV-1 have been documented (Alexander, 1997).

Even though, ND is endemic in Nigeria with widespread epidemic outbreaks; there has been no report of PPMV-1 related outbreak in chickens. The husbandry practices where there is co-mingling of multi species of birds including pigeon and other wild birds with domestic chicken in LBM and rural household makes transmission of velogenic NDV from pigeon to chicken and other species inevitable.

This study to our knowledge reports for the first time the detection and molecular characterization of PPMV-1 from chicken in Nigeria. More worrisome is the recovery of the PPMV-1 from a chicken that died showing signs of ND. This finding highlights the vulnerability of domestic poultry to PPMV-1 infection.

CONCLUSION

There is need for a continuous and sustained surveillance for ND in domestic, wild and free-living birds. This should be followed by advanced genomic analysis of the pathogen to understand the evolutionary dynamics of the virus. Farmers, live bird vendors should be educated on the danger of mixing multi-species of birds in same cages and premises.

Good management practices like; properly constructed poultry houses that will screen off pigeons and other wild and free-living birds around poultry houses should be emphasised and encouraged. Also, feeds should be properly stored and farm utensils clean and avoid exposure to contamination by dropping from wild and free-living birds around the poultry premises

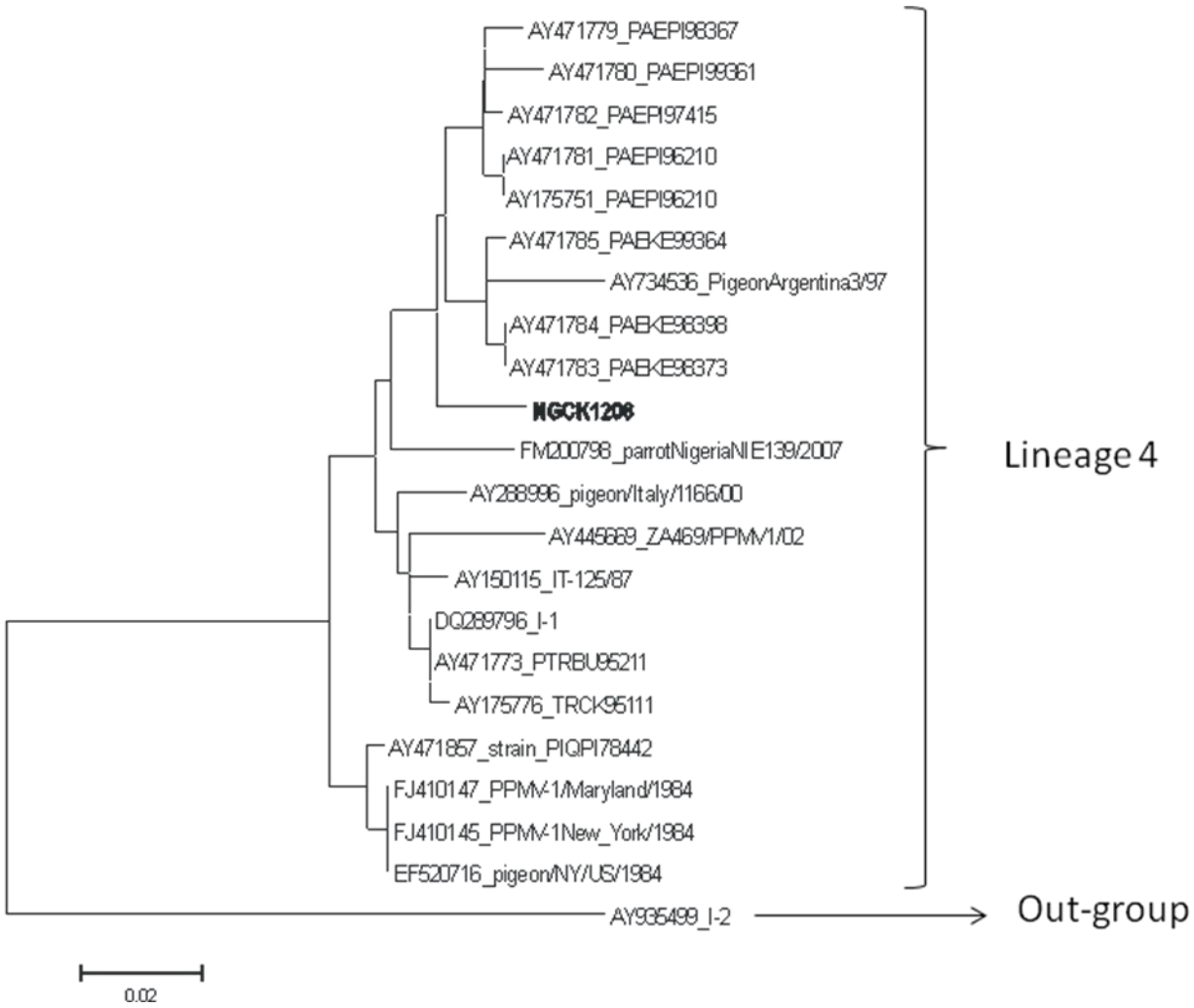

Fig. 1 A neighbor joining tree showing the Nigerian PPMV-1 in this study (in bold-face) clustering with representatives of Lineage 4

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SURVIVAL PATTERN OF CAMPYLOBACTER IN VARIOUS FAECAL MATRICES

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INTRODUCTION:

Campylobacter is the single largest cause of sporadic bacterial gastrointestinal infections worldwide occurring more times than the combined total cases for *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* (Blaser, 1997; Centers for Disease Control and Prevention (CDC), 2008). Infection is almost always derived through the faecal-oral route with as little as 500 *Campylobacter* cells initiating disease in an individual (Black *et al.*, 1988).

In the United Kingdom (UK), almost half of all bacterial infectious intestinal disease is caused by *Campylobacter* (The University of Aberdeen., 2008). In the United States of America, the Centre for Disease Control (CDC), estimates over 2.4 million persons are affected every year or 0.8% of the entire population with an approximate 124 deaths from *Campylobacter* infections (CDC, 2010). Lack of a national surveillance program for campylobacteriosis in many developing countries prevents determination of an accurate data of incidence. Isolation rates range from 5-20%, while case-control community-based studies in Egypt and other countries, have provided estimates of 40,000-60,000 cases in children <5 years of age (Oberhelman and Taylor, 2000; Rao *et al.*, 2001).

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Poultry, livestock and wild animals have all been implicated in the harbouring and spread of *Campylobacter,* though studies of sporadic outbreaks of *Campylobacter* infections have implicated poultry as the biggest source of infection to humans (Altekruse *et al.*, 1999; Sheppard, *et al.*, 2009).

Campylobacter growth outside its host and in the environment is limited because of its thermophilic nature and also in its susceptibility to many environmental stresses, such as ambient temperature (Holler *et al.*, 1998), atmospheric oxygen (Hoffman *et al.*, 1979), and drying (Doyle and Roman, 1982). Without the ability to produce spores or any other known physiologically based protective mechanism, the main survival strategy for Campylobacter appears to be the production of large numbers, in the hope that once in the 'environment', enough will survive to infect another host (Jones, 2001).

Survival mechanisms for *Campylobacter* seem to be limited compared to other more established model organisms like *Escherichia coli* and *Bacillus subtilis* (Park, 2002). This might be due to the fact that its physiology is still poorly understood and survival strategies not yet fully known owing to its seeming lack of adaptive response features (Park., 2000).

Phase variable gene expression in *Campylobacter*, as a mechanism, can be seen in virulence (Purdy *et al.*, 2000), tolerance to temperature (Chan *et al.*, 2001) and subtypes noticed during poultry processing (Newell *et al.*, 2001). The presence of hyper-variable sequences in *Campylobacter*, also gives it the freedom to generate diversity through phenotypic selection (Parkhill *et al.*, 2000). When exposed to certain stressors, certain genes are switched on in a limited number of cells which survive and perpetuate that resistant phenotype.

In an experiment conducted by Sinton *et al.* (2007), survival studies of indicator and pathogenic bacteria in bovine faeces on pasture, was carried

out. Results seen after the first three weeks showed no increase in counts for *C. jejuni* with a 90% inactivation in 6.2 days from first day of deposition.
In another experiment, Moriarty *et al.* (2010) measured the survival rates for *E. coli*, *Enterococci* and *Campylobacter* species in sheep faeces on pasture. Growth was strongly influenced by moisture content while inactivation, by dehydration.Also, *Campylobacter* species were rapidly inactivated at a rate which tended to be faster at higher temperatures.
In carrying out this experiment, our aims were:

a. To evaluate the survival of *Campylobacter*, by assessing its detection in various faecal matrices by culture on selective media.
b. To determine the effect of temperature differences on the survival pattern of *Campylobacter* in faecal matter.
c. To compare adaptive survival ability between natural and inoculated *Campylobacter* strains in the different faecal matrices.

In this study, culture and plate counting of colonies was used in the isolation, identification and enumeration of *Campylobacter* species while MLST was used in strain identification.

METHODOLOGY

Freshly voided pooled faecal samples were collected from cattle, sheep and chicken from farm around Aberdeenshire, and immediately transported to the laboratory.
Approximately 3kg of the faecal sample was placed into a bucket and thoroughly mixed using gloved hands. Two 750 g portions were placed into 2 Tupperware boxes, to be used as naturally contaminated samples.
Previously sequenced and archived *Campylobacter* (ST-827- University of Aberdeen ID C184E), was used as the inoculum for spiked faecal samples. Ten millilitres of the inoculum in phosphate buffered saline (PBS) solution was in-pipetted into various places in the remaining faeces (~1500 g) faeces,

and thoroughly mixed using gloved hands. The inoculated faeces were split into 2 batches (each 750 g).

One naturally contaminated (1A) and one inoculated (1B) were placed into the fridge at approximately 4^{o}C and one naturally contaminated (1C) and one inoculated (1D) were placed in a normal atmosphere incubator at 15^{o}C.

Sampling:

On the sampling days, 10 grams of the faecal sample was serially diluted and plated onto selective media plates, charcoal cefoperazone deoxycholate agar (CCDA). The CCDA plates were incubated under microaerophilic (5% oxygen, 10% carbon dioxide, 85% nitrogen) conditions at 37^{0}C for 2 days after which presumptive Campylobacter colonies were counted. To confirm the colonies as *Campylobacter,* a latex agglutination test (M46CE; Microgen, Camberley, UK) was carried out according to manufacturer's instructions.

Sampling was performed at day 0, 3, 7, 14, 21 and 28.

Molecular Typing:

Isolates which had tested positive for *Campylobacter* were then subjected to DNA extraction.

An isolate each was selected from the 'start' point of each experiment and 'end' point, for all the non-inoculated samples while only isolates from the 'end' point were selected from inoculated samples.

DNA was extracted from colony growth by lysis in the presence of Chelex 100 resin (CAT No.142– 1253; Bio-Rad Laboratories, CA, USA) as described previously (Gormley *et al.*, 2008).

MLST PCR and sequencing reactions were carried out using the methods described by Dingle *et al* (2001), while sequencing was carried out at Oxford University.

Seven housekeeping genes: *pgm* (phosphoglucomutase), *glyA* (serine hydroxymethyl transferase), *gltA* (citrate synthase), *glnA* (glutamine

synthetase), *tkt* (transketolase), *uncA* (the ATP synthase subunit) and *aspA* (aspartase A), were amplified by PCR.

Primers for five of the loci (*pgm*, *glyA*, *gltA*, *glnA*, and *aspA*) were used as previously described (Miller *et al.*, 2005). Primers *tkt* and *uncA* were modified at The University of Aberdeen and described below.

Table 1. Modified primers *tkt* and *uncA*

Locus	Dideoxyoligonucleotide primer	Amplicon size (bp)
tkt-F	GCWGATATTTTAASKGTTTTAAGTTATC	653
tkt-R	TGACTKCCTTCAAGCTCTC	
uncA-F	TGTTGCMATWGGWCAAAAGC	660
uncA-R	CTTTGTCCRCGTTCAAGTTG	

Table 2. Other primers utilized as described by Miller *et al.* (2005)

Locus	Dideoxyoligonucleotide primer	Amplicon size (bp)
asp-F	GAGAGAAAAGCWGAAGAATTTAAAGAT	676
asp-R	TTTTTTCATTWGCRSTAATACCATC	
glnA-F	TGATAGGMACTTGGCAYCATATYAC	751
glnA-R	ARRCTCATATGMACATGCATACCA	
gltA-F	GARTGGCTTGCKGAAAAYAARCTTT	706
gltA-R	TATAAACCCTATGYCCAAAGCCCAT	
glyA-F	ATTCAGGTTCTCAAGCTAATCAAGG	716
glyA-R	GCTAAATCYGCATCTTTKCCRCTAAA	
pgm-F	CATTGCGTGTDGTTTTAGATGTVGC	720
pgm-R	AATTTTCHGTBCCAGAATAGCGAAA	

PCR mastermix for the amplification of the seven *Campylobacter* loci was as follows; 1.25µl of a 10 x NH4 reaction buffer, 0.63µl of MgCl (50mM), 0.1µl of dNTPs (25mM), 0.06µl of F+R Primers, 0.06µl of Taq DNA polymerase 5 U/µl, 8.91µl of nuclease-free water and 1.5µl of the DNA template.

The amplification cycle was denaturation at 95°C for 2 minutes, followed by 35 annealing cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C with a final extension step of 72°C for 5 minutes.

Following PCR, each of the 96 plate wells had 12.5 ml of PEG precipitation mix (20% (w/v) PEG 8000, 2.5 M $NaCl_2$) added, briefly vortexed and centrifuged at 710 x g then left at room temperature for 1 hour. The plates were then centrifuged for 80 minutes at 1400 x g. The supernatant was discarded and product washed with 150 µl of 70% ethanol and allowed to dry. 50µl of sterile distilled water (SDW) was added mixed, briefly spun and stored at -20°C or immediately used in the sequencing protocol.

Sequencing protocol:

The mastermix for the MLST sequencing reaction of the seven MLST loci was; 2.0µl of 5x sequencing buffer, 0.45µl of BigDye Terminator v3.1, 0.1µl of F or R primers, 5.45µl of nuclease-free water and 2.0µl of the previous PCR product.

Reaction conditions were 96°C for 2 minutes, followed by 25 cycles of 96°C for 10 s and 2 minutes at 60°C. The products were purified by adding 80µl of SEQ precipitation mix (187.5 ml of absolute ethanol, 9 ml of 3M sodium acetate pH 5.3, 43.5 ml of Sigma water) to each product. The plates were mixed, briefly centrifuged and stored at -20°C for 30 minutes then spun for 40 minutes at 1400 x g and the supernatant discarded. The DNA pellet was washed with 150 µl of 70% ethanol, allowed to dry then sent to Oxford University, UK, for sequencing.

Sequencing of a single strand only was done for all the samples. The forward strand was used.

Sequences were uploaded to BioNumerics (v6; Applied Maths) to identify sequence errors and mis-called bases, and to identify alleles for each product using the database at PubMLST website [http://pubmlst.org/campylobacter/ (accessed July 2012)]. Each of the

seven sequences was given an allele number from which sequence types were enumerated.

Line graphs were constructed to show decay patterns over time for all the samples.

RESULTS AND DISCUSSION

Faecal samples for each experiment were sourced from multiple animals within the flock. This took care of the possibility of having high *Campylobacter* shedders, low shedders and possibly non-shedders among the animals.

Line graphs for the animal species sampled showing $\log_{10}$ colony forming units against the sampling days are given below.

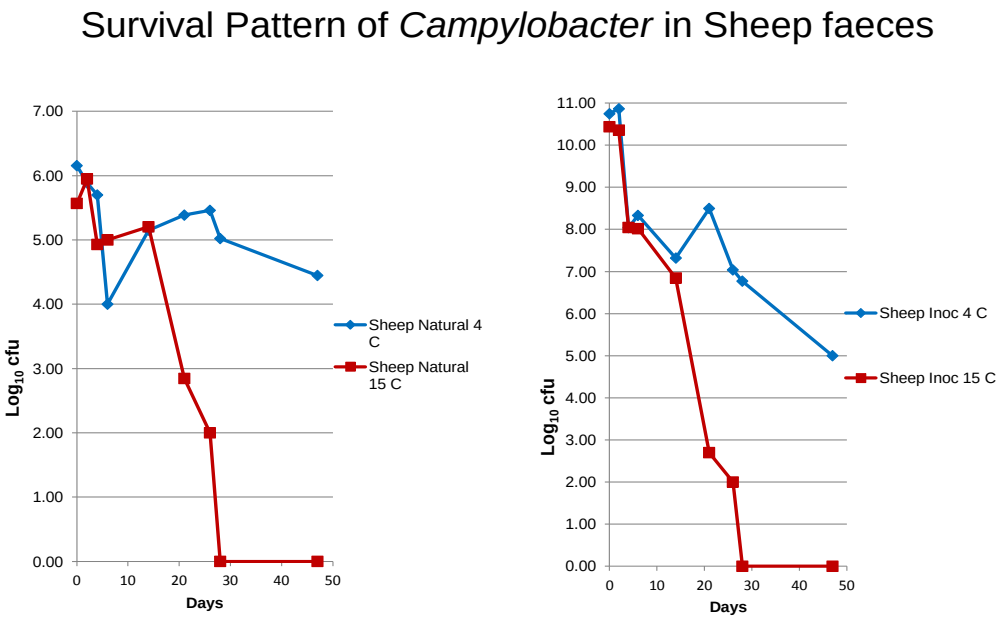
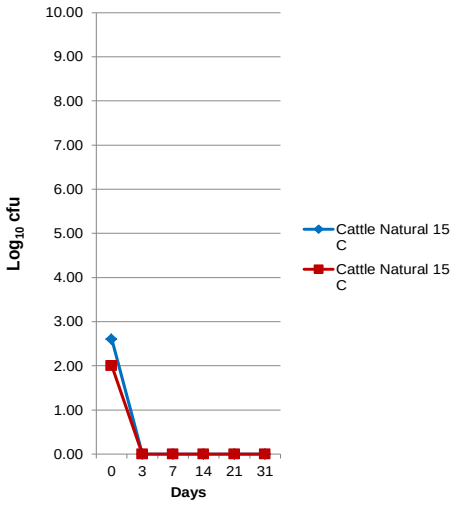


Figure 1 **Figure 2**

*Figure 1 and Figure 2. Graphs of Naturally contaminated and inoculated sheep faeces at 4°C and 15°C

Survival Pattern of *Campylobacter* in Cattle faeces



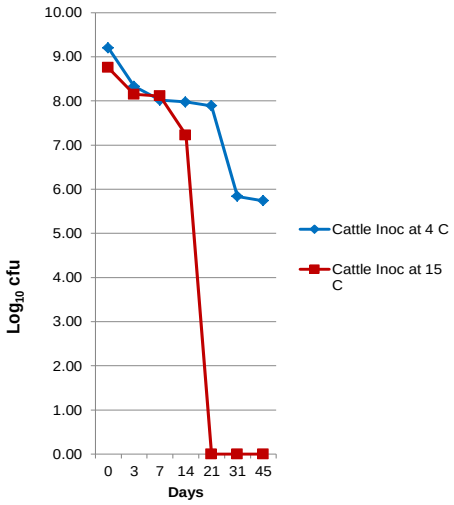


Figure 3 **Figure 4**

*Figure 3 and Figure 4. Graphs of Naturally contaminated and inoculated cattle faeces at 4°C and 15°C

Survival Pattern of *Campylobacter* in Chicken faeces

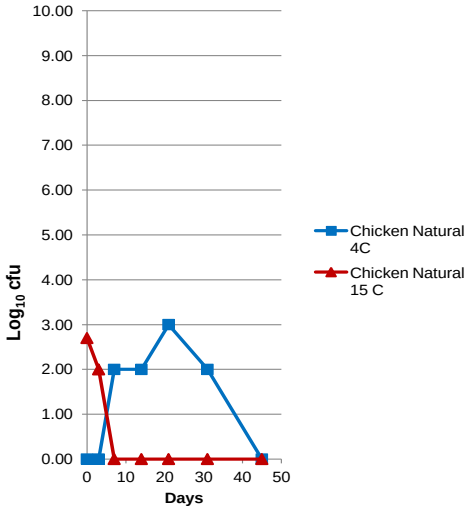


Figure 5.

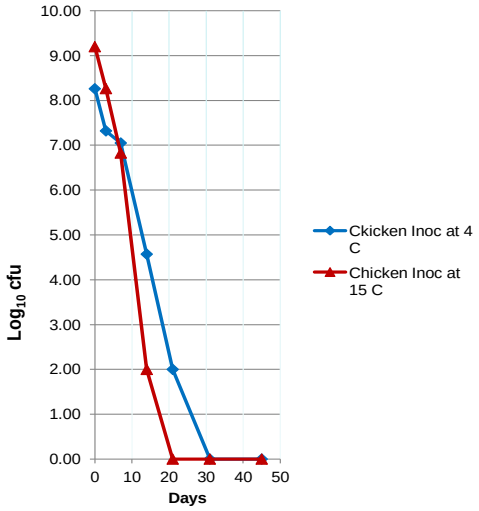


Figure 6.

*Figure 5 and Figure 6. Graphs of Naturally contaminated and inoculated chicken faeces at 4°C and 15°C

For the sheep sample at 4^0C, both the inoculated and naturally contaminated samples showed an initial decline in colony counts which increased by 1 order of magnitude by the 4^{th} sampling date. From then on, a decline in colony counts was detected but still showed up to 2.8 x 10^4 cfu's per gram of faeces at the point of termination of the experiment for the naturally contaminated sample and 1.0 x 10^5 for the inoculated sample.

At 15^0C, the survival pattern showed a slight drop in colony count of 2 orders of magnitude in the inoculated sample and 1 order of magnitude for the naturally contaminated sample by the 3^{rd} sampling day followed by a reduced decline rate, then a rapid regression till no colonies were detected by direct plating or enrichment on the 8^{th} sampling day.

In cattle, the naturally contaminated batch had an initial count of 4.0 x 10^2 cfu's per gram of faeces. By the 2^{nd} sampling day, both the sample at 4^0C

and 15^0C had no detectable colony counts by direct plate or enrichment. Inoculated cattle faeces had *Campylobacter* figures of 1.6 x 10^9 cfu's per gram of faeces on the first day of sampling day 0. Values on the graph show a declining rate of 1 order of magnitude for the sample at 4^0C and 2 orders of magnitude for the sample at 15^0C between the first sampling day and the fourth sampling day. At day 21, no *Campylobacter* was detected in the sample kept at 15^0C. The sample at 4^0C still had detectable *Campylobacter* at 5.5 x 10^5 cfu's at the termination of the experiment on day 45 (Figure 4).

The naturally contaminated chicken samples presented a unique picture (Figure 5). While the sample kept at 15^0C showed an initial cfu count of 5.0 x 10^2 at day 0, a rapid decline occurred and was undetectable by the third sampling point. The sample at 4^0C showed no detectable *Campylobacter* colonies on day 0 by direct plate but was detectable by direct plate on the fifth sampling day. Thereafter, it declined to undetectable limits by day 47. Very low counts within the faecal matrix could be the explanation for this pattern. Initial colony counts for inoculated chicken faeces gave a value as high as 1.6 x 10^9 cfu's per gram of faeces at day 0 (Fig.6). Counts for both the samples stored at 4^0C and 15^0C, showed a similar decline pattern reaching undetectable limits by day 21 for the sample kept at 15^0C and day 28 for the sample kept at 4^0C.

From the results of the experiments carried out, *Campylobacter* was determined to be able to survive outside its natural host for varying lengths of time in sheep, cattle and chicken faeces.

Storage temperatures of 4^0C and 15^0C across all the samples had a noticeable effect on the survival rate and pattern of *Campylobacter*. Survival rate was longer at 4^0C in all cases between the naturally colonized and inoculated samples. It was also seen that samples deliberately spiked with *Campylobacter* survived longer over the naturally colonized samples.

MLST results

The 7 housekeeping genes, *pgm*, *glyA*, *gltA*, *glnA*, *aspA,tkt* and *uncA* were amplified for each sample chosen for analysis. A total of 20 isolates were analyzed which represented the start and end points for all the experiments. Using Bionumerics (v6; Applied Maths at PubMLST website http://pubmlst.org/campylobacter/ accessed July 2012), allele numbers were assigned to each sequenced loci. This is enumerated in the table below.

Allele	Naturally Contaminated						Inoculated		
	Start 4⁰C	End 4⁰C		Start 15⁰C	End 15⁰C		INOC. ST	End 4⁰C	End 15⁰C
aspA	0	0		0	0		33	0	0
glnA	0	0		0	0		39	263	0
gltA	76	0		0	0		30	30	2
glyA	82	82		37	82		82	82	62
pgm	104	104		60	104		104	106	11
tkt	0	51	0	0	56	51	0		
uncA	36	0		5	17		17	17	248
ST	888	854	273	854	828	646	1489		
CC	828	828	206	828	828	828	354		
Strain	C.coli	C.coli		C.jejuni	C.coli		C.coli	C.coli	
	C.jejuni								

Diversity of clonal complexes

From observations of the table, allocation of allele numbers for certain loci was not possible. However, the available allele numbers were queried against the database at http://pubmlst.org/campylobacter/ and the closest matched sequence type (ST) and clonal complex (CC) derived. In the sheep samples, all the clonal complexes belonged to ST-828 except two which were

from CC ST-206 and ST-354. Strain type were predominantly *C. coli* with only two being *C. jejuni*.

CONCLUSION

The results obtained from the various experiments showed the ability of *Campylobacter* both as natural contaminants in the faeces and as inoculated additions, to survive in sheep, cattle and chicken faecal matrices. The survival pattern though was varied amongst the matrices and has been shown. The effect of temperature on survival of *Campylobacter* was studied and seen to be profound. An increase of 11^0C from 4^0C produced a significant decrease in survival length and detection. Faecal matter kept at 15^0C dried up faster compromising the survival of *Campylobacter.*

Between indigenous *Campylobacter* and inoculated strains, survival was studied and found to be possible for both but at high initial concentrations.

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I want to appreciate my supervisor, Dr. Ken Forbes who showed me how to get it right and so made this thesis possible. To Dr. Emma Sproston for guidance right at the start**,** the Executive Director, NVRI Vom for his support towards my studies. Finally, to my family, classmates, colleagues and friends for the show of love

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REVERSE ZOONOSIS: THE TRANSMISSION OF PANDEMIC A/H1N1 INFLUENZA VIRUS FROM HUMAN TO PIGS IN NIGERIA

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INTRODUCTION

Ecological and biological barrier in disease transmission between human and animals has narrowed over the years, propel in part by climate change, habitat alterations, and intensification of livestock production in peri-urban zones (Meseko *et al.*, 2013). These activities have increased intermingling of human and animals in shared environment. There is therefore a present concern with respect to many diseases that can be transmitted at the human-animal interface.

Over 70% of emerging infectious diseases are of animal sources and are directly transmitted in nature to human and approximately 60% of all human diseases are zoonotic (World Bank, 2012; CDC, 2013). Episodes of transmission of infectious diseases from human to animal are also on record, prominent among others was the Spanish influenza pandemic of 1918 that caused over 50 million deaths in human and was also transmitted to pigs (Taubenberger and Morens, 2006). The virus was eventually isolated for the first time in pigs (CDC, 2010).

The H1N1 virus was established in pigs as classical swine influenza,

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Seminar presented 1st August, 2013 at NVRI Auditorium

circulating globally and described as American, Asian, and European lineage. Till date no known descriptions in literature or database exist with respect to swine influenza lineage in Africa. This is thus a major gap in Knowledge considering there are over 30 million pigs in the region (FAO, 2009; Capua and Cattoli, 2010)

When 2009 pandemic H1N1 influenza was reported first in Mexico, the virus was described as a progeny of the classical swine influenza virus sharing genes from avian, swine and human (Garten, *et al.*, 2009). The dearth of data on classical swine influenza in Africa and the global spread of pandemic H1N1 necessitate influenza virus investigation at the human-animal interface in Nigeria, a region described as zoonotic hotspot (ILRI, 2012).

METHODOLOGY

Field sampling in the sentinel surveillance for influenza-like illness at the human-animal interface was carried out in a multi complex pig farm estate in Nigeria over a period of two years. Two hundred and twenty seven clinical cases of respiratory infection were observed in pigs and 40 cases of influenza-like illness were also sampled from human handlers of pigs. Molecular screening by Real time RT-PCR and subtype identification of influenza A virus was carried out. This was followed by virus isolation in specific antibody negative chicken embryonated eggs. Thereafter isolates were visually identified by negative staining Electron Microscope. Molecular characterization of selected isolates by DNA sequencing was subsequently carried out. Nucleotide BLAST of the HA and other genes was performed and phylogenetic trees were constructed on MEGA 5 using the Neighbor Joining method.

RESULTS AND DISCUSSION

Thirty one (13.77%) samples from pigs were positive by real time RT-PCR (Figure 1). Twenty nine of those (12.9%) were isolated in chicken embryonated eggs. Typing with subtype specific primers and probes and

haemagglutination inhibition with homologous antisera confirmed 18 (8%) as 2009 pandemic influenza A/H1N1.

Further molecular characterization of selected isolates by sequencing and analysis in Seqscape, MEGA5 and BLAST showed 99% nucleotide homology with related pandemic influenza virus earlier detected in human host in San Diego, USA and neighbouring West African countries of Ghana, Cameroon including earlier human isolates in Nigeria (Dalhatu *et al.,* 2012). The Nigerian swine isolates of pandemic influenza A/H1N1 are monophyletic and clustered with other 2009 A/H1N1 influenza pandemic in the Phylogenetic tree (Figure 2).

Point by point analysis of nucleotides showed mutations at 13 positions and three amino acid substitutions in the HA gene alone in comparison with prototype A/California/07/2009. Significantly, aspartate (Q) to arginine (R) substitution at the antigenic binding domain of HA in position 240 (H1-numbering) 226 (H3 numbering) was observed.

Phylogenetically, the virus and related virus in the global Genebank and Global Initiative for Sharing Influenza Data (GISAID) shares it's HA gene with swine and human influenza, the NA gene with avian influenza while the internal genes were derived from swine and avian lineages of influenza A/H1N1.

This is the first isolation of pandemic 2009 influenza A/H1N1 in pigs in Nigeria and the second in Africa (Njabo *et al.*, 2012). Full genomic data have been deposited at the Genbank and assigned accession numbers: JX442481- HA, JX4442482- NA, JX482555-PB2, JX482556-PB1, JX482557-MA, JX482558-NP, JX482559-PA and JX48260-NS (Bao *et al*, 2008). Data obtained and information gathered from this study underscores the importance of surveillance of influenza virus at the human-animal interface. Continuous circulation of this mutated virus in susceptible and replication efficient host (pigs) may result in increase mutations and substitution or re-assortment with other circulating virus form human and

other species leading to further antigenic molecular/biological changes and the emergence of novel, zoonotic influenza virus with pandemic potential. Epidemiological information, antigen and genetic data obtained in this study are useful for influenza virus diagnosis, control and pandemic preparedness in Nigeria and globally.

CONCLUSION

2009 pandemic influenza A/H1N1 is primarily a human virus. This study and similar investigation across the globe showed evidence of transmission of the virus from human to pigs in a phenomenon known as reverse zoonoses. This demonstrates the importance of One Health and cross-sectoral approach in emerging infectious disease control and public health policies.

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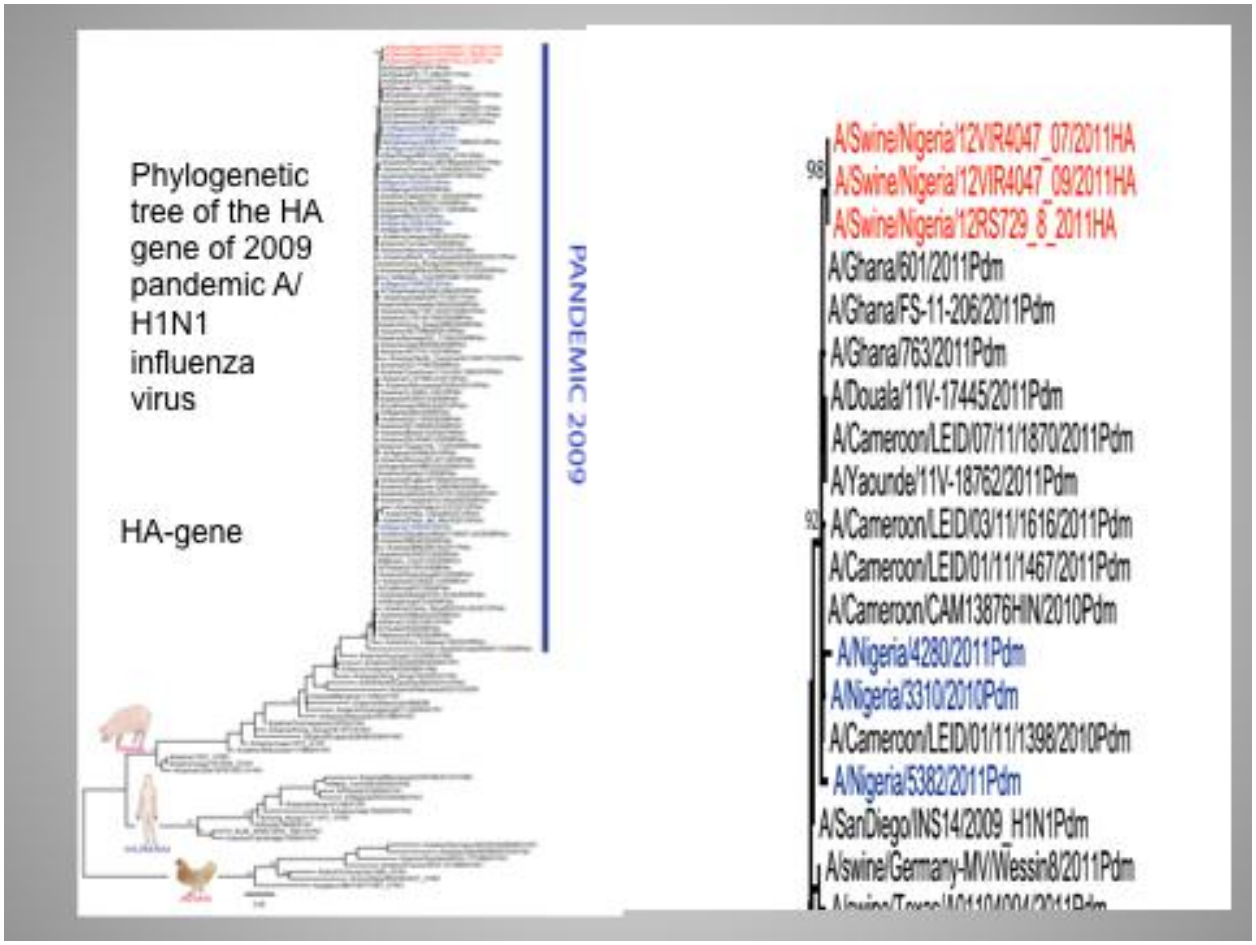

Figure 1: Phylogenetic tree of the HA gene of 2009 pandemic A/H1N1 Influenza virus

DETECTION OF AFRICAN SWINE FEVER VIRUS FROM FORMALIN FIXED AND NON-FIXED TISSUES BY POLYMERASE CHAIN REACTION

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INTRODUCTION

Molecular based analysis has a vast application in bio-medical research especially in the utilization of stored or preserved samples. Formalin fixing and paraffin embedding of tissue samples is one of the techniques for preserving the morphological integrity of tissues for a relatively long time prior to analysis. These preserved samples are a vast repository of genetic information of interest to both biological and medical researchers especially in a situation where fresh or frozen tissues are not available (Wandeler et al., 2007), or where the diagnostic integrity of such samples can be compromised by poor infrastructures.

However, the use of molecular DNA based techniques on formalin treated tissues has been with enormous challenges because of the capacity of formaldehyde to form cross links between nucleic acids and proteins that causes fragmentation of genomic material (Kayser et al., 1988; Perlmutter et al., 2004). These fragmentations lead to the inability to amplify large molecular products by polymerase chain reaction (PCR) (Gilbert et al., 2007 and Gillio-Tos et al., 2007). Moreover the success of PCR is hinged on the quality of the DNA extract

African swine fever (ASF) is an acute DNA viral disease of pigs with high morbidity and mortality and thereby affecting food security at household and commercial levels. Molecular technique has been one of the rapid diagnostic procedures employed in the diagnosis of ASF virus. African swine fever virus is a large and complex enveloped DNA virus and member of the

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family Asfarviridae with a genome size range of 170 -190 Kbp. The aim of this work was to detect ASF genome from formalin fixed tissues and non-formalin fixed tissues using a conventional DNA extraction procedure and commercial kits in view of the need for a rapid and qualitative DNA extraction protocol on the type of samples submitted to the laboratory for ASF diagnosis.

MATERIALS AND METHODS

This study utilized African swine fever positive and negative tissues retrieved from -80^{o}C ultra low freezer of the Applied Biotechnology Department, National Veterinary Research Institute, Vom Nigeria. Four positive tissues (mesenteric lymph nodes, lungs, liver and spleen) and four negatives earlier diagnosed for ASF by polymerase chain reaction were used in this study. Each of the four positive samples were divided in two (Group 1,2), Group 1 were divided in triplicates and fixed in 10% formalin for 3 days at room temperature while group 2 were also divided in triplicates but not fixed (Non-fixed). A group of four samples confirmed negative for ASF was also obtained to serve as control. DNA was extracted from all samples (formalin fixed [FFT], non-fixed [NFT] and negative tissues).

Approximately 10 - 15µm tissue snip from the total 28 samples were put into 1.8ml tube and DNA extracted using the commercial kits (QIAamp DNA mini Kit [Qiagen, Germany], DNeasy Blood and Tissue Kit [Qiagen, Germany] and ZR Genomic DNA™ Tissue MiniPrep [Zymo Research, CA, USA]) according to manufacturer's instruction. DNA was also extracted using the conventional method reported by Huang et al., (2010) with some modifications. Briefly, the formalin fixed tissues were first washed twice in 100%, 95% and 75% ethanol for 6 hr interval after which they were also washed twice in distilled water at 6 hr each. The tissues were digested overnight at 55^{o}C using a total concentration of 40mg/ml Proteinase K added twice. DNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and precipitated with isopropanol and 3M Sodium acetate, allowed to dry at room temperature and the resultant pellet dissolved with

50 µl distilled water. Extraction was confirmed by agarose gel electrophoresis and the DNA purity measured at A_{260}/A_{280} wavelength kept at + 4°C prior to use.

To assess the yield (purity) of extracted DNA, the BioPhotometer Spectrophotometer (BioPhotometer; Eppendorf Scientific, Hamburg, Germany) was used. The DNA quality was evaluated by DNA amplification using the OIE pAS diagnostic primers and amplification conditions for 278 bp product. Data obtained from the triplicate samples were analyzed using GraphPad prism version 5.00, GraphPad Software, San Diego California USA.

RESULT

No DNA material was detected in agarose gel electrophoresis for the formalin fixed tissues using the three commercial kits (Figure 1). Allowing the tissue to digest further for 48 hrs at an increased volume of proteinase K produced no amplification.

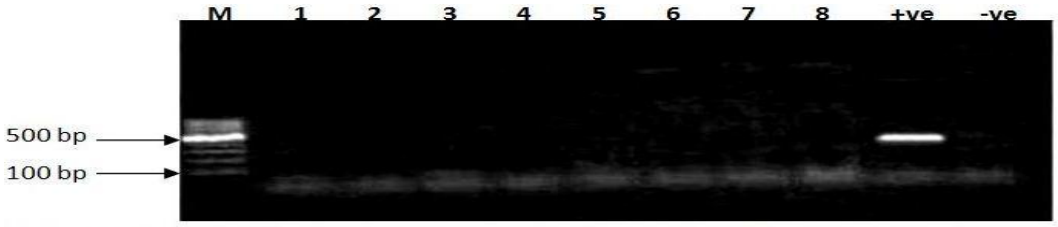

Figure 2: Gel electrophoresis showing ASV amplification: Lane M, Molecular marker of 100 bp. Lane 1-8, formalin fixed tissues DNA from commercial Kits.

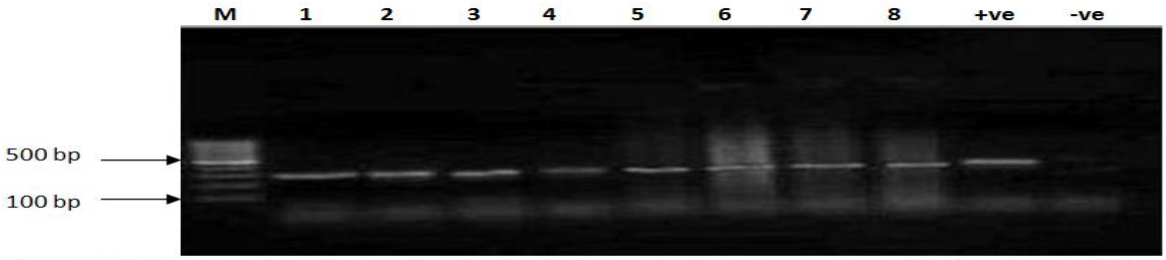

Figure 3: PCR gel showing 278 bp of ASFV: The first lane M, Molecular marker of 100 bp. Lane 1-8 Non-fixed tissues and lane 5-8 Formalin fixed tissues products from modified conventional method respectively.

However, DNA was obtained from the formalin fixed tissues using the method of Huang et al., (2010) with some modifications (Figure2). The purity

of DNA extract measured at A_{260}/A_{280} ranged from 0.86 – 1.68 and 3.22 - 5.32 µg DNA/mg for Formalin fixed and Non – fixed tissues, respectively (Table 1). The overall DNA quality of the formalin fixed tissues was 1.24 µg DNA/mg compared to 4.21 µg DNA/mg for Non-fixed tissues. For the modified conventional method, checking the DNA on agarose electrophoresis gel showed smears.

Table I: Analytical DNA yield in mesenteric lymph node, lungs, liver and spleen, expressed as µg DNA/mg of tissue for formalin and non-formalin fixed tissues

	DNA yield (purity) [mean ± SD (n=)]				
Treatment	Mesenteric LN	Lungs	Liver	Spleen	Overall
Formalin Fixed	1.51 ± 0.082 (3)	0.91 ± 0.058 (3)	0.86 ± 0.13 (3)	1.68 ± 0.014 (3)	1.24 ± 0.416(12)
Non-Fixed	5.32± 0.123 (3)	3.22± 0.082 (3)	4.11± 0.018(3)	4.20± 0.163(3)	4.21 ± 0.860(12)

Table 2: Analytical DNA yield in mesenteric lymph node, lungs, liver and spleen, expressed as µg DNA/ mg of tissue. Fixation time of 3 days followed by conventional method and commercial Kit extraction

	DNA yield (purity) [mean ± SD (n=)]				
Treatment	Mesenteric LN	Lungs	Liver	Spleen	Overall
Conventional Method	3.22±0.132(3)*	2.30±0.072(3)*	2.68 ±0.13(3)*	2.96±0.014(3)*	2.79±0.394(12)
QIAamp DNA mini Kit	1.46 ±0.054 (3)	0.95±0.042 (3)	1.18±0.018 (3)	1.22 ±0.163 (3)	1.20±0.209(12)
DNeasy Blood & Tissue	1.04 ±0.123 (3)	0.91 ± 0.022(3)	.25±0.053(3)	1.12 ±0.021 (3)	1.08±0.143(12)
ZR Genomic DNA™	1.52 ± 0.16 (3)	0.91 ±0.058 (3)	1.22±0.022(3)	1.29 ±0.166 (3)	1.24±0.252(12)

The data of DNA yield was subjected to analysis of variance for any possible difference between the different extraction kit and treatments. The differences were considered statistically significant when probability was less than 0.05. Moreover the result revealed that the there was not statistical significance between the commercial kits used but statistical significance between the commercial kits and conventional method $p<0.05$ and $p<1.00$

DISCUSSION

Diagnostic methods using DNA from formalin fixed tissues are becoming relevant to resolve retrospective questions from preserved tissues because of their repository genomic value. We tested African swine fever virus DNA extracts from 3 commercially used DNA extraction kits and a conventional method for the quality of DNA for amplification of 278 bp gene segment of ASFV by conventional PCR. Although formalin has the ability to fragment nucleic acid, DNA was extracted and amplified (Gilbert et al., 2007). Besides, limited number of samples was used in this study for the different commercial DNA extraction kits but the result obtained agrees with previous result of non-fixed ASF tissues (Owolodun et al., 2010).

The commercial kits used have been optimized and widely used for DNA isolation from samples (Klaassen et al., 2004; Koidl et al., 2008; Giammarioli et al., 2011). DNA extracted from non-fixed tissues using the above methods yielded the expected product of 278 bp for ASFV. This agrees with previous findings using the various kits for the detection of ASFV from suspected porcine tissues (Bastos et al., 2003; Owolodun et al., 2010). Although the sensitivities of the different commercial kits was not considered but they have been used by different authors for the extraction of DNA from ASF samples.

Using the modified conventional method of Huang et al., (2010) we got high yield (purity) DNA following prolonged washing with ethanol and proteinase K digestion however, this method is laborious and time consuming and may contain residues which will affect downstream applications. These treatment yielded better results than the commercial kits suggesting that proteinase K treatment plays an important role in proper purification of DNA fragments. Thus the electrophoresis gel showed smears which probably corresponded to DNA degradation due to the effect of formalin. The lack of smears and bands on the commercial kit suggested the role of proteinase K in the tissue digestion (Cao et al., 2003; Gilbert et al., 2007).

Although the purity of the DNA extracted was not as high as compared to NFT but the amplification yielded the desired product size. The desired amplicon size for ASFV was 278 bp, it would have been difficult if we were to amplify a product above 1kb due the genome fragmentation (Cao et al., 2003). Comparatively, the DNA yield from NFT (4.21 µg DNA/mg) was higher than those from FFT (1.24 µg DNA/mg). This may be attributed to the influence of formalin and loss of DNA during the washing steps. This is in agreement with previous report by Huijsmans et al., (2010). Our findings showed no significant differences in DNA yield among the commercial kits but significant difference with the conventional method. This differs from studies by Mirmomeni et al., (2010) who reported no significant difference between phenol-chloroform method, salting out using ammonium acetate and commercial kit.

In summary, the use of commercial Kits for DNA extraction from ASFV formalin fixed tissues appeared to be unsuitable in terms of quantity and quality of DNA. We therefore recommend that the use of conventional method be considered for ASF DNA extraction from formalin fixed tissues. Further studies using more samples is required to ascertain the suitability of commercial kits in DNA extraction from ASF formalin fixed tissues.

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A DECADE OF RABIES DIAGNOSIS: NVRI PERSPECTIVE

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INTRODUCTION

Rabies is an acute viral encephalomyelitis that affects all mammals including man (OIE, 2007). It is caused by rabies and Rabies-related lyssaviruses, in the family *Rhabdoviridae* (Smith, 1996). Although preventable by vaccination, fatality rate is almost 100 percent (Rupprecht *et al.,* 2002); only 6 people survived rabies globally. Information on the disease in Nigeria and Africa is sparse due to misdiagnoses and underreporting, absence of multidisciplinary approach to its control, lack of surveillance and enforcement of movement control as factors that could promote the spread of the disease.

HISTORICAL BACKGROUND

The first reported case of rabies was in a man bitten by dog before 23rd century B.C. (Koprowski, 1966). First case of rabies in dog in Africa was in Ethiopia in 1884, while the first reported case in human in Nigeria was in 1912 (Boulger and Hardy, 1960) and the first laboratory diagnosis in Nigeria was in 1925. Early existence of the disease in Africa can be inferred from its various local vernacular names (Obeogbulem, 1994).

EPIDEMIOLOGY

Rabies is found all over the world except in countries and regions with strict quarantine system, rigorous eradication or natural barriers like mountains oceans and rivers plus regular vaccinations. Domestic dog remains the

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likeliest carrier and the principal source of human rabies in Africa and Asia. Globally, about 55, 000 human deaths occurs annually and one person dies every 10 minutes, and 10 million post-exposure treatments annually (WHO, 2005). Rabies is endemic in Nigeria, and all published reports and survey have established dogs as the major vector of rabies (Ogunkoya, 1997; Tomori, 1980). Others include cats, rodents, hyena, cattle, sheep, goat, pig, horses, etc. Cases of human and animal rabies have been recorded from all the ecological zones and sub regions of Nigeria annually, despite the availability of vaccines. Three (75%) of the 4 genotypes of lyssaviruses found in Africa are present in Nigeria (Shope *et al.*, 1970; Ogunkoya *et al.*, 1984). These are the classical rabies virus (genotype 1), Lagos bat virus (genotype 2) and Mokola virus (genotype 3). No fewer than 10,000 Nigerians were exposed to rabies annually (Nawathe, 1980). It is also a highly neglected disease in Nigeria due to poor surveillance, low level of awareness, poverty, poor reporting system (Fagbami *et al.*, 1981) and limited political will. Genetic characterization of rabies viruses from Nigerian dogs demonstrated close relationship with 93.2 - 100% sequence identity, but could be differentiated into one major group of viruses recovered from dogs from the North Central, North East and South West regions respectively (Ogo *et al.*, 2008).

TRANSMISSION

Transmission is mainly by bite and scratch of infected animal. Licking or contamination of superficial wounds and mucus membrane can also transmit the disease. Transmission by inhalation of aerosols, organ transplant, transmammary and transplacental routes have been reported.

DIAGNOSIS

Diagnosis of rabies is achieved by antigen detection tests, amplification and detection of nucleotide fragments and serological tests. In the past (2003 to 2005), diagnostic methods used in National Veterinary Research Institute (NVRI), Vom include histopathology and Seller's staining techniques and

mouse inoculation test (MIT). Precisely, the dFAT started on 27th March, 2006 in CDL, NVRI. This was probably motivated by a "Training in rabies diagnosis" held on 8th & 9th September, 2005 by an expert from the Ahmadu Bello University, Zaria, at the instance of the CDL, NVRI, Vom. However, there seemed to be a 3-year (2006 – 2009) period of transition from the former to the current diagnostic techniques in the Institute. During this period, 2 to 3 of these techniques were being employed concurrently.

In 2010, when the south-south twinning between the NVRI and OIE Reference Laboratory at Onderstepoort Veterinary Institute, South Africa commenced, the dFAT was fully adapted as the routine technique for rabies diagnosis in NVRI. It is rapid, sensitive, specific and a reliable test, and the MIT is used as a backup, for routine diagnosis of rabies in animal samples. The dFAT is the gold standard technique for diagnosis of rabies approved by WHO and OIE, and involves the staining of a tissue smear (usually brain) with an FITC-labeled anti-rabies immunoglobulin, following acetone fixation. Specimens submitted for laboratory diagnosis include head, fresh brain or salivary gland of rabid suspect animal in cold condition or in 50% glycerol saline or formalin. Saliva collected in virus transport medium can also be submitted. Specimens of animal carcasses, especially dogs, submitted for necropsy at the Central Diagnostic Laboratory (CDL) are also routinely examined for rabies.

Vital information on specimens received such as: sex, age, species, vaccination record, ownership, source, human/animal exposure, etc are documented in a specimen submission form and a central specimen register. Until 2009, only the hippocampus was used for rabies diagnosis in NVRI. Currently however, brainstem, with thalamus, cerebellum, and hippocampus are the portions used for laboratory diagnosis, as rabies virus is not homogeneously present in the brain (Bishop *et al.* 2003, Bingham & Merwe, 2002)

In the last decade, a total of 2,494 animal specimens have been submitted to the CDL for rabies diagnosis. This consists of 2, 343(94.0%) domestic

dog, 146(5.8%) livestock and 5(0.2%) wild animals (Figures 1 and 2). As expected, specimen submission from the central states is indirectly proportional to their proximity to the diagnostic laboratory. Similarly, it was observed that the numbers of positive specimens are commensurate with specimen submission (Figure 3). In 2012 alone, 84.40% of the total samples submitted had history of human contact, 58.15% of which was positive. Out of those without human contact, 25.71% was positive.

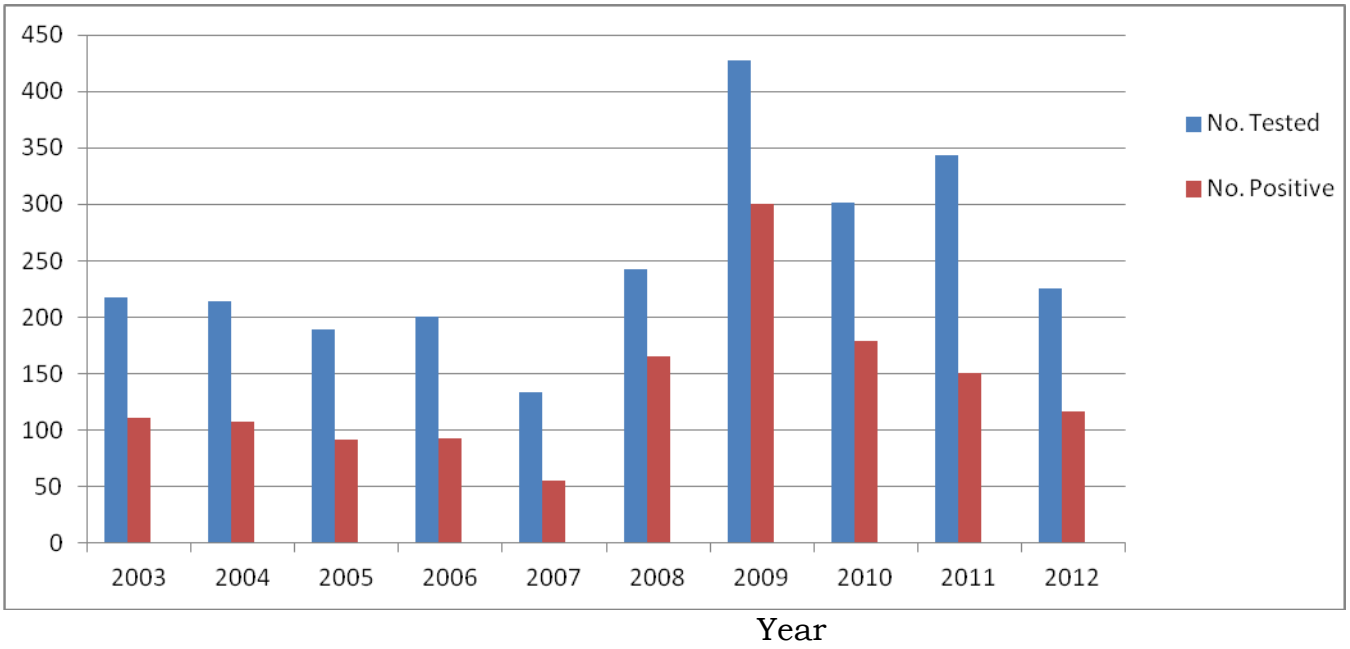

Figure 1: Data on Rabies Diagnosis, 2003 to 2012

NVRI has also built capacity and is upgrading facilities for cell culture isolation, virus neutralization assays, Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) and monoclonal antibody typing for rabies diagnosis and research. It is pertinent to also mention that, rabies diagnosis in NVRI has been free of charge since 2009.

MANAGEMENT

Immediately after exposure however, thorough wound cleansing is important in the management, followed by Post exposure prophylaxis (PEP)

on Day 0, 3, 7, 14 and 28 or day 0, 7, 21 with prompt administration of rabies hyperimmune globulin (OIE, 2007). This reduces mortality from 100 to 0 %.

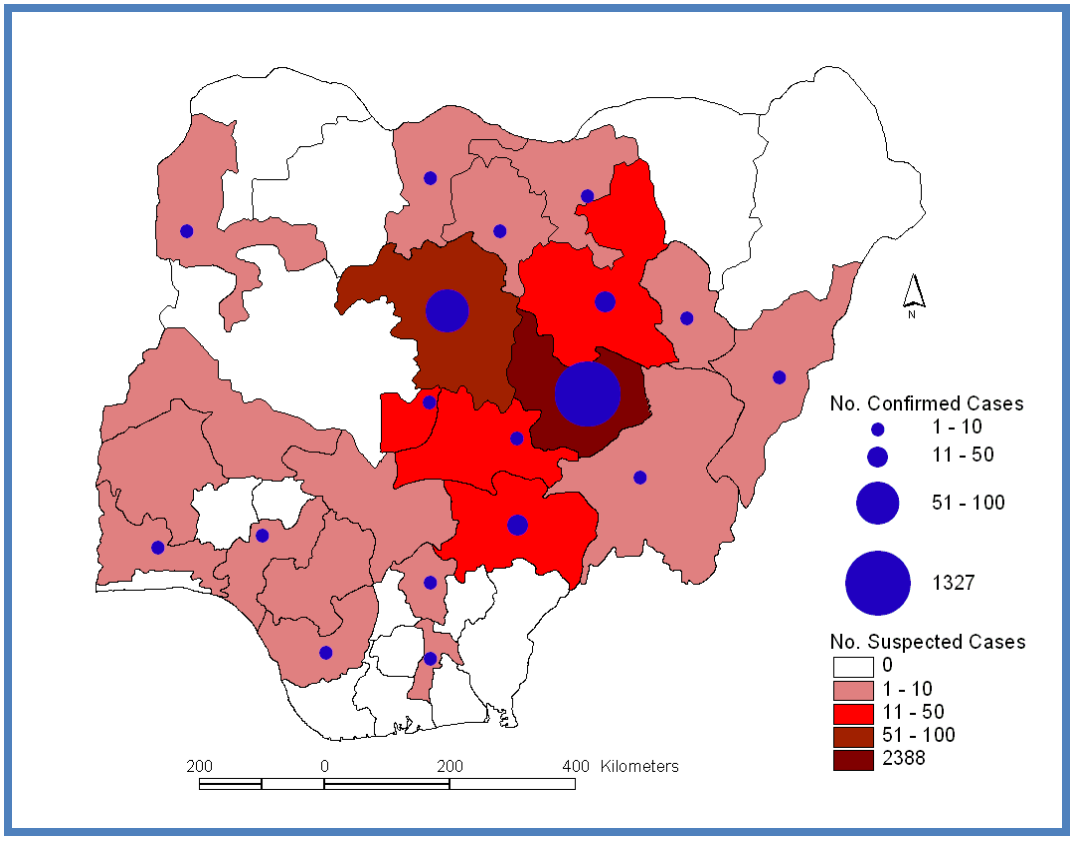

Figure 2: Sample submission by States, 2003 to 2012

PREVENTION AND CONTROL

Prevention and control of rabies is best achieved by vaccination of dogs and cats (Bingham, 2005).

1) Vaccine Production

NVRI has been producing animal vaccines for more than 50 years. Currently, the antirabies vaccines produced in the institute are egg based.

These are the flurry Low Egg Passage for dogs and Flurry high egg passage vaccine for cats, cattle, horses, and puppies. However, the Institute is in the process of developing killed Adjuvanted Rabies cell culture based vaccine.

2) Collaborative Activities

The 2-year south-south collaboration activities of NVRI with the Onderstepoort Veterinary Institute (OVI), South Africa that commenced in 2010 were successfully completed in 2011. This in to enable NVRI adapt new techniques in rabies diagnosis and research, and to obtain the OIE certification as regional laboratory for rabies for west and central Africa after applying duly and satisfying prescribed conditions.

The institute has also been assisting the Nigerian Veterinary Medical Association (NVMA) Plateau State Chapter, with antirabies vaccines for free mass vaccination of dogs and cats every September to coincide with the World Rabies Day. In 2009 to 2012, the Plateau state Chapter of the NVMA carried out antirabies campaign in Jos, Bukuru and Mangu metropolis, in the state. There was mass carnival through the cities, sponsored adverts and jingles on the local radio to sensitize the public. Interactive session was also held with veterinarians on the state radio and television stations on the danger of rabies and need to vaccinate dogs and cats against the disease. Over three thousand pets, guard and hunting dogs were vaccinated with certificate of vaccination issued.

CONSTRAINTS

Low level of awareness/poverty, inadequate funding for vaccine production, research and surveillance are the main constraints of rabies control in Nigeria. Little or no attention is paid to rabies and its control during annual national capital appropriation, leading to the neglect of the disease.

CONCLUSION

Correct and reliable diagnosis is critical to rabies control. NVRI, Vom has played laudable role in the diagnosis of rabies in the last decade. The fight

against the disease should be seen as everyone's concern. It is therefore the responsibility of the government at all levels, non-governmental organizations, veterinary and public health authorities, veterinarians in private sectors, environmental and law enforcement agencies, dog owners and the general public. Sustained collaboration among all stakeholders is essential for successful control of rabies. There is also need for proper education of the public on importance of rabies, mass dog vaccination campaign across the country, sufficient funding for vaccine production, disease surveillance and sample submissions for diagnosis.

RECOMMENDATIONS

To achieve effective control and eradication of rabies in Nigeria, it is essential to undertake the following: Promotion of correct laboratory diagnosis and vaccination of dogs against rabies, raising public awareness on the disease, provision of adequate funding for determination of the existing number, distribution and ecology of dogs in Nigeria, promotion of collaboration among all stakeholders, promotion of responsible dog ownership and enforcement of relevant legislation.

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DETECTION OF LEAD IN LIVER AND KIDNEY OF SLAUGHTERED CATTLE AT SOKOTO CENTRAL ABATTOIR SOKOTO STATE NIGERIA

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INTRODUCTION

Heavy Metals are those elements which have density more than 5,000 kg/m^3 (Passow *et al., 1961;* Hawkes 1997), atomic weight 63.546g to 200.590g (Kennish, 1992) and a specific gravity greater than 4.0 (Connell and Miller, 1984). Although heavy metals contaminate ground water, soil, pasture (vegetation), and air, these metals when consumed by animals and human tend to bio-accumulate in their tissues and organs, and excess accumulation will lead to severe health hazard (Kennish, 1992).

Historically the fall of Roman Empire was believed to be as a result of utensils coated with lead, which resulted in lead poisoning in the Empire (Andrada *et al.*, 2006).

Once the environment is contaminated with heavy metals, they remain so for years and increase the chances of becoming toxic to humans and animals (Chin *et al.*, 2008). Recent studies have shown that poor handling of some modern products such as paints, petrol, battery, engine oil, (Bastarche, 2003), mercury amalgam dental filling (Ellender *et al.*, 1978; Chin *et al.*, 2008), cosmetics (Hardy *et al.*, 1998), and ground water residues (Ghosh *et al.*, 2004) resulted to chronic exposure to heavy metals.

Food crops and pasture grown on soil containing toxic heavy metals or irrigated with water containing heavy metals accumulate heavy metals in them and serves as a source of heavy metals exposure to animals and

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humans (Ward and Savage, 1994). Some occupations that involve direct contact of the workers with heavy metals like, painting, dental surgery, and welding, plumbing, mechanics etc (Olah and Tolgyessy, 1985) are at greater

risk of exposure to heavy metal poisoning. Indiscriminate dumping of waste materials, and sewage water containing heavy metals on land gradually increase the toxic concentration of heavy metals in the soil and these are increasingly taken up by the plants and finally find its way into the food chain causing severe health hazard to both animals and human (Haiyan and Stuanes, 2003).

Animals reared on pasture fertilized with materials containing heavy metals or fed with feed containing heavy metals become a good source of heavy metal residues in their edible products such as meat, fish, eggs, milk etc.

Heavy metal contamination in meat and other edible tissues is a matter of great concern for food safety. Some heavy metals are toxic in nature and even at relatively low concentrations can cause adverse effects (Santhi *et al.*, 2008). Different researchers have reported the instances of contamination of heavy metals in meat products during processing (Brito *et al.*, 2005; Santhi *et al.*, 2008). Heavy metals residues were found in meat and meat product of livestock fed with contaminated feed and reared in proximity to environment polluted with heavy metals. (Sabir *et al.*, 2003; Miranda *et al.*, 2005). This is of serious public health problem that needs an immediate attention of health regulatory authorities and researchers as well

MATERIALS AND METHOD

Study Area

The study area is Sokoto central abattoir, which is located in Sokoto North local government area of Sokoto State Nigeria. Sokoto state is geographically located in the North Western part of Nigeria, between longitudes 4^{o}8'E and 6^{o}54' E and latitudes 12^{o} N and 13^{o}58'N. The State share boundaries with Niger Republic to the North, Kebbi State to the West and Zamfara state to the East. Sokoto State covers a total land area of about 32,000 square

Kilometres with an estimated human population of 3,696,999 (NPC, 2006). The State rank second in the Nigeria livestock population with an estimated 3 million cattle, 3 million sheep, 5 million goats, 4,600 camels, 52,000 donkeys and host of other species of local and exotic poultry species (MOCIT, 2002; Mamman, 2005).

Study Design

The study is cross- sectional, and abattoir based, and covered a period of seven weeks of sample collection.

Samples Collection and Preservation:

Samples (kidney and liver) were purchased from randomly selected slaughtered cattle from Sokoto central abattoir for a period of seven weeks. The age of the selected slaughtered cattle was determined. About 100 g of liver from any lobe and a whole kidney (either right or left) of each selected animal were packed in a sterile polythene bags, properly labelled with permanent marker, and transported to Veterinary Public Health and Preventive Medicine laboratory, where it was frozen and stored at 20°C. The frozen samples were packed on ice block in a cooler and transported to National Research Institute for Chemical Technology Zaria, Kaduna State, Nigeria for further processing and analysis.

Processing of Samples

Digestion of Samples

Liver and kidney samples were dried at 45^{o} C using oven, after drying, individual sample was crushed into fine powder using mortar and pestle, and 1.0g of the fine powder sample was weighed into porcelain crucible. The crucible and the fine powdered samples were ignited in a muffle furnace at 500^{o}C for six to eight hours. The samples were then removed from the furnace and allowed to cool in desiccators, and weighed again. The difference between the weight of the crucible and ash and the weight of the crucible alone was used to calculate the percentage ash content of the sample. Then, 5cm^{3} of 1M trioxonitrate (v) acid (HNO_3) solution was added to the left-over ash and evaporated to dryness on a hot plate and returned

to the furnace for heating again at 400^{o}C for 15-20 minutes until perfect grayish-white ash was obtained. The samples were then allowed to cool in desiccators. To the cooled ash 15 cm^{3} hydrochloric acid (HCl) was then added to dissolve it and the solution was filtered into 100 cm^{3} volumetric flask. The volume was made to the 100cm^{3} with distilled water.

Spectrophotometry Techniques for Lead

In the prepared liver and kidney samples, lead was determined using AA-6800, Shimadzu atomic absorption spectrophotometer according to manufacturer's instructions The concentration of lead in each sample was determined using the calibration curve (Szkoda and Żmudzki, 2005).

RESULTS AND DISCUSSION

The mean concentration of lead in liver samples was higher among the age group of 0-2 years with the mean concentration of 0.61396 mg/kg, and lower among the age group of ≥9 years with the mean concentration of 0.2653 mg/kg. In kidney samples, the mean concentration of lead was also higher among the age group of 0-2 years with the concentration of 0.17436 mg/kg and lower among the age group of ≥9 with the concentration of 0.1253mg/kg.

Generally the mean concentration of lead in liver samples were higher than in kidney samples of all the age groups of slaughtered cattle at Sokoto Central abattoir, since liver serve as an organ of detoxification, and lead is a toxic metal this may be the reason why the concentration of lead was higher in liver than in the kidney, another reason why liver has high concentration of lead than the kidney may also be as a result of excretion of lead to the bile which later is been re-absorbed into the liver (enterohepatic biliary cycles) which in the process liver may contain high level of lead than the kidney. Markovac and Gold Stein (1988) also reported that lead competes with irons for binding, and liver is a very good source of iron, it may also be one of the reasons why the concentration of lead was higher in liver than in the kidney.

Table showing mean concentration of Lead (Pb) (mg/Kg) in Liver and kidney samples of different age groups of cattle.

Age Range (years)	Mean conc of Pb liver (mg/kg)	kidney (mg/kg)	Permissible limit by FAO (mg/kg)
0-2	0.6140	0.1744	0.1
3-5	0.3125	0.1427	”
6-8	0.2913	0.1326	”
≥9	0.2684	0.1313	”

The concentrations of lead in liver samples of different age groups were higher than 0.1 mg/kg recommended permissible limit of intake of lead by Food and Agricultural Organisation (FAO) (2002) and Egyptian Organisation for Standardisation (EOS) (1993). While the concentration of lead in kidney was higher than the permissible level of lead (0.1 mg/kg) in age group of 0-2 years of slaughtered cattle, while the mean concentration in age group of 3-5 years, 6-8 years and ≥9 years old are within the permissible level of lead recommended by FAO (2002) and (EOS) (1993).

Cattle and other ruminant animals serve as bio- indicators of environmental contamination with heavy metals (Miranda *et al.*, 2009). The presence of lead in tested samples serves as an indication that the environment where these cattle are reared before they were purchased for slaughtering is contaminated with lead materials. And consumption of liver and kidney from tested animals may pose a serious health problem to man as a result of bio-accumulation of lead in the body tissues, and this varies with individual and the duration of lead exposure (Kosnett *et al.*, 2007).

Symptoms are nonspecific, and someone with elevated lead levels may have no symptoms (Mycyk *et al.*, 2005). Some of the symptoms include hyperactivity, or slow growth in the young. Lead can also cause high blood pressure, nervous disorders, oligospermia in male, abortion in female, and amnesia in adults, in severe cases; it can lead to seizures, coma, and death (Wagner, 1995; Kocak *et al.*, 2005). .

The bone is considered to be a sink for lead and it may contain 90-98% of the total body burden of lead. When bones and blood from the affected animals are used as bone and blood meal in animal feed, the lead content may bio-accumulate in tissue of the animals fed with such feeds and cause lead poisoning (Kosnett *et al.*, 2007).

Lead is also excreted from the faeces of affected cattle in high amount (90%) (Jensen 1983), when such faeces are used as manure to fertilised land for the growth of pasture and crops for animals and human consumption, the lead in the manure will contaminate the plants (Blakley, 1984) and when such plants are consumed by animals and man, it may cause lead poisoning in animals, and a very serious public health implication in human.

CONCLUSION

Lead was detected in all the liver and kidney samples collected from the slaughtered bovine carcasses at Sokoto Central abattoir. It shows that these animals were exposed to lead either in their feed (pasture), water, or inhalation of exhaust fume from automobile. kidney and liver are consumed by humans, which may serve as source of exposure. Further research is required to established the link between the probable source of lead exposure to these animals

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COST EFFICIENCY OF HERD HEALTH MANAGEMENT PROGRAMS

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INTRODUCTION

Veterinary Herd Health Management (VHHM) programs were introduced in the Netherlands in the 1970's (Sol and Renkema, 1984) with primary objectives of optimization of herd health, productivity, quality of products, and profitability of the dairy enterprise (Blood et al., 1978; Brand et al., 1996). The herd health programs are centered around regularly planned farm visits, based on a protocol approach to operational farm management functions (Brand et al., 1996).

The nature of veterinary work in dairy health management in Europe has changed over the past years and will change even more in the near future. Herd health programs are becoming a more commonly adopted trend in the dairy industry in recent times as planning for health is gaining momentum as an important element of modern dairy farming. As dairy farmers strive for further efficiency in production, driven by market economics, the risks and consequences of poor health and suboptimal production also become greater (Sibley, 2006). VHHM programs are believed to play a substantial role in the aim of farmers to produce products of high quality for a low cost price. At present, several veterinarians (vets) provide VHHM to farmers at

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specific costs. Ideally, VHHM combines animal health, food safety, animal welfare and public health with farm management and economics (Noordhuizen and Wentink, 2001; LeBlanc et al., 2006) but in reality, some farms or veterinarians embrace these concepts by active participation, while others do not.

For more than 30 years, Veterinary Herd Health Management programs (VHHM) have been carried out in many dairy farms in the Netherlands, and have been shown to be effective regarding experimental studies in the past. A study that followed afterwards showed approximately eight per cent increase compared with the initial income level, using 1974/75 as a base year (Hogeveen et al., 1992). However, no current information is available on the costs and benefits of these programs.

This study evaluates the economic relationship between the VHHM program and farm performance on dairy farms in the Netherlands.

METHODOLOGY

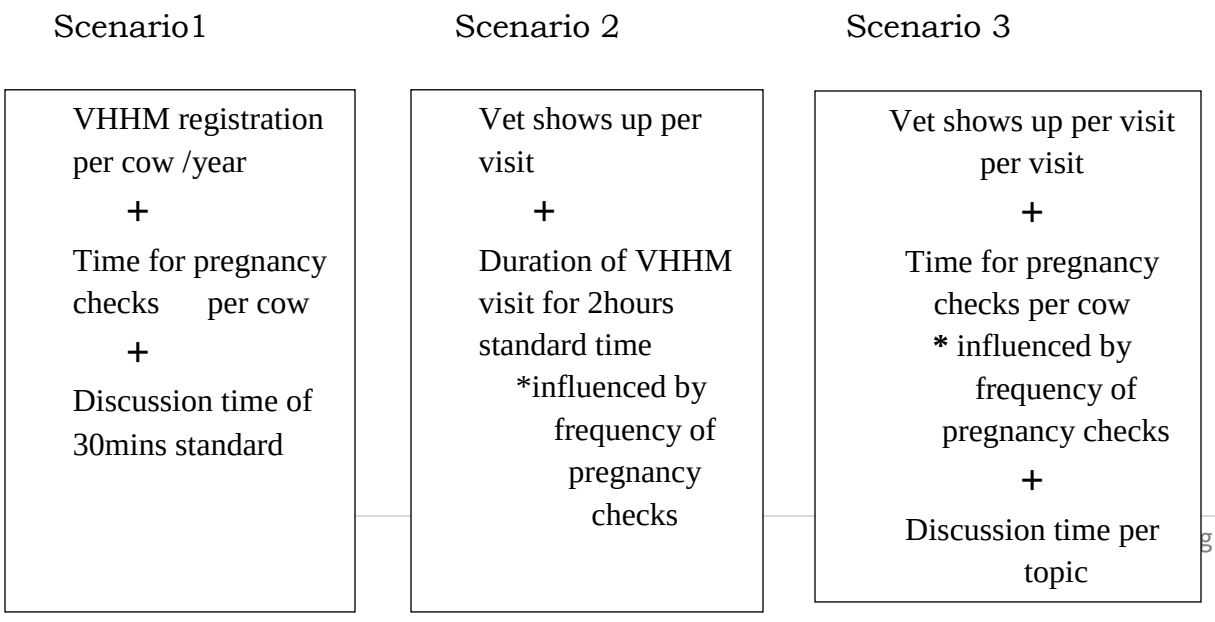
A total of 5,000 farms in the Netherlands, which have at least 40 milking cows and participating in the Milk Production Registration (MPR) by CRV (Arnhem, The Netherlands) for over two years, were randomly selected and sent questionnaires via email (Derks et al., 2013). In the questionnaires, farmers were asked several questions on participation in a VHHM program and activities during the program. For returned questionnaires that were fully completed, available farm data concerning each of these farms was obtained from the CRV database which included number of cows, production data, reproduction data, herd health data, culling data and economic data. These parameters from participating (VHHM) and non-participating (NVHHM) groups were summarized and their means compared using independent sample t-tests. Subgroup analysis of the data of the VHHM group was done

to evaluate the different activities and participation levels within the VHHM program. The number of visits by the vet, frequency of pregnancy checks and topics discussed were summarized. For each of these parameters (frequency of visits, frequency of pregnancy checks, number of topics discussed), their association with net returns was tested using chi-square test, and where significant, a post hoc test was done.

In order to calculate the costs associated with the VHHM program as described in the questionnaire, data, reflection of current practice, expert opinion and literature was used.

Three scenarios were developed to estimate the total costs of the program per farm based on activities and time spent during the program (Figure1). Estimates of the current costs incurred by the participation in this program were calculated using a normative model, and various parameters influencing net returns were also examined. The activities within the participating farms were also assessed in relation to their economic output. The scenario 3 was used for the statistical analysis.

Figure1. Different scenarios to estimate the costs of the VHHM program based on data from the questionnaire, and information from expert opinion.



Three multivariable models were then created with net returns (per cow) of each farm as outcome which was assumed to be the most important economic variable present. The first model is with the farm data net returns as outcome, the second is with net returns adjusted for VHHM costs as outcome, and the third with net returns adjusted for both VHHM costs and costs of rearing heifers as outcome. Other variables included in each model were VHHM as a fixed factor; and Age at first calving (AFC), Somatic Cell Count (SCC), NR56, and Age at Culling (AC) as co-variates respectively with subscript for each model. ϵ is the residual. Statistical analyses conducted using IBM SPSS statistics 20.

RESULTS AND DISCUSSION

Data from 1,013 farms were received and analyzed and had an average farm size of 83 cows. Of the 1013 farms, 695 (68.6%) were VHHM participants and 318 (31.4%) were non-participants. 211 veterinary practices were listed, and 42% of the farms had 40-70 cows, 47% had 71-120 cows and 11% of farms had more than 120 cows. The VHHM group showed better performance with regard to production, but different outcomes were observed with regard to reproduction parameters. The VHHM group also had a lower somatic cell count and higher net returns per cow per year.

Within the VHHM group, over 90 % of the farms were visited at least once in 6 weeks and most farms discussed at least 3 topics. In 97% of the farms the veterinarian did the pregnancy checks. In relation to net returns, the farms that had pregnancy checks by the veterinarian only when there are problems showed lower returns than farms that checked always ($p<0.01$). The number of topics discussed was not associated with net returns. The

net return was also different between groups for number of visits. Following a post hoc test, the net returns for the farms with less than 6 visits a year were significantly lower than those with 8 visits or 13 visits a year ($p<0.01$).

The normative models for each scenario were compared. There was no significant difference in the costs (€/cow) of VHHM program as calculated based on the different scenarios. The estimated cost of VHHM program per cow per year was €20. There was a benefit cost ratio of €4.8 per cow in net returns for participants, and a gain of €95 for the participants after adjusting for the cost of the program; and with further correction for higher costs of replacement heifers on VHHM farmers, there was a benefit cost ratio of €4.2. The returns per costs portrays that the program is efficient. However there is a lot of unexplained variation in the net returns, and this may be due to diverse approaches by the veterinarians.

In the three multivariate regression models created, the estimates for β in VHHM decreases with further correction of the net returns. Based on the MPR data, the difference in mean net returns for VHHM versus NVHHM corrected for the influence of AFC, SCC, NR56, and AC (i.e. β) is €48, but in models 2 and 3, it is €30 and 28€ respectively (Table 1). The effect of the VHHM program appears, although positive, was only significant ($P<0.05$) in the first model. All the co-variates are negatively associated with the net returns.

Table 1: Outcomes from the multivariable model with VHHM as fixed factor

	Model 1	Model 2	Model 3
Outcome predicted	Net returns	Net returns adjusted for VHHM program cost	Net returns adjusted for VHHM program cost and cost of replacing heifer
Parameter	**β CI p-value**	**β CI p-value**	**β CI p-value**
Intercept	4225 3993; 4457 <0.01	4197 3954; 4439 <0.01	3782 3541; 4022 <0.01
VHHM (yes/no)	48.8 -81; -16.7 <0.01	30 -63.9; 2,9 0.07	28 -61.3; 5.1 0.10
AFC (days)	-1,10 -1.5; -0.9 <0.01	-1.2 -1.5; -0.9 <0.01	-1.2 -1.5; -0.92 <0.01
SCC(cells/ml) (*1000)	-1,24 -1.5; -0.9 <0.01	-1.2 -1.6; -0.9 <0.01	-1.3 -1.6; -1.0 <0.01
NR56 (%)	-261.7 -388; -135 <0.01	-264 -398.9; -129.9 <0.01	-278 -412; -144.6 <0.01
AC (days)	-0.2 -0.3; -0.1 <0.01	-0.18 -0.2; -0.1 <0.01	-0.1 -0.137; -0.021 0.01
R squared	0.28	0.27	0.24

NVHMM is the reference category, CI –confidence interval.

As expected, the influence of VHHM program on net returns reduced as the net returns was adjusted with the costs of replacement heifers since the VHHM had a higher culling rate. However, the other co-variates showed negative relationships with the net returns, and these were higher in the NVHHM group. With only 24-28 percent of the variation of the net returns being explained by these parameters, there is once again proof that the eventual production rate is influenced by many more factors which have not been included in this study.

In general, the gains of a VHHM program are expected to be a higher milk production, lower disease levels and better reproduction. Studies on costs of disease have shown that most of the costs of disease are in milk production losses and culling (Noordhuizen and Van Meurs 1985; Sol et al., 1984)**.** These two factors were implicitly taken into account in this study. Costs of treatments and farmers labour were known and because incidence of disease was lacking, we could not make an estimation of these costs. It was difficult to estimate the disease cost of the farms as the only available data on health was mean SCC, for which is it challenging to peg the severity of the diseases (Sol and Renkema, 1984) and estimate the cost of reducing the disease impact. Hence the approach of the normative model for the cost of the program was used.

CONCLUSION

The VHHM program is related to a higher milk production (kg/cow/year) of farms and consequently a better net return. Even when the net return was corrected for the costs of the VHHM program and the higher costs of replacement heifers on VHHM farmers, there was a benefit cost ratio of €4.2 per euro spent for the cost of the program which makes it efficient. However,

participation of VHHM does not significantly predict the net returns of a farm.

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MORINGA MULTI-NUTRIENT BLOCKS: FORMULATION, PRODUCTION, AND FEEDING TRIAL UNDER A TROPICAL ENVIRONMENT

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INTRODUCTION

The use of browse plants as supplements is a recent development. Browse plants offer a considerable protein for ruminant nutrition. In his review on browse supplementation experiments involving a Napier diet with Gliricidia, Leucaena spp and Sesbania, Topps (1992) noted that they increased dry matter intake and weight gains. Similarly, Bannulin et al., (1986) also noticed an increase in digestibility of crude protein (CP) and dry matter (DM) when sheep were fed tropical pasture supplement with Leuceana. However, some browse plants have been found to have anti-nutritional factors that limit their use as feed supplements. For instance, condensed tannins have been reported to be present in Gliricidia,leucaena, Acacia spp and Albizia spp (D' mello, 1992). Tannins are known to be potent protectors of the proteins from microbial degradation in the rumen thereby increasing the quantity of bypass proteins (Ehoche *et al.*, 1983). On the whole, substantial amount of proteins, vitamins and minerals are supplied when ruminants browse on plants or when used as supplements.

Formulation, development, production and use of multi-nutrient blocks utilizing *Moringa oleifera* as a basic ingridient will be a long term relieve to

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feed shortage especially during the dry and wet seasons for the ruminants. Nutrition is a critical factor that could limit ruminant production. Reports exist on the formation of multi-nutrient blocks, crumbs/cakes as partial substitutes for cereal-based concentrates for animals during all phases of production. It was reported that such mini-blocks could be a complete feed for animals by including a source of forage in the formation. The use of forage legumes as supplement has been suggested as an alternative to concentrates. However, many tropical fodder legumes contain secondary plant compounds such as tannins, saponins, cynogens, mimosine and coumarins which limit nutrient utilization. Moringa oleifera Lam, a non-leguminous multipurpose tree with a high crude protein in the leaves (251g/kg DM) and negligible content of tannins and other anti-nutritive compounds offers an alternative source of protein to ruminants wherever they prosper(Topps,1992).

In Nigeria, *Moringa oleifera* is grown in the backyard and as fodders and the leaves are mainly used as food. Evaluation of the blood profile of animals may give some insight as to the potentials of a dietary treatment to meet the metabolic needs of the animals since according to Church *et al*. (1984), dietary components have measurable effects on blood constituents such that significant changes in their values can be used to draw inference on the nutritive value of feeds offered to the animals. The assertion that most of the available information on haematological parameters of goats in the humid tropics is based on disease prognosis is also applicable to sheep. Thus, data on blood profile of yankasa (YK) sheep offered multi-nutrient blocks containing varying levels of *Moringa oleifera* powder is scanty. The objective of this study therefore, was to evaluate the nutrient intake, digestibility, nitrogen balance and haematological profile of YYR as affected by dietary inclusions of *Moringa oleifera* multi-nutrient blocks as supplement to Signal grass (*Brachiaria decumbens*)

MATERIALS AND METHODS

Experimental site

The study was conducted at the experimental Unit (EU) in the Livestock Investigation Department (LID) of the National Veterinary Research Institute (NVRI) Vom, Plateau State, Nigeria, located at latitude 8^0 45/ and longitude 9^0 43/ North. The mean annual temperature is 20.8^{0}C and mean annual rain fall 1400mm with a marked dry season (November – May).

Experimental feed

Air-dried leaves of *Moringa oleifera* were purchased from a recognized *Moringa* plantation in Nasarawa, near Vom. The air-dried leaves were ground through 2 mm mesh harmer mill and stored in polythen airtight bags until required for used. The concentrate was milled at the Farm's Milling Unit (FMU). The basal diet was Signal grass (*Brachiaria documbens)* sourced from the Institute's Farm. Their nutrient compositions are given in Table 1.

Moringa Multi – nutrient block ingredients, formulation and assessment

Four different formulae were tested using four ingredients: *Moringa oleifera* leaf powder (MOLP); Lime powder (LP); Salt (S), with Cement (C) as a possible binder. The MOLP was prepared from *Moringa* Leaves. The other ingredients were purchased from a reputable livestock feed ingredients store in Bukuru, Jos, Nigeria. The blocks were prepared manually from 10kg of the ingredient for each formulation. The S and C in each formulation were mixed together, and these mixtures were separately mixed with water at the ratio of 1:2 (w/v; Aye and Adegun, 2010) before being incorporated into the second mix, comprising of MOLP and LP. Cylindrical plastic moulds (13cm x13cm with a height of 10cm) were used in casting the blocks to give 1 kg per block. The blocks were monitored and assessed for hardness and

compactness over 7, 14 and 21 days respectively by 4 persons independently using a subjective scale: Soft (+) Medium (++) and Good (+++). Hardness was determined by pressing with the thumb in the middle of the block while compactness was measured by the ease to break the block by hand (Mohammed *et al*. 2007). About 50 g samples from each of the four investigated formulation were separately pulverized, pooled together and sampled for their respective proximate contents (AOAC, 1995). The second phase of the experimental work was the testing of the moulded blocks on yearling yankasa rams at the LID, NVRI, Vom. Yearling Yankasa rams (n= 25) averagely weighing 23.10kg were used in a completely randomnized design experiment. They were divided into five groups of five animals each after balancing for weight and each group randomly assigned to five treatments namely:

Treatment 1:*B. decumbens* + concentrate (Control)

Treatment 2: *B. decumbens* + concentrate + 5% *Moringa* Block

Treatment 3: *B. decumbens* + concentrate + 7% *Moringa* Block

Treatment 4: *B. decumbens* + concentrate + 9% *Moringa* Block

Treatment 5: *B. decumbens* + concentrate + 11% *Moringa* Block

The experiment was conducted during the raining season of 2012(June-September) and lasted for 70 days.

Digestibility Trial

In the last week of the experiment, total faecal and urinary outputs were collected from each animal daily and weighed. 10% of daily faecal outputs were dried, bulked together and stored until needed for proximate analysis, while 10% of the daily urine output preserved with 50% sulphuric acid was frozen till it was required for nitrogen analysis.

Chemical Analysis

Feed and faeccal samples were oven-dried, ground to pass through 1mm screen and analysed for proximate compositions (AOAC, 1995). Nitrogen in

urine was determined by Microkjedahl methods. Results obtained were used to calculate the nutrients intake, digestibility, N balance and N retention.

Haematological studies

Blood was collected from the jugular vein of the experimental animals at the termination of the experiment in a vial containing ethylene diamine tetra-acetic acid (EDTA). The bottles were immediately capped and the content mixed gently for about a minute by repeated inversion or rocking. Blood samples were analysed immediately after collection for packed cell volume (PCV) and haemoglobin (Hb) concentration as described by Jain (1993). Red blood cells (RBC), white blood cell (WBC) as well as the differential WBC counts were determined using the Neubauer Haemocytometer after appropriate dilution (Lamb, 1981). Values for the constants: mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin (MCH) and mean corpuscular volume were calculated from RBC, Hb and PCV values as described by Jain (1993).

Statistical Analysis

The data collected were subjected to analysis of variance (ANOVA) using the General Linear Model(GLM) procedure of MINITAB(2000) and where significant F-values for treatment effect were found, means were compared by Least Significant Difference (LSD). Linear correlation and regression analyses were carried out according to SAS (1999).

RESULTS AND DISCUSSION

Nutrient composition

The proximate compositions of *Moringa* and *Brachiaria* indicated in Table 1 show that both plants had differing dry matter (DM) contents (90.06% and 69.36% respectively) with *Moringa* higher in DM than *Brachiaria*.The crude protein (CP) content of *Moringa* at 15.93% is higher than that for *Brachiaria* (7.54%) and it exceeds the minimum protein requirements for ruminant recommended by ARC (1985). The CP of *Moringa* (15.93%) obtained in this

study fell within the range (7.8-20.5%) obtained by Aganga and Tshwenyane (2004). However, the CP value obtained in this study differs from values reported by other authors. For instance, Manh *et al.* (2005) obtained CP value of 26.4% for *Moringa*. Variability in the nutrient content of browses has been attributed to within species differences, plant parts, season, harvesting regime, location, soil type and age (Norton, 1994).

Crude fiber (CF) content in *Brachiaria* was similar to 33.4% reported by Aganga and Tswenyane (2004) and was higher than the value obtained for *Moringa* in this study. This is consistent with the observation of Okoli *et al.* (2003) that CF content of tropical grasses is usually higher than that of browse shrubs and trees.

Nutrient Intake and Digestibility

DM intake was highest ($P<0.05$) in treatment 1 but it is comparable with values for treatment 2 to 5 (Table 2). The higher DM intake for treatment 1 could be due to the fact that small ruminants have affinity for Signal grass and since it was offered as sole feed, animals have no choice than to eat what was served (Babayemi and Bamikole, 2006). With the exception of CF, nutrient digestibility were significantly higher ($P<0.05$) in supplemented diets than in the control. Among the *Moringa* – supplemented diets, DM and ADF digestibility were highest in treatment 5 while CF and EE digestibility were best in treatment 3 (Table 2).

CP digestibility in treatments with dietary levels of *Moringa* were statiscally similar and were all significantly higher ($P<0.05$) than the control. This is probably because *Moringa* fodder consists of more degradable components especially crude protein than *Brachiaria decumbens* and this could serve as supplement to the latter in ruminant diets. The higher CF digestibility in treatments 3 and 4 equally suggests an increase in the rumen activities of fibrolytic bacteria in the rumen probably as a result of the availability of

essential nutrients especially protein, energy and minerals in balanced proportions to enhance microbial growth and multiplication.

The significant (P<0.05) positive relationship between *Moringa* inclusion and both CP and NFE intakes is probably a reflection of the increasing quality of the diets with increasing level of *Moringa* supplementation since according to Ventura *et al.* (1975), forage quality increases as nutrient intake increases.

Nitrogen Balance

N- intake increased progressively from treatments 1 to 4 and only dropped slightly in treatment 5, hence it has significant (P<0.05) direct relationship with dietary level of *Moringa*. This is probably due to increased CP intake with increasing level of *Moringa* inclusion in the experimental diets. However, this is at variance with the 9.2g day^{-1} obtained by Alli – Balogun *et al* (2003) for Yankasa/WAD sheep crosses fed grasses supplemented with cassava foliage or groundnut hay. The difference might not be unconnected with the method of feeding, materials fed and genotypic difference.

Both faecal and urinary- N did not differ (P>0.05) among the diets in agreement with Black *et al.* (1978) that faecal and urinary- N were not significantly affected by N intake. All the treatments gave positive N- balance and N- retention values, except treatment 1 which gave negative N- retention value, an indication that protein requirement for maintenance in the experimental animals was adequate by the dietary treatment, except treatment 1 which did not enjoy *Moringa* benefits. Treatment 4 had the highest (P<0.05) N- balance and N- retention. The implications of treatment 4 having the best N- balance and N- retention in this study is that, optimum level of supplementation of *Brachiaria decumbens* with *M.oleifera* is probably at 9% and that at higher inclusion levels, efficiency of protein utilization decreases. Nitrogen balance has been described as a good indicator of the

protein value of a diet when the amino acid supply is balanced with the energy supply (Babayemi and Bamikole, 2006).

Haematological Studies

Except for mean corpuscular haemoglobin (MHC), mean corpuscular value, and neutrophils (N) which were apparently significantly different ($P<0.05$), other erythrocyte indices were apparently similar across the treatments. Even though, the red blood cell (RBC) counts did not differ significantly ($P>0.05$), only value obtained for treatment 1 did not fall within the normal physiological range of 9.0 – $15.0x10^{12/L}$ for healthy sheep (Jain, 1993). RBC counts aid in the characterization of anaemia. Thus the abnormally low values recorded for treatment 1 is an indication of a likely high susceptibility to anaemia – related disease conditions by these animals. This is corroborated by the fact that animals in this treatment also recorded MCV value that was comparatively at the high end of the normal physiological range of 28.0 – 40.0 FL (Jain, 1993) which according to Merck (1979) increases the probability of the release of immature red blood cells into the circulatory system.

Out of all the differential leucocyte parameters, only neutrophil counts showed significant difference ($P<0.05$). It was pointed out that varying lymphocyte values indicated different levels of immune status of farm animals and Lazzaro (2001) further explained that depressed levels of lymphocytes might indicate a depleted immune system or elevated neutrophil in an active infection. However, lymphocyte and neutrophil counts obtained in this study fell within the physiological range of 40.0-75.0% and 20.0-70.0% respectively for healthy sheep (Jain, 1993). Neutrophil and lymphocyte counts had non-significant ($P>0.05$) negative correlation ($r = -0.22$). All other haematological parameters(Table 4) had variable, significant and non-significant correlations ($P<0.05$) with Moringa inclusions, suggesting that, the probability of predicting haematological

profile of Yankasa sheep from levels of dietary inclusion of *M. oleifera* is high.

CONCLUSION

It was concluded that the optimum inclusion level of *Moringa* at which the best nitrogen balance, nitrogen retention and haematological profile was recorded was at 9% Moringa inclusion. The inclusion of Moringa in the diets significantly improved the blood profile of YYR and invariably enhanced their performance.

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TABLE : PROXIMATE COMPOSITION (%) OF MORINGA OLEIFERA, BRACHIARIA DECUMBENS AND CONCENTRATE

COMPOSITION (%)	M. Oleifera	B. documbens	concentrate	±SEM
DM	90.06	69.36	92.87	3.51
CP	15.93	7.54	12.28	6.43
EE	1.06	1.06	4.87	0.59
ASH	4.89	12.15	6.39	4.31
NFE	66.28	54.62	32.81	1.50
CF	11.84	33.87	9.27	7.12

MLP= Moringa Leaf Powder

TABLE 8: MLP NUTRIENT DIGESTIBILITY

Parameters %	% Levels of MLP T1(0%)	T2(5%)	T3(7%)	T4(9%)	T5(11%)	±SEM
DM	80.51^{a}	84.76^{b}	87.67^{b}	86.08^{b}	89.01^{b}	1.42
CP	15.21^{a}	20.11^{b}	20.21^{b}	21.12^{b}	20.18^{b}	2.03
ASH	25.01^{a}	27.96^{b}	28.88^{b}	27.84^{b}	27.69^{b}	2.66
EE	1.22^{a}	1.98^{b}	2.13^{b}	1.88^{b}	1.99^{b}	1.34
CF	4.21^{a}	4.87^{a}	4.93^{a}	5.03^{a}	4.79^{a}	1.48
NDF	20.03^{a}	21.30^{b}	21.21^{b}	22.15^{b}	23.12^{b}	1.52
ADF	10.61^{a}	11.36^{b}	10.59^{b}	11.12^{b}	11.39^{b}	1.05
Lignin	3.44^{a}	2.64^{b}	1.98^{b}	2.02^{b}	2.34^{b}	0.81

a, b Means along the same row with different superscripts are significantly different (P< 0.05); SEM= Standard error of the mean

TABLE 10: NITROGEN BALANCE (g day-1)

Parameter (%)	% Level of MLP T1(0%)	T2(5%)	T3(7%)	T4(9%)	T5(11%)	±SEM
N – intake	2.72[a]	3.22[b]	3.23[b]	3.38[b]	3.23[b]	1.41
Feacal – N	2.17[a]	2.38[a]	1.85[a]	1.78[a]	1.50[a]	1.62
Urinary – N	0.60[a]	0.64[a]	0.59[a]	0.71[a]	0.85[a]	2.10
N – retention	-0.05[a]	0.20[b]	0.79[b]	0.89[b]	0.85[b]	0.81

a, b Means along the same row with different superscripts are significantly different (P< 0.05); SEM= Standard error of the mean

TABLE : MATRIX OF CORRELATION COEFFICIENTS AMONG HEAMATOLOGICAL PARAMETERS OF SHEEP

	PCV	WBC	HB	RBC	HCHC	MCH	MVC	N	L
PCV	1.000	0.554**	0.997**	0.882**	0.870**	0.390	0.434	0.390	0.632
WBC		1.000	0.573**	0.597**	0.631**	0.371	0.381	0.375	0.234
HB			1.000	0.901**	0.878**	0.392*	0.431	0.379	0.650
RBC				1.000	0.928**	0.499**	0.526**	0.415	0.666**
HCHC					1.000	0.617**	0.643**	0.512**	0.641**
MCH						1.000	0.993*	0.607**	0.144
MVC							1.000	0.629**	0.167
N								1.000	-0.215
L									1.000

**,*=Significant at 0.01 and 0.05% level of probability respectively

SERO-PREVALENCE AND BRUCELLA INFECTION IN KACHIA GRAZING RESERVE KADUNA STATE, NIGERIA

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INTRODUCTION

Brucellosis is a major zoonotic bacterial disease of global importance (Cutler *et al.*, 2005). It is caused by bacteria belonging to the genus *Brucella.* The primary clinical manifestation of brucellosis in animals is related to the reproductive tract, with abortion being the most common manifestation (Godfroid *et al.,* 2010). In Nigeria, brucellosis is recognized as an endemic disease and previous studies showed a range of 0.4 to 26% seroprevalence in the traditional nomadic Fulani herds (Ocholi *et al.*, 1993). The objectives of this study were to (i) determine the prevalence of *Brucella* antibodies in cattle and other small ruminants in Kachia Grazing Reserve (KGR) located in Kaduna State, Northern Nigeria (ii) isolate and characterize *Brucella* organisms in animals in the KGR. (iii) to compare Rose Bengal Plate Test and Lateral Flow Assay.

METHODOLOGY

The study was carried out between March and July 2011 in KGR. A 3-level multi-stage cluster sampling method was used to sample the target animal population. A total of 2,037 serum samples were collected from four (4) blocks, consisting of 1,685 from cattle, 280 from sheep and from 72 goats. Twenty three vaginal swabs, 18 milk samples from lactating cows and one hygroma fluid were also collected for isolation of *Brucella* organisms. Serum samples were tested for *Brucella* antibodies by the Rose Bengal plate test (RBPT), serum agglutination test (SAT), milk samples were also tested for *Brucella* antibodies using milk ring test (MRT) all as described by (Alton et al., 1988) and *Brucella* lateral flow assay (LFA) (Abdeol *et al.,* 2008).Samples

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for isolation were cultured as described by (Alton et al., 1988). Recovered *Brucella* isolate was identified according to standard protocol (Godfroid *et al.*, 2011). The strength of association between sex and prevalence of *Brucella* antibodies was analysed using Chi-square (X^2), with Yates correction. The test was carried out at 5% level of significance (Yates, 1934).

RESULTS AND DISCUSSION

Out of the 1,685 cattle sampled, 10 were positive for *Brucella* antibodies with RBPT giving a prevalence of 0.59 % (Table 1). The sex distribution of the 10 sero-positive samples was 9 (0.53%) females and 1 (0.06%) male. There was a significant ($p<0.05$) association between sex and presence of *Brucella* antibodies. All cattle positive for *Brucella* antibodies were more than one year old. All bovine sera positive for *Brucella* antibodies with RBPT were also positive with *Brucella* LFA. Seven (70%) of the 10 RBPT- positive samples were also SAT- positive. Four (22.22%) of the 18 milk samples tested with MRT, were positive for *Brucella*. Out of the 280 sheep sera tested with RBPT only one (0.36%) female was positive for *Brucella* antibody. None of the serum samples from goats was seropositive. *Brucella* organism was isolated from the hygroma fluid (Table 2) giving an isolate rate of 2.4%. The isolate was typed and identified as *Brucella abortus* biovar 1.

The low prevalence rate recorded in this study could be due to the large land mass of the grazing reserve (33, 411 square meters). Each herd (household) has a specific grazing area, thus limiting contact between infected and non infected herd since brucellosis is mainly a herd problem (FAO, 2004); and one way it is transmitted is by contact between infected and non infected cattle. The implication of the presence of seropositive cattle in the grazing reserve is that these animals could serve as source of infection to non infected animals in the herd.

A higher infection rate was recorded among female (0.53%) than male (0.06%) cattle and the association between infection rate and sex was

significant (P<0.05). The higher prevalence rate recorded in female's cattle than males is not surprising since female cattle are known to be the main source and foci of *Brucella* infection (Godfroid *et al.,* 2010). Brucellae are known to have a high affinity to the alcohol D-erythritol found in higher volume in the gravid uterus than the seminal vesicles, making the infection more common in female than male cattle (Walker, 2004).

Only one female sheep was seropositive with RBPT. Although, *Brucella* species are known to have a specific host range, cross infection is known to occur (Godfroid, *et al.,* 2010). It is therefore, likely that the sheep was infected from contact with infected cattle. Presence of *Brucella* antibodies in milk recorded in this study is of public health importance as consumption of raw and inadequately pasteurized milk constitute one of the means of acquiring brucellosis (Acha and Szyfres, 2003).

Results of RBPT require a confirmation by Compliment Fixation Test (CFT) or Enzyme Linked Immunosorbent Assay (ELISA) as recommended by (OIE, 2009). These tests (CFT and ELISA), require specialize training and expensive laboratory equipment and reagents. The LFA could be an alternative test, it has several practical advantages which include the fact that the use of the LFA does not require specific training, expertise, electricity or expensive equipment, and the test devices may be stored without the need for refrigeration. The test results are obtained almost instantaneously and by visual inspection with the unaided eye (Abdeol *et al.,* 2008). The positive result recorded by both RBPT and SAT in this study may indicate a point of transition from acute to chronic infection.

Ocholi *et al.* (2004) recovered 23 *Brucella* isolates from vaginal swabs, milk and aborted tissues from cattle, sheep and horses in studies carried out in 10 states of Nigeria. The low isolation rate in this study could be due to the fact that there was no case of recent abortion at the study as there is a high tendency of isolating *Brucella* from aborted tissues (Alton *et al.,* 1988).

Table 1: Prevalence of Brucella antibodies in cattle in Kachia Grazing Reserve by Rose Bengal plate test

	No (%) Positive				
	Block 1	**Block 2**	**Block 3**	**Block4**	**Total**
Number	624	666	260	135	1685
Sex					
Males	0(0)	1(1.15)	0	0	1(0.06)
Females	7(1.12)	2(0.30)	0	0	9(0.53)
Total	7(1.12)	3(0.45)			10(0.59)
Age					
Young	0	0	0	0	0 (0)
Adult	7(1.12)	3(0.45)	0	0	10(0.59)

Table 2: Isolation rate of *Brucella* from cattle in kachia grazing reserve

	No. culture	No. (%) positive
Hygroma fluid	1	1 (100)
Vaginal swabs	23	0 (0)
Milk sample	18	0 (0)
Total	42	1 (2.4)

CONCLUSION

The results of the present study revealed that the prevalence of *Brucella* antibodies in cattle, sheep and goats in Kachia Grazing Reserve was 0.59%,

0.4% and 0% respectively by RBPT diagnostic technique. Isolation of *Brucella abortus* biovar 1 from hygroma fluid provided a definitive diagnosis of the infection in the study area.

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MOLECULAR DETECTION OF LEPTOSPIRA IN RODENTS (*Rattus norvegicus*); IMPLICATION FOR PUBLIC HEALTH

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BACKGROUND

Leptospira, the causative organism of Leptospirosis belongs to the phylum of Spirochaetes, family *Leptospiraceae*, order spirochaetales (Adler and Moctezuma, 2010), and comprises of both saprophytic and pathogenic species. Pathogenic species infect both humans and animals (Xue *et al.*, 2008).

Leptospirosis is considered one of the most widespread zoonosis worldwide, with the exception of Antarctica (Adler and Moctezuma, 2010) but its most significant impact is in tropical and subtropical countries with a global estimate of severe cases amounting to over 500,000 occurring annually (Ahmed *et al.*, 2009). The disease burden might be significantly higher as a result of under developed diagnostic capacity as well as human behavioral changes (Levett, 2004).

Leptospirosis is often considered an occupational disease affecting animal handlers (especially rodents, whether for research, food or as pets), Veterinarians, abattoir and sewer workers (Hartskeerl *et al.*, 2011). Wild or domestic animals especially rodents, cattle, pigs and dogs may be carriers (Adler and Moctezuma, 2009) and infection can be acquired either through direct contact with the animals or through environmental contamination with infected animals urine (*Lau et al.*, 2010). The natural host often remains asymptomatic, but serves as a reservoir for infection in alternative

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hosts such as man who succumb to clinical disease (Fenner *et al.*, 2010).

The disease Leptospirosis manifests as mild to severe with symptoms mimicking those of endemic infections such as Malaria, hepatitis, enteric fevers and dengue fever (Hartskeerl *et al.*, 2011),thus it is often misdiagnosed by clinicians.

Different diagnostic methods have been developed over the years for the diagnosis of *Leptospira*. The sensitivity of PCR assays has overshadowed the need for culturing to isolate infecting Leptospires (Stoddard *et al.*, 2009). Some Real time PCR assays have been developed for the specific detection of pathogenic *Leptospira*, one such target is 16s rRNA gene which is present in all Leptospires while another targets the LipL32 gene that is also considered to be only present in pathogenic strains (Stoddard *et al.*, 2009).

This study was aimed at detection, identification and molecular characterization of *Leptospira* in rodents in the UK by the use of molecular techniques applied directly on clinical material.

MATERIALS AND METHODS

250 rodent kidneys in the repository of the University of East London were used in this study. Processing was done by removing the outer portion and excising a small internal piece of tissue (up to 25mg).

DNA extraction was done using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturer's instructions. Real time PCR was performed on Leptospiral DNA extracts first for the 16s rRNA gene according to protocol of Fenner *et al* (2010). DNA of *Leptospira interrogans* from the university of East London Bacteria isolate Bank and ultra pure water were used as positive and negative controls respectively.

Samples positive for both 16s rRNA gene and LipL32 outer membrane lipoprotein were considered positive pathogenic *Leptospira*. Identification of confirmed *Leptospira* positive samples was performed using eight different Housekeeping genes (accA2, ccmF, czcA, gcvP, groEL, polA, recF and secY).

Positive samples were submitted for sequence typing analysis at the Genome Centre, Queen Mary's University of London. Reference Sequences of Leptospires were retrieved from the Gene bank. Multiple alignment, phylogenetic and molecular evolutionary analyses were conducted using MEGA 5 (Tamura *et al.*, 2011).

RESULTS

As this experiment targets the pathogenic specie, only samples positive for both 16srRNA and LipL32 were considered positive for pathogenic Leptospira. 22 (9%) of the 250 rodent samples used in this analysis were positive for leptospira spp. 11 were *L. borgpetersenii*, 1 was *L. kirshneri*, and another that was not conclusively identified. Thirteen positive RNA obtained had nucleotide sequences that were 82-93% homologous and clustered together on the phylogenetic tree. However, *L.kirschneri* was an outlier for the pol A gene.

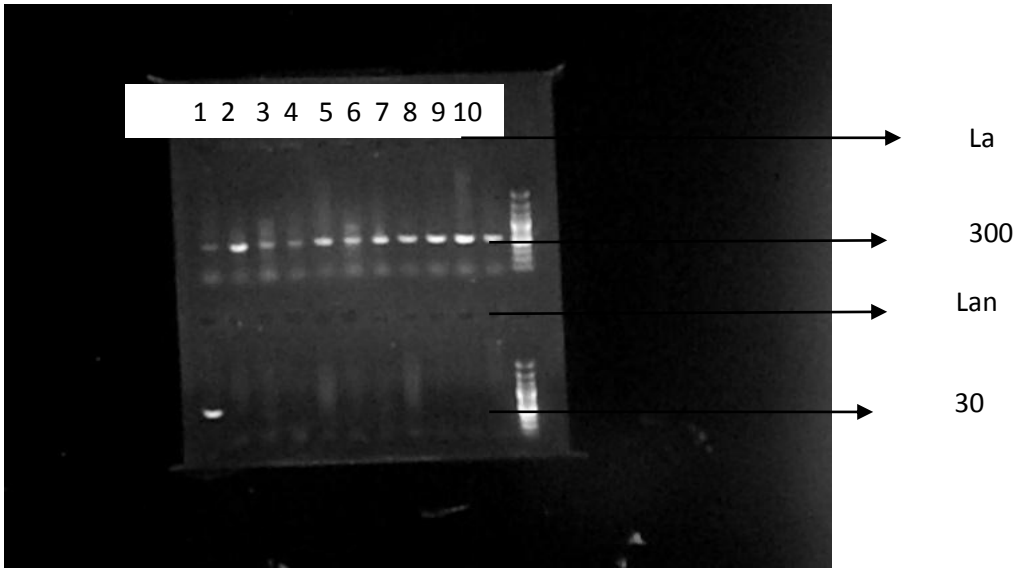

Fig1. Agarose -gel electrophoresis showing amplification products, Molecular size marker 100bp ladder. Lane 1: 2-9 positive samples,11: positive control; lane 2: 1 positive sample.

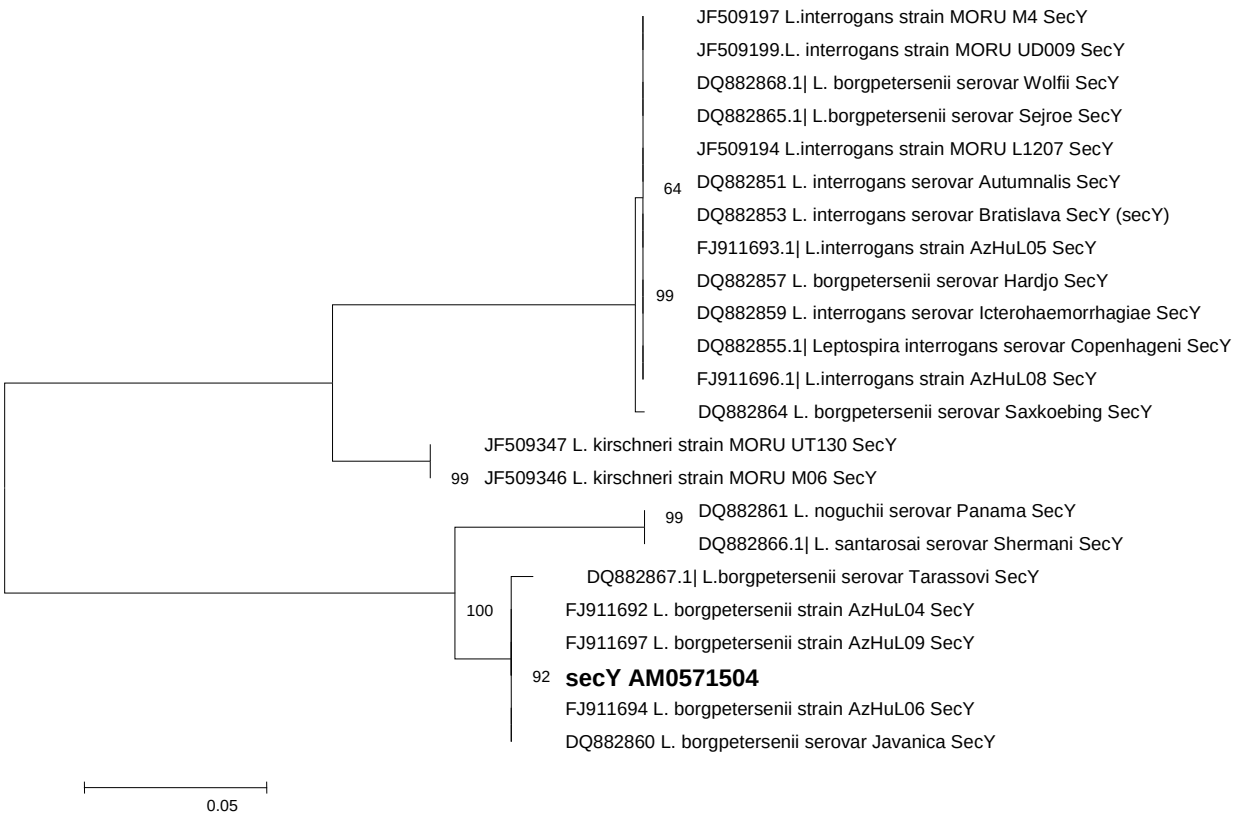

Fig 2: Phylogenetic tree constructed from sequences of secY gene of *Leptospira* strains. The identification numbers of the strains, the serogroups and the serovars are indicated with the isolate in this study in bold. The maximum Likelihood method was used for sequence comparison.

DISCUSSION

The highly conserved nature of the LipL32 among pathogenic Leptpspires accounts for its recommended use for differentiation of non pathogenic and pathogenic *Leptospira* , this potential was demonstrated in this study by its ability to detect pathogenic species and supports previous reports (Stoddard *et al.*, 2009). Interestingly, the samples identifies by the secY genes were grouped alongside those isolated from human patient in Portugal (Accession Numbers FJ 911692, FJ 911697, FJ 911694) by Goncalves *et al* (2010), this suggests that the isolates identified from rats in this study are probably strains pathogenic in humans.

Rodents play important roles as reservoirs and transmitters of zoonotic pathogens like Leptospira which causes Leptospirosis in human s. Bush burning and habitat alteration cause rats to migrate to human dwelling thereby contaminating food stuff in the process. There is also the risk of occupational exposures by researchers and technicians who handle animals.

Leptospira borgpetersenii and *Leptospira kirschneri* are the two major pathogenic species implicated in animal infection which can also be transferable to human (Salaun *et al.*, 2006), detection of these species goes further to suggest the probable role of rodents in the epidemiology of Leptospirosis serving as source of environmental contamination and infection to other animals and humans and the role of rats in the epidemiology of Leptospira in the UK.

CONCLUSION AND RECOMMENDATIONS

With our findings we are able to attest to earlier reports that gene PCR-sequencing is able to characterize *Leptospira* up to species level and quite interestingly even directly from tissue sample without necessarily growing in media thereby reducing the time required for diagnosis. This emphasizes its potential in discerning strain's ancestral relationship during outbreaks or for epidemiological surveillance for adequate disease control measures.

It is important to investigate the zoonotic risks of Leptospira in Nigeria and suggest preventive and control policies for public health. There is also a need to replicate this study in a wide variety of animal species to establish the prevelance and molecular characteristics and most critically the possible pathogenicity of the so called intermediate *Leptospira* spp.

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DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX INFECTION AMONG SLAUGHTERED CATTLE AT JOS SOUTH ABATTOIR, PLATEAU STATE, NIGERIA

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INTRODUCTION

Bovine tuberculosis is still widespread in Africa, parts of Asia and some Middle Eastern countries (Kazwala *et al.*, 2001). In Africa, the occurrence of bovine tuberculosis due to *M. bovis* in humans is difficult to determine accurately because of technical problems in isolating the microorganism (Cadmus *et al.*, 1983). In West and Central Africa, where bovine TB is prevalent in animals, human TB cases due to *M. bovis* occur (Shitaye *et al.*, 2006) resulting from ingesting contaminated unpasteurized milk, raw meat and by inhaling cough spray from infected livestock (Ayele *et al.*, 2004).

Bovine TB in humans is becoming increasingly important in countries like Nigeria as humans and animals are sharing the same micro-environment and dwelling premises especially in rural areas (Cadmus *et al.*, 2006). There is increasing contact between humans and animals worldwide due to

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increasing population density and growth especially in poor developing countries where livestock offers important socioeconomic, cultural, and religious pathways out of poverty (Whipple *et al.*, 1996). Therefore, information generated from this study will aid in the development of guidelines and recommendations to public health regulatory bodies with the aim of reducing the transmission of bovine tuberculosis

METHODS

A total of 168 lung tissues with or without lesions from 485 slaughtered cattle were tested for acid fast bacilli using Ziehl-Neelsen staining technique (ZN) and a duplex polymerase chain reaction. Cattle were selected consecutively until sample size was achieved. A structured interviewers' administered questionnaire was used to obtain demographic characteristic of cattle with respect to the breed of cattle, sex, and age (years). Other information include body conformation of cattle: Categorized into: Apparently healthy (AH); slightly emaciated (SE) or emaciated (E) depending on visual assessment of their body conformation. Lesions from tissue were scored as 1+, if only one organ was affected and the nodules were miliary, that is, still embedded; 2+, if only one organ, but with pronounced nodular lesions; 3+, if more than one organ were affected. Data was entered and analyzed using Epi info 3.5.3 version software and Microsoft Excel to determine frequency, proportions, prevalence ratio, prevalence odds ratio and Chi Square at 95% confidence interval was used to assess risk factors for *Mycobacterium tuberculosis* complex infection. Descriptive analytical statistics, bi-variate and multi-variate analyses were done.

Laboratory method

Sample processing for tissue biopsies

Tissue biopsy was used directly to make a smear on a clean grease free micro slide using a sterile wire loop. Smears were processed as described by Ochei *et al.*, (2008) and examined using ×100 objective.

Polymerase Chain Reaction Technique

DNA extraction from tuberculosis and non tuberculosis lung tissue

DNA extraction was carried out using kit extraction (Zymo Research® country South Africa) according to manufacturer's instruction. The duplex PCR (two sets of primers) that has the capacity to detect both MTB complex organisms and *M. bovis* at the same time was employed. The Polymerase chain reaction was carried out according to the method of Eduardo *et al.*, (2009).The primers ISN1 (5'CGTGAGGGCATCGAGGTGGC-3') and reverse ISN2 (5'-CGTAGGCGTCGGTGACAAA-3) amplifying a 260 bp genomic fragment of insertion sequence *ISN* specific for MTB complex were used in master mix containing 16µl nuclease free water, 25µl of 2X Dream tag DNA polymerase, 1µl of template DNA, 1µl (10pmol/ml) of JB21 and JB22 (Forward and Reverse) each respectively, 1µl of INS1 and INS2 (Forward and Reverse) each respectively. Amplification was initiated by initial denaturation at 95^0C for 5 minutes and this was followed by 30 cycles of 96^0C for 1 minute (final denaturation), 68^0C for 1 minute (Annealing), and 72^0C for 1 minute (Initial extension). This was followed by 72^0C for 7 minutes (final extension).The PCR was carried out in Thermal cycler (Applied Biosystem- Gene Amp PCR system 2700)

Electrophoresis

PCR products were fractionated electrophorectically in 1.5% agarose gel in Ix TBE buffer, pH 8.3 for 1 hour 30 minutes, and visualised under UV light using an image documentation system (Syngene) after staining with ethidium bromide. The staining was done by immersing the gel in a solution of ethidium bromide in a shaker for 15 minutes. The size of the amplicon was determined by comparison with 260 bp DNA ladder.

Ethical consideration

Ethical clearance was obtained from the Health Research Ethics Committee in Plateau State (PSSH/ADM/ETH, CO/2012/40) and Permission was obtained from the Head of Veterinary Services, Plateau State.

RESULTS

The mean age of the cattle was 5.6 ± 1.3 years and 64.3% were females. Majority were indigenous (58.5%), White Fulani breed of cattle (58.5%) and about half (54.8%) of the cattle were slightly emaciated (Table 1). As body conformation score decreased from apparently healthy to slightly emaciated and then to emaciated, the risk of a positive AFB test increased. The cattle that were slightly emaciated and emaciated are eight times (POR= 8.17; 95% CI: 1.67-44.97) and six times (POR= 6.09; 95% CI: 1.62-26.94) respectively, at greater risk of a positive result to AFB test when compared to apparently healthy animals. There was no significant difference in the prevalence of bovine tuberculosis, as regards age, sex, breed and source of cattle (Table 2). Lungs with lesions were 52 times more likely to test positive to AFB test compared to those tissues without lesions (AOR=52.3; 95 % CI: 16.4-191.8) (Table 3). The prevalence rate of *Mycobacterium tuberculosis complex* by Ziehl-Neelsen staining technique was 21.4%, while the prevalence rate of *Mycobacterium tuberculosis* complex was 16.7% by duplex polymerase chain reaction (Table 4)

DISCUSSION

Based on abattoir records, a total of 485 cattle were slaughtered at Jos South Abattoir from the study period of May-June, 2012. Almost all the cattle slaughtered were from the nomadic herds located within Plateau State. Sometimes cattle from neighboring countries like Chad, Cameroun, and Niger were brought to the abattoir for slaughter which corresponds with the report of Corbett *et al.*, 2003.

A prevalence rate of *Mycobacterium tuberculosis complex* 21.4% by acid fast bacilli test and 16.3% by duplex polymerase chain reaction were recorded in

this study. This is higher than the prevalence rate of 4.4% recorded by Damina *et al.*, (2011) a research work conducted at Jos South abattoir and Bukuru cattle market in Plateau State, whereby samples collected from cattle were analyzed using a PCR technique known as deletion analysis. A higher prevalence rate of bovine TB in this study might be connected with the failure to adopt the test and slaughter policy in Nigeria, and the influx of infected cattle from neighboring countries (Cameroon, Chad and Niger) as a result of lack of control of border and inadequate quarantine measure.

Cattle of age >5 and more females were slaughtered during the study period and were the most affected by tuberculosis lesions. This may be due to the fact that cattle owners in the study area prefer to keep more females than male animals in their herds. Such animals are usually disposed and taken to the abattoir for slaughter when they can no longer reproduce due to old age. Bikom *et al.*, (2007) also reported that the female cattle stay longer in the herd for the purpose of reproduction. However, the white Fulani cattle were slaughtered more during the study period.

There was no significant difference in the prevalence rate of bovine tuberculosis in cattle with respect to age, sex, breed and source of cattle which corresponds to the findings reported by (Ameen *et al.*, 2008) a work conducted in Ethiopia, but different from the literature reported in Republic of Ireland (Eduardo *et al.*, 2009).

A significant difference of $P<0.05$ in the distribution of lesions in lung tissue of cattle shows that bovine tuberculosis is dependent on granulomatous lesions which may be associated with the route of infection. The presence of caseous and/or calcified lesions and even lesions resembling tuberculosis lesion may not always be found to be of mycobacterium origin. In contrast, calcified lesions can be caused by any other intracellular organisms or parasites which may also be a reason for the 6 (18.2%) that failed to be detected by the duplex polymerase chain reaction technique among the 33 lung tissues with visible lesions. It may also have been affected by the

sampling technique during smear preparation as *Mycobacterium* is not evenly distributed in the tissue sample. In most cases tuberculosis lesions, in cattle in particular, may not always be observed; therefore the absence of visible lesions may lead to failure in *Mycobacterium tuberculosis* complex detection as isolation of *M. bovis* has been confirmed from non-visible lesions (Whipple *et al.*, 1996). This corresponds with the findings of 9 (6.7%) lung tissues without visible lesions which were found positive for bovine tuberculosis.

As body conformation decreases from apparently healthy to slightly emaciated and then to emaciated the risk of a positive result to AFB test was increased at $P<0.05$. The animals that were slightly emaciated and emaciated were 8 and 6 times respectively at the risk of a positive result to AFB test when compared to apparently healthy animals by bivariate analysis.

Polymerase chain reaction analysis is a much faster method of identifying members of the *Mycobacterium tuberculosis* complex compared to conventional method like cultural isolation and biochemical tests which are time consuming and in most cases facilities for such tests are not easily available in developing countries (Shah *et al.*, 2002). Agarose gel electrophoresis of duplex PCR (using two sets of primers) products obtained from lungs of cattle and amplified at band (260bp) with the ISN1 & ISN2 primers confirmed the presence of MTB complex. However, Shah *et al.*, (2002) reported that the detection of tuberculosis in cattle either by Acid fast bacilli test or PCR is suggestive of *M. bovis*, hence further molecular work like spoligotyping and variable numbers of tandem repeats (VNTR) to differentiate strains are recommended.

CONCLUSION

The presence of MTB complex in cattle signifies its potential risk to public health. Acid fast bacilli positivity increased with the presence of lesion on

tissue from slaughtered cattle. Health education of the public on bovine TB is urgently required. Also, active surveillance for MTB complex and enforcement of test and slaughter policies at abattoirs are recommended.

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Table 1: Characteristics of cattle slaughtered at Jos South abattoir, Plateau State, Nigeria, 2012

Characteristics	Frequency	Percent
Age of cattle (years):		
< 5	83	49.4
>5	85	50.6
Breed of Cattle:		
Red Fulani	70	41.7
White Fulani	98	58.3
Sex:		
Female	108	64.3
Male	60	35.7
Source of Cattle:		
Indigenous	98	58.3
Neighboring Country	70	41.7
Body Conformation:		
Apparently healthy	52	31.0
Emaciated	24	14.3
Slightly Emaciated	94	54.8
Presence of lesion on lung tissue		
Yes	33	19.6
No	135	80.4
Scores for lesion		
+1	27	84.4
+2	5	15.6
Mean age for cattle is 5.6 ± 1.3 years		

Table 2: Association between socio-demographic factors and result of acid fast bacilli test for cattle slaughtered at Jos South Abattoir, Plateau State, Nigeria, 2012

Characteristics	No. Positive (%)	No. Negative (%)	POR*(95%CI)
Age group (years):			
≤5	19 (22.9)	64 (77.1)	1.19(0.53-2.65)
≥5	17 (20.0)	68 (80.0)	
Sex:			
Female	26 (24.1)	82 (75.9)	1.59(0.66-3.86)
Male	10 (16.7)	50 (83.3)	
Breed of Cattle:			
Red Fulani	20 (24.1)	50 (71.4)	2.05 (0.91-4.62)
White Fulani	16 (16.7)	82 (83.3)	
Source of Cattle:			
Indigenous	16 (16.3)	82 (83.7)	0.49 (0.22-1.09)
Neighboring Country	20 (28.6)	50 (71.4)	
Presence of lesion on tissue:			
Yes	27 (81.8)	6 (18.2)	63.0 (20.7-191.8)
No	9 (6.7)	126 (93.3)	
Body condition:			
Apparently healthy	3 (5.8)	49 (94.2)	1.00 (referent)
Slightly emaciated	8 (33.3)	16 (16.7))	8.17 (1.67-44.97)
Emaciated	25 (27.2)	67 (72.8)	6.09 (1.62-26.94)

*POR- Prevalence Odds Ratio

Table 3: Unconditional logistic regression of socio-demographic factors and result of acid fast bacilli test for cattle slaughtered at Jos South Abattoir, Plateau State, Nigeria, 2012

Characteristics	*APOR (95%Confidence Interval)	p value
Presence of lesion:		
Yes		
No	52.31 (16.38-191.82)	0.00
Body conformation of cattle:		
Apparently Healthy	0.54 (0.13-2.32)	0.41
Emaciated		

*APOR – adjusted Prevalence Odds Ratio

*Age and Sex had been controlled for under the unconditional logistic regression model

Table 4: Ziehl Neelsen test results and detection of MTBC from lung tissuespositive for AFB test by Polymerase Chain Reaction Technique among cattle slaughtered at Jos South abattoir, Plateau State, Nigeria 2012

Result	ZN Test Frequency	Percent (%)	PCR Frequency	Percent (%)
Positive	36	21.4*	30	83.3
Negative	132	78.6	6	16.7*

MTBC: *Mycobacterium tuberculosis* complex, AFB: Acid fast bacilli

*Prevalence rate by ZN test = 21.4%

*Prevalence rate by duplex PCR = 16.7%

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