Viral Causes of Acute Febrile Jaundice in Selected Provinces of Zambia

Boniface Kabungo
Ministry of Health
AaroAyato Takada
Department of Disease Control, University of Zambia, School of Veterinary Medicine
Masahiro Kajihara
Research Center for Zoonosis Control, Hokkaido University, Japan
Akina Mori
Research Center for Zoonosis Control, Hokkaido University, Japan
Katendi Changula
3Department of Disease Control, University of Zambia, School of Veterinary Medicine
John Amos Mulemena
6Mulungushi University, School of Medicine and Health Sciences
Kingsley Kamvuma (kamvumak@yahoo.com)
6Mulungushi University, School of Medicine and Health Sciences
Benson M. Hamooya
Mulungushi University, School of Medicine and Health Sciences

Research Article

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Abstract

Background

Following the yellow fever (YF) risk assessment conducted in 2013, Ministry of Health in collaboration with WHO successfully implemented YF case based surveillance among the YF suspects in the high risk areas of Zambia. To date, none of the patients has been confirmed as a case of YF and the epidemiology of flavi-viruses has not been comprehensively investigated in Zambia. As YF may be hardly distinguished clinically from other febrile diseases, because early in the clinical course YF may appear similar to other diseases but YF will diverge clinically as the disease course progresses. This study was designed to investigate the viral causes of febrile jaundice among YF suspects in selected provinces of Zambia.

Method

We conducted a retrospective study on 93 archived serum samples previously collected from patients meeting a case definition of YF suspect from January 2014 to July 2015 presented in selected health facilities. Yellow Fever, Dengue Fever, West Nile, pan Flavivirus, and Hepatitis A viruses were tested by reverse transcriptase polymerase chain reaction (RT_PCR) and Hepatitis B virus using PCR, while Hepatitis C and Hepatitis E viruses were tested by nested polymerase chain reaction (nPCR). Samples were also tested for YF and dengue fever (DF) antibodies using in-house immunoglobulin M enzyme linked immunosorbent assay (Ig M ELISA) and immunoglobulin M rapid test respectively. STATA version 12 was used for data analysis.

Results

Fourteen percent (13/93) of the serum samples were identified as YF IgM positive. None of the samples tested positive for DF IgM ELISA. All 93 serum samples tested negative for the flaviviruses by RT-PCR. However, 8.6% (8/93) showed acute Hepatitis A and 2/20 (10%) of pooled sera tested positive for HBV. The median age of patients with Hepatitis A was 9.5 years old and for those without evidence of HAV infection was 19 years old. Approximately 85 (91.4%) of patients had acute diseases of unknown origin.

Conclusion

The study revealed that YF IgM was prevalent among study participants. However, the causes of fever and jaundice in Zambia may include viral hepatitis and needs to be considered if flaviviral diseases are suspected.

Background

The genus *Flavivirus* of the family *Flaviviridae* consists of approximately 73 virus species. This genus include yellow fever virus (YFV), dengue virus (DENV) and West Nile virus (WNV) which are of public health importance [1]. The Zika virus (Zikv) which was first discovered from rhesus monkey in the Zika forest of Uganda has emerged as a global threat with an accelerated geographic spread noted in the last 5 years [2] [3]. Flaviviruses are associated with the zoonotic diseases of which the majority are transmitted by arthropods (mosquitoes and ticks) [4]. Several outbreaks of the flaviviral disease have been reported in Angola and Democratic Republic of Congo (DRC) in the past [5] [6]. Currently, large YF outbreaks have been occurring in Angola and DRC with the potential to spread worldwide [7] [8] [9] [10]. Although Zambia was reclassified as a low risk zone of YF by World Health organization (WHO) in 2010, no outbreak of flaviviral disease has ever been documented for the past 60 years. YF disease was first described in North-Western and Western provinces of Zambia in 60-70 years ago [11]. The recent data suggest the presence of suitable vectors of flaviviruses and inconclusive low level circulation of flaviviruses in Zambia [12] [13] [14]. Following the reclassification, the Zambian Ministry of Health (MOH) in collaboration with WHO successfully instituted a risk assessment in 2013 followed by YF case based surveillance in January, 2014. This system has been capturing all YF suspects in Lusaka, Copperbelt, Muchinga, North-Western and Western provinces of Zambia. According to WHO recommendations, a clinical case definition of YF suspect is defined as “Any case presenting with acute onset of fever, with jaundice appearing within 2 weeks onset of symptoms” [15] [16]. Despite patients meeting the case definition of YF suspect, none of the YF suspects tested at the University Teaching Hospital has been confirmed as a case of YF. Hence, it is important to ascertain whether other viruses could be causing acute febrile jaundice in cases presenting to our health facilities. In Zambia, the epidemiology of flaviviruses has not been comprehensively investigated. In addition, viral hepatitis being an important differential diagnosis of YF illness was included in this study. Therefore, the main objective of this study was to investigate the viral causes of febrile jaundice in patients presenting to health facilities in selected provinces of Zambia.

Methods

Study design and Sites

This was a Laboratory retrospective re-analysis of samples collected from patients meeting the case definition of YF between January, 2014 and July, 2015 [15] [16]. The study was conducted in health facilities in selected provinces of Zambia (Lusaka, Copperbelt, Muchinga, North-Western and Western), See figure 1. Samples collected were shipped to reference laboratory (UTH Virology Laboratory) under cold chain within a week of collection. Criteria of selecting the provinces were based on the fact that North-Western and Western provinces share a common border with endemic countries and also to supplement YF risk assessment’s findings conducted in 2013 [17]. A case of Chikungunya fever caused by non-flavivirus and presence of suitable vectors have been previously documented on the Copperbelt province [18]. *Flaviviruses* and Chikungunya may be causes of febrile illness and have a common transmission vector and co-circulate in the same area [18]. The increasingly international travel of people and trade across endemic countries are a potential risk of emerging infectious

diseases in Lusaka province whilst Muchinga was included due to its new provincial status. The other provinces had no unique characteristics (no cases has been previously reported) for them to be included in the study and given the financial constraints of the project. Therefore, it was not possible to include all the provinces.

Sample collection

Archived serum samples from YF suspects regardless of sex and age were included in the study. The samples were previously collected for YF surveillance. About 3 to 4 mL of blood was collected by venipuncture into plain containers (Becton Dickinson and company, USA) and transported on cold chain to local health facilities and storage. The serum samples were then shipped on cold chain to Virology Laboratory at the University Teaching Hospital (UTHVL). In the laboratory, sera were stored at – 40 ^{o}C.

Laboratory analysis

Serological assays

All serum samples were first tested at the UTHVL by YFV IgM antibody capture ELISA (in-house ELISA of Institute of Pasteur, Senegal) as previously described [19]. A P/N (positive control [or sample] optical density $OD_{405\ and\ 620}$/negative control $OD_{405\ and\ 620}$) ratio ≥ 2.0 was considered positive whilst < 2.0 was considered as negative.

Serum samples were analyzed using commercial IgM DENV rapid kits (CTK BIOTECH, San Diego, USA) according to the manufacturer. DENV IgM capture ELISA is the qualitative presumptive detection of elevated IgM antibodies to dengue infection.

PCR and Sequence analysis

Serum was stored at -40^{o}C in the University Teaching Hospital Virology Laboratory (UTHVL) and was transported on dry ice to the University of Zambia (UNZA), School of Veterinary Medicine in Virology Laboratory for PCR analysis. The 93 serum samples collected from YF suspects in 5 provinces between January, 2014 and July, 2015 were grouped into 20 pooled sera (i.e., 3-5 samples/pool) and subjected for PCR analysis. Thereafter, we analyzed samples individually for those pools that tested positive. Viral nucleic acid was extracted from serum using QIAamp viral RNA kits (Qiagen, Hilden, Germany). All PCR assays were performed in a volume of 10 µL with 1.0 µL nucleic acid as a template as previously described [20]. Nested PCR contained 1.0 µL of PCR product of the first round RT-PCR as a template. Cycling conditions, primer sequences and amplicon lengths of PCR assays are shown in Table 1. RT-PCR assays for flaviviruses including YFV [20], DENV [21] [22] and WN virus were performed using AB Applied Biosystem Thermo cycler (Applied Biosystem, USA) according to manufacturer's instructions. The Pan- Flavi RT-PCR assay was also used to optimize the detection of a wide range of flaviviruses and complement flavivirus-specific assays. The pooled sera were also tested for HAV, HBV, HCV and HEV [23]. The individual samples for positive HAV pooled sera were also re tested by RT-PCR. The positive samples were re-amplified, the product excised from the gel and purified with extraction kit (Promega, USA) before the DNA generated were sequenced using 3130 Genetic Analyzer (AB Applied Biosystem, HITACHI, Japan). Sequence data were compared using the Blast program.

Statistical analysis

Data were captured from the laboratory request forms and analysed by using STATA Version 12 (Stata Corp, College Station, TX, USA). The outcome variable of interest was presence or absence of flaviviruses and predictor variables were gender, age groups, vaccination status, travel history and provinces. Descriptive statistics were employed to describe the basic features of the data. One-sample test of proportion was used to determine statistical difference of two proportions arising from the same sample. To test for significance difference between YF IgM (yes or no) and other categorical independent variables, a chi-square test was used. We used Wilcoxon rank-sum test to ascertain the statistical difference between two medians. Logistic regression was used to determine factors associated with YF IgM cases. All tests were set at 5% statistical significance.

Results

Demographic characteristics and clinical manifestations of YF suspects

Table 2 below shows the basic characteristics of the study participants. Overall, 48 (51.6%) were male and the median age was 19 years (IQR: 7-33). The ages were further classified into six age groups. The majority suspects were aged 6-14 years (23.7%) and followed by those aged 0-5 years (20.4%) and 16-25 years (20.4%). None of the patients reported having been vaccinated against YF vaccine and 1.1% of the total patients had history of travel to Tanzania. The majority of the YF suspected cases were reported from North-Western (31.2%) and Western provinces (47.3%).

Sample collection time

Thirty eighty (41%) samples were collected within 5 days of the onset of fever and jaundice and there was a statistical difference (p=0.0004). It was also noticed that a significant proportion (66%, p<0.0001) of samples were collected within 10 days of the onset of fever and jaundice. See table 3.

Serological Epidemiology of YFV

Table 4 below shows the relationship of YF IgM cases with indpendent variables. The YF IgM was detected in 14% of the YF suspects. The median age of patients with positive and negative presumptive results was 14 years (IQR: 8-27) and 19 years (IQR: 7-35.5), respectively. The medians were statistically significant (p < 0.001). The majority of patients with YF IgM presumptive positive results were females (61.5%). The YF suspects with presumptive positive results against YF IgM antibodies were reported from the following provinces; Copperbelt (23.1%), Muchinga (7.7%), North-Western (23.1%) and Western (46.2%). The age groups with the majority cases of presumptive YF IgM were 6-14 years (30.8%) and 0-5 years (23.1%).

Predictors of YF IgM cases

Table 5 below shows the univariate and multivariate analysis of variables associated with YF IgM cases. Patients from the Western province were more likely to have presumptive YF IgM cases as compared to those from Copperbelt province (aOR 1.11; 95%CI 0.14, 0.94, p=0.04). All other covariates were comparable. Hence, the only variable associated with YF IgM cases was location (province).

Serological Epidemiology DENV Immunoglobulin M

Among the 90 samples tested, all the patients tested negative for DENV IgM antibodies.

Molecular Epidemiology of Flaviviruses and hepatitis viruses

All the serum samples tested negative for flaviviruses including YFV, dengue virus, and West Nile virus by PCR assays. Eight (8.6%) of the patients had evidence of acute Hepatitis A, consisting of 3 (37.5%) males and the median age was 9.5 years (IQR: 3.5-27 years). The median age for those YF suspects who were not infected with acute Hepatitis A was 19 years (IQR: 7-36 years). The medians were statistically significant different ($p < 0.001$). These cases were detected from the following provinces; Lusaka had 1 case (12.5%), Copperbelt had 2 cases (25%), Muchinga had 1 case (12.5%), North-Western had 2 cases (25%) and Western had 2 cases (25%). We also genetically confirmed Hepatitis B virus in 2/20 pooled sera of YF suspects' samples. In addition, none of the patients tested positive for Hepatitis C virus and Hepatitis E virus by PCR assays. The aetiologies of 85/93 (91.4%) of cases presenting with acute febrile jaundice remained unknown.

Discussion

Although the current evidence from other sub-Saharan countries close to Zambia strongly suggests that flaviviruses would be among the main causes of acute febrile jaundice, these viruses have not been comprehensively investigated in Zambia [6] [24] [25].

The main finding from our data is that the flaviviruses may not contribute to hospital morbidity in the study areas. However, this does not prove that flaviviruses are absent due to the limited sample size and given that a significant amount of samples were collected after 5 days of the onset of fever and jaundice. Moreover, the previous studies suggest inconclusive low circulation of flaviviruses in North-Western and Western provinces of Zambia and the presence of suitable vectors [12] [13] [14]. Instead of the flaviviruses, some cases of viral hepatitis were found.

The case definition of YF suspect used to screen for potential flaviviruses was intentionally broad. The clinical complications of flaviviruses are frequently observed in other febrile illnesses in Africa such as bacterial and viral diseases. For example, fever with jaundice is a common presentation of viral hepatitis. In the process, the broad case definition facilitated the sampling of patients with other acute febrile undifferentiated illnesses in this study. The WHO clinical case definition of YF suspect seems to be more sensitive but not sufficiently specific since symptoms of flaviviruses may mimic other febrile illnesses [26] [27].

Although, 14% of the YF suspects tested had results suggesting a possibility of recent infection, the PCR testing failed to identify flaviviruses in all samples. Hence, results reported here represent both cases that were seropositive and negative but tested negative for PCRs. However, a large proportion of samples were collected after 5 days of onset of symptoms. This could have affected the results in that the viremia in YF is usually brief. Of note also is that the sensitivity and specificity of the in-house ELISA used in this study was not 100% (Institute Pasteur de Dakar, Senegal). IgM ELISA reported in several studies suggests that few of the cases are misclassified [28] [29]. Furthermore, other study have shown that using IgM ELISA alone may give false positive results [30]. Thus, it is possible that some if not all results were false positive cases. Moreover, no antibodies to DENV another *flavivirus* were detected and WNV Ig M and ZKV IgM tests were not done in this study due to non-availability of reagents. The serology results may also probably be due to a cross-reaction from a similar yet previously unidentified non-flaviviruses including *Alphavirus* genus [31] [32] [33]. Therefore, the possibility of cross-reactivity with other pathogens cannot be excluded. However, this cross-reactivity was a limitation in this study since the absence of evaluated flaviviruses were largely tested by the molecular methods [34].

The data from our study may not absolutely confirm the absence of flaviviruses as the cause of acute febrile jaundice in selected provinces of Zambia. However, the finding is consistent with other previous study done in Ghana where none of the patients with viral haemorrhagic fever (VHF) symptoms tested positive [35]. These findings may also support the current data that none of the presumptive YF IgM positive cases detected at UTHVL has been confirmed as a case of YF.

Our findings have shown some cases of hepatitis A in selected provinces of Zambia. This is slightly lower than the hepatitis A prevalence obtained in Ghana [36]. The dissimilarity could be due to differences in the sample size and study design. This study had a similar finding with previous study conducted in Tunisia where Hepatitis A was more common in children below 15 years [37]. However, our study does not agree with other findings in the previous study done in Egypt where the average age of symptomatic hepatitis A had shifted to adult age due to better sanitation [38],[29]. The proportion of female febrile jaundiced patients who were positive for HAV (62.5 %) was not similar to that of the male febrile jaundiced patients (37.5%). Additionally, age category and provinces were also not statistically associated with HAV infection in this study. Despite our finding confirming HAV as a frequent cause of acute febrile jaundice, further investigation is needed to determine the risk factors (social economic indicators) and expand our understanding of the epidemiology in Zambia. In places where HAV is not documented as a health problem, it may be neglected as a cause of acute hepatitis delaying implementation of preventive measures and leading to increased morbidity.

We also found cases of Hepatitis B infection although we were unable to determine whether these were acute or chronic infection due to lack of IgM anti-HBc test kits during our study. Hence, the exact incidence of acute hepatitis B was very difficult to assess. Furthermore, hepatitis B can also persist in a chronic

asymptomatic infection; therefore, the jaundice observed in our cases may possibly be due to other unknown causes such as drug toxicity, severe malaria and leptospirosis.

In Africa where there is a risk of flaviviruses, the areas are typically endemic with other pathogens that cause acute undifferentiated febrile illness in patients and their specific aetiologies are often unknown [35] [39]. Therefore, it is not surprisingly to note that large proportion of febrile jaundice cases still remain largely unknown with aetiological agents and need further investigations.

This study was conducted in five provinces of Zambia over one and half years we cannot be confident that these findings can be generalized to the whole country. The small sample size was another study limitation. Furthermore, we were not able to exclude the possibility of drug toxicity or bacterial infections as cause of acute febrile jaundice. It is also important to note that the viruses with low viral load close to technical limit of detection may go undiagnosed due to the dilution effect of pooled sera. However, we mixed 3-5 serum samples to prepare 20 pooled samples. Therefore, the influence of pooling on the PCR sensitivity was limited. In addition, majority of the samples were collected after 5 days of onset of fever and jaundice.

Further research to understand other causes of febrile jaundice in Zambia should be promoted in the context of on-going surveillance. There is a need to perform both serological and molecular surveillance systems in patients presenting with YF like illnesses which may provide timely and comprehensive information regarding virus introduction and circulation.

Conclusion

Yellow fever IgM was prevalent among the study participants. However, the causes of fever and jaundice in Zambia may include viral hepatitis such as Hepatitis A and Hepatitis B. Moreover, hepatitis A is not laboratory diagnosed in Zambia which stresses the importance of promoting awareness among health workers and improving diagnostic tests to reliably assess the disease burden in Zambia. Furthermore, investigation should be made to screen for viral hepatitis when flaviviral diseases are suspected. In addition, a large proportion of acute febrile cases remain undiagnosed with unknown aetiologies.

Abbreviations

DENV
dengue virus
DF
dengue fever
ELISA
Enzyme linked immunosorbent assay
HAV
Hepatitis A virus
HBV
Hepatitis B virus
HCV
Hepatitis C virus
HEV
Hepatitis E virus
IgM
Immunoglobulin M
MOH
Ministry of Health
RT-PCR
Reverse transcriptase polymerase chain reaction
nPCR
nested polymerase chain reaction
OD
Optical density

Declarations

Ethical considerations

The study was approved by the Institutional Review Board of Excellence in Research Ethnics and Science Converge (ERES Converge IRB) Ref. No. 2015-Apr-003 and National Health Research Authority Ref MH/101/23/10/1. Permission to conduct a research and use of the archived samples was obtained from management of University Teaching Hospital and Ministry of Health. Study numbers were used to identify the archived samples.

Competing interest

The author declares that they have no competing interest.

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Authors' contributions

BK, conceived and designed the study. BK, MK, AM, ML and CM collected the data. AM, AT, MK, AM and KC were responsible for coordination and implementation of study. BK, WC, JAM, SKM, BMH and KK conducted data analysis. All authors read and approved the final manuscript.

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Tables

Table 1: Primers and cycling conditions of PCR assays

		Anealing Temp (°C)	Extension time (sec)	Forward primer (5'-3')	Reverese primer (5'-3')	Aplicon length (bp)	Refer
HAV	OneStep RT-PCR Kit (Qiagen)	58	60	+300: GCTGTAGGAGTCTAAATTGGGGAC	-516: ACTCAATGCATCCACTGGATGAG	257	NIID Manu
HBV	Ex Taq Hot Start Verison (TaKaRa)	53	90	TSB1: TCACCATATTCTTGGGAACA	TSB2: TTCCTGAACTGGAGCCACCA	480	NIID Manu
HCV	OneStep RT-PCR Kit (Qiagen)	55	30	#32S: CTGTGAGGAACTACTGTCTT	#36AS: AACACTACTCGGCTAGCAGT	221	NIID Manu
HEV	Ex Taq Hot Start Verison (TaKaRa)	55	40	HEV-F1: TAYCGHAAYCAAGGHTGGCG	HEV-R2: TGYTGGTTRTCRTARTCCTG	564	Li 20 EID
YFV	OneStep RT-PCR Kit (Qiagen)	57	30	YFV-29F: AATCGAGTTGCTAGGCAATAAACAC	YFV-141R: TCCCTGAGCTTTACGACCAGA	112	Drose et al. 2002 Clin Micro
WNV	OneStep RT-PCR Kit (Qiagen)	53	60	Fla-U5004:ggAACDTCMggHTCNCCHAT	Fla-5457:gTgAARTgDgCYTCRTCCAT	467	NIID Manu
p-flavi	OneStep RT-PCR Kit (Qiagen)	51	30	Flavi all S PanFV: TACAACATgATggggAARAgAgARAA PanFV: TACAACATgATgggRAAACgTgAGA	Flavi AS 2 PanFV: gTgTCCCAgCCNgCKgTgTCATCWgC	267	NIID Manu
DENV	M-MLV Reverse Transcription (Invitrogen); Ex Taq Hot Start Verison (TaKaRa)	55	120	D1: TCAATATGCTGAAACGCGCGAGAAACCG	D2: TTGCACCAACAGTCAATGTCTTCAGGTTC	511	Lanc et al. 1992 Clin Micro
DENV	OneStep RT-PCR Kit (Qiagen)	51	30	Flavi all S: TACAACATgATGGGGAARAGAGARAA DEN4 F: TACAACATGATGGGRAAACGTGAGAA	Flavi all AS 2: GTGTCCCAGCCNGCKGTGTCATCWGC	511	Patel al. 20 Virol

* We followed the manual provided by National Institute of Infectious Diseases, Japan

Table 2: Demographic characteristic of yellow fever suspects

Characteristic	no. of Patients	Percentage (%)
Total Tests	93	100
Sex		
Male	48	51.6
Female	45	48.4
Travel History		
Yes	1	1.1
No	92	98.9
Vaccination Status		
Yes	0	0
No	93	100
Age		
0-5yrs	19	20.4
6-15yrs	22	23.7
16-25yrs	19	20.4
26-35yrs	11	11.8
36-45yrs	12	12.9
>45yrs	10	10.8
Residence (provinces)		
Copperbelt	4	4.3
Lusaka	5	5.4
Muchinga	11	11.8
North-Western	29	31.2
Western	44	47.3

no: number

Table 3. Sample collection time from the onset of fever and jaundice

Characteristics	Frequency	Percent (%)	p-value[o]
Sample collected within 5 days			
Yes	38	41	0.0004
No	55	59	
Sample collected within 10 days			
Yes	61	66	<0.0001
No	32	34	

[o] One-sample test of proportion

Table 4: Comparison of YF IgM cases with independent variables

	number (%)		
Characteristics	**YF IgM+**	**YF IgM-**	***p*-value**
Number	13 (14)	80 (86)	
Median age (IQR)	14 (8, 27)	19 (7, 35.5)	<0.001[w]
Sex			
Male	5 (38.5)	43 (53.8)	0.306[c]
Female	8 (61.5)	37 (46.2)	
Total	13 (100)	80 (100)	
Age category (in years)			
0-5	3 (23.1)	16 (20.0)	0.947[c]
6-15	4 (30.8)	18 (22.5)	
16-25	2 (15.4)	17 (21.2)	
26-35	2 (15.4)	9 (11.2)	
36-45	1 (7.7)	11 (13.8)	
>45	1 (7.7)	9 (11.2)	
Total	13 (100)	80 (100)	
Provinces			
Lusaka	0 (0)	4 (5.0)	0.04[c]
Copperbelt	3 (23.1)	2 (2.5)	
Muchinga	1 (7.7)	10 (12.5)	
North western	3 (23.1)	26 (32.5)	
Western	6 (46.2)	38 (47.5)	
Total	13 (100)	80(100)	

YF IgM: Yellow fever Immunoglobulin M, **+**: positive, **-**: Negative, [c:] chi-square, [w:] Wilcoxon-rank-sum test

Table 5: Predictors of YF IgM cases in the study population using univariate and multivariate logistic regression

Variable	Odds Ratio[u] (95% CI)	*p*-value	adjusted Odds[m] Ratio OR(95%CI)	p- value
Sex				
Male	1.00	0.344	1.00	0.177
Female	1.67 (0.58, 4.86)		0.44(0.13, 1.45)	
Age category (in years)				
0-5	1.00		1.00	
6-15	1.75 (0.42, 7.25)	0.440	1.63 (0.36, 7.39)	0.523
16-25	0.20 (0.20, 2.06)	0.181	0.12 (0.01, 1.50)	0.100
26-35	0.83 (0.12, 5.50)	0.850	0.76 (0.16, 5.64)	0.784
36-45	0.34 (0.04, 3.49)	0.364	0.38 (0.04, 4.00)	0.424
>45	0.94 (0.14, 6.28)	0.947	1.07 (0.15, 7.91)	0.944
Provinces				
Copper belt	1.00		1.00	
Muchinga	0.20 (0.02, 1.81)	0.153	0.16 (0.01, 1.85)	0.141
North western	0.25 (0.04, 1.56)	0.138	0.21 (0.02, 1.88)	0.163
Western	0.15 (1.03, 0.94)	0.043	1.11 (0.14, 0.94)	0.043

CI: Confidence Interval, **OR**: Odds Ratio, [u]: Univariate, [m]: Multivariate

Figures

Figure 1

Map of Zambia with locations of the study sites (Source: Google maps, 2015)