Survey of bovine tuberculosis in Nigerian beef cattle

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Abstract

Bovine tuberculosis (bTB) is a zoonotic disease caused by *Mycobacterium tuberculosis* complex (MTBC) that primarily affects cattle, but also other domestic and wild mammals. In Nigeria, abattoir monitoring of gross bTB lesions is the only control method being applied in all animals. This study aims to investigate tubercle bacilli infection in slaughtered cattle found with visible tuberculosis-like lesions. Lesions suggesting bTB were detected in 226 cattle during abattoir monitoring in Zamfara State, Nigeria. Tissue samples collected from the affected carcasses were subjected to Ziehl-Neelsen stain (ZN). Of the 226 carcasses with lesions, 37 (16.4%) were positive by the Ziehl-Neelsen stain (ZN), and MTBC was detected from 34 (91.9%) of the 37 ZN-positive samples. Molecular typing by region of difference (RD) deletion analysis revealed the genotype of *Mycobacterium bovis*, *Mycobacterium caprae* and *Mycobacterium tuberculosis*. Infection was most significantly associated with age of the animals (OR = 3.49; CI: 1.29-9.47 [$p = 0.002$]). The findings indicate a serious threat for health as well as for TB control in Nigeria.
Keywords: Bovine tuberculosis, Cattle, Nigeria.

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Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium tuberculosis* complex (MTBC), is a zoonotic disease that primarily affects cattle, but also other domestic and wild mammals. *Mycobacterium bovis* is the bacilli within the MTBC mostly detected in tuberculous lesions from cattle (Krajewska *et al*., 2017; Waters *et al*., 2017; Yahyaoui-Azmi *et al*., 2017; Ku *et al*., 2018). Once infection is established in a herd, *M. bovis* can be transmitted between animals via aerosols, suckling, direct contact, and by sharing of contaminated water and feeds (El-Sayed *et al*., 2016; Yahyaoui-Azmi *et al*., 2017). Economic losses caused by bTB are mainly as a result of reduced productivity of affected animals and the condemnation (partial or total) of the carcasses at slaughter (Olea-Popelka *et al*., 2017).

BTB is endemic across sub-Saharan Africa due to the lack of proper financial resources that renders the control programs absent or ineffective. The disease significantly affects the livelihoods of rearing communities for its negative impacts on animal health, welfare and animal trade at international level. Only 7 out of 55 African countries followed adequate control programs, the remaining 48 countries including Nigeria control bTB inadequately or not at all (El-Sayed *et al*., 2016). Officially, postmortem examination of carcasses without systematic antemortem test-and-slaughter is the only current bTB control strategy being applied in Nigeria.

Previous studies (Ameen *et al*., 2008; Cadmus *et al*., 2009; Raufu and Ameh, 2010; Damina *et al*., 2011; Ahmad *et al*., 2017) undertaken at Nigerian abattoirs have reported bTB in cattle based on macroscopic lesions at an estimated range of 0.54% – 6.1%, indicating an increased trend in the prevalence. However, laboratory analyses to confirm infection in animals found with gross lesions are rarely performed unless for research purposes.

Diagnosis of bTB by identification of *M. bovis* has been enhanced with the aid of molecular techniques such as PCR and its variations (Carvalho *et al*., 2015). Deletion typing, as one of the PCR variants, has been found to be an important molecular tool in differentiating *M. bovis* from the other members of the MTBC, determined by discriminating the pattern (i.e. either presence or absence) of the genomic region of difference (Brosch *et al*., 2002; Mostowy *et al*., 2002; Warren *et al*., 2006; van Ingen *et al*., 2009). Hence, identification of tubercle bacilli responsible for the gross pathology is required for epidemiological investigations, control and eradication of bTB. This study therefore aimed at detecting tubercle bacilli in slaughtered cattle identified with gross TB lesions in an abattoir in Nigeria.

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Materials and Methods

Study area and sample collection

The study was conducted in Gusau abattoir, Zamfara State, northwest of Nigeria. It is the only authorized slaughterhouse in the State that is manned by trained personnel (veterinarians).

Two hundred and twenty six cattle carcasses, examined according to the guidelines of Corner (1994), were identified with bovine TB-compatible lesions from April to June 2014. Lesion samples were collected in sterile containers for laboratory investigations, conveyed to Bacterial Zoonoses Laboratory at Ahmadu Bello University Zaria, Nigeria and stored at -20ºC until processing. Acid-fast bacilli (AFB) were identified using Ziehl-Neelsen stain (ZN), and RD deletion analysis was performed on all AFB positive samples. Individual animal data such as age was determined by dentition; breed and sex were also recorded.

Tissue processing and Ziehl-Neelsen stain

Prior to processing, frozen samples were thawed overnight, removed from containers, and put into sterile Petri dishes. Excess fatty tissues were trimmed off using sterile scalpel blade and forceps, before samples were macerated into tiny pieces. Approximately 5g of sliced tissues were crushed using sterile Mortar and pestle before being homogenized in 10 ml of sterile normal saline solution for 10 min. Thereafter, homogenates were decontaminated with 2 ml of 4% NaOH for 15 min, neutralized with 1% (0.1 N) hydrochloric acid (HCl) using phenol red as an indicator, and concentrated by centrifugation at 3000 x g for 15 min. The supernatant was discarded, and an aliquot of the pellet from each whole-processed sample was used to prepare a smear for ZN. Loops of the pellet were gently smeared on a clean, grease-free microscopic slide, air-dried and heat-fixed on a hot plate at 70^0C for 30 min. ZN was performed to screen AFB positive samples as described by Lumb et al. (2013) with the following modifications: concentration of the acid-alcohol used was raised to 5% instead of 3% and decolourization time was extended from 5 to 10 min. The stained slides were then viewed under x 100 objective of a light microscope to determine the presence and morphology of acid-fast bacilli.

Determination of MTBC using RD deletion typing

The homogenates of the samples that showed acid-fast bacilli were subjected to multiplex RD polymerase chain reaction (PCR). Thus genomic DNA was extracted from the homogenates ($n = 37$) according to the described protocols of Miller et al. (1997). Molecular identification of MTBC was performed in DNA-LABS at Kaduna Nigeria as previously described (Warren et al., 2006).

Briefly, PCR amplification was performed at a final reaction volume of 20 µl consisting of 1 µl DNA template re-suspended in HotStat PCR Premix (1 unit Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 250 µM of each dNTPs, 1xPCR buffer and 1.5 mM $MgCl_2$), 25 pM of 0.5 µl each of the four 3-sequence primers for the RD present or absent in the genome of members of the MTBC (Table 1) and 13 µl sterile nuclease free water. Positive control with DNA template of reference-typed strain (M. bovis BCG Pasteur) and negative control mixture (Master mix without DNA template) were included in the amplification run. The reaction was carried out in PTC-100^{TM} Programmable Thermal Controller (MJ Research, Inc. USA) at the following conditions: initial denaturation at 95^0C for 15 min followed by 45 cycles of denaturation at 94^0C for 1 min, annealing at 62^0C for 1 min and extension at 72^0C for 1 min, with a final extension step at 72^0C for 10 min. Amplification products (6 µl) were separated in 3% agarose gels (Green Bio Research, USA) stained with 5 µl ethidium bromide (Invitrogen, USA) at 120 V for 4 hours using Horizontal Gel Electrophoresis Apparatus (GIBCOBRL Model H5-Gaithersburg, USA). Gels were visualized, photographed and analyzed by comparing band sizes between M: DNA-Marker (100 bp Plus DNA ladder) (Bioneer, Inc. USA) and Lane 3-onwards (samples amplified products). As previously described (Table 1), assessment of the presence or absence of RD by deletion analysis was used to differentiate species of the MTBC (Brosch et al., 2002; Warren et al., 2006; Mwakapuja et al., 2013).

After laboratory analyses, animals presumed tuberculous due to bTB-compatible lesions were classified as TB positive or negative based on positive/negative findings from ZN and deletion typing. Infection was confirmed in animals that were positive for both ZN and deletion typing, while negative in those initially identified only as acid-fast positive but showed no proper amplicons in deletion typing analysis.

Statistical analysis

Data obtained from field and laboratory was coded and analyzed using MS Excel 2016 and IBM SPSS Statistics software for Mac (Version 24) and expressed as frequencies and percentages, unless otherwise stated. Distribution of infection in different groups was compared by Chi-square or Fisher Exact Test. Robust univariate and multivariate logistic regression was used to evaluate the associations between infection, age, breed and sex of the animals in this study to demystify risk factor(s) for bovine TB. During multivariate regression analysis, adjustments were made for likely confounding factors (age and sex). Odds ratios (OR) with 95% confidence intervals (CI) were calculated to estimate the association between groups of interest to identify risk factors for infection. A p value < 0.05 was considered to have statistical significance.

Table 1. Target region of difference (RD), primer sequences and corresponding amplicons for the identification of species from the *M. tuberculosis* complex (MTBC).

Target RD	Primers sequence	*M. bovis*	*M. tuberculosis*	*M. caprae*	Reference
1	AAGCGGTTGCCGCCGACCGACC	RD1 present (146 bp)	RD1 present (146 bp)	RD1 present (146 bp)	
1	CTGGCTATATTCCTGGGCCCGG				
1	GAGGCGATCTGGCGGTTTGGGG				
4	ATGTGCGAGCTGAGCGATG	RD4 absent (268 bp)	RD4 present (172 bp)	RD4 present (172 bp)	
4	TGTACTATGCTGACCCATGCG				
4	AAAGGAGCACCATCGTCCAC				Warren *et al.* (2006)
9	CAAGTTGCCGTTTCGAGCC	RD9 absent (108 bp)	RD9 present (235 bp)	RD9 absent (108 bp)	
9	CAATGTTTGTTGCGCTGC				
9	GCTACCCTCGACCAAGTGTT				
12	GGGAGCCCAGCATTTACCTC	RD12 absent (306 bp)	RD12 present (369 bp)	RD12 absent (306 bp)	
12	GTGTTGCGGGAATTACTCGC				
12	AGCAGGAGCGGTTGGATATTC				

bp: base pairs.

Results

The age, breed and sex distribution of infected animals is shown on Table 2.

Acid-fast bacilli were detected from thirty seven of all the cattle found with lesions (Fig. 1), while MTBC were identified from thirty four of thirty seven acid-fast positive cattle (Fig. 2a, b, c).

Thus the ZN and RD deletion typing positive rates were 16.4% (37/226) and 91.9% (34/37) respectively. More so, 8% (3/37) of the acid-fast samples turned out to be negative for RD deletion typing.

All the samples confirmed as MTBC infection were positive to RD1 deletion typing, while 88.2% (*n* = 30) were identified to be *M. bovis* as they were negative to RD4, RD9 and RD12 PCR.

In addition, three acid-fast samples (8.8%) were identified to be *M. caprae* as they were positive to RD4 but negative to RD9 and RD12 PCR. While one (2.9%) of the acid-fast samples was identified to be *M. tuberculosis* as it was positive to the RD4, RD9 and RD12 PCR.

The difference in the amplicons sizes indicates presence or absence of regions of difference in the genomic DNA amplified, which differentiates species of the *Mycobacterium tuberculosis* complex (Table 1). The odd of being infected is about four times higher in adult than juvenile animals (OR = 3.94; CI: 1.47-10.57).

Though not statistically significant (*p* = 0.19), Red Bororo and Sokoto Gudali breeds have 2.30 and 1.12 times higher potential of being infected than White Fulani breed. However, male animals have lesser chance of infection than females (Table 3).

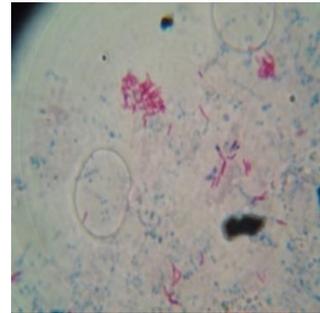A similar trend was observed when confounding parameters were adjusted (Table 4).

Fig. 1. Acid-fast bacilli appeared pinkish in a bluish background, detected in tuberculous lung from a cow (x100).
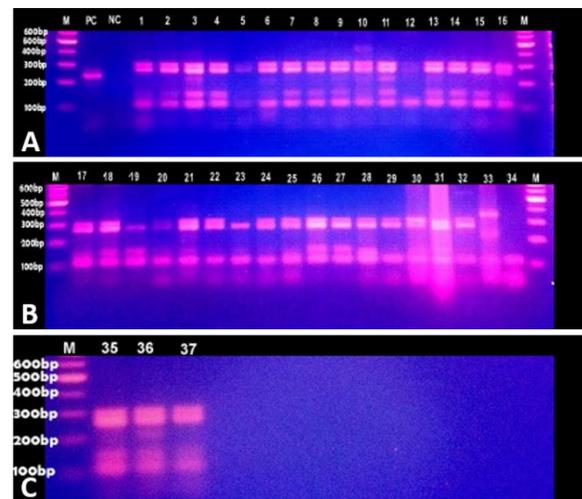

Fig. 2. (A, B, and C): Electrophoretic fractionation of amplicons in 3% agarose. M = 100 bp DNA ladder. PC = Positive control. NC = Negative control. Lane 1-4, 6-11, 13-18, 21, 22, 24 - 32 and 35-37 = *Mycobacterium bovis*. Lane 19, 20 and 23 = *Mycobacterium caprae*. Lane 33 = *Mybacterium tuberculosis*. Lane 5, 12 and 34 = samples negative for RD PCR.

Table 2. Summary table of acid-fast (AF) bacilli and *Mycobacterium tuberculosis* complex (MTBC) detected by ZN and Region of difference deletion typing from slaughtered cattle (with tuberculous lesions) in Nigeria.

Variables		n	AF Positive			AF Positive with MTBC			*Mycobacterium* spp.		
			No.	%	95% CI	No.	%	95% CI	*M. b.*	*M. c.*	*M. t.*
Age	Adult	149	32	14.16	10.21-19.31	30	78.38	62.80-88.61	27	3	
	Juvenile	77	5	2.21	0.95-5.07	4	13.51	5.91- 27.98	3		1
Breed	White Fulani	129	25	11.06	7.61- 15.82	22	59.46	43.49-73.65	18	3	1
	Sokoto Gudali	78	8	3.54	1.80- 6.83	8	21.62	11.39-37.20	8		
	Red Bororo	19	4	1.77	0.69- 4.46	4	10.81	4.29- 24.71	4		
Sex	Female	147	29	12.83	9.08- 17.82	27	72.97	57.02-84.60	23	3	1
	Male	79	8	3.54	1.80- 6.83	7	18.92	9.48- 34.20	7		

(n): number of tuberculous cattle investigated; (CI): Exact binomial confidence intervals; (*M. b.*): *Mycobacterium bovis*; (*M. c.*): *Mycobacterium caprae*; (*M. t.*): *Mycobacterium tuberculosis*.

Table 3. Univariate analysis of factors associated with the presence of acid-fast (AF) bacilli in tuberculous cattle slaughtered at an abattoir in Nigeria.

Variables		Tuberculous cattle			OR	95% CI	*p* -value
		AF positive	AF negative	Total			
Age	Juvenile	5	72	77	1		0.004
	Adult	32	117	149	3.94*	1.47 - 10.57	
Breed	White Fulani	25	104	129	1		0.190
	Sokoto Gudali	8	70	78	1.12	0.34 - 3.67	
	Red Bororo	4	15	19	2.30	0.612 - 8.64	
Sex	Female	29	118	147	1		0.089
	Male	8	71	79	0.46	0.20 - 1.06	

(OR): odds ratio; (CI): confidence interval; (*): Significant at *p* < 0.05.

Table 4. Multivariate logistic regression model for the presence of acid-fast (AF) bacilli in tuberculous cattle slaughtered at an abattoir in Nigeria.

Variables		AF Positive	Adjusted odds ratio	95% CI	Likelihood ratio test
Age	Adult	32	3.49*	1.29- 9.47	0.002
	Juvenile	5	1		
Breed	White Fulani	25	1		0.19
	Sokoto Gudali	8	1.11	0.33- 3.76	
	Red Bororo	4	1.75	0.45- 6.80	
Sex	Female	29	1		0.06
	Male	8	0.6	0.25- 1.46	

(CI): confidence interval; (*): Significant at *p* < 0.05.

There is no difference in odd ratio in the rate of infection across ages and breed among acid-fast (AF) positive cattle with MTBC infection, but a non-statistical significance (*p* = 0.53) with reduced odd of infection was observed for male cattle (OR = 0.52; CI: 0.04-6.58) (Table 5).

This non-significant odd was reduced (Adjusted OR = 0.11; CI: 0.005-2.55) after controlling for age and breed of the animals (Table 6). There were significantly increased odds of infection among female, adult, Sokoto Gudali and Red Bororo cattle. Although age of the animals retained a statistically significant association (*p* = 0.004; 0.002), breed and sex are not significant factors in bovine TB infection (*p* = 0.19; 0.06).

Therefore, this shows by implication that age of animals is the main predictor associated with much higher levels of tubercle bacilli infection.

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Table 5. Univariate analysis of factors associated with the presence of *Mycobacterium tuberculosis* complex (MTBC) infection in tuberculous cattle slaughtered at an abattoir in Nigeria.

Variables		Acid-fast positive cattle		OR	95% CI	*p* –value
		MTBC infection	No MTBC infection			
Age	Adult	29	3	1		0.63
	Juvenile	5		0	0	
Breed	White Fulani	22	3	1		0
	Sokoto Gudali	8		2203	0	
	Red Bororo	4		1	0	
Sex	Female	27	2	1		0.53
	Male	7	1	0.52	0.04- 6.58	

(OR): odds ratio; (CI): confidence interval.

Table 6. Multivariate logistic regression model for *Mycobacterium tuberculosis* complex (MTBC) infection in tuberculous cattle slaughtered at an abattoir in Nigeria.

Variables		Acid-fast positive cattle with MTBC infection	Adjusted odds ratio	95% CI	Likelihood ratio test
Age	Adult	29	1		0.34
	Juvenile	5	0	0	
Breed	White Fulani	22	1		0.29
	Sokoto Gudali	8	4679	0	
	Red Bororo	4	0.83	0	
Sex	Female	27	1		0.62
	Male	7	0.11	0.005- 2.55	

(CI): confidence interval.

Discussion

Postmortem examination of carcasses in abattoirs detects only the more advanced forms of tuberculosis. Gross visible lesions in bTB developed as immune response tries to wall off the infection whereas early stages of the disease can easily be missed out, but this is avoidable if ante-mortem tests are also employed. Quite possibly, errors do occur in macroscopic lesions analyses of bTB-like pathology in abattoirs, in which case, diseased animals are slaughtered early and with recent infection (Carvalho *et al*., 2015).

However, tubercle bacilli were isolated from cattle with no visible lesions suggesting that *M. bovis* can be detected from cattle with or without lesions (Ku *et al*., 2018).

The proportion of samples positive by acid-fast staining (16.4%) was low as compared to the about 30% reported from a different part of Nigeria (Chukwu *et al*., 2013), and also lower than the 58% reported elsewhere in Africa (Koro *et al*., 2013). This may be related to the adjusted concentration of acid-alcohol solution (5% instead of the regular 3%) and extended decolorization time of 10 min employed during the staining procedure in the present study. This might have helped to rule out other weak acid-fast non-tuberculous mycobacteria.

This study has shown that 92% of the acid-fast organisms to be *M. tuberculosis* complex, a figure higher than the 79.6% reported using deletion typing in Nigeria (Damina *et al*., 2011) and 68% reported from Morocco (Yahyaoui-Azmi *et al*., 2017), but close to the 92.6% reported from Ethiopia (Ameni *et al*., 2010). Most of the lesions found in this study are caused by *M. bovis*, similar to the findings reported from abattoirs in Morocco by Yahyaoui-Azmi *et al*. (2017).

Similarly, a study in Morocco found *M. bovis* and *M. caprae* in lesions from cattle (Yahyaoui-Azmi *et al*., 2017), whereas previous studies in Nigeria, Cameroon, Ethiopia, Zambia, and India found *M. tuberculosis* in tuberculous lesions from cattle (Damina *et al*., 2011; Aylate *et al*., 2013; Malama *et al*., 2014; Alemu *et al*., 2016; Egbe *et al*., 2016; Anne *et al*., 2017). Furthermore, infection with human tubercle bacilli among domesticated animals occurs mainly in urban areas, where human and animals coexist in the same microenvironment (Krajewska *et al*., 2017).

Krajewska *et al*. (2012) reported a case of an active TB in a Polish woman that eventually transmitted *Mycobacterium tuberculosis* to a calf in her farm. *M. tuberculosis* could be transmitted to cattle through various routes including inhalation and ingestion of

feeds contaminated by sputum from infected farmers (Ameni *et al*., 2013; Alemu *et al*., 2016).

Our study revealed the risk of having bTB to be significantly associated with the age of the animal. This finding agrees with the published observations made elsewhere in Africa (Dejene *et al*., 2016; Egbe *et al*., 2016).

From our findings, female animals appear to have a higher risk of infection than males, similar to the findings of Worku *et al*. (2016) in Ethiopia. This might be due to the stress induced by calving that makes cows immunologically weak, and more vulnerable to infection than bulls.

The present study has also shown that Red Bororo and Sokoto Gudali breeds appear to have a higher risk (not significant) of contracting bTB as compared to White Fulani breed.

Cattle breed has been considered among other factors to be associated with the persistence of bovine TB infection (Alvarez *et al*., 2014).

Thus it requires a breed substitution of increased host resistance, among other viable options, to limit the spread of infection between herds and regions (Richardson *et al*., 2016).

The evidence of *M. tuberculosis* infection in cattle highlights a serious public health concern, suggesting a bi-directional transmission between animals and man. In certain non-industrialized nations, bTB is recognized as a public health problem due to persistent direct contact of humans with animal reservoir hosts and consumption of poorly processed animal products. In Nigeria, animals are not housed in segregated holdings but share both space and resources with man. This narrows the gap between species aggravating intra- and inter-species interactions, thus enhancing TB transmission through close repeated contacts and food chain.

Conclusion

This study has documented a high occurrence of *M. bovis* in beef cattle, with older animals having increased risk of bTB infection in Nigeria.

There is therefore the need for sound control and eradication programs in the country, as well as improved standards of meat inspection. In addition to reducing transmission through food chains and direct contact, there is also a need to intensify efforts on public enlightenment campaigns regarding potential dangers of the disease.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

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