revealed that antiviral agents such as ganciclovir or foscarnet should be used to treat CMV disease (Boeckh 2011). Neonatal HSV infection, caused by exposure to HSV in the genital tract during delivery, often results in severe fever and has a survival rate of 50% only. Antiviral therapy with high-dose acyclovir reportedly led to a reduction in the mortality of infants with disseminated disease (Corey and Wald 2009). The number of genital HSV infections among adults has increased in the last 30 years (Corey 2002). Varicella zoster virus (VZV), which belongs to the subfamily Alphaherpesvirinae, is commonly treated by nucleosides such as acyclovir, famciclovir, and valacyclovir (Rajan and Rivers 2001). These antiviral drugs have been approved for the treatment of herpes zoster and have shown beneficial results in reducing the duration of established postherpetic neuralgia. Although acyclovir anti-viral activity against herpes virus HHV8 could not be detected in vitro, some anti-herpes virus 6 activity was observed (De Clercq et al. 2001). One case report also showed that ganciclovir was effective in the treatment of adenovirus-associated hemorrhagic cystitis (Chen et al. 1997).

As described above, acyclovir seems to be potentially beneficial in the treatment of IM and IM-like illness. Therefore, we conducted this retrospective study involving IM and IM-like illness patients whose treatment empirically included or excluded acyclovir. We especially investigated the cases where patients were given acyclovir for the treatment of suspected viral infection upon admission to our hospital, and we examined the effects of this drug on patient condition.

Methods

Patients

In this retrospective cohort study, we collected data involving patients who were hospitalized for fever (> 37.5°C) and lymphadenopathy, from 2008 to 2010, at the Tohoku University Hospital Infectious Disease Ward. Since "conventional" IM diagnostic criteria only apply to EBV-caused IM, we enrolled 25 patients in this study, including patients with IM-like illness caused by other viruses, with the following inclusion criteria: duration of fever was defined as > 37.5°C for at least 1 day; sputum examination, including tuberculosis smear and PCR, did not indicate any bacterial infection; blood bacterial cultures were also negative for aerobes, anaerobes, and fungi; no antibodies against toxoplasma, chlamydia pneumonia, and mycoplasma were detected; antinuclear antibodies, indicating collagen diseases, did not exceed normal levels; sarcoidosis was excluded; HIV antibody and HIV RT-RNA levels were below the detection limit; lymphoma was not detected in any of the patients who underwent bone-marrow puncture and 18F-2-deoxy-fluoro-D-glucose positron emission tomography.

SRL laboratories (Tokyo, Japan) were used to perform HHV 1-8 viral PCR, assess HIV RNA quantity, conduct the CMV antigenemia (pp65 antigenemia, a component of the shell surrounding the virus nucleoprotein core) assay, and measure anti-EBV VCA IgG, IgM, and IgA as well as anti-EBV EA IgG and IgA and Epstein-Barr virus nuclear antigen (EBNA) antibody levels. EBV antibodies were measured using the fluorescent antibody technique, and antibodies against other viruses were measured using enzyme immunoassay (EIA). HIV antibody titrations were assessed at a Tohoku University clinical laboratory using the EIA method.

Statistical analysis

We expressed analyzed data as mean ± standard deviation. Comparisons between pre-treatment and post-treatment of the acyclovir and control groups were calculated by subtracting the post-treatment value from the pre-treatment value. The Mann-Whitney's U test was used to calculate the P values of the 2 groups. The Kruskal-Wallis test was used to evaluate clinical improvement. A P value < 0.05 was considered statistically significant. Analysis of data was performed using EXCEL software.

Results

Eight of the 25 patients were given acyclovir with their consent, as part of conventional therapy. Patient characteristics are presented in Table 1. Mean age, physical examination results, and laboratory results were compared between control patients (CP) and acyclovir patients (AP). Whereas all 8 APs were men, 13 of the CPs were men and 4 were women ($P = 0.13$). In general, clinical presentations including palpable surface lymph nodes (lymphadenopathy), severe throat pain (pharyngeal pain), continuous headache (headache), major multiple polyarthralgia (arthralgia), rash covering more than 1% of the body surface area (rash), and multiple mucosal bleeding (mucosal bleeding) were not significantly different between CPs and APs ($P > 0.05$). The largest liver and spleen diameter measurements observed also were not significantly different between CPs and APs ($P = 0.90$ and $P = 0.37$, respectively). Only the neck flexion test results were significantly different between the 2 groups ($P = 0.01$), and the 3 APs with positive neck flexion test results were clinically suspected of viral meningitis based on collected spinal fluid. Based on blood samples collected upon admission, laboratory test results showed that hemoglobin concentration, white blood cell count (WBC), white blood cell differentiation, platelet cell count, as well as C-reactive protein (CRP), serum amyloid A (SAA) proteins, ferritin, soluble IL-2 receptor (sIL-2R), lactate dehydrogenase (LDH), D-dimer, glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) levels were not significantly different between the 2 groups ($P > 0.05$).

Antibody titer tests were performed, and viral DNA was detected using PCR in order to identify the causes of fever; results are shown in Table 2. Patients with acute EBV infection were defined as those with anti-EBV VCA IgM positive (> 10) and EBNA negative (< 10) results. Accordingly, 5 CPs and 1 AP met the clinical criteria identifying acute EBV infection as the cause of IM. Since CMV antigenemia test results were negative for all patients, IM-like illness was unlikely caused by CMV. PCR results were positive for EBV DNA (> 10 copies/500,000 cells) in peripheral blood mononuclear cell (PBMC) samples of 2 CPs and 3 APs. Since PCR results were negative for HSV,

Table 1. Patient characteristics.

	Control	Acyclovir	*P*
	Mean ± S.D.	Mean ± S.D.	
n	17	8	
Male	13	8	0.13
Female	4	0	
Age (years)	31 ± 3	42 ± 5.2	0.06
Lymphadenopathy	14	7	0.74
Pharyngeal pain	11	7	0.24
Headache	13	6	0.94
Neck flexion test	0	3	0.01
Arthralgia	4	5	0.06
Rash	8	7	0.054
Mucosal bleeding	10	7	0.15
Liver (cm)	18 ± 0.5	17 ± 0.70	0.90
Spleen (cm)	10 ± 0.7	10 ± 1.2	0.37
Hemoglobin (g/dL)	13.7 ± 0.39	14.2 ± 0.39	0.49
White blood cell count ($10^3/\mu L$)	3,747 ± 909	8,675 ± 1,741	0.45
Neutrophil ($10^3/\mu L$)	4,203 ± 695	6,648 ± 1,608	0.11
Lymphocyte ($10^3/\mu L$)	2,058 ± 413	1,145 ± 244	0.16
Platelet cells count ($10^3/\mu L$)	215 ± 14	219 ± 32	0.89
CRP (mg/dL)	5.2 ± 1.2	8.7 ± 2.9	0.20
SAA (μg/mL)	349 ± 80	1,027 ± 519	0.07
Feritin (ng/mL)	581 ± 145	2,088 ± 1,632	0.20
sIL-2R (U/mL)	1,349 ± 160	1,044 ± 248	0.30
LDH (IU/L)	422 ± 60	409 ± 91	0.90
D-dimer (μg/mL)	3.4 ± 0.8	2 ± 0.6	0.24
GOT (IU/L)	64 ± 9.2	116 ± 63	0.29
GPT (IU/L)	84 ± 16	112 ± 71	0.61

CRP, c-reactive protein; SAA, serum amyloid A; sIL-2R, soluble interleukin-2 receptor; LDH, lactate dehydrogenase; GOT, glutamicoxaloacetic transaminase; GPT, glutamic-pyruvic transaminase.

Table 2. Antibody titer and viral DNA PCR.

		Control			Acyclovir		
		+	−	Unknown	+	−	Unknown
Antibody	EBV VCA IgG	11	5	1	7	1	0
	EBV VCA IgM	5	11	1	1	7	0
	EBV VCA IgA	2	12	3	0	8	0
	EBV EA IgG	1	14	2	0	8	0
	EBV EA IgA	0	15	2	0	8	0
	EBNA	7	9	1	4	4	0
Virus PCR	EBV	2	4	11	3	3	2

PCR of viral DNA was conducted on peripheral blood mononuclear cells.
VCA, viral capsid antigen; EA, early antigen; EBNA, epstein barr virus nuclear antigen.

CMV, VZV, or HHV DNA (< 10 copies/500,000 cells) in all patients, the pathogen was only identified in the EBV patients.

Acyclovir (750 mg/day) was daily administered intravenously with normal saline, as part of the treatment. The duration of treatment was similar between CPs and APs. Adverse effects of acyclovir such as skin eruption, liver injury, and shock were not observed in any patients. Antibiotic therapy was administered to 4 CPs and 4 APs, and gamma globulin was administered to 2 APs. The anti-

Table 3. Comparison between pre-treatment and post-treatment laboratory examination results.

	Control (n = 17)	Acyclovir (n = 8)	P
	Mean ± S.D.	Mean ± S.D.	
Hemoglobin (g/dL)	0.45 ± 0.19	1.1 ± 0.38	0.17
White blood cell count ($10^3/\mu L$)	888 ± 848	2,625 ± 1,186	0.25
Neutrophil ($10^3/\mu L$)	1,158 ± 591	2,940 ± 1,006	0.09
Lymphocyte ($10^3/\mu L$)	−460 ± 187	−163 ± 276	0.94
Platelet cell count ($10^3/\mu L$)	−61 ± 13	−50 ± 17	0.33
CRP (mg/dL)	3.2 ± 0.99	3.2 ± 0.86	0.40
SAA (μg/mL)	98 ± 37	505 ± 204	0.02
Ferritin (ng/mL)	25 ± 161	21 ± 49	0.75
sIL-2R (U/mL)	341 ± 123	−51 ± 84	0.55
LDH (IU/L)	−33 ± 29	42 ± 21	0.09

Table 4. Duration of hospitalization and of fever.

	Control (n = 17)	Acyclovir (n = 8)	P
	Days ± S.D.	Days ± S.D.	
Hospitalizaion (days)	27 ± 7.7	16 ± 3.7	0.36
Fever (days)	18 ± 6.5	4.5 ± 1.8	0.04

Table 5. Clinical presentation following treatment with acyclovir.

		No change	Improved	Worsened	P
Lymphadenopathy	Control	1	3	13	0.01
	Acyclovir	0	6	2	
Pharyngeal pain	Control	2	8	7	0.16
	Acyclovir	1	6	1	
Headache	Control	1	4	12	<0.01
	Acyclovir	1	6	1	
Arthralgia	Control	5	5	7	<0.01
	Acyclovir	1	6	1	
Rash	Control	6	4	7	0.17
	Acyclovir	1	5	2	
Mucosal bleeding	Control	3	5	9	<0.01
	Acyclovir	1	6	1	

P value is calculated by Kruskal-Wallis test.

biotic and gamma globulin treatments did not affect the patients' laboratory results.

Blood was collected from CPs and APs over a period of 2-3 days following acyclovir treatment. When we subtracted the laboratory examination post-treatment values from the pre-treatment values, the decline in WBC count was less prominent in the CP group (888 ± 848 × $10^3/\mu L$) compared to the AP group (2,625 ± 1,186 × $10^3/\mu L$; P = 0.25), as shown in Table 3, suggesting a beneficial effect of acyclovir on the condition of APs. This difference was mainly due to the much more prominent decrease in neutrophil count in APs (2,940 ± 1,006 × $10^3/\mu L$) compared to CPs (1,158 ± 591 × $10^3/\mu L$; P = 0.09). Although the reduction in CRP and ferritin levels was similar in APs and CPs (CRP, P = 0.40; ferritin, P = 0.75), SAA levels were significantly lower in APs (505 ± 204 μg/mL) than in CPs (98 ± 37 μg/mL; P = 0.02). Acyclovir administration also caused an increase in sIL-2R levels in APs (−51 ± 84 U/mL) and a reduction in CPs (341 ± 123 U/mL), although the change was not statistically significant (P = 0.55).

The duration of hospitalization was more prominently reduced in APs (16 ± 3.7 days) than in CPs (27 ± 7.7 days; P = 0.36), as was the duration of fever in APs (4.5 ± 1.8 days) compared to that in CPs (18 ± 6.5 days; P = 0.04), as shown in Table 4. The mean hospitalization period among EBV-IM patients was 18 days for the 1 AP and 12 ± 15.3

days for the 5 CPs. The mean hospitalization period among IM-like illness patients was 16 ± 4.3 days for APs and 31 ± 9.8 days for CPs.

Clinical presentation following treatment with acyclovir is indicated in Table 5. Bilateral lymphadenopathy symptoms worsened in 13 of the 17 CPs, but dramatically improved in 6 APs ($P = 0.01$). Pharyngeal pain was alleviated in 8 CPs and 6 APs ($P = 0.16$), and headache had subsided in significantly more APs than CPs ($P < 0.01$). Moreover, improvement in arthralgia symptoms was observed in 5 CPs and 6 APs ($P < 0.01$), and mucosal bleeding improved more significantly among APs than CPs ($P < 0.01$). On the other hand, rash was similarly observed in CPs and APs ($P = 0.17$). In general, acyclovir reduced tissue inflammation and symptoms associated with it such as lymphadenopathy, headache, arthralgia, and mucosal bleeding.

Discussion

In this study, we demonstrated the benefits of empirical acyclovir treatment of IM and IM-like illness patients. Acyclovir reduced the duration of hospitalization and of fever; it also led to a rapid decline in the levels of the acute inflammatory marker SAA, within a period of time shorter than the half-life time of ferritin and CRP, which is a chronic inflammation marker. Additionally, acyclovir-mediated improvement in vital signs such as fever, which is indicative of increased inflammatory cytokine secretion as part of the immunological response against the pathogen, was correlated with the laboratory findings. The recovery of neutrophil count was also more prominent than that of lymphocyte count in APs.

The mechanism behind the beneficial effects of acyclovir on AP condition is unknown. One possible explanation is that acyclovir anti-viral activity also inhibits the DNA replication of viruses other than HHV1 to 3. According to a previous study, acyclovir did not exhibit activity against HHV-7 infection (Zhang et al. 1999). On the other hand, acyclovir combined with interferon-beta demonstrated anti-CMV activity in vitro (Spector et al. 1982). Acyclovir antiviral activity against EBV was also reported in vitro (Long et al. 2003). Some studies have shown that acyclovir combined with prednisolone inhibits replication of oropharyngeal EBV without affecting the duration of IM clinical symptoms (Ernberg and Andersson 1986; Tynell et al. 1996). However, no studies have yet reported clinical data indicating that acyclovir is effective in the treatment of EBV-IM and IM-like illness in vivo.

Another possible explanation is that acyclovir exhibits anti-inflammatory activity through suppressing virus-induced cytokine secretion. Studies have reported that HSV enhances the expression of cytokines such as interferon-gamma and tumor necrosis factor-alpha, and that EBV can immortalize B cells activated by cytokines (Wendel-Hansen et al. 1994; Chen et al. 2000). Interestingly, a previous study has reported that acyclovir blocks the expression of inflammatory cytokines (Halford et al. 1997). Moreover, recent reports have shown that antibiotics such as minocycline or macrolides demonstrate anti-inflammatory activity in addition to their known function (Amin et al. 1996).

However, the present study had several disadvantages. For instance, it included a small number of patients, and only clinical features and conventional markers were examined. Therefore, future studies should include a larger number of cases and additional clinical markers such as galectin-9 or osteopontin, which reflect disease activity, should be examined (Chagan-Yasutan et al. 2009; Saitoh et al. 2012).

In conclusion, treatment with acyclovir reduced the duration of hospitalization and of fever without having any evident adverse effects. Therefore, acyclovir treatment may be effective in treating patients suspected of having a viral infection. Additionally, after experiencing a severe earthquake followed by a tsunami in March of 2011 (Shibahara 2011), we observed that, in such resource-limited conditions, acyclovir treatment could constitute one of the initial empirical therapies for IM-like patients.

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Conflict of Interest

The authors state that they have no conflict of interest.

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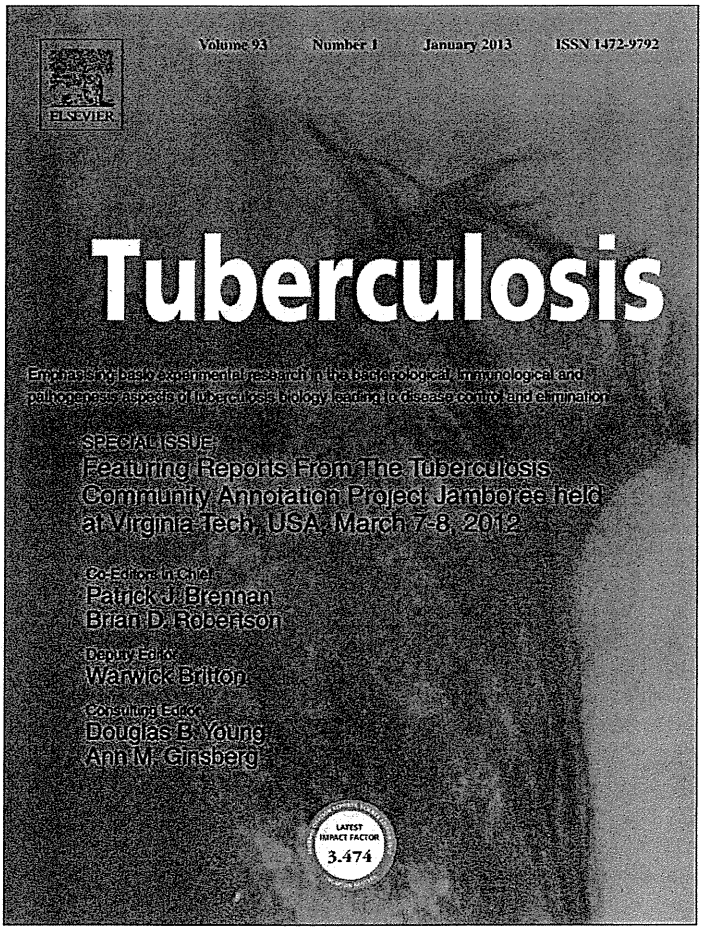

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MOLECULAR ASPECT

Characterization of extensively drug-resistant *Mycobacterium tuberculosis* in Nepal

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SUMMARY

The emergence of extensively drug-resistant tuberculosis (XDR-TB) has raised public health concern for global control of TB. Although molecular characterization of drug resistance-associated mutations in multidrug-resistant isolates in Nepal has been made, mutations in XDR isolates and their genotypes have not been reported previously. In this study, we identified and characterized 13 XDR *Mycobacterium tuberculosis* isolates from clinical isolates in Nepal. The most prevalent mutations involved in rifampicin, isoniazid, ofloxacin, and kanamycin/capreomycin resistance were Ser531Leu in *rpo*B gene (92.3%), Ser315Thr in *katG* gene (92.3%), Asp94Gly in *gyr*A gene (53.9%) and A1400G in *rrs* gene (61.5%), respectively. Spoligotyping and multilocus sequence typing revealed that 69% belonged to Beijing family, especially modern types. Further typing with 26-loci variable number of tandem repeats suggested the current spread of XDR *M. tuberculosis*. Our result highlights the need to reinforce the TB policy in Nepal with regard to control and detection strategies.

1. Introduction

Worldwide emergence of multi- and extensively drug-resistant tuberculosis (MDR and XDR-TB) has become a major obstacle to TB control. XDR-TB is a form of TB caused by *Mycobacterium tuberculosis* (MTB) strains, which is resistant to isoniazid (INH) and rifampicin (RIF), defined as multidrug-resistant MTB (MDR-MTB), as well as fluoroquinolone (FQ) and any of the second-line anti-TB injectable drugs, amikacin (AMK), kanamycin (KAN), or capreomycin (CAP). By the end of 2010, 68 countries had reported at least one case of XDR-TB.[1] XDR-TB is the result of an adverse treatment outcome of MDR-TB; many cases are never diagnosed due to limitations in laboratory capacity to test for second-line drug resistance.[2] Treatment of XDR-TB patients is more challenging and less successful than that of patients with other types of TB.[3] An extremely high death rate from XDR-TB was reported in patients co-infected with HIV in South Africa.[2,4]

In Nepal, TB is a major public health problem. The incidence of all forms of TB was estimated to be 173/100,000 population while the incidence of new smear-positive cases was at 77/100,000 in 2008. The four surveys conducted between 1996 and 2007 have indicated the fluctuating prevalence of MDR-TB among new cases of between 1.1% and 3.7% (1.1% in 1996, 3.7% in 1999, 1.4% in 2001 and 2.9% in 2007). The latest estimate of MDR-TB is 2.9% and 11.7% among new and recurrent cases, respectively.[1,5,6] Although the prevalence of drug-resistance confirming mutations in MDR-TB isolates in Nepal have been reported recently,[6] to the best of our knowledge, no published data on mutations and genotypes of XDR-MTB strains are currently available from Nepal.

Molecular epidemiological studies of *M. tuberculosis* strains have identified variability in the phylogeography of strains globally.[7,8] Beijing strains are most prevalent globally and also associated with enhanced acquisition of drug resistance; however their resistance patterns varied regionally.[9] Drug resistance in *M. tuberculosis* is commonly caused by mutations in various genes. Previous works have indicated that mutations within 81-bp core region of the RNA polymerase β-subunit gene (*rpo*B) gene are the cause of RIF resistance in more than 90% of cases.[10,11] In contrast, several different loci are known to be involved in INH resistance,

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especially *katG* and *inhA*.[6,10] Mutations in a conserved quinolone resistance-determining region (QRDR) of the *gyrA* or *gyrB* genes encoding DNA gyrase are often involved in fluoroquinolone (FQ) resistance.[12] Resistance to aminoglycosides (KAN and AMK) and CAP is attributed to mutations in 16s rRNA (*rrs*) gene.[13,14]

The present study documents drug resistance-associated mutations in XDR isolates from Nepal. To gain an insight into the epidemiology of these isolates, genotyping by using spoligotyping, multilocus sequence typing (MLST) and variable number of tandem repeats (VNTR) were also performed.

2. Materials and methods

2.1. M. tuberculosis isolates

A total of 109 MDR *M. tuberculosis* clinical isolates were randomly selected from isolates bank at German Nepal Tuberculosis Project (GENETUP), Nepal, collected over a 3-year period from 2007 to 2010. Each isolates were recovered from individual patients with pulmonary TB.

2.2. Antibiotic susceptibility testing

Testing for susceptibility to first- and second-line drugs was carried out at GENETUP using the conventional proportional method on Löwenstein–Jensen medium according to the World Health Organization guidelines[15] with the following critical drug concentrations: INH (Cat No. 2261/0801; Fatol Arzneimittel GmbH, Schiffweiler, Germany); 0.2 μg/ml, RIF (Cat No. 004030; Fatol); 40 μg/ml, streptomycin (STR) (Cat No. S6501; Sigma–Aldrich, St. Louis, MO); 4 μg/ml, ethambutol (EMB) (Cat No. 1237/0806; Fatol); 2 μg/ml, ofloxacin (OFX; Cat No. O8757; Sigma–Aldrich); 2 μg/ml, KAN (Cat No. 60615; Sigma–Aldrich); 30 μg/ml and CAP (Cat No. C4142; Sigma–Aldrich); 40 μg/ml.

2.3. DNA extraction

DNA was prepared for PCR by mechanical disruption, as described previously.[6] Briefly, the colonies were suspended in TE buffer consisting of 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA in a 2 ml screw-cap vial, one-fourth of which was filled with 0.5 g glass beads (0.1 mm) (Bio Spec Products Inc., Bartlesville, OK). Mycobacterial cells were disrupted by shaking with 0.5 ml chloroform on a cell disrupter (Micro smash; Tomy Seiko Co. Ltd., Tokyo, Japan) for 1 min. After centrifugation, the DNA in the upper layer was concentrated by ethanol precipitation and dissolved in 100 μl TE buffer.

2.4. PCR amplification and DNA sequencing of drug resistance-associated genes

PCR reactions were performed in a 20 μl mixture consisted of 0.25 mM each of dNTPs, 0.5 M betaine, 0.5 μM of each primer (Primers for rrs, gyrA and gyrB in Table 1 and those in Poudel A et al.[6] for *rpoB*, *katG* and *inhA* gene segment amplification). One U GoTaq DNA Polymerase (Promega, Madison, WI), GoTaq buffer and 1 μl DNA template. The reactions were carried out in a thermal cycler (Bio-Rad Laboratories, Ipswich, MA) under the following conditions: initial denaturation at 96 °C for 60 s followed by 35 cycles of denaturation at 96 °C for 10 s, annealing at 55 °C for 10 s and extension at 72 °C for 30 s with a final extension at 72 °C for 5 min. PCR products were sequenced according to the manufacturer's instructions with the same primers used for PCR and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies Corp., Carlsbad, CA) using an ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp.). The resulting sequences were compared with wild-type sequences of *M. tuberculosis* H37Rv using Bio-Edit software (version 7.0.9) (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Table 1
Nucleotide sequence of primers used for PCR and sequencing.

Locus	Primer	Nucleotide sequence (5′–3′)	Target region	Product size (bp)
gyrA	TB *gyrA* S	AGCGCAGCTACATCGACTATGCG	220–339	321
	TB *gyrA* AS	CTTCGGTGTACCTCATCGCCGCC		
gyrB	TB gyrB S	CGGCACGTAAGGCACGAGAG	1373–1770	398
	TB *gyrB* AS	GAACCGGAACAACAACGTCAAC		
rrs	TB *rrs* S	AGTCCCGCAACGAGCGCAACCC	1350–1550	665
	TB *rrs* AS	GATGCTCGCAACCACTATCCA		

2.5. Phylogenetic markers

Spoligotyping was performed according to the standard protocol.[16] and the spoligotype in the binary format was compared with the SpolDB4 database.[17] Another molecular epidemiological investigation was performed by PCR amplification of the 26 variable *M. tuberculosis* microsatellites and assigned an allele number based on the number of repeats as described previously.[18] A combined spoligotype-VNTR UPGMA3 dendrogram was computed and drawn using Bionumerics 6.0 version software (Applied Maths, Sint-Marten-Latems, Belgium). MLST targeting 10 chromosomal positions were performed according to Filliol et al.[19]

3. Results

3.1. Drug-susceptibility patterns

Among 109 MDR-MTB isolates obtained, 13 were found to be XDR (Table 2). Three of the patients having XDR-TBs (84, 90 and 123) were naive for MDR treatment. Of the remaining 96 isolates, 41, 1, and 1 were mono-resistant to OFX, KAN, and CAP, respectively, and categorized as pre-XDR-MTB.

3.2. Geographical distribution of XDR M. tuberculosis isolates

The XDR-MTB isolates were originated from patients living in five main cities of Nepal (Figure 1): Kathmandu ($n = 7$), Pokhara ($n = 3$), Butwal ($n = 1$), Bhairahawa ($n = 1$) and Dhangadhi ($n = 1$). The number of XDR-TB in Kathmandu correlates well with its high population.

3.3. Mutations identified in the rpoB, katG, inhA, gyrA, gyrB and rrs genes

Sequence analysis identified the most frequent mutations conferring Ser to Leu amino acid substitution at position 531 (Ser531Leu) in *rpo*B (12/13), Ser315Thr in *kat*G (12/13), Asp94Gly in *gyr*A (7/13), and a mutation from A to G at nucleotide position 1400 (A1400G) in *rrs* (9/13). Other mutations with lower rates were seen in *rpo*B (Asp516Val; 1/13), *inhA* regulatory region (C-15T; 1/13), *gyrA* (Ser91Pro; 1/13, Asp94Ala; 2/13, Asp94Asn; 1/13, Asp94His; 1/13, and Asp94Tyr; 1/13), and *rrs* (C1401T and G1483T; two each), while none had mutations in the quinolone resistance-determining region of *gyr*B (Table 2).

3.4. Spoligotyping and MLST

Among XDR-TB isolates, Spoligotyping revealed the predominance of Beijing family strains (9/13). In addition, 1 strain of CAS

Table 2
Antimicrobial susceptibility profile and mutation pattern of the different drug-target genes or regions among XDR isolates.

Strain no.	Drug susceptibility profile*							Mutation pattern in different drug-target genes or regions†						Spoligotype based clade with ST	Geographical location	Age of patient
	RFP	INH	STR	EMB	OFX	KAN	CAP	*rpoB*	*katG*	*inhA* regulatory region	*gyrA*	*gyrB*	*rrs*			
84	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt§	Asp94Gly	wt	A1400G	Beijing (Modern)	Kathmandu	21
86	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	C1401T	Beijing (Modern)	Kathmandu	16
90	R	R	R	R	R	R	R	Asp516Val	Ser315Thr	wt	Ser91Pro	wt	A1400G	Beijing (Ancient)	Kathmandu	26
103	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	C1401T	Beijing (Modern)	Kathmandu	24
108	R	R	S	S	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	CAS	Kathmandu	40
118	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Kathmandu	25
123	R	R	R	R	R	R	R	Ser531Leu	wt	C-15T	Asp94Asn	wt	G1483T	Beijing (Modern)	Kathmandu	21
139	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Ala	wt	A1400G	Beijing (Modern)	Pokhara	25
140	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Ala	wt	A1400G	T2	Pokhara	33
142	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Tyr	wt	G1483T	New	Pokhara	45
151	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94His	wt	A1400G	T1	Bhairahawa	40
155	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Butwal	18
161	R	R	R	R	R	R	R	Ser531Leu	Ser315Thr	wt	Asp94Gly	wt	A1400G	Beijing (Modern)	Dhangadhi	32

* INH, isoniazid; RFP, rifampicin; STR, streptomycin; EMB, ethambutol; OFX, ofloxacin; KAN, kanamycin; CAP, capreomycin; R, resistant; S, susceptible.
† Mutations in rpoB, katG and gyrA are presented as amino acid changes with codon position; mutations in rrs gene and inhA promoter region are presented as nucleotide changes with mutation position.
§ WT, wild type.

family, 2 strains of T family (T1 and T2) and 1 strain of undefined type were also identified. MLST confirmed 8 isolates with Beijing spoligotype belonged to modern types (Table 2).

3.5. Cluster analysis by VNTR

VNTR typing grouped the isolates into seven unique patterns and two clusters (Figure 2). Each cluster contained three isolates of the Beijing family. Among the clustered isolates, 86 and 103 in a cluster (cluster 1) had the same profile of drug resistance-associated mutations (*rpoB*-Ser531Leu, *katG*-Ser315Thr, *gyrA*-Asp94Gly, and *rrs*-C1401T), whereas 84 carried a distinct mutation in *rrs* (A1400G). Similarly, 118 and 161 in another cluster (cluster 2) had the same profile of drug resistance-associated mutations (*rpoB*-Ser531Leu, *katG*-Ser315Thr, *gyrA*-Asp94Gly, and *rrs*-A1400G) and 123 showed a distinct mutation pattern (C-15T at *inhA* regulatory region instead of *katG*-Ser315Thr for INH resistance, *gyrA*-Asp94Asn for FQ resistance, and *rrs*-G1483T for KAN/CAP resistance).

4. Discussion

In this study, we investigated drug resistance-associated mutations and genotypes of XDR-MTB isolates in Nepal. This study also raises concerns over the high proportion of pre-XDR-TB in Nepal. The high rate of pre-XDR-MTB isolates implied the inappropriate usage of drugs, especially FQs, including OFX. OFX is the most commonly prescribed antibiotic for respiratory tract infection in Nepal and this might lead to the emergence of pre-XDR-TB with resistance to OFX. As drug resistance in *M. tuberculosis* is due to the stepwise accumulation of mutations in the genome, this pool of pre-XDR-MTB isolates are always at the risk of developing XDR-TB.

Figure 1. Geographical location of XDR-TB isolation. Cities where XDR-MTB has been isolated are indicated by a closed circle.

Sequence analysis of the hot spot regions of various genetic loci showed that the most common mutations among XDR isolates were Ser531Leu of *rpoB*, Ser315Thr of *katG*, Asp94Gly of *gyrA* and A1400G of *rrs* for RIF, INH, OFX and KAN/CAP resistance, respectively. Other studies have also reported similar mutations among XDR-TB isolates from different countries.[20–23] As mutations such conferring amino acid substitutions, Ser531Leu in *rpoB* and Ser315Thr in *katG* with low fitness costs are known to dominate the drug-resistant isolates.[24]

Genotyping of the isolate by spoligotyping and MLST pointed out the predominance of strains belonging to the modern type Beijing genotypes. The similar involvement of XDR-MTB by modern type Beijing genotypes has been reported from South Africa,[4] India,[20] and China,[23] while the ancient type Beijing family predominates in Japan.[25] Over-representation of Beijing genotype in XDR-MTB in this study compared to the lower prevalence of this genotype in non-MDR and MDR isolates (33 and 51%, respectively; data not shown) supported the previous study that this genotype has been associated with drug resistance,[4,26] because of its higher mutation rates and lower fitness costs with specific mutations.[24] The significantly low average age of patients suffering from Beijing genotype MTB compared to patients suffering from MTB with other genotypes (23.1 ± 4.8 vs 39.5 ± 4.9 years old; Table 2) may suggest the higher transmissibility of Beijing genotype XDR-MTB among the young generation because of their frequent movement.[27]

Although the numbers of isolates were small, complete matches of VNTR, including three hypervariable loci (QUB 11a, QUB 3232, QUB 3336) and drug resistance-associated mutations between two isolates in each cluster, suggested the possible transmission of XDR-TB in Nepal. MDR treatment of a patient who was the source of strain No. 103 started 3rd, April 2006 and the duration of MDR treatment was 12 month. In contrast, that of strain No. 86 started 29th, October 2009 and the duration of MDR treatment was 1 month. By these facts, we arrived at the idea that patient with strain No. 103 might be a source of transmission of XDR-TB and that with

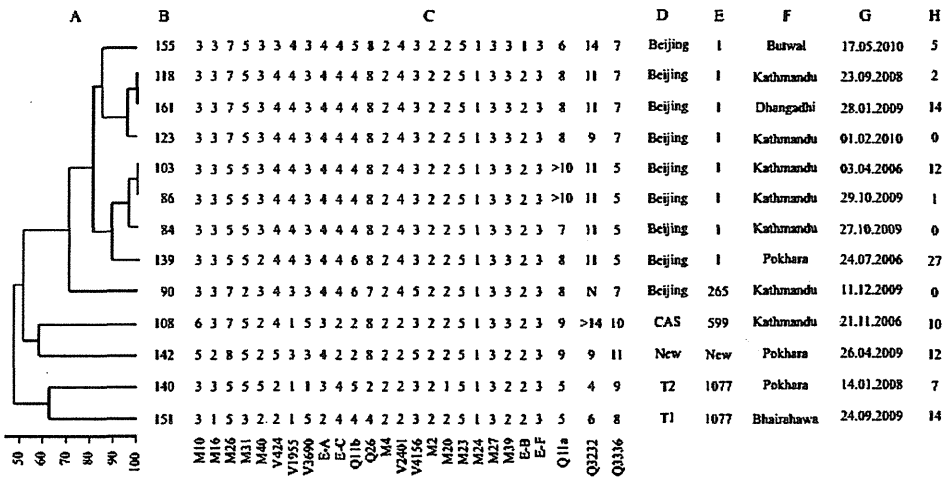

Figure 2. Dendrogram and schematic representation of VNTR typing and spoligotyping results obtained with 13 XDR-TB isolates in Nepal. Column A: dendrogram (UPGMA method, distance matrix average of spoligotyping-based and VNTR) built with Bionumerics version 6, B: strain identification, C: 26 loci VNTR results, D: spoligotyping-based defined clades; E: spoligotyping international type, F: geographical location, G: MDR treatment start date and H: duration of MDR treatment.

strain No. 86 might be a recipient. Alternatively, there might be common transmission source(s) to these patients. Situation was different in another cluster. MDR treatment of a patient who was the source of strain No. 118 started 23rd, September 2008 and the duration of MDR treatment was 2 month and primary XDR-TB was suspected. In contrast, that of strain No. 161 started 28th, January 2009 and the duration of MDR treatment was 14 month. The existence of common infection source of these strains was supposed. The transmission of XDR-TB was also speculated from the fact that three patients (from whom strain No. 84, 90 and 123 were isolated) were naive for MDR-TB treatment. It is interesting that transmission of XDR-TB were speculated not only within Kathmandu but also between Kathmandu and Dhangadhi, apart more than 650 km (Figure 1). As Kathmandu is the capital of Nepal and people come and go frequently from different parts of Nepal, transmission between people living in Kathmandu and those living far from Kathmandu might be possible. Indeed, the patient from whom strain No. 161 was isolated has a history of traveling to Kathmandu. The possibility of transmission of XDR-TB seemed to be high, especially in cluster 1 (including strains No. 86 and 103), because the *rrs*-C1401T mutation carried by both strain No. 86 and 106 was rare between KAN/CAP-resistant isolates.[10,13] On the other hand, care should be taken when concluding XDR-TB transmission in cluster 2 (including strains No. 118 and 161) as both of the mutations, *gyrA*-Asp94Gly and *rrs*-A1400G, have been reported to be rather common in OFX- and KAN/CAP-resistant MTB, respectively, and the distance between the two cities is great. The high rate of pre-XDR-TB in MDR-TB might suggest the acquisition of XDR phenotype during successive transmission as these strains belong to the Beijing family, known to have higher mutation-acquiring capacity. The high number of MDR-TB patients who stop treatment in Nepal could also explain this high drug resistance acquisition rate.[5] Both the possibility of direct transmission and acquired resistance should be considered equally for XDR-TB in Nepal.

5. Conclusion

The majority of XDR-MTB isolates in this study belonged to the Beijing family. Infections of this family were more common among younger generation than those belonging to other spoligotype families. In addition, the identical pattern of VNTR and drug resistance-associated mutations suggested the possible transmission of Beijing genotype XDR-MTB among people in Nepal. Our findings emphasize the urgent need to identify patients suffering from XDR-TB with Beijing genotype MTB and to treat them in isolated wards for a better control program to prevent the spread of this incurable disease.

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RESEARCH ARTICLE Open Access

Mycobacterium bovis infection at the interface between domestic and wild animals in Zambia

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Abstract

Background: In Zambia, the presence of bovine tuberculosis in both wild and domestic animals has long been acknowledged and mutual transmission between them has been predicted without any direct evidence. Elucidation of the circulating *Mycobacterium bovis* strains at wild and domestic animals interphase area in Zambia, where bovine tuberculosis was diagnosed in wildlife seemed to be important.

Results: A PCR identified 15 and 37 *M. bovis* isolates from lechwe and cattle, respectively. Spoligotype analysis revealed that *M. bovis* strains from lechwe and cattle in Kafue basin clustered into a major node SB0120, where isolates outside the Kafue basin clustered into different nodes of SB0131 and SB0948. The comparatively higher variety of strains in cattle compared to lechwe elucidated by Mycobacterial Interspersed Repetitive Units–Variable Number Tandem Repeats analyses are consistent with cattle being the probable source of *M. bovis* in wild and domestic animals interphase area in Zambia.

Conclusions: These results provide strong evidence of *M. bovis* strains transfer between cattle and lechwe, with the latter having developed into a sylvatic reservoir host.

Keywords: Bovine tuberculosis, Cattle, *Mycobacterium bovis*, Strains, Wildlife, *Kobus leche Kafuensis*

Background

Majority of the mycobacterial species that cause human and animal tuberculosis are grouped together as members of the *Mycobacterium tuberculosis* complex (MTC) [1,2]. This *Mycobacterium tuberculosis* complex includes very closely related species of mycobacteria among them: *M. tuberculosis, M. africanum, M. microti, M. bovis, M. caprae, M. canettii* and *M. pinnipedii* [1]. Although *M. tuberculosis* infection is the most common cause of human tuberculosis, part of other proportion of cases are due to *M. bovis* [3,4].

Zoonotic tuberculosis is caused mainly by *M. bovis* that has been shown to have a very wide host range [4-6]. The specie has been documented throughout the world with a similar impact in terms of disease occurrence [5]. In Zambia, BTB is not homogenously distributed, however, high prevalence rates have been recorded within and around the Kafue basin where there is extensive overlap in terms of grazing land for both wild and domestic animals [7-9]. Additionally, the Kafue lechwe antelopes (*Kobus leche Kafuensis*) found in the Kafue basin have been described as feral reservoirs of BTB in Zambia [10,11]. The disease has a historical presence in the Kafue basin that predates the identification of the area as a protected ecosystem and Ramsar Site no.530 [12]. Despite the continued reduction in annual rainfall figures under the effects of global warming, the Kafue basin still remains as one of the few lucurstrine wetland ecosystems in Zambia and Africa, supporting a surging cattle population estimated at 300,000 animals, [13] at a carrying density of 50 animals per square kilometre and approximately 38,000 lechwe antelopes [12] on a 6,000 square kilometre wetland [14]. The area is characterised by high BTB with a herd level prevalence of around 50%, whereas a comparatively lower herd prevalence averaging 5.6% has been determined in areas outside the

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basin [7,15]. Likewise, the corresponding Kafue lechwe antelopes have been shown to have a higher BTB prevalence rate [11,16], raising questions on a possible interspecies transmission of the disease between cattle and Kafue lechwe antelopes. This is however hampered by the lack of direct evidence to conclusively ascertain this assertion.

Sequencing of the whole genome of the members of MTC [17-21] has shown a high level of sequence homogeneity among the members (99.95%). Thus a careful and detailed comparative exploration into the individual genomes of the members of this complex was employed to mine out significant differences for diagnostic capabilities. Comparative genome analysis informed us that *M. bovis* has a smaller genome compared with *M. tuberculosis* [1]. Furthermore, *M. bovis* has over time lost off some genes compared to *M. tuberculosis*. These genomic insertion-deletions are commonly referred to as Regions of difference (RD) and have been used in speciation of members of this complex as well as in explaining the evolution of the MTCs [1,17,19]. Spoligotyping diagnostic technique highlights intra species differences determined by the loss of spacers at a direct repeat region in MTCs, thereby creating a fingerprint typical of a particular specie [22]. Additionally, it is a more rapid and specific method of MTC speciation apart from being less laborious compared with biochemical, phenotypic and IS*6110*-restriction fragment length polymorphism (RFLP) analysis [22]. Spoligotyping results are very practical and reproducible across different laboratories internationally [22,23]. The technique has developed considerably and it is marked by a system of nomenclature and strain data capture and identification with a huge geographical and epidemiological relevance worldwide [24]. Most strains of *M. bovis* have one copy of IS*6110* and spoligotyping is in general more discriminative when used with methods based on PCR amplification of the loci containing variable number tandem repeats (VNTRs) [25,26].

The target of this study was to molecularly characterize a population sample of *M. bovis* from cattle and kafue lechwe antelopes in Zambia to determine the genetic diversity and relatedness of the isolates from domestic animals and wildlife.

Results and discussion

Isolation and confirmation of *M. bovis* by MTCD-MPCR

A total of 315 specimens from cattle and 75 from lechwe antelopes were analysed to initially determine the prevalence of MTC species. The samples were collected based on observations of gross pathological lesions upon examination. The observations included generalized lesions involving the lungs, pleural and mediastinal lymph nodes in both lechwe and cattle. These tuberculous lesions were observed by other workers who were investigating gross pathological distribution of tuberculous lesions in both cattle and lechwe [9,27,28]. From the samples analysed, 52 MTC strains (Table 1) were obtained with 37 isolates from 315 cattle and 15 from 75 lechwe. Following isolation *M. bovis* was confirmed by screening using the MTCD-MPCR analysis as previously observed [11,29]. The MTCD-MPCR technique is very useful in the differentiation of MTC at whatever level of diagnosis as it is simple and specific [29-31]. The presence of *M. bovis* in wildlife may translate into a perpetual focus of the disease [10] considering that BTB control in wild animals is a very difficult undertaking [32,33]. The only workable solution would be to intensify BTB testing in domestic animals so that reactors are removed. Furthermore traditional cattle herders must also be informed of the dangers of grazing their animals in areas where lechwe antelopes are present.

Spoligotyping and Multiple locus variable number of tandem repeats analysis

Spoligotyping of the 52 *M. bovis* isolates revealed their molecular clonality (Table 2). Two major spoligotype patterns (SB0120 and SB0131) were observed accounting for 36 isolates (69.2%), and 15 isolates (28.8%) cattle and lechwe respectively. Two isolates not identifiable with the two major clusters was given an SB0948 under the global spoligotype patterns diversity provided by the international data base on spoligotyping [24]. This only accounted for 4% of the observed isolates. *M. bovis* strains from both cattle and lechwe of the Kafue basin were found to share the same spoligotype (SB0120). This spoligotype was previously reported to be the major strain circulating in cattle around the Kafue basin, although by that time, no strains were determined from wild animals [34]. *M. bovis* strains isolated outside the Kafue basin were found to share a different cluster (SB0131). All the 52 isolates lacked spacers 3, 9, 16 and 39 to 43, a characteristic feature that distinguishes *M. bovis* from *M. tuberculosis* [22]. Molecular studies have previously shown that clonality implies active transmission of disease [35]. Thus our results in the Kafue basin where there was high clonality between isolates from lechwe and cattle, suggest an active transmission of *M. bovis* between the two animal species. The spoligotyping results showed that SB0131, clustered outside the Kafue basin suggesting that none of the lechwe antelopes harboured *M. bovis* with such a spoligo pattern.

Table 1 Results of the MTCD-MPCR of the isolated *Mycobacterial* isolates from cattle and lechwe

Animal species	Total sampled	MTCD-MPCR *M. bovis* +ve
Cattle	315	37
Lechwe antelope	75	15
Total	390	52

Table 2 Spoligotyping results by area of origin across animal species

Animal species	Area of origin	Strain type	Clade	STI	No of isolates
Lechwe	Blue lagoon National park	SB0120	BOVIS 1_BCG	482	4
	Lochinvar National park	SB0120	BOVIS 1_BCG	482	11
Cattle	Namwala (Southern Zambia)	SB0120	BOVIS 1_BCG	482	20
		SB0131	BOVIS 1	594	4
	Lusaka (Central Zambia)	SB0131	BOVIS 1	594	1
	Chongwe (Central Zambia)	SB0120	BOVIS 1_BCG	482	1
		SB0131	BOVIS 1	594	1
	Kabwe (Central Zambia)	SB0131	BOVIS 1	594	8
	Mumbwa (Central Zambia)	SB0948	No data	New	1
	Nampundwe (Central Zambia)	SB0948	No data	New	1
Total					52

MLVA analysis identified 2 distinct clusters, which are corresponding to either SB0131 or SB0120 (Figure 1). Within the cluster possessing the spoligo pattern SB0131, there were 2 groups and 1 strain distinguishable one another by 2 loci, MIRU23 and QUB3232. The cluster possessing the Spoligotype SB0120 was differentiated in 6 groups. Nineteen strains, 14 from lechwe and 5 from cattle, consisted of a major group and other 5 groups or singletons were discriminated from the major group by a single MLVA locus difference each. The spoligotype SB0948 seems to be a descendant pattern of SB0120 lacking spacer 1 and also clustered in SB0120 group with 1 locus (MIRU24) difference. The high isolation frequency of spoligotype SB0120 in cattle and lechwe conforms to the finding of previous works from Zambia [34] and may demonstrate dominance of this spoligotype despite the small sample size. The SB0120 spoligotype has a considerable degree of geographical

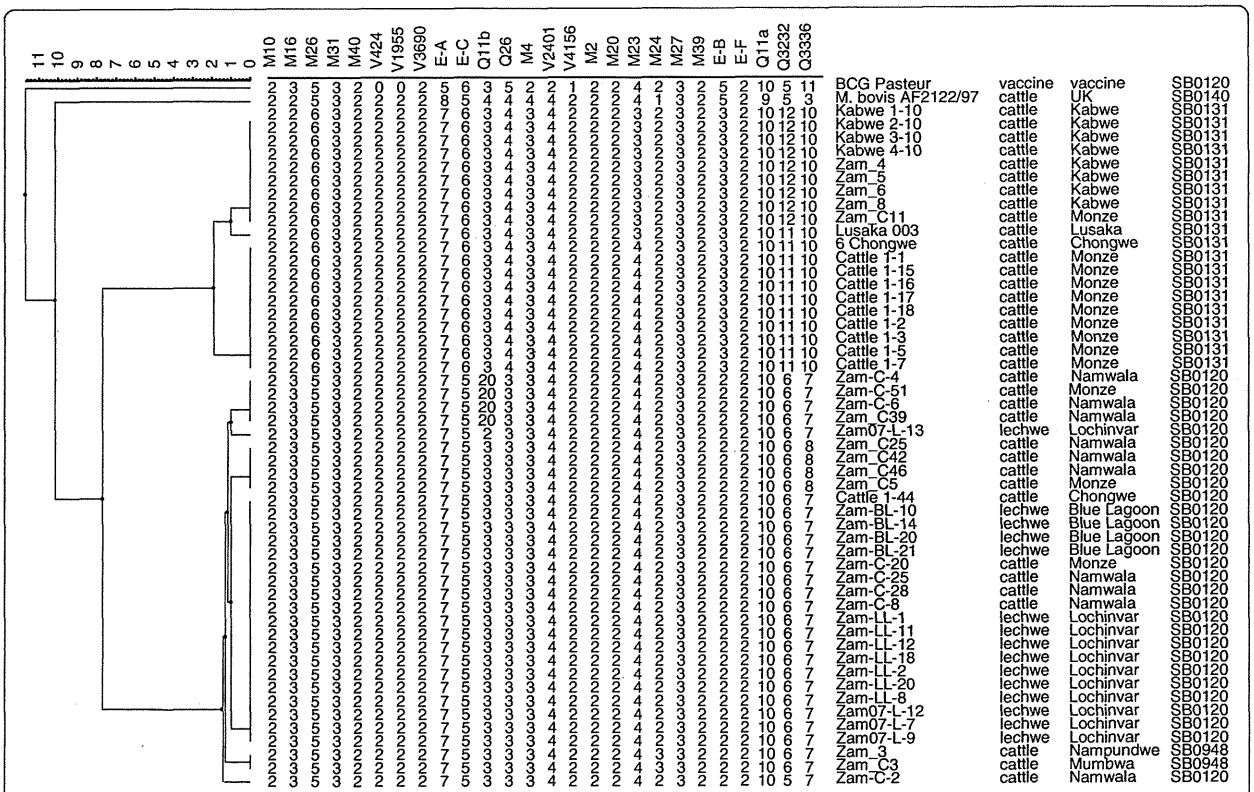

Figure 1 Dendrogram based on the *M. bovis* MLVA clusters distribution data. The major spoligotype SB0120 observed in cattle and lechwe are presented with varying MLVA types. The spoligotype SB0131 is also indicated with 3 MLVA types.

dispersion in Zambia and other African countries such as Algeria [36] and South Africa [37]. This spoligotype is also common in continental Europe [37-39]. Such a strain could have been introduced through livestock development schemes of trying to improve African beef local breeds. This finding has been highlighted by different workers [36,40,41]. Spoligotype SB0131 has been reported in this work for the first time in Zambia. This spoligotype has been documented in South Africa [42], which has a close livestock trade link with Zambia.

To our knowledge, this is the first study conducting molecular characterization of *M. bovis* strains from cattle and lechwe in Zambia. However, routine studies have already previously revealed the presence of BTB in these animals [7,11,16,34]. In this study, results have shown genetic relatedness between *M. bovis* in cattle and lechwe antelopes of the Kafue basin. It is important to note that the area around Lochnivar National park was used for ranching purposes by the early settlers from South Africa. This area was only gazetted as an animal sanctuary in 1972. It is the only place where Kafue lechwe antelopes antelopes are confined. The animals are semi-aquatic living in large groups near water and as such are confined to the area shown in Figure 2 (dotted circle).

Characterization of *M. bovis* strains based on molecular tools is important in understanding the epidemiology of BTB [25,36,41]. These results are significant in understanding the transmission and dispersion of *M. bovis* strains within Zambia (Figure 2), given the high level of internal migration by the local people from the Southern part of the country to the Central and Northern regions of Zambia with their cattle. This type of internal migration may lead to the dispersion of the SB0120 strain which in this present study was found confined to the Southern regions of Zambia.

Conclusion

The current study has described the possible source of *M. bovis* in wildlife and the transmission of limited strains of *M. bovis* between cattle and lechwe. The identified maintenance and spread of *M. bovis* may become a dynamic and highly active process considering that human activities allow movement of cattle from one location to another.

Methods

Study area and sampling

All the samples used in this study were collected in Zambia from 2006 to 2010. The bulk of the samples were obtained at abattoirs from cattle of the Kafue Basin and from other areas supplying cattle to the main abattoirs of Lusaka, the capital city of Zambia (Figure 3). The animals were coming from the Southern province and Central areas of Zambia. Carcasses were followed along the slaughter line and screened for any visible tuberculous lesions. All animals suspected to have tuberculous lesions were examined further by collecting

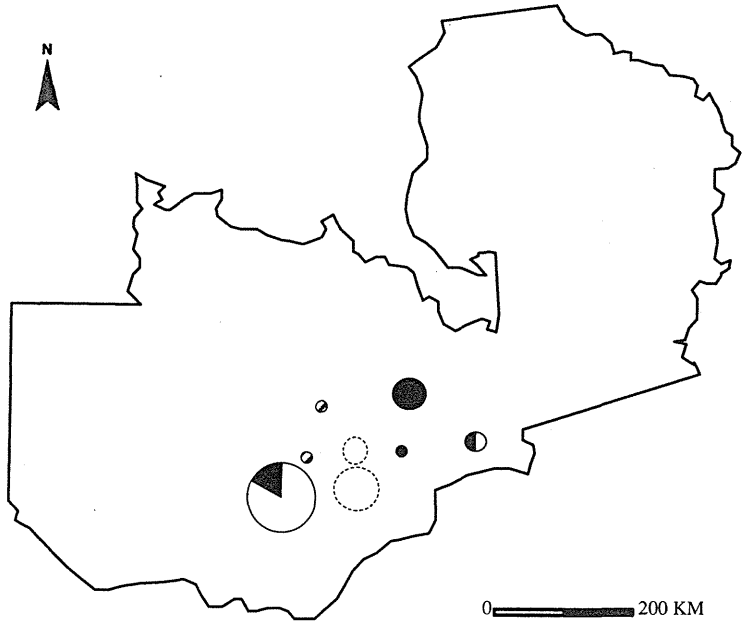

Figure 2 Spread of *M. bovis* strains in Zambia. Circle and dotted circle indicates the *M. bovis* from cattle and lechwe, respectively. White portion in circles indicates the *M. bovis* isolates with spoligotype SB0120, Black portion of circles indicates the *M. bovis* isolates with spoligotype SB0131, and the shaded circle indicates the *M. bovis* with spoligotype SB0948. Size of circle indicates the strain number.

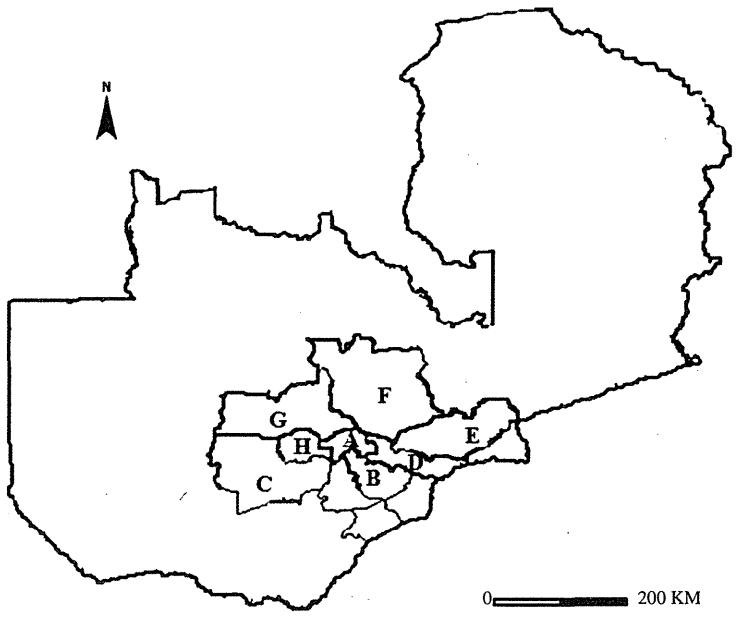

Figure 3 Study area in Zambia. A: Blue lagoon National park, **B**: Lochnivar National park, **C**: Namwala, **D**: Lusaka, **E**: Chongwe, **F**: Kabwe, **G**: Mumbwa, **H**: Nampundwe. **A**, **B**, **C**, **G** and **H** are an interphase area consisting of a wildlife sanctuary and a traditional cattle grazing area; while Area **D**, **E** and **F** are a farming area consisting of commercial and subsistence cattle farms.

samples for laboratory analysis. The wildlife samples were obtained from Kafue lechwe antelopes, which were sampled over the same period as cattle through a special research license from the Zambia Wildlife Authority. The sample size depended on the number of animals allocated for scientific studies with the selection being done at random by gun stunning. The animals were collected from Blue lagoon national park (Figure 3: area A) and Lochnivar national park (Figure 3: area B). The animals were examined for gross lesions according to the standard post mortem examination procedures [43]. The organs and tissues with suspected TB lesions were collected together with accompanying demographical data of area of origin, type of organ or tissue as well as the type of gross pathological postmortem disposition. These specimens were placed in sterile self-zipping histopathological bags, placed into a cooler box with ice packs before transportation to the laboratory for analysis.

Cultivation of Mycobacterial and DNA extraction

The collected samples of TB suspect were analyzed and cultured for growth as previously described [16]. Briefly, the tissues were trimmed of fat and then a 500 mg sample was minced with sterile scissors and homogenized in a sterilized glass homogenizer. A milliliter of phosphate buffer (pH 6.8) was added and thoroughly mixed after which 1 mL of 5% sodium hydroxide was added. After incubation for 15 min at room temperature, 10 mL of phosphate buffer was added and then centrifuged at 1500 *xg* for 20 min. The pellet was collected and then resuspended in a final volume of 0.5 mL of phosphate buffer which was then used for inoculation onto 2% Ogawa medium. Bacterial growth was then monitored up to 8 weeks at 37°C. The resulting cultures were tentatively identified as probable *Mycobacterium tuberculosis*-complex by their slow growth and colony morphology. Purity and acid-fastness of the colonies were checked by Ziehl Neelsen staining. DNA was extracted from Mycobacterial colonies using DNAzol reagent (Invitrogen, Carlsbad, CA, USA) and mechanical disruption as previously described [44] according to the manufacturer's instructions and dissolved in 50 μL TE buffer consisting of 10 mM Tris/HCL (pH 8.0) and 1 mM EDTA.

MTC discrimination by multiplex PCR

The *Mycobacterium tuberculosis* complex-discriminating multiplex PCR (MTCD-MPCR) targeting genetic regions *cfp32* (a specific gene for MTC), RD9 (region of difference 9; seen only in *M. tuberculosis* and *M. canettii*) and RD12 (region of difference 12; deleted in *M. bovis*, *M. caprae* and *M. canettii*) was used for species differentiation according to the previously publication [27]. The reaction mixture consisted of 7.4 μL H_20, 2 μL 10 x Taq buffer, 2 μL dNTPs (2.5 mM each), 0.2 μL Taq (Takara Bio Inc, Shiga, Japan), 1 μL of DNA sample, 2.2 μL of 10 μM cfp32 primers, 0.7 μL of 5 μM RD9 primers and 0.8 μL of 5 μM RD 12 primers. The PCR was performed using the following program: denaturation for 1 min at 98°C followed by

35 cycles of 5 sec at 98°C, 20 sec at 58°C and 1 min at 68°C with final elongation at 72°C for 5 min in a thermal cycler (iCycler, Bio-Rad Laboratories Inc., Hercules, CA). The PCR products were separated by electrophoresis in a 2% agarose gel in TAE buffer, and then visualized after staining with ethidium bromide.

Spoligotyping of M. bovis isolates using micro-spoligoarrays

Spoligotyping was performed according to the procedure by Kamerbeek and co-workers with slight modifications [22]. Forty-three spacer-sequence probes were covalently bound to the membrane (Pall Co., NY, USA). The primers used were DRa (GGTTTTGGGTCTGACGAC) and DRb (CCGAGAGGGGACGGAAAC). A hot start PCR was done by mixing 1 μL each of the 10 μM primer, 7.3 μL H_2O, 3 μL of 5 x colorless Go Taq buffer (Promega™, Fitchburg, WI), 1.5 μL of PCR DIG Labeling Mix (Roche), 0.2 μL of Go Taq DNA Polymerase (5 units/μL; Promega) and 1 μL of DNA sample in 15 μL total reaction mix per tube. The PCR reaction was initiated by denaturation at a temperature of 98°C for 1 min, followed by 40 cycles of 98°C for 5 sec, 55°C for 10 sec and 72°C for 30 sec with a final elongation step at 72°C for 1 min in a thermal cycler. The 500 times diluted PCR product in hybridization buffer was heat denatured at 95°C for 5 minutes and immediately cooled on ice to leave the DNA single stranded. Hybridization was performed by placing the nitrocellulose membranes (Pall Co., NY, USA) for incubation at 60°C for 1 hour. After which, the membrane was washed in TBST-E (0.1% Tween-20 and 1mM EDTA-2Na in TBS) at 60°C for 1 min and then10 min, finally 1 min. The membrane was then dried at room temperature. DIG on the nitrocellulose membranes were reacted with a 1000 times diluted Ant-Digoxigenin-POD [poly], Fab fragments (Roche), with TBSTE-E at room temperature for 30 min. Then the membranes were sequentially washed in TBST-E at room temperature for 1 min, 10 min and 1 min. Then, POD on the membranes was detected by TMB solution (TMB Peroxidase Substrate Kit, Vector Labs Inc™, Burlingame, CA) according to the manufacturer's protocol.

Multiple locus variable number of tandem repeats (MLVA) assay of M. bovis isolates

The isolates were further genotyped by PCR amplification using primers targeting 26 VNTR loci [45]. Two different PCR reaction mixtures were conducted according to the loci. Locus MIRU10, 16, 24, 26, 27, 39, 40, ETR-B, F, VNTR-424, 3690 were done under betaine 1.0 M, whilst locus MIRU2, 4, 20, 23, 31, ETR-A, C, QUB-11a, 11b, 26, 3232, 3336, VNTR-1955, 2401, 4156 were performed under GCII buffer (Takara). The PCR reaction mixture under 1.0 M betaine buffer was conducted in a mixture consisted of 6.3 μL H_2O, 0.4 μL of dNTP (10 mM each), 3.0 μL of 5x colorless Go Taq buffer, 3.0 μL of betaine (5 M), 0.6 μL of Primer 1 and 0.6 μL of Primer 2, 0.1 μL of Go Taq (5 units/ μL) DNA Polymerase, and 1 μL of DNA sample. An initial denaturation step of 95°C for 5 min was followed by 32 cycles at 95°C for 15 sec, 58°C for 20 sec and 72°C for 1 min with a final elongation step at 72°C for 1 min in a thermalcycler. The PCR reaction mixture under the GCII buffer consisted of 4.8 μL H_2O, 0.4 μL of dNTP (10 mM each), 7.5 μL of 2 x GCII buffer, 0.6 μL of primers, 0.1 μL of Go Taq (5 units/ μL) DNA Polymerase (Promega), and 1 μL of DNA sample. An initial denaturation step at 95°C for 5 min was followed by 32 cycles of 95°C for 15 sec, 50°C for 20 sec and 72°C for 45 sec with a final elongation step at 72°C for 1 min in a thermalcycler. All the samples were electrophoresed on a 2% agarose gel to identify the repeat numbers.

Abbreviations

PCR: Polymerase chain reaction; BTB: Bovine tuberculosis; MTCD-MPCR: *Mycobacterium tuberculosis* complex-discriminating multiplex PCR; MIRU-VNTR: Mycobacterial interspersed repetitive units–variable-number tandem repeats; RD: Regions of difference.

Competing interests

All authors have no competing interests.

Authors' contributions

HBM and MM undertook sample collection, laboratory experiments, analyzing the results and drafting the manuscript. CN contributed the data analysis. YF, HS and AI were responsible for laboratory experiments. WM contributed to the design of field data collection. ASM and YS contributed to the design, writing of the manuscript and coordinated the study. All authors read and approved the manuscript.

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HBM, MM, CN, WM and ASM are veterinarians.

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DRUG DISCOVERY AND RESISTANCE

Molecular mechanism of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* from Bangladesh

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SUMMARY

Despite having 100% coverage of directly observed treatment short-course, multi drug-resistant (MDR) tuberculosis (TB) is still increasing in Bangladesh. Early detection of MDR-TB by rapid molecular test and early initiation of treatment will effectively stop this trend. To develop rapid diagnostic tools, molecular characterization of genes conferring *Mycobacterium tuberculosis* resistance to rifampicin (RIF) and isoniazid (INH) will be required. Hence, this study elucidated the molecular mechanism RIF and INH resistance in 218 MDR strains from hospitalized ($n = 161$) and non-hospitalized ($n = 57$) TB patients in Bangladesh. Mutations in *rpoB* gene were detected in 207 (95.0%) with majority at codon 531 (52.3%). Mutations in *katG* or *inhA* or both were detected in 206 (94.5%) with majority at codon 315 of *katG* (83.9%). It was noteworthy that a novel C to T mutation at position −34 and G to A mutations at position −47 in *inhA* regulatory region were found, respectively, in combination with mutation at codon 315 of *katG*. This is the first comprehensive molecular analysis of *rpoB* and *katG* genes and *inhA* regulatory regions of MDR isolates from Bangladesh. This study provides basic data for the construction of low cost tailor-made molecular system for rapid diagnosis of MDR-TB in Bangladesh.

1. Introduction

Tuberculosis (TB) is one of the important re-emerging infectious diseases. Due to its infectious nature, one third of the world's population is latently infected with the causative agent *Mycobacterium tuberculosis* and annual new cases of TB worldwide counts approximately 9 million. This disease is associated with more deaths than any other single infectious agents.[1] The World Health Organization (WHO) estimated prevalence of 277/100,000 in the South–East Asian Region (SEAR) with 90% of patients living in six high TB burden countries: India, China, Indonesia, Pakistan, Bangladesh and the Philippines. The target country of this study, Bangladesh, is one of the six high TB burden countries in SEAR and the 7th highest burden country worldwide. WHO estimated 330,000 new cases of TB with 64,000 deaths in this country in 2010.[2]

TB is a treatable infectious disease, however, its treatment fails with the emergence of multidrug resistance (MDR). The global rate of MDR-TB is increasing due to the lack of treatment compliance, supply of poor quality drugs and recent direct transmission from MDR patients. Hence, MDR-TB has become an issue of increasing importance in both developed and developing country.[3,4] In Bangladesh, MDR-TB increases from 2% to 5.5% with progress of time.[5,6] WHO estimated 2.2% and 14.7% MDR-TB, respectively, among new and previously treated TB cases.[4]

Mycobacteria adopt various mechanisms for its survival in humans under anti-TB treatment. It acquires mutations in genes encoding drug target protein to inhibit the binding of a drug,[7] encoding cell wall proteins contributing impermeability to reduce the entry of drugs, as well as encoding membrane protein that acts as efflux pump.[8,9]

Identification of mutations responsible for drug resistance seems to be a suitable approach for development of molecular tools

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