Infection, Genetics and Evolution 17 (2013) 16–22

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Molecular investigation of multiple strain infections in patients with tuberculosis in Mubende district, Uganda

Adrian Muwonge [a,g,*], Clovice Kankya [c], Francisco Olea-Popelka [d], Demelash Biffa [e], Willy Ssengooba [b], Djønne Berit [f], Eystein Skjerve [a], Tone B. Johansen [f]

[a] Department of Food Safety and Infection Biology, Centre for Epidemiology and Biostatistics, Norwegian School of Veterinary Science, P.O. Box 8146 Dep., 0033 Oslo, Norway
[b] Mycobacteriology Laboratory, Department of Medical Microbiology, Makerere University, P.O. BOX 7072, Kampala, Uganda
[c] Department of Public Health and Preventive Medicine, College Of Veterinary Medicine, Animal Resources & Biosecurity, Makerere University, P.O. Box 7062, Kampala, Uganda
[d] Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1678, USA
[e] College of Medicine, University of Arizona, 1656 E. Mabel St., P.O. Box 245221, Tucson, AZ 85724, USA
[f] Norwegian Veterinary Institute, P.O. Box 750, N-0106 Oslo, Norway
[g] The Roslin Institute, College of Medicine and Veterinary Medicine, University of Edinburgh, Easter Bush, Roslin, Midlothian EH25 9RG, United Kingdom

ARTICLE INFO

Article history:
Received 28 January 2013
Received in revised form 18 March 2013
Accepted 19 March 2013
Available online 30 March 2013

Keywords:
Mixed infections
Mycobacterium tuberculosis
Mubende
Uganda

ABSTRACT

Multiple strain tuberculosis (TB) infections are now an acceptable facet of tuberculosis epidemiology. Identification of patients infected with more than one strain gives an insight in disease dynamics at individual and population level. This study therefore aimed at identifying multiple strain infections among TB infected patients. Furthermore, to determine factors associated with multiple strain infections in Mubende district of Uganda.

A total of 72 *Mycobacterium tuberculosis* isolates from patients at Mubende regional referral hospital were characterized using 15 loci MIRU-VNTR, Spoligotyping and deletion analysis. Genotypic and epidemiological data were analyzed using MIRU-VNTR *plus*, Bionumerics software version 6.1 and an exact logistic regression model respectively.

Eight (11.1%) of the 72 patients had mixed TB infections. Five were exclusively pulmonary mixed infections while three had both pulmonary and extra-pulmonary infections (Compartmentalized TB infections). Unlike previous studies that have linked this phenomenon to Beijing strains, multiple strains in this study belonged to T2-Uganda, X2 and T1 lineages. Two of the pulmonary mixed infections were resistant to rifampicin or isoniazid. All except one were HIV positive, newly diagnosed cases and urban residents of Mubende district.

The study revealed that one in nine urban dwelling, HIV/TB co-infected patient were infected with more than one *M. tuberculosis* strains. The molecular findings give indications of a vital component of the disease dynamics that is most likely under looked at clinical level.

1. Introduction

One third of the world's population is exposed to *Mycobacterium tuberculosis* (*M. tuberculosis*), most of who are residents of developing nations (WHO, 2012). Uganda is ranked 16th among the twenty-two high burden countries, with an incidence and mortality rate of 299 and 84 cases per 100,000 per year respectively (WHO, 2010). In the recent past, infection by *M. tuberculosis* has been assumed to be caused by a single strain, and the concept of several *M. tuberculosis* strains within the same patient was considered very rare and anecdotal at best (García de Viedma et al., 2003). It was believed that such single strain infection would confer protective immunity against exogenous re-infection (Warren et al., 2004). This assumption was probably based on molecular epidemiology that in the past aimed at detecting recent transmission of *M. tuberculosis* based on identification of cases infected with *M. tuberculosis* isolates sharing identical fingerprints (strains), without considering the presence of different strains within these patients (Pérez-Lago et al., 2012). Multiple strain infection analysis has revealed that tuberculosis infections can be more complex than the schematic vision of one strain infecting one host. Multiple strains can occur in the same or different anatomical compartments also referred to as compartmentalization. Multiple strain infections can either be due to mixed infections or clonal diversity (polyclonal infections) (García de Viedma et al., 2003; Shamputa et al., 2004; Pérez-Lago et al., 2012). Reports on this phenomenon suggest that

* Corresponding author at: Department of Food Safety and Infection Biology, Centre for Epidemiology and Biostatistics, Norwegian School of Veterinary Science, P.O. BOX 8146 Dep., 0033 Oslo, Norway.
E-mail addresses: adrian.muwonge@nvh.no, edroxuk@yahoo.co.uk (A. Muwonge).

1567-1348/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.meegid.2013.03.039

it is difficult to draw clear distinctions between clonal diversity and mixed infections in clinical settings. This is because mixed strain infections are typically characterized by more than one amplification product using PCR based typing tools given that the sample collection and culturing process are stringent enough to avoid contamination (Dickman et al., 2010; Nabyonga et al., 2011; Navarro et al., 2011; Cohen et al., 2012). However, there is a vast difference between how these two mechanisms produce within-host diversity; clonal diversity involves sporadic occurrences of polymorphism due to sequential adaptive mutations(Microevolutions) while mixed infections involves a host acquiring an entirely new *M. tuberculosis* genome by sequential or simultaneous exposure to different strains (Cohen et al., 2012). The rate at which mixed infection occur depend on the diversity of strains in a community as well as transmission pressure (Warren et al., 2004; Cohen et al., 2011, 2012). At individual level, host immunity seems to be one of the key drivers of the within-host diversity of *M. tuberculosis*. For example, protective immunity to TB depends on both $CD4^+$ and $CD8^+$ T-cells which rarely eradicate the infection but rather this process promotes granuloma formation (Rahman et al., 2009). Not only do the granulomas favor mycobacterial survival and mutation strategies, but also lead to chronicity which buys the pathogen time (Iwashiro et al., 2001; Rushbrook et al., 2005; Kinter et al., 2007; Rahman et al., 2009).This would explain the bacteria's ability to adapt thus become protected in the absence of timely treatment and the association of this phenomenon with HIV/TB co-infection (García de Viedma et al., 2005; Cohen et al., 2012). Currently, reports of mixed *M. tuberculosis* infections are accumulating for various geographic settings. Unfortunately there has been little or no effort to examine the clinical and public health implications (Cohen et al., 2012). The possibility that a patient may be infected with both drug resistant and susceptible strains, of which only one is detectable through drug resistance testing has both clinical and public health implications (Dickman et al., 2010; Lew et al., 2008).

Since members of *M. tuberculosis* complex (MTC) have highly conserved genomes, high definition tools are needed to reveal the subtle changes within the infecting mycobacterial population. The accurate identification of different strains at individual and population level provides an insight in disease dynamics which are essential in clinical diagnostics, treatment and population control strategies. Mycobacterial Interspersed Repetitive Units-variable number of tandem repeat MIRU-VNTR analysis use variations in copy of repeats in highly variable regions of the MTC genome to shows changes in the genome over relatively shorter time periods (Savine et al., 2002; García de Viedma et al., 2005; Supply et al., 2006). Mubende is the poorest district in the central region of Uganda with an HIV prevalence of 18% (Rwabwogo, 2007). This state of double jeopardy is expected to create an immune compromised sub population that is more likely to be susceptible to multiple strain TB infections. This study therefore aimed at identifying mixed infections in TB patients in Mubende district of Uganda. Furthermore to determine factors that could be associated with mixed infections in this area.

2. Materials and methods

2.1. Ethical considerations

Full ethical clearance was obtained from the Uganda National Council for Science and Technology (UNCST) which is the body mandated to give ethical clearance for biomedical research in Uganda. The ethical clearance number is HS 879. Prior to this study, healthcare authorities and the research team were briefed about the ethical issues. Due to logistical and facility setup oral consent was obtained from participating patients (documented on the information sheets) this was in line with the research ethical mandate given UNCST. Furthermore, data was anonymously analyzed as stipulated by the UNCST guidelines of research involving human as research participants (2.2/b-e/2007).

2.2. Study area

Mubende district is located in the central region (00°0 33 27^2"N, 31°0 23 42^2"E) of Uganda, administratively divided into two counties namely; Buwekula and Kassanda. The counties are further divided into ten sub-counties; Bagezza, Butologo, Kasambya, Kitenga, Kiyuni, Madudu, Bukuya, Kassanda, Kiganda and Myanzi (Rwabwogo, 2007). Mubende is inhabited by approximately 750,000 people, sixty percent of whom live below the poverty line in population dense urban and peri-urban areas with an HIV and HIV/TB co-infection prevalence of 18% and 60%, respectively (Rwabwogo, 2007; Arone, 2009; Globalgiving, 2010). In Mubende, TB case detection is as low as 37% far below the national target with treatment adherence reported to be the main challenge to the control strategy (Globalgiving, 2010).

2.3. Study design and population

This was a cross sectional study conducted between February and July 2011 whose inclusion criterion was patients who presented with cervical lymphadenitis and/or a cough that had persisted for at least two weeks at Mubende regional referral hospital. Given that the nation tuberculosis program is only run at the referral hospital in this district and that the persistent ailments are treated at this facility. The sample collected over the six months period we believe is a cross sectional window into the population dynamics. A total of 344 patients met the above criterion; 41 at the Outpatient Department (OPD), 216 at the tuberculosis ward, 8 at the pediatric ward and 79 at the HIV/AIDS department run by the SUSTAIN program under the Joint Clinical Research Council (JCRC). In addition to sample collection, bio data (sex, age, marital status, weight height etc.) and clinical history such as HIV status was collected. A questionnaire was administered by a nursing officer to obtain information about the geographical and factors associated with TB prevalence.

2.4. Sample collection, culturing and identification of mycobacteria

Sputum and/or lymph node aspirates were collected by qualified and experienced medical personnel. One sample was collected per patient who had either a cough or lymphadenitis. It is noteworthy that there were five individuals from which both pulmonary (sputum) and extra pulmonary (lymph node aspirates) were collected since they had presented with lymphadenitis and a two week persistent cough. The samples were collected in sterile labeled containers and delivered to the Biosafety Level Three (BSL3) tuberculosis laboratories at the department of medical microbiology, Makerere University College of Health sciences. All collected samples were subjected to culture using standard mycobacteriology operating procedures (Kent et al., 1985; Siddiqui SH, 2007). Each sample was cultured on both liquid media and solid media. Cultures were incubated for up to eight weeks on Lowenstein Jensen (LJ) slants (BD BBL™; Franklin lakes, NJ, USA) and six weeks in *Mycobacterium* Growth Indicator Tube (MGIT) (BD BBL™ MGIT960, Franklin lakes, NJ, USA) according to the manufactures' manual. Smears were made from the remaining portion of the concentrated samples and stained for fluorescent smear microscopy. Participants' results for smear microscopy were communicated to their health facilities for routine management. Cultures positive for mycobacteria were subjected to Capilia Neo™ assay (TAUN, Numazu, Japan) to differentiate between the *Mycobacterium*

tuberculosis Complex (MTC) and non tuberculous mycobacteria (Hirano et al., 2004). Absence of colonies on LJ medium or fluorescence on MGIT was reported as a negative result. A culture was considered contaminated if all LJ slants or MGIT tubes from all cultures of the sample revealed growth of organisms other than mycobacteria. Positive results were taken if at least one colony on solid media or one positive tube using liquid media revealed acid fast bacilli on Ziehl–Neelsen (ZN) microscopy and were identified as MTC on the Capilia Neo™ assay. Pure secondary cultures were then forwarded for DNA harvesting. Results were entered into a computerized laboratory access database and linked with individual patient records on the sample reporting form.

2.5. Molecular analysis

Genomic DNA was extracted by heat inactivation of culture material dissolved in TE buffer and direct use of the supernatant as template. Mycobacterial colonies grown on solid media were harvested as a loop full of colony material and suspended in 200 µl of 10 mM Tris–HCl, 0.1 mM EDTA (pH 7.0) in a screwed cap tube and heated at 95 °C for 20 min, left to cool and kept at −20 °C before shipped to Genoscreen® (Lille, France) and the National Veterinary Institute, Oslo. The standard PCR amplification of the genomic regions of difference was performed as previously described (Warren et al., 2006) at the National Norwegian Veterinary Institute, Oslo. A set of primers that included RD1, RD4, RD9 and RD12 was used.

Spoligotyping and MIRU-VNTR with the standardized 15 loci were done by Genoscreen® based on the protocol described by Supply et al. (2006). For spoligotyping, amplification of the spacers was done using the primers DRa and DRb, which enable amplification of the whole DR region. Chromosomal DNA of *M. tuberculosis* strain H37Rv and *Mycobacterium bovis* BCG were used as positive controls and water as a negative control.

For MIRU-VNTR, the fifteen loci were amplified using triplex PCR with fluorescent primers, and the DNA fragments were separated by capillary electrophoresis. Determining the size of the PCR product and assigning the suitable alleles was done using the customized module with GeneMapper® Software (Applied Biosystems, Oslo, Norway). The results were reported in Roman numerals representing the number of repeats per loci.

2.6. Drug resistance assay

The MTC cultures were subjected to drug susceptibility testing using the BACTEC MGIT960TM according to the manufactures' manual (Siddiqui SH, 2007). This is an automated system for detection of mycobacterial growth and drug susceptibility testing of *M. tuberculosis* (Siddiqui SH, 2007). Four first line drugs were tested; Isoniazid, rifampicin, ethambutol and streptomycin. Only the four most used first line TB drugs in Mubende district were evaluated because of logistical reasons.

2.7. Multiple strain infection identification

The following scenarios qualified to be categorized as multiple strain infections.

- If an isolate from any of the anatomical compartments exhibited multiple alleles at any MIRU-VNTR loci.
- If two different genotypes were recovered from any of the two anatomical compartments

Generally the term 'multiple stain infection' can refer to either mixed infections or polyclonal infections or both. A patient would qualify as mixed strain infection if the recovered isolates had more than one allele at more than one MIRU-VNTR locus and as a clonal population infection (polyclonal infections) when there was more than one allele at a single MIRU-VNTR locus (Shamputa et al., 2006a; Martin et al., 2007).

2.8. Data assembly and analysis

Information obtained from the questionnaires, HIV status from case history and corresponding presence or absence of multiple strain infection results for individual patients were entered and validated in Microsoft Excel® 2007. The Data were then imported into Stata (Stata/SE 11 for windows, Stata Corp, College Station, TX) for appropriate statistical analysis. Variables with $P < 0.25$ in univariable analyses were included in a multivariable exact regression for identification of factors associated with the occurrence of multiple strain of *M. tuberculosis* among TB patients. Since few patients had the feature of interest (multiple strain infections), an exact logistic regression was used. It should be noted that an exact logistic model gives the conditional maximum likelihood of an event (multiple strain infections) within the sub population of the sample formed by the different factors included in the model.

Molecular results from Genoscreen® and the National Veterinary Institute were entered and validated in Excel® 2007. These were then copied into MIRU-VNTR *plus* (Weniger et al., 2010) SpolDB4.0 (Weniger et al., 2010) to establish the lineage, Genotypes, sub-types and spoligotype international types (SIT) designations and generate dendograms.

3. Results

3.1. Summary of TB status in study population

M. tuberculosis was isolated from 74 patients (21.5%), of these 51 (68%) were newly diagnosed cases and 23 (32%) were previously treated TB cases. Majority of patients had pulmonary TB 65(88%) and 48 (65%) were co-infected with HIV. Two of the isolates were eliminated from the following analysis due to poor DNA quality. The records on HIV status revealed that 24.7% (85/344), 17% (59/344) and 59% (203/344) were HIV positive, negative and unknown status respectively.

3.2. Multiple strain infections

Seventy-two isolates were included in the analysis for this study. Eight (11.1%) of the 72 patients were found to be infected with more than one *M. tuberculosis* strains. All except one were recovered from newly diagnosed cases. In three patients (#6695, #8034 and #8871), different genotypes/strains were detected in sputum (pulmonary compartment) and lymph nodes aspirates (extra-pulmonary compartment) (Fig. 1 and Table 1). Isolates from patient #8034 belonged to two different lineages (Fig. 1). On the other hand, isolates from patients #6695 and #8871 belonged to the same lineage. However, the MIRU-VNTR signature of the isolates recovered from each anatomical compartment of both #6695 and #8871 were different. The two strains from each of the patient's anatomical compartments were different at five loci (Fig. 1 and Table 1). Critical examination and comparison of the MIRU-VNTR signatures reveals that the extra-pulmonary (8871L) and pulmonary (8871S) *M. tuberculosis* strains of patient #8871 were identical to pulmonary recovered isolates of patients #9202 and #6973, respectively (Fig. 1). There were no identical strains with those recovered from #6695 although #6697 is closely related to the extra-pulmonary *M. tuberculosis* strain of patient #6695 (Fig. 1). It is noteworthy that two of these compartmentalized multiple strain infected individuals were co-infected with HIV and residents of urban areas of Mubende district (Mubende town

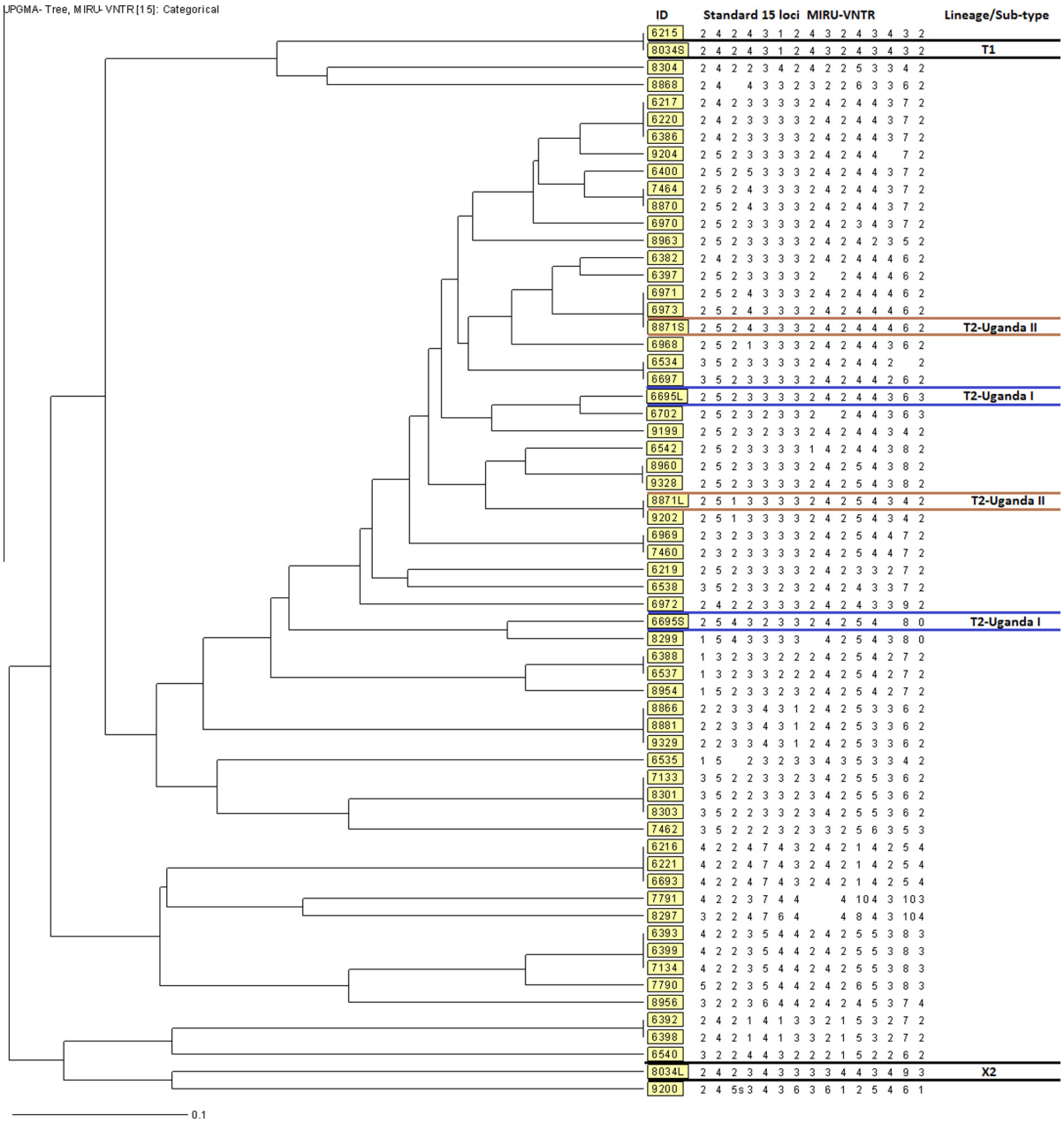

Fig. 1. UPGMA tree based on the standard 15 loci MIRU-VNTR of isolates recovered from TB patients at Mubende referral hospital. The L and S embedded in the ID indicate the anatomical source lymph node aspirate and sputum respectively. # 8034, #8871, #6695.

council and Bagezza). In the remaining five patients, multiple strain infection was detected by presence of multiple alleles at MIRU-VNTR loci (Table 1).

These pulmonary multiple strain infected individuals were also co-infected with HIV and furthermore, #7792 and #9201 showed resistance to rifampicin and isoniazid, respectively (Table 1).

The univariable association between multiple strain infections and demographic factors like cigarette smoking, gender is shown in Table 2. On the other hand the conditional maximum likelihood from the multivariable exact regression (Table 3) suggest that female gender was associated to multiple strain infections (OR = 6.88; $P = 0.046$).

4. Discussion

Multiple strain infections in tuberculosis are now an acceptable facet of tuberculosis picture and therefore identifying patients infected with multiple TB strains should be of paramount importance in clinical practice, public health and molecular epidemiology. The analysis of the multiple strain infection's complex gives a more robust understanding of individual and population disease dynamics and can be of major importance in evaluating treatment regimens as well as population control strategies.

This study revealed that one in nine TB patients (11%) was infected with multiple strains of *M. tuberculosis*. This prevalence was higher than the 2.7% and 7.1% reported in the Ugandan and Spanish capital cities but lower that the 16% and 19% reported in two South African cities (Warren et al., 2004; Dickman et al., 2010; Cohen et al., 2011; Navarro et al., 2011). In the study carried out in Cape Town, South Africa multiple infections were associated with the Beijing strains (Warren et al., 2004) which is in contrast with the findings in this study since strains involved in multiple infections belonged to T2, T1 and X2 lineages.

Table 1
Patient at Mubende regional referral hospital harboring more than one strain of *M. tuberculosis*, isolate characterization and lineage assignment done using spoligotyping and multiple strain identification using MIRU-VNTR standardized 15 loci.

	Patient status				DST	SIT	MIRI-VNTR loci														
ID	HIV	SEX	CASE	ST			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
8034	+	F	ND	S	S	53	2	4	2	4	3	1	2	4	3	2	4	3	4	3	2
				Ln	S	137	2	4	2	3	3	3	3	2	3	4	4	3	4	9	3
6695	Un	F	ND	S	S	52	2	5	2	3	3	3	3	2	4	2	4	4	3	6	3
				Ln	S	52	2	5	4	3	2	3	3	2	4	2	5	4		8	0
8871	+	F	ND	S	S	420	2	5	1	3	3	3	3	2	4	2	5	4	3	4	2
				Ln	S	420	2	5	2	4	3	3	3	2	4	2	4	4	4	6	2
8869	+	F	ND	S	S		2,3	5,10	3	4,7	3,4	2,4	3	2	4	2,4	3	2,3	2	1,4	6
8302	+	M	ND	S	S		2	4,5	1,3	3,4	3	4	2	2,6	4	2	1	2	2	1,3	5
7792	+	F	ND	S	R^r		2,3	5,10	3	4,7	3,4	2,4	3,4	2,5	4	2,4	3	2,3	2	1,4	6
9201	+	F	ND	S	R^i		2	4,5	1	4	1,3	3,4	4	4	2	1,2	2	2	2,4		
6214	+	M	PT	S	S		2	4,5	3,4	3,4	1,3	3	2	4	2,3	2,4	4,5	0,3	3	2,3	5

ID = patient number, ST = sample type. MIRU-VNTR Loci: 1 = 580, 2 = 2996, 3 = 802, 4 = 4052, 5 = 960, 6 = 1644, 7 = 3192, 8 = 424, 9 = 577, 10 = 2165, 11 = 2401, 12 = 3690, 13 = 4156, 14 = 2163b, 15 = 1955. SIT = spoligo international shared type. Un = unknown status. F = female, M = male. S = sputum. Ln = lymph node. Case reference ND is newly diagnosed patient, PT = previously treated. DST = drug susceptibility test S = susceptible to the four drugs tested, R^r = resistant to rifampicin, R^i = resistant to isoniazid.

Table 2
Exact univariable association of factors to Mixed *M. tuberculosis* infections among TB patients at Mubende referral hospital.

Variable	Label	TB +ve	Polymorphism	Percentage%	*P* value
Sex	Male	50	2	4	
	Female	22	6	27	0.03
Age	Youth (<20)	7	0	0	–
	Adult(20–45)	54	8	14.8	0.86
	Old (>45)	11	0	0	–
Marital status	Single	35	1	2.8	–
	Married	30	4	13	0.24
	Divorced	7	2	28.6	0.47
Family size	$\geqslant 5$	11	1	10	–
	$\leqslant 6 \geqslant 10$	50	6	12	0.99
	<10	11	1	9	0.99
Cigarette Smoking	No	35	6	17	–
	Yes	37	2	5.4	0.09
Alcohol consumption	No	22	3	13.6	
	Yes	50	4	8	0.68
TB episode	Primary	49	6	12	
	Secondary	23	1	4.3	0.48
Sample type	Lymph node aspirate	11	1	9	
	Sputum	61	7	11.5	0.22

Table 3
Exact multivariable logistic regression of factors associated with mixed M. tuberculosis infections among TB patients in Mubende district.

Variable	Level	Sufficient statistic	OR (95%CI)	*P* value
Sex	Male	–		
	Female	6	6.88 (1.02–81.04)	0.046
Marital status	Single	–		
	Married	4	6.47 (0.55–356.83)	0.18
	Divorced	2	13(0.50–106.35)	0.15
Sample type	Sputum	–		
	Lymph node aspirate	1	1.33 (0.02–18.77)	1.00

N = 71 and the overall model *P* score = 0.0394.

Six of the eight patients with multiple strain infections were urban dwellers. This might reflect high TB transmission rates and heterogeneity of strains in these urban areas (Cohen et al., 2012). This hypothesis is also supported by the proportion of newly diagnosed cases that exhibited the multiple strain infection phenomena, an attribute that is reported to indicate high transmission rates (Pérez-Lago et al., 2012).

Almost all the patients that exhibited the multiple strain infection phenomenon were co-infected with HIV, this finding is similar to reports from KwaZulu-Natal where all multiple strain TB infected individuals were also HIV positive (Cohen et al., 2011). This seems to corroborate the conclusion on the association between multiple strain TB infection and HIV/TB co-infection (García de Viedma et al., 2005; Cohen et al., 2012). In this study it is plausible to suggest that HIV-induced immune deficiency could play a major role given the high HIV and HIV/TB co-infection prevalence in this district (Arone, 2009; Globalgiving, 2010).

Three of the eight exhibited compartmentalized multiple strain infections which were characterized by different genotypes/strains being recovered from single pure colonies grown from pulmonary and extra-pulmonary isolates of the same patient. #6695 and #8871 were infected with strains that belong to a locally predominant lineage. Given that most patients are infected through the aerosol route, and that dissemination usually occurs from the pulmonary site to extra pulmonary site (García de Viedma et al., 2003), one could speculate that the strains isolated from the pulmonary compartment could be the progenitors of the strains in the extra-pulmonary compartment by microevolution. However, based on the categorization between polyclonal and mixed

infection when MIRU-VNTR is used (Shamputa et al., 2006b; Martin et al., 2007), these two patients were mixed strains *M. tuberculosis* infections. The strains from both anatomical compartments of #6695 were unique to this individual. The same is not true for #8871, because the strains recovered from both anatomical compartments were identical to those recovered from the pulmonary compartments of #9202 and 6973#. Given the stringent laboratory practice and the fact that these isolates (#8871, #9202, #6973) were in different batches i.e. they were submitted and analyzed on different dates, this rules out the possibility of cross contamination. This scenario therefore seems to affirm the prevalence of a heterogeneous pool of strain in this area which is most likely one of the drivers of mixed infections. Similarly, patient #8034 was a case of mixed infection; however this case was infected with strains that belong to two different lineages.

Five of the eight patients gave double alleles at more than one on loci therefore according to the categorization between polyclonal and mixed infection when MIRU-VNTR is used (Shamputa et al., 2006b; Martin et al., 2007), these two were also categorized as mixed infections. Under less stringent sampling conditions double alleles can indicate cross-contamination, but given that both collection and culture protocol were carried out under stringent conditions these findings reflect the actual disease status. All the five patients were cases of pulmonary infections. Since tuberculosis is an air borne disease and the respiratory apparatus being the tissue of interface the pulmonary is expected to be the dominant type of multiple strain infections especially in areas with high transmission rates (Warren et al., 2004). #7792 and #9201 exhibited resistance to rifampicin and isoniazid, respectively. Since the presence of underlying resistance including mono-resistance erodes treatment success and increases risks of acquired resistance with standardized combined therapy (Lew et al., 2008), it is anticipated that mixed infections with resistant strains may be associated with poor treatment out comes (Cohen et al., 2012). Therefore this finding has clinical implications in Mubende district where drug resistance has already been reported as an emerging problem (Globalgiving, 2010).

The findings in this study also suggest a link between gender and multiple strain TB infections because the exact regression model showed that the female gender was associated with multiple strain TB infections. This is however in contrast with the findings in South Africa (Cohen et al., 2011). It is however noteworthy that the exact regression model in this study gives a conditional maximum likelihood of the phenomenon occurring, therefore a larger and more robust study is required to clearly validate this association. In Mubende district, this association could be due to the fact that women take the social responsibility of 'caretaker-ship' in the event of a sick family member being admitted to hospital where they share wards with other patients and care takers. This is also true when TB patients are admitted to the TB clinic. Therefore this social responsibility could be exposing women to a variety of *M. tuberculosis* strains in such high risk and crowded conditions.

This study has documented the prevalence of multiple strain infections in TB patients in Uganda. One of the draw backs of this study is the few cases of multiple strain infections which inherently made it hard to make comparisons between and within groups. Similarly, it is possible that the picture of multiple strain infections is bigger than detect given that 15 loci were used instead of the 24 loci MIRU-VNTR analysis. However, the molecular findings give indications of a vital component of the disease dynamics that is most likely under looked at clinical level and that could have major implications for treatment outcome.

5. Conclusions

Findings reveal that one in nine urban dwelling, HIV/TB co-infected patient were infected with more than one *M. tuberculosis* clonal variants. The molecular findings indicate a vital component of the disease dynamics that is most likely under looked at clinical level and furthermore highlight the need to further investigate the potential high risk of exposure to females at population level.

Conflict of interests

This was purely research work and therefore to the best of our knowledge there was no competing interest.

Authors' contribution

AM: contributed to the conception, study design, data collection, laboratory analysis, interpretation, statistical, epidemiological analysis and drafting of the manuscript. TBJ: laboratory analysis and interpretation and drafting of the manuscript. CK: data collection and field work, drafting and writing of the manuscript. WS: laboratory analysis and interpretation, writing of the manuscript. FOP & DB: statistical epidemiological analysis and writing of the manuscript. DB & ES: conception, study design, laboratory analysis, and mobilization of funds and writing of the manuscript.

Acknowledgements

This work was carried out by the generous financial supports obtained from the Norwegian Educational loan fund as quota scholarship to Dr. Adrian Muwonge, as well as from core funding source of the Norwegian School of Veterinary Science and the National Veterinary Institute. The authors are grateful to the participant who contributed to all of the studies and the teams of investigators involved. These include Dr Fred Miti, Dr Kenneth Batsikana, Sr. Thereza Kitandwe, Ms. Sylvia Nabukeera, Ms. Stella Mumbi, Mr. Muhindo Emmanuel. We are also grateful to the Dr. Wilson Mubiru the director of district health services and Dr. Edward Nkurunziza the medical superintendent of Mubende regional referral hospital and Dr. Martin Zziwa for all the support offered during the study. We are also grateful to Ms Vigdis Edvardsen for the laboratory work at the National Veterinary Institute.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2013.03.039. These data include Google maps of the most important areas described in this article.

References

Arone, K., 2009. Mubende Orphans Support Organization (MOSO), Uganda. In: Arone, K. (Ed.), Orphanage.org. Mubende, Uganda.

Cohen, T., Wilson, D., Wallengren, K., Samuel, E.Y., Murray, M., 2011. Mixed-strain *Mycobacterium tuberculosis* infections among patients dying in a hospital in KwaZulu-Natal, South Africa. Journal of Clinical Microbiology 49, 385–388.

Cohen, T., van Helden, P.D., Wilson, D., Colijn, C., McLaughlin, M.M., Abubakar, I., Warren, R.M., 2012. Mixed-strain *Mycobacterium tuberculosis* infections and the implications for tuberculosis treatment and control. Clinical Microbiology Reviews 25, 708–719.

Dickman, K., Nabyonga, L., Kateete, D., Katabazi, F., Asiimwe, B., Mayanja, H., Okwera, A., Whalen, C., Joloba, M., 2010. Detection of multiple strains of *Mycobacterium tuberculosis* using MIRU-VNTR in patients with pulmonary tuberculosis in Kampala, Uganda. BMC Infectious Diseases 10, 349.

García de Viedma, D., Marín, M., Ruiz Serrano, M.J., Alcalá, L., Bouza, E., 2003. Polyclonal and compartmentalized infection by *Mycobacterium tuberculosis* in patients with both respiratory and extrarespiratory involvement. Journal of Infectious Diseases 187, 695–699.

García de Viedma, D., Alonso Rodriguez, N., Andrés, S., Ruiz Serrano, M.J., Bouza, E., 2005. Characterization of clonal complexity in tuberculosis by mycobacterial interspersed repetitive unit-variable-number tandem repeat typing. Journal of Clinical Microbiology 43, 5660–5664.

Globalgiving, 2010. Advocacy for 8000 TB Affected Persons in Mubende. Uganda Health. Globalgiving.co.uk, United Kingdon.

Hirano, K., Aono, A., Takahashi, M., Abe, C., 2004. Mutations including IS6110 insertion in the gene encoding the MPB64 protein of capilia TB-negative *Mycobacterium tuberculosis* isolates. Journal of Clinical Microbiology 42, 390–392.

Iwashiro, M., Messer, R.J., Peterson, K.E., Stromnes, I.M., Sugie, T., Hasenkrug, K.J., 2001. Immunosuppression by $CD4^+$ regulatory T cells induced by chronic retroviral infection. Proceedings of the National Academy of Sciences 98, 9226–9230.

Kinter, A., McNally, J., Riggin, L., Jackson, R., Roby, G., Fauci, A.S., 2007. Suppression of HIV-specific T cell activity by lymph node $CD25^+$ regulatory T cells from HIV-infected individuals. Proceedings of the National Academy of Sciences 104, 3390–3395.

Lew, W., Pai, M., Oxlade, O., Martin, D., Menzies, D., 2008. Initial drug resistance and tuberculosis treatment outcomes: systematic review and meta-analysis. Annals of Internal Medicine 149, 123–134.

Martin, A., Herranz, M., Serrano, M., Bouza, E., de Viedma, D., 2007. Rapid clonal analysis of recurrent tuberculosis by direct MIRU-VNTR typing on stored isolates. BMC Microbiology 7, 73.

Nabyonga, L., Kateete, D., Katabazi, F., Odong, P., Whalen, C., Dickman, K., Moses, J., 2011. Determination of circulating Mycobacterium tuberculosis strains and transmission patterns among pulmonary TB patients in Kawempe municipality, Uganda, using MIRU-VNTR. BMC Research Notes 4, 280.

Navarro, Y., Herranz, M., Pérez-Lago, L., Martínez Lirola, M., Ruiz-Serrano, M.J., Bouza, E., García de Viedma, D., 2011. Systematic survey of clonal complexity in tuberculosis at a populational level and detailed characterization of the isolates involved. Journal of Clinical Microbiology 49, 4131–4137.

Kent, P.T., Kubia, G.P., 1985. Public health mycobacteriology: a guide for the level III laboratory. Atlanta, Ga.: U.S. Dept. of Health and Human Services, Public Health Service, Centers for Disease Control, Viii, 207.

Pérez-Lago, L., Herranz, M., Bouza, E., García de Viedma, D., 2012. Dynamic and complex *Mycobacterium tuberculosis* microevolution unrevealed by standard genotyping. Tuberculosis 92, 232–235.

Rahman, S., Gudetta, B., Fink, J., Granath, A., Ashenafi, S., Aseffa, A., Derbew, M., Svensson, M., Andersson, J., Brighenti, S.G., 2009. Compartmentalization of immune responses in human tuberculosis: few $CD8^+$ effector T cells but elevated levels of $FoxP3^+$ regulatory T cells in the granulomatous lesions. The American Journal of Pathology 174, 2211–2224.

Rushbrook, S.M., Ward, S.M., Unitt, E., Vowler, S.L., Lucas, M., Klenerman, P., Alexander, G.J.M., 2005. Regulatory T cells suppress in vitro proliferation of virus-specific $CD8^+$ T cells during persistent hepatitis C virus infection. Journal of Virology 79, 7852–7859.

Rwabwogo, M.O., 2007. Uganda district information handbook. Fountain Publishers, Kampala, Uganda.

Savine, E., Warren, R.M., van der Spuy, G.D., Beyers, N., van Helden, P.D., Locht, C., Supply, P., 2002. Stability of variable-number tandem repeats of mycobacterial interspersed repetitive units from 12 Loci in serial isolates of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology 40, 4561–4566.

Shamputa, I.C., Rigouts, L., Eyongeta, L.A., El Aila, N.A., van Deun, A., Salim, A.H., Willery, E., Locht, C., Supply, P., Portaels, F., 2004. Genotypic and phenotypic heterogeneity among *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients. Journal of Clinical Microbiology 42, 5528–5536.

Shamputa, I., Jugheli, L., Sadradze, N., Willery, E., Portaels, F., Supply, P., Rigouts, L., 2006a. Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respiratory Research 7, 99.

Shamputa, I., Jugheli, L., Sadradze, N., Willery, E., Portaels, F., Supply, P., Rigouts, L., 2006b. Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respiratory Research 7, 99.

Siddiqui SH, S.R., 2007. MGIT Procedure Manual; For BACTEC MGIT 960 TB System, Specially Prepared for FIND MGIT demonstration Project. In: Diagnostics, F.f.I.N. (Ed.), Foundation for Innovative New Diagnostics. Mycobacteria Growth Indicator Tube (MGIT) Culture and Drug Susceptibility Demonstration Projects.

Supply, P., Allix, C., Lesjean, S., Cardoso-Oelemann, M., Rüsch-Gerdes, S., Willery, E., Savine, E., de Haas, P., van Deutekom, H., Roring, S., Bifani, P., Kurepina, N., Kreiswirth, B., Sola, C., Rastogi, N., Vatin, V., Gutierrez, M.C., Fauville, M., Niemann, S., Skuce, R., Kremer, K., Locht, C., van Soolingen, D., 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology 44, 4498–4510.

Warren, R.M., Victor, T.C., Streicher, E.M., Richardson, M., Beyers, N., van Pittius, N.C.G., van Helden, P.D., 2004. Patients with active tuberculosis often have different strains in the same sputum specimen. American Journal of Respiratory and Critical Care Medicine 169, 610–614.

Warren, R.M., Gey van Pittius, N.C., Barnard, M., Hesseling, A., Engelke, E., de Kock, M., Gutierrez, M.C., Chege, G.K., Victor, T.C., Hoal, E.G., van Helden, P.D., 2006. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference [Short Communication]. The International Journal of Tuberculosis and Lung Disease 10, 818–822.

Weniger, T., Krawczyk, J., Supply, P., Niemann, S., Harmsen, D., 2010. MIRU-VNTRplus: a web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. Nucleic Acids Research 38, W326–W331.

WHO, 2010. Global Tuberculosis Report. WHO (WHO/HTM/TB/2010.7).

WHO, 2012. Global Tuberculosis Report. WHO (WHO/HTM/TB/2012.6).