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# Molecular characterization of *Mycobacterium avium* subspecies *hominissuis* isolated from humans, cattle and pigs in the Uganda cattle corridor using VNTR analysis

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## ABSTRACT

*Background:* Members of the *Mycobacterium avium* complex (MAC) cause disease in both human and animals. Their ubiquitous nature makes them both successful microbes and difficult to source track. The precise characterization of MAC species is a fundamental step in epidemiological studies and evaluating of possible reservoirs. This study aimed at identifying and characterizing *Mycobacterium avium* subsp. *hominissuis* isolated from human, slaughter cattle and pigs in various parts of the Uganda cattle corridor (UCC) at two temporal points using variable number of tandem repeat (VNTR) analysis.

*Methods:* A total of 46 *M. avium* isolates; 31 from 997 pigs, 12 from 43 humans biopsies and three from 61 cattle lesions were identified to subspecies level using IS*1245* and IS*901* PCR, thereafter characterized using VNTR. Twelve loci from two previously described VNTR methods were used and molecular results were analyzed and interpreted using Bionumerics 6.1.

*Principal findings:* 37 of the isolates were identified as *M. avium* subsp. *hominissuis* and four as *M. avium* subsp. *avium*, while five could not be differentiated, possibly due to mixed infection. There was distinct clustering that coincides with the temporal and spatial differences of the isolates. The isolates from humans and cattle in the North Eastern parts of the UCC shared identical VNTR genotypes. The panel of loci gave an overall discriminatory power of 0.88. Some loci were absent in several isolates, probably reflecting differences in isolates from Uganda/Africa compared to isolates previously analyzed by these methods in Europe and Asia.

*Conclusions:* The findings indicate a molecular difference between *M. avium* subsp. *hominissuis* isolates from pigs in Mubende and cattle and human in the rest of the UCC. Although human and cattle shared VNTR genotypes in the North Eastern parts of the UCC, it is most likely a reflection of a shared environmental source.

## 1. Introduction

*Mycobacterium avium* subsp. *avium* and subsp. *hominissuis* belong to the *Mycobacterium avium* complex (MAC). This group of environmental mycobacteria is the most frequently encountered non-tuberculous mycobacteria (NTM) in patients in the western world. MAC includes *M. avium* subsp. *silvaticum*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *avium* (Falkinham, 1996; Biet et al., 2005; Turenne et al., 2007). *M. avium* subsp. *avium* has mostly been isolated from birds with tuberculous lesions, but can occasionally cause lesions in humans, pigs and other mammals (Cousins et al., 2004; Pate et al., 2005). In pigs, mycobacterial infections are usually caused by *M. avium* subsp. *hominissuis* and characteristically manifests as inflammatory reactions resulting in granulomatous lesions mainly along the digestive tract system (Pate et al., 2005; Cvetnic et al., 2007; Agdestein et al., 2011). However, general disease and reproductive disorders have been reported (Wellenberg et al., 2010; Eisenberg et al., 2012). *M. avium* subsp. *hominissuis* has been isolated from tuberculous lesions of cattle in Uganda (Oloya et al., 2007, 2008), although their role in tuberculous lesion development in cattle is yet to be established.

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Over the past few decades members of MAC have emerged as pathogens of human diseases in industrialized countries, and the most common manifestations include lymphadenitis in children, pulmonary tuberculosis-like disease in patients with predisposing conditions and disseminated infections which occur mainly in AIDS patients (Biet et al., 2005; Jarzembowski and Young, 2008; van Ingen et al., 2009). Most members of MAC are ubiquitous in the environment with a wide range of sources including water, soil and animal bedding (Cvetnic et al., 2007; Krizova et al., 2010; Ofukwu and Akwuobu, 2010; Agdestein et al., 2011). Therefore infection is usually assumed to come from the environment (Biet et al., 2005; Falkinham, 2009). However, the presence of similar isolates among animals and humans has led to speculations regarding the ability of some members of MAC to be potential zoonotic pathogens (Komijn et al., 1999; Johansen et al., 2007; Ofukwu and Akwuobu, 2010). The recent findings by (Agdestein et al., 2011) seem to suggest that pigs could play a role in the dissemination of *M. avium* subsp. *hominissuis,* given their ability to shed in feces.

In Africa, as in other parts of the developing world, research tends to focus on diseases caused by *M. tuberculosis* complex and less on diseases caused by NTM. But, in Zambia these infections have been reported in chronically ill underweight patients who consumed tap municipal water (Patricia et al., 2009). Similarly, members of MAC have been isolated from pastoral eco systems, pigs, cattle and humans in Uganda (Oloya et al., 2007, 2008; Kankya et al., 2011; Muwonge et al., 2012). Unfortunately little has been done to characterize and compare isolates in order to elucidate transmission dynamics in this area. Multi locus variable number of tandem repeats (VNTR) analysis is a typing method that has proven to be a rapid and reliable typing method with a high discriminatory power for isolates in the *M. tuberculosis* complex, but has also been used for other types of mycobacteria (Supply et al., 2006; Ichikawa et al., 2010). The method is increasingly used for molecular epidemiological analysis of *M. avium* (Thibault et al., 2007; Inagaki et al., 2009). Mycobacterial interspersed repetitive units (MIRUs) are repeats formed by a replicative mechanism confined to each locus (Mijs et al., 2002; Supply et al., 2006), therefore within a population of bacterial isolates the variation in number of copies of the repeat elements at a specific locus indicates the diversity (Tirkkonen et al., 2010). The precise differentiation of MAC species is a fundamental step in epidemiological studies and in the detection of possible reservoirs (Biet et al., 2005). Therefore, this study aimed at identifying and characterizing *M. avium* subsp. *hominissuis* isolated from human, slaughter cattle and pigs in various parts of the Uganda cattle corridor at two temporal points using twelve VNTR loci from two previously described methods. Furthermore, compare the molecular signatures recovered in Uganda with previously documented signatures elsewhere with the same methods.

## 2. Materials and methods

### 2.1. Ethical clearance

Full ethical clearance was obtained from the Uganda National Council for Science and Technology (UNCST) is the body mandated to give ethical clearance for biomedical research in Uganda. The ethical clearance number is HS 879 covers all samples used in this study as this is part of an ongoing study to document mycobacteria in the Uganda cattle corridor. Prior to the human study component, Healthcare authorities and the research team were briefed about the ethical issues. Oral consent was obtained from participating patients, next of kin or caretakers of minors instead of written consent. All the adjustments were approved as per 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). It was not necessary to anonymize samples from animals as they were obtained as part of routine meat inspection.

### 2.2. Mycobacterial isolates

Forty-six isolates of *M. avium* from the Ugandan cattle corridor (UCC) were analyzed. Of these, 31 isolates were from 997 cervical lymph nodes of slaughter pigs isolated between 2008 and 2009 in Mubende district in the central area of the UCC (Muwonge et al., 2012), 12 from 43 cervical lymph nodes biopsies of patients in Karamoja in the North eastern part of the UCC between 2004 and 2005 (Oloya et al., 2008) and three isolated from 61 lesion samples in slaughtered cattle collected in 2004–2005 from pastoral communities (Karamoja, Nakasongola, Masindi and Mbarara) 2004 (Oloya et al., 2007). The procedures for sampling, culturing and species determination have been previously described (Oloya et al., 2007, 2008; Muwonge et al., 2012). It is noteworthy that both culture methods used in these studies did not use Mycobactin in growth medium, therefore all the MAC recovered in these studies are considered MAC other than *M. avium* subsp. *paratuberculosis*.

### 2.3. Identification of M. avium subsp. avium and M. avium subsp. hominissuis

One loop-full of colonies from a pure subculture of *M. avium* were suspended in TE buffer and killed by heat treatment at 96 °C for 20 min. Genomic DNA were isolated and used to classify the isolates of *M. avium* to subspecies level using IS*1245* (Primers P41 and P40) and IS*901* PCR (Primer 901a and primer 901c) as previously described (Johansen et al., 2005; Muwonge et al., 2012) using Amplitaq® enzyme (Life Technologies, Carlsbad, CA). The conditions for IS*1245* PCR were as follows: Initial denaturation at 94 °C for 3 min; followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. The IS*901* PCR was performed using annealing temperature of 55 °C with 35 cycles, otherwise the conditions were identical. The resultant PCR products were analyzed on 2% (w/v) agarose gel, stained with GelRed™ (Biotium Inc., Hayward, CA) and visualized by UV illumination. The reference strain *M. avium* subsp. *avium* ATTC25291 was used as a positive control, Milli-Q water as a negative control.

*M. avium* subsp. *avium* isolates were also confirmed by 16SrRNA sequencing as briefly described; Genomic DNA was characterized based on the 16S rRNA gene using the following primers16S8F (AGAGTTTGATCMTGGYTCAG) and 16SM259 (TTTC ACGAACAACGC GACA). The control strain for this analysis was the ATCC 25291. The obtained sequences were edited and analyzed in the bioinformatics software Bio-edit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the sequences were blasted at the NCBI Blast database (National Centre for Biotechnology Information). The species identification was strictly determined based on the maximum score and maximum identity values of the reference sequences in NCBI Blast alignment. Isolates with the maximum scores and maximum identities of 100% were considered matches.

### 2.4. VNTR typing

A total of 12 loci were used for the multi-locus variable number of tandem repeats (VNTR) analysis. Eight MIRU-VNTR loci described by (Thibault et al., 2007) were combined with four MATR-VNTR loci described by Inagaki et al. (2009). From the panel of MATR-VNTR loci, MATR-1, MATR-7, MATR-13 and MATR-14 were chosen as targets, as they were the most discriminatory loci in the Japanese analysis. The primers used are as described by

Thibault et al. (2007) and Inagaki et al. (2009). Procedures were as described earlier with slight modifications. The PCR was performed using HotStar Taq® polymerase (Qiagen, Hilden, Germany). PCR conditions were as follows: Initial denaturation at 95 °C for 15 min; 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C; and 1 cycle of 7 min at 72 °C. To detect differences in repeat numbers, the size of the PCR products in base pairs were determined using Agilent Bioanalyzer® (Agilent Technologies, Santa Clara, CA).

### 2.5. Comparative analysis of VNTR-typing

Literature available does not seem to have studies that have combined MATR and MIRU into a single panel to characterize *M. avium* subsp. *hominissuis.* Therefore in order to compare Ugandan isolates to European and Japanese isolates, the analysis was exclusively restricted to each set of loci used in this study i.e. the European and Japanese isolates were compared to the Ugandan isolates based on 8-MIRU and 4-MATR respectively. The data for France, Slovenia and Finland was retrieved from supplementary and/or published material by Radomski et al. (2010), Pate et al. (2011) and Tirkkonen et al. (2010) respectively. On the other hand the data for Japan is retrieved from work published by Inagaki et al. (2009). K10 is the *M. avium* subsp. *paratuberculosis* reference strain.

### 2.6. Data analysis

The PCR fragment size was converted to the corresponding number of repeats for each of the 12 loci. The molecular signature revealed by the repeats for each isolate was entered and stored in an excel file. The data was then transferred into Bionumerics 6.1 for cluster analysis using the unrooted UPGMA tree of *M. avium* subsp. *hominissuis.* Furthermore, a minimum spanning tree visualizing the molecular variability of *M. avium* subsp. *hominissuis* was also constructed based on the 8-MIRU. The molecular variability shown in the minimum spanning tree is based on a single locus variation (SLV).

The allelic diversity (h) for each locus was calculated using a method described by Selander et al. (1986). The formula;

$$h = 1 - \sum x_i^2 \left[\frac{n}{n-1}\right]$$

where $x$ is the frequency of the $i$th allele at the locus, n is the number of isolates in the analysis, and $n/(n-1)$ being the correction factor for bias in small samples. It should be noted that the allelic diversity used for the international comparison was extracted from the published material, however, these too where computed using the same formula above.

The discriminatory power (DI) on the other hand was computed using the Hunter–Gaston discriminatory index (Hunter and Gaston, 1988), which was calculated using the formula:

$$DI = 1 - 1\frac{1}{N(N-1)}\sum_{i}^{s} x = 1nj(nj-1)$$

where $N$ is the total number of strains in the typing analysis, $S$ is the number of clusters and $n_j$ is the of strains belonging to the $j$th cluster.

## 3. Results

### 3.1. Identification

All isolates from UCC recovered in the period between 2004 and 2005 ($n = 15$) from humans ($n = 12$) and cattle ($n = 3$) were identified as *M. avium* subsp. *hominissuis* based on presence and absence of IS*1245* and IS*901* respectively on PCR analysis. Of the 31 isolates recovered from pigs in Mubende in the period between 2008 and 2009; five gave conflicting results (faint bands) on repeated IS*901* PCR and were excluded from the remaining analysis as no more DNA was available for further analysis. Among the remaining 26 isolates, four isolates were identified as *M. avium* subsp. *avium* based on the presence of both IS*1245* and IS*901* and 16rRNA sequencing, the rest were identified as *M. avium* subsp. *hominissuis* (Fig. 1, Tables 1 and 2 and Table S1).

### 3.2. VNTR typing

The VNTR analysis performed on 41 isolates; 37 *M. avium* subsp. *hominissuis* and 4 *M. avium* subsp. *avium.* These produced allelic profiles of 11 and 5 different genotypes of *M. avium* subsp. *hominissuis* and *M. avium* subsp. *avium* respectively. Five *M. avium* subsp. *hominissuis* and two *M. avium* subsp. *avium* exhibited multiple amplifications on at least one locus (Table 1), hence not assigned specific genotypes. There were four clusters; two small clusters each containing two isolates and two large clusters containing eleven and ten isolates each respectively. The locus repeats range and mode for each locus used was as follows; locus TR292 (range 0–2, mode 3), TRX3 (0–3, mode 5), TR25 (0–3, mode 7), TR47 (1–5, mode 3), TR10 (0–5, mode 5), MATR1 (1–2, mode 2), MATR7 (0–8, mode 5), MATR 13 (0–1, mode 2), MATR 14 (0–4, mode 4). The following loci produced an allelic diversity $\leqslant 0.5$; TR292, TR47 and MATR13 (Table 2). TRX3 and TR25 were the most polymorphic loci, closely followed by MATR 1 (Fig. 2 & Table 3). The panel of twelve loci used for VNTR analysis gave a total discriminatory power of 0.88.

#### 3.2.1. Host, spatial and temporal characteristics within the UCC

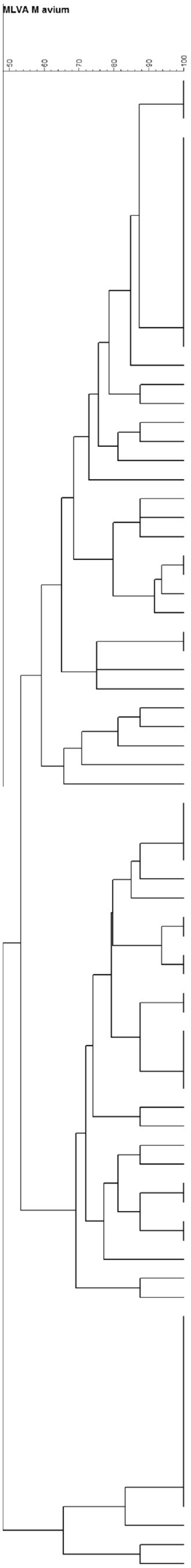
All human and cattle isolates lacked MATR14, which is not the case for all porcine recovered isolates. Furthermore, all but one human sample lacked TR3 (Fig. 1 & Table 1), while all the *M. avium* subsp. *avium* lacked MATR 7 (Table 2). The UPGMA tree reveals two distinct *M. avium* subsp. *hominissuis* clusters that appear to coincide with the host, temporal and spatial differences in the isolates (Fig. 1). It should be noted that seven of the human isolates from Karamoja had an identical VNTR profile with cattle in the same area (Table 1 and Fig. 3B). The same profile was also isolated from Nakasongola while a closely related isolate (B24-JO) was recovered from Masindi district (Fig. 3B). Porcine *M. avium* subsp. *hominissuis* isolates in Mubende appear to be slightly more heterogeneous than cattle and human isolates recovered from the rest of the UCC (Fig. 3).

### 3.3. Comparative analysis of M. avium subsp. hominissuis

#### 3.3.1. Allelic diversity

In general, when compared to K10, the *M. avium* subsp. *hominissuis* isolates from Uganda exhibited similar allelic diversity characteristics as the European isolates (France and Slovenia) (Fig. 2). In contrast to K10, all *M. avium* subsp. *hominissuis* isolates do not show any allelic diversity at TR3 and TR7. On the other hand, unlike the European isolates, Ugandan isolates exhibited a high allelic diversity at TR25 and TR10. K10 and the European isolates exhibit some allelic diversity at TR32, the same is not true for the Ugandan isolates. It is also noteworthy that all porcine isolates from Uganda showed no repeats for locus TR292, while the same is not true for porcine isolates from the rest of the European countries (Fig. 1).

A comparison of the four MATR loci reveals varying levels of allelic diversity at three of the four loci (Table 3). The Ugandan and Japanese samples exhibited similar allelic diversity characteristics at MATR 7, and almost a third and half of what was produced by the Japanese isolates at MATR1 and MATR13 respectively. All



| Loci | | | | | | | | Isolate no. | Country | Origin | Host | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 292 | X3 | 25 | 47 | 3 | 7 | 10 | 32 | | | | | |
| 0 | 5 | 3 | 3 | 1 | 1 | 1 | 8 | 51c | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 1 | 8 | 68 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 1 | 8 | 74c2 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 11 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 162 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | | 3 | 1 | 1 | 2 | 8 | 24 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 25 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 26 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 27 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 28 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 33 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | | 3 | 1 | 1 | 2 | 8 | 34 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 49 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 8 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | AM87B | Finland | | Porcine | Tirkkonen |
| 0 | 0 | 3 | 3 | 1 | 1 | 2 | 8 | 19 | Uganda | Mubende | Porcine | Muwonge |
| 2 | 5 | 3 | 3 | 1 | 1 | 2 | 8 | 2002.07886.00 | France | | Porcine | Radomski |
| 2 | 5 | 2 | 3 | 1 | 1 | 2 | 8 | AM45 | Finland | | Porcine | Tirkkonen |
| 0 | 3 | 3 | 3 | 1 | 1 | 2 | 8 | 17 | Uganda | Mubende | Porcine | Muwonge |
| 3 | 3 | 3 | 3 | 1 | 1 | 2 | 8 | M11 | Slovenia | | Porcine | Pate |
| 3 | 2 | 3 | 3 | 1 | 1 | 2 | 8 | 2007,127 | France | | Human | Radomski |
| 3 | 5 | 3 | 3 | 1 | 1 | 3 | 8 | 2000.05848.00 | France | | Cattle | Radomski |
| 3 | 4 | 3 | 2 | 1 | 1 | 2 | 8 | M15 | Slovenia | | Porcine | Pate |
| 2 | 4 | 3 | 2 | 1 | 1 | 2 | 8 | AM67 | Finland | | Porcine | Tirkkonen |
| 1 | 4 | 3 | 2 | 1 | 1 | 2 | 8 | M2 | Slovenia | | Porcine | Pate |
| 1 | 5 | 3 | 2 | 1 | 1 | 2 | 8 | M3 | Slovenia | | Porcine | Pate |
| | 5 | 3 | 2 | 1 | 1 | 2 | 8 | 1995.08166.01 | France | | Porcine | Radomski |
| 2 | 5 | 3 | 2 | 1 | 1 | 2 | 8 | 1999.05289.00 | France | | Porcine | Radomski |
| 0 | 5 | 3 | 2 | 1 | 1 | 2 | 8 | AM34B | Finland | | Porcine | Tirkkonen |
| 0 | 5 | 2 | 1 | 1 | 1 | 2 | 8 | 165 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 5 | 2 | 1 | 1 | 1 | 2 | 8 | 166 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 3 | 0 | 1 | 1 | 1 | 2 | 8 | UM5 | Uganda | Mubende | Porcine | Muwonge |
| 0 | 1 | 3 | 1 | 1 | 1 | 2 | 8 | 54 C | Uganda | Mubende | Porcine | Muwonge |
| 2 | 2 | 3 | 3 | 1 | 1 | 1 | 8 | 1996.09691.00 | France | | Porcine | Radomski |
| 2 | 2 | 3 | 3 | 1 | 1 | 2 | 8 | 2007,1265 | France | | Human | Radomski |
| 2 | 2 | 4 | 3 | 1 | 1 | 2 | 8 | H0914/04 | Finland | | Human | Tirkkonen |
| 2 | 3 | 3 | 3 | 1 | 1 | 5 | 8 | 2007.00624.00 | France | | Porcine | Radomski |
| 1 | 2 | 4 | 3 | 1 | 1 | 5 | 8 | 2007,1255 | France | | Human | Radomski |
| 2 | 2 | 2 | 2 | 1 | 1 | 2 | 8 | 1995.06877.00 | France | | Porcine | Radomski |
| 2 | 2 | 2 | 2 | 1 | 1 | 2 | 8 | 2007,1266 | France | | Human | Radomski |
| 2 | 2 | 2 | 2 | 1 | 1 | 2 | 8 | 2007,1267 | France | | Human | Radomski |
| 2 | 2 | 2 | 2 | 1 | 1 | 2 | 8 | AM30B | Finland | | Porcine | Tirkkonen |
| 2 | 2 | 2 | 2 | 1 | 1 | 5 | 8 | 2007,1253 | France | | Porcine | Radomski |
| 2 | 2 | 2 | 2 | 1 | 1 | 2 | 7 | 1995.08166.02 | France | | Porcine | Radomski |
| 2 | 3 | 2 | 2 | 1 | 1 | 2 | 8 | 1999.03873.00 | France | | Porcine | Radomski |
| 2 | 3 | 2 | 2 | 1 | 1 | 2 | 8 | AM85 | Finland | | Porcine | Tirkkonen |
| 3 | 3 | 2 | 2 | 1 | 1 | 2 | 8 | M9 | Slovenia | | Porcine | Pate |
| | 3 | 2 | 2 | 1 | 1 | 2 | 8 | 2000.09692.00 | France | | Porcine | Radomski |
| 2 | 4 | 2 | 3 | 1 | 1 | 2 | 8 | AM84B | Finland | | Porcine | Tirkkonen |
| 2 | 4 | 2 | 3 | 1 | 1 | 2 | 8 | H11149/04 | Finland | | Human | Tirkkonen |
| 2 | 4 | 2 | 2 | 1 | 1 | 2 | 8 | 1995.02051.00 | France | | Porcine | Radomski |
| 2 | 4 | 2 | 2 | 1 | 1 | 2 | 8 | 1996.06520.00 | France | | Cattle | Radomski |
| 2 | 4 | 2 | 2 | 1 | 1 | 2 | 8 | 2007,126 | France | | Human | Radomski |
| 2 | 4 | 2 | 2 | 1 | 1 | 2 | 8 | AM49 | Finland | | Porcine | Tirkkonen |
| 2 | 4 | 2 | 2 | 1 | 1 | 2 | 5 | 1995.01088.00 | France | | Porcine | Radomski |
| 2 | 7 | 2 | 2 | 1 | 1 | 2 | 5 | 1997.07613.00 | France | | Porcine | Radomski |
| 3 | 5 | 2 | 2 | 1 | 1 | 2 | 8 | M17 | Slovenia | | Porcine | Pate |
| 3 | 5 | 2 | 2 | 1 | 1 | 2 | 9 | M18 | Slovenia | | Human | Pate |
| 2 | 5 | 2 | 2 | 1 | 1 | 2 | 8 | 2007.00512.00 | France | | Porcine | Radomski |
| 2 | 5 | 2 | 2 | 1 | 1 | 2 | 8 | AM23B | Finland | | Porcine | Tirkkonen |
| 2 | 5 | 2 | 2 | 1 | 1 | 2 | 9 | 2007,1258 | France | | Human | Radomski |
| 2 | 5 | 2 | 2 | 1 | 1 | 2 | 9 | H1078/03 | Finland | | Human | Tirkkonen |
| 2 | 5 | 2 | 2 | 1 | 1 | 5 | 9 | 2007,1252 | France | | Human | Radomski |
| 3 | 4 | 2 | 2 | 1 | 1 | 1 | 8 | M13 | Slovenia | | Porcine | Pate |
| 3 | 4 | 2 | 2 | 1 | 1 | 2 | 8 | M14 | Slovenia | | Porcine | Pate |
| 3 | 3 | 2 | 3 | | 1 | | 8 | 11 JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | 13-Z JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | 15B-1 JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | 17 JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | 18 JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | 24-I JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | 33 JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | B- 14 JO | Uganda | Karamoja | Human | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | B13-2 JO | Uganda | Nakasongola | Cattle | Oloya |
| 3 | 3 | 2 | 3 | | 1 | | 8 | B24 JO | Uganda | Masindi | Cattle | Oloya |
| 3 | 3 | 2 | 3 | | 1 | 5 | 8 | B24-2 JO | Uganda | Karamoja | Cattle | Oloya |
| 3 | 3 | 2 | 5 | | 1 | | 8 | 15-II JO | Uganda | Karamoja | Human | Oloya |
| 4 | 2 | 2 | 3 | 1 | 1 | 2 | 8 | M21 | Slovenia | | Porcine | Pate |
| 4 | 3 | 2 | 3 | 1 | 1 | 2 | 8 | M22 | Slovenia | | Porcine | Pate |

**Fig. 1.** An unrooted UPGMA tree showing the genetic relationships between *M. avium* subsp. *avium* and *hominisuis* isolated from humans, cattle and pigs from the Ugandan cattle corridor as compared to isolates from Finland, France and Slovenia. This dendrogram is based on 8-MIRU allelic profiles of 32 isolates, 14 genotypes, 24 genotypes, and 11 genotypes from Uganda, Finland, France and Slovenia respectively, generated in Bionumerics 6.1.

**Table 1**
The isolates that showed polymorphism at the different loci used in this study.

| ID | Host | Origin | Subspecie | Loci | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | TR292 | TRX3 | TR25 | TR47 | TR3 | TR7 | TR10 | TR32 | MATR1 | MATR7 | MATR13 | MATR14 |
| 6 | PG | MBD | *Mah* | 0 | 2 | 3 | 3 | 1 | 1 | 2 | 8 | 2 | 5 + 9 | 2 | 4 |
| 38 | PG | MBD | *Mah* | 0 | 3 + 5 | 3 | 3 | 1 | 1 | 2 | 8 | 1 + 2 | | 0 | 1 |
| 50 | PG | MBD | *Maa* | 0 | 4 | 2 | 3 | 1 | 1 | 1 | 8 | 1 + 2 | | 0 | 1 |
| 161 | PG | MBD | *Maa* | 0 | 2 + 7 | 3 | 3 | 1 | 1 | 1 | 8 | | | | |
| 29-0 | HN | KJA | *Mah* | 3 | 5 | 2 | 3 | | 1 | 0 | 8 | 2 | | 0 | |
| 43-JO | HN | KJA | *Mah* | | 1 | 1 | 3 | | 1 | 0 | | | | | |
| B-5 | HN | KJA | *Mah* | 3 | 3 + 7 | 2 | 3 | 1 | 1 | 5 | 8 | 2 | 3 | 2 | |

Host: PG is pig and HN is human, Origin: MBD is Mubende district, KJA is Karamoja district. Subspecies Mah is *Mycobacterium avium* subsp. *hominisuiss*, Maa is *Mycobacterium avium* subsp. *avium.*

**Table 2**
The VNTR profile of *M. avium* subsp. *avium* isolated from Mubende district.

| ID | Host | Origin | Loci | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | TR292 | TRX3 | TR25 | TR47 | TR3 | TR7 | TR10 | TR32 | MATR1 | MATR7 | MATR13 | MATR14 |
| 112 c | PG | MBD | 0 | 3 | 2 | 3 | 1 | 1 | 1 | 8 | 2 | | | |
| 115 | PG | MBD | 0 | 3 | 2 | 3 | 1 | 1 | 1 | 8 | 1 | | 2 | 1 |
| 159 | PG | MBD | 0 | 3 | 1 | 3 | 1 | 1 | 1 | 8 | | | – | |
| 164 | PG | MBD | 0 | 5 | 2 | 3 | 1 | 1 | 2 | 8 | 2 | | 2 | |

Host: PG is pig, Origin: MBD is Mubende district.

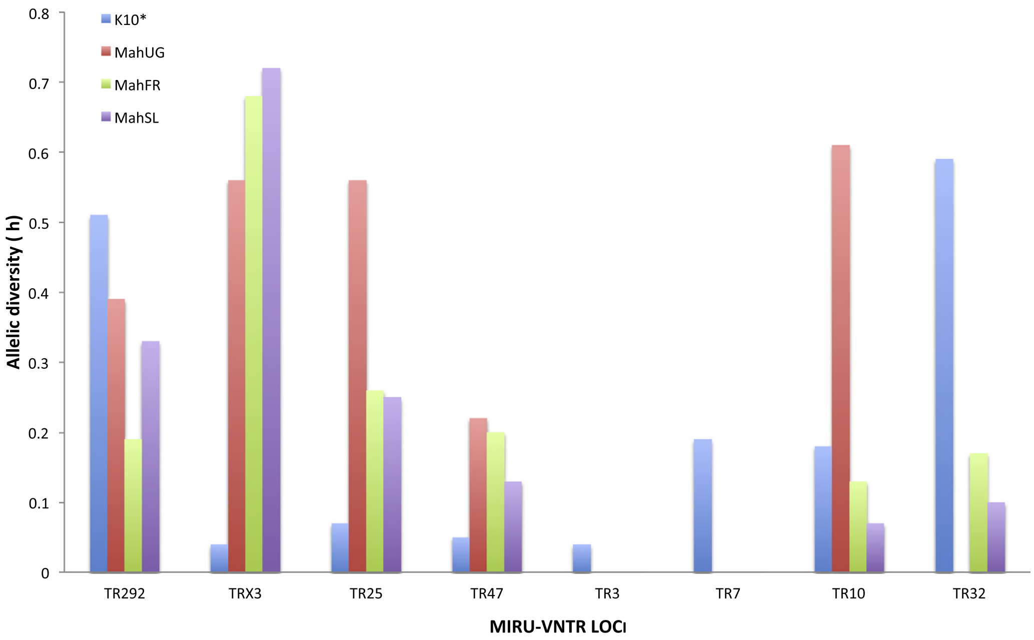


**Fig. 2.** Bar chat showing a comparison of allelic diversity based on 8-MIRU – loci for *M. avium* subsp. *hominissuis* isolates recovered from Uganda, France (Radomski et al., 2010) and Slovenia (Pate et al., 2011).

Ugandan *M. avium* subsp. *hominissuis* isolates lacked MATR14 (Fig. 1 & Table 1).

### 3.3.2. Geographic comparison

In general, *M. avium* subsp. *hominissuis* clustered according to their geographic source (Figs. 1 & 3B). This was however not true for all isolates; for example isolate AM87B from Finland had an identical MIRU-VNTR genotype with a Mubende porcine cluster from Uganda (Figs. 1 and 3). Similarly a number of shared MIRU-VNTR genotypes between the European countries are revealed by Figs. 1 & 3B. Although *M. avium* subsp. *hominissuis* has been recovered from cattle and humans, majority of the isolates are recovered from porcine. (Fig. 3A). The minimum spanning trees also reveal MIRU-VNTR genotype that spans across species as well as European boundaries (Fig. 3).

## 4. Discussion

In this study four of the most allelic diverse MATR loci used by Inagaki et al. (2009) and eight MIRU loci used by Thibault et al. (2007) were used to characterize *M. avium* isolated from humans in Karamoja, cattle in Karamoja, Masindi, Nakasongola districts and pigs in Mubende district all located in the Uganda cattle corridor (UCC). The loci adequately typed *M. avium* subsp. *hominissuis* strains isolated from pigs in Mubende between 2008 and 2009 and cattle and humans in Karamoja between 2004 and 2005.

**Table 3**
A comparison of MATR-VNTR allelic distribution among *M. avium* subsp. *hominissuis* isolated from Uganda and Japan.

| Locus | Source | Number of isolates with these no. of copies | | | | | | | | | | | Allelic diversity (h) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| MATR1 | Mah$^{UG}$ | | 3 | 33 | | | | | | | | | 0.13 |
| | Ma$^{JP}$ | 1 | 30 | 38 | | | | | | | | | 0.51 |
| MATR7 | Mah$^{UG}$ | 1 | 2 | | 13 | | 17 | | | 1 | | | 0.68 |
| | Ma$^{JP}$ | | 20 | 27 | 3 | | 5 | 14 | 1 | | | | 0.71 |
| MATR13 | Mah$^{UG}$ | 4 | 32 | | | | | | | | | | 0.22 |
| | Ma$^{JP}$ | 38 | 3 | 29 | | | | | | | | | 0.52 |
| MATR14 | Mah$^{UG}$ | 1 | 4 | | 1 | 4 | | | | | | | – |
| | Ma$^{JP}$ | | 1 | 45 | 22 | 2 | | | | | | | 0.48 |

Mah$^{UG}$ Ma$^{JP}$(Inagaki et al., 2009) are isolates of *M. avium* subsp. *hominissuis*, *M. avium* sp. from Uganda and Japan respectively, MATR14 allelic diversity is not included because it was absent from majority of the Ugandan isolates.

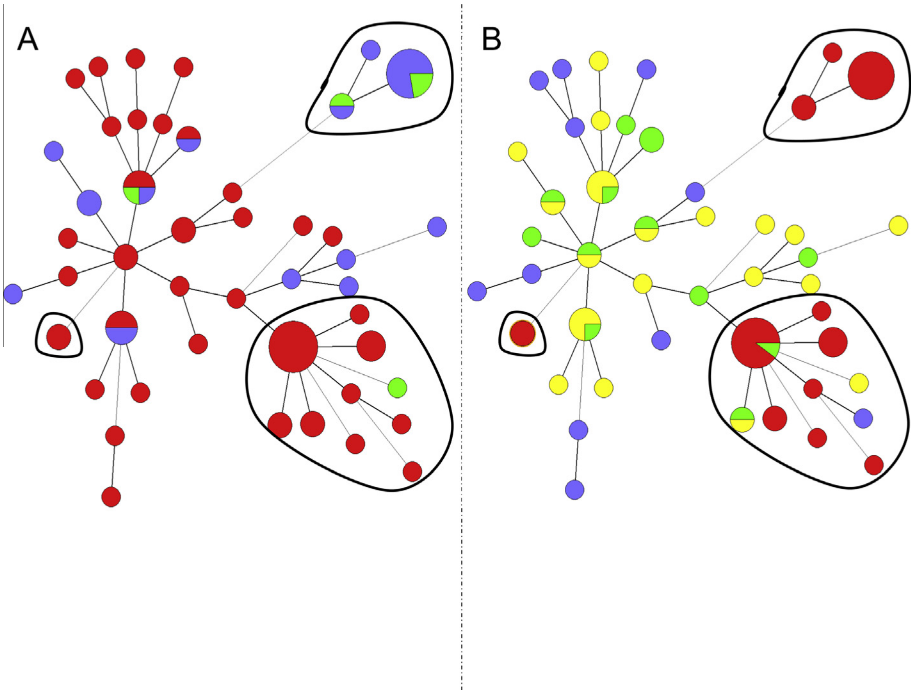


**Fig. 3.** Minimum spanning trees based on 8 MIRU loci of *M. avium*. subsp. *hominissuis*. The trees are constructed using 32 isolates, 14 genotypes, 24 genotypes, and 11 genotypes from Uganda, Finland, France and Slovenia respectively. The molecular relationship is computed based on a single loci variation (SLV). Tree (A): is a host-based tree; red, blue and green represent porcine, human and cattle isolates respectively. Tree (B): is a country-based tree; red, yellow, green and blue represent isolates from Uganda, France, Finland and Slovenia respectively. The distance between VNTR-genotypes and thickness of the connecting line represents the degree of variation between any two connected variants. The size of the circle represents the frequency of a VNTR-genotype. The highlights show where majority of the Uganda isolates lay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The genetic diversity analysis of isolates of human, bovine and porcine origin revealed homology in *M. avium* subsp. *hominissuis* isolates recovered from the North Eastern parts of the UCC. Seven human isolates shared a VNTR genotype with one of the isolates from cattle while one human and one cattle isolate mutual exclusively share a VNTR genotype. Although these identical genotypes occur in the North Eastern parts of the UCC, their molecular proximity does not seem to fit with their spatial information. This is because human isolates are recovered from Karamoja and cattle isolates were from Masindi and Nakasongola district (Oloya et al., 2007, 2008). This finding seems to point towards a common environmental source within the UCC rather than direct transmission between these hosts. The limited access to safe water for human use means that pastoralists share water sources with cattle, especially the soiled swampy areas believed to contain a high load of mycobacteria including MAC in rainy seasons (Oloya et al., 2008; Kankya et al., 2011). Although *M. avium* subsp. *hominissuis* was not recovered from human hosts in Mubende district (Muwonge et al., 2013), other studies have documented presence of identical isolates from human and pigs (Mijs et al., 2002). The findings in this study indicate a geographic molecular difference in *M. avium* subsp. *hominissuis* isolates. The Mubende district isolates are different from the rest of the Ugandan isolates at five of the eight MIRU loci hence the two clusters, which given the difference in sampling time could also reflect temporal difference in the isolates.

All isolates from areas of the UCC other than Mubende district lacked MATR14 and TR3. This could be an indication that these loci might not generally be present in isolates from this part of the UCC or in isolates recovered from cattle and human. The latter is not supported by the comparison with European isolates, which shows the presence of TR3 in all cattle and human recovered isolates. Although the studies in Japan document presence of MATR14 in all samples, it seems to support the absence of TR3. The Japanese study attributed the presence and absence of TR3 to geographic differences of the isolates (Inagaki et al., 2009).

Overall, loci TR3, TR7 and TR32 gave no allelic diversity, implying that they contributed little to the discriminatory power of this typing tool. Therefore, TR3 and TR7 could be the least desirable for MIRU-VNTR typing of *M. avium* isolates in Uganda. In contrast, the isolates from France and Slovenia produce some level of allelic diversity for these three loci although in general this was below 0.5 (Radomski et al., 2010; Pate et al., 2011). The rest of the MIRU loci gave variable allelic diversity that contributed sufficiently to the discriminatory power of this tool. It should however be noted that Ugandan isolates produced significantly higher allelic diversities at TR25 and TR10 compared to isolates from France and Slovenia (Thibault et al., 2007; Radomski et al., 2010; Pate et al., 2011). This furthermore highlights the geographic molecular differences of *M. avium* subsp. *hominissuis* isolates. MATR7 produced the highest allelic diversity among the MATR a finding that is in agreement with (Inagaki et al., 2009). On the other hand MATR1 and MATR13 produced almost half the allelic diversity reported in the same study (Inagaki et al., 2009). This too tends to support the geographic molecular difference of isolates. TRX3 produced multiple amplifications and thus variable repeats for some isolates, an attribute that has been previously reported in Slovenian and Japanese isolates (Pate et al., 2011; Inagaki et al., 2009). All porcine isolates lacked repeats on TR292 that seems to form part of the distinguishing feature from human and cattle recovered isolates in Uganda. If validated in larger studies, this could potentially form a molecular marker that differentiates *M. avium* subsp. *hominissuis* of pig origin in this area. This is however in contrast to findings from European studies which have reported repeats at this loci ranging from complete absence to as high as four repeats (Tirkkonen et al., 2010; Radomski et al., 2010; Pate et al., 2011) .

The reports from Japan, France and Slovenia give a discriminatory power of 0.95, 0.89 and 0.87 respectively, while the combined panel in the current study gave a discriminatory power of 0.88. Therefore the four extra MATR gave a mere 0.014 unit increment to the discriminatory power over the study in Slovenia (Pate et al., 2011). The increment would probably have been significant if the rest of the MATR had given the same allelic diversity as that in the Japanese study (Inagaki et al., 2009).

Seven of the isolates produced multiple repeats at three of the twelve loci used in this study, a phenomenon which could indicate multiple strain infections. On the other hand, one of the loci (TRX3) has been reported to be prone to multiple repeats (Inagaki et al., 2009), this therefore rules out its contribution to the multiple infection state in this case. This leaves only MATR, which have not been extensively explored out side Japan, therefore, this observation could as well be a function of the loci (MATR1 and MATR7) as is the case for TRX3 rather than a reflection of multiple strains. On the other hand, five isolates that were not typed could be mixed infections because the samples produced faint bands on repeated attempts for IS1245 and IS901 PCR.

In general, most of the isolates clustered according to the geographic area of isolation, an observation which seems to suggest that the epidemiology of *M. avium* subsp. *hominissuis* is usually local in nature. There were however some trans-boundary as well as trans-continental VNTR genotypic similarities. The identical VNTR genotype between Ugandan and Finnish isolates is most likely to be a case of "homoplastic genotypes" rather than an epidemiological linkage given the spatial difference in the isolates. On the other hand the identical VNTR genotypes between France and Finland could in fact represent an epidemiologic linkage between the two geographic areas as well as the porcine and human hosts.

The clustering among porcine isolates in Mubende district can be traced back to Madudu Sub County. Some of the isolates in this cluster were also recovered from Butologo and Kiyini Sub County. Butologo is to the north, while Kiyuni is to the west of Madudu Sub County, suggesting that environmental sources in Madudu could be harboring *M. avium* subsp. *hominissuis* to which scavenging pigs from the neighboring sub counties are exposed. This seems to be in agreement with the findings from the environment and slaughter pigs in the same district (Kankya et al., 2011; Muwonge et al., 2012).

The study could not compare pig and human isolates from Mubende district, because *M. avium* was not recovered from humans in this district (Muwonge et al., 2013). Furthermore, the panel of loci used in this study was not able to discriminate between *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis*. The authors were unable to run further experiments to investigate the reason why this was the case. Literature however seems to suggest that the poor differentiation between the two sub species by this panel of loci could lay in the selection of genomic segments used for this purpose. Pate et al. (2011) noted that the up-to-date reporting on this tool is done based on the currently accessible complete genome of *M. avium* subsp. *paratuberculosis* strain K10 and the *M. avium* subsp. *hominissuis* strain 104 (Turenne et al., 2007; Pate et al., 2011) even when salient genomic differences as well as allelic diversity characteristics have been noted. None-the-less, *M. avium* subsp. *avium* were further identified by 16S rDNA sequencing in the current study. Since this was the first attempt in this area to characterize isolates with this panel of loci, the tool fairly discriminated between *M. avium* subsp. *hominissuis* isolates. It however leaves room for improvement with other combinations or novel geographically and/or host specific loci.

## 5. Conclusions

The findings in this study revealed a molecular heterogeneity between *M. avium* subsp. *hominissuis* isolates from within Mubende and between districts in the UCC. There was homology in VNTR genotypes isolated from human and cattle isolates in North Eastern UCC, suggesting a shared environmental source or human exposure from cattle. Although local isolates showed a similar allelic profile to those in Europe, there was a marked difference in the allelic diversity at two of the 8-MIRU. This indicates subtle differences in the Ugandan *M. avium* subsp. *hominissuis* isolates which is most likely to influence the discriminatory power of the tool. It is therefore imperative to continue mining the *M. avium* genome for new VNTR loci with high allelic diversity specific for this geographic area.

## Authors' contributions

A.M. contributed to the conception, design, and data collection, laboratory work, drafting and writing of the manuscript. J.O. contributed to data collection and manuscript drafting. T.B.J., C.K. contributed to laboratory work, data analysis and drafting of the manuscript. S.N. contributed to the laboratory analysis and drafting of the manuscript. J.G., E.S. and B.D. contributed to the acquisition of funds, design of study and drafting of the manuscript. All authors have read and approved the final manuscript.

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## 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.2013.11.012.

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