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).

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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*