Infection, Genetics and Evolution 12 (2012) 846–852

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Genetic diversity of *Mycobacterium avium* subsp. *hominissuis* strains isolated from humans, pigs, and human living environment

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ARTICLE INFO

Article history:
Available online 2 July 2011

Keywords:
Mycobacterium avium subsp. *hominissuis*
Variable numbers of tandem repeat
Minimum spanning tree
Genetic diversity
Molecular epidemiology
Insertion sequence

ABSTRACT

Mycobacterium avium subsp. *hominissuis* (MAH) strains are genetically diverse and cause infections in pigs and humans. To elucidate the geographical and host-dependent variations in the genetic diversity of MAH, we performed variable numbers of tandem repeat (VNTR) analysis targeting 19 loci for MAH samples from humans ($n = 146$), bathroom environments ($n = 37$), and pigs ($n = 75$) in Japan; these data were then compared with previously reported VNTR data from other countries. The minimum spanning tree (MST) and the multi-dimensional scaling (MDS) analyses based on the VNTR data indicated a high degree of genetic relatedness between isolates from humans and bathrooms in Japan, but a low degree of similarity with the isolates from France and Finland. Moreover, the comparison showed a higher similarity of isolates from Japanese pigs with those from French humans and pigs and Finnish humans and pigs than with other isolates from humans and bathrooms in Japan. The singularity of the Japanese MAH was characterized as the prevalence of *hsp65* sequevar code 15 and IS*Mav6* for the human and bathroom isolates; however, none of the isolates obtained from the pigs belonged to the code 15 or possessed IS*Mav6*. The genetic diversity of MAH and its regional variations imply a possible regional or local specific source of infection and route of transmission of MAH for humans.

1. Introduction

Nontuberculous mycobacteria (NTM) are normal inhabitants of both natural and human-engineered environments, wherein they encounter humans and animals (Falkinham, 1996, 2010; Feazel et al., 2009; Winthrop, 2010). They are known as environmental opportunistic pathogens of humans and animals, which are transmitted between the environment, wildlife, livestock, and humans (Biet et al., 2005; Cayrou et al., 2010). Although NTM-associated disease has been reported primarily among immunocompromised persons, it is being increasingly observed among those without any predisposing conditions (Petrini, 2006; Prevots et al., 2010; Primm et al., 2004). The increase in the number of cases of nontuberculous mycobacteriosis has led to increasing awareness of the pathogens as a global public health concern. Therefore, the better understanding of its ecology, reservoirs, and vehicle for human infection is indispensable for developing disease control and/or eradication strategies.

In many countries, especially in developed countries, the most frequent agents of nontuberculous mycobacteriosis belong to the *Mycobacterium avium* complex (MAC) (Glassroth, 2008; Sakatani, 1999; Winthrop, 2010). In particular, *M. avium* subsp. *hominissuis* (MAH) is a frequent agent of human mycobacteriosis (Ichikawa et al., 2009; Johansen et al., 2007; Maekura et al., 2005; Mijs et al., 2002). *M. avium* is thermophilic (du Moulin et al., 1988), resistant to chemical germicides (Taylor et al., 2000; Wendt et al., 1980), and readily aerosolized (Angenent et al., 2005; Parker et al., 1983). Because of the physiological characteristics of *M. avium*, it is plausible that manmade alterations to the aquatic environment (e.g., hot-water systems, hot tubs, and showers, where humans can be exposed to aerosolized bacterial cells) have increased the risk of *M. avium* infection (Feazel et al., 2009; Mangione et al., 2001). Indeed, numerous studies have attempted to establish the link between *M. avium* infection and its putative anthropogenic reservoirs (Falkinham, 2010). There are some reports of matching

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doi:10.1016/j.meegid.2011.06.018

DNA fingerprint between patient and human residential settings, that is, drinking water (Aronson et al., 1999; Hilborn et al., 2008; von Reyn et al., 1994), shower water and showerheads (Falkinham et al., 2008; Mangione et al., 2001), hot tubs and bathrooms (Embil et al., 1997; Falkinham et al., 2008; Kahana et al., 1997; Mangione et al., 2001; Nishiuchi et al., 2007, 2009). Besides the environmental surveillances, the zoonotic aspects of *M. avium* have been extensively investigated in the field to determine the exposure risk for humans. Several studies have reported close genetic relatedness between human and pig isolates (Johansen et al., 2007; Komijn et al., 1999; Mobius et al., 2006; Ramasoota et al., 2001; Tirkkonen et al., 2007, 2010), suggesting either a common source of infection or a possible transmission from pigs to humans, although this possibility has never been demonstrated. Currently, for these molecular epidemiological investigations, fingerprinting methods, such as restriction fragment length polymorphism (RFLP), based on the IS*1311* and IS*1245* insertion sequences and pulse-field gel electrophoresis (PFGE) have been used as the standard typing method. The methods are technically demanding and time consuming. Moreover, the critical drawback of the methods is a lack of interlaboratory reproducibility, which makes comparison with different experiments and laboratories difficult. Due to the technical limitations, worldwide data accumulation and/or comparison of the available fingerprinting data from different studies are not currently feasible. As the results, current molecular epidemiological studies of *M. avium* are restricted to the small size or local population.

In order to resolve these issues, and to facilitate the comparison of different studies and data accumulation, the variable-numbers of tandem-repeat (VNTR) analysis, which is a simple polymerase chain reaction (PCR)-based molecular typing method, has recently been introduced as a promising molecular epidemiological tool for *M. avium*, especially for MAH (Inagaki et al., 2009; Radomski et al., 2010; Thibault et al., 2007). This method has been successfully used to genotype *Mycobacterium tuberculosis* strains and has facilitated the unraveling of the global epidemiological aspects of the pathogen, such as the worldwide prevalence of the certain genotypes (Hanekom et al., 2007; Wada et al., 2009), phylogeographical distribution of the certain lineages (Kang et al., 2010; Maeda et al., 2010; Mokrousov et al., 2002), and global genetic diversity of the pathogen (Allix-Beguec et al., 2008; Mokrousov et al., 2008). Moreover, the data have been used to study its evolution (Mokrousov et al., 2005; Supply et al., 2003; Wada and Iwamoto, 2009; Wirth et al., 2008) and the history of its host adaptation (Mokrousov, 2007, 2008). The breakthrough in the study of *M. tuberculosis* can be expected to occur for MAH. Applying VNTR to molecular epidemiological studies of MAH would provide a promising direction for obtaining insights into global epidemiology and could contribute to developing disease control.

In this work, we examined the geographical variations of the genetic diversity of MAH by comparing our VNTR data obtained from human, pig, and a human living environment (bathroom) isolates in Japan with previously reported data of French isolates (Radomski et al., 2010) and Finnish isolates (Tirkkonen et al., 2010) from human and pig. We also aimed to elucidate the genetic characteristics of MAH isolates from different sources (human, pig, and bathroom) and to show the genetic relatedness among them, and to determine whether specific sublineages or genotypes of MAH are associated with human pulmonary infection.

2. Materials and methods

2.1. Bacterial isolates

We used 258 strains for this study: 146 isolates from human patients, 37 isolates from bathrooms, and 75 isolates from pigs. They were identified as *M. avium* by the sequencing of 16S rRNA gene. The *hsp65* sequencing analyses that were performed in this study confirmed all of the isolates belonged to MAH. These isolates were collected from Osaka (western Japan) and Hokkaido (northern Japan), which are about 1000 km apart from each other. The human isolates were obtained from the sputa of patients with pulmonary MAC infection, except for seven isolates from other clinical specimens such as bronchial lavage fluid, at Kinki-chuo Chest Medical Center (93 isolates from 93 patients) in 2007, Hokkaido University Hospital (31 isolates from 31 patients) between 2002 and 2007, and Toneyama National Hospital (22 isolates from 20 patients) between 2001 and 2010. Kinki-chuo Chest Medical Center and Toneyama National Hospital serve mainly the inhabitants of the Osaka area, whereas Hokkaido University Hospital mainly serves the inhabitants of the Hokkaido area. All the sampled patients at Kinki-chuo Chest Medical Center and Toneyama National Hospital were negative for human immunodeficiency virus (HIV). The HIV status of 31 patients at Hokkaido University Hospital was unknown, but most of them were considered negative for HIV infection because of its low incidence in Japan. The 37 isolates from bathrooms were selected from the strains collection at Osaka City University, according to their VNTR genotyping profiles. These isolates were derived from the 27 residential bathrooms of 26 patients and one healthy volunteer during the previously conducted environmental surveillance study (Nishiuchi et al., 2007, 2009). These isolates represented all of the different VNTR genotyping profiles observed in the isolates obtained through the environmental surveillance study. Details regarding isolates from the previous study are summarized in the Supplementary Table S1. Seventy *M. avium* strains were isolated from 70 slaughtered pigs from an Osaka slaughterhouse where pigs were gathered from 10 different prefectures located in the western part of Japan, between 2004 and 2007. Five strains were isolated from three slaughtered pigs from a slaughterhouse in Iwamizawa, Hokkaido in 2006.

2.2. DNA extraction

A loopful of colonies from each strain was suspended in 300 µl of 1× TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA) and boiled for 10 min. The crude lysates were used for further PCR amplification.

2.3. VNTR typing and minimum spanning tree (MST)

The genotypic data of 19 loci that comprised mycobacterial interspersed repetitive units (MIRU)-VNTR targeting 8 loci (Thibault et al., 2007) and *M. avium* tandem repeats (MATR)-VNTR (Inagaki et al., 2009) targeting 15 loci were analyzed. Three loci overlapped between MIRU-VNTR and MATR-VNTR: MIRU-292 and MATR-2, MIRU-X3 and MATR-3, and MIRU-10 and MATR-9. We excluded the TR-3 locus, which is one of the 8 loci MIRU-VNTR, from our genotyping analysis owing to the large number of isolates that did not yield PCR products at this locus, an observation also reported by Inagaki et al. (2009) for *M. avium* clinical isolates in Japan. This locus is also known as a monomorphic locus for MAH (Pate et al., 2010; Radomski et al., 2010). Therefore, the present study involving a 7-loci MIRU-VNTR analysis is equivalent to the previously reported study on 8-loci MIRU-VNTR. The allelic diversity of each VNTR locus was evaluated by Nei's diversity index, i.e. the polymorphic information content (PCI) (Keim et al., 2000). The level of genotypic diversity based on each VNTR loci set was calculated using the Hunter–Gaston discriminatory index (HGDI) (Hunter and Gaston, 1988).

The MIRU-VNTR data from previous reports (Radomski et al., 2010; Tirkkonen et al., 2010) were retrieved for the geographical