Species identification of non-tuberculous mycobacteria from humans and cattle of Chad

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Summary

In Chad, during a study on tuberculosis in humans and cattle, 52 non-tuberculous mycobacteria (NTM) strains were isolated. By means of INNO-LiPA, PRA-*hsp65* amplification and sequencing of 16S rDNA, NTM species of 25/52 isolates were identified. *M. fortuitum* complex (8) was the most frequent species, followed by *M. nonchromogenicum* (4) and *M. avium* complex (4). PRA method could identify *M. fortuitum* 3rd variant among isolates derived from cattle specimens. This finding could confirm the existence of farcy in the Chadian cattle population as *M. fortuitum* 3rd variant and putitative pathogen *M. farcinogenes* can't be distinguished by the methods used in this study. Half of the NTM isolates could not be specified and we considered them as contaminants from the environment.

Keywords: non-tuberculous mycobacteria, Chad, molecular methods, INNO-LiPA assay, PRA amplification, sequencing of the 16S gene

Spezies Identifikation von nicht-Tuberkulosen Mykobakterien bei Mensch und Rind im Tschad

Während einer Studie im Tschad, welche die Tuberkulose bei Mensch und Rind untersucht, wurden 52 nicht-Tuberkulose Mykobakterien (NTM) isoliert. Mit Hilfe von INNO-LiPA tests, PRA-*hsp65* Amplifizierung und Sequenzierung der 16S rDNS, konnten 25/52 Isolate identifiziert werden. *M. fortuitum* complex (8) war die häufigste Spezies, gefolgt von *M. nonchromogenicum* (4) und *M. avium* complex (4). Die PRA-Methode konnte bei Isolaten von Kühen die dritte Variante von *M. fortuitum* identifizieren. Dieser Befund könnte die Existenz von Farcy in der tschadischen Kuhpopulation bedeuten, weil die dritte Variante von *M. fortuitum* vom vermuteten Farcy-Erreger *M. farcinogenes* nicht mit den in dieser Studie angewendeten Methoden unterschieden werden kann. Die Hälfte der NTM-Isolate konnte nicht identifiziert werden und wir betrachten diese als Kontaminationen aus der Umwelt.

Schlüsselwörter: Nicht-Tuberkulose Mykobakterien (NTM), Tschad, molekulare Methoden, INNO-LiPA, PRA Amplifizierung, Sequenzierung des 16S Gens

Introduction

With the increase in human tuberculosis cases and the advent of HIV/AIDS, there has been resurgence in interest in diseases caused by non-tuberculous mycobacteria (NTM). NTM are subdivided into rapid and slow growers. Their ecologic niche is the environment, as they have been found in soil, plants, house dust and water. In contrast, animals are not considered as an important reservoir for NTM (Saiman, 2004). However, they can cause infections in humans and animals (Phillips and von Reyn, 2001; Hamid et al., 1991; Alander-Damsten et al., 2003; Valheim et al., 2001). Mycobacteria cause a variety of illnesses, which have profound individual and public health implications. The clinical symptomatology of these diseases is not different from classical tuberculosis (Dvorska et al., 2001), but their therapy is problematic due to the high resistance to antituberculous drugs seen for most ubiquitous mycobacteria (Schutt-Gerowitt, 1995).

Reports on NTM infections in humans and animals in Africa are scarce. Most published studies are from South Africa, and specifically on investigations in the South African gold mines where *Mycobacterium kansasii* and *M. scrofulaceum* were the main causes of mycobacterial diseases (Churchyard et al., 1999; Corbett et al., 1999) and the first case of infection with *M. marinum* since 1987 was reported (Mousdicas and Saxe, 1987). For the others part of Africa,

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information can be found in studies on AIDS patients. In Burkina Faso, Ledru et al. (1996) found that 6.5% of mycobacterial isolations from AIDS patients were NTM without further specification, and in Nigeria, Idigbe et al. (1994) identified 20% *M. avium* and 10% *M. kansasii* among their isolates. In livestock, the serological investigation detected antibodies to *M. paratuberculosis* in camels and goats in Kenya (Paling et al., 1988). *M. farcinogenes* was described as main causal agent of bovine farcy in Sudan (Hamid et al., 2002).

In Chad, during a study of two years on tuberculosis in humans and animals, numerous *Mycobacterium tuberculosis* complex (MTC) and NTM isolates were obtained. The purpose of the present article is to report the different NTM species found among mycobacterial strains from Chad.

Materials and Methods

Isolates and study sites

1) Specimens collected in 5 Chadian health centres (sputum and urine) and in one slaughterhouse (tubercles from lymph nodes, lung, spleen, liver and pleural cavity of condemned cattle's carcass) in N'Djaména, were subjected to decontamination and cultivation. Obtained mycobacterial isolates were identified by biochemical testing (Kent and Kubica, 1985). On the basis of biochemical tests results, the isolates were categorised in *M. tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM). These preliminary studies were performed at the "Laboratoire de Recherches Vétérinaires et Zootechniques de Farcha (LRVZ/F)" in Chad.
2) Thirty six NTM had been sent to the Institut Pasteur (IP) in Paris for identification NTM species by molecular method.
3) Sixteen NTM strains were characterized at the National Centre of Mycobacteria (NCM) Zurich.

Molecular methods

1) The INNO-LiPA assay was carried out according to manufacturer's instructions and using the reagents provided with the LiPA kit (Versant® INNO-LiPA HCV II). The protocol consisted of PCR amplification, hybridization of the PCR products to the strips, detection and interpretation of the results (Suffys et al., 2001)
2) PRA amplification was performed according to the procedure described by Telenti et al. (1993). This method amplified a 439-bp fragment of the *hsp65* gene.
3) Real-time PCR
   DNA extraction and subsequently amplification and identification were carried out according to the procedure described by Kraus et al. (2001). This method allowed the classification of NTM and MTC strains which were all previously categorised as MTC by biochemistry.
4) Sequencing of the 16S gene
   The obtained PCR products were used to perform the sequencing of the 16S gene. The sequence processing was done with computer software from ABI PRISM™ 310 (Applied Biosystems). Alignments of mycobacterial 16S rDNA sequences were done with the Model 310 (version 3.4.1.) alignment tool. All probe sequences were subsequently matched with sequences in the GenBank by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) to detect sequence similarity. A similarity of 98 to 99% suggests that the obtained sequence likely derives from this species (Turenne et al., 2001). The search was performed at the National Centre of Mycobacteria in Zurich.

Results

At the LRVZ of N'Djaména, biochemical testing revealed a total of 52 NTM isolates, which were further characterized by three different molecular methods (INNO-LiPA, PRA-*hsp65* and 16S (rDNA). We analyzed 36 isolates by INNO-LiPA and PRA-*hsp65* at the NTM and 16 isolates by only sequencing of the 16S (rDNA) at the NCM. 25 of 52 isolates resulted in the identification of NTM isolates by at least one of these tools (Tab. 1).

M. fortuitum complex was identified for eight isolates from seven cattle and one human origins and was found the most. Six of them were classified as *M. fortuitum* supsp. perigrum (with INNO-LiPA) of which three were further characterized as *M. fortuitum* 3rd variant by PRA-*hsp65*. *Mycobacterium avium* complex was found for four isolates of which one and one of human (626 UR) and cattle origin (502 GG; Tab. 1) respectively, were classified as *M. intracellulare*. We received also four *M. nonchromogenicum* of cattle origin of which two and two were classified as subsp. mucogenicum and type I, respectively. The three remaining isolates of cattle origin were identified as *Mycobacterium* IWGMT.90093, *M. smiae* and *M. Szulgai/trivialé/brumae*. Further human isolates were *M. mariokaense* (two), *M. celatum* (one), *M. chelonae, Mycobacterium* sp.N120 and also *M. smiae* (one) (Tab. 1).

Discussion and conclusion

M. fortuitum complex was the most frequent NTM species (8/25) in this study and among them, 3 isolates from cattle were identified as the 3rd variant by PRA.

Table 1: Results of NTM species identification of human and cattle origin from Chad with the three methods INNO-LiPA, PRA-hsp65 and16S (rRNA) sequencing. NI: not identified. N/D: not done.

N° of strain	Origin of specimen	INNO-LiPA	PRA-*hsp65*	16S rDNA (% identity)
407CR/G	human	*M. fortuitum* subsp. *peregrinum*	*M. peregrinum/M. porcinum*	*N/D*
219GG	Cattle (Mbororo)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* subsp. *peregrinum*	*N/D*
446GG	Cattle (Mbororo)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum*	*N/D*
455GG	Cattle (Mbororo)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* 3rd variant	*N/D*
454GG	Cattle (Arabe)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* 3rd variant	*N/D*
548PM	Cattle (Arabe)	*M. fortuitum* subsp. *peregrinum*	*M. fortuitum* 3rd variant	*N/D*
483PM/P	Cattle (Arabe)	*N/D*	*N/D*	*M. fortuitum* (99 %)
490GG/P	Cattle (Arabe)	*N/D*	*N/D*	*M. fortuitum* (99 %)
582PM	Cattle (Arabe)	NI	*M. nonchromogenicum* subsp. *mucogenicum*	*N/D*
441PM	Cattle (Mbororo)	NI	*M. nonchromogenicum* subsp. *mucogenicum*	*N/D*
464FOIE	Cattle (Arabe)	NI	*M. nonchromgenicum* type I	*N/D*
522PM	Cattle (Mbororo)	NI	*M. nonchromogenicum* type I	*N/D*
663PM	Cattle (Mbororo)	*M. avium complex*	*M. intracellulare/MAI/scrofulaceum*	*N/D*
502GG	Cattle (Arabe)	*M. avium complex*	*M. intracellulare*	*N/D*
444GG	Cattle (Arabe)	*M. avium complex*	–	*N/D*
626UR	human	*M. intracellulare*	*M. intracell/MAI/scrofulaceum*	*N/D*
661GG/G	Cattle (Arabe)	*N/D*	*N/D*	*M. simiae* (99%)
277CR/G	human	*N/D*	*N/D*	*M. simiae* (100%)
280CR/P	human	*N/D*	*N/D*	*M. moriokaense* (99%)
381UR/P	human	*N/D*	*N/D*	*M. moriokaense* (98%)
637GG/G	Cattle (Arabe)	*N/D*	*N/D*	*Mycobacterium* IWGMT.90093 (99%)
269CR/P	human	*N/D*	*N/D*	*M. celatum* (98%)
430CR/G	human	*N/D*	*N/D*	*M. chelonae* (99%)
685CR/P	human	*N/D*	*N/D*	*Mycobacterium* sp.N120 (98%)
559PM	Cattle (Arabe)	NI	*Szulgai/trivialé/brumae*	N/D

Two studies have demonstrated the exact identity of 16S rDNA of *M. senegalense, M. farcinogenes* and *M. fortuitum* 3rd variant (Kirschner et al., 1992; Turenne et al., 2001). This finding is interesting because it confirms the existence of bovine farcy in the Chadian cattle population which has not been done since 1963 (Perpézat et al., 1963). However, we can't draw any conclusion if the responsible pathogen for farcy is *M. farcinogenes* (like in Sudan) or *M. senegalense* (like a case found in Chad), because of the lack of discrimination of the methods used (Hamid et al., 2002).

Only four isolates were identified as *M. avium* complex and none was *M. kansasii*, while usually these two mycobacteria are the most common NTM in clinical specimens and biopsies (Shih et al., 1997; Marras and Daley, 2002; Thorel, 1980; Pate et al., 2004). Concerning the remaining mycobacteria found in our study, they are rarely isolated but most of them were described as potential pathogens. *Mycobacterium simiae* is commonly found in the environment and was rarely associated with human disease. However, some cases of disease caused by *M. simiae* in AIDS and non-AIDS patients have been reported (Vandercam et al., 1996; Huminer et al., 1993; Bell et al., 1983; Lavy and Yoshpe-Purer, 1982). *M. celatum* was described as cause of fatal pulmonary infection in an old woman (Bux-Gewehr et al., 1998) and of disseminated infection in domestic ferret (Valheim et al., 2001). *M. chelonae* was frequently isolated from patients with cystic fibrosis (Hjelt et al., 1994; Tomashefski et al., 1996; Fauroux et al., 1997). *M. terrae* complex is composed of *M. nonchromogenicum, M. terrae* and *M. triviale*. They are uncommon colonizers of human epithelia and generally regarded as non-pathogenic (Lee et al., 2004). However, *M. nonchromogenicum* may occasionally cause human disease such as pulmonary infection and tenosynovitis (Peters and Morice, 1991).

We obtained many NTM usually found in the environment by sequencing because this method is not focused on pathogen NTM strains. However, we have not confirmed our sequencing results by another molecular method as suggested (Hafner et al. 2004). Generally, NTM isolates should be further characterised because NTM infections can cause disease and first line antituberculosis drug treatment may be not efficacious. In another hand, it will be interesting for veterinarians to know about the importance of NTM in cattle, particularly in interpretation of tuberculin test.

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Identification d'espèces de mycobactéries non tuberculeuses chez l'homme et le bétail au Tchad

Au Tchad, lors d'une étude de la tuberculose humaine et animale, 52 souches de mycobactéries non tuberculeuses (MNT) ont été isolées. La caractérisation génétique des isolats a été réalisée au moyen des tests INNO-LIPA, PRA-hsp65 et le séquençage du 16 rDNA. 25/52 isolats ont pu être identifié. *M. fortuitum* le complexe (8) était l'espèce la plus fréquente, suivie par *M. nonchromogenicum* (4) et *M. avium* le complexe (4). La méthode PRA a pu spécifier *M. fortuitum* variante 3 chez le bétail. Cette découverte peut apporter une preuve supplémentaire sur l'existence du farcin dans le cheptel tchadien, sachant que *M. fortuitum* variante 3 et *M. farcinogenes* ne peuvent pas être distingués par les méthodes utilisées dans cette étude. L'autre moitié des MNT n'ont pas pu être spécifié et nous les avons considéré comme étant des polluants environnementaux.

Identificazione nell'uomo e nel manzo di specie di micobatteri non tubercolari nel Ciad

Durante uno studio in Ciad dove veniva studiata la tubercolosi nell'uomo e nel manzo sono stati isolati 85 micobatteri non tubercolari (NTM). Con l'aiuto del test INNO-LiPA, PRA-hsp65 amplificazione e sequenziamento del 16S r ADN, sono stati identificati 25/52 isolati. La specie più frequente era il complesso *M. fortuitum* (8) seguito da *M. nonchromogenicum* (4) e dal complesso *M. avium* (4). Il metodo PRA ha identificato la terza variante di *M. fortuitum* in isolati nelle mucche. Questo risultato potrebbe significare l'esistenza del farcino bovino nella popolazione di mucche del Ciad poiché la terza variante di *M. fortuitum* non può essere differenziata con i metodi utilizzati in questo studio dal probabile agente patogeno *M. farcinogenes.* Non è stato possibile identificare la metà degli isolati NTM e consideriamo questo come una contaminazione dall'ambiente.

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