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Endosymbiotic *Mycobacterium chelonae* in a *Vermamoeba vermiformis* strain isolated from the nasal mucosa of an HIV patient in Lima, Peru

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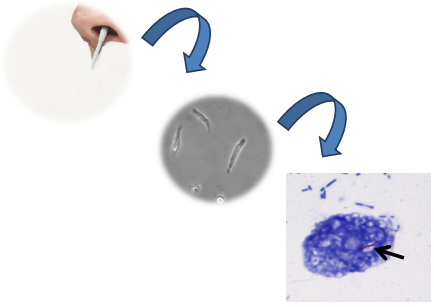
HIGHLIGHTS

- *Mycobacterium chelonae* was identified as an endosymbiont of a FLA strain belonging to the species *Vermamoeba vermiformis*.
- Pathogenic agents such as *Mycobacterium chelonae* are using FLA to propagate and survive in the environment.
- Awareness should be raised within clinicians and public health workers worldwide.

GRAPHICAL ABSTRACT



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ABSTRACT

In March 2010, a 35 year-old HIV/AIDS female patient was admitted to hospital to start treatment with Highly Active Antiretroviral Therapy (HAART) since during a routine control a dramatic decrease in the $CD4^+$ levels was detected. At this stage, a nasal swab from each nostril was collected from the patient to include it in the samples for the case study mentioned above. Moreover, it is important to mention that the patient was diagnosed in 2009 with invasive pneumococcal disease, acute cholecystitis, pancreatitis and pulmonary tuberculosis. The collected nasal swabs from both nostrils were positive for *Vermamoeba vermiformis* species which was identified using morphological and PCR/DNA sequencing approaches. Basic Local Alignment Search Tool (BLAST) homology and phylogenetic analysis confirmed the amoebic strain to belong to *V. vermiformis* species. Molecular identification of the *Mycobacterium* strain was carried out using a bacterial universal primer pair for the 16S rDNA gene at the genus level and the *rpoB* gene was amplified and sequenced as previously described to identify the *Mycobacterium* species (Shin et al., 2008; Sheen et al., 2013). Homology and phylogenetic analyses of the *rpoB* gene confirmed the species as *Mycobacterium chelonae*. In parallel, collected swabs were tested by PCR and were positive for the presence of *V. vermiformis* and *M. chelonae*. This work describes the identification of an emerging bacterial pathogen, *M. chelonae* from a Free-Living Amoebae (FLA) strain belonging to the species *V. vermiformis* that colonized the nasal cavities of an HIV/AIDS patient, previously diagnosed with TB. Awareness within

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clinicians and public health professionals should be raised, as pathogenic agents such as *M. chelonae* may be using FLA to propagate and survive in the environment.

1. Introduction

Free-Living Amoebae (FLA) include opportunistic pathogenic and non-pathogenic species that are distributed worldwide in many environmental sources such as water, soil and dust (Schuster and Visvesvara, 2004; Thomas et al., 2008). The complex ecology of FLA and their role in spreading pathogenic microorganisms through environmental systems (mostly water and dust related) have recently raised considerable interest (Kuiper et al., 2004; Thomas et al., 2008, 2010; Gryseels et al., 2012).

Among the so called Amoebae Resisting Bacteria (ARB) important pathogens such as *Legionella pneumophila*, Non-Tuberculosis Mycobacteria (NTM) and *Pseudomonas aeruginosa* among others have been reported to be able to survive within FLA belonging to *Acanthamoeba*, *Vermamoeba* and *Echinamoeba* genera (Kuiper et al., 2004; Thomas et al., 2010; Mba Medie et al., 2011; Gryseels et al., 2012). Infections caused by members of the *Mycobacterium chelonae–abscessus* complex represent serious emerging public health problem, and their role has expanded, with growing numbers of therapeutic interventions that disrupt the competency of the human immune system and HIV/AIDS individuals. Excepting one species forming this complex, all of them have been implicated in human disease (Adekambi et al., 2004; Simmon et al., 2011; da Costa et al., 2013).

This work describes the identification of an emerging bacterial pathogen, *M. chelonae* from a FLA strain belonging to the species *Vermamoeba vermiformis* that was isolated from the nasal swab of a HIV/AIDS patient previously diagnosed with TB. Previous reports have demonstrated that some *Mycobacterium* species are able to survive within FLA of *Acanthamoeba* genus including *M. chelonae* species implicated in cases of human infections (Adekambi et al., 2004; Munayco et al., 2008; da Costa et al., 2013; Sheen et al., 2013).

To the best of our knowledge, this is the first report of an endosymbiotic relationship between the FLA species *V. vermiformis* and *M. chelonae* in the literature. Interestingly, this FLA species is widely distributed in the environment and even some strains have been isolated in coinfection cases of amoebic keratitis (Aimard et al., 1998).

2. Material and methods

During 2010–2011, nasal swabs samples were collected from 107 HIV/AIDS patients that were admitted to hospital as part of a survey to establish whether FLA colonize the nasal cavities of HIV/AIDS patients in Lima, Peru. Informed consents were obtained from all the individuals whose nasal swabs samples were included in the study and also agreed for the samples to be collected and used for research purposes. Moreover, all procedures were performed in compliance with relevant laws and institutional guidelines from the Universidad Peruana Cayetano Heredia, Lima, Peru.

In March 2010, a 35 year-old HIV/AIDS female patient was admitted to hospital to start treatment with Highly Active Antiretroviral Therapy (HAART) since during a routine control a dramatic decrease in the $CD4^+$ levels (from 648 at the end of 2009 to 55 $CD4^+$ cells/mm^3 in March 2010) was detected. At this stage, a nasal swab from each nostril was collected from the patient to include it in the samples for the case study mentioned above. Moreover, it is important to mention that the patient was diagnosed in 2009 with invasive pneumococcal disease, acute cholecystitis, pancreatitis and pulmonary tuberculosis (TB; baciloscopy positive, BK+). At that time, she did not show any sign of sinusitis or skin lesions but received first line treatment for TB (isoniazid, rifampicin, pyrazinamide and ethambutol). The last visit to hospital of this patient was recorded in 2012, she did not respond to HAART or TB treatment and her CD4+ levels were 54 $CD4^+$ cells/mm^3. After this year, the patient never returned to hospital.

Nasal swabs were collected from each nostril of the patient (2 samples per nostril) and kept in Page's Amoeba Saline Solution (PAS) until further processing in the laboratory (Page, 1988). These swabs were cultured onto 2% Non-Nutrient Agar (NNA) plates covered with a layer of heat killed *Escherichia coli* in order to check for the presence of amoebic growth. Plates were sealed and monitored daily for the presence of amoebae under an inverted microscope for up to 20 days. Blocks of agar were excised from the positive plates and transferred to another NNA plates without any bacteria in order to obtain an amoebic culture without any contaminant.

After that, amoebae were grown axenically in PYG liquid medium (0.75% proteose peptone [wt/vol], 0.75% yeast extract [wt/vol], 1.5% glucose [wt/vol]) containing gentamicin 50 μg/ml (Sigma, Madrid, Spain) and 0.2 ml of the BBL™ MGIT™ PANTA™ Antibiotic Mixture (BD, Madrid, Spain).

DNA from cultures identified as positive for amoebae by microscopy was extracted by placing 1–2 ml of amoebic cultures directly into the Maxwell® 16 Tissue DNA Purification Kit sample cartridge (Promega, Madrid, Spain). *Acanthamoeba* genomic DNA was purified using the Maxwell® 16 Instrument as described in the Maxwell® 16 DNA Purification Kits Technical Manual #TM284 (Promega, Madrid, Spain). DNA yield and purity were determined using the NanoDrop® 1000 spectrophotometer (Fisher Scientific, Madrid, Spain).

After DNA extraction, PCR amplification of FLA 18S rDNA was performed using a universal primer pair as previously described (Smirnov et al., 2011). At the same time, swabs were directly checked also by PCR.

PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced using a MEGABACE 1000 automatic sequencer (Healthcare Biosciences, Barcelona, Spain) in the University of La Laguna Sequencing Services (Servicio de Secuenciación SEGAI, University of La Laguna). The obtained sequences were aligned using Mega 5.0 software program (Tamura et al., 2011).

3. Results and discussion

The collected nasal swabs from both nostrils were positive for *V. vermiformis* species (Fig. 1A) which was identified using morphological and PCR/DNA sequencing approaches (Page, 1988; Smirnov et al., 2011). Homology and phylogenetic analysis confirmed the amoebic strain to belong to *V. vermiformis* species.

Interestingly, when amoebae were growing in axenic conditions, it was observed that the medium presented a high number of the typical clumps of bacilli that mycobacteria form in liquid culture. Furthermore and due to the presence of BBL™ MGIT™ PANTA™ Antibiotic Mixture (a mixture which is intended for the detection and recovery of mycobacteria, eliminating the rest of contaminants) in the medium, these bacteria were highly suspicious to belong to the mycobacteria family.

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