**Mycobacterium leprae**: pathogenic agent in leprosy. Discovery of new species *Mycobacterium lepromatosis*. Perspectives in research and diagnosis of leprosy

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**ABSTRACT**

**Background:** *M. leprae* was presumed as an aetiologic agent in DLL, without specific microbiologic studies in the past. The sequencing of the genome through genetic testing led to the discovery of a new species *M. lepromatosis* by Han et al. in 2008.

**Materials and Methods:** The genetic analysis of the phylogeny of bacteria, based on the analysis of the 16S rRNA coding regions and other genes (e.g., rpoB, rpoT, hsp65, mmaA, fibF-rpsO) appears to be helpful in species discrimination. Twenty genes and pseudogenes (22,818 bp of sequence) proved that in the phylogenetic tree *M. leprae* and *M. lepromatosis* are closely related. On the other hand, the differences were great, which gave rise to the distinction between the two species. Detected insertions in both species of mycobacteria in the rRNA and mmaA genes were similar to each other and were found only in the human genome, which confirms the close relationship during *M. leprae* and *M. lepromatosis* evolution of genomes in the human species.

**Results:** The first population-based study analysing the presence of both mycobacteria indicates that *M. lepromatosis* came to America with human populations migrating from Asia through the Strait of Bering, in contrast to *M. leprae*, which came to America with the colonists and as a result of the slave trade.

**Conclusions:** The latest experiments with *M. lepromatosis* showed that it is a specific agent in DLL, not only in the endemic area in Mexico, but also in different parts of the world. *M. lepromatosis* was also found in other forms of multibacillary leprosy, or as a dual infection with *M. leprae*, as well. There are great expectations that genetic methods with the sequencing of the whole genome will lead to a better understanding of some of the mysteries behind leprosy.

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**Key words:** *Mycobacterium leprae*, *Mycobacterium lepromatosis*, Lucio’s phenomenon, gene sequencing, genetic diversity, phylogeny

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**THE SITUATION OF LEPROSY TODAY**

Leprosy is a chronic, systemic, and infectious disease caused by *Mycobacterium leprae*, affecting principally the peripheral nerves and the skin. Complications due to neuropathy can result in deformity and disability with remaining stigmatisation.

The highest prevalence of leprosy cases worldwide is present in the developing countries, mainly in South-East Asia (India), Latin America (Brazil, Mexico), and Central Africa.

The introduction of effective multidrug therapy by the WHO in the year 1982 decreased the number of cases from 14 million to about 250,000 in recent years [1–3].

There is a dilemma: the number of new cases detected yearly is persistently high with active leprosy, often with grade-2 disabilities. The disparity between prevalence and...
new case detection over the years emphasises the lasting epidemiological threat of leprosy [4]. The chain of transmission has not been broken [5–7].

INTRODUCTION

The epidemiological agent of leprosy is *M. leprae*, an obligate intracellular pathogen, discovered by G. Hansen in 1873, and genome sequenced by S.T. Cole in 2001 [8].

*M. leprae* differs from other human bacilli by non-cultivation *in vitro*, extremely slow generation time (14 days), and long incubation period (2–14 years). *M. leprae* has a unique ability to infect peripheral nerves with the invasion of Schwann cells and vascular endothelial cells [9–12]. Transmission of the disease is not conclusively proven, although it is probably person-to-person via nasal droplet infection or by long contact with infected persons [6, 13]. The role of environmental reservoirs is not completely resolved.

Shepard in 1960 [14] demonstrated the multiplication of *M. leprae* by inoculation into footpads of mice and later into the nude (nu/nu) mouse. This material has been used as a culture medium for different investigations to identify the organism of *M. leprae*, genetic characterisations, and others. The experimental model of animal leprosy was demonstrated by Kirchheimer and Storrs in 1971 [15]. Nine-banded armadillos inoculated with *M. leprae* develop fully disseminated infection with the involvement of internal organs and nerves and characteristic histopathological picture of leprosy [9].

Armadillos, living in some parts of the Southern United States, are a large reservoir for *M. leprae*. Leprosy patients residing in these areas have the possibility of exposure to armadillo-born *M. leprae*. The latest investigations revealed that the infective strains of *M. leprae* are identical both in animals and patients. There is a supposition that leprosy may be a zoonosis in this part of the US [16].

Leprosy presents great diversity in clinical and histopathological manifestations due to immunological and genetic response. Classification identifies on the one hand tuberculoid leprosy (TT) with a high degree of cell-mediated immunity and solitary skin lesions often with undetectable bacilli (paucibacillary leprosy) (Fig. 1). On the other hand, lepromatous leprosy (LL) presents numerous nodular lesions, containing a large number of bacilli (multibacillary leprosy) with a characteristic anergy to *M. leprae* and low degree of cell-mediated immunity [17–19] (Fig. 2).

Leprosy reactions are the most dangerous events in the course of the disease among 30–50% of patients. They represent acute inflammatory and immunological complications, leading to severe nerve impairment and disability. Type I reaction occurs in paucibacillary leprosy with exacerbation of existing lesions without constitutional symptoms. The cardinal features of type II reaction are deep, painful nodules, often ulcerating. Erythema nodosum leprosum (ENL) is a systemic disease with high fever, malaise, and involvement of any organ or tissue where the bacilli are found. (*neuritis, iridocyclitis, orchitis*, and others) (Figs. 3, 4) [9, 20, 21].
Concerning the history and nomenclature, the disease was reported by Lucio and Alvadoro in 1852, and later by Latapi and Chévez-Zamora in 1948. DLL is also called Diffuse Leprosy of Lucio and Latapi or Lucio's phenomenon/erythema necroticans. The Caribbean DLL is a severe and rare form of leprosy with high mortality, common in Mexico. Occasional cases were reported worldwide, including Asia, Africa, North America, and Brazil. The clinical pictures of DLL appear as diffuse, shiny infiltrations of the skin with no reactional phase in the initial stage. Gradually spreading erythema (purple in colour) is a signal of haemorrhagic infarcts. Plagues and blister formations, and later painful necrotic ulcers are healing with atrophic, stellar scars. This form of reaction is called Lucio’s phenomenon/erythema necroticans (Fig. 5). Clinically, there are additional symptoms occurring in multibacillary leprosy such as: dysaesthesia, anhidrosis, madarosis, alopecia, and destructive rhinitis. Histological findings suggest that Lucio’s phenomenon is a vascular disorder produced by massive, direct invasion of vascular endothelial cells by \textit{M. leprae}. Dilatation of vessels, endothelial proliferation, luminal occlusion, thrombosis, and ischaemic necrosis are the cardinal features [22–26].

Vasculonecrosis reactions are present in Lucio’s phenomenon and in ENL. Despite the possibility of distinguishing them through clinical and histopathological characteristics, both are often used as synonyms. This may have therapeutic implications, justifying the use of different drugs in these two kinds of reaction. For instance: Thalidomide gives excellent results in ENL but is worthless in Lucio’s phenomenon [27].

Erythema necroticans, being confined to multibacillary lepromatous leprosy, needs not only clinical and histopathological endorsement for the diagnosis but also genetic and molecular investigations. The next step for better understanding of the unclear pathogenesis of Lucio’s phenomenon is the discovery of a new species of \textit{M. leprae} — \textit{Mycobacterium lepromatosis} by Han et al. in 2008 [28].

**MYCOBACTERIUM LEPRAE AND MYCOBACTERIUM LEPROMATOSIS COMPARISON AND PHYLOGENY**

In 2001, owing to \textit{M. leprae} whole genome sequencing and comparing it with the genomes of other mycobacteria, it was discovered that the genome of the leprosy mycobacte-
Leprosy had significantly reduced. This reduction was the result of massive loss of large parts of the genome, leaving only a gene essential for the transmission, infectivity, and survival in human cells. A key role in this process was played by repetitive sequences (RLEP, REPLEP, LEPREP, and LEPRPT). This suggests targeted mycobacterial speciation [8, 29].

Compared to other mycobacterial species, the Mycobacterium leprae genome is small. For example, the genome of leprosy consists of 3.2 Mbps (Mb - million base pairs) as compared to the genome of Mycobacterium tuberculosis, the volume of which amounts to 4.4 Mb [30].

Leprosy as a disease of man has been known since ancient times [31]. Lucio’s phenomenon, an aggressive form of leprosy, was described in 1852. Only thanks to genetic testing made in 2008, was it discovered that it is caused by a separate species [28].

Genetic analysis of the phylogeny of bacteria is based on the analysis of conservative (almost invariant) regions (DNA sequences) of the bacterial genome [32]. They are usually the 16S rRNA coding regions and other genes (e.g. rpoB-RNA polymerase β subunit, rpoT-RNA polymerase δ factor, hsp65-65kDa heat shock protein, mmaA-metyl mycolic acid synthases 3 and 4, and fibF-rpsO-riboflavin kinase and ribosomal protein S15). Using this methodical approach, it is possible to detect the presence of bacterial species whose genome sequence is not yet sequenced [28].

The difference between M. leprae and M. lepromatosis in the studied regions was 2.1% and was the basis for the distinction of a new species. Testing the rRNA segment taken from a sample from a DLL patient had 1,504 bp and, comparing to the corresponding section in the M. leprae, showed 97.9% identity. As a result of the careful analysis of rRNA sequences 19 nucleotide insertion was detected. This insertion was present in a place that in the genome of leprosy is occupied by 16 nucleotide insertion, which is highly characteristic of this species. The two insertion sequences were not similar to each other, which is also proved by the distinction between M. leprae and M. lepromatosis. It is interesting that very similar sequences were found in the human genome, which may indicate a long history of common evolution of mycobacterial and the human species [33].

Gene sequences of mmaA and riff-rpsO from an analysed case of the M. lepromatosis showed a great difference in comparison to the sequence of M. leprae, which were 14% and 8.5%, respectively. In addition, the analysis of mmaA gene sequences from both mycobacteria revealed a 21 nucleotide sequence similar to the rRNA insertion described above, which was found only in the human genome, confirming the close relationship during Mycobacterium genome evolution in the human species. Compared to other mycobacteria (including M. avium, M. tuberculosis), the differences in the genes mmaA and riff-rpsO were even higher (19–27%). It should be noted that in the rpoT gene a three-times-repeated CGAGCCAATACAGCA sequence unique to M. lepromatosis was detected [33]. In the future, if the presence of the repeated sequence is confirmed using a larger group of cases, this sequence repetition could be a specific marker for M. lepromatosis. However, hsp65 and rpoB genes as compared to the sequences of other mycobacteria showed differences in the sequence of about 7%. The differences described above give rise to a diagnosis of a new species of mycobacteria [28].

Thanks to further research analysing sequences of 20 genes and pseudogenes (22,818 bp) it was found that in the phylogenetic tree M. leprae and M. lepromatosis are closely related. However, the difference between the two species gives rise to their distinction. G+C content of the sequences in the both mycobacteria was almost identical and amounted to 58.6% (M. lepromatosis) and 58.8% (M. leprae), respectively. The similarity between the sequences tested in these mycobacteria was 93% on average for the coding sequence and 79.1% in the case of sequences of pseudogenes. The overall sequence similarity was only 90.9%, which provides a basis to distinguish the two species such as M. leprae and M. lepromatosis. In order to investigate phylogenetic relationships between both mycobacteria, synonymous (dS) and non-synonymous (dN) mutations in protein-coding sequences were used. Studies have shown that they have evolved from a common ancestor about 10 million years ago, after the massive reduction in its genome, as evidenced by the strong resemblance of the evolutionarily neutral pseudogenes between the two species of mycobacteria. Through the genetic analysis of M. leprae and M. tuberculosis it was found that the evolution of the ancestor of M. leprae and M. lepromatosis diverged about 66 million years ago with M. tuberculosis [34].

The above research and analysis show that both species evolved from a common ancestor, the massive genome reduction of which occurred before the separation of the two species of mycobacteria. In addition, the evolution of mycobacteria was carried out in close contact with an infected human species more than 100,000 years ago.

It should be noted that the above-described research made it possible to distinguish the two species of mycobacteria and enabled the analysis of epidemiological and clinical correlation [35]. In the future, this may help in the treatment and control of the spread of these mycobacterial infections. Despite the success in identifying and distinguishing between the two species of mycobacteria, the described populations of patients showing clinical signs of infection of M. leprae and M. lepromatosis PCR tests were negative in some cases. This is probably due to degradation of DNA in random samples [33].
The leprosy genome is very stable because it shows very little variation between the isolates from different parts of the world [31]. Single nucleotide polymorphisms (SNP) occur with a frequency of 1/48,000 base pairs in the genome of M. leprae. However, in the M. tuberculosis genome SNP is already found at a frequency of 1/300 bp. These results point to a genome stability and make it extremely useful for tracking the migration of human populations over tens of thousands of years, so called phylogeographic analysis. These studies have recently challenged the widely held view that leprosy was taken to America with human populations migrating through the Bering Strait. Genetic studies have shown that leprosy came to North America with European colonists. However, leprosy mycobacterium reached South America as a result of the slave trade from the African continent [31, 34]. Until recently, it was suspected that leprosy in Europe was introduced by Greek soldiers from the army of Alexander the Great while returning from the Indian campaign. Population genetic studies of patients with leprosy showed the dissemination of M. leprae from Africa through the Middle East to Europe [31]. In contrast to M. leprae migration, the first population-based study analysing the presence of both mycobacteria in Mexico indicates that M. lepromatosis came to America with human populations migrating from Asia through the Bering Strait [34, 36].

M. lepromatosis: The Latest Clinico-pathologic Investigations

Han et al. [36] differentiated the leprosy agents among 120 patients with various forms of the disease in Mexico, 2012. The aetiological species was confirmed in 87 patients, 33 were PCR-negative. The investigators tested DNA extracted from archived skin biopsies, using PCRs that targeted the unique 16SrRNA genes and others.

M. lepromatosis was found in 63.2% of patients, M. leprae in 20.7%, and dual infection in 16.1% of cases. The result verified that M. lepromatosis was the specific cause of all DLL patients and was also found in other forms of multibacillary leprosy (LL), but not in paucibacillary leprosy (TT). The coexistence of M. leprae and M. lepromatosis probably develops in endemic countries like Mexico.

The clinical picture in patients infected with M. lepromatosis showed a diffused cutaneous infiltrate with no nodules and evident skin anaesthesia, whereas those infected with M. leprae presented macules, numerous nodules, and plaques. Patients with dual infection showed features of both groups. The younger age predominated in M. lepromatosis infected cases [36].

Ongoing studies confirm that new species M. lepromatosis exists beyond Mexico and was identified in Singapore (endemic area) causing DLL in two patients, with dual, fatal infection. M. lepromatosis gene sequences from Singapore patients matched 99.9% with a known Mexican strain, and they matched the corresponding M. leprae sequences 89.2% [37]. Another report comes from Canada presenting leprosy-like illness associated with M. lepromatosis [38]. New cases will be reported soon from Burma and Brazil [personal communication with X.Y. Han].

Conclusions

Leprosy remains a major global health problem and is not going to disappear. The infection is curable, but not preventable. The reported new active cases registered each year remain the same, or are even increasing in endemic countries. Progress has been made concerning the immunology and immunopathology of leprosy, but still should be focused on genomic and molecular identification. The discovery of M. lepromatosis by X. Y. Han in DLL and other forms of leprosy have important implications for the disease spectrum and its clinical picture. Further epidemiological and clinical evidence should be gathered with replication of these findings in different areas of the world. Propagation of M. leprae restricted to animal models of armadillo and gene knockout mice is the basic resource for genetic studies. Inoculation of M. leprae and M. lepromatosis to these animals seems to be useful in comparison of the results for both species. The whole genome sequencing and the further differentiation between M. leprae and M. lepromatosis is a priority. The results are expected in the near future by virtue of the rapid development of massive parallel sequencing techniques.

References

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