

spread of the proven sandfly vector *Ph. (Laroussius) perniciosus* and the competent sandfly vector *Ph. (Transphlebotomus) mascittii* into Germany (5). It has been hypothesized that sandflies have always been sporadically present in central Europe, but that climate change will lead to extended distribution (10).

It is tempting to assume that climate change resulted in cutaneous leishmaniasis at 46°N in France. In any event, our case and those reported by others should make clinicians aware of the possibility of cutaneous leishmaniasis outside the well-known disease-endemic areas.

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## Visceral Leishmaniasis during Italian Renaissance, 1522–1562

**To the Editor:** Leishmaniasis, an infectious disease caused by parasites of the genus *Leishmania*, is transmitted to humans through the bite of a female sandfly. The 3 forms of leishmaniasis are visceral (VL) and cutaneous (CL), which are typical of the Old World, and mucocutaneous leishmaniasis, which occurs primarily in Central and South America. VL (also called kala-azar) is caused by species of the *L. donovani* complex (including *L. infantum*), and CL is mainly caused by *L. major* or *L. tropica* (1). In Italy,

VL and CL are caused by *L. infantum*. The origin and spread of leishmaniasis are a matter of debate. Widespread in antiquity, visceral leishmaniasis has been identified only in mummies from ancient Egypt and upper Nubia (2). Similarly, only 4 cases of mucocutaneous leishmaniasis have been identified in skulls from northern Chile (3).

We describe the identification of *L. infantum* infection in Eleonora from Toledo (1522–1562), wife of Cosimo I de' Medici and member of one of the major political Italian families during the Renaissance. The positive identification of *Leishmania* infection was achieved in bone samples by 2 independent approaches. First, a molecular ancient DNA (aDNA) analysis identified a specific 123-bp fragment of a conserved region of the minicircle molecule of the parasite's kinetoplast mitochondrial DNA (4,5) which on direct sequencing showed a *Leishmania*-specific sequence compatible with *L. infantum* (Figure; online Appendix Figure, [wwwnc.cdc.gov/EID/article/18/1/10-2001-FA1.htm](http://wwwnc.cdc.gov/EID/article/18/1/10-2001-FA1.htm)). This PCR result was independently replicated in 2 laboratories and additionally supported by the second approach, a protein assay showing a concomitant positive reaction by detecting IgG against *L. infantum* by Western blot sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Direct sequencing of the *Leishmania* aDNA identified a strain with high homology to *L. infantum*. Accordingly, we obtained a 98% concordance rate between our sequence and that of *L. infantum* (expect rate 6e-47, identity rate 113/118) (online Appendix Figure). The rates for other *Leishmania* species indicated that concordance for those species was less probable.

For the protein assay, fractionated proteins from a lysate of late-log-phase promastigotes of *L. infantum* ZMON-1 (World Health Organization

code MHOM/TN/1980/IPT-1) were electroblotted onto nitrocellulose membrane, and antibody detection was conducted on a Bio-Rad (Hercules, CA, USA) Multiscreen apparatus (6). Antibodies against *L. infantum* selectively reacted in a supernatant of protein extract from Eleonora, thereby confirming the immunologic identification of the protozoal infection. The response of IgG against *L. infantum* whole-parasite antigens revealed specific recognition of 8 polypeptides ranging from 14–16 kD to 184 kD. This pattern of bands is consistent with a symptomatic form of VL as shown by the 14 to 16-kD bands.

Although it was initially proposed that the antigenicity of ancient proteins may be altered by diagenesis, further investigations have shown that ancient immunoglobulins can persist across geologic times (7). Potential pitfalls in protein-based detection of ancient pathogens have been addressed by incorporating proper controls during the analysis. False-positive data, which can result either from contamination of ancient material by modern materials or from lack of microbe specificity of the test (7), have been ruled out by

the parallel testing of several blanks (buffer without ancient material) and by testing, in parallel, samples of ancient bone tissue harvested from persons who died of known diseases other than leishmaniasis (e.g., plague). All negative controls used in aDNA and protein research and all blanks yielded negative results. To avoid contamination, we used no positive controls.

The disease history of Eleonora from Toledo is as follows. Her clinical history was dominated by a large number of pregnancies. When 18–32 years of age, she gave birth to as many as 11 infants. On the basis of additional clinical reports of court doctors, it was assumed that pulmonary tuberculosis developed when she was 29 years of age (8). In the last years of her life, Eleonora from Toledo had various severe ailments. Irregular bouts of fever, wasting and constant vomiting, stomach pain, weight loss, anemia, and hemorrhage were recorded. Autopsy revealed that her most damaged organs were the lungs and that the lung lesions were consistent with a chronic pulmonary infection. Hepatomagaly and splenomegaly

were also recorded (9). Although these signs and symptoms could have come from the tuberculosis infection, they are also consistent with those in patients with symptomatic VL, i.e., progressive fever, weight loss, splenomegaly, hepatomegaly, hypergammaglobulinemia, and pancytopenia. Complications include immunosuppression, secondary bacterial infections, hemorrhage, and anemia (10). All these observations lend support to the notion that Eleonora from Toledo was not immunocompetent. In addition to a supposed tuberculosis co-infection, VL infection may have been a key event leading to her death at age 40.

Our molecular and serologic identification of *Leishmania* infection in a historically prominent person from southern Europe has major relevance. This information might be useful for monitoring the infection and its pathogen throughout history and might provide data on the host–pathogen interaction over different periods.

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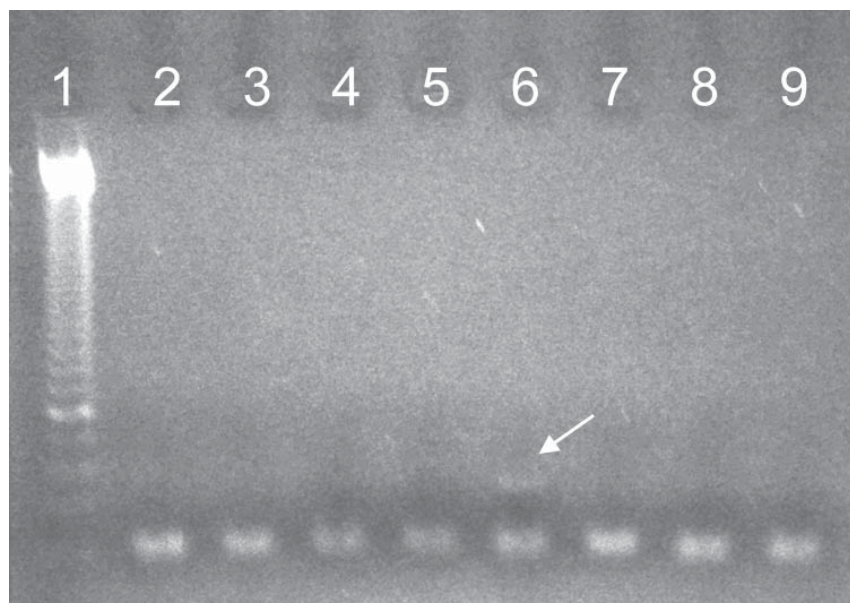


Figure. PCR amplification of a 123-bp fragment of kinetoplastid mitochondrial DNA of *Leishmania* spp. from Eleonora from Toledo (lane 6, arrow). Lane 1, molecular mass standard; lanes 2–5, ancient controls; lanes 7–9, blank controls.

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## Plague Epidemic in the Kingdom of Naples, 1656–1658

**To the Editor:** In 1656, an epidemic of plague occurred in the Kingdom of Naples, Italy. Earlier the disease had spread from Algiers to Spain; in June 1647, it appeared in Valencia, and in the spring of 1648, it appeared in Aragon and several other Spanish areas of Valencia, Andalusia, and Catalonia. In 1652, plague had spread to Sardinia and then to the cities and territories of Naples, Rome, and Genoa. Within the Kingdom of Naples, plague first reached the town of Naples in the spring of 1656. Despite measures restricting population movement, by the summer of 1656, the disease had reached several provinces in southern Italy (1,2).

Historical records indicate that the epidemic in Barletta, in southern Italy, developed after the arrival of a ship from Naples. On May 26, 1656, the ship Sant' Andrea arrived from Naples at the port of Barletta. However, after sanitary inspection, the ship was prevented from landing and obliged to depart, but this measure was not sufficient to prevent the disease from entering the port. The Barletta epidemic peaked in October, after which the number of cases diminished; and on June 22, 1657, Barletta was declared free of plague. Of this city's original population of 20,000, the disease killed 7,000–

12,000 persons. It is hypothesized that throughout the Kingdom, the plague killed ≈1,250,000 persons (1,2).

Since the 14th century, noble families of Barletta had been buried in tombs in underground tunnels of Sant' Andrea church. During restoration of the church in 2009, more underground tunnels containing many skeletons were discovered. It has been hypothesized that the church had also been used as a cemetery during the plague epidemic. During an inspection of the skeletons, 5 skulls of young persons were identified and collected. For a negative control, the skull of a person buried in a tomb before the epidemic was also collected.

The skulls were radiographed to identify unerupted teeth (Figure), which were then aseptically extracted. After classification, each tooth was cut along a sagittal line to uncover the dental pulp, which was then hydrated in sterile phosphate-buffered saline (pH 7.2) for 48 h at 37°C. The DNA was extracted by using DNAeasy Blood and Tissue Kits (QIAGEN, Hilden, Germany) and by modifying the first step, which was conducted overnight at 56°C with 600 μL of ATL buffer (QIAGEN) and 50 μL of proteinase K. To verify the presence of inhibiting substance, the control DNA extracts were screened by using a PCR for human mitochondrial DNA (3).

To investigate the cause of the deaths, we adopted a PCR suicide method and searched for *Yersinia pestis*. We amplified the *pla* gene for *Y. pestis* by using Sybr green PCR in real time with a modification of a previous protocol (4) coupled with conventional PCR according to Drancourt et al. (5). Conventional PCRs were adopted for *Bacillus anthracis* by targeting the *pag* and *capC* genes (6) and for *Salmonella enterica* serovar Typhi by targeting the *narG* gene (7). To prevent cross-contamination, we conducted all PCRs with a negative control and in the absence of positive controls. Melting curve analysis and agarose