# Chapter 9
# Past Plague

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**Abstract** The recent discovery, by two independent teams, of *Yersinia pestis* DNA in human remains dating from two historical plague pandemics, has generated renewed interest in the epidemiology of past plague epidemics. A scenario involving one of the three different *Y. pestis* pathovars identified at the time in each of the three pandemics was proposed in 1951. Palaeomicrobiologic and genetic data support an alternative scenario, with an Orientalis-like strain originating from Asia being responsible for all three plague pandemics.

## 9.1 Introduction

Plague caused by *Yersinia pestis* has been responsible for millions of deaths for at least two millennia (Perry and Fetherston 1997). In recent times, renewed interest in plague has been generated due to the emergence of multi-resistant strains of *Y. pestis* (Chanteau et al. 1998; Galimand et al. 1997) and the growing recognition of the potential of *Y. pestis* as an agent of biological warfare (Inglesby et al. 2000) (http://www.bt.cdc.gov).

Plague, a zoonose, primarily affects rodents. Man, an incidental host, is infected by rat fleas (Perry and Fetherston 1997). The flea acquires *Y. pestis* from the blood of a bacteraemic reservoir animal. The infection is restricted to the gastrointestinal tract of the flea. Typically, plague is thought to exist indefinitely in rodent populations in so-called enzootic (maintenance) cycles that involve transmission between partially resistant rodents (enzootic or maintenance hosts) and their fleas. Not infrequently, the disease spreads from enzootic to more susceptible animals (epizootic or amplifying hosts), causing rapidly spreading die-offs (epizootics) (Gage et al. 1995). Anthropophilic rodent fleas may transmit *Y. pestis* to humans and are believed to be responsible for human plague. Following the third pandemic of

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plague at the end of the nineteenth century, Yersin (1894), Simond (1898) and Raybaud (Gauthier and Raybaud 1903) established that rat fleas transmitted *Y. pestis* to human subjects. This was later endorsed by the then Indian National Advisory Committee on plague (1907). Transmission by human ectoparasites, including the body louse *Pediculus humanus corporis*, has also been observed during twentieth century epidemics (Drancourt et al. 2006). Recent experimental data confirmed the potential role of body lice as a vector of plague (Houhamdi et al. 2006). This fact may explain the particular epidemiological patterns that emerged during large historical pandemics (Drancourt et al. 2006)

Based on the description of outbreaks associated with bubonic lesions, history can trace three great pandemics of plague (Perry and Fetherston 1997). We know no other cause of bubonic fever outbreak, and it has been speculated that these pandemics were caused by the same organism. The first great pandemic was that known by historians as the Justinian plague (541–544 A.D.). A detailed account of the outbreak of plague in Constantinople (modern Istanbul, Turkey) was given by Procopius of Caesarea in his book "De bello persico" (Procopius 1914). According to this source, the outbreak involved bubonic plague and had killed an estimated 50% of the inhabitants of the Byzantine Empire by the year A.D. 565. Resurgence of this pandemic was noted every 8–12 years until the eighth century. In 1347, plague re-emerged and this second pandemic claimed an estimated 17–28 million human lives (Ziegler 1991). It was described properly as an outbreak of bubonic fever by the fourteenth century physician and plague witness Guy de Chaulliac in 1363 (Enselme 1969). Resurgences were observed until the end of the eighteenth century. In France, the last resurgence of this second pandemic occurred in Marseilles and claimed 40–50% deaths among the 100,000 inhabitants (Signoli et al. 1996). The third pandemic (1855) established stable enzootic foci in every continent except Antarctica. The infectious nature of plague was not understood until Alexandre Yersin cultured Gram-negative bacilli from enlarged lymph node aspirate obtained from a case of bubonic plague during the Hong Kong epidemics in the mid-1890s (Yersin 1894). At around the same time, the Japanese bacteriologist Shibasaburo Kitasato independently announced the isolation of the plague bacillus. However, the initial description of the microorganism he isolated might have included a contaminating pneumococcus (Kitasato 1894). The fact that Yersin inoculated high inoculum, buboe-derived material at room temperature instead of using an incubator may have been decisive in his success in isolation of the further named *Yersinia pestis*.

## 9.2 The Bacterium

*Yersinia pestis* is a non-motile, non-sporulating, Gram-negative biochemically unreactive member of the family *Enterobacteriaceae* of γ-proteobacteria. Although encapsulated, *Y. pestis* produces an envelope that contains the unique fraction 1 (Fr1) glycoprotein surface antigen. It dies rapidly if exposed to temperatures exceeding 40°C or desiccation. Three *Y. pestis* biotypes have been recognised on the basis of

their abilities to convert nitrate to nitrite and to ferment glycerol (Perry and Fetherston 1997). The biotype Antiqua has both characteristics. The biotype Medievalis ferments glycerol but does not form nitrite. The biotype Orientalis forms nitrite but does not ferment glycerol. A fourth biotype, Microtus, has been proposed to accommodate Chinese isolates from *Microtus* sp. The latter biotype differs from Medievalis by its inability to ferment arabinose (Zhou et al. 2004). Russian authors developed an alternative classification scheme based on 18 phenotypic characters, which distinguished six subspecies found in the former Soviet Union with defined pathogenicity in guinea-pigs and geographic partition in former Soviet states (Anisimov et al. 2004) (Table 9.1).

Genetic analyses at the population level have indicated that *Y. pestis* diverged from its closely related *Yersinia pseudotuberculosis* ancestor [probably serotype O: 1b (41) 1,500–20,000 years ago (Achtman et al. 1999, 2004) along one branch (branch 0) supporting the human-avirulent Microtus isolate 91001 and pestoides isolates, and then diverged into two main branches: branch 1 comprising Orientalis and the African Antiqua isolates, and branch 2 comprising the Medievalis and the Asian Antiqua isolates (Achtman et al. 2004; Chain et al. 2006) (Fig. 9.1). These analyses, as well as single nucleotide polymorphism (SNP)-based analysis of complete genomes, therefore indicated that Antiqua is an inaccurate phylogenetic representation (Chain et al. 2004).

Isolates of the African Antiqua biotype are currently found in Central Africa, whereas those belonging to the Asian Antiqua biotype are found in south-eastern Russia, Manchuria, Mongolia and central and northern Asia; the biotype Medievalis is currently found around the Caspian Sea, Iranian Kurdistan and Southeastern Russia in Western Kazakhstan between the Volga and Ural rivers; the biotype Microtus is found in China and Tibet; and the biotype Orientalis is disseminated worldwide (Perry and Fetherston 1997) (Fig. 9.2).

Comparative genomics of five complete *Y. pestis* genomes including two Antiqua isolates (Chain et al. 2004) and one each of the Medievalis (Deng et al. 2002), Orientalis (Parkhill et al. 2001) and Microtus (Song et al. 2004) biotypes, along with the closely related *Y. pseudotuberculosis* genome (Chain et al. 2004), found unique features for each *Y. pestis* biotype (Table 9.2). The Orientalis biotype genome is unique in having not only a decreased number of coding sequences, but also a decreased number of predicted inactivated genes, a decreased number of RNA operons (6 copies of rRNA operons instead of 7; 70 tRNA genes instead of 72–73), and the accumulation of insertion sequences (44 copies of *IS* 100 insertion sequences instead of 30–75, 62 copies of *IS* 1541 instead of 43–67). It shares some of these characteristics in common with the African Antiqua strain. This evolution is typical of the bacteria causing human outbreaks as they have a higher rate of multiplication (Wren 2000).

Diversity among *Y. pestis* strains was first assessed by pulsed-field gel electrophoresis (PFGE). Analyses of a limited number of isolates have demonstrated that the "pulsotypes" were closely related to their corresponding biotypes (Lucier and Brubaker 1992; Rakin and Heesemann 1995). Strains of ribotype B were all of biovar Orientalis origin and were found over five continents, whereas those of ribotype

**Table 9.1** Phenotypic typing of *Yersinia pestis* isolates adapted from Russian authors (Anisimov et al. 2004)

| | Fermentation of Dependence on nutrition factors | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Y. pestis* subspecies | Rhamnose | Mellbiose | Arabinose | Glycerol | Melezitose | Nitrate reduction | Urease activity | Pesticin 1 production | Susceptibility to pesticin 1 | Fibrinolytic activity | Coagulase activity | Leucine | Methionine | Arginine | Thiamine | Cysteine | Phenylalanine | Threonine | Tyrosine | Virulence for guinea pigs | Region of circulation | Biovar |
| *pestis* | − | − | + | − | − | + | ± | + | − | + | + | ± | ± | − | − | ± | ± | + | − | + | Worldwide | Orientalis |
| *pestis* | − | − | + | + | − | + | − | + | − | + | + | ± | + | − | − | ± | ± | + | − | + | Central Africa, central and northern Asia, China (Manchuria), Mongolia | Antiqua |
| *causasica* | + | + | + | + | − | + | − | − | + | − | − | ± | + | + | + | ± | + | + | NA | − | Transcaucasian highland, Mountain Dagestan | Antiqua |
| *altaica* | + | + | − | + | NA[a] | − | − | + | + | + | + | + | − | + | − | + | + | NA | NA | − | Mountain Altai | Medievalis |
| *hissaria* | + | + | − | + | ± | − | ± | + | ± | + | + | + | + | − | − | + | + | − | − | − | Issarian Ridge | Medievalis |
| *ulegeica* | + | + | + | + | NA | − | − | + | ± | + | + | − | − | − | − | + | + | NA | NA | − | Northeast Mongolia, Gobi Desert | Medievalis |
| *talassica* | + | + | − | + | − | − | + | + | − | + | + | + | NA | + | NA | + | + | NA | + | − | Talassian Ridge | Medievalis |

[a] Not available

**Fig. 9.1** A view of 1720 Marseilles' plague outbreak as painted by a contemporary witness, M. Serre

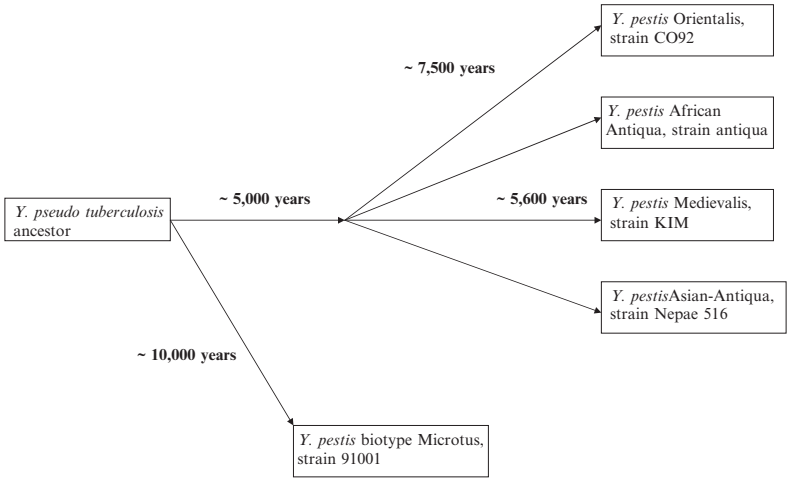


**Fig. 9.2** Phylogenetic representation of the various biotypes of *Yersinia pestis* (adapted from Achtman et al. 2004 and Chain et al. 2006). The sequenced reference strain for each biotype is indicated

O were either biovar Medievalis or biotype Antiqua and were found in suspected remaining foci of the first and second pandemics (Guiyoule et al. 1994). However, individual isolates within one biotype demonstrated heterogeneity on PFGE. The latter suggests spontaneous rearrangements of DNA occurring in various isolates. Ribotyping subdivided 70 strains of *Y. pestis* into 16 ribotypes and found that two ribotypes (B and O) comprised the majority of isolates. The analysis of variable

**Table 9.2** Comparative genomics of *Yersinia* spp. genomes

| | | | *Y. pestis* | | | *Y. pseudotuberculosis* |
|---|---|---|---|---|---|---|
| Strain | CO92 | Antiqua | KIM | Nepal 516 | 91001 | IP32593 |
| Biotype | Orientalis | African-Antiqua | Medievalis | Asian-Antiqua | Mictotus | / |
| Chromosome size (Mbp) | 4653 | 47 | 46 | 453 | 4595 | 4.744 |
| G+C content | 47.64 | 47.7 | 47.64 | 47.58 | 47.65 | 47.61 |
| Coding sequences | 4,012 | 4,138 | 4,198 | 3,956 | 4,037 | 3,974 |
| Average gene length (bp) | 998 | 953 | 940 | 958 | 966 | / |
| Predicted inactivated genes | 36 | 85 | 86 | 49 | 98 | NA |
| 16S–23S–5S rRNAs | 6 | 7 | 7 | 7 | 7[a] | 7 |
| Transfer RNAs | 70 | 68 | 73 | 72 | 72 | 85 |
| pMT size (bp) | 96,210 | 96,471 | 100,990 | 100,918 | 106,642 | / |
| pCDb size (bp) | 70,305 | 70,299 | 70,504 | NAa | 70,159 | 68,526 |
| pPCP size (bp) | 9,612 | 10,777 | 10,961 | 10,778 | 9,609 | / |
| Total IS elements | 134 | 176 | 111 | 129 | 103 | 20 |
| IS 100 elements | 44 | 75 | 35 | 32 | 30 | 5 |
| IS 285 elements | 21 | 24 | 19 | 25 | 23 | 7 |
| IS 1541 elements | 62 | 67 | 49 | 64 | 43 | 5 |
| IS 1661 elements | 7 | 10 | 8 | 8 | 7 | 3 |
| Reference | Le et al. 2001 | Ingelsby et al. 2000 | Kitasato 1894 | Ingelsby et al. 2000 | Lowell et al. 2005 | Lucier and Brubaker 1992 |

[a] Not available

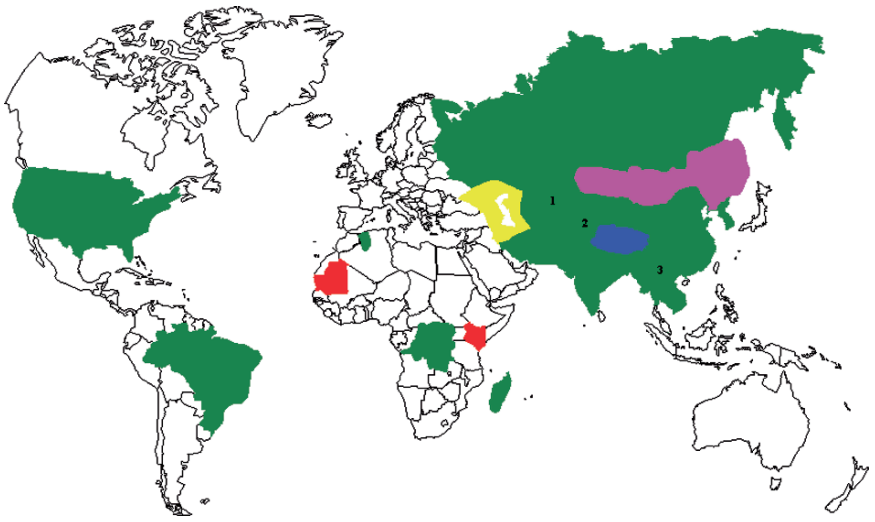


**Fig. 9.3** Putative sources of historical plague pandemics (indicated by *numbers 1–3*) illustrates limited spread of Antiqua, Medievalis and Microtus biotypes and worldwide distribution of the Orientalis biotype. *Red* African Antiqua, *pink* Asian Antiqua, *yellow* Medievalis, *green* Orientalis, *blue* Microtus. This overall distribution was compiled from references Pollitzer 1954, Chanteau et al. 1998 and Inglesby et al. 2000

number tandem repeats (VNTR) on electrophoresis gels offered promising results for the typing of *Y. pestis* (Le et al. 2001). When applied to a large collection of 180 isolates originating from three continents, the analysis of 25 markers found 61 genotypes and distributed the three biotypes within three main branches (Pourcel et al. 2004). This method showed promise in the identification of sources of plague in modern cases of human plague (Lowell et al. 2005). However, VNTR analysis is restricted to cultured strains that yield high quality DNA.

Recently, we have developed a sequence-based method named multispacer sequence typing (MST) based on complete genome data by sequencing several intergenic spacers, some including tandem repeats and some SNPs (Drancourt et al. 2004). This has been shown to be effective in discriminating among biotypes in a large collection of *Y. pestis* isolates (Drancourt et al. 2004) (Fig. 9.3). Moreover, MST was successfully applied to the genotyping of uncultured strains from ancient, buried specimens (Drancourt et al. 1998). MST analyses single mutations in addition to tandem repeats – two molecular events with the same evolutionary significance.

## 9.3 Geographical Sources of Historical Plague Pandemics

The geographical origin of the first pandemic remains controversial. It is widely believed that plague was present in Ethiopia in 541 A.D. and spread quickly from Pelusium at the eastern limit of the Nile Delta, Egypt, through the Middle East to

the Mediterranean basin. Although contemporary sources suggest either an Egyptian (Procopius 1914), or Ethiopian origin, from the kingdom of Axum, whether Eastern Africa was the ultimate source of the first pandemic remains doubtful. Other authors have suggested that first pandemic had its origins among wild gerbil populations in eastern Asia (McKeown 1988). Recent molecular evidence suggests that the first pandemic focus may have been Asia rather than Africa (Zhou et al. 2004).

The second pandemic probably originated in the steppes of Central Asia, where there was an epidemic of marmots. Chwolson, a Russian archaeologist, found inscriptions related to plague on memorial stones dating back to 1338–1339 in Nestorian graveyards near Issyk Kul Lake. However, it remains unclear how and when *Y. pestis* circulating in the marmot populations of the Middle and Central Asian mountains and Tibet, penetrated into the mountain savannas of Eastern and Central Africa. Ziegler (1991) and Pollitzer (1954) suggested that wild rodent populations in the area near Lake Issyk Koul in the district of Semiriechinsk in Central Asia provided the cradle for the epidemic that broke out in 1338. From there, it probably spread eastwards and southwards to China and India, respectively, and westwards to the Crimea, from thence to the rest of the Old World (Ziegler 1991). The plague might have reached the Nile outflows along its densely populated valley from Egypt through Nubia and Ethiopia, which were also embraced by the second pandemic (McKeown 1988).

The third pandemic started in 1855 in the Chinese province of Yünnan, where troop movements during the war in that area caused a rapid spread of the disease to the southern coast of China (Perry and Fetherston 1997; Simond 1898). Plague reached Hong Kong and Canton in 1894, Bombay in 1898 and, by 1899–1900, steamships had disseminated the disease worldwide (Fig. 9.2). Most scientists believe that plague was introduced relatively recently into America by human beings migrating from Asia. Altogether, there is currently no convincing evidence that the three plague pandemics emerged elsewhere than in Asia.

## 9.4 Palaeomicrobiology of Plague

*Yersinia pestis*-specific sequences have now been found, by two independent teams, in human remains dating back to first (Justinian) and second ("Black Death") pandemics (Drancourt et al. 1994; Wiechmann and Grupe 2005; Signoli et al. 1996; Drancourt et al. 1998) (Fig. 9.5). We initially found specific *Y. pestis* sequences, i.e. chromosome-borne *rpo*B and plasmid-borne *pla* genes, in four individuals thought to have died during the 1590 and 1722 plague epidemics in the Marseilles area, but not in seven negative controls. We based our molecular analyses on dental pulp for several reasons, including its susceptibility to septicaemic pathogens, durability, protection against external contamination and ease of manipulation in the laboratory (Drancourt et al. 1998). Using the suicide PCR protocol, in which primers are used only once, we further detected specific sequences in the dental pulp specimens of three individuals thought to have died of plague during the fourteenth century Black

Death pandemic. All negative controls remained negative and original sequences due to point mutations were found in *pla* (Raoult et al. 2000). The typhus agent *Rickettsia prowazekii* and the anthrax agent *Bacillus anthracis* were not detected in these specimens. Furthermore, we were able to detect specific *Y. pestis* sequences in three individuals thought to have died in the fifth–sixth century Justinian plague, along with confirming previous results in Black Death individuals (Drancourt et al. 2004). Later data were independently confirmed by the recovery of two specific *pla* gene sequences from two sixth century individuals from Upper Bavaria, by another research team using total tooth DNA (Wiechmann and Grupe 2005). Interestingly, using the same primers negative results were found when we investigated louse-borne infection in Napoleon's soldiers, and when Papagrigorakis and collaborators investigated the Plague of Athens, which revealed *Salmonella enterica* Typhi (Papagrigorakis et al. 2006). An English team aimed to detect the 16S rRNA gene of *Yersinia pestis* in 61 individuals collected in five burial sites suspected of plague from the thirteenth to the seventeenth century in Northern Europe (Gilbert et al. 2004). Although the authors claimed they failed to detect *Y. pestis*, two specimens yielded sequences that matched enterobacterial 16S rRNA gene sequence, including *Y. pestis*, with 99% and 100% similarity, respectively. Because *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* share 100% sequence similarity in the targeted 16S rRNA gene region, the data were non-interpretable but they do not positively exclude the presence of *Y. pestis* in these two particular specimens. Either differences in technical protocols or lack of plague in these individuals may explain discrepancies between studies (Gilbert et al. 2004). A specific 93-bp region of the *Y. pestis caf*1 gene was also detected by PCR and Southern hybridisation in 2/12 seventeenth century individuals suspected of ancient plague and 0/12 controls (Pusch et al. 2004). In these individuals, detection of the *Y. pestis* F1 capsular antigen was achieved in 10/12 individuals but in none of the 12 controls or contemporary soil specimens (Pusch et al. 2004). The fact that molecular evidence of *Y. pestis*-specific DNA sequences have now be obtained by two independent teams, and some original sequences have been identified in agreement with criteria for authenticity in palaeomicrobiology (Drancourt and Raoult 2005), should end the controversy regarding the etiology of historical plagues.

## 9.5 The Causative Strains of Pandemics

All strains isolated from areas unaffected by plague before the third pandemic are of biotype Orientalis. In ancient enzootic foci, other biotypes were found (Antiqua, Medievalis, Microtus). On the basis of this geographical repartition of biotypes in plague foci, and putative historical data regarding the potential geographical source of past plague epidemics, Devignat hypothesised in 1951 that each of the three known biovars Antiqua, Medievalis, and Orientalis caused the first, second, and third pandemics, respectively (Devignat 1951). This contention was based on the hypothesis that contemporary foci have been the primary sources for each pandemic, a contention without any scientific basis as acknowledged by Devignat himself.

This speculation did not receive any significant confirmation but remained unchallenged and over time became established as a common hypotheses (Achtman et al. 1999; Devignat 1951). Moreover (see above), the original source of plague for the second and the third pandemics was clearly Asian, and the source of the first is disputed between Africa and Asia.

Recent genetic data challenge this hypothesis. Suppression-subtractive hybridisation techniques have demonstrated that Antiqua isolates share a 15,603 bp chromosomal fragment in common with *Y. pseudotuberculosis* and some Microtus isolates (Radnedge et al 2001). Further genome sequencing of two Antiqua isolates refined phylogenetic representation by delineating an Asian Antiqua subgroup 1, closely related to Orientalis, and an African Antiqua subgroup 2, closely related to Medievalis (Chain et al. 2006). Thorough investigation of a 156 isolate *Y. pestis* collection based on synonymous SNPs, VNTR and insertion of IS*100* elements found the *Y. pestis* species to comprise eight populations, which did not match with the one biotype / one pandemic theory (Achtman et al. 2004). Indeed, while one Orientalis population was clearly associated with last pandemic, biovars Antiqua and Medievalis were found to be too polyphyletic to be unambiguously associated with first and second pandemics, respectively (Achtman et al. 2004). Evidence gathered from DNA microarray analysis of genome dynamics in *Y. pestis* indicated that Orientalis evolved in China directly from Antiqua, not from Medievalis (Zhou et al. 2004). Also, micro-evolution analysis of *Y. pestis* clearly indicated a greater diversity of *Y. pestis* isolates in Asia than in Africa, and high diversity is often a good indicator of the geographical source of microbes (Achtman et al. 2004). Altogether, genetic as well as contemporary investigations lead us to consider Asia as the source of the pandemic strain (or strains).

## 9.6 Identifying the Causative Strains in Human Remains

In order to identify the genotype involved in the three pandemics, we applied MST to dental pulp collected from the remains of eight persons who likely died in the first and second pandemics (Fig. 9.4). In the 46 PCR experiments we performed, we obtained 10 *Y. pestis* sequences in seven of eight persons' remains and no sequences were found in the 51 PCR experiments with negative control teeth of 17 persons ($P < 10^{-4}$). YP1 PCR yielded an amplicon in one of six tested persons; its sequence revealed complete similarity with the homologous region in *Y. pestis* Orientalis over 390 positions. YP8 PCR yielded an amplicon with identical sequence in six of six tested persons, which exhibited 99% sequence similarity with the homologous region in *Y. pestis* Orientalis over 178 positions. YP3 PCR yielded an amplicon in three of seven tested persons; its sequence yielded complete sequence identity with that of the homologous region in *Y. pestis* Orientalis over 364 positions in two persons and a 98% similarity with the homologous region in *Y. pestis* CO92 strain over 283 positions in the last case. This amplicon exhibited

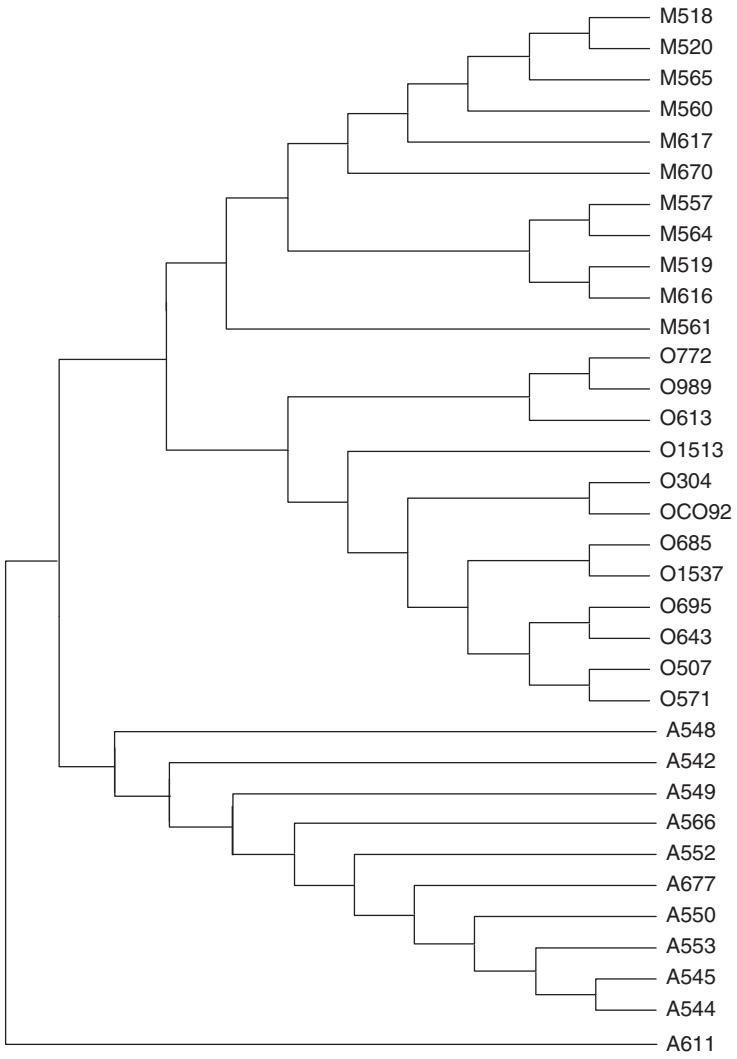


**Fig. 9.4** Multispacer sequence typing (MST)-based tree of *Yersinia pestis*. *O* Orientalis biotype, *M* Medievalis, *A* Antiqua, followed by the number of the strain, including reference strain CO92

two specific nucleotide substitutions that were consistently obtained in six clones. MST therefore indicated that the historical strains were more closely related to biotype Orientalis than to the other two biotypes of *Y. pestis* (Drancourt et al. 2004). We further amplified and sequenced the *glp*D gene encoding glycerol-3-phosphate dehydrogenase in five individuals from the two historical pandemics and found the 93-bp deletion reported to be specific for the Orientalis biotype, thus confirming the


**Fig. 9.5** A view of the 1720 plague mass grave in Martigues, southern France

MST data (Motin et al. 2002). Palaeomicrobiology data have recently been further augmented by the discovery that a YpF Φ filamentous phage is stably integrated in Orientalis isolates but formed unstable episomes in Antiqua and Medievalis isolates (Derbise et al. 2007). This phage contributes to the pathogenicity of *Y. pestis* in mice and may confer a selective advantage to *Y. pestis* under natural conditions. This illustrates the fact that palaeomicrobiology studies can describe unique characteristics of ancient pathogens, further elucidated by studying modern isolates. At this point, palaeomicrobiology investigations thus indicate that the Orientalis biotype has pandemic potential, whereas other biotypes may have a more limited diffusion potential.

According to the data presented herein, we now hypothesise that the three plague epidemics originated in Asia and were caused by *Y. pestis* biotype Orientalis. We hypothesise that the continuous evolution of the *Y. pestis* genome conferred unique biological properties on biotype Orientalis, including the capacity to promote pandemia, whereas the Antiqua, Medievalis and Microtus biotypes were local variants with limited epidemic potential. Such unique biological properties may be linked to *Y. pestis*–vector relationships or increased capacity to induce septicaemia in its host.

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