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Plague and *Yersinia pestis*: some knowns and unknowns

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Abstract:

Since its first identification in 1894 during the third pandemic in Hong Kong, there has been significant progress of understanding the lifestyle of *Yersinia pestis*, the pathogen that is responsible for plague. Although we now have some understanding of the pathogen's physiology, genetics, genomics, evolution, gene regulation, pathogenesis and immunity, there are many unknown aspects of the pathogen and its disease development. Here, we focus on some of the knowns and unknowns relating to *Y. pestis* and plague. We notably focus on some key *Y pestis* physiological and virulence traits that are important for its mammal-flea-mammal life cycle but also its emergence from the enteropathogen *Yersinia pseudotuberculosis*. Some aspects of the genetic diversity of *Y. pestis*, the distribution and ecology of plague as well as the medical countermeasures to protect our population are also provided. Lastly, we present some biosafety and biosecurity information related to *Y. pestis* and plague.

Keywords: *Yersinia pestis*, plague, physiology, evolution, pathogenesis, immunity, vaccine, diagnostic, treatment, bioterrorism

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Human plague, caused by *Yersinia pestis*, a bacterium which first and foremost occurs in wildlife rodent species but occasionally spills over to people: hence the title "Plague and *Yersinia pestis*". Plague has brought about three pandemics in history, including the first pandemic Justinian plague (from around 541 CE), the second Black Death (from around 1347 CE), and the third modern plague (from around 1880 CE). Since the identification of etiological agent for plague by Dr. Alexander Yersin during the third pandemic in Hong Kong of China in 1894[1], more than 120 years have passed, and we have, to some extent, understood the physiology, pathogenesis and evolution of *Y. pestis*. We have developed technologies for plague diagnosis and treatment. Plague is effectively controlled in most endemic regions except some parts of Africa. However, the wide distribution of natural plague foci in Asia, Euroasia, Africa and the Americas, with occasional occurrences in both rodents and humans or following long-distance travel of plague infected individuals, into large cities reminds us of the threat of a plague outbreak. In this review, we will summarize what we have known and what we have not known about plague and its causative agent, *Y. pestis*. The key issues need to be solved in future were also proposed (Box).

Known 1: Physiology of *Y. pestis*

Growth properties and cellular structures

Y. pestis is a Gram-negative, nonsporulating and nonmotile coccobacillus, which grows in broth culture. It often exhibits bipolar staining with Giemsa or Wayson dye. *Y. pestis* grows on a variety of media under aerobic or facultatively anaerobic conditions at temperatures betwwen 4 to 40 °C (optimum at 26-28 °C) and can survive within a pH range from 5.0 to 9.6 (optimum approximately 7.5) [2].

It typically grows as grey-white translucent, non-hemolytic colonies within 24 h on blood or chocolate agar and as yellow and opaque colonies with irregular, 'fried egg' shiny appearance after 48 h. In some but not all broth culture, it aggregates with flocs typically attached to the sides of the tube, often projecting stalactite shape and leaving a clear broth at 48 h [3].

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Y. pestis has typical cell structures and antigen compositions like other enteric bacteria and produces a short-chain, rough lipopolysaccharide (referred as lipo-oligosaccharide) that lacks the O antigen due to the absence of some genes from the O-antigen cluster [4]. This unique feature might be an adaptive evolution that contributes to systemic infection of this pathogen. *Y. pestis* lacks a true capsule but frequently produces a unique glycoprotein envelope known as the surface capsule at temperature above 33°C [5].

Genomic composition

The deadly pathogen*, Y. pestis,* is a clone that emerged from the self-limiting gastroenteric pathogen *Y. pseudotuberculosis* [6-9]. About 97% similarity is shared at the chromosomal DNA level [6] and like other pathogenic *Yersiniae*, *Y. pestis* contains the plasmid pCD1 (70-75 kb) [10]. During evolution, *Y. pestis* horizontally acquired two additional plasmids, pMT1 (100-110 kb) and pPCP1 (9.5kb), and a high pathogenicity island consisting by 32 chromosomal genes are unique to *Y. pestis*. Some determinants encoded by plasmids pMT1 and pPCP1 facilitate *Y. pestis*-specific tissue invasion, survival in flea vectors or possibly its heavy growth in host blood[11-15].

Gene modification and loss are attributed to modifications of cellular structural or regulatory networks or elimination of activities no longer required for *Y. pestis* new lifecycle [16]. For example, mutation or interruption of *yadA*, *inv* and *ail* encoding adhesin or invasin makes it attenuate the activities usually attributed to enteropathogenic virulence [17-19]. Urease is essential for pathogenesis in other members of *Yersinia* spp. (*Y. enterocolitica* and *Y. pseudotuberculosis*) [20] but *Y. pestis* does not exhibit ureolytic activity due to a premature stop codon of the *ureD* gene [21]. Even though the *ure* operon is transcriptionally upregulated at 26°C, this locus might not be directly involved in the *Y. pestis* life cycle but likely reduces the toxicity to fleas, increasing the chances of maintaining an infection in a larger population [22].

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Nutrition & metabolism traits

Almost all *Y. pestis* strains require media supplementation with isoleucine, valine, methionine, phenylalanine, and glycine (or threonine) for successful growth but the nutrient requirements differ depending on the temperature with additional nutrients such as biotin, thiamine, pantothenate, and glutamate required at 37 °C.

Y. pestis strains have different abilities to ferment glycerol and arabinose and reduce nitrate. Based on this, they have been classified into five biovars (antiqua, mediaevalis, orientalis, microtus and Intermedium) [23, 24]. The strains of the former three biovars are highly virulent to animals or humans, while the biovar microtus strain, including 'pestoides' one, is avirulent or opportunistic to larger mammals but virulent to small rodents [25].

Y. pestis has a complete Embden-Meyerh pathway but no functional pentose-phosphate pathway due to missense mutations in the glucose 6-phosphate dehydrogenase-encoding gene *zwf*. Unlike *Y. pseudotuberculosis*, the glyoxylate bypass pathway is constitutively expressed in *Y. pestis* due to de-repression from the IclR transcriptional repressor [26] which may explain its ability to metabolise acetate and fatty acids provided by the host.

Unknown 1: the regulation of physiology of *Y. pestis in vitro* and *in vivo*

In contrast to its ancestor *Y. pseudotuberculosis*, a self-limiting gastroenteric pathogen, *Y. pestis* has evolved to be a deadly pathogen occupying different niches [6, 7]. This organism circulates only within a narrow host range between rodent reservoir hosts and flea vectors in nature. The first challenge for *Y. pestis* survival in its lifecycle is sensing and adapting to temperature shifts but avoiding host innate immune cells during the early stage of infection and in host blood after releasing from innate immune cells including macrophages [27] is problematic as *Y. pestis* develops into a systemic infection. During its complex life cycle, the intense or even life-threatening environmental changes are concomitant with a series of dynamic regulatory physiological responses which we are still far from understanding in terms of *Y. pestis* physiology and pathogenesis at the transcriptional and posttranscriptional

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level [28].

Key physiological traits

Temperature sensing

Y. pestis alternatively grows in the flea or in warm-blood mammalian hosts during its lifecycle. Fleas are often infected with *Y. pestis* by sucking blood of a bacteremic mammal. The bacteria form a biofilm mass in the flea foregut at temperatures below 25°C [29]. Obviously, *Y. pestis* distinguish the temperature shift between environmental temperatures and the body temperatures of hosts during the transmission process. Intriguingly, most of the putative virulence factors are transcriptionally regulated by temperature shifts and active at either 26°C or 37°C. Under the control of a fourU RNA thermometer switch[30], the histone-like regulator YmoA negatively modulates the virulence effectors of the type III secretion system (T3SS) through the transcriptional activator LcrF [31]. This thermosensing pattern is quickly responsive and energy-efficient to match the changing temperature environments encountered by *Y. pestis*.

Low-calcium response

The low-calcium response (LCR) in *Y. pestis* can be induced *in vitro* under low Ca^{2+} or Ca^{2+}-free condition at 37 °C but not 26 °C, where growth cessation was coordinated with upregulating the T3SS [32]. The phenomenon of triggering the expression and secretion function of the T3SS is used to stimulate the *in vivo* signal of *Y. pestis* contact with host cells during infection [33]. However, growth cessation might be an artifact that only occurs *in vitro*. Expression of components of the T3SS encoded on pCD1 shared among all three pathogenic Yersiniae initiates the secretion of *Yersinia* outer proteins (Yops) into host cell cytoplasm, which prevents macrophage phagocytosis and inhibits the host immune response [34].

Survival within macrophage

It is generally considered that the survival inside macrophage vacuoles is critical

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in the early stages of the *Y. pestis* lifestyle within warm-blooded hosts [35-37]. However, there are reports that *Y. pestis* isolated from fleas showed resistance to phagocytosis[38] and the host innate immune cells demonstrated different responses to flea-transmitted and needle-inoculated *Y. pestis* [39]. This reminds us that we should be prudent to explain the experimental results from needle-injected challenge of laboratory animals for understanding the natural infection through flea feeding. The intracellular microenvironments of macrophage may provide a temporary shelter for the organism and meanwhile induce the synthesis of antiphagocytosis factors to be ready for the subsequent release into the extracellular environment [40]. PhoP/PhoQ, a two-component regulatory system, is probably important for *Y. pestis* survival within macrophage *via* its pleiotrophic effects on gene expression [41].

Counteraction of biometal sequestration

During *Y. pestis* infection, the host's microenvironments are thought to sequester key biometals such as iron, zinc and manganese. *Y. pestis* utilizes an iron-scavenging siderphore Yersiniabactin (Ybt) and iron transporters Yfe and Feo to overcome the iron deprivation [42-45]. Besides the zinc transporter ZnuABC, Ybt siderophore also contributes to zinc acquisition as the second zinc transporters in both the mammalian and flea hosts during infection [45-47]. Two Mn transporters,Yfe and MntH, that are functional in *Y. pestis*, play an important roles in bubonic plague progression [48].

Regulation of physiological stress response *in vitro* and *in vivo*

The complex lifestyle requires *Y. pestis* to monitor environmental cues and regulate stress-responses accordingly to ensure environmental adaptation in their hosts or vectors.

The stressful conditions *in vitro* stimulating intracellular or external microenvironments encountered by *Y. pestis* during its infection and life cycle were used in studies on regulation of stress responses. The research on stimulons (temperature, osmolarity, ion, oxidative, acid and nutrition) and regulons (Fur, PhoP, OmpR and OxyR) refines the regulatory responses and identify the differentially

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regulated genes that are important for *Y. pestis* physiology and pathogenesis [49]. The integrated analysis showed that the expression of putative virulence locus in *Y. pestis* (*hms*, *caf1*, T3SS and *psa*) is responsive to a wide range of environmental stresses and multiple regulatory proteins. Other genes responsible for cellular metabolism were also active upon exposure to multiple stresses, including energy metabolism, sulfur metabolism, ribosome protein biosynthesis, iron uptake, heme synthesis and utilization to chemotaxis and motility [49].

The virulence-associated genes or loci such as the T3SS, *pgm* locus, pH6 antigen and *pla* encoded by pPCP1 were found to be regulated in established bubonic or pneumonia plague. Iron deprivation and NO-induced stress are speculated to be more reactive based on the regulation of the corresponding pathways in the rat bubo [50]. In the intracellular *Y. pestis*, the genes associated with antioxidant stresses were strongly induced [51]. In flea transmission model, the regulation of genes involving in innate immunity and pathogenicity might facilitate survival of *Y. pestis* during the period of transmission from flea to the host [38].

Although we understand some details relating to regulatory networks and their physiological consequences there is still much to uncover. For example, *Y. pestis* can escape from innate immunity defense by inhibiting cytokine production or the LPS-induced inflammatory response [52, 53] but more comprehensive analysis of (epi)genomic comparison should be performed on *Y. pestis* strains with different host ranges, to further reveal the physiological basis to this extraordinary aspect of its lifestyle. The interactions either between chromosome and plasmids, especially the horizontal acquired plasmids, or between plasmids or the contribution of small open reading frames (sORFs) [54], would strengthen our understanding of this phenomenon.

Known 2: Genetic diversity of *Y. pestis*

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Y. pestis has been known as a genetically monomorphic species since the advent of molecular genotyping [6, 55]. By using Multilocus Sequence Typing (MLST) methods [56], which is frequently used in pathogen genotyping and spread analysis, it is apparent that within-species diversity cannot be determined, *i.e*, all strains of *Y. pestis* had an identical sequence type based on the sequences of six house-keeping genes [6].

To determine the genetic diversity of *Y. pestis* before the appearance of next generation sequencing technology (NGS), researchers investigated multiple types of genomic variations including different regions (DFRs), variable number of tandem repeats (VNTRs) and clustered regularly interspaced short palindromic repeats (CRISPRs) whilst developing corresponding genotyping methods. The DFR method was built on presence/absence of 23 genome fragments and could distinguish 909 *Y. pestis* natural isolates into 32 genotypes [57]. According to Platonov (2001) [58], DFR-Typing of 275 *Y. pestis* strains from the CIS Natural Foci found 56 novel genomovars, indicating that the discriminatory power of this method is high enough to distinguish between subspecies, populations and even strains circulating in certain natural plague focus. There are three CRISPR spacer arrays in the *Y. pestis* chromosome. According to the composition of spacers in these CRISPR loci, 125 *Y. pestis* representative isolates from China, the former Soviet Union and Mongolia, could be classified into 12 types [59]. Multiple loci VNTR analysis (MLVA) methods provide quite high discrimination power, as integrated variation information of multiple VNTR loci have high mutation rates, and therefore could be used in outbreak investigation to provide clues on source-tracing [60]. However, the rapidly mutated VNTR loci are prone to parallel or reverse changes, which makes the deep branches of the phylogeny less robust, hence it is not suitable for inferring long-term evolutionary dynamics of the bacterial population.

Advances in NGS technology have reduced the cost and speed of bacterial whole genome sequencing resulting in hundreds of *Y. pestis* genomes of natural isolates

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being deciphered, and their robust high-resolution genealogy was rebuilt based on genome-wide variations through population genetic methods. Results from whole genome sequencing verified that *Y. pestis* is a young and monophyletic species, which evolved from *Y. pseudotuberculosis* between 2,600-28,000 years ago, and the average pairwise genetic distance among any two natural isolates of *Y. pestis* is 126 SNPs [61]. It was reported that *Y. pestis* has a decay genome which resulted from adapting to the nutrient-rich environment of its new survival niche, the blood of rodent hosts. The genome content composition supports this hypothesis: compared with other species, *Y. pestis* has a relatively closed pan-genome with a length of 5.4 Mb but a rapidly reduced core genome size of 3.5 Mb, based on genomes of 133 *Y. pestis* global isolates with average genome size of 4.6 Mb.

Currently, 33 phylogroups in five main branches of *Y. pestis* are identified (Fig. 1). Branch 0 is a root lineage of *Y. pestis*, which contains several ‘untypical’ groups: the 0.PE2 and 0.PE5 groups that have been recognized as subspecies of *Y. pestis* and termed ’pestoides‘ [62]; strains of the 0.PE4 group are assigned to biovar microtus that are known as highly virulent for their main hosts (*Microtus* spp.) and laboratory mice, but attenuated in larger mammals such as guinea pigs and humans; the 0.PE3 group contains only the Angola strain that has the largest number of strain-specific SNPs (n=437), compared with the average number of 126 SNPs across all species, although the reason for this is currently unknown [61, 63]. The 0.PE7 group represents the oldest group among all modern *Y. pestis* natural isolates and includes two strains that were both isolated from Qing-Tibet plateau of China in the 1960s [61]. One 0.PE7 strain was isolated from a human plague case, suggesting *Y. pestis* acquired pathogenicity to humans in the very early stages of its evolution, which has subsequently been confirmed by aDNA sequencing analysis[9].

The Justinian Plague was the first plague pandemic to be described in historical records and was most likely caused by an extinct lineage, 0.ANT4 (Fig. 1) [64]. Then after the appearance of the other three 0.ANT lineages (0.ANT2, 3 and 5), a ‘Big

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Bang' node of *Y. pestis* emerged between 1330-1340, with Branch 1-4, radiated from it [64, 65]. According to synthesized analysis of archaeological, historical and ancient genomic data, Spyrou et al found that ancient Y. pestis from tombs of the Kara-Djigach (the Tian Shan region of North Kyrgyzstan), exactly located at the Big Bang node [66]. Concerned with the fact that after the Big Bang, one SNP accumulated along Branch 1 in a Laishevo ancient genome (LAI009), and one more SNPs after LAI009 were observed from the remnants of the Black Death strain originating at the beginning of the 2nd Pandemic [65, 67, 68], the Big Bang event occurred immediately preceding the Black Death. Because the Kara-Djigach genomes come from a region, in which 0.ANT - including 0.ANT3, the closest old 'cousin' of the Big Bang – are currently circulating, it has been inferred that the Big Bang itself must have occurred in one of marmot reservoirs of the Tian Shan region. Thus, the spatio-temporal origins of the Big Bang have been established. One of the newly born lineages out of the Big Bang was Branch 1, whose strains are associated with both the 2nd Pandemic (commencing with the Black Death) and the 3rd Pandemic (commencing in Yunnan in 1772 and becoming global in 1894). Branch 1 is currently most widely distributed lineage of *Y. pestis* that currently thrives in natural plague foci in Asia, Africa, and America, and most likely also thrived in Europe in the late-medieval and possibly early modern periods [69]. In particular, the 2nd Pandemic waves were caused, with one exception, by now-defunct Branch1A and its sub-lineages, while the 3rd Pandemics was spread by 1.ORI lineage strains (deriving from Branch 1B, which appears to have left Europe in the 1360s) across the globe through steamship transportation at the end of the 19th century [70] . There are 90 SNPs and about 380 years separating the Big Bang and the Great Plague of Marseille (1720-2), implying about one mutation in 4.2 years within Branch 1A. Branch 2 is split into 2.ANT and 2.MED lineages, with the strains of the former are circulating in Nepal, China and Mongolia, while 2.MED is found all over Asia, all the way from Caucasus, Caspian, Volga-Ural region in the west, via western Kazakhstan, Turkmenistan, northern Kyrgyzstan, into China and Mongolia. The reported number of genomes of Branch 2 was the second largest in *Y. pestis*, only less than that of

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Branch 1. Strains of Branch 3 were only found in Gansu Province and Qinghai Province of China and Mongolia, with 12 genomes being reported (as of April 2022). And strains of Branch 4 were only found in Russia and Mongolia, with 11 genomes being reported (as of April 2022) [61, 71, 72].

The genomes of ancient DNA (aDNA) could provide genetic information for historically extinct populations and therefore are important in bacterial evolutionary research as a molecular fossil [73]. Following the successful sequencing of the first whole aDNA genome from the Black Death victims in London (1349) in 2011, there have been more than 110 publicly available ancient genomes of *Y. pestis* (as of April 2022) and 7 extinct lineages have been identified (Fig. 1) [65, 74]. This extends our knowledge of human plague infection to the Neolithic and Bronze Age and onwards to early and later medieval periods across Euro-Asia. Furthermore, studies of ancient genomes have yielded valuable information on the early adaptive evolution of this bacterium, such as the development of flea-borne transmission, and also revealed changes of genomic profiles during pandemics such as convergent evolutionary signals, the 49-kb deletion and *pla* decay at the end of the first two pandemics (Fig. 1) [65, 75].

Unknown 2: evolutionary dynamics and driven force

Although the genealogy of *Y. pestis* has been well defined based on population genomic studies, the driving force shaping such genealogy and the detailed dynamics during its evolution are still vague. Whole-genome wide SNP analysis suggested the evolutionary process of *Y. pestis* was generally neutral, i.e., most of the observed mutations were accumulated randomly [61]. However, after considering multiple types of variation such as indels and gene gain/loss, it might change our current understanding as was the case with *Salmonella enterica* serovar Paratyphi A. The population genetic analysis combining variations of SNPs, indels and accessory

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genomes provided evidence of transient Darwinian selection during its evolution [76]. In addition, by using SNPs and indels five mutation hotspots have been identified with strong selection signals in 78 *Y. pestis* isolates [77]. Mutations at one hotspot, the *rpoZ* gene, might affect the vector behaviors and were proved to be closely related with climate changes. There were 19 out of all 130 mutations (14.6%) involved in mutation hotspots showing selection signals [77], which suggested natural selection might play a more important role than previously thought.

Therefore, reconstructing the evolutionary dynamics using combined variations, including SNPs, indels, gene gain/loss *etc.*, is still a challenge. Another challenge is the association between genetic variations and niche factors, including climate, soil components, hosts, and vectors *etc.*, for inferring natural selection forces. By understanding how *Y. pestis* interacts with the environment, we can begin to understand its natural survival strategies and learn how the genome is shaped, hence promoting the development of novel countermeasures for plague prevention and control.

The evolution of virulence in *Y. pestis* is also an important and unsolved issue. *Y. pestis* is a clone that derives from its ancestor species *Y. pseudotuberculosis* with few intermediate lineages between these two species, and therefore it is difficult to infer the step-by-step evolutionary scenario. Through comparative genomic analysis, we know that *Y. pestis* acquired two plasmids (pMT1 and pPCP1) including a subsequent gain of *Yersinia* murine toxin (*ymt*) gene in pMT1 plasmid and meanwhile inactivated numerous functional genes (*ureD*, *rcsA*, *flhD*, *pde2*, and *pde3*) (Fig. 1) [7, 75, 78, 79]. However, in what order these events occurred and their fitness advantages during the evolution are not clear. Additionally, there is still no satisfactory explanation for the distinct animal virulence across different phylogroups of *Y. pestis*, except for few clues which relate selection pressures with fine genomic changes, such as frameshift indels and copy number variations [80].

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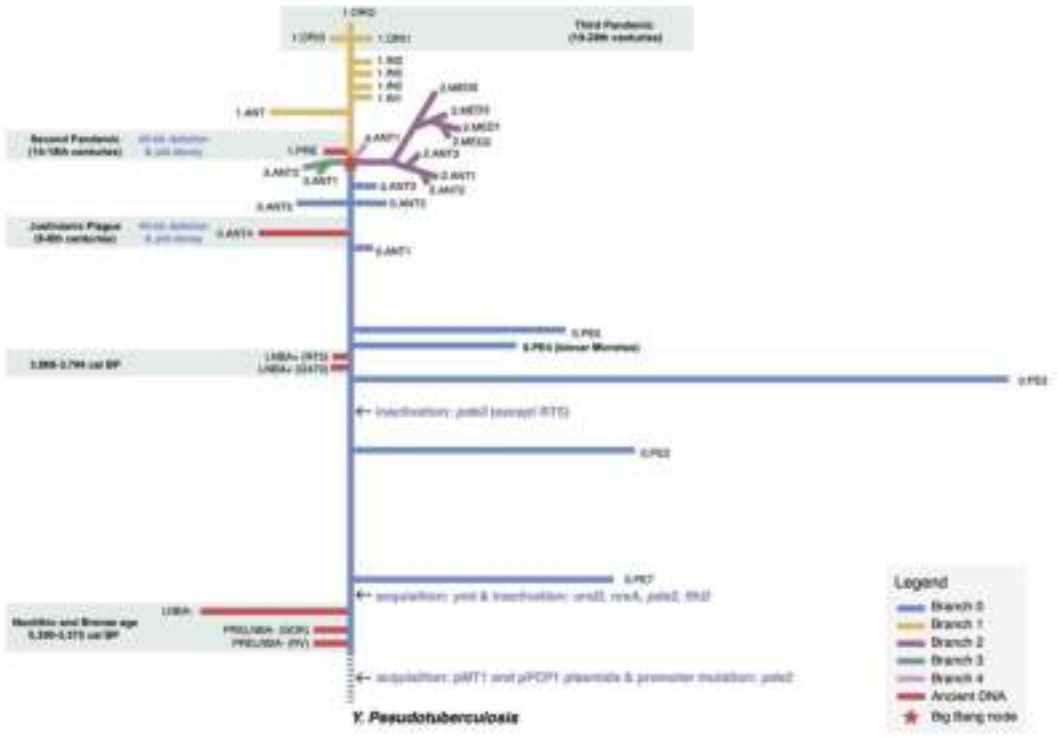

Fig. 1. Schematic phylogenetic tree of *Y. pestis* with *Y. pseudotuberculosis* at the root representing the ancestor of *Y. pestis*. Five major branches (Branch 0-4) are shown in different colors with ancient DNA related lineages colored in red (see color legend at the bottom-right corner). The "Big Bang" node giving rise to Branch 1-4, is marked by a star. Shaded areas indicate phylogroups associated with prehistoric plague or three historically recorded pandemics. The key events of plasmid acquisition and gene gain/inactivation/loss during evolution of *Y. pestis* are also displayed.

Known 3: Distribution and ecology of natural plague foci

The existence of plague natural foci has long been recognized and as far back as 1910–1911 during the Manchurian plague epidemic, which resulted in 50,000 to 60,000 deaths. Zabolotny D. K. proposed that the epidemics were caused by a spillover of the *Y. pestis* population from the natural *Marmota sibirica* plague foci [81].

Natural foci would consist of *Y. pestis*, hosts, vectors and the local environment

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and would be based on the food chain and spatial interaction between *Y. pestis*, hosts and vectors which then form the biomes of plague in evolution and finally establish the biogeographic community suited to the specific local environment [82]. The global distribution of plague is extensive and has been observed on all continents except Antarctica with natural foci distributed widely in Asia, Africa and the Americas (Fig.2) and covering wet and dry regions, grasslands, deserts, plateaus and plains[83-86].

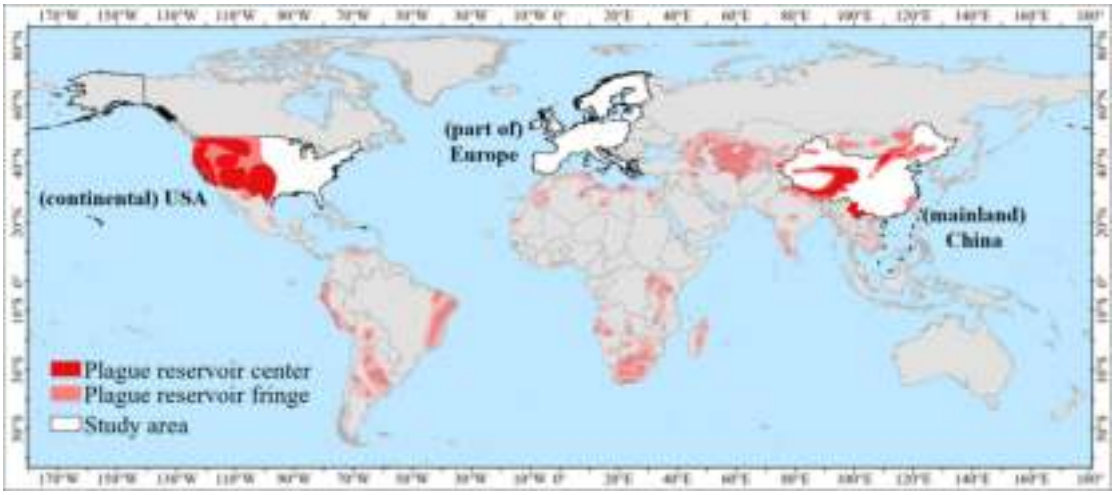

Figure 2 Map of the observed plague reservoir around the world during the last 50-80 years. This figure was adapted with modifications from Liu et al.[86]

In Asia, the active foci distribute in Central Asia (mainly in the Lake Balkhash region of Kazakhstan), Mongolia, China and Vietnam [83, 87, 88] and in Africa, the natural foci exist in broad areas mainly in the southern and eastern regions but is particularly prevalent in Madagascar, Tanzania, and the Democratic Republic of Congo [83]. In the Americas plague is endemic in Brazil, Bolivia, Ecuador, Peru and the United States [83] and in Europe and Oceania, there are no natural plague foci today, but most European countries were severely affected during the Second and third pandemics [89].

Mammals are the most common host species with 351 species globally that can act as hosts [90]. Among all these species, 279 species of rodents have been identified as plague carriers. According to the strong adaptability to a wide variety of lifestyle and

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environments and high reproductive capacity, rodents have a worldwide distribution (excluding Antarctica) and play an integral role in the *Y. pestis* circulation processes [84, 91]. *Y. pestis* circulation within natural foci is thought to be guaranteed with the existence of hosts through 3 processes: preservation, accumulation, and dissemination [84]. The primary rodent hosts that persist in the region are generally resistant to the bacteria and experience low level bacteremia when infected and results in the preservation process of pathogen. The accumulation process occurs when *Y. pestis* transmit to sensitive secondary and tertiary rodent hosts from primary rodent hosts via fleas and the secondary and tertiary rodent hosts generate a high level of bacteremia in their blood. Rodent overcrowding is a key contributing factor to dissemination of *Y. pestis* [92].

Due to geographical and climatic diversity host distribution varies globally with approximately 70 species known as main reservoir species concentrated in the regions corresponding to current plague foci, such as the western North America, eastern South America, eastern Africa, Central Asia and Southeast Asia (Fig.3) [84].

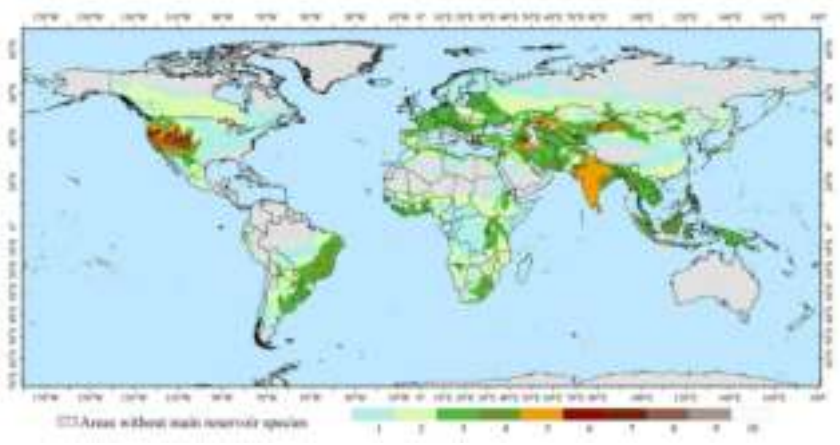

Figure 3 Geographic distribution of the main reservoir species (≈70 species) in different foci around the world at the spatial resolution of 0.5 arc degree [84].

In natural foci of plague, only fleas are its main vectors. Under natural conditions, the plague pathogen has been detected in 280 species and subspecies of fleas belonging to 62 genera [93].

Except the indispensable roles of hosts, vectors and *Y. pestis* for developing

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natural plague foci, environmental and ecological parameters also play key roles in this process. At the end of last century, some scholars proposed that global climate dynamics are responsible for outbreaks of infectious diseases, and others warn that long-term global warming could increase the risks of acquiring such diseases. Parmenter *et al* (1999) proposed the trophic cascade model in ecology and hypothesized that increased precipitation during the winter-spring period in arid and semi-arid regions would increase the ecosystem productivity, and further gave rise to greater population of mammal plague hosts and insect vectors [94]. Subsequent articles related to the trophic cascade model proved the validity of the hypothesis [95], and found additional complexity between the elements of the model, such as the lag effect of precipitation, bottom-up regulation and immediate climate effects on plague prevalence [96, 97].

Maintenance of plague foci depends on a whole suite of rodent hosts and their associated fleas [98] and climate affects the occurrence of plague by affecting rodent distribution, vector burden, population density, and ultimately susceptible population exposure to *Y. pestis* [99-101]. Unfavorable climatic conditions or extreme weather events due to climate change can lead to reduced productivity and famine, leading to human migration followed by rodents in search of water and food, which elevates human-rodent interactions and finally increase the risk of human plague [102, 103]. Additionally, climate can also influence replication cycles of pathogens or alter the mode of transmission, which in turn can lead to changes in the prevalence of plague [104].

The patterns of climate change differ from local to regional and ultimately to the global level with humidity, rainfall, temperature, etc. studied in detail at the local scale. Studies investigating climate and plague epidemiology in India during 1898 and 1949 revealed that the timing of plague outbreaks was associated with seasonal changes in humidity [105] while work examining the climate drivers of plague in the West Nile Region of Uganda found that the number of suspected cases in this region were negatively associated with dry season rainfall and positively with rainfall prior to the plague season. At high elevations, plague risk was positively associated with

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rainfall during winter and spring and negatively associated with rainfall throughout June [106, 107]. Conversely, environmental predictors of plague in Vietnam found that the risk of plague increased during the dry season when rainfall was low [108]. In addition, a study investigating the factors influencing global transmission velocity of plague during the Third Pandemic found that temperature exhibited a nonlinear, U-shaped association with spread speed [109].

There are also studies at the regional scale. The research on the human plague in the USA found that the Pacific Decadal Oscillation, together with previous plague levels and above-normal temperatures, explained much of the plague variability [110]. The research on the connection between the North Atlantic Oscillation (NAO) and plague in pre-industrial Europe found that the pathway from climate change to plague incidence is distinctive in its spatial, temporal, and non-linear patterns. The NAO-plague correlation in Atlantic-Central Europe primarily remained positive, while the correlation in Mediterranean Europe switched between positive and negative alternately [111].

Unknown 3: Ecological interactions of *Y. pestis*, the hosts and natural environments for long-term survival of natural plague foci.

Because of the scarcity or complete absence of controlled studies before the turn of the last century, there are obstacles in seeking the associations between the global scale climate drivers and plague outbreaks. However, the research on the relationship between volcanism and global plague pandemics suggested that all three pandemics coincided with periods of significant volcanic activity, and a series of connections between volcanism and plague are possible [112].

The ability of *Y. pestis* to colonizes and propagates in the flea gut prior to transmission to a new host is well established [113, 114] and within an inveterate rodent population, enzootic (maintenance) plague episodes ensure *Y. pestis* is passed through a partially resistant enzootic host population by fleas and upon transmission into epizootic (amplifying) hosts plague rapidly spreads [115]. Plague is therefore circulating in associated hosts prior to re-emergence in the human population [116,

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117]. However, although this rat/flea model has endured as the plague reservoir for transmission to humans, it is likely to constitute only one mode of transmission since the general picture has been hugely oversimplified [118].

A remarkable aspect of epizootic plague biology is the fact that *Y. pestis* displays interepizootic cryptic periods of quiescence [119, 120] between plague outbreaks in rodents or humans. In locations where there have been no human cases or mass rodent die-offs [116, 121] plague often re-emerges decades later, such as in Algeria, Libya, Madagascar, and India 57, 25, 60 and 30 years respectively. In China, in Xinjiang province the Junggar Basin plague focus was discovered in 2005 following continuous surveillance and this plague focus was in the cryptic quiescence period since the year of 1949 [122].

Reports of long interepizootic periods during which time *Y. pestis* is absent from host and vector populations have largely been overlooked, possibly due the fact that it has been suggested that once *Y. pestis* evolved to colonize insect and mammalian vectors it became host dependent [123]. However, it is more likely that *Y. pestis* adopts a 'sit and wait' lifestyle [124] in the environment where it can survive under highly diverse ecological conditions which would compare favorably with the lifestyle of the near identical (~98% DNA identity) free-living soil borne gastrointestinal pathogen ancestor, *Yersinia pseudotuberculosis* from which it evolved [6, 125-127].

Where then does *Y. pestis* persist during these quiescent periods and which factor(s) result in epizootics? There are several hypotheses to explain this phenomenon, including spill-over of plague from its primary hosts to highly susceptible rodents with high mortality [128]. This would exacerbate transmission from low level and undetectable epizootics within the main host by changes in ecological and environmental conditions resulting in unexpected fluctuations of host/vector abundance and/or behavior [115, 128, 129]. There may also be the formation of the non-culturable L-form of *Y. pestis* in soil or survival in protozoa or biofilm formation either abiotically or biotically on the surface of nematodes [130-133]. A number of microorganisms are resistant to soil-dwelling free-living

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amoebae predation [134] and this is also true for *Y. pestis* [135]. Markman *et al*., [136] and Benavides-Montañ o and Vadyvalooa, [137] showed that *Y. pestis* is resistant to trophozoite predation and can survive and replicate intracellularly in *Dictyostelium discoideum* and *Acanthamoeba castellani* respectively. *Y. pseudotuberculosis* and *Y. enterocolitica* can also survive in *A. castellani* trophozoites and cysts. However, there is a need for field-based investigations to corroborate these hypotheses [138-141] .

Another facet of interaction between *Y. pestis*, hosts, vectors and environmental/ecological parameters is which natural drivers select the variants of the bacterium and how the gene mutations in these variants impact the persistence of plague foci. For example, colder and drier winters may select for *rpoZ* mutants which rapidly form biofilms and subsequently promote rapid transmission by infected fleas [77]. Further works need to initiate to elucidate the existence mechanisms of natural plague foci.

Knowns 4: Transmission of *Yersinia pestis* by fleas

Bubonic plague as a vector-borne disease and the key role of the flea in its transmission were recognized soon after the discovery of *Y. pestis*. Several possible transmission mechanisms were considered, initially using a model in which groups of fleas were allowed to feed on a rodent dying of septicemic plague and then transferred to naïve rodents [142]. Transmission resulted if the fleas were transferred within a few days after their infections blood meal but waned rapidly beyond that. This is now referred to as early-phase transmission. A few years later, a second mode of transmission was discovered– the well-known “blocked flea” transmission model [114].

Both modes of transmission stem from the marked propensity of *Y. pestis* to rapidly autoaggregate in the flea digestive tract. Large masses of bacteria can form in the midgut and localize to the proventricular valve in the flea foregut within a few hours after an infectious blood meal [143, 144]. Initially, the bacteria appear to be associated with a semi-fluid, serous matrix, and in some fleas an aggregate can extensively occupy the lumen of the proventriculus within a few days. Although long

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assumed to be mechanical (transmission via contaminated mouthparts), the infected foregut is the more likely source of bacteria transmitted during the early phase. According to this model, a heavy proventricular infection is sufficient to transiently impede blood flow, resulting in some backflow of contaminated blood into the bite site [143, 145]. Because the early proventricular aggregates are relatively soft and fragile, however, they are readily dislodged and washed back into the midgut by the incoming blood pressure, terminating early-phase transmission. The second mode of transmission, the well-described blocked flea mechanism, is phenomenologically the same (regurgitation from the foregut) except in this case the proventricular aggregate is more cohesive and firmly entrenched in the proventriculus such as it severely impedes the passage of blood into the midgut.

Fundamentally, the flea-borne transmission phases reflect a continuum of *Y. pestis* biofilm development in the proventriculus. Maturation of the biofilm over the following days notably involves production of a polysaccharide extracellular matrix that acts to stabilize the *Y. pestis* aggregates, making them more cohesive and firmly fixed to the proventriculus [146]. A transmission-competent partially blocked stage develops first [147]. At the end stage of complete blockage, proventricular biofilm prevents feeding, but continuous probing and feeding attempts by a blocked flea result in more efficient regurgitative transmission.

Biofilm development is a common strategy of many bacteria to adapt to nutrient-limited moist environments and is characterized by the formation of surface-adherent dense microcolonies that are embedded within an extracellular polymeric matrix. Metabolic adaptation of *Y. pestis* to the protein- and lipid-rich but carbohydrate-poor flea gut induces biofilm. The regulatory and development pathways underlying this process are complex (reviewed recently in [148]), but culminate in upregulation of the Hms genes, which act to produce the aforementioned polysaccharide component of the biofilm matrix [143, 149]. *Y. pestis* mutants lacking the *hmsHFRS* operon are unable to block fleas because unable to synthesize the polysaccharide extracellular matrix stabilizing *Y. pestis* aggregates in the proventriculus [144, 150]. However, a *hmsR* mutant is transmissible by the

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early-phase mechanism [151, 152].

Differential biochemical characteristics of vertebrate blood and the speed at which it is digested can strongly affect the ability of *Y. pestis* to colonize the flea gut and be transmitted [145, 153]. For example, rodent fleas infected using rat blood are much better early-phase transmitters than fleas infected using mouse blood [145]. Furthermore, an augmenting effect of Hms-dependent biofilm formation on transmission (corresponding to evidence of partial or complete blockage) can be seen as early as three days after an infectious rat blood meal, suggesting that early-phase and proventricular blockage-dependent transmission can overlap temporally. The initial bacterial aggregates following an infected rat blood meal are associated with a viscous mixture of undigested hemoglobin and red blood cell components that results in a much more tenacious proventricular colonization as well as reflux of the infected mixture into the esophagus [145]. This condition enhances transmission in the early phase.

Reliance on flea vectors for transmission between mammalian hosts is an evolutionary recent adaptation, arising within the last 3,000 to 6,000 years since *Y. pestis* emerged from its closely related progenitor, *Y. pseudotuberculosis*. This abrupt switch to arthropod-borne transmission was possible because it required just a few, discrete genetic changes [75]. Pseudogenization and functional loss of three genes extended the pre-existing biofilm capability of the *Y. pseudotuberculosis* progenitor to the flea gut environment because their loss resulted in increased intracellular levels of cyclic-di-GMP, a universal bacterial inducer of biofilm development. Two other genetic changes were also important. One was another gene loss that eliminated an enzyme activity (urease) that is toxic to fleas [22], but it is not related to flea blockage. The other was the acquisition of a new gene, present on the *Y. pestis*-specific plasmid, that encodes a phospholipase D enzyme (Ymt) that protects Gram-negative bacteria from a toxic product produced by the flea [154, 155]. Making these same five changes in a modern *Y. pseudotuberculosis* strain was sufficient to enable it to block fleas [75].

The evolutionary road to flea-borne transmission was a stepwise process. The earliest *Y. pestis* strains, characterized by genome sequencing from infected human

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skeletal remains dating from the Stone Age, already had most if not all the plague-related virulence factors. However, most of the Stone Age strains had only one of the three biofilm-enhancing genetic changes, still had urease, and had not yet acquired Ymt [156]. This led to the conclusion that these strains could be transmitted by fleas but to an extent that was not compatible to sustain a vector borne cycle for a long period of time, at least in relation to blockage. However, it is now known that the importance of Ymt for bacterial survival in the flea gut is host-blood-dependent. Ymt-negative *Y. pestis* survives well in fleas infected using blood of the brown rat (*Rattus norvegicus*) [155]. This new finding, together with the increased early-phase transmission efficiency associated with brown rat blood infections and the biofilm-enhancing effect of the one genetic change, suggests that a rudimentary rat flea-brown rat transmission cycle was possible. Interestingly, the northern China habitat of *R. norvegicus* overlaps geographically with the emergence of *Y. pestis*. Acquisition of Ymt and accumulation of the remaining gene losses described above occurred by the Bronze Age, fully enabling flea-borne transmission, and significantly extending the host range in which it could occur [155, 157].

Unknowns 4: Complex interaction between *Yersinia pestis* and fleas

While much has been learned, many gaps remain in our understanding of *Y. pestis* transmission. The general transcriptional responses of *Y. pestis* in adapting to the flea gut and of the flea to infection have been characterized [38, 158], but specific gene induction steps and molecular mechanisms required to produce a transmissible infection have yet to be fully elucidated. Key factors at the flea-*Y. pestis*-mammalian dermal interface that determine successful transmission have not been well characterized. Vector competence differences between flea species and how this relates to enzootic and epizootic plague are not well understood. Thus, questions ranging from individual *Y. pestis*-flea interactions to the complex ecology of plague, involving many flea vectors and hosts, are still ripe fields for further research.

A prime topic is biofilm formation in the flea. What is the molecular and biophysical basis for the rapid aggregation of *Y. pestis* upon entering the flea gut and

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what is the makeup of the extracellular matrix that surrounds these aggregates? These features of its vector-specific life stage may protect *Y. pestis* from antibacterial factors in the lumen of the flea digestive tract, which is a hostile environment for Gram-negative bacteria. Furthermore, *Y. pestis* is transmitted in association with the extracellular matrix. There are clear differences in pathogenesis following flea-bite transmission versus intradermal injection of *in vitro* culture-grown *Y. pestis*. For example, a chronic intradermal infection often follows transmission by fleas but is not seen following intradermal injection [159]. Differences in antigenic makeup between flea- and culture-derived bacteria as well as the presence of extracellular matrix may be responsible. Flea saliva is also injected into the bite site but its effect on nascent infection and immunity have not been thoroughly investigated. Mice and rats mount an immune response to flea salivary components, but this does not appear to significantly affect productive transmission [160].

How the biochemical characteristics of host blood can so strongly influence infectivity and transmission is another unknown. The rate at which a particular type of blood is digested by the flea and the concomitant generation of antibacterial digestion products appear to be important, but the molecular mechanisms are yet to be discovered. For example, the importance of the protective effect of Ymt varies with blood meal source [155], but the identity of the relevant target(s) of this phospholipase D in the flea gut remains elusive.

Y. pestis is a generalist, able to produce a transmissible infection in many different flea species by the same mechanism. However, fleas vary widely in their vector competence and transmission efficiency by both early-phase and blockage mechanisms [161]. The reasons for this are not clear, although differences in digestion kinetics and processing of host blood, feeding frequency and excretion rates, and foregut anatomy are likely influencing factors [143]. The incidence and rate of complete blockage development are much lower for some fleas than for others, leading to proposals that early-phase transmission is more ecologically important in certain flea-rodent transmission cycles. However, blockage rate comparisons between different fleas have been based on few studies, small sample sizes, and variable

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experimental conditions, making conclusions tentative and sometimes discordant or misleading [162].

Quantitative evaluation of the relative efficiency of the early-phase and blockage-dependent transmission modes for different flea merits systematic reexamination, using more standardized protocols with appropriate controls [142, 161]. The infectious blood source should also be taken into account. For example, recent early-phase transmission comparisons have all been based on fleas infected using brown rat blood. As described above, this blood source induces an initial severe foregut infection highly favorable for early-phase transmission, which is not seen with other host bloods. Ideally, future studies would start with a single cohort of fleas infected using their usual blood source, which would then be monitored for early-phase transmission, blockage development, and blockage-dependent transmission at different times after infection. These studies would provide more reliable vector competence data for mathematical models of enzootic and epizootic plague.

Known 5: virulence factors and their roles in pathogenesis of *Y. pestis*

Three pathogenic yersiniae share several virulence mechanisms, among which type III secretion system (T3SS), encoded by a 70-kb plasmid (pCD1 in *Y. pestis* and pYV in *Y. enterocolitica* and *Y. pseudotuberculosis*), is essential for full virulence in all three pathogens. Although closely related in terms of evolution, these pathogens are extremely diversified in clinical symptoms, ecological niches, and typical infection routes. *Y. pestis* is an etiological agent of plague that has caused more than 200 million deaths in the past three pandemics, whereas the other two cause only self-limited gastrointestinal diseases. In addition to acquisition of two virulence-associated plasmids of pMT1 and pPCP1, massive gene losses played more important roles than gene acquisitions in the virulence evolution of *Y. pestis* from its acknowledged ancestor *Y. pseudotuberculosis* [163].

5.1 Type III secretion system (T3SS)

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Gram-negative bacterial T3SS assembles a macromolecular device called injectisome capable of delivering virulence effectors into eukaryotic cells. *Yersinia* T3SS is temperature and contact dependent, and the secretion of effectors are triggered at mammalian temperature only when contacts with host cells occurs. Virulence factors of *Y. pestis* exhibit various enzymatic activities that can disrupt eukaryotic cytoskeleton and host immune singling to promote the bacterial survival and replication.

LcrV is the component of needle tip structure of T3SS injectisome, and it also induces the production of suppressive interleukin (IL)-10 [164]. YopH is a potent tyrosine phosphatase that dephosphorylates a variety of functionally distinct substrates. YopH inhibits T cell and B-lymphocyte activation via dephosphorylation of Lck and ZAP70, the major signal transducer for the T cell antigen receptor (TCR), enabling the blockage of the first step of TCR and suppress immune response against *Yersinia* [165]. YopE, YopT, and YpkA, all of which belong to a large family of bacterial toxins that target the Rho family of small GTP-binding proteins (Rho GTPases), inhibit phagocytosis by professional phagocytes. YopE inactivates multiple Rho GTPases, including RhoA, Rac1, and Cdc42 [166, 167]. YpkA is inactive in bacteria and is activated by binding to the coactivator actin in eukaryotic cells [168]. Upon activation, YpkA undergoes autophosphorylation and phosphorylates Gαq (the α subunit of heterotrimeric G proteins) [169], vasodilator-stimulated phosphoprotein (VASP)[170], otubain-1, among others, to disrupt actin cytoskeleton. YopT acts as a papain-like cysteine protease that removes the prenyl group from RhoA, RhoG, Rac1, and Cdc42, which releases these GTPases from the membrane and lead to their inactivation [171]. YopT has been shown to contribute to the anti-phagocytic activity of bacteria but not essential for the virulence of pathogenic *Yersinia*. This may be due to the fact that YopT is functionally redundant in the presence of YopE and YpkA [166].

Recognition of *Y. pestis* by host nod-like receptors (NLRs), including NLRC4, NLRP3 and Pyrin, induces the formation of inflammasomes, leading to the caspase-1 activation, IL-1β processing and secretion and cell death. YopM enables bacteria to avoid this host innate immunity strategy by directly binding caspase-1 to inhibit caspase-1 activation and inflammasome maturation [172]. Furthermore, YopM

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interacts with Pyrin and kinases RSK1 and PKN1, the negative regulators of Pyrin, to inhibit the activation of the Pyrin inflammasome that is triggered by the RhoA-inactivating enzymatic activities of YopE and YopT [173, 174]. YopM has a nuclear localization signal at its carboxyl terminus [175] and *Y. pestis* infections cause a systemic depletion of natural killer cells in mice in YopM-dependent manner [176].

YopJ functions as an acetyltransferase that inactivates nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways by acetylating MAPK kinase [177]. Once translocated into host cells, Yop effectors act in a finely tuned and coordinated manner to hijack various host signaling pathways to thwart the innate immune response [166]. It has also been reported that YopJ showed deubiquitination activity towards molecules in NF-κB signaling pathway including TRAF2, TRAF6, and IκB to inhibit the inflammatory response [178]. Mutation of *yopJ* in *Y. pestis* showed no obvious virulence attenuation, although greater virulence attenuations have been observed in enteropathogenic *Yersinia* [179-181].

YopK plays important regulatory roles in Yop translocation and controls both the rate and fidelity of Yop injection into host cells, and a *yopk* mutant exhibits Yops-translocation phenotype [182, 183]. This regulatory mechanism is partially exerted by interaction of YopK with the YopB-YopD translocon, which prevents the recognition of the *Yersinia* T3SS and inhibit the NLRP3 and NLRC4 inflammasome activation [184]. YopK inhibits the attachment of *Y. pestis* to the host cells by binding to the extracellular matrix protein MATN2 and disruption YopK-MATN2 interaction results in Yops hyper-translocation phenotype in similar to the *yopk* mutant [185]. YopK also interacts with the receptor for activated C kinase (RACK1) and that this interaction promotes the phagocytosis resistance of *Y. pseudotuberculosis* [186].

5.2 Lipopolysaccharide and Pla

Y. pestis produces a short-chain, rough lipopolysaccharide (LPS) that lacks the O antigen due to mutations of genes involved in LPS biosynthesis. The O antigen confers resistance to complement- mediated phagocyte bacterial killing and is important for virulence of *Y. enterocolitica* and *Y. pseudotuberculosis*. Pla is a pPCP1-encoded protein protease that can cleave plasminogen to plasmin, which further degrades extracellular matrix proteins to promote the bacterial dissemination.

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The proteolytic activities of Pla require rough LPS, but are inhibited by the O antigen, highlighting the selective advantage of rough LPS for *Y. pestis* [187]. Pla is also an adhesin that is specific for laminin and heparan sulfate proteoglycan and cleaves the C3 component [15, 188]. However, Pla is not required for serum resistance. As the temperature shifts from 26 °C to 37 °C, lipid A of *Y. pestis* switches from a hexa-acylated to tetra-acylated form due to the loss of *lpxL* that is responsible for adding of the secondary acyl chains to the tetra-acylated lipid A precursor [53]. The tetra-acylated lipid A is poorly recognized by Toll-like receptor 4 (TLR4), which render much lowered toxic compared to the other *Enterobacteriaceae* grown at 37 °C [52] and facilitates the pathogen to escape the host immune surveillance [189].

5.3 F1 Capsule

The F1 capsule is composed of linearly polymerized Caf1 subunit that is encoded by the pMT1 plasmid. F1 appears to have some role in anti-phagocytosis although the primary *Y. pestis* virulence factor that blocks uptake by phagocytosis is T3SS [190], and strains with spontaneous mutations or deletion of *caf1* are still virulent [191, 192]. The F1 antigen contributes to virulence in naturally acquired bubonic plague but is not essential for pneumonic plague in mouse or guinea pig infection model [13, 190, 193-196]. An anti-F1 antibody provides high protection against F1-positive but not F1-negative strains [197].

5.4 Adhesion and invasion

Y. pestis is lymphophilic and prefers to attack certain type of cells, such as innate immune cells including macrophages, neutrophiles and dendritic cells (DC) [198] as well as the lung alveolar epithelial cells. The pH6 antigen (PsaA) adhesin contributes to the cell-type preference of *Y. pestis*. Two major receptors for Psa were found to be β1-linked galactosyl residues in glycosphingolipids and phosphocholine and phosphatidylcholine in phospholipids, which are present on alveolar epithelial cells [199, 200]. However, an *in vitro* cell infection assay showed that PsaA promotes resistance to phagocytosis rather than adhesion to mouse macrophages [201]. PsaA facilitates the delivery of Yops by T3SS by mediating intimate contacts between host cells and bacteria, as do other adhesions, such as the attachment invasion locus (Ail) and Pla [202]. Mutation of *psaA* locus results in a 100-fold increase in LD_{50} via

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intravenous challenge, but no attenuation by subcutaneous route of infection [203, 204].

Ail is a chromosomally encoded small-membrane protein common to all three pathogenic *Yersinia*. The major adhesins YadA and Inv in *Y. pseudotuberculosis* are inactivated in *Y. pestis*, thus Ail is the primary adhesin of *Y. pestis*. An *ail* negative mutant of the KIM5 (*pgm*$^-$) was attenuated over 10^3-fold in intravenously challenged mice and deletion of *ail* in CO92 resulted in approximately 10^5-fold attenuation in rat model of bubonic and pneumonic plague [205-207]. Ail binds to the ECM components fibronectin, laminin, and heparin [208, 209], and is critical for invasion and Yop delivery into host cells. Ail also binds to the complement inhibitor C4b-binding protein and vitronectin, a host protein that is critical in cell attachment, fibrinolysis, and inhibition of the complement system [207, 210, 211]. *Y. pestis* Ail shows higher binding affinity to ECM substrates than *Y. pseudotuberculosis* Ail because of two amino acid differences, and the rough LPS of *Y. pestis* increases the accessibility of Ail to eukaryotic cells.

5.5 Yersiniabactin

Iron utilization by *Y. pestis* predominately relies on the yersiniabactin (Ybt), which is encoded in a high-pathogenicity island within 102-kb pigmentation (*pgm*) chromosomal locus common to the pathogenic *Yersinia* species. The *ybt* locus includes genes for Ybt siderophore synthesis (high-molecular-weight protein 1 (HMWP1), HMWP2, YbtD, YbtE, YbtS, YbtT, and YbtU) and Ybt uptake (YbtQ, YbtP, and Psn). Ybt has high affinity for ferric iron and is necessary for acquiring iron from transferrin and lactoferrin by yersiniae [212]. The *pgm*$^-$ mutant or specific Ybt synthesis or uptake mutants are avirulent in subcutaneously challenged mice but are fully virulent in intravenously challenged mice [45, 213-215]. In a natural context of infection (i.e. fleabite) a Ybt-negative mutant is also attenuated in virulence but remains capable to cause fatal disease in some mice [45].

Unknown 5: Early interactions between *Y. pestis* and the host cells: still largely a mystery

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Y . pestis is a facultative intracellular pathogen that predominantly lives an extracellular life. In a typical fleabite transmission, *Y. pestis* bacilli are readily engulfed by professional phagocytes when initially entering the host. Those taken up by PMNs can temporally survive and replicate within 3 days post infection (dpi); however, the living *Y. pestis* bacilli can only be found in macrophages at 5 dpi [216]. The released bacteria from the disrupted host cells embark on an extracellular life thereafter. Thus, rapid adaption to the adverse mammalian host environments is critical for *Y. pestis* through arming itself with a variety of virulence factors, including F1 capsule and the T3SS to avoid phagocytosis and to paralyze the host immune response. An impressive feature of plague infection is that *Y. pestis* replicates massively without evoking a significant host innate immune response during the initial period of infection, termed as pre-inflammatory phase. Studies of bubonic plague rodent models revealed that *Y. pestis* can replicate rapidly in draining lymph nodes (dLNs) near the infection sites with no detectable inflammation at the early stage (6–36 hours post infection (hpi)) of infection, and then escape from the bubo and disseminate via lymph circulation to establish systemic infections [50, 217, 218]. Pathology studies on mouse primary pneumonic plague confirmed the biphasic feature, in which the infection begins with an immune-suppressive state in the first 24–36 hpi and rapidly progresses to a highly proinflammatory state by 48 hpi [219].

Although the rapid adaption to the adverse mammalian host environments is critical for the pathogenesis *Y. pestis,* reports on the early interactions between the bacteria and the host immune cells are limited and remains to be clarified. It has been shown that *Y. pestis* preferentially targets host immune cells during infections. Dendritic cells (DCs), macrophages and neutrophils are the most frequently injected cells; however, T and B lymphocyte are less selected [198], even though it delivers Yop effectors to almost all types of cells in tissue cultures [198, 220]. In an intradermal (i.d.) infection of *Y. pestis*, a larger number of PMNs were shown to be recruited by 4 hpi and the majority of cell-associated bacteria were associated with PMNs but very few bacteria interact with DCs [221]. Neutrophil depletion and CCR7 knockout mouse experiments indicated that dissemination of *Y. pestis* from the dermis to the dLNs may not rely on DCs and PMNs. Another study showed that intracellular *Y. pestis* bacteria can survive and replicate in cultured human PMNs, and a high percentage of the infected PMNs underwent apoptosis within 12 hpi. The PMNs containing *Y. pestis*

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can be recognized and internalized by autologous macrophages, in which *Y. pestis* survives and replicates following efferocytosis [222].

Recently, in order to describe the critical events occurring during the initial interactions between *Y. pestis* and the host innate immune cells, single-cell RNA sequencing (scRNA-seq) was utilized to characterize the composition and alterations of immune cells in the functional dynamics of dLNs during the early stages of bubonic plague which revealed a dynamic immune landscape at single-cell resolution of murine dLNs in the response to *Y. pestis* infection [223]. The data suggested that DCs responded to the presence of *Y. pestis* within 2 hpi, followed by the activation of Mφs/Mons and recruitment of polymorphonuclear neutrophils (PMNs) to dLNs at 24 hpi. PMNs could be recruited to lymph nodes by CCL9 secreted by Mφs/Mons through CCR1–CCL9 interaction. When mice were treated with BX471, a CCR1 antagonist, prior to infection, the number of PMNs in dLNs at 24 hpi decreased significantly in comparison to the untreated mice, suggesting PMN recruitment by Mφs/Mons through CCR1 is important for host defense against plague. It is critical important to confirm these finding based on the scRNA-seq study in an animal bubonic model in future investigations.

Y. pestis biovar microtus strains are highly virulent to mice but avirulent to humans and other larger mammals. The underlying mechanisms responsible for this unique host-specific pathogenesis is far from clear and difficult to investigate due to biosafety and ethics issues. *In vitro* infection studies showed that human macrophages could clear intracellular *Y. pestis* microtus 201, more efficiently than the murine macrophage RAW264.7 cells [224]. Given that the survival in macrophages at the early stage is critical for the later establishment of infection of *Y. pestis*, it is possible that the key events occurring during this period could lead to the host-specific pathogenesis of biovar microtus strains. Higher bactericidal activity of human macrophages to *Y. pestis* 201 could partially explain the virulence attenuation of biovar microtus strains in humans. Biovar microtus strains of *Y. pestis* is more closely related to its progenitor *Y. pseudotuberculosis* than the strains of other biovars based on the phylogenetic studies [70]. The products of the biovar-specific genome contents could be involved in either the direct bacteria-host interactions or control of the

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adaptions to the hostile environments inside the host to contribute to the variation in virulence in different mammalian hosts.

Known 6: Diagnosis and treatment of plague

The diagnosis of plague depends on epidemiological information, clinical manifestations, physical and laboratory examination. Plague patients usually have contact history with infected animals or patients. In the clinic *Y. pestis* infections usually present either as bubonic plague with regional lymphadenopathy, primary pneumonic plague following direct inhalation of infectious respiratory droplets or aerosolized *Y. pestis* and primary septicemic plague resulting from cutaneous exposure [225]. Some other less common forms of plague, include local cutaneous ulcers at the entry site of *Y. pestis* in humans, plague pharyngitis, plague endophthalmitis, meningeal plague, secondary septicemia plague and secondary pneumonic plague. Isolation and identification of *Y. pestis* from clinical specimens are critical for accurate diagnosis in the laboratory (https://www.cdc.gov/plague/healthcare/clinicians.html) [226]. F1 capsular antigen and its antibody are usually targets for immunological detection methods [227], including F1 antigen hemagglutination inhibition test, passive hemagglutination test, enzyme-linked immunosorbent assays and direct fluorescent antibody testing. Polymerase chain reaction (PCR) is often used for directly detecting *Y. pestis* in clinical specimens. Some point-of-care testing such as immunochromatographic assays (ICA) have been developed for on-site detection of *Y. pestis* or its antibody.

The WHO (*Plague Manual: Epidemiology, Distribution, Surveillance and Control*) [225] and WHO guidelines for plague management [228] recommend the treatment scheme for the different form of plague.

U.S. centers for disease control and prevention recommended a new guideline for antimicrobial treatment and prophylaxis of plague [229]. This guideline gave detailed treatment schemes for different types of plague. To treat pneumonic plague [229], the

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first-line antibiotics for adults include fluoroquinolone (ciprofloxacin, levofloxacin, and moxifloxacin) and aminoglycoside (gentamicin and streptomycin), while the alternatives are tetracycline (doxycycline), amphenicol (chloramphenicol), some fluoroquinolone (ofloxacin and gemifloxacin), aminoglycoside (amikacin, tobramycin, and plazomicin) and sulfonamide (trimethoprim-sulfamethoxazole). For children aged ≥1 month to ≤17 years, the recommended first-line antibiotics are fluoroquinolones (ciprofloxacin and levofloxacin) and aminoglycosides (gentamicin and streptomycin). Alternatives are tetracycline (doxycycline), chloramphenicol, fluoroquinolones (moxifloxacin and ofloxacin), aminoglycosides (amikacin and tobramycin) and sulfonamides (trimethoprim-sulfamethoxazole). Further recommendations for treatment of bubonic plague and other less common forms of plague in adults and children including prophylaxis for pre- and post-exposed adults and children have also described[229].

However, *Y. pestis* isolates with multiple drug resistance by a transferable plasmid have previously been reported in Madagascar [230, 231]. *Y. pestis* is also considered to be a category A agent with bioengineered multi-drug resistant *Y. pestis* with the potential to be used as a bioterrorism weapon [232, 233]. There is therefore a need to develop precision treatment schemes using antibiotic combinations or other biotherapeutics, including monoclonal antibodies [234]. A potent inhibitor, targeting LpxC (uridine diphosphate-3-O-(R-3-hydroxymyristoyl)-N-acetyl-D-glucosamine deacetylase), was reported to be potentially effective for treating plague [235-238]. Other potential drugs, including cationic antimicrobial peptides, antivirulence drugs, predatory bacteria, and phages and phage endolysins etc., and immunotherapies are also under exploration [237].

Unknown 6: On-site diagnostic techniques; precision treatment using different kinds of antibiotics or other biotherapeutics.

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Although we have comprehensive immunological and nucleic acid-based techniques for plague diagnosis in the laboratory, we still lack rapid, sensitive and specific point-of-care testing for on-site detection and ultrasensitive assays in the laboratory for detecting traces of *Y. pestis*. Single molecule detection methods are being developed [239, 240]. A microsphere labeled with carbon dots (CDs) and a colloidal gold-based lateral flow immunochromatographic assay combined with CRISPR/Cas-based nucleic detection for highly specific and sensitive detection of bacterial pathogens has been developed[241, 242]. These highly sensitive techniques are also easily developed as a point-of-care testing for onsite screening of potential pathogens.

Known 7: Live attenuated vaccines and subunit vaccines

Attempts to create effective plague vaccines started soon after the microorganism was first identified. Most approaches involved inactivation of bacterial culture of wild-type isolates and the result was a commercial preparation of USP vaccine composed of formaldehyde-killed *Yersinia pestis* 195/P that was used in the West for several decades. The major contributor to immunity elicited by this vaccine was likely provided by significant expression of the capsular antigen F1; however, this vaccine was reactogenic with short-term immunity. Importantly, it did not convey robust defense against pulmonary exposure to *Y. pestis*. Currently, it is not available [243].

The live plague vaccine (LPV) introduced by Girard and Robic *via* selection of attenuated variant EV76 was a breakthrough event, and it saved numerous lives in Madagascar when it was first tested during the 1930 plague epidemic. Derivatives of this vaccine were widely used as the LPV prototype in many countries. This vaccine was never approved in the West due to the safety concerns but was widely used in the USSR [244] where it was administered to millions of individuals without any serious complications. LPV is currently in use in several counties of the former Soviet Union to immunize plague workers, or those living in plague endemic territories when

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plague is active [245]. The LPV in lyophilized form is easy to produce, store and utilize. However, its major disadvantage is short-term protection that requires an annual booster. The reason for this phenomenon is not well understood, as live vaccines often elicit prolonged or even life-time immunity. Unraveling the mechanism of this LPV deficiency in human vaccination will be crucial for the development of an improved version of LPV, and likely for the development of a novel subunit plague vaccine [245]. Of note, a study of humans vaccinated with EV NIIEG (EV76 version) by using a panel of highly pure recombinant *Y. pestis* antigens showed that both humoral and cellular immune responses to LcrV were generally poor. This was true even for donors who received multiple annual boosters over a long period of time [246]. Since LcrV is a major protective antigen of *Y. pestis* (section 5.1), it is speculated that modification of LPV to elicit an enhanced response to LcrV may increase the level and length of protection.

Since the first publication demonstrating that recombinant LcrV could provide protection in mice against infection with *Y. pestis* [247], there were several hundred articles published that described successful use of this antigen alone or in combination with F1 in development of plague subunit vaccines (PSV) [248]. The LcrV provided most of the protection, while the addition of F1 further increased the level of immunity. The LcrV/F1 vaccines were proven to work well in a number of formulations, such as purified antigens with different adjuvants, micro- and nanoparticles, viral and bacterial vectors and plants. [248]. The most common animal models to test the protective properties of this specific PSV against bubonic and pneumonic plague were mice, rats, guinea pigs, and non-human primates. However, this subunit vaccine still needs optimization for human use, mostly with regard to selecting the most efficient adjuvant and/or the method of expression of these antigens. The different versions of LcrV/F1 subunits vaccines have undergone (or are in the process) phase 1 and 2 clinical trials; however, no phase 3 clinical trials have been conducted on them thus far (https://clinicaltrials.gov/ct2/show/NCT00246467; https://clinicaltrials.gov/ct2/show/NCT00332956;

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https://clinicaltrials.gov/ct2/show/NCT01381744;
https://clinicaltrials.gov/ct2/show/NCT05330624).

Unknown 7: Effective vaccine for long-term protection; immune correlates

The EV76-based LPV is attenuated mostly due to the lack of pigmentation locus (Pgm), containing a high pathogenicity island. Therefore, some efforts were focused on improvement of such vaccines by introducing additional mutations. For example, the *lpxM* (*msbB*) mutant of EV NIIEG affecting the lipid A structure was less reactogenic for animals and provided prolonged multiplication in lymphoid tissue. These characteristics potentially could improve both protective properties and immunity longevity of the existing LPV [249]. However, the usefulness of this approach should be verified in clinical trials. Mutations in many other genes have also been evaluated in attempts to rationally attenuate *Y. pestis* for vaccine use, both alone and in combination with the Pgm-negative variants [244, 250]. The most promising variant was a triple deletion mutant of *Y. pestis* CO92 impaired in *lpp*, *msbB*, and *ail* [251]. This mutant not only elicited strong humoral and cellular immune responses and robust protection in different animal models but was safe enough for Centers for Disease Control and Prevention to remove it from the select agent list.

Overall, the central advantage of live vaccines compared to other vaccine types is their ability to elicit strong immune responses to several antigens, including those expressed *in vivo*, mimicking to some extent the initial course of natural infection. In addition, live vaccines are known to stimulate robust T cell immunity that contributes to protection against plague [252]. While capsular antigen F1 is a well-established protective antigen, the response to this polymeric abundant protein is overwhelming, and might be detrimental to the overall immunity. To increase the contribution of other antigens in the LPV, it might be beneficial to reduce the expression of F1 to decrease its immunodominance.

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Currently, plague subunit vaccines in clinical trials consist of the F1/LcrV antigens. Taking into account that capsule negative *Y. pestis* strains are fully virulent, found in nature, or can be easily created, the LcrV is an essential component of the vaccine. However, existing natural polymorphisms of LcrV [253, 254] may require the addition of different variants of this antigen in vaccine formulations. Moreover, there are engineered versions of LcrV that can avoid cross-protection [255]. Therefore, identification and inclusion of additional *Y. pestis* antigens, particularly those eliciting robust T cell response, should be an immediate priority for development of PSVs. There are several candidates with limited ability to elicit protective immunity, for example, YopD and YopE. The most reliable antigen is likely to be YscF, a polymeric subunit of the Type 3 Secretion System (T3SS) apparatus. The inclusion of YscF into Adenovirus 5-based vaccine in addition to F1 and LcrV (Ad5-YscF-F1-LcrV) contributed to enhanced protection provided by this tri-valent PSV alone or in combination with purified antigens or LPV. This vaccine candidate provided a high level of protection against bubonic and pneumonic plague even after a single dose, while both homologous and heterologous boosting conferred 100% efficacy in different animal models [256-258]. Moreover, a hybrid combination YscF-F1-LcrV (YFV) in Ad5, where the antigens were separated with the linker GGGS with the expression optimized for humans, can already serve as a prototype candidate for the mRNA vaccine.

Another important aspect to consider during vaccine design and testing is immune correlates of protection, particularly in humans although thus far this issue is not well determined for the candidate plague vaccines. This will likely be particularly important during optimization of PSV formulations, development of novel LPVs or for development of other types of vaccines such as mRNA vaccines. The rubric defining the parameters of humoral and cellular immunity, results of direct protection studies in different animal models, the longevity of immunity and the evaluation of safety for the candidate plague vaccines remains to be determined for human use. Nevertheless, one quantitative characteristic was introduced for the testing of

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LcrV-based PSV. The LcrV protein is located at the tip of the T3SS needle guiding the injection of virulence effectors and impairing this function of LcrV disables the T3SS that is essential for virulence. The ability of anti-LcrV antibodies to block T3SS function can be evaluated with different reporters injected by T3SS to the host cell [259] although this approach has not yet been standardized. Moreover, LcrV is a multifunctional virulence factor, which besides its involvement with translocation of effectors via T3SS, contributes to immunomodulatory activity of *Y. pestis* [260, 261]. Therefore, focusing strictly on titers of antibodies capable of diminishing T3SS translocation function may miss the other important features of anti-LcrV antibodies related to the neutralization of immunomodulatory properties of secreted LcrV. The contribution of this part in protection against plague is totally unknown.

Overall, as outlined in recent WHO recommendations, plague vaccines should have not more than two doses of administration, provide long-lasting protection in both humoral and cellular immunity categories, not require cold chain storage, be injected by a needle-free method and have robust safety profiles including for immunocompromised individuals [262]. While these criteria remain an aspiration, a prime-boost regimen involving LPV as the initial vaccination followed by PSV boost could represent an effective, rapid solution in case a significant plague outbreak suddenly occurs.

Known 8: biosafety and biosecurity of plague

Plague as a bioweapon: The earliest documented use of bioweapons date back to the middle of 14th century. During 1343 to 1347, the Genoese's city Caffa (now Feodosiya, Ukraine) was besieged by Mongol (Tartars) troops. An epidemic of plague struck the Tartars and caused severe mortality in their camps. The Tartars then catapulted their deceased plague infected compatriots into Caffa causing plague outbreaks in the city [263].

In the 1930s, a secret branch of the Imperial Japanese Army known as the Kwantung

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Army Epidemic Prevention and Water Supply Department (known as Unit 731), had developed *Y. pestis* as bioweapons. They airdropped clay vessels filled with infected fleas or *Y. pestis* contaminated food into populated areas in China with an attack on Ningbo City in 1940 killing112 civilians [264].

In April 10, 1972, 78 nations signed the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction (BWC). The enforcement of BWC legally terminated the offensive plans of bioweapons around the world. However, some countries such as the former USSR had earlier weaponized *Y. pestis* but their offensive bioweapon programs was officially closed in 1992 [265, 266].

***Y. pestis* as a bioterrorism agent:** After the anthrax letter attacks in USA[267], pathogens-based bioterrorism has become an immediate threat to the global security. Bioterrorism is defined as ‘‘the deliberate release of viruses, bacteria or other agents used to cause illness or death in people, animals, or plants. It is aimed at creating casualties, terror, societal disruption, or economic loss, inspired by ideological, religious or political beliefs” [268]. Contrary to biological warfare, in a bioterrorism attack, biological agents are intentionally released against a civilian population.

The US Department of Health and Human Services and Department of Agriculture have identified certain ‘Select Agents’ or ‘Biological Agents’, which are pathogens and toxins having the potential to pose a severe threat to public health and safety. As *Y. pestis* is easy to disseminate and transmit, and causes a high mortality rate in humans, it is designated as category A ‘select agents’ [269]. *Y. pestis* has been tagged as a high priority agent which might be on the top of the list of potential bioterrorism agents and could pose great risk to national and international biosecurity.

Biosafety regulations for *Y. pestis*: Several laboratory-acquired infection cases have been reported in the United States, some of which were fatal. The most recent lethal case was due to an attenuated strain KIM D27 infecting a laboratory researcher. The condition of hereditary hemochromatosis coupled with diabetes in the researcher is

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believed to have contributed to the fatal course of the disease [270]. Occupational infections of plague have also been reported in veterinary staff, pet owners and marmot hunters [271, 272].

Due to the risk of laboratory-acquired infection approved local laboratory containment practices are recommended for all manipulations of suspect cultures, animal necropsies, and for experimental animal studies. Characterized strains of reduced virulence such as *Y. pestis* strain A1122 and KIM can be manipulated at lower containment levels [273]. However, these general recommendations in the U.S. Department of Health and Human Services for example are not fully accepted by other countries. For example, in France suspected plague samples are first dealt with at a lower containment level but all culture must be work at the higher level. For avirulent/vaccinal strains (with long history) could be handle in BSL1 with strict respect of the regulation of BSL1 to avoid the dissemination of the bacteria (e.g. lab coat, gloves, overshoes).

Unknown 8: Rapid recognition and control of plague in deliberate release of *Y. pestis*

Deliberate release of *Y. pestis*: Theoretically, deliberate release of *Y. pestis* can be categorized as biological warfare, bioterrorism and biocrime. According to BWC of 1972, nations are prohibited to undertake research to produce biological weapons or to produce and stockpile them. Although BWC has no inspection mechanism, it is unlikely that any soverign nation would now pursue a bioweapon program. However, *Y. pestis* related biocrime has been documented and in 1933, Dr. Taranath Bhatacharyna, a physician with bacteriology knowledges in Calcutta together with Benoyendra Chandra Pandey, injected a lethal dose of *Y. pestis* in the arm of Amarendra Pandey (Benoyendra's half-brother). Three years after, both perpetrators were convicted and sentenced to death [269].

Possible ways of deliberate release of *Y. pestis*: The release of fleas infected with *Y. pestis* could be a viable method of releasing plague. Assuming it was successful, an

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attack with fleas would primarily cause bubonic plague [264]. Due to the low mortality rate of bubonic plague, infected fleas will not be an efficient way to deliberately release *Y. pestis.* However, such an attack might potentially generate a plague wild-life reservoir in a plague-free region and may result in long-term ecological and economic consequences, in addition to being a public health issue.

Pneumonic plague related dispersal would be an ideal way to intentionally spread *Y. pestis*. An aerosol release of 50 kg of *Y. pestis* over a city of 5 million people would result in 150,000 initial clinical cases, and 36,000 deaths [274]. However, this previously modelled scenario should be revisited according to our increment of experiences against epidemics and diagnostic and treatment tools in hands [275, 276].

Another potential dispersal method for deliberate release of *Y. pestis* would be through a suicide attacker which would lead to an explosion in cases of pneumonic plague originating from a single source or multiple sources with secondary infections. Without adequate precautions modern transportation systems could then readily deliver plague rapidly across the globe [277].

Countermeasures for deliberate release of *Y. pestis*: The most important issue combating deliberate release of *Y. pestis* is timely and effective responses of public health systems. As to the identification, scientists are obligated to develop assays to identify wild-type and genetically modified strains rapidly and accurately. The third-generation genome sequencing platform like NanoPore will be of great value in this respect.

However, in the hypothetical scenario of the deliberate release of *Y. pestis*, timely and accurate recognition of the pathogen would be extremely difficult due to the overall complexity of the outbreak. In this case, symptoms surveillance system will be of help to identify potential patients as early as possible. Any unusual and clustered symptoms will trigger alerts and subsequent investigations and reduce the response time for combating the possible intentional release of *Y. pestis*.

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