The rise of pneumonic plague in Madagascar: current plague outbreak breaks usual seasonal mould

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Abstract
Madagascar has just emerged from the grip of an acute urban pneumonic plague outbreak, which began in August 2017, before the usual plague season of October–April and outside the traditional plague foci in the northern and central highlands. The World Health Organization reported a total of 2417 confirmed, probable and suspected cases, including 209 deaths between 1 August and 26 November 2017. The severity and scope of this outbreak, which has affected those in higher socioeconomic groups as well as those living in poverty, along with factors including the potential for use of multi-drug-resistant strains of plague in bioterrorism, highlights the ongoing threat posed by this ancient disease. Factors likely to have contributed to transmission include human behaviour, including burial practices and movement of people, poor urban planning leading to overcrowding and ready transmission by airborne droplets, climatic factors and genomic subtypes. The outbreak demonstrates the importance of identifying targeted pneumonic plague therapies and of developing vaccines that can be administered in planned programmes in developing countries such as Madagascar where plague is endemic. The dominance of pneumonic plague in this outbreak suggests that we need to focus more urgently on the danger of person-to-person transmission, as well as the problem of transmission of plague from zoonotic sources.

OVERVIEW
Plague is an infectious disease caused by the zoonotic bacillus Yersinia pestis, a non-motile, Gram-negative bacillus of the family Enterobacteriaceae [1]. Plague is mainly a disease of small mammals and their fleas, but can be transmitted between animals and humans via flea bites. It has caused three pandemics, the ‘Justinian plague’ between 540 and 750 AD [2], the second pandemic, also known as the ‘Black Death’, which lasted for more than 300 years after first appearing in 1334 in China and eventually reducing the population of Europe by 40–60 % in a wave of epidemics [3–6], and the third and current pandemic, which probably arose in Yunnan Province in China around 1855 and eventually spread to every inhabited continent except Oceania, causing large epidemics [7]. Most cases since the 1990s have arisen in Africa, while the disease is currently most endemic in Madagascar, Democratic Republic of Congo (DRC) and Peru [6].

Plague remains a cause of public health concern due to its virulence in humans and other mammalian species. There are three clinical forms in humans, bubonic plague, pneumonic plague and septicaemic plague [6]. Bubonic plague is the most common and is transmitted from infected rats via flea bite transmission. Y. pestis multiplies at the site of the flea bite and is disseminated via the lymphatic system causing swollen lymph nodes termed ‘buboes’ after 2–6 days, and other symptoms such as fever, headache and dizziness [8]. Pneumonic plague can be transmitted person-to-person via inhalation of respiratory droplets or small particles. It is rare but is the most virulent form and is invariably fatal if left untreated; it is the dominant form in the current Madagascar outbreak. Septicaemic plague can arise if infection spreads through the bloodstream, and is fatal in 30–70 % of untreated patients [8]. Humans are most vulnerable to infection during plague epizootics [9]. Antimicrobial treatment early in infection is crucial for survival.

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Abbreviations: CU, chaperone/ usher; DRC, Democratic Republic of Congo; FDA, Federal Drug Administration; HCW, healthcare worker; MDR, multi-drug-resistant; MLVA, multiple-locus variable number tandem repeat analysis; OMP, outer membrane protein; PLA, plasminogen activator; PPE, personal protective equipment; SNP, single nucleotide polymorphism; T3S, type III secretion system; WHO, World Health Organization.
There is an ongoing outbreak of plague in Madagascar. Between 1 August and 26 November 2017, the World Health Organization (WHO) reported a total of 2417 confirmed, probable and suspected cases, including 209 deaths [10]. The outbreak has affected 57 out of the 114 districts in Madagascar [10]. A case in the Seychelles was linked with recent travel to Madagascar. WHO has reported that the outbreak has now been contained, but that continued vigilance is vital [10].

Plague is endemic in Madagascar, with an annual upsurge of predominantly bubonic plague during the plague season (September–April). Madagascar accounts for approximately one-third of global plague cases annually. The main Y. pestis zoonotic reservoir is Rattus rattus. The rat flea Xenopsylla cheopis is the main vector [8]. Another rat flea, Synopsyllus fonquerniei, is also important, particularly in high-altitude areas [8]. The traditional plague foci have centred on two large areas in the northern and central highlands; persistence of plague in these areas has been linked to phylogeographical factors, climatic conditions and human behaviour [8, 11]. The persistence of a sub-group of wild rats with immune resistance to Y. pestis infection has been postulated to contribute to the stability of these foci [12]. A third focus of disease has been the coastal city of Mahajanga, in which there were 1702 clinically suspected cases of bubonic plague between 1995 and 1998 [13]. However, this does not appear to be a stable focus [8, 13]. The current outbreak differs from the usual pattern of seasonal outbreaks. The predominant form is pneumonic plague, accounting for 77% of cases, and the outbreak occurred in a non-endemic area and in large urban centres, including the capital city Antananarivo [10, 14].

In this review, we consider possible causes of transmission of the current plague outbreak, along with an overview of plague with particular attention to Madagascar and potential therapies and vaccines that could target the pneumonic plague that currently dominates. Major topics covered in the review are also summarized in Table 1.

**Microbiology and genomics of plague**

Historically, it has been postulated that one of three biovars was responsible for each of the three plague pandemics [15]. Antiqua has been associated with the first, Medievalis with the second and Orientalis with the current plague pandemic. These biovars are distinguished according to their ability to ferment glycerol and arabinose and to reduce nitrate. Subsequently, a new biovar termed Microtus (or Pestoides) was proposed, with unique pathogenic, biochemical and molecular features [16]. Genomic studies have helped in strain discrimination within the biovars and challenged this traditional assignment of pandemics to biovars. For example, multiple spacer typing has shown that the genotype Orientalis was involved in all three pandemics [17]. Single nucleotide polymorphism (SNP) analyses of samples from skeletons of Black Death victims in plague pits in Europe resulted in identification of two previously unknown related clades which were ancestral to modern Orientalis and Medievalis biovar isolates [18]. Enterobacterial repetitive intergenic consensus (ERIC)-PCR has also established that Antiqua biovars were recovered from patients in a 2002 plague outbreak in India, while Medievalis biovars were recovered from a rodent trapped during an environmental surveillance operation on the Deccan Plateau in 1998 [19].

SNP analysis has allowed definition of a phylogenetic tree rooted on *Yersinia pseudotuberculosis*, the parental species of which *Y. pestis* is a monomorphic clone. The tree has an ancestral branch 0 from which branches 1 and 2 containing geographically diverse *Y. pestis* populations were derived, namely 1.ORI (Orientalis), 2.MED (Medievalis), 1. ANT (African Antiqua) and 2.ANT (east Asian Antiqua) [20]. Microtus was assigned to another four populations (0.PE1 to 0.PE4). The 1.ORI branch is the most important with respect to Madagascar. The earliest node in the 1.ORI branch resulted in three sub-branches: 1.ORI1 reached the United States, 1.ORI2 reached Europe, South America, Africa and Southeast Asia, and 1.ORI3 spread to Madagascar and Turkey [20]. 1.ORI3 was introduced to Madagascar in 1898 and remains endemic [8, 21]. For example, the 1.ORI3-k SNP genotype was identified as the cause of a pneumonic plague outbreak in northern Madagascar in 2011 [22]. Phylogeographical patterns of *Y. pestis* arise due to spread of one or a small number of genotypes, followed by localized differentiation within countries [11, 20, 21, 23]. Madagascar is no exception, and thus has many phylogenetically and geographically distinct plague subpopulations [11, 21, 24]. A combination of SNP and multiple-locus variable number tandem repeat analysis (MLVA) phylogeny studies has suggested that there are 18 major phylogenetic subgroups of *Y. pestis* in Madagascar, arranged into two overall groups, I and II [11].

Virulence of plague in Madagascar and elsewhere is mediated by several determinants, both chromosomally encoded and encoded on its three plasmids, the 70 kb pYV/pCD1, the 100 kb pFra/pMT1 and the 9.5 kb pPst/pPCP1/pPla [25–30]. The essential virulence factor plasminogen activator (PLA), a surface protease involved in facilitation of dissemination of *Y. pestis* and evasion of the innate immune system, is encoded on pPst/pPCP1/pPla, along with pesticin and coagulase, which are also involved in bacterial transmission from the flea. The Fraction 1 antigen (F1-antigen), a capsule-associated protein involved both in *Y. pestis* adhesion to epithelial cells and in evasion of phagocytosis, is encoded on pFra/pMT1, along with a phospholipase D, a murine toxin (MT) involved in protecting the bacteria from flea gut digestive enzymes [31]. The virulence (V)-antigen and the type III secretion system (T3S) are encoded on the pYV/pCD1 plasmid. (T3S) is essential for virulence and is involved in direct injection of bacterial proteins into human cells [32]. The V antigen is a *Yersinia* outer protein (Yop) essential in virulence. When expressed intracellularly it has a role in regulation of (T3S) while, when it is expressed on
<table>
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<th>Plague topic</th>
<th>Summary</th>
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| **Zoonotic reservoirs and vectors** | **Reservoirs**  
- *Rattus rattus*  
- *Rattus norvegicus* (urban settings)  
- *Arvicanthis niloticus* (DRC, Uganda)  
- *Crocidura* species (DRC, Uganda)  
- *Peromyscus maniculatus* (USA)  
**Vectors**  
- *Xenopsylla cheopis*  
- *Synopsyllus fonquerniei* (high altitudes) |
| **Transmission causes/risk factors** | Seasonal outbreaks  
- Altitude >800 m  
- Climatic factors: high rainfall, heat, El Niño Southern Oscillation, Pacific Decadal Oscillation  
**Human behaviour (pneumonic plague)**  
- Funerary practices  
- Movement of people  
- Reliance on traditional medicine  
- Delays in seeking healthcare  
**Inadequacies in urban planning – overcrowding**  
**Inadequacies in infection prevention and control in healthcare settings**  
**Uncertain antibiotic access** |
| **Methods of diagnosis** | **Reference standard**: bacteriological isolation from a clinical sample with microscopic evaluation and/or culturing  
**Disadvantage**: impractical in the field in endemic regions  
**Serological testing**, e.g. ELISA  
**Advantage**: effective surveillance method where there is high seroprevalence  
**Disadvantage**: impractical in the field in endemic regions  
**F1 dipstick test**  
**Advantage**: simple and quick immunoassay; practical for field conditions  
**Disadvantage**: cannot be used to test environmental samples or samples from fleas as F1 antigen is synthesized primarily at 37°C; ineffective in detection of F1-negative strains; could be overcome by introduction of PLA dipstick test  
**Possible PCR-based diagnostic methods**  
- Real-time detection of the pPCP1 and pMT1 plasmids  
- Single-tube nested-PCR for rapid detection of the F1-encoding cagT1 gene |
| **Treatment** | Immediate treatment of suspected patients with aminoglycosides (e.g. streptomycin, gentamicin), tetracyclines (e.g. doxycycline), chloramphenicol, sulphonamides and/or fluoroquinolones (e.g. levofloxacin) according to national guidelines  
**Ketolide antibiotic cethromycin** (MDR strains) (mouse model)  
**LpxC inhibitor LPC-069** (mouse model)  
**Screen of FDA-approved drugs**:  
- Doxapram (DXP)  
- Amoxapine (AXPN)  
- Trifluoperazine (TFP) |
| **Possible vaccines** | **Subunit vaccines**  
- Subunit vaccine comprising F1 and rV antigens (phase 2a clinical trial; rhesus macaques)  
- Monomeric and soluble F1V mutant (F1mutV) (less tendency to aggregate); nanoparticle vaccine with F1mutV arrayed on phage T4 (mouse model)  
- Ail and OmpA antibodies (mouse model – bubonic plague)  
- Pla antibodies (mouse model – pneumonic plague)  
- YopE peptide subunit vaccine (mouse model)  
**Live attenuated vaccines**  
- Microtus strain 201 (rhesus macaque model)  
- VTnF1-modified version of *Y. pseudotuberculosis* strain IP32953 encapsulated in *V. pestis* F1 (mouse model – bubonic and pneumonic)  
- *Y. pestis* CO92 with *pla* and Braun lipoprotein (*lpp*) genes deleted (mouse model – bubonic and pneumonic)  
**DNA vaccines**  
- IL-12 (adjuvant) combined on bicistronic plasmids with either an F1-V fusion protein or V-Ag only – humoral and cell-mediated immune responses in mouse model |
the Y. pestis cell surface it is essential for contact between the host cell and a bacterial injectisome [33–35].

Some chromosomally encoded proteins are also essential in virulence, such as the Ail/OmpX/PagC/Lom family of outer membrane proteins (OMPs) which mediate bacterial attachment and delivery of Yops to host cells and help evade the innate immune system [36]. Several chromosomally encoded chaperone/usher (CU) systems have potential pathogenic roles in Y. pestis [37]. The pH 6 antigen (Psa) CU system, which encodes virulence-associated pili or fimbrae, has been implicated in adhesion and formation of biofilms [37] and with circumvention of the host immune response, for example by inactivation of cationic antimicrobial peptides (CAMPs) [38]. Results of studies in mouse models of plague suggest that the Psa system, along with the caf gene CU system encoding the F1 capsule, may be particularly important in pneumonic plague pathogenesis [39]. High-throughput, signature-tagged mutagenic studies identified genes encoding new Y. pestis virulence factors in a mouse pneumonic plague model, including rbsA, encoding a putative sugar transport system ATP-binding protein, vasK, encoding a component of the type VI secretion system, Braun lipoprotein (lpp) and acyltransferase genes (msbB) [40]. These virulence and pathogenic factors are potential plague vaccine targets.

### Global/regional epidemiology of plague

While the global incidence of plague has declined in recent years, it remains a dangerous disease, as illustrated by the severity of the current outbreak in Madagascar. Between 2010 and 2015, WHO received reports of 3248 plague cases resulting in 584 deaths [41]. The dominant animal reservoir varies between regions. The majority of cases occur in Africa, where plague is regularly reported in Madagascar, DRC, Uganda and Tanzania, and is usually a disease of poverty, associated with living conditions that facilitate close contact with rodents, strained healthcare infrastructure, uncertain antibiotics access and delays in seeking healthcare [41].

Madagascar has a relatively effective surveillance system, but while it has seen a decline in overall incidence, death rates have increased due to a higher preponderance of pneumonic plague [8]. The traditional plague foci are characterized by altitudes of greater than 800 m, while the plague season (October–April) is hot and rainy [8]. R. rattus is the main Y. pestis zoonotic reservoir in Madagascar, although R. norvegicus has increased in importance in urban settings [8].

In DRC, reports of plague have declined, although this is more likely to be due to a breakdown in surveillance systems due to conflict situations rather than to a real decrease in cases. Numerous outbreaks of pneumonic plague have been reported in DRC over the last 15 years, including 87 cases in 2005, 117 cases in 2006 and an outbreak in 2014 with a case fatality rate in excess of 41 % [41–43]. Uganda and DRC share a common plague focus in the West Nile region in north-western Uganda [44]. As in the focal regions of Madagascar, this region is characterized by high altitudes (generally >1300 m) and high rainfall, although temperatures tend to be lower than in the surrounding lowlands. An active surveillance and laboratory-testing study in Uganda between 2008 and 2016 showed that there were 255 suspected human plague cases in this endemic region, predominantly bubonic [44]. There was an overall 7 % mortality rate among suspected cases. However, among the one-third of cases which were laboratory-confirmed there was a 26 % mortality rate, higher than in Africa generally but similar to that in the USA [45]. Cases were episodic and clustered both spatially and temporally. Laboratory confirmation of plague was significantly associated with large numbers of dead rats in the vicinity [44]. In another study, results showed that other potentially important enzootic host species, Arvicanthis niloticus and Crocidura species, increased during the plague season within the plague focus, but not outside it [46]. Another study of 1092 suspected human plague cases identified between January 2004 and December 2012 in the same region of Uganda revealed that there were two distinct 1.ANT genetic subpopulations which differed with respect to geography and elevation [47]. Thus, these genetic subtypes appeared to be maintained in highly localized enzootic cycles, which could be associated with different vectors and/or hosts. This type of information would be important to gather in the current outbreak in Madagascar to help pinpoint ecological and environmental risk factors.

### Table 1. cont.

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<th>Plague topic</th>
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<td>Control measures</td>
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<td>• LerV DNA vaccine (mouse model)</td>
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<td>• F1 and truncated V protein in racoon poxvirus – endangered species protection in USA</td>
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<tr>
<td>Improve safety and effectiveness of sample collection, transport and storage</td>
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<td>Insecticide treatment of bubonic plague patients’ bedding and clothing</td>
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<tr>
<td>Effective infection prevention and control by healthcare workers (PPE, hygiene)</td>
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<td>Prophylactic antibiotic treatment of healthcare workers. Isolation of pneumonic plague patients</td>
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<td>Mask provision to pneumonic plague patients and their healthcare workers to reduce droplet transmission; bedding, clothing, sputum and excreta treatment with chlorinated solution</td>
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### Plague topic Summary

Control measures

- Improve safety and effectiveness of sample collection, transport and storage
- Start antibiotic treatment immediately; do not wait for confirmation (WHO, CDC, Medicins sans Frontieres and ECDC guidelines)
- Insecticide treatment of bubonic plague patients’ bedding and clothing
- Effective infection prevention and control by healthcare workers (PPE, hygiene)
- Prophylactic antibiotic treatment of healthcare workers. Isolation of pneumonic plague patients
- Mask provision to pneumonic plague patients and their healthcare workers to reduce droplet transmission; bedding, clothing, sputum and excreta treatment with chlorinated solution
Outside Africa, plague is found in every continent except Oceania. For example, in the western USA plague has been established since the early 1900s as a consequence of industrial shipping associated with entry of *Y. pestis*-infected rodents [48, 49]. According to the Centers for Disease Control and Prevention (CDC), the last urban plague epidemic in the USA was in Los Angeles between 1924 and 1925 [49]. Currently, plague is mainly associated with sylvan foci in rural areas in the west [48, 49]. There were 106 confirmed or probable cases in humans between 1900 and 2012, of which more than 80% were bubonic; there have been between one and 17 cases per year in recent decades [49]. However, modelling of the ecological plague niche in sylvan and domestic animals between 2000 and 2015 showed that areas including central Colorado, north-central New Mexico, and south-western and north-eastern California remain at relatively high risk of human exposure [48]. Risk factors include the presence of *Peromyscus maniculatus*, the most important enzootic host in the region, as well as factors that are also relevant in Madagascar such as altitude, precipitation and distance to artificial surfaces [48].

**Possible causes of transmission of the current outbreak**

The current outbreak in Madagascar differs from the typical seasonal outbreaks in several respects. Most cases have been reported in major urban centres including Antananarivo and the eastern coastal city of Toamasina, rather than in the traditional plague foci, and have been pneumonic rather than bubonic. Also, normally the plague epidemic season lasts from September to April, whereas the first confirmed case in this outbreak was identified in August.

The index case was a 31-year-old man from Toamasina who developed malaria-like symptoms on 23 August 2017 while on a visit to a plague-endemic area [50, 51]. He developed respiratory symptoms on 27 August and later died in hospital in the Moramanga district. In total, 31 contacts subsequently became ill, of whom four died. Confirmation of the outbreak came with the death from pneumonic plague of a 47-year-old woman in hospital in Antananarivo on 11 September, which prompted a comprehensive field investigation by public health authorities. A male patient from the Seychelles who was working in Madagascar died in hospital on 27 September from confirmed pneumonic plague. Immediate contacts of this individual were given precautionary prophylactic antibiotic treatment. Outside the major urban centres, pneumonic plague cases have sporadically arisen throughout Madagascar in 57 of the 114 districts.

Human behaviour is likely to be an important factor contributing to transmission in this outbreak. For the index case, initially there was no suspicion of plague and so his body was prepared for burial using traditional methods, without any special precautions. Funerary practices have been previously observed to coincide with plague onset in Madagascar, in particular spread of pneumonic plague [8, 52]. Movement of people may also be an important contributor. Previous genomic studies have shown that there were multiple *Y. pestis* isolate transfers from the traditional plague focal regions to Mahajanga city harbour, probably mediated by human transport of goods in vehicles containing infected rats and their fleas [21, 53]. Thus, Mahajanga was re-colonized by *Y. pestis* strains originating in the traditional plague focal regions [54]. Also, the index case in the current outbreak first displayed respiratory symptoms when travelling in a public taxi, with potential to spread the disease directly. Pneumonic transmission occurs person-to-person via respiratory droplets, facilitated by the densely populated nature of the urban centres. During a 2015 pneumonic plague outbreak in Moramanga, outside the normal plague season and in a previously plague-free area, there was high human-to-human transmission [55]. It is also possible that plague has been circulating within the rodent populations in some affected areas at a low level [54]. Normally plague is considered a disease of poverty [21, 53, 56]. In the current outbreak, media reports suggest that the disease is also affecting people in higher socioeconomic groups [57]. The rise of importance of *R. norvegicus* in urban settings in the spread of plague is one indication that poorly controlled urban development may be a contributory factor [8, 54]. Another contributor to transmission is a reliance on traditional healers, thus delaying antibiotic treatment [8]. Also, there is lack of adequate infection prevention and control procedures in some healthcare settings, which could facilitate transmission [41].

Climatic factors may also contribute to transmission of plague in Madagascar. For example increases in the strength of the El Niño Southern Oscillation (ENSO), a large-scale climate phenomenon, impacts on regional climate in Madagascar and has been linked to plague anomalies [58]. Large-scale climatic factors have also been linked to plague levels in other countries; for example, the Pacific Decadal Oscillation (PDO) has been shown to impact on precipitation and temperature in the western USA and hence influence plague hosts and vectors, with knock-on effects in increased human cases [59].

The genomic characteristics of the causative strain(s) may also contribute to transmission in this outbreak. Of the 18 major phylogenetic subgroups of *Y. pestis* in Madagascar identified by SNP and MLVA analyses, the Group I subgroup s has been the most widespread, found in the plague focal regions in the north–central highlands and in Antananarivo, and implicated in plague re-emergence in Mahajanga in the 1990s [11]. It is a persistent subgroup, associated with many different animal species linked to the spread of plague. Other subgroups have dominated in other regions. Various different subgroups were associated with the Befato region, while subgroup q dominated in the Moramanga region, where an outbreak of pneumonic plague was encountered in 2015 [11, 55]. Isolates taken from patients during that outbreak were assigned to the q3 subgroup [55]. While long-term plague maintenance in endemic regions of Madagascar has been associated with persistent, endemic *Y. pestis* transmission cycles, rather than wide epidemic spread,
subgroup q has been associated with dispersal over long distances in previous plague outbreaks [11]. Identification of the dominant subgroups in the present outbreak would be important in clarifying transmission patterns.

Readiness to contain and fight plague

The Ministry of Public Health instigated a robust response in Madagascar to the current outbreak, with the support of WHO, which resulted in a decline in cases since mid-October. This included new case investigation, the isolation and treatment of all pneumonic cases, enhanced case finding, contact tracing and monitoring, free prophylactic antibiotic treatment, increases in epidemiological surveillance in affected areas, increased rodent and vector control, awareness raising, information provision on safe and dignified burial procedures, and enhanced exit screening provisions at Antananarivo’s international airport [60].

Meanwhile, a case in the Seychelles was linked to recent travel to Madagascar [50]. This resulted in major efforts to ensure the Seychelles is prepared to reduce possible spread. WHO has equipped their Ministry of Health with supplies including 400 sets of personal protective equipment (PPE) for healthcare workers (HCWs). Training on the new equipment is being provided to HCWs to help reinforce existing infection prevention and control measures. Examination of current infection prevention and control plans, systems and protocols is also being carried out to ensure they are adequate to respond to further suspected cases [6].

Methodologies of diagnosis

Early diagnosis of plague is vital so that appropriate antibiotic treatment can be started without delay. The ‘reference standard’ for diagnosis would be bacteriological isolation of the Y. pestis strain from a clinical sample, such as sputum, lymph node aspirate, blood culture or pus from a bubo, and microscopic evaluation and/or culturing. In practice, bacteriological testing is impractical in the field in locations such as the endemic regions of Madagascar. Serological testing, for example by ELISA-based testing for seroconversion as indicated by the anti-F1 IgG titre in paired serum samples early in disease and 4–6 weeks later, is an effective surveillance method where there is high seroprevalence. However, again in the field ELISA may not be practical or affordable [61]. The need for affordable and accessible diagnostic methods under field conditions has led to the development of rapid test formats such as the F1 dipstick test, a simple and quick immunoassay developed in Madagascar [62]. With support from WHO, the F1 dipstick test is carried out widely in the field in Africa and South America [41]. Limitations of the test are that it cannot be used to test environmental samples or samples from fleas as the F1 antigen is synthesized primarily at 37 °C, and that it is ineffective in detection of F1-negative strains [63]. These limitations could potentially be overcome by introduction of a dipstick test to detect PLA, which is synthesized at 20 °C as well as at 37 °C and so is effective in both environmental samples and samples from fleas, in addition to the F1 dipstick test [63].

Possible PCR-based diagnostic methods include real-time PCR detection of the pPCP1 (pla gene) and pMT1 (cafl, Ymt genes) plasmids [64] and use of single-tube nested-PCR (STNPCR) for rapid detection of the F1-encoding cafl gene [65].

Guidelines for clinical practice

Despite the availability of the rapid F1 dipstick test for use in the field, difficulties remain around safe and efficient collection and transport of high-quality samples under field conditions, for example in Madagascar. Effective bacterial isolation processes are vital for antimicrobial drug susceptibility testing. Appropriate sample collection, preservation and transport methods are fundamentally important, and are often compromised in endemic developing countries. For example, during two pneumonic plague outbreaks in DRC in 2005 and 2006, only six paired serum samples were obtained during the first outbreak out of a total of 130 cases, while no paired samples were collected during the second outbreak [42]. Isolation of Y. pestis from sputum samples was compromised by limitations including prior antibiotic treatment of patients, non-sterile conditions of specimen collection, and long delays in handling and transport of samples [42]. It has been suggested that existing WHO guidelines for collection of sputum samples in tuberculosis control programmes in developing countries such as Madagascar and DRC could be adapted to improve safety and effectiveness of sputum sample collection in cases of suspected pneumonic plague [66]. WHO recommends that all patients with suspected plague should have pus samples from buboes, sputum and serum samples taken; they have issued interim technical guidance on safe collection of these types of samples [67, 68].

Organizations including WHO, the CDC, Medicins sans Frontieres and European Centre for Disease Control and Prevention (ECDC) all recommend starting antibiotic treatment immediately when plague is suspected, without waiting for confirmation of diagnosis or antimicrobial susceptibility profiles [69–72]. Bubonic plague patients’ bedding and clothing should be treated with insecticide, and infection prevention and control measures in terms of hygiene and use of PPE should be observed by HCWs [72]. HCWs caring for plague patients should receive prophylactic antibiotics for 7 days or for as long as they are exposed to infected patients [6]. Pneumonic plague patients should be isolated, masks should be provided for both patients and HCWs to reduce droplet transmission, bedding, clothing, sputum and excreta should be treated with chlorinated solution, and infection prevention and control measures should be observed by HCWs [6, 70, 72].

Current guidelines recommend immediate treatment of suspected plague patients with aminoglycosides such as streptomycin or gentamicin, tetracyclines such as doxycycline, chloramphenicol, sulphonamides and/or fluoroquinolones such as levofloxacin, according to national recommendations. For example, in the USA, intravenous gentamicin and/or levofloxacin is the usual first-line treatment [70].
Streptomycin is considered the ‘reference standard’ first-choice treatment for pneumonic or septicaemic plague, but is not widely available in the USA and some other countries [69, 70]. Duration of treatment is typically 10–14 days [69, 70, 73].

**Antibiotic resistance and potential new treatments**

Prompt antibiotic treatment within 24–36 h of infection has been shown to dramatically reduce mortality rates in all the clinical forms of plague. However, antibiotic-resistant strains of *Y. pestis* have been recovered in Madagascar [74, 75]. Plasmid-mediated resistance either to streptomycin only (plasmid pIP203) or to eight separate antimicrobial agents (plasmid pIP1202) was observed in *Y. pestis* isolates taken in 1995 from two different patients in separate areas of Madagascar [74, 75]. These plasmids are members of the highly conjugative IncA/C family [76]. The emergence of antibiotic resistance in *Y. pestis* raised public health concerns, particularly when it was demonstrated under laboratory conditions that high-frequency conjugative genetic exchange of multi-drug-resistant (MDR) plasmids could occur between *Escherichia coli* and co-infecting *Y. pestis* in the flea midgut [77]. However, in studies on *Y. pestis* isolated in Madagascar between 1995 and 2007, there has been no evidence of MDR strains [78, 79]. Results of a 2012 study showed no evidence of resistance to eight antibiotics in 392 *Y. pestis* isolates taken in 17 countries in North America, South America, Asia and Africa, including Madagascar [79].

There remains the potential that antibiotic-resistant strains could be exploited in bioterrorism, prompting research into alternative antibiotic treatments for plague. It is also important to be alert for any future emergence of MDR isolates, for example in the current outbreak. A study on genes in *Y. pestis* that could potentially mediate antibiotic resistance if over-expressed identified the *robXp* gene, which encodes a transcription-regulator, as a suppressor of the flouroquinolone ofloxacin activity when over-expressed, as well as a low-level suppressor of other antibiotics often used in the treatment of plague [80]. Studies have also been carried out via mutagenesis and inducible plasmid-mediated target gene expression in order to determine *Y. pestis* gene essentiality, i.e. identification of genes essential for viability, to help in targeting of anti-plague therapies [81]. The uridine monophosphate kinase PyrH has been identified as an essential *Y. pestis* protein by this approach [81]. The most likely method of delivery in bioterrorism would be as an aerosol, resulting in pneumonic plague via inhalation of the infectious agent. Development of non-human primate models of viable *Y. pestis* aerosols should be helpful in furthering our understanding of plague and development of new therapies and vaccines [82]. *In vitro* pharmacodynamic model studies on alternative treatments to streptomycin showed that gentamicin, ampicillin, meropenem, ciprofloxacin and moxifloxacin were as effective as streptomycin in killing pan-susceptible *Y. pestis* and were less prone to resistance amplification [73]. Results of pneumonic plague rat model studies on alternative antibiotics to combat MDR strains suggest that the ketolide antibiotic tigecycline is highly effective in protecting animals from infection if administered within 24 h post-infection, with few toxic effects [83, 84]. Another promising candidate compound is LPC-069, a biphenylacetylene-based inhibitor of the LpxC component of the lipid A biosynthetic pathway [85]. In a mouse model of bubonic plague, LPC-069 was a safe and effective treatment [85].

A potentially useful and cost-effective approach to identifying further plague therapeutics is systematic screening of agents not necessarily classified as antibiotics that have been approved by the Food and Drug Administration (FDA) for other indications. One such screen of a library of 780 FDA-approved drugs was carried out to identify drug candidates that induced resistance to *Y. pestis* strain CO92 in RAW 264.7 murine macrophages, with further testing of efficacious compounds in a murine model of pneumonic plague [86]. Three drugs, doxapram, amoxapine and trifluoperazine, were shown to increase animal survivability [86].

**Vaccines**

There are no currently licensed plague vaccines available. However, the potential for further occurrences of MDR isolates, the threat of bioterrorism and the difficulties inherent in administering therapeutic agents in remote regions of developing countries such as Madagascar all suggest that vaccination programmes may be the best option in reducing or preventing future outbreaks. Current research strategies are focused on subunit vaccines containing recombinant forms of the antigens F1 and low calcium response (LcrV or V) rather than whole-cell vaccines, due to issues surrounding reliability and safety [87–90]. Augmentation of the plague F1-V vaccine with the immunomodulatory CpG oligodeoxynucleotide has been shown in murine models of pneumonic and bubonic plague [91]. Recently, a randomized phase 2a clinical trial established good immunogenicity and safety of a novel subunit plague vaccine comprising F1 and rV antigens in a group of 240 healthy adults aged 18–55 years [92]. In a study on rhesus macaques, the F1 and rV subunit vaccine induced strong humoral immunity and a lower level of cellular immunity with good safety performance [93].

Issues with F1 and V recombinant vaccines include a tendency of F1 to aggregate, and sub-optimal induction of cell-mediated immune responses, which are needed in addition to humoral responses to effectively combat *Y. pestis* infection [94]. To address these problems, two new proposed vaccines were recently designed. These included a monomeric and soluble F1V mutant (F1mutV), with less tendency to aggregate but similar immunogenicity to WT F1V, and a nanoparticle vaccine in which the F1mutV was arrayed on phage T4, and which induced both antibody- and cell-mediated immune responses in *Y. pestis* CO92-challenged mice [94]. Possible alternative vaccine targets include OMPs and Pla. Ail and OmpA antibodies have been shown to protect in a bubonic plague murine model after challenge with F1(-) mutant or WT CO92, while Pla
antibodies were protective against pneumatic plague [95]. The potential value of adding YopE to subunit vaccines has also been established in murine studies in which YopE was identified as a dominant antigen recognized by CD8 T cells [96]. Immunization of mice with a YopE peptide, YopE (69–77), protected mice from lethal pulmonary challenge with Y. pestis [96].

Research into possible live attenuated vaccines is also ongoing. In a rhesus macaque model of bubonic plague infection, the Microtus strain 201 was highly attenuated when delivered subcutaneously. It resulted in an F1-specific antibody titre and protective cell-mediated immune responses [97]. Another live, irreversibly attenuated vaccine candidate involved production of a modified version of the Y. pseudo-tuberculosis strain IP32953 encapsulated in Y. pestis F1 [98, 99]. A single oral dose of the final vaccine version, VTnF1, gave efficient and prolonged protection against both bubonic and pneumatic plague in mice caused both by WT and F1-deleted Y. pestis, with a good safety profile [98]. A mutant version of Y. pestis CO92 in which both pla and the Braun lipoprotein (lpp) genes were deleted has also been shown to be attenuated in induction of both bubonic and pneumatic plague when administered either subcutaneously or intranasally to mice [100]. This live attenuated strain protected mice against challenge with Y. pestis WT CO92 by induction of both humoral and cell-mediated immune responses [100].

Other possibilities include DNA vaccines, such as two vaccines in which IL-12 was combined on bicistronic plasmids with either an F1-V fusion protein or V-Ag only [101]. These vaccines induced both humoral and cell-mediated immune responses in mice, suggesting the efficacy of IL-12 as a molecular adjuvant [101]. An LcrV DNA vaccine has also been shown to induce a strong, protective CD8 T-cell response in mice subjected to a lethal intranasal challenge of Y. pestis [102]. Meanwhile, vaccines based on expression of F1 and truncated V protein in racoon poxivirus are proving potentially valuable in protection of endangered species in the USA, including prairie dogs and black-footed ferrets, from the effects of sylvatic plague [103, 104].

CONCLUSIONS

The current plague outbreak in Madagascar highlights the rise in importance of pneumatic plague and how its transmission from person to person can have devastating impacts in the context of overcrowded urban communities. We need to be vigilant against future outbreaks of this ancient disease and continue in efforts to identify novel therapeutics, especially in the context of possible MDR strains, and particularly in developing safe and effective vaccines.

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