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5. Zoonotic Diseases

5.1. [Overview](#)

Zoonotic infections (also called zoonoses or anthroozoonotic infections) are human diseases acquired from a vertebrate animal. As a matter of fact, all arthropod-borne diseases with an animal host belong to the group of zoonotic infections, whether yellow fever, West Nile fever, Japanese encephalitis, tick-borne encephalitis or Rift Valley fever, to only name a few. Zoonotic infections also include haemorrhagic fevers such as EBOLA or Marburg, which primarily infect bats and secondarily African apes, Lassa fever and South American haemorrhagic fevers due to arenaviruses, which affect specific rodents, Nipah viral encephalitis, which is harbored in bats and may cause a lethal bronchopneumonia in pigs, as well as avian influenza (H5N1). They also include SARS, leptospirosis, plague, anthrax, and many other parasitic, viral and bacterial zoonoses. Several of these diseases are newly emerged and the general perception of their public health significance extends far beyond their actual incidence, due to their extremely high case fatality rate (60% for avian influenza, 50% for Nipah, 50% to 90% for EBOLA outbreaks). Many zoonotic infections actually are promoted by human behavior such as bush-meat hunting (EBOLA fever), the farming and trade of live wild animals (SARS), close and repeated contacts with infected animals (avian influenza), deforestation, which brings humans closer to infected vectors and animal reservoirs (leishmaniasis), or building of dams, that favors the proliferation of mosquitoes (Rift Valley fever). It is probable that bush-meat hunting was at the origin of HIV/AIDS [1] [2]. Rabies remains a major public health problem in the world's poorest areas, especially in Africa and South and South-East Asia, where most human cases follow stray dog bites.

This chapter will focus on rabies, anthrax, whose interest was renewed when used as a bioterrorism agent, plague, and hepatitis E, an acute viral hepatitis with a high case fatality rate in pregnant women whose most likely reservoir is pigs. Rift Valley Fever is another zoonotic infection with an important, albeit geographically limited, impact.

5.2. [Anthrax](#)

5.2.1. *Disease burden and epidemiology*

Anthrax, a deadly zoonotic disease due to *Bacillus anthracis*, has been known since antiquity [3]. The fifth and sixth plagues in the Bible's book of Exodus may have been outbreaks of anthrax in cattle and humans. Naturally occurring anthrax in humans is acquired from contact with anthrax-infected animals or anthrax-contaminated animal products, which allows one to distinguish agricultural

anthrax, a most significant problem in developing countries especially among veterinarians, agricultural workers and butchers, and industrial anthrax, resulting from exposure to contaminated sheep wool or goat hair that are processed into yarns used in the textile and carpet industry, as well as cattle hides that are processed into leather goods, or bones used for the manufacture of gelatin and/or fertilizer.

Anthrax infection in humans occurs by three major routes, the skin, the respiratory tract or the gastrointestinal tract, generating three different primary forms of the disease, the cutaneous, the inhalational and the gastro-intestinal forms [4] [5]. Cutaneous anthrax presents as a small pruritic papule that develops within a week into a vesicle usually on an exposed part of the body such as the face, the neck or arm. Edema and erythema often develop around the lesion. The vesicle eventually ruptures, revealing a depressed ulcer crater that develops into a black eschar. The case fatality rate of cutaneous anthrax usually is about 20% if untreated. Inhalational anthrax presents within one to five days with nonspecific symptoms, fatigue, myalgia and slight fever, which are followed by a sudden severe respiratory distress with dyspnea, cyanosis, and stridor leading to a lethal shock syndrome associated with pulmonary haemorrhage and mediastinal edema. Systemic infection with *B anthracis* resulting from inhalation causes a 100% case fatality rate. As to gastrointestinal anthrax, it develops within 2 to 5 days following ingestion of contaminated meat with nausea, vomiting, fever, abdominal pain and diarrhoea, eventually leading to toxemia, shock and death in 25% to 75% of cases.

The incidence of natural anthrax in industrialized countries remains quite low and the disease is not a major public health problem in the world. Thus, between 1900 and 2005, only 82 cases of inhalational anthrax were reported in the USA [6]. Occasional anthrax epidemics nevertheless did occur, such as the Zimbabwe epidemic in the early 1980s with approximately 10 000 cases reported. The scene changed in 2001 with the bioweapon attacks in the Eastern USA. Modelling studies showed that anthrax spores used as a bioweapon against civilian populations could generate catastrophic consequences [7] [8]. It was for example estimated that the airborne delivery of 50 kgs of anthrax spores over a large city could lead to 125 000 severe clinical cases of anthrax and 95 000 deaths. This renewed the general interest for the field and prompted the development of new anthrax vaccines.

Control of anthrax in humans and animals is based on control measures in livestock in endemic areas, such as the safe disposal of anthrax carcasses and vaccination of at-risk cattle herds. Incineration of carcasses is a manner to prevent contamination of the surrounding soil. Local conditions in many endemic countries however make these simple control measures difficult to implement. In industrialized countries, prevention lies in good agricultural and industrial hygiene.

5.2.2. Bacteriology

B anthracis, the agent of anthrax, is a large gram-positive, spore-forming, nonmotile bacillus with little if any genetic variability. In tissues, the bacteria are encapsulated and appear singly or in short chains of a few bacilli. The spores are extremely resistant in the environment and may survive for decades in certain soil conditions. They eventually are ingested by cattle or wild animals such as deer [9] when grazing on contaminated land.

B anthracis has two major virulence factors that are carried by two distinct plasmids, pX01 which carries the tripartite toxin genes *cya* (edema factor), *lef* (lethal factor) and *pagA* (protective antigen), and pX02, which carries the gene encoding the polyglutamate capsular filaments. The tripartite exotoxin consists of the 83 kD protective antigen (PA), the 90 kD lethal factor (LF), and the 89 kD edema factor (EF) [10]. PA binds to cellular receptors and mediates the entry into the cytosol of both

LF, a Zn⁺ metalloprotease that cleaves mitogen-activated protein kinase kinases (MAPK), and EF, an adenylate cyclase that converts ATP to cyclic AMP (cAMP) and promotes lethal tissue edema [11] [12]. The lethal toxin is composed of PA combined with LF while the edema toxin is made from the combination of PA and EF. Both LF and EF inhibit acquired and innate immune responses, allowing the bacteria to replicate unchecked in the host [13].

The polyglutamate capsule plays a major role as an invasiveness factor. Bacteria which lack plasmid pX02 and therefore are unencapsulated are attenuated for animals and can be used as live attenuated vaccines, as initially demonstrated by Sterne [14] [15] [16] (For a review, see [17]).

5.2.3. Vaccines

Anthrax vaccines are available for both animals and humans. However, in humans, their use has been confined to high-risk groups such as occupationally exposed workers and military personnel.

A few live attenuated *B. anthracis* strains have been developed as vaccines, such as the unencapsulated Sterne strain for subcutaneous immunization of domestic animals or the unencapsulated SST-1 strain used in Russia and the Langzhou avirulent strain A16R developed in China. The latter are given by skin scarification as a single or a double immunization followed by yearly booster immunizations [18].

The human anthrax vaccine licensed in the USA is made from cell-free filtrates of bacterial cultures of an unencapsulated, nonvirulent strain of *B. anthracis* adsorbed to aluminium hydroxide (Anthrax Vaccine Adsorbed/BioThrax, Emergent BioSolutions Inc). [19] [20]. To develop and maintain protective immunity in humans, these vaccines must be administered subcutaneously six times over 18 months, followed by yearly booster injections [21] [22]. A recent study showed that by using the IM route of immunization rather than the SC route, effective immunization required a three-dose schedule rather than the original four-dose schedule [23]. Still, these vaccines have shown only partial protection from infection with some strains of *B. anthracis* in animal models [24]. After the intentional release of anthrax spores in 2001, it was clear that a more effective, easily administered, and safer vaccine was needed for emergency situations [25] [26] [27] [28] for both pre- and post-exposure prophylaxis.

While the poly-D-glutamic acid capsule is nonimmunogenic [29], the PA component of the toxin has been shown to induce a protective antibody response in numerous studies using animal models of infection [27] [30] [31] [32] and including inhalational anthrax [33]. Recent research has focused on the design of a recombinant PA (rPA) vaccine which would eliminate the need for filtered culture supernatants or whole *B. anthracis* lysates, as well as produce a more consistent immune response. Thus, rPA given to healthy adults in two IM injections four weeks apart with the adjuvant alhydrogel was well tolerated and highly immunogenic [34]. PA is the main component of the two licensed anthrax vaccines, Anthrax Vaccine Adsorbed (AVA) in the USA and Anthrax Vaccine Precipitated (AVP) in the UK.

Several new human adjuvants have been studied to be included in anthrax vaccines, including monophosphorylated lipid A (MPL A), saponin QS-21, and muramyl tripeptide linked with dipalmitol phosphatidylethanolamine. In recent attempts at developing mucosal anthrax vaccines a variety of other adjuvants were tested including soy phosphatidyl choline, cholera toxin (CT), and CpG oligonucleotides [35] [36]. The use of soybean oil-and-water nanoemulsions (NEs) (NanoBio Corporation, Ann Arbor, MI) as a mucosal adjuvant appears very promising: the candidate vaccine generated long-term, high-titer neutralizing anti-PA IgAs and IgGs in mucosal secretions and provided significant protection of the animals against intranasal challenge with *B anthracis* spores after only two intranasal immunizations [37].

Live recombinant anthrax vaccines using bacterial or vaccinia virus vectors are also being developed, as well as recombinant HBc particles expressing a PA epitope [38] [39]. The demonstration that spore components could offer additional protection in animal models has moreover lead to the development of a dual component candidate anthrax vaccine that combines rPA with formaldehyde-inactivated spores and was shown to be significantly protective against intra-nasal spore challenge in mice [40]. In a similar approach, PA was combined with LF and poly-gamma-glutamic acid (gamma PDGA) and administered by the intra nasal route in mice, inducing high level protective bactericidal antibodies [41]. Similarly, a trivalent vaccine composed of rPA added with inactivated LF and EF induced long-lasting protective immunity in rabbits [42].

5.3. *Hepatitis E (HEV)*

5.3.1. *Disease burden*

Hepatitis E was first identified as an acute non-A non-B viral hepatitis. It has since been recognized as a major cause of acute hepatitis in young adults throughout much of Asia, Africa and Latin America [43] [44] [45] [46] [47]. The disease is endemic in many parts of the world, including the Indian subcontinent, northwest China, and the Central Asian Republics. In these regions, HEV is transmitted predominantly through the fecal-oral route, especially through the consumption of fecally contaminated drinking water. In India, the lifetime infection risk is more than 60%, which translates into hundreds of thousands of cases annually [48]. The highest rates of infection occur in regions with poorest sanitation. A minor mode of transmission could be through blood transfusion [49].

High prevalence of anti-HEV antibodies has been reported in blood donors from non-endemic regions [50] [51] [52], which could be due to zoonotic transmission of the virus. HEV-related viruses have been found in pigs [53] [54] [55], deer [56], and wild boar [57] as well as in rodents and chickens [58]. Direct transmission has been reported from animals to humans through consumption of undercooked deer meat [59] or uncooked liver from a wild boar [60]. Humans who consume contaminated pork products or are involved in the rearing of pigs are potentially at risk of HEV infection [59] [61] [62].

HEV infection occurs mostly in young to middle age adult population, i.e. between the ages of 15 and 40 years. The presence of anti-HEV antibodies has been detected in only less than 5% of children under the age of 10, contrary to what is observed with HAV infection. Clinical symptoms of hepatitis E are typical of acute viral hepatitis including jaundice, abdominal pain, fever and hepatomegaly

lasting 1 to 4 weeks. The existence of a chronic form of HEV infection has recently been reported in organ transplant recipients [63]. In rare cases, patients may present with severe disease progressing to fatal liver failure. This is mostly observed in chronic liver patients [64] and in pregnant women in their third trimester, who often develop encephalopathy with cerebral edema and disseminated intravascular coagulation [65] [66]. The case fatality rate among these women may be as high as 25%, whereas it only is 0.2% to 1% in the general population [67] [68]. The selective suppression of NFκB p65 in pregnant women, causing liver degeneration, severe immunodeficiency and multi-organ failure has been suggested [69] but the precise cellular/molecular mechanisms involved are not clear.

5.3.2. Virology

HEV is a small (32-34 nm in diameter), spherical, nonenveloped virus with a 5.2 kb positive-sense, 5'-capped single-stranded RNA genome. It belongs to the genus *Hepevirus* in the *Hepeviridae* family. Its molecular organization, deduced from the cloning and sequencing of the genome [70] [71], shows a high degree of sequence conservation among isolates from different origin. At least four phylogenetically distinct HEV genotypes have been defined [47] [65] [72], although all HEV strains share at least one major serologically cross-reactive epitope, so that they all belong to the same serotype. Genotype 1 includes Asian and African human HEV strains, genotype 2 includes a Mexican and African strains. Genotype 4 is prevalent in Western industrialized countries whereas genotype 3 is mostly found in Far Eastern Asian countries. Outbreaks due to HEV genotype 1 or 2 are the results of efficient human-to-human fecal-oral transmission. In contrast, genotypes 3 and 4 are prevalent in domestic animals such as swine, and only occasionally infect humans, probably due to less efficient cross-species transmission.

The HEV genome includes three partially overlapping open reading frames (ORF). ORF1 encodes a large nonstructural protein with methyltransferase, cysteine protease, RNA helicase and RNA polymerase activities. ORF 2 encodes the 660 amino acid long viral capsid protein, and ORF3 encodes a 123 amino acid protein which seems to interact with various intracellular pathways to create an environment favorable for virus replication. The capsid protein of HEV (pORF2) is glycosylated. The glycosylation seems to be required for the production of infectious viral particles and replication in macaques [73].

5.3.3. Vaccines

Since there is no robust system to grow HEV in cell culture, inactivated or live attenuated vaccines were considered as not feasible until recently [74] [75], when the successful replication of a genotype 3 HEV isolate was obtained in PLC/PRF/5 cells from nonhuman primate origin [76] [77].

The available HEV vaccine is made of a 56 kD pORF2 segment protein (genotype 1). The truncated protein produced in insect cells using a recombinant baculovirus efficiently self-assembles into virus-like particles (VLPs) that expose the dominant HEV neutralization epitope [78] and elicit a protective antibody response in a monkey challenge model [79] [80]. Cynomolgous monkeys were successfully protected against challenge by passive immunization with human convalescent serum or by active immunization with the ORF2 VLP vaccine. These results prompted a randomized clinical trial of the vaccine's efficacy in volunteers from the Nepalese Army, a population at high risk for hepatitis E

[81]. The VLP vaccine was administered in three doses at months 0, 1 and 6 to 898 subjects who were followed up for a median of over 800 days in parallel with 896 subjects in the placebo group [82]. Hepatitis E developed in 66 subjects in the placebo group versus 3 in the vaccine group, which translates into a 95.5% vaccine efficacy. Moreover, the increase by a factor of 10 in anti HEV IgG levels after the administration of the third dose of vaccine was evidence that the first two vaccine doses elicited a strong immune memory.

Another HEV vaccine based on the 50 kD recombinant capsid protein went through Phase III clinical trials at the Xiamen University in China [83]. The self-assembled recombinant virus particle was analyzed at a 22-A resolution basis by cryo-electron microscopy and image reconstruction, yielding the first image of a T=1 particle with 30 morphological units showing protruding dimers at the icosahedral two-fold axes.

The combination of the HEV VLP vaccine with an inactivated HAV vaccine was studied in mice and showed that a dual HAV / HEV vaccination was feasible [84].

5.4. *Plague*

5.4.1. *Disease burden*

Plague is an exceptionally virulent, vector-borne zoonotic disease transmitted from rodents, especially rats, through the bites of infected fleas, most often the rat flea, *Xenopsylla cheopsis*. Many different species of mammals, including rats, squirrels, mice, prairie dogs and gerbils actually are animal reservoirs for the agent of plague, *Yersinia pestis*, which persists in the environment as the result of a stable and constant rodent-flea infection cycle, causing a fatal disease in murine and sciurid populations [85]. The reduction of rodent populations, whether as a consequence of the disease or of rodent control measures, compels fleas to seek new warm-blooded mammalian hosts, incidentally including humans.

The first major epidemic of plague to be historically recorded occurred in China in 224 BC. In Europe, plague was endemic in all of the Roman Empire, with severe outbreaks occurring occasionally, such as the outbreak which occurred in Rome in the third century AD, giving rise to one of the worst persecutions of Christians. Plague later came in long-lasting, dreaded pandemic waves [86]. The first documented pandemic, the Justinian plague, killed several million people in the Byzantine Empire during the 6th to 8th century. The second pandemic, the “Black Death”, started in the middle of the 14th century and persisted over several hundred years, killing about 30% of the European population and culminating with the Great Plague of London in 1665. The third pandemic started in China in the middle of the 19th century and caused 10 million deaths in India alone.

Although the dramatic epidemics of urban plague have disappeared, due to improved sanitation and public health surveillance, plague still is a significant health problem in Africa, Asia and South America, which report around 2 000 cases every year with a global case fatality rate of 5% to 15%. The disease is endemic to Africa, India, and the southwestern states of the USA, and isolated

outbreaks continue to this day in many regions of the world [87] [88] [89]. Africa, mainly The Democratic Republic of Congo and Madagascar, account for 96% of world cases since 1990. The identification of naturally occurring multiple-drug-resistant strains of *Y pestis* in Madagascar [90] [91], as well as the discovery of high frequency conjugative transfer of antibiotic resistance genes to *Y pestis* in the flea midgut [92] are matters of serious concern. Moreover, plague has attracted a considerable attention because of its possible use as an agent of biological warfare and terrorism [93].

Plague assumes three major clinical forms in humans: bubonic, pneumonic, and septicemic. Flea bites usually cause bubonic plague, whose name comes from the bubo, a painful swelling of the bite site-draining lymph nodes which often become hemorrhagic and necrotic (hence the name ‘Black Death’). Without prompt antibiotic treatment, approximately 50% of bubonic cases rapidly progress to sepsis and death. About 30% of fleabites directly lead to sepsis, without prior evidence of a bubo [94]. Sepsis is characterized by circulatory collapse, coagulopathy, hemorrhage, respiratory distress, shock, and organ failure, leading to death in about 40% of cases. The most feared form is pneumonic plague because this form can readily be transmitted from person-to-person via inhalation of contaminated airborne droplets [95]. Symptoms begin with rigor, severe headache and malaise then quickly advance to fever, difficulty breathing, and cough that yields infectious, bright red sputum teeming with bacteria. The case fatality rate is close to 100% if no antibiotic treatment is given within the first 48 hours following symptoms onset [96]. The study of experimental *Y pestis* aerosols in animal models showed that 1µm particle aerosols resulted in both primary pneumonia and infection of the upper respiratory tract whereas 12 µm particles infection resulted in the attack of the nasal mucosa and nasal-associated lymphoid tissues (NALT) prior to bacteremic dissemination and secondary pneumonia [97].

The pathology of plague is very similar in rodents, nonhuman primates and humans [98] [99] [100] [101].

5.4.2. Bacteriology

Yersinia pestis, first identified by Alexandre Yersin in 1894, is a Gram-negative, nonmotile bacterium that belongs to the family *Enterobacteriaceae*. Three species in the genus *Yersinia* are pathogenic for humans: *Y pestis*, *Y pseudotuberculosis* and *Y enterocolitica*, the latter two being the cause of self-limiting enteropathogenic infections characterized by diarrhoea, fever and abdominal pain. The extreme virulence of *Y pestis* mostly results from its virulence factors that impair the host innate immunity response, including phagocytosis, and allow the bacteria to multiply and spread unchecked in the host [102] [103] [104] [105].

The major mechanism that impairs the host phagocytosis response is a 70-kb plasmid (pCD1)-encoded Type III secretion system which is activated by growth of the bacterium at 37°C and whose function is to directly translocate *Yersinia* outer proteins (Yops) to neighboring host cells, mostly dendritic cells, macrophages and neutrophils, in which they disrupt signaling pathways, suppress cytokine production, debilitate the antibacterial defense mechanisms and promote apoptosis [106]. The pCD1 plasmid also carries the *lcrV* gene, which encodes the 37-kD low calcium response virulence antigen, LcrV-Ag, that serves as a positive regulator of the type III secretion system [107]. *Y pestis* lacking LcrV is avirulent in mouse models of plague disease. In addition, LcrV can activate

Toll-like receptor 2 and trigger the release of IL-10 [108] [109], a cytokine that suppresses innate immune functions [110]. LcrV also prevents the release of proinflammatory cytokines tumor necrosis factor (TNF)- α and γ -interferon in murine and human macrophages [111].

Other *Y pestis* virulence factors include the F1 pilus antigen, a 17 kD polypeptide encoded by the *caf* gene that is carried on a large 100-kb plasmid (pMT1, or pFra). F1 is the major protein component of the outer capsule encompassing *Y pestis* bacilli and is believed to help avoid phagocytosis [112]. YopH, a protein tyrosine phosphatase that is part of the type III secretion system [113], Pla, a plasminogen activator protease encoded by plasmid pPCP1, as well as murein (or Braun) lipoprotein (Lpp), which links the outer bacterial membrane to the peptidoglycan layer in *Enterobacteriaceae* [114] are also virulence factors, as judged by the fact that their mutation or deletion attenuates the virulence of *Y pestis* in rodents, which is currently used as a basis for the development of live attenuated vaccine strains.

5.4.3. Vaccines

The first widely used plague vaccine was developed by Haffkine in 1897 using a heat-killed culture of *Y pestis*. The vaccine conferred significant protection against bubonic plague but induced severe adverse reactions including high fever in the majority of vaccinees. Moreover, later studies in rodents and nonhuman primates showed that the vaccine was unable to elicit protection against pneumonic plague. A formalin-killed whole-cell vaccine was developed in the mid-20th century in the USA and used to protect US military personnel against bubonic plague during the Vietnam War [115] [116], but it also caused severe adverse reactions and was unable to elicit protection against pneumonic plague. Its use was discontinued in 1999 [117].

The development of live attenuated plague vaccines began at the beginning of the 20th century using partially attenuated, pigmentation negative (pgm negative) *Y pestis* strains such as the Girard and Robic EV strain [118] [119] and later derivatives [120]. Between 1934 and 1940, mass vaccination campaigns in Madagascar dramatically reduced the annual plague incidence (from 3500 to 200 cases). In spite of frequently reported side effects and residual virulence in nonhuman primates [121], the live attenuated EV 76 and EV 88 strains are still in use in Russia and Central Asian republics today [122], both for protecting humans and camels. Other live attenuated plague vaccines that are in early development include a DeltaYopH strain, which protected mice against high-dose parenteral or aerosol challenge after a single intranasal administration [113], a Deltalpp mutant [114] and a YadC mutant [123], as well as an IpxM mutant of the already partially attenuated EV strain of *Y pestis* [124]. In addition, the use of *Yersinia pseudotuberculosis* as a Jennerian vaccine against plague has been entertained because it shares high genetic identity with *Y pestis*, is less virulent and can be administered by the oral route [125].

The development of subunit plague vaccines started in the 1950s, focusing on the use of the capsular F1 (CafI) pilus antigen. Vaccination with F1 protected rats, mice and nonhuman primates against subcutaneous and aerosol challenge with virulent *Y pestis* [126] [127] [128]. However, *Y pestis* variants lacking *cafI* were found which not only were fully virulent in animal models of bubonic and pneumonic plague, but also broke through the immune responses generated with F1 subunit vaccines [129] [130] [131].

Unlike F1, the Lcr V antigen was found to be critical for *Y pestis* virulence [109] [132] [133] and, when used as a subunit vaccine, generated high titers of antibodies that conferred protective immunity

against bubonic and pneumonic plague in mice, guinea pigs and nonhuman primates, whether the strain used for challenge was F1 positive or not [134] [135] [136] [137]. Passive immunization with an anti-Lcr V monoclonal antibody was shown to protect mice against aerolized Y pestis, even when administered 48 h postinfection [138]. Finally, anti-Lcr V antibodies were demonstrated to neutralize Y pestis-mediated macrophage cytotoxicity in a dose-dependent manner, which could be used as an in vitro assay as a correlate of protective immunity [139]. In view of the immune modulatory properties of Lcr V, concerns were raised regarding its safety as a vaccine in humans. A truncated version of the Lcr V antigen, V10, which lacks amino acids 271 to 300, was developed that showed reduced immune modulatory properties while offering full protection of mice against bubonic and pneumonic plague [123] [140] [141] and could be used advantageously in place of the full molecule.

Candidate vaccines containing either a combination of F1 and Lcr V antigens or a recombinant F1-Lcr V fusion protein in alum or alhydrogel formulations have been developed that efficiently protect mice against pulmonary Y pestis challenge [142] [143] [144] [145] [146] [147] and elicit long-lasting protective antibodies able to neutralize Y pestis-mediated cytotoxicity of macrophages in cynomolgus macaques [148]. The F1-V vaccine also protected black-footed ferrets against oral challenge with Y pestis [149]. Both vaccines appeared to be safe and immunogenic in human trials [150] [151]. A spray-freeze-dried F1-V fusion protein powder vaccine was recently developed that could be administered either by the IM or the ID route with similar protective efficacy in mice. The vaccine could also be administered by the intranasal route but an extra dose was required to achieve the same level of protection [152]. The DynPort Vaccine Company (DVC) is managing the advanced development of a rF1-V vaccine for the US Department of Defense (DOD) [151].

The possibility of developing a dual vaccine against anthrax and plague was investigated in a murine model by combining equal amounts of the anthrax rPA antigen and the Y pestis F1-V antigen. The vaccine was able to elicit a robust IgG and IgG1 response in mice against both antigens when administered by the SC route and a robust IgG2 response when administered by the intranasal route with appropriate adjuvants. Circulating antibody levels were still detectable at 6 months post primary immunization [153].

The US Army Medical Research Institute of Infectious Diseases (USAMRIID) demonstrated however that while the F1/V vaccines efficiently protected cynomolgus macaques against aerosolized Y pestis challenge, they failed to do so in African green monkeys, which raises the question of their eventual efficacy in humans [154]. A number of approaches are underway to increase the efficacy of the subunit F1/V vaccines [155], such as the introduction of point mutations in the V antigen [156] or the use of other adjuvant formulations than alhydrogel. Thus, the use of flagellin as an adjuvant was tested by generating a flagellin-F1-V triple fusion protein that elicited robust antigen-specific humoral immunity in mice and two species of nonhuman primates and fully protected mice against intranasal Y pestis challenge [157]. The flagellin-F1-V antigen showed remarkable stability at temperatures between 4° and 25°C. In another approach, a promising intranasal vaccine against pneumonic plague was developed using lipid A mimetics as adjuvants, which showed high protective efficacy in both mice and rats [158]. In still another approach, rF1 and V antigens were separately microencapsulated in polymeric microspheres, mixed together and used to immunize mice by either the IM or intranasal route, resulting in high levels of serum IgGs, secretion of cytokines by the spleen and draining lymph nodes and protection against high level Y pestis challenge after a single immunization [159].

Protective efficacy of DNA vaccines was studied using plasmids that encoded the F1 and V antigens together with interleukin 12 (IL-12) as an adjuvant. DNA vaccines were administered either by the intranasal or the IM route, but protection was reached only after three weekly doses followed by protein boosts [160] [161].

Improving the efficacy of F1- and/or Lcr V-based vaccines by delivering the antigens via live attenuated recombinant vectors was also attempted using attenuated *Salmonella enteritica* serovar Typhimurium (*Salomonella typhimurium*) as a vector [162] [163] [164] [165] [166]. A single oral dose of a *Samonella*-F1-antigen recombinant protected mice against bubonic plague challenge, but not

against pneumonic plague challenge. Protection against pneumonic plague challenge required the dual expression of both the F1 and the V antigen by the Salmonella vector [166]. The use of a vesicular stomatitis virus (VSV) vector expressing the V antigen and administered in a prime-boost regimen also protected mice against intranasal challenge with *Y pestis* [167]. The same protective efficacy was obtained with a single-dose IM immunization with an adenovirus recombinant expressing the V antigen [168].

Mice immunized by SC immunization with F1-V antigen purified from *Nicotiana tabacum* leaves expressing the F1-V fusion antigen in chloroplasts were boosted by oral delivery of the transgenic plants, which induced effective protection against aerosolized *Y pestis* challenge [169].

In spite of all these efforts, and the wealth of investigational approaches, we still currently lack a safe, effective and licensed vaccine for pneumonic plague, which is nearly always fatal and can be intentionally transmitted by weaponized strains of *Y pestis*. None of the live attenuated or live recombinant plague vaccine candidates is ready yet for an application for a license, which will have to be in accordance with the FDA 'Animal Rule' that requires safety and immunogenicity data in humans along with robust efficacy data in more than one animal model.

5.5. [Rabies](#)

5.5.1. *Disease burden*

Rabies is a viral encephalitis transmitted from animal to animal and from animal to man through saliva. Animal bites introduce the virus into muscle and nerve ending-rich tissues from which it penetrates into nerve cells where it replicates and progressively travels through the spinal cord to the brain. This process usually requires weeks or even months, depending upon the distance from the bite site to the brain. Replication of the virus in the brain causes hydrophobia, hallucinations, aggressive behavior, and paralysis, eventually leading to coma and death. The virus also spreads to salivary glands and the skin, cornea, nasal and intestinal mucosa and other organs including kidneys. The disease thrived from most ancient times (its first written description can be found in the Babylon Codex, 23 centuries BC) to the end of the 19th century when, in 1885, Louis Pasteur and collaborators succeeded in the first cure of human rabies through post-exposure vaccination [170]. More than 120 years later, however, the disease still continues to affect mankind, especially in developing countries in Africa, in South and South-East Asia, and to a lesser extent Latin America.

In nature, rabies is a disease of wild carnivores, involving dogs, cats, wolves, foxes, coyotes, jackals, raccoons, skunks and also bats as reservoirs and vectors. All mammalian species are believed to be susceptible, including nonhuman primates. Human infection almost always results from the bite of an infected animal, although transmission of the virus through transplantation of infected corneas and other organs (heart, liver and kidney) has been reported. On rare occasions, the virus was also reported to be transmitted by aerosols in caves populated by rabies-infected bats. According to WHO, more than 3.3 billion people are at risk for rabies in over 85 countries worldwide [171] [172]. About 55 000 deaths from rabies are estimated to occur every year, 99% of which are the consequence of dog bites [173] [174]. Of these 55 000 deaths, 31 000 are estimated to occur in Asia (20 000 in India alone) and 24 000 in Africa. The annual incidence of animal bites in many countries can be as high as 100-200 bites per 100 000 population. In 2005, more than 12 million individuals received a post-exposure prophylaxis (PEP) treatment against rabies, preventing an estimated 280 000 deaths [175].

Canine rabies is still widespread in stray dogs in Asia, Africa and parts of Latin America. Control of rabies in these countries is often hampered by religious beliefs and cultural habits. For example,

Buddhist and Hindu ethics restrain culling of the canine population. India and Thailand have prohibited the euthanasia of stray dogs by municipalities. In these countries, stray dogs account for >90% of human rabies exposures, especially among 5-14 years old children in rural or peri-urban areas. Human rabies has been endemic in India for immemorial times, but the actual incidence of the disease never had been carefully studied. A recent survey on 10.8 million persons in mainland India led to conclude that the annual incidence of rabies was 2 per 100 000 population [176]. In most developing countries, however, the true incidence of rabies is largely underestimated, due to poor reporting.

In countries where an effective rabies control has been implemented, various wildlife species including bats have become the main reservoir of rabies and most human cases are secondary to bites by rabid bats [177] [178]. In the USA, the predominant vectors for rabies are skunks in the western and central states, raccoons in the eastern states, coyotes in the far south and foxes at the Canadian border and in Alaska. In Western Europe, prior to the implementation of wildlife vaccination, 83% of rabid animals were foxes. Most of Western Europe is now rabies-free, and several countries in Central and Eastern Europe are almost rabies-free, but rabies is still a problem in the Baltic countries, Ukraine, Russia and Independent States of former USSR.

5.5.2. Virology

Rabies virus is an enveloped, bullet-shaped virus which belongs to the genus *Lyssavirus* in the family *Rhabdoviridae*. The negative sense viral RNA genome encodes five viral proteins, the N (nucleocapsid), P, M (membrane), G (envelope glycoprotein) and L (replicase) proteins. Seven virus genotypes have been described, genotype 1 corresponding to the rabies virus and genotypes 2 to 7 to bat lyssaviruses. These are: genotype 2 (Lagos bat virus), genotype 3 (Central Africa Mokola virus), genotype 4 (South Africa Duvenhage virus), genotypes 5 and 6 (European bat lyssaviruses) and genotype 7 (Australian bat virus). New Lyssaviruses have been identified (e.g. central Asia) and the addition of new genotypes is under consideration. All these viruses can be pathogenic for humans.

The G protein, which forms spikes at the surface of the virion, is responsible for the attachment of the virus to virus receptors and bears the neutralization epitopes. There seems to be little cross neutralization between genotype 1 (rabies virus) and genotypes 2 (Lagos bat virus) and 3 (Mokola virus).

5.5.3. Vaccines

For more than 70 years after Pasteur's original work, inactivated vaccines were produced from sheep, goat or rabbit brains and contained nerve tissue, a cause of severe neurological adverse events. The Semple vaccine, which was produced on sheep or goat brains and inactivated with phenol was until recently commonly used in humans in many countries in Africa and Asia. The first rabies vaccine produced in animal tissues with low myelin content was prepared in the 1960s by Fuenzalida and colleagues [179] starting from infected suckling mouse brains, again using phenol as the inactivating agent. Suckling mouse brain vaccines (SMBV) were the most frequently used vaccines for many years, until their production started being discontinued a few years ago, especially in major SMBV producing countries such as Brazil and Mexico, to be replaced by a number of inactivated rabies vaccines produced on either primary or continuous cell lines or embryonated eggs.

The first modern rabies vaccine (Human diploid cell vaccine, HDCV) was developed at the Wistar Institute in Philadelphia by propagating the rabies virus on the human diploid cell line WI-38 and

using beta-propiolactone for its inactivation [180]. This was followed by a purified vaccine produced in Vero cell cultures (PVRV), and also inactivated by beta-propiolactone, which was developed by Sanofi Pasteur [181] [182]; and a purified vaccine produced on primary chick embryo cells (PCECV), developed by Novartis. The three vaccines show comparable tolerance and immunogenicity and their efficacy was demonstrated in several PEP clinical trials. In addition, a primary baby hamster kidney cell culture vaccine (PHKCV), a purified duck embryo vaccine (PDEV), three Vero cell-based vaccines and one HDCV have been developed in China and India.

These vaccines can be used either for preventive immunization or for PEP. Preventive immunization is recommended for certain professional groups such as veterinarians and for travelers to rabies-endemic countries. There is no doubt that preventive vaccination of children in areas where rabies is endemic should also be given thorough consideration. The recommended preventive immunization regimen consists in three IM doses at 0, 7, and 21 or 28 days. The longevity of rabies neutralizing antibody anamnestic response in vaccinated persons was examined on 118 Thai volunteers who had received a cell culture vaccine 5 to 20 years previously and who each received a booster ID injection of 0.1 mL PVRV. All volunteers except one had detectable neutralizing antibody titers on day 0 and responded to the ID booster immunization with an accelerated antibody response, indicative of a long-lasting immune memory conferred by the vaccine [183].

Rabies vaccines are mostly used for PEP after bites from suspect rabid animals, as the time the virus takes to travel to the brain can be up to 2 months, which allows the immune system to mount an immune barrier in response to PEP before symptoms occur. The recommended schedule for PEP is five doses at 0, 3, 7, 14 and 28 days, often coupled to passive immunization with rabies immunoglobulins (RIGs). Two types of RIG are currently available, human IgGs (HRIGs) and highly purified equine IgGs (ERIGs). The overall shortage of RIGs and their current cost however represent a real public health threat in many countries. The use of rabies monoclonal antibodies (MRIGs) to replace RIG in PEP has been studied by various groups [184] and one such cocktail of two human monoclonals was recently tested in a Phase I study [185].

The relatively high cost of a full PEP regimen using IM immunizations also prompted attempts at using a reduced dose of vaccine for PEP, replacing IM by ID inoculations and using 0.1 mL (1/5th of a dose) of vaccine [186] [187]. Various ID post-exposure regimens (PEP) have undergone extensive evaluation, especially by the Thai Red Cross (TRC) in Thailand, using two ID injections on days 0, 3 and 7 followed by two injections day 28 [188] [189] [190]. The immunogenicity, tolerance and efficacy of PEP using either PCECV or PVRV administered ID at 0.1 mL per site according to the WHO recommended protocols [191] have been well documented [192] [193] [194]. Post-exposure ID vaccination is also routinely practiced in India, the Philippines and Sri Lanka, where it reduced the cost of PEP intervention by about 80% [175] [195].

The possibility of using the ID route of immunization for rabies pre-exposure vaccination was eventually tested in a randomized, open-label Phase II trial in schoolchildren in Thailand using 1/5th of a dose of purified chick embryo cell vaccine (PCECV) on days 0, 7, and 28 or 0, 7 and 21: 100% of the children developed protective rabies-neutralizing antibody titers [196] [197]. An abbreviated pre-exposure vaccination schedule using two ID injections at two sites on day 0 was found to lead to persistence of neutralizing antibodies one year later [198]. The possibility of routinely immunizing children in rabies-endemic countries using a simplified ID vaccination regimen is being explored in Thailand, again showing that a two-site ID immunization done on the same day using 0.1 mL of vaccine in each site could prime the human host immune memory for at least one to three years [199] [200].

It should be realized, however, that there will not be any easy solution to the problem of rabies in rabies-endemic countries if no attempt is made at eliminating the virus from its animal reservoirs. Vaccinating dogs against rabies has already been demonstrated as a highly efficacious preventive measure [\[174\]](#) [\[201\]](#). Similarly, oral vaccination of foxes and other wildlife using either live attenuated rabies virus mutants or a live vaccinia virus recombinant that expressed the rabies G glycoprotein (RaboralTM) was highly successful at eliminating rabies from Western Europe. Different strategies, kinds and shapes of baits have been developed for targeting the major wild rabies reservoirs: stray dogs, foxes, coyotes and raccoon dogs [\[202\]](#) [\[203\]](#) [\[204\]](#) [\[205\]](#).

No disease exceeds the case fatality rate of rabies. Progress must continue towards the elimination of human rabies, itself depending on wildlife rabies control and canine rabies elimination [\[206\]](#). The declaration of an Annual World Rabies Day, September 8, should hopefully raise public awareness of the severity of the disease [\[207\]](#).