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2. Acute Respiratory infections

2.1. [Overview](#)

2.1.1. *Respiratory disease burden*

Acute respiratory infections (ARIs) continue to be the leading cause of acute illnesses worldwide and remain the most important cause of infant and young children mortality, accounting for about two million deaths each year [1] [2] [3] and ranking first among causes of disability-adjusted life-years (DALYs) lost in developing countries (94.6 millions, 6.3% of total[4]. The populations most at risk for developing a fatal respiratory disease are the very young, the elderly, and the immunocompromised. While upper respiratory infections (URIs) are very frequent but seldom life-threatening, lower respiratory infections (LRIs) are responsible for more severe illnesses such as influenza, pneumonia, tuberculosis, and bronchiolitis that are the leading contributors to ARIs' mortality [5]. Pneumonia, with a global burden of 5 000 childhood deaths every day, is a tangible threat that needs to be dealt with accordingly.

The incidence of ARIs in children aged less than 5 years is estimated to be 0.29 and 0.05 episodes per child-year in developing and industrialized countries, respectively, which translates into 151 million and 5 million new episodes each year, respectively [6]. Most cases occur in India (43 million), China (21 million), Pakistan (10 million), Bangladesh, Indonesia and Nigeria (56 million each). Pneumonia is responsible for about 21% of all deaths in children aged less than 5 years, leading to estimate that of every 1000 children born alive, 12-20 die from pneumonia before their fifth birthday [4].

The main etiological agents responsible for ARIs in children include *Streptococcus pneumoniae*, *Haemophilus influenzae* type b (*Hib*), *Staphylococcus aureus* and other bacterial species, respiratory syncytial virus (RSV), measles virus, human parainfluenza viruses type 1, 2, and 3 (PIV-1, PIV-2 and PIV-3), influenza virus and varicella virus.

2.1.2. *Bacterial diseases*

S pneumoniae (pneumococcus) was identified in 30%-50% of bacterial pneumonia cases in developing countries in the 1990s, followed by Hib (10%-30% of cases), then *Staphylococcus aureus* and *Klebsiella pneumoniae* [6]. Non-typhable *H influenzae* (NTHI), and non-typhoid *Salmonella* spp have also been implicated in some but not all studies. Other organisms, such as *Mycoplasma pneumoniae*, *Chlamydia* spp, *Pseudomonas* spp and *Escherichia coli* also can cause pneumonia. The most common syndromes associated with *M pneumoniae* infections are acute bronchitis, pharyngitis and otitis, but 10% of infected children develop pneumonia [7].

The introduction of Hib conjugate vaccines has resulted in a truly remarkable decline in Hib disease where the vaccine has been introduced. However, the vaccine is not yet routinely made available to a majority of children worldwide. As a result, 400 000 deaths are still estimated to occur from Hib disease each year [8]. In view of their safety and remarkable efficacy, the WHO has recommended the global implementation of the Hib conjugate vaccines [9].

S pneumoniae is estimated to cause more than one-third of the 2 million deaths due to ARIs, especially in developing countries where the bacterium is one of the most important bacterial pathogens of infancy and early childhood [10]. Virtually every child in the world is colonized with one or more strains of pneumococcus and becomes a nasopharyngeal carrier during his first few years of its life. Many children will go on to develop otitis media, and a few will eventually develop invasive pneumococcal disease including bacteremic pneumonia and/or meningitis [11]. The introduction of the conjugate

pneumococcal vaccine in routine infant immunization should have a major impact on pneumonia in children less than five years of age worldwide [12], as already documented in the USA [13].

Last but not least, tuberculosis (TB) continues to be a leading cause of deaths worldwide, with an estimated one third of humanity infected and about 1.7 million deaths each year, a global toll of 4650 lives daily. The emergence of *Mycobacterium tuberculosis* (Mtb) strains carrying drug-resistance mutations against first-line drugs (MDR-TB) and, more recently, against both first- and second-line drugs (XDR-TB), shows that it will most probably be impossible to contain the TB pandemic with drugs alone. More than one hundred new TB vaccine candidates have been tested in animal models and some have moved into clinical trials. Testing such a wide variety of vaccine types using different strategies will obviously require time and a lot of coordination, especially as surrogate markers of protection still remain mostly unknown at this time.

2.1.3. Viral diseases

Among the viral agents of ARIs, measles virus was still responsible, in 2002, and in spite of the inclusion of the live attenuated measles vaccine in the Expanded Program of Immunization (EPI), of some 213 000 deaths worldwide, essentially due to insufficient vaccine coverage [14]. The situation has fortunately been substantially improved lately [15].

But the leading cause of serious respiratory illness in young children is respiratory syncytial virus (RSV), the agent of infantile bronchiolitis, which is associated with substantial morbidity and mortality [16]. Parainfluenza viruses (PIV-1, PIV-2 and PIV-3), especially PIV-3, are second in incidence immediately after RSV. All children by the age of 2 years have had at least one episode of PIV and/or RSV illness. In addition, both viruses can cause severe disease in the elderly, especially in patients with a chronic respiratory or cardiac condition. Although the disease burden due to these pathogens has not been accurately quantified in developing countries, extrapolation from known figures in industrialized countries, such as 125,000 reported cases of RSV per year in the USA [17], leads to the impressive global estimates of 64 million cases and 160,000 deaths per year from RSV infection worldwide. RSV was identified in 15-40% of pneumonia or bronchiolitis cases admitted to hospital in developing countries, followed by influenza viruses, parainfluenza viruses, human metapneumovirus and adenovirus [18]. The elderly also are at risk for severe RSV disease, and 14 000 to 60 000 RSV-related hospitalizations of the elderly are reported to occur annually in the USA [19].

Human metapneumovirus, a member of the *Paramyxoviridae* family, is a recognized cause of a large fraction of severe ARIs in infant, elderly and immunocompromised population [20] [21] [22] [23]. Other viruses that cause respiratory infections are coronaviruses, adenoviruses and rhinoviruses. Recently discovered coronaviruses HCoV-HKU1 and HCoV-NL63 are significant pathogens that contribute to the hospitalization of children for ARI [24] [25] [26]. Among other members of the *Coronaviridae* family are human coronaviruses HCoV-229E and HCoV-OC43, agents of the common cold, the feline infectious peritonitis virus (FIPV), the avian infectious bronchitis virus (IBV) and the pig transmissible gastroenteritis virus (TGEV).

Another recently identified coronavirus is that of the severe acute respiratory syndrome (SARS), SARS-CoV, which emerged in southern China in late 2002 and spread in the spring of 2003 to some 30 countries within Asia, Europe and North America [27] [28]. The epidemic finally came to a stop in July 2003 through strict implementation of quarantine and isolation procedures and thanks to international collaboration under the coordination of WHO [29]. At that date, 8,096 cases had been identified worldwide and 774 patients had died, a 9.6% mortality rate. SARS-CoV belongs to a newly identified group in the family *Coronaviridae*, which are enveloped RNA viruses whose envelope is characterized by crown-like proteinic spikes, and whose genome is an exceptionally long 29 727 nucleotides single-stranded positive RNA molecule that encodes 23 different proteins. The viral spike (S) protein is responsible for the induction of virus neutralizing antibodies [30] [31] [32].

Although there is evidence that SARS-CoV emerged from a nonhuman source, no animal reservoir has yet been identified with certainty. Masked palm civet cats and raccoon dogs have been found to be

carriers of the virus [33] [34], and Chinese wild-animal traders, especially civet cats traders, showed high seroprevalence figures. Horseshoe bats carry a SARS-CoV-like virus and might be the virus reservoir [35] [36] [37]. Less than one year after SARS first appeared, half a dozen candidate vaccines already were in development [38], including whole inactivated virus vaccines, live recombinant vector-based vaccines, VLPs, and subunit vaccines. More recently, a live attenuated virus has been developed [39]. Most of these efforts have however been put on hold, in view of the current elimination of the disease.

Regarding influenza virus, the average global burden of inter-pandemic influenza may be on the order of 1 billion cases per year, leading to 300,000-500,000 deaths worldwide [40] [41]. In the elderly, influenza-related pneumonia remains a leading cause of infectious disease-related deaths. The threat of an avian influenza pandemic has been looming ever since the emergence in 1997 in Hong Kong of the H5N1 avian influenza virus, especially since the reappearance of human cases in 2003-2004. The new H5N1 variant is highly pathogenic for poultry and wild birds and can lethally infect cats and humans. At this time, however, it still is not possible to predict which virus is going to eventually cause a pandemic and when it is going to happen [42] [43], but the preparation of pandemic influenza vaccines is being actively pursued, generating broad new knowledge on how to possibly improve seasonal influenza vaccine immunogenicity [44] [45].

Viruses are a common cause of ARIs in children worldwide. However, the substantial reduction in ARI mortality observed in developing countries that have implemented simple case management including provision of antibiotics to children with ARI suggests that bacterial pneumonia contributes to a large proportion of deaths in these populations [46]. Available data suggest that dual infections with viral and bacterial pathogens may be quite common, as seen by the fact that, in the industrialized world, epidemics of RSV and/or influenza coincide with epidemics of *S pneumoniae* year after year [47]. While influenza virus is the most commonly met pathogen in this context, other respiratory viruses, including RSV, measles virus, parainfluenza viruses, or adenoviruses may also predispose to secondary bacterial infections. Several different bacterial species may be implicated, including *H influenzae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Mycoplasma pneumoniae*, and, above all, *S pneumoniae* [48]. Half or more of the flu-associated mortality in the 1918-1919 Spanish Flu epidemic is believed to have resulted from pneumococcal superinfections [49] [50].

The same is true for developing countries. As an example, the observation was made in South Africa that children vaccinated with the 7-valent conjugate pneumococcal vaccine showed 31% reduction in virus-associated pneumonias requiring hospitalization, strongly emphasizing the presumed importance of dual infections involving *S pneumoniae* [51]. Dual infection seems to increase the severity of the disease and to result in higher mortality. This might be due to inhibition of pulmonary antibacterial defenses during recovery from viral infection [52].

Finally, it should be emphasized that nosocomial or hospital-acquired pneumonia is a major public health problem: pneumonia is the second most common type of all nosocomial infections, with an associated case fatality rate of 20%–50%.

2.2. [Influenza](#)

The repetitive occurrence of yearly, seasonal influenza epidemics is due to the fact that influenza viruses are continuously changing antigenically. This was well illustrated by the emergence of the influenza A H3N2 ‘Fujian’ strain, which appeared in July 2003 in the southern hemisphere then spread to the northern hemisphere a few months later to become a dominant strain [53]. To face this continuous change, virus strains to be included in the vaccine are updated annually so as to match the circulating virus strains. An alternative would be to develop “universal” influenza vaccines which would cover all possible influenzavirus strains (see below). Meanwhile, the recent emergence of highly pathogenic avian influenza H5N1 in poultry farms and markets in several countries in Asia, from where it spread to Africa and Europe, and its transmission to humans with a 60% case fatality rate, has revived the fear of a possible new pandemic of influenza with high mortality and planetary consequences [33] [54] [55].

2.2.1. Disease burden

2.2.1.1. Seasonal influenza

The burden of influenza is currently estimated to be 25-50 million cases per year (~ 20% of the population) in the USA alone, leading to 150 000-200 000 hospitalizations and 30,000-40,000 deaths [56]. If one extrapolates these figures to the rest of the world, the average global burden of seasonal influenza comes to be on the order of 600 million cases, 3 million cases of severe illness and 250 000-500 000 deaths per year. Hospitalization rates from severe illness can be as high as 3 per 1000 for 6 to 23 months old children and as high as 9 per 1000 for children younger than 6 months [57].

Influenza is highly contagious [58] and is readily transmitted via aerosols and droplets from the respiratory tract of infected persons by direct contact, through coughing or sneezing, or by hands contaminated with respiratory secretions. Adults are most infectious from 1 day before symptom onset up to 7 days afterwards. When influenza is introduced into a household, 20-60% of exposed persons will eventually show virologic or serologic evidence of infection. The disease can affect all age groups, but rates of infections are highest among young children who shed virus and are a potential source of infection in older age cohorts [59], whereas rates of serious illness, complications and death are highest in persons aged 65 years and older, as well as in persons with chronic cardiac or respiratory conditions. Data collected in Michigan (USA) in Japan and in Russia indicate that mass vaccination of school-aged children correlates with a reduced rate of respiratory illness in all age groups, suggesting that larger-scale immunization in childhood could favourably affect influenza epidemics [60].

Epidemics and outbreaks of influenza occur in different seasonal patterns depending on the region in the world: in temperate climate zones, seasonal epidemics typically begin in the late Fall and peak in mid-winter, infecting about 5-15% of the population each season. In tropical zones, seasonal patterns are less pronounced and the virus can be isolated year-round [61]. The disease, characteristically a febrile illness with respiratory symptoms, ranges in severity from mild to debilitating and can lead to lethal primary fulminant pneumonia, particularly in persons with underlying pulmonary or cardiopulmonary pathologies.

The repetitive occurrence of yearly influenza epidemics is maintained through the ongoing process of “antigenic drift”, which results from the accumulation of point mutations in the genes that encode the two viral surface proteins haemagglutinin (HA) and neuraminidase (NA), and leads to the constant emergence of new virus variants against which there is little or no pre-existing immunity in the population. For this reason, major seasonal epidemics of influenza continue to occur each year and the virus strains to be included in the vaccine of the year must be chosen to match the emerging new variants [40] [62]. Recent molecular studies suggest that new virus strains emerge in East and South-East Asian countries, from which they spread around the world, first to Europe and North America which are reached within 6-9 months then to South America [63].

2.2.1.2. Pandemic influenza

At unpredictable intervals, due to the segmented nature of the influenza virus genome, circulating human influenza virus A strains also can acquire new genes from an avian or other animal influenza virus. This process is believed to occur most readily in pigs, as pigs have the complete set of sialylated receptors for avian, swine and human influenza virus strains. Co-infection in pigs can thus result in the emergence of a virus with a completely new glycoprotein subtype, which is referred to as an “*antigenic shift*”. If the reassortant virus can efficiently spread into the human population, a worldwide pandemic can occur, as was the case in 1918, 1957, and 1968 [43] [64].

The impact of a new influenza pandemic has grossly been estimated at 1-2 billion cases of flu, 5-12 million cases of severe illness, and 1.5-3.5 million deaths worldwide [65]. It could result in 1 million to 2.3 million hospitalizations and 250 000 to 650 000 deaths in industrialized nations alone. In the USA, the impact of a new pandemic, assuming it would be of a similar magnitude as the 1957 or the 1968 pandemics, and not like the 1918 pandemic, is projected to be 18-42 million outpatient visits, 314 000-

734 000 hospitalizations and 89 000–207 000 deaths. Its impact would be even more devastating in developing countries [66]. Historians estimate that more than 50 million people died in the 1918-1920 influenza pandemic [67] [68].

Of note is the observation that in three studies of blood cultures taken from living soldiers with pandemic influenza-associated pneumonia in 1918, pneumococci were isolated from 46% of patients. The data from influenza hospitalization in children and from pandemic influenza mortality in 1918 suggest a significant role for pneumococci in influenza-associated pneumonia [69]. This is most likely due to inhibition of macrophage-mediated antibacterial defense during the recovery stage from influenza infection, which is characterized by pulmonary infiltration of T cells that secrete high amounts of IFN- γ [52]. This suggests that the prevention of pneumococcal super-infection should be an essential part of pandemic influenza preparedness [70].

The emergence of the avian H5N1 influenza virus A strain with pandemic potential occurred in 1997 in Hong Kong, SAR, resulting in the death of 6 of the 18 affected patients, mainly young adults [71]. The virus was fortunately not able to spread from person-to-person and the outbreak was controlled through massive culling of poultry. The H5N1 strain however reemerged in 2003–2004 in China, Japan, South Korea then Thailand, Vietnam, Indonesia, Cambodia and Malaysia, leading to massive culling and attempts at vaccination of poultry. More than 60% of human patients diagnosed with the virus died. In May, 2005, a highly pathogenic (HP) H5N1 variant emerged in wild birds in Quinghai Lake, China [72] [73] [74], that not only killed domestic poultry but also wild aquatic birds. The HP strain also is pathogenic for ferrets, cats and tigers. Cats can be infected both by the respiratory route and by feeding on virus-infected birds. The HP H5N1 strain spread to a great many countries in Asia, Africa and Europe where it was repeatedly recovered from migratory birds and was the cause of multiple outbreaks in poultry [75]. A significant part of transmission has been linked to commercial trade of poultry and derived products [76].

Cases of human H5N1 infections have been reported in many countries but no human-to-human transmission has been evidenced so far, except in a couple of instances among close contacts. As of October, 2008, a total of 387 confirmed human cases from 15 countries resulting in 245 deaths had been reported to WHO, of which more than 100 cases and 50 deaths had occurred in Vietnam. The virus seems to now have become endemic in several countries, including Nigeria, Egypt, Bangladesh, Vietnam and Indonesia, while showing continuous sequence evolution leading to the emergence of different molecular clades and sub-clades [77]. Significant concerns therefore remain that the virus could adapt to infect humans or exchange genetic material with co-circulating human influenza viruses, resulting in the emergence of a highly pathogenic pandemic strain [78].

Other avian influenza viruses have occasionally caused a human outbreak, such as a H9N2 strain in 1999 in Hong Kong, a H7N7 strain in 2003 in the Netherlands, which caused 89 confirmed human cases with conjunctivitis and one death, and H7N2 and H7N3 in 2003–2004 in North America. No one can know how severe the next pandemic will be, nor which influenza virus will cause it –it could be an H2, H5, H7, H9 or another subtype. A report from the Centers for Disease Control and Prevention (CDC) indicates that North American H7N2 and H7N3 strains have now partially adapted to the human sialic acid receptor (see below), a characteristics that would enhance the virus potential to infect and spread among humans [79]. At the same time, the H5N1 virus continues to be a zoonotic virus, not a human-adapted one, and human infections remain rare. Still, given the alarming 60% average case fatality rate of H5N1 influenza cases in humans so far, it probably is prudent to prepare for the ‘worst case’ scenario [78].

2.2.2. Virology

Influenza viruses are enveloped viruses with a segmented genome made of eight single-stranded negative RNA segments, most of which encode only one viral protein (for a review, see [80]). They form the family *Orthomyxoviridae*, which includes three genera, Influenzavirus A, Influenzavirus B, and Influenzavirus C, that differ antigenically by two of the structural proteins, the matrix (M) protein and the nucleoprotein (NP). Influenza A viruses are further divided into subtypes according to the

antigenicity of their major envelope glycoproteins, the haemagglutinin (HA) and neuraminidase (NA). Sixteen HA subtypes (H1 to H16) and nine NA subtypes (N1 to N9) have been identified. The nomenclature of human influenza virus strains includes the type of the isolate, the place where it was isolated, the year of isolation, an identification number and for influenza A viruses, the subtype of both HA and NA, e.g. “*A/Panama/2007/99 (H3N2)*”[\[40\]](#).

Influenza A viruses of the H1N1, H1N2, H3N2 and H2N2 subtypes naturally infect humans, but only the first three types are currently circulating in the human population. In contrast, influenza A viruses of all 16 HA subtypes and all 9 NA subtypes have been recovered from aquatic birds, which serve as a natural virus reservoir and a potential source of new genes for pandemic influenza viruses. Study of the epidemiological dynamics of human influenza A virus shows that genomic segments encoding the non-structural proteins (NS1 and NS2), the matrix proteins (M1 and M2), and two of the polymerase proteins, PB2 and PA, have remained unchanged since the global pandemic of 1918 [\[81\]](#) [\[82\]](#). In contrast, new RNA segments encoding the haemagglutinin (HA) and neuraminidase (NA) glycoproteins, as well as the PB1 polymerase, have been acquired through reassortment with avian influenza virus, coinciding with the global pandemics in 1957 (H2N2) and 1968 (H3). Seasonal epidemics of influenza A virus since 1968 have been dominated by H3N2 viruses and characterized by punctual mutations, natural selection and frequent reassortment that underlie antigenic drift [\[83\]](#) [\[84\]](#) [\[85\]](#).

Influenza A viruses also can infect poultry, pigs, horses, dogs, and sea mammals. Interspecies transmission has been well documented [\[86\]](#). Aquatic birds, in which the virus multiplies in the gut, usually have an asymptomatic infection and excrete the virus in their faeces. They can transport the virus over large geographical distances. Influenza B viruses appear to naturally infect only humans, although infection of seals was documented in the Netherlands [\[87\]](#). Influenza C viruses only appear to infect humans and pigs and usually cause sporadic cases of upper respiratory disease.

The HA glycoprotein molecules are present at the surface of the influenza virion in the form of a trimeric HA0 precursor which must undergo proteolytic cleavage to generate functional subunits HA1 and HA2. HA1 bears the receptor-binding site and neutralization epitopes, whereas HA2 is responsible for the fusion of the viral envelope with the host-cell membrane at acidic pH [\[88\]](#) [\[89\]](#) [\[90\]](#) [\[91\]](#). Classical avian virus strains have a HA0 cleavage site which is trypsin-like, hence their tropism for the gastrointestinal tract. In contrast, highly virulent avian strains such as the 2004 H5N1 strains from Thailand and Viet Nam or the HP H5N1 isolates have acquired through spontaneous mutations an ubiquitous furin-like cleavage site, which allows them to multiply in many tissues including the respiratory tract [\[92\]](#). A recent post-mortem study of two fatal H5N1 human cases showed that in addition to the lungs, the virus had disseminated to other organs including the intestine, T lymphocytes in lymph nodes and circulating monocytes and macrophages, as well as cerebral neurons [\[93\]](#). The virus could also be transmitted from mother to foetus across the placenta. Viremia and extra-respiratory complications appear to be more common with the avian H5N1 virus than with usual human influenzaviruses [\[94\]](#).

Influenza virus entry into cells of the respiratory tract is mediated by binding of the trimeric haemagglutinin spikes on the virion to specific sialic acid receptors at the cell surface. The difference between the avian (H5) and human (H1, H3) haemagglutinins lies in their specificity for α -2,3-Gal or α -2,6-Gal sialic acid linkages, respectively [\[95\]](#). In mammals, cells with α -2,3 receptors only occur deep in the lungs, whereas α -2,6 receptors are found in the upper respiratory tract, explaining why the H5N1 influenza subtype may cause infection of the lower respiratory tract and severe pneumonia in humans but does not spread readily from human-to-human [\[96\]](#). Two amino acid mutations that caused a switch in receptor binding preference from the human α -2,6 to the avian α -2,3 sialic acid resulted in a 1918 H1N1 virus incapable of respiratory droplet transmission between ferrets, suggesting that a predominant α -2,6 sialic acid-binding preference is essential for optimal transmission of influenza viruses among mammals [\[97\]](#). Three mutations in the H5 haemagglutinin were both necessary and sufficient to change its receptor binding specificity from the avian α -2,3 to an human α -2,6 pattern [\[98\]](#).

The pathogenicity of influenza A virus is clearly due to a polygenic effect [\[99\]](#) [\[100\]](#). Studies of virulence of avian influenza virus strains shows that in addition to the HA cleavage site (see above),

changes in NS1 are important determinants [100] [101], as well as mutations in the PA and PB1 genes [102]. Similarly, the HA, NS, NP, PA, PB1 and PB2 proteins contribute to the virulence of influenza A virus strains in mice, pigs and monkeys (reviewed in [99]). The NS1 protein is critical for the pathogenicity of H5N1 influenza viruses in mammalian hosts as it antagonizes host cell interferon induction and the double-stranded RNA-mediated activation of the NF-kappaB and IFN pathways [103] [104] [105].

2.2.3. Vaccines

The currently available influenza vaccines are made from either inactivated, detergent-split or whole virion influenza virus or live attenuated influenza virus (LAIV) propagated in the allantoic cavity of 9-12 days embryonated chicken eggs from certified farms under strict veterinary control [106]. They include two currently circulating influenza A virus strains and one influenza B virus strain. These trivalent vaccines, which have been used for decades in industrialized countries to prevent seasonal influenza infection, provide a high benefit/cost ratio in terms of preventing hospitalizations and deaths, as shown in numerous studies on vaccination of the elderly and of individuals at high risk for severe outcomes of influenza [40]. Recommended recipients of influenza vaccines are people at high risk of influenza-related complications, namely adults and children with chronic health conditions such as cardiac or pulmonary disorders, asthma, cancer, immunodeficiency or immunosuppression, people who are residents of nursing homes and other chronic care facilities, people over 65 years of age, healthy children aged 6-23 months [107] [108] and pregnant women. Household contacts of individuals at high-risk, as well as providers of health care in facility or community settings who are potentially capable of transmitting the virus to vulnerable populations should also be vaccinated, as well as those providing regular child care to children under 24 months of age.

WHO estimates that there globally are about 1.2 billion people at high risk for severe influenza outcomes: 385 million elderly over 65 years of age, 140 million infants, and 700 million children and adults with an underlying chronic health problem. In addition, 24 million health-care workers ought to be immunized to prevent spreading of the disease to the high-risk population.

2.2.3.1. Seasonal inactivated vaccines.

Continual antigenic drift of the virus means that a new vaccine updated to contain the most current circulating strains is needed every year to protect against new seasonal infections. This is done by reassortment or through the use of the techniques of reverse genetics, which allow one to transfer the HA and NA genomic segments from a circulating wild-type virus into a well characterized, high-yield egg-adapted or cell-culture-adapted influenza virus strain such as the PR8 virus strain, then to select the reassortant virus with the desired HA and NA gene combination. The reassortant virus is usually grown in the allantoic cavity of embryonated hen's eggs, although some vaccines are now produced using mammalian cell lines such as MDCK, PERC-6 or Vero cells.

Monovalent vaccine lots are inactivated using either formalin or β -propiolactone, then purified and combined to make the final trivalent vaccine [109] [110]. Most seasonal vaccines produced today are 'split-vaccines' (subvirion vaccines), which result from treatment of the virions with a detergent or a solvent to dissolve the viral lipid envelope. Less frequently used are whole-virion vaccines made of intact inactivated virus, which may be more reactogenic but have consistently proved to be better immunogens and confer more efficient protective responses than split vaccines [111].

Other inactivation procedures have been reported, such as treatment with hydrophobic 1,5-iodonaphtyl-azide (INA), which selectively targets the transmembrane segments of proteins in the viral envelope while preserving the structure of the antigens on the surface of the virus. INA is activated by UV irradiation, resulting in a non-infectious intact influenza virus particle with full neuraminidase activity able to induce potent anti-influenza virus serum neutralizing antibody and T cell responses and to provide protection against heterosubtypic challenge after a single Intranasal immunization, similar to live attenuated virus immunization [112].

The trivalent split inactivated influenza vaccines have a remarkable safety profile, including in 6 to 23 months old children [113], as recently confirmed in a retrospective study bearing on 45,356 vaccinations in children that age [108]. Multiple studies show the vaccine to be approximately 60% to 90% efficacious against influenza illness in healthy children and adults, depending on the antigenic match between the circulating and vaccine viral strains [114] [115] [116]. Vaccine effectiveness was found to actually vary substantially across successive seasons from a low 10% to a high 52% depending on the antigenic match between the circulating virus strains and the strains used in the vaccine [117]. Vaccination was also found to decrease the incidence of pneumonia, physician visits, hospitalizations and deaths in the elderly [118] [119] [120]. In vaccine-naïve children less than 9 years old, a two dose schedule is required [121] [122]. In contrast, a second dose of vaccine in elderly individuals does not boost immunity [123].

The inactivated split influenza vaccine also is quite immunogenic when administered as a full dose (15µg HA) to elderly volunteers by the ID route using a Beckton-Dickinson microneedle delivery system [124] [125]. The use of the ID route was found to enhance the immunogenicity of the vaccine in the elderly population as compared to the classical IM route of administration, as judged by higher geometric mean antibody titers and higher percentage of vaccinees with a > 4-fold rise in HA antibody titer [126]. Similar data were generated in recipients of renal transplants. A randomized, open-label study in 112 healthy children aged 3 to 18 years showed that the immunogenicity of ID vaccination at one fifth of a dose was comparable to that of standard-dose IM vaccination in children as young as 3 years of age [127]. Attempts at vaccinating healthy adults by the intranasal route using the inactivated split vaccine in combination with detoxified *E coli* enterotoxin (LTK63) as an adjuvant also are in progress [128] [129].

At this time, the vaccination of people at high risk of complications from influenza is a key public health strategy in industrialized countries. Almost 350 million trivalent vaccines are distributed worldwide each year. Universal influenza vaccination has been proposed as one strategy to improve vaccination coverage and disease prevention. Most economic analyses show that universal vaccination of healthy adults and children would be cost-effective. Also, herd immunity resulting from vaccination of school-age children would substantially reduce the incidence of disease and mortality among adults [129] [130]. The world's total vaccine production capacity at this time, however, is only about 350 million doses of trivalent vaccine, of which close to 50% are produced by just one manufacturer, Sanofi Pasteur.

2.2.3.2. Live attenuated influenza vaccines (LAIVs)

The second major approach to influenza vaccines has been the development of cold-adapted (*ca*) virus strains which grow well in primary chicken kidney cells and embryonated eggs at 25–33°C, have a reduced replication titre at 37°C, and show attenuated virulence in ferrets. Cold adaptation was found to be a reliable and efficient procedure for the derivation of LAIVs, which are obtained by reassorting the HA and NA genomic segments from a circulating influenza A virus strain with the other six genomic segments from a well characterized laboratory strain that carries the *ca* mutation. Thus, a bivalent *ca* LAIV was developed by Microgen and used for decades in Russia to immunize more than 100 million people every year.

A trivalent *ca* LAIV (Flumist™) has been developed for intra-nasal spray delivery by MedImmune and Wyeth in the USA where it officially is licensed for 2-59 years-old persons. The Flumist™ vaccine was proven highly efficacious in Phase III trials, showing a 92% overall protection rate over a 2-year study in children [131] [132]. It was well tolerated and effective in preventing culture-confirmed influenza illness in children as young as 6 months of age who attended day care centers [133] and was shown to provide herd immunity to adults when used in children [134]. It also demonstrated superior efficacy compared to the trivalent inactivated vaccine in preventing influenza illness in children 12 to 59 months of age [135], in young children with recurrent respiratory tract infections [136] and in children with asthma [137] [138]. One dose of Flumist™ induced significant protection against influenza illness and pneumonia in 5 to 18 years old children when administered during an influenza outbreak due to a

circulating influenza virus strain distinct from the vaccine strain, whereas the trivalent inactivated vaccine was not able to provide protection [139]. Nasal shedding of LAIV in individuals 5-49 years of age was found to generally be of short duration (days 1-10 post immunization) and at low titers. LAIV recipients should therefore avoid contact with severely immunosuppressed persons [140].

The Flumist™ vaccine shows remarkable genetic stability, but it has to be kept at -18°C. A new, heat-stable version of the vaccine has been developed, which showed good efficacy in clinical trials in Asia and Europe, but was associated with an increase in asthma episodes in young children [141]. The current vaccine was also tested for safety in infants aged 6 to 24 weeks, who received two doses of vaccine intranasally 1 month apart: the vaccine was generally well tolerated [142]. In children, LAIV provided sustained protection against influenza illness caused by antigenically related strains for 9-12 months, and showed meaningful efficacy although at a lesser level through a second season without revaccination [143].

Another *ca* LAIV also is in development at Applied Microbiology in Austria by growth of the virus in Vero cells at 25 °C [144]. Still another cold-adapted LAIV grown in Madin-Darby canine kidney (MDCK) cells on microcarrier beads in serum-free medium is at an advanced preclinical development stage at the Vector Scientific Center in the Russian Federation.

Preliminary studies indicate that the IFN γ -ELISPOT assay, a measure of cell-mediated immunity, may be a more sensitive measure of influenza immune memory responses to LAIVs than serum antibody. The role of cell-mediated immunity in actual protection against culture-confirmed clinical influenza by LAIV was investigated in a large efficacy trial involving 2172 6 to 36 months-old children in the Philippines and Thailand who were vaccinated with an intranasal dose of 10⁷ FFU of LAIV: the majority of infants with >100 IFN- γ spot-forming cells/10⁶ peripheral blood mononuclear cells were protected against influenza [145].

2.2.3.3. Other types of influenza vaccines

2.2.3.3.1. Virosomes

Berna Biotech, now Crucell, is commercializing an influenza vaccine formulated in virosomes, with the surface spikes of the three currently circulating influenza virus strains inserted into the vesicle membrane of liposomes. A nasal formulation of the vaccine had to be withdrawn from the market, due to undesirable neurological side effects (Bell's palsy) linked to the presence of the *E. coli* labile toxin (LT) used as an adjuvant, most likely because the B subunit of LT could bind to GM1 ganglioside receptors in neuronal tissues associated with the olfactory tract. Other formulations of inactivated influenza vaccine for mucosal delivery are in progress including immunostimulating complexes (ISCOMs).

2.2.3.3.2. Synthetic vaccines

Yeda, an Israeli R&D company, is developing a synthetic peptide influenza vaccine for nasal administration. The vaccine has shown protective efficacy in humanized mice and is planned to enter clinical trials soon.

2.2.3.3.3. M2e-based vaccines

A "universal" human influenza virus A vaccine was initially developed at the University of Ghent, Belgium, based on transmembrane viral protein M2, which is scarcely present on the virion but is abundantly expressed on virus-infected cells [146]. The extracellular M2 domain, M2e, a 23 amino acid-long peptide, is remarkably conserved between H1N1, H2N2 and H3N2 influenza A virus strains [147]. Passive administration of anti-M2e antibodies affords significant protection against influenza A virus challenge in animal models. The mechanism is believed to be NK cell-mediated antibody-dependent cellular cytotoxicity (ADCC), not virus neutralization.

A recombinant particulate vaccine has been engineered by genetically fusing copies of the influenza virus M2e domain to the hepatitis B core antigen (HBc). The (M2)-HBc fusion protein was found to spontaneously assemble into highly immunogenic virus-like particles (VLPs) that provided complete protection against a lethal influenza A virus challenge in mice and ferrets [148]. The VLP vaccine, ACAM-FLU-A™, which is developed by Acambis, Cambridge, MA, USA, was recently tested in a two-dose immunization schedule clinical study in 18-40 years-old volunteers.

Another formulation involving a fusion protein based on the CTA1-DD adjuvant and containing tandem repeats of the M2e sequence, still is at a preclinical stage of development [149] [150]. These vaccines could theoretically serve as universal influenza A vaccine in view of the high degree of conservation of the M2e sequence among human influenza A viruses. The question of whether they would also protect against a pandemic virus is however open, as the M2e sequence in avian virus strains including H5N1 appears to be divergent from that in humans viruses.

The M2e peptide has also been conjugated to other protein carriers, such as the *Neisseria meningitidis* outer membrane protein complex (OMPC) and derivatives of the cholera toxin. Another approach has been developed at VaxInnate, USA, linking four tandem copies of M2e to *Salmonella typhimurium* flagellin type 2 protein, a potent TLR5 ligand. The resulting STF2.4xM2e fusion protein, which can be produced in high yields in *E coli* fermentors, was shown to induce a robust and long-lasting protective antibody response in mice [151].

2.2.3.3.4. Subunit vaccines

A subunit vaccine made of the conserved NP internal protein, a known target for cytotoxic T cells, which is covalently coupled to the M2e peptide and to the ISS adjuvant, is in development at Dynavax Technologies, Berkeley, CA, USA. ISS, a TLR9 activator, has been shown to activate NK cell secretion of IFN- γ [152] and was successfully tested as an adjuvant in the Heplisav™ hepatitis B vaccine.

2.2.3.3.5. VLPs

Influenza VLPs are produced in insect cell using recombinant baculovirus vectors that express viral proteins HA, NA and M1 [153] [154]. Influenza VLPs induced mucosal IgG and cellular immune responses in mice, as well as long-lived antibody-secreting cells that were detected in the bone marrow of the immunized animals [155].

A two-dose immunization regimen with influenza VLPs developed by Novavax, Rockville, MD, USA, was highly immunogenic in mice and ferrets, even at the low dose of 6 μ g HA [156]. In contrast, an experimental trivalent influenza vaccine made of HA0 haemagglutinins H1, B and H3 produced in serum-free insect cell cultures using three recombinant baculoviruses (Protein Science), required 75 μ g of each of the three HAs to elicit HAI antibody responses in only 51%, 65% and 81% of vaccine recipients, respectively [157].

2.2.3.3.6. Live recombinant vaccines

Other influenza vaccines, such as DNA vaccines, live recombinant vaccines based on non-replicative Ad5 [158], modified vaccinia Ankara (MVA) virus [159] or Newcastle disease virus vectors [160] are still at a preclinical or early clinical development stage.

2.2.3.4. Pandemic Influenza vaccines

No current influenza vaccine would offer protection against a pandemic triggered by an emerging avian virus strain, such as H5N1, H7N7 or H7N2. Specific 'pandemic' vaccines need therefore to be prepared for that purpose. In addition, two doses of pandemic strain vaccine spaced by one month will likely be necessary as no humans have any underlying immunity to avian influenza virus strains [161]. Given the world's current vaccine production capacity, if a monovalent inactivated pandemic influenza vaccine were produced according to the formulation of seasonal vaccines, i.e. with 15 μ g haemagglutinin per

dose, only about 475 million people could be vaccinated. The WHO has been active at encouraging pandemic influenza vaccine R&D and reviewing progresses through a number of meetings. Updated results on clinical trials of pandemic influenza vaccines can be found at [\[162\]](#).

2.2.3.4.1. *Inactivated split vaccines*

Immunogenicity trials with candidate split inactivated vaccines prepared with a H5N1 virus strain (A/Vietnam/2004 H5N1) showed disappointingly low immune potency, as two 45 µg haemagglutinin doses of split vaccine at four weeks interval elicited only 38% seroconversion among 2-9 years old children and two doses of 90 µg H5 haemagglutinin only 35% responses in elderly volunteers [\[163\]](#) [\[164\]](#) [\[165\]](#). The need to enhance the immunogenicity of inactivated H5N1 vaccines and to search for 'antigen-sparing' formulations was therefore obvious [\[78\]](#) [\[166\]](#) [\[167\]](#).

Attempts at using aluminium salts as adjuvants in inactivated H5N1 influenza vaccines had a limited effect on the potency of the vaccines, as two doses of 30µg or even 45 µg HA were required for induction of six-month antibody persistence [\[168\]](#). In contrast, remarkable results were obtained with adjuvants such as polyoxidonium (Microgen), MF59 (Novartis), AS03 (GSK) or AF03 (Sanofi Pasteur), which are based on oil-in-water emulsions. These adjuvants considerably increased the immunogenicity of the inactivated split H5N1 vaccines, allowing a significant reduction in the dose of haemagglutinin needed to elicit protection.

Thus, two doses of MF59-adjuvanted Novartis vaccine (Fluad™) containing 7.5 µg of H5 haemagglutinin elicited a protective influenza neutralizing antibody response in 77% of recipients [\[169\]](#); the antibody response to two doses of vaccine containing 15µg HA (H5N1) with MF59 were higher than the response to 45 µg of vaccine alone [\[170\]](#). Quite similarly, two doses of AS03-adjuvanted GSK H5N1 vaccine containing 3.8 µg and 7.5 µg H5 haemagglutinin elicited haemagglutination-inhibiting (HAI) antibodies in 82% and 90% of the volunteers, as compared to 4% and 16% with the non adjuvanted vaccine controls, respectively [\[171\]](#) [\[172\]](#); and two doses of AF03-adjuvanted H5N1 Sanofi Pasteur vaccine containing 1.9 µg or 3.75 µg of antigen generated a high level seroprotective immune response in over 70% and over 80% of the participants, respectively [\[44\]](#). In addition, the antibodies elicited by the adjuvanted vaccines were broadly cross-neutralizing antibodies that neutralized virus strains belonging to the various H5N1 virus clades in cell culture and could protect ferrets from challenge with virus strains from different H5N1 clades [\[173\]](#).

There is therefore no more theoretical impossibility to manufacture 1.5-2 billion doses of split pandemic influenza vaccine using the currently available manufacture facilities if need be. Further improvements aimed at up-scaling vaccine production in case of a pandemic would be to develop cell-culture vaccines, which would bypass the bottleneck of limited egg supply. Influenza virus can be adapted to grow in a variety of cell lines including Vero cells and PER.C-6 cells, which would allow large-scale production of the virus.

The possibility is also entertained that the addition of adjuvants to seasonal influenza vaccines might be of benefit to populations at risk such as persons with underlying chronic diseases [\[174\]](#). Inactivated whole-virion vaccines adjuvanted with aluminium hydroxide were found to have better immunogenicity in naive individuals than split vaccines, but may be associated with febrile reactions, particularly in children [\[175\]](#). New adjuvants based on TLR ligands are being developed that could eventually be used for influenza vaccines, such as the deoxynucleotide IC31™ (Intercell, Austria) [\[176\]](#) or the *Salmonella typhimurium* flagellin type 2 (STF2, VaxInnate Corp, USA).

2.2.3.4.2. *Inactivated whole-virion vaccines*

Candidate pandemic inactivated whole-virion vaccines also have been produced by several companies, including Baxter, Austria, Nobilon International, The Netherlands, Sinovac, China, and Omnivest, Hungary. Adjuvantation with aluminium hydroxide was found to be devoid of effect on the immunogenicity of the Vero cell-derived Baxter H5N1 vaccine in mice and humans [\[177\]](#), whereas it

provided high immunogenicity to the Nobilon vaccine in ferrets. The Omnivest H5N1 vaccine (Fluval™), an egg-based whole-virion vaccine adjuvanted with aluminium phosphate, was found to elicit significant protective cross-clade immune responses in 70% of volunteers, including 60-90 years-old persons and children, after only a single IM dose of 6µg HA [178]. The Baxter vaccine [177] and the Sinovac vaccine [175] both necessitated two doses of vaccine with somewhat higher antigenic content 3-4 weeks apart to elicit protective neutralizing antibody levels in volunteers (For review, see [45]).

2.2.3.4.3. LAIVs

Candidate pandemic LAIVs have also been prepared. A single-dose immunization of ferrets with a candidate Flumist LAIV prepared from the A/Hong Kong/97 (H5N1) strain provided 100% protection against challenge of the animals with a variety of H5N1 strains belonging to different clades. A series of LAIVs were produced by MedImmune (USA) by reassortment between avian influenza A viruses H5N1, H7N3 or H9N2 and the *ca* Ann Arbor virus strain (H1N1). Phase I clinical trials of resulting *ca* reassortants were performed on volunteers kept in an isolation unit at Johns Hopkins Medical Center, Baltimore, MD, USA. Virus shedding into nasal secretions was detected for one day in 60% of volunteers and for up to four days in another 25%. After a second immunization a few weeks later, no viral shedding could be detected. A systematic comparison between avian-human and human-human *ca* reassortants in human volunteers however evidenced reduced infectivity and immunogenicity of the avian-human LAIVs, as compared with human-human H1N1 or H3N2 LAIVs. The H5 LAIV was the least immunogenic of the five *ca* reassortants tested (for a review, see [45]). A two-dose immunization with the H5N1/influenza A/Ann Arbor 60 *ca* (H3N2) reassortant LAIV fully protected mice and ferrets against pulmonary replication of homologous and heterologous wild-type H5N1 viruses [179].

Another avian-human *ca* reassortant influenza virus strain was developed as LAIV by Microgen, Russia [180], that elicited strong cross-clade protection in mice. The resulting LAIV strain, which carries a H5N2 antigenicity, was tested in Phase I and Phase II clinical trials using two immunizations 21 days apart and showed reasonably good immunogenicity.

Still another type of H5 LAIV was developed by Green Hills Biotech, Vienna, Austria, by deleting the NS1 viral gene, a virulence factor known to antagonize interferon, from a H5N1 avian strain. The resulting Δ NS1 H5N1 strain can only be grown in Vero cells, shows attenuation in mice and ferrets and provides protection of the animals against virulent challenge with wild-type H5N1 virus strains [181].

2.2.3.4.4. VLPs

A H5N1 VLP containing the HA, NA and M1 viral proteins was developed at Novavax, USA, and found to elicit strong HAI and cell-mediated immune responses in mice and ferrets and to protect 100% of the animals against cross-clade H5N1 virus challenge. A two-dose Phase I trial is planned to take place shortly. A new recombinant VLP candidate vaccine was recently developed by LigoCyte Pharmaceuticals in collaboration with the Batelle Biomedical Research Center based on the expression of a fusion protein between the murine leukemia virus Gag protein and the influenzavirus HA glycoprotein in a baculovirus-insect cell expression system. The resulting HA-pseudotyped Gag VLPs were purified as 100 nm spherical particles that elicited robust anti-influenza immunity in ferrets and protected the animals against cross-clade H5N1 virus challenge [182].

2.2.3.4.5. Live vectored vaccines

Live recombinant H5N1 vaccines also have been developed but still are at preclinical development stage. These include a mixture of three Ad5 recombinants that expressed the HA, M1 and NP proteins from a H5N1 strain, respectively; the vaccine elicited strong protection against lethal H5N1 challenge in mice and chickens [183].

An MVA recombinant that expressed the H5 HA protein elicited protection against challenge with three antigenically distinct strains of H5N1 influenza viruses in mice [184]. Another MVA recombinant which will express all five M1, M2, HA, NA and NP proteins is being developed and should be tested shortly.

Last, but not least, a NDV recombinant that expresses the H5 HA protein was found to be highly immunogenic and protective in chickens and could be used as bivalent vaccine against both Newcastle disease and highly pathogenic H5N1 avian influenza virus infection in poultry [160] [185].

2.2.3.4.6. Subunit vaccines

A subunit recombinant H5 vaccine based on HA protein expressed in a baculovirus expression system was well tolerated in human volunteers but showed mediocre immunogenicity [186], probably due to lack of oligomerization of the antigen [187].

2.2.3.5. 'Pre-pandemic' vaccines

Many plans have been made on how to mitigate an influenza pandemic once it breaks out, including stockpiling of antiviral drugs, and restricting population movements until a vaccine is available for the specific strain that has broken out. As strain-specific vaccines would be available only several months into the pandemic and would be in short supply, the possibility has been entertained of using 'pre-pandemic' vaccines which would not match the exact pandemic strain but an earlier variant. As seen above, H5N1 vaccines do show cross-clade protection in animal models and thus might confer sufficient protection against death or severe disease due to a new emerging pandemic H5N1 variant. The World Health Organization is planning to stockpile more than 100 million doses of pre-pandemic H5N1 vaccines and several nations are considering the same.

2.3. **Respiratory syncytial virus and parainfluenza viruses**

Respiratory syncytial virus (RSV) and parainfluenza viruses (PIV) belong to the same family Paramyxoviridae. Their importance as respiratory pathogens in young children has been recognized for over 40 years, yet the development of vaccines against RSV or PIV has been hampered by several factors, including the risk of potentiation of naturally occurring disease, as was observed in the early 1960s (see below).

RSV is the most important cause of viral lower respiratory tract illness (LRI) in infants and children worldwide [16], being responsible for 70 000 to 126 000 infant hospitalizations for pneumonia or bronchiolitis every year in the USA alone. The elderly also are at risk for severe RSV disease [19] [188] and 14 000 to 62 000 RSV-associated hospitalizations of the elderly occur annually in the USA [189].

Human parainfluenza viruses types 1, 2 and 3 (PIV1, PIV2 and PIV3, respectively) are second only to RSV as important causes of viral LRI in young children [10], being recovered from 18% young children [190], with upper respiratory illness (URI), 22% with LRI and 64% with croup [191]. PIV-1 and PIV-2 are the principal causes of croup, which occurs mostly in children 6 to 48 months of age, whereas PIV-3 causes bronchiolitis and pneumonia predominantly in children less than 12 months of age.

2.3.1. Disease burden

2.3.1.1. RSV infection

Human RSV infection, the single most important cause of severe respiratory illness in infants and young children and the major cause of infantile bronchiolitis, is the most frequent cause of hospitalization of infants and young children in industrialized countries [192]. In the USA alone, from 85 000 to 144 000

infants with RSV infection are hospitalized annually [17], resulting in 20%-25% of pneumonia cases and up to 70% of bronchiolitis cases in the hospital [193] [194]. Global RSV disease burden is estimated at 64 million cases and 160 000 deaths every year.

RSV disease spectrum actually includes a wide array of symptoms, from rhinitis and otitis media to pneumonia and bronchiolitis. Humans are the only known reservoir for RSV. Spread of the virus from contaminated nasal secretions occurs via large respiratory droplets, and close contact with an infected individual or contaminated surface is required for transmission. The virus can persist for several hours on toys or other objects, which explains the high rate of nosocomial RSV infections, particularly in paediatric wards. In the USA, nearly all children by 24 months of age have been infected at least once with RSV, and about half have experienced two infections [195].

Children who experience RSV infection early in life run a high risk of subsequent recurrent wheezing and asthma [196] [197] [198], especially premature infants and infants with bronchopulmonary dysplasia, for whom preventive passive immunization with anti-RSV monoclonal antibodies such as Palivizumab is highly recommended [199] [200] [201]. RSV-infected infant sera and nasal secretions show a marked increase in levels of Th-2 cytokines and chemokines, including IL-4 and MIP-1 α , as well as of non-neutralizing IgE antibodies. The RSV G protein is believed to induce the release of large amounts of Th-2 cytokines and MIP-1 α from CD4+ T cells and mast cells, basophils and monocytes, that trigger increased pulmonary eosinophilia and asthma exacerbation [202]. Repeated RSV infection in a mouse model similarly induces persistent airway inflammation and hyperresponsiveness which are characteristics of asthma [203] [204] [205].

Infants who had been immunized with a formalin-inactivated RSV vaccine in the 1960s similarly experienced enhanced RSV disease and pulmonary eosinophilia upon subsequent RSV infection, leading to numerous hospitalizations and two deaths [206] [207] [208], probably due to skewing of the immune response towards a Th-2 response and failure of the vaccine to induce a CD8+ T cell response [209] [210].

RSV also is a significant problem in the elderly [188], in persons with cardiopulmonary diseases [211] and in immunocompromized individuals [212]. RSV attack rates in nursing homes in the USA are approximately 5%-10% per year with a 2%-8% case fatality rate, amounting to approximately 10 000 deaths per year among persons >64 years of age [213]. Among elderly persons followed for 3 consecutive winters, RSV infection accounted for 10.6% of hospitalizations for pneumonia, 11.4% of hospitalizations for obstructive pulmonary disease, 5.4% for congestive heart failure and 7.2% for asthma [214].

Few population-based estimates of the incidence of RSV disease in developing countries are available, although existing data clearly indicate that the virus accounts for a high proportion of ARIs in children. Studies in Brazil, Colombia and Thailand suggest that RSV causes 20–30% of ARI cases in children from 1–4 years of age, a proportion similar to that in industrialized countries. Another confusing aspect of the epidemiology of RSV infection is the seasonality of the disease. In Europe and North America, RSV disease occurs as well-defined seasonal outbreaks during the winter and spring months. Studies in developing countries with temperate climates, such as Argentina, have shown a similar seasonal pattern. On the other hand, studies in tropical countries often have reported an increase in RSV in the rainy season but this has not been a constant finding. Cultural and behavioral patterns in the community might also affect the acquisition and spread of RSV infection.

2.3.1.2. *Parainfluenza virus infection*

Parainfluenza viruses also cause a spectrum of respiratory illnesses, from upper respiratory infections, 30–50% of which are complicated by otitis media, to lower respiratory infections, about 0.3% of which require hospitalization. Most children are infected by human parainfluenza virus type 3 (PIV-3) by the age of two years and by parainfluenza virus types 1 and 2 (PIV-1 and PIV-2) by the age of five years [191]. PIV-3 infections are second only to RSV infections as a viral cause of serious ARI in young children. Pneumonia and bronchiolitis from PIV-3 infection occur primarily in the first 6-12 months of life, as is the case for RSV infection [190]. Croup is the signature clinical manifestation of infection with other parainfluenza viruses, especially PIV-1, and is the chief cause of hospitalization from

parainfluenza infections in children two to six years of age [191]. The proportions of hospitalizations associated with PIV infection vary widely in hospital-based studies. Consequently, the annual estimated rates of hospitalization fall within a broad range: PIV-1 is estimated to account for 5,800 to 28,900 annual hospitalizations in the USA, PIV-2 for 1,800 to 15,600 hospitalizations, and PIV-3 for 8,700 to 52 000 hospitalizations. Along with RSV, parainfluenza viruses are also leading causes of hospitalization in elderly with community-acquired respiratory disease.

PIV-1 causes large, well-defined outbreaks, marked by sharp biennial rises in cases of croup in the autumn of odd-numbered years. Outbreaks of infection with PIV-2, though more erratic, usually follow type 1 outbreaks. Outbreaks of PIV-3 infections occur on a yearly basis, mainly in spring and summer. Although PIV-1, -2 and -3 have been described as a cause of ARI in developing countries, the corresponding disease burden has not been accurately determined in these countries.

Reinfection with any of the parainfluenza viruses and/or with RSV can occur throughout life [215], usually resulting in mild upper respiratory infections in young adults, but causing severe disease in immunocompromized patients [16] [216] [217].

2.3.2. Virology

RSV and parainfluenza viruses belong to the family Paramyxoviridae. These are enveloped viruses with a negative-sense single-stranded RNA genome.

Human RSV, together with its close relative bovine RSV, belongs to the subfamily Pneumovirinae, genus Pneumovirus. Its genome is a 15 222 nucleotide-long, negative-sense RNA molecule which encodes 11 viral proteins, among which the nucleoprotein (N), the fusion protein (F), the surface glycoprotein (G), the matrix protein (M) and several non-structural proteins including the L protein (replicase) and virulence factors NS1 and NS2 that mediate resistance to IFN- α/β [218]. The tight association of the RNA molecule with the viral N protein forms a nucleocapsid wrapped inside the viral envelope, from which protrude viral proteins F, G and SH. The RSV G protein was shown to be a structural and functional mimetic of fractalkine, a proinflammatory CX3C chemokine that mediates leucocyte migration and adhesion [219], which explains its role in pathogenesis [220] [221] [222].

The fusion protein F and attachment glycoprotein G are the only two components that induce RSV neutralizing antibodies. The sequence of the F protein is highly conserved among RSV isolates. In contrast, that of the G protein is relatively variable [223]: two serogroups of RSV strains have been described, the A and B groups, based on differences in the antigenicity of the G glycoprotein. Current efforts are directed towards the development of a vaccine that will incorporate strains in both groups, or will be directed against the conserved F protein (for a review, see [224]).

Parainfluenza viruses belong to the subfamily Paramyxovirinae, itself subdivided into three genera: Respirivirus (PIV-1, PIV-3, and Sendai virus (SeV)), Rubulavirus (PIV-2, PIV-4 and mumps virus) and Morbillivirus (measles virus, rinderpest virus and canine distemper virus (CDV)). Their genome, a ~15 500 nucleotide-long negative-sense RNA molecule, encodes two envelope glycoproteins, the haemagglutinin-neuraminidase (HN), and the fusion protein (F), itself cleaved into F1 and F2 subunits, a matrix protein (M), a nucleocapsid protein (N) and several nonstructural proteins including the viral replicase (L) [225]. All parainfluenza viruses except PIV-1 express a non-structural V protein that blocks IFN signalling in the infected cell and acts therefore as a virulence factor [226].

2.3.3. Vaccines

2.3.3.1. General considerations

Development of vaccines to prevent RSV infection have been complicated by the fact that host immune responses appear to play a significant role in the pathogenesis of the disease. Early attempts at vaccinating children in the 1960s with a formalin-inactivated RSV vaccine showed that vaccinated children suffered from more severe disease on subsequent exposure to the virus as compared to unvaccinated controls (see above). These early trials resulted in the hospitalization of 80% of vaccinees

and two deaths [206] [207] [208]. The enhanced severity of disease has been reproduced in animal models and is thought to result from inadequate levels of serum-neutralizing antibodies, lack of a cellular immune response, and excessive induction of a Th2 immune response with pulmonary eosinophilia and increased production of IL-4, IL-5 and MIP-1 α [209] [210].

In addition, naturally acquired immunity to RSV is neither complete nor durable and recurrent infections occur frequently during the first three years of life. Older children and adults, however, usually are protected against severe RSV disease, suggesting that protection does develop after primary infection.

Passive immunization in the form of RSV-neutralizing immune globulin or humanized monoclonal antibodies given prophylactically has been shown to prevent RSV infection in newborns with underlying cardiopulmonary disease, and in small, premature infants [199] [200] [201]. This demonstrates that humoral antibody plays a major role in protection against infection. In general, secretory IgAs and serum antibodies appear to protect against infection of the upper and lower respiratory tracts, respectively, while T-cell immunity targeted to internal viral proteins appears to help terminate viral infections.

Although live attenuated RSV vaccines seem preferable for immunization of naive infants, nonreplicative vaccines may be useful for immunization of the elderly and older, high-risk children, as well as for maternal immunization. In addition, RSV vaccines should induce a balanced Th1-Th2 response and cover the two antigenically distinct serogroups RSVA and RSVB.

2.3.3.2. Subunit RSV vaccines

Three types of RSV subunit vaccines have been evaluated in clinical trials [227].

Candidate vaccines based on purified F protein (PFP-1, PFP-2 and PFP-3) prepared from RSV-infected cells were tested in a variety of rodent and nonhuman primate models and found to induce protection against RSV challenge [228]. These candidates were tested in human clinical trials involving elderly volunteers [229] [230], pregnant women [231] and children with chronic lung disease [232] [233]. The vaccines were found to be safe and moderately immunogenic but the incidence of lower RSV ARI was not significantly diminished in the vaccinees. Vaccination of women in the 30th to 40th week of pregnancy induced RSV anti-F antibodies titres that were persistently fourfold higher in newborns to the vaccinated mothers than to those who had received a placebo and was not followed by increase in frequency or morbidity of respiratory illnesses in the seropositive infants.

Another subunit vaccine consisting of co-purified F, G, and M proteins from RSV A was tested in healthy adult volunteers in the presence of either alum or polyphosphazene (PCPP) as an adjuvant. Neutralizing antibody responses to RSV A and RSV B were detected in 76–93% of the vaccinees, but waned after one year, suggesting that annual immunization with this vaccine would be necessary [234].

Still another subunit approach was investigated using the central domain of the RSV G protein, whose sequence is relatively conserved among serogroup A and B viruses. A vaccine candidate, BBG2Na, was developed by fusing this domain (G2Na) to the albumin-binding region (BB) of streptococcal protein G and producing the fusion protein in a bacterial expression system. The candidate vaccine elicited a protective immune response in animals [235], and was moderately immunogenic in adult human volunteers [236]. Its clinical development had to be interrupted due to the appearance of unexpected type 3 hypersensitivity side effects (purpura) in a couple of immunized volunteers.

2.3.3.3. Live attenuated RSV and PIV-3 vaccines

The development of reverse genetic systems for RSV and parainfluenza viruses has provided for the generation of a number of genetically designed vaccine candidates that harbor mutations or deletions in an effort to attenuate virus replication without compromising immunogenicity [237]. Achieving an

appropriate balance between attenuation and immunogenicity has however been a major obstacle to the development of these vaccines.

A temperature-sensitive (ts) human PIV-3 (HPIV-3) strain, cp45, was selected after 45 passages of the virus in African green monkey cells at low temperature and evaluated as an intranasal vaccine in Phase I/II trials in RSV seropositive and seronegative children and in young infants. The HPIV-3 cp45 vaccine candidate was well tolerated and immunogenic in seronegative infants as young as 1 month of age [238] [239] [240] and showed little risk of transmission to unvaccinated children and toddlers [241]. Rcp45 is a promising HPIV-3 candidate vaccine that is likely to soon be evaluated in efficacy trials [16].

A cold-passaged (cp) derivative of RSV still caused mild respiratory illness in young children: the strain was further attenuated by chemical mutagenesis to produce the cpts 248/404 strain, which was, however, still reactogenic in 1-2 months-old infants [242] and had to be further mutagenized to produce suitably attenuated vaccine candidate strain rAcp248/404/1030- Δ SH [243]. Its immunogenicity remains to be tested. Another set of engineered candidate vaccines that have a deletion of the NS2 gene in common (rAcp248/404- Δ NS2) have been found to be overattenuated for children [244]. Meanwhile, a combination RSV and HPIV-3 intranasal vaccine was tested in 6-18 months-old children, using as a vaccine a mixture of the RSV cpts 248/404 and the HPIV-3 cp45 strains: both vaccines were found to be as immunogenic after simultaneous administration as after separate administration [245].

Another live attenuated candidate PIV-3 vaccine was developed using the Kansas strain of bovine PIV-3 (BPIV-3). BPIV-3 is closely related antigenically to HPIV-3, it can protect monkeys against challenge with HPIV-3, and it replicates poorly in humans, making a perfect Jennerian vaccine candidate. BPIV-3 was well tolerated and immunogenic in seronegative children and infants as young as 2 months old [246] [247] but the magnitude of the anti-HN response was lower in children who received the BPIV-3 vaccine than after immunization with the HPIV-3 cp45 strain.

2.3.3.4. Live chimeric and recombinant vaccines

A chimeric bovine/human PIV-3 (B/HPIV-3) strain was engineered by substituting in a BPIV-3 genome the HPIV-3 F and HN genes and the F/NH intergenic sequences to their bovine equivalent. The resulting B/H chimeric virus retained the attenuated phenotype of BPIV-3 and was highly immunogenic in rhesus monkeys [248].

The B/HPIV-3 chimeric strain was then used as a vector to express the F, or F and G open reading frames of RSV subgroup A or B [249], thus providing a candidate intranasal vaccine against both RSV and PIV-3 infections [250]. African green monkeys immunized with the B/HPIV-3 chimera expressing either the native or soluble RSV F protein produced RSV-neutralizing antibodies and were fully protected against challenge with wild-type RSV [251]. The live attenuated nasal RSV/PIV-3 candidate vaccine (MEDI 534TM) was shown to be safe and well tolerated in Phase I clinical studies conducted by MedImmune in the USA in adults and seropositive children 1-9 years of age. The vaccine is presently entering Phase I/IIa clinical trials in 2 month-old infants and in 6-24 month-old children. The PIV-3-vectored RSV candidate vaccine could be a vaccine of choice to prevent RSV and PIV-3 infections in young infants.

Other virus vectors have been used to deliver RSV F and/or G proteins. Recombinant vaccinia virus and adenoviruses expressing RSV F, RSV G or RSV F and G were constructed and tested in animal models including chimpanzees but showed mediocre immunogenicity [252] [253]. Sendai virus (SeV), the murine PIV-1, which had been shown to be safe in human volunteers and to protect African green monkeys against human PIV-1 challenge, was also used as a vector to express RSV fusion protein F. The recombinant SeV-RSV F induced RSV-neutralizing antibodies and RSV-specific CTLs and protected cotton rats and mice against challenge with RSV of both A and B subgroups [254] [255]. Similarly, a SeV recombinant expressing the haemagglutinin-neuraminidase (HN) gene from HPIV-3 induced protection against both PIV-1 and PIV-3 challenge [256]. Sendai virus, however, does not seem to be sufficiently attenuated to be used as a Jennerian vaccine in human infants.

Venezuelan equine encephalitis virus (VEEV) replicon particles (VRPs) expressing RSV F or G similarly induced RSV-specific T cell and neutralizing antibody responses and protected mice and cotton rats against RSV challenge [257] [258].

2.4. [Streptococcus pneumoniae](#)

2.4.1. Introduction

Streptococcus pneumoniae, or pneumococcus, is a leading cause of morbidity and mortality among children worldwide and particularly in developing countries [259] [260] [261]. It was estimated that 10.6 million children less than 5 years present with pneumococcal disease every year [262]. By far the most common form of the disease is bacteremic pneumococcal pneumonia, whose highest incidence is associated with both extremes of age (children < 2 years of age and adults >65 years of age), the next most common form being pneumococcal meningitis, especially in infants and young children, followed in order of decreasing incidence by blood stream infection (or sepsis) and otitis media. Sinusitis and, more rarely, endocarditis and peritonitis also have been reported. In developing countries, the bacterium is the leading bacterial cause of childhood ARI mortality and the leading cause of non-epidemic childhood meningitis [263] [264].

The capsular polysaccharides (PS) on the surface of *S pneumoniae*, which are its primary factor of virulence, also are the basis for the serotyping classification of the bacterium among 40 serogroups comprising 90 serotypes [265], only 20 of which are responsible for 70% of invasive pneumococcal disease. The most common serogroups worldwide are 6, 14, 19 and 23 [266], but other serogroups such as 1, 5 or 8, contribute much to invasive pneumococcal disease in young children in developing countries. A matter of concern is the increasing antibiotic resistance of *S pneumoniae*, both in older individuals, where it accounts for an increasing proportion of pneumococcal infections and in children less than 2 years of age, where it has added to the urgency of developing more effective pneumococcal vaccines for this age group [5].

2.4.2. Disease burden

Although all age groups may be affected, the highest rate of pneumococcal disease occurs in young children and in the elderly population. In addition, persons suffering from a wide range of chronic conditions and immune deficiencies are at increased risk.

The only natural reservoir of *S pneumoniae* is the human nasopharynx, from which it can be transmitted through respiratory droplets to other individuals. Virtually every child in the world is colonized with one or more strains of *S pneumoniae* and becomes a carrier during his first years of life. In most cases carriage is asymptomatic; disease occurs in only a minority of persons, the bacteria spreading locally from the rhinopharynx into the sinuses and middle ear cavity or to the lungs, or causing systemic infections including bacteremia and meningitis. Infection of the blood stream and subsequent infection of secondary sites is referred to as invasive pneumococcal disease.

In some developing countries, as for example Southern India, 50% of infants have been colonized by *S pneumoniae* by 2 months of age and 80% are carriers by the age of 6 months [267]. A study in South Africa showed that the prevalence of carriage was 30%, 44%, 51% and 61% in children aged 6 weeks, 10 weeks, 14 weeks and 9 months, respectively [268]. In industrialized countries, carriage occurs on the average at about six months of age.

Pneumococcal disease is estimated to cause more than one third of the 2 million global annual child deaths following ARIs [4]. In industrialized countries, the highest levels of infection occur in children less than 2 years of age, being the highest in the second 6 months of life. Thus, prior to the introduction of the conjugate pneumococcal vaccine, the annual average incidence of invasive pneumococcal disease in the USA was 167 cases per 100,000 population in children less than 12 months of age, with a peak at 235 cases per 100,000 among children 6 to 11 months old, and 203 per 100,000 population among children 12 to 23 months old. Children from minority groups suffered disproportionately, with annual

incidence figures in children less than 2 years of age of 400, 642 and 2396 per 100,000 among the black, Alaskan Native and Native American populations, respectively [261]. Reported incidence figures are lower in Europe, ranging from 14 cases per 100,000 in Germany and The Netherlands to more than 90 per 100,000 in Spain. *S. pneumoniae* is recognized as the first cause of infant and young children mortality, the leading cause of meningitis and the first cause of bacteremia in less than 2 years old children in France [269], where incidence of pneumonia is estimated at 100,000 cases per year, causing 3500-11,000 deaths, essentially in the elderly, and that of otitis media at 200,000 cases per year [270].

The true burden of pneumococcal infections in children less than 5 years old is much less documented in developing countries, where disease surveillance systems and diagnostic facilities are lacking and children with invasive infections are diagnosed only if hospitalized. In several surveys done in sub-Saharan Africa, *S. pneumoniae* was found to account for about 25% to >30% of the cases of meningitis in less than 5 years old children, with a case fatality rate of more than 50% [271]. Conservative estimates have put the incidence of invasive pneumococcal disease in The Gambia at 500 per 100,000 in children in their first year of life and 250 per 100,000 in children less than 5 years of age [272]. A recent study in Kenya reported an annual incidence of presentation to the hospital with pneumococcal bacteremia of 597 per 100,000 children younger than 5 years of age [273]. Case fatality rates for invasive pneumococcal disease range from 5% to 20% for bacteremia and from 40% to >50% for meningitis. From 25% to 56% of children who survive meningitis suffer from long-term neurologic sequelae [274].

Less severe but more frequent forms of pneumococcal disease include middle-ear infection, sinusitis or recurrent bronchitis. Thus, in the USA alone, seven million cases of otitis media are attributed to pneumococci each year.

Among adults in industrialized countries, pneumococcal pneumonia still accounts for at least 30% of all cases of community-acquired pneumonia admitted to the hospital, with a case fatality rate of 11% to 44%. Annual incidence of community-acquired pneumonia in 2002 in the USA was 18.3 cases per 100,000 elderly persons, and the incidence of pneumococcal pneumonia among the elderly population was at least 5.5 cases per 100,000 population [275]. Again, substantially higher incidence rates were reported in blacks than in whites, and even higher rates in native Americans and Alaskan natives [276]. Similarly, a very high incidence rate was reported in the aboriginal Australian population [277]. *S. pneumoniae* also is an underappreciated cause of nosocomial pneumonia in hospital wards and intensive care units as well as in nursing homes and long-term care institutions. Important risk factors are age, chronic heart and lung disease, cigarette smoking, and asplenia [278].

The burden of invasive pneumococcal infections in adults in developing countries is poorly known, mostly due to failure to obtain blood cultures from patients with pneumonia. *S. pneumoniae* has been the leading nonmycobacterial cause of pneumonia among HIV-infected persons both in developed and developing countries [11] [279] [280]. The impact of HIV infection on pneumococcal disease can clearly be evidenced from the clinical study in HIV-infected young adults in Uganda, which reported an annual incidence of 1700 cases of invasive pneumococcal disease per 100,000 population [281], as well as from an earlier survey among HIV-positive commercial sex workers in Kenya, where annual incidence of pneumococcal disease was found to be 4250 per 100,000 people [282]. HIV-infected children seem to be 20-40 times more likely to contract pneumococcal disease than uninfected children.

Influenza also increases the risk of secondary pneumococcal infection. Based on evidence from past influenza pandemics, the attack rate for secondary pneumococcal pneumonia in a pandemic setting is anticipated to reach 13% [49].

2.4.3. Bacteriology

S. pneumoniae is a Gram-positive encapsulated diplococcus. The external capsular polysaccharide (PS) of the bacterium is the primary factor of virulence, other virulence factors being pneumolysin, which leads to pore formation and osmotic lysis of epithelial cells, autolysin, and pneumococcal surface protein A (PspA), which interferes with phagocytosis and immune function in the host.

Pneumococci are transmitted by direct contact with respiratory secretions from patients or healthy carriers. Although transient nasopharyngeal colonization rather than disease is the normal outcome of exposure to pneumococci, bacterial spread to the sinuses or the middle ear, or bacteraemia following penetration of the mucosal layer, may occur in persons susceptible to the involved serotype. Pneumococcal resistance to essential anti-microbials such as penicillins, cephalosporins and macrolides is a serious and rapidly increasing problem worldwide.

Capsular PS also are the basis for the serotyping classification of the bacterium among 91 serotypes. The distribution of disease-causing serotypes varies between geographic regions and by age and disease within regions. The most common serogroups worldwide are 6, 14, 19 and 23 but some serogroups, such as 1, 5 or 8, contribute much to invasive pneumococcal disease in young children in developing countries. Approximately 90% of the most frequent isolates belong to 23 serogroups or serotypes and have been included in the 23-valent pneumococcal vaccine.

2.4.4. Vaccines

Current *S pneumoniae* vaccines are based on the use of the bacterial capsular polysaccharides (PS), which induce type-specific antibodies that activate and fix complement and promote bacterial opsonization and phagocytosis [11] [261]. The two types of currently licensed vaccines [283] are the pneumococcal polysaccharide vaccine (PPV), based on purified capsular PS, and pneumococcal conjugate vaccines (PCV), obtained by chemical conjugation of the capsular PS to a protein carrier [284].

2.4.4.1. 23-valent polysaccharide vaccine (PPV23)

The PPV23 vaccine contains 25 µg of the purified capsular PS from each of the 23 different *S pneumoniae* serotypes that together account for 90% of cases of severe pneumococcal disease in industrialized countries. Two vaccines are currently manufactured, Pneumovax 23™ by Merck and Pneumo 23™ by SanofiPasteur. Relatively good antibody responses are elicited following a single IM injection in 60-80% of healthy adults and normal children over two years of age. Pneumococcal vaccination of children 2 to 5 years of age was 62% effective in preventing invasive pneumococcal disease due to vaccine serotypes [285].

In a case control study in Connecticut, the effectiveness of pneumococcal vaccination with PPV23 in immunocompetent adults was estimated to be 61% against pneumococcal bacteremia, but in immunocompromised patients this figure fell to only 21% [286]. In spite of such suboptimal immunogenicity, administration of a single dose of PPV23 continues to be recommended for solid-organ transplant recipients [287]. The PPV23 vaccine also is recommended for people over 65 years of age, particularly those living in institutions. Several studies have shown that PPV23 is effective in preventing invasive pneumococcal disease [288], but it remains unclear whether it has a significant protective effect against pneumonia [289] [290]. A recent meta-analysis concluded that there actually was little evidence of PS vaccine protection against pneumonia among elderly or adults with chronic illness [291].

Also, PPV23 is unable to elicit immune memory, so that a second dose of vaccine does not boost antibody levels. PPV23 does not provide protection against mucosal infection, and is thus unable to reduce nasopharyngeal carriage of pneumococci. Moreover, studies of PPV23 in adults and children have shown that a state of immune tolerance, or hyporesponsiveness, can develop to repeated PS vaccine exposures [292]. Last, but not least, PPV is poorly immunogenic in less than 2 years old children and is thus inadapted to infants and young children.

On another hand, a number of studies have confirmed the safety of PPV during pregnancy and documented the use of the vaccine for the vaccination of pregnant or breast-feeding mothers for preventing pneumococcal pneumonia in young infants [293] [294].

2.4.4.2. *Pneumococcal conjugate vaccines*

Pneumococcal conjugate vaccines (PCVs) are based on the covalent coupling of the capsular PS from diverse *S pneumoniae* serotypes to a variety of protein carriers. These vaccines elicit higher antibody levels in infants, young children, the elderly and immunodeficient persons than the PPV23 vaccine, as well as significant immune memory resulting in an anamnestic response on subsequent booster immunizations. Moreover, they suppress nasopharyngeal carriage of the pathogen, thus decreasing bacterial transmission in the community and generating herd immunity. Conjugate vaccines immunization followed by PS vaccine boosting might provide a foundation for lifelong protection against pneumococcal disease and/or maintain high levels opsonophagocytic antibody titers in elderly adults while broadening serotype coverage [295] [296].

The first PCV, Prevnar™ or Prevenar™ (Wyeth), was licensed in the USA in 2000 and recommended for routine use in children younger than 2 years of age, to whom it is administered in a 3 doses schedule, when possible in combination with usual routine vaccination, followed by a booster dose at 15-18 months. Alternatively, the vaccine can be administered in a two-dose immunization schedule at 3 and 5 months of age, followed by a booster immunization at 11-12 months of age [297] [298]. The vaccine contains poly- or oligo-saccharides from seven *S pneumoniae* serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), each conjugated to genetically detoxified diphtheria toxin CRM 197.

Four large clinical trials of the 7-valent PCV7 and of a closely related, unlicensed 9-valent PCV9 in the USA, South Africa, and The Gambia have reported vaccine efficacy of between 77% and 97% against severe invasive pneumococcal disease caused by vaccine serotypes and of 19% to 37% against radiologically confirmed pneumonia [299] [300] [301] [302]. Efficacy of the vaccine against otitis media was reported to be 57% against vaccine serotypes [303]. Introduction of PCV7 in the USA resulted in a dramatic decline in the rates of invasive pneumococcal disease among children <5 years of age, which dropped from 97 cases per 100,000 population during 1998-1999 to 24 cases per 100,000 in 2005; disease caused by vaccine-type strains fell from 80 cases per 100,000 population to 4.6 [304]. A significantly decreased incidence of pneumococcal otitis media and acute bacterial rhinosinusitis was also noted [305] [306]. In children not at high risk for invasive disease, the effectiveness of the full 4-dose schedule vaccine against vaccine serotypes was estimated to be 91%. Substantial protection against invasive pneumococcal disease and clinical pneumonia was also noted in HIV-infected infants [307].

A significant reduction in *S pneumococcus* disease incidence also was seen in unvaccinated individuals as a result of herd immunity [308] [309]. Thus, in adults 65 years old and older, invasive disease dropped by about one-third since introduction of the conjugate vaccine for children, and a drop of similar magnitude was seen in hospitalizations for pneumococcal bacteraemia [310]. Paradoxically, after 5 years of wide use of Prevnar for infant immunization in the USA, more cases seem to be prevented through the indirect effects of herd immunity than by vaccine-induced immunity in the vaccinees [311].

Additional Phase III clinical trials also have been performed using 9-valent and 11-valent PCVs that are not expected to reach the market. Trial of PCV9 in South Africa on 40,000 subjects showed 83% and 65% efficacy after 2.3 years of follow-up in HIV-infected and uninfected children, respectively. The figures still were 77.8% and 38.8% after 6.16 years of follow-up [312]. In The Gambia, PCV9 reduced radiologically-confirmed pneumonia by 37% and invasive disease caused by vaccine serotypes by 77% [51]. The Gambian trial of PCV9 also showed a 16% reduction in hospital admissions and deaths from any cause in children 3-29 months of age who received the vaccine compared with those who had not received the vaccine. PCV9 could successfully be administered to infants in a two-dose schedule at 2 and 4 months of age followed by boosting at 12 months of age [313].

In a recent cost-effectiveness analysis of PCV7, it was projected that, in the 72 GAVI-eligible countries, pneumococcal vaccination with a conjugate vaccine would prevent between 262,000 and 407,000 deaths in children aged 3-29 months, depending on the level of vaccine coverage, and not counting herd immunity effects [314]. At a price of \$5 per dose, the vaccines would be a highly cost-effective

purchase. In more affluent societies, where the cost of the vaccine is definitely higher, studies of cost-benefit ratio of large scale PCV vaccination always have been highly positive [315].

These results speak very favorably in favor of the currently available conjugate vaccine and lead to expect an even greater vaccine effectiveness in infants and young children with the eventual licensing and use of the 10-valent (GSK) and 13-valent (Wyeth) conjugate vaccines which are in Phase III clinical trials at this time and are expected to be licensed in the coming years. The currently licensed 7-valent vaccine, Prevnar™, does not contain some of the serotypes that cause severe disease in developing countries, notably serotypes 1 and 5 [316]. Serotype 1 is the most common among children over 2 years of age in many countries in Asia and Africa [317]. Serotypes 1 and 5 are predominant in Nepal, whereas serotypes 14 and 19 are predominant in Sri Lanka [318]. The new conjugate vaccines will provide more optimal serotype coverage in these countries. The protein carrier used by Wyeth is CRM197, a genetically detoxified mutant of diphtheria toxin, whereas that used by GSK is the *H. influenzae* protein D [319] [320].

The success of PCV7 has partly been offset by the observation that introduction of PCV can lead to replacement of the vaccine serotypes by other, nonvaccine *S pneumoniae* serotypes. Carriage of non-vaccine type strains increased among children receiving the Prevnar™ vaccine such that the overall prevalence of pneumococcal carriage was not different in vaccinated and unvaccinated children, new serotypes taking up the mucosal territory vacated by the pneumococcal serotypes included in the vaccine [268] [321] [322]. So far, strain replacement has only had relatively modest effects on disease, except in certain settings [323]. Replacement disease was nevertheless observed in the otitis media trial in Finland, where the vaccine group had 33% more episodes of otitis media caused by serotypes not included in the vaccine [303]. Of positive note was the significant reduction in the disparity in disease rates between black and white children following PCV introduction in the USA [324]. Serotype replacement makes it essential that there be continued monitoring and surveillance of pneumococcal colonization and invasive disease [325].

2.4.4.3. Protein vaccines

Newer vaccine approaches have been developed, based on the use of conserved external surface proteins such as PspA and PspC, which have a choline-binding function, pneumococcal surface adhesin A (PsaA), a metal-binding transporter, surface proteins PiaA and PiuA, or virulence factors such as pneumolysin or autolysin. Some of these antigens have been identified by a reverse vaccinology approach, including the pneumococcal pilus, a potent immunogen that elicits cross-protection against various *S pneumoniae* serotypes [326] [327]. These new vaccines would circumvent the complexity of manufacture of conjugate vaccines and be serotype-independent [328]. Several of these candidate vaccines induced protection against systemic challenge in animal models [329]. PspA and PsaA have been tested in Phase I trials and found to elicit protective anti-*S pneumoniae* antibodies [330] [331] [332].

Two other surface proteins, BVH 3 and BVH 11, have been identified that can elicit protective anti-pneumococcal antibodies in the mouse model, and a recombinant 100 kD fusion protein, BVH3/11V, was tested by ID BioMedical in a Phase I trial in toddlers and elderly volunteers. A 2-dose immunization regimen was able to induce a 50-fold increase in anti-*S. pneumoniae* antibody levels. Phase II clinical studies in infants and elderly persons have been initiated. The advantage of this approach is that the BVH proteins seem to be conserved among the 90 serotypes of *S pneumoniae* and therefore could constitute a universal pneumococcal vaccine [333].

2.5. Tuberculosis

2.5.1. Introduction

An estimated one third of the world population (two billion people) is exposed to the risk of tuberculosis (TB), whose main causative agent, *Mycobacterium tuberculosis* (Mtb), infects approximately 9 million

new individuals and causes 1.7 million deaths every year [334] [335]. This problem is compounded by the global emergence of multi drug-resistant (MDR) Mtb strains [336], the evidence that diabetes predisposes people to TB infection, and the increased susceptibility of HIV-infected individuals to TB, which all make the need for improved TB control even more urgent.

The currently available TB vaccine is bacillus Calmette-Guérin (BCG), a *M bovis* derivative which is routinely used in children because it is effective at protecting them against severe extrapulmonary forms of the disease, such as TB meningitis. BCG, which also elicits protection against leprosy [337], does not however appear able to provide protection against pulmonary TB in adults [338] [339]. BCG vaccination may provide protection only against primary infection but has a limited effect on reactivation TB in already infected individuals or on TB re-infection in adults. It also has been shown that the “take” of BCG may be impaired due to previous exposure to environmental mycobacteria [340] such as *M avium* [341], which are frequent in tropical countries. There is, therefore, an urgent need to develop better or improved TB vaccines, but it would be unethical and impractical to test new vaccine strategies that would not include BCG in early infancy [342] [343]. Therefore, most new TB vaccine development is being done in a BCG prime-vaccine boost strategy.

2.5.2. Disease burden

The global death toll of TB was 1.7 million deaths in 2006, 200 000 of which were from HIV-associated TB [344].

M tb currently infects about 2 billion people worldwide and causes an estimated 8.8 million new cases every year, especially in the sub-Saharan African continent, in Southeast Asia and in Eastern Europe [345]. In view of underreporting and lack of systematic surveillance, the real incidence of TB is suspected to actually be perhaps as high as 14 million new TB cases each year. About one half of the new cases occur in China, India, Pakistan, Bangladesh, Indonesia and The Philippines. In Africa, the single most important factor determining the increased incidence of TB in the last 10 years is HIV infection: in some regions, up to 75% of new TB cases are in HIV-infected people [346]. Paradoxically, TB seems to be severely aggravated in these dually infected patients when active antiretroviral therapy is initiated.

In addition, about 500 000 new cases of multiple-drug resistant TB (MDR-TB) are reported each year worldwide, with high incidence in parts of Russia, Latvia and Estonia (up to 10% of new TB cases) and Azerbaijan (22% of new cases). The newly discovered extensively drug-resistant Mtb strains (XDR-TB) emerged in 2005 in KwaZulu Natal (South Africa) among HIV-TB patients, probably as a consequence of lack of observance of therapy [347] [348] [349]. XDR-TB strains are now been found in >45 different countries, mostly in HIV-infected patients [350]. XDR-TB is resistant to almost all drugs used to treat TB, including isoniazid, rifampicin, fluoroquinolones and amikacin, kanamycin or capreomycin [351].

TB is highly contagious. Left untreated, each patient with active TB will infect on average between 10 and 15 people every year. TB spreads readily from person to person, due to the production of small particle droplets when a patient coughs and to the low dose of bacilli needed to initiate infection. Transmission is common in households, schools, hospitals, prisons, crowded work places, refugee camps and shelters. In fact, the incidence of TB in industrialized countries, which generally increased from the early 1980s to the early 1990s, has been decreasing ever since. As an example, in the USA, 13 767 cases were reported in 2006, a 3.2% decrease from 2005 and a 53% decrease from 1992 [352]. However, TB is a poverty-related disease: it has long been recognized that war, malnutrition, population displacement and crowded living and working conditions favor the spread of TB among humans, whereas periods of improvement in societal conditions and hygiene favor its rapid decline.

The global spread of the disease is also facilitated by migrations and movements of populations: thus, in industrialized countries, about one-half of TB cases occur in foreign-born or migrant persons. The incidence of TB in 2003 in France was for example 5.6 cases per 100,000 population in the native population, 31.7 per 100,000 in persons born in North African countries and 187.7 per 100,000 in persons born in TB-endemic sub-Saharan African countries [353]. But the major bottleneck for higher

success rates in controlling TB is the fact that currently only about 40% of all sputum-positive TB are detected. Thus, the majority of TB cases remains untreated or is treated only at a very late and highly infectious stage, causing enormous individual hardship as well as creating a public health time bomb. For all these reasons, added to the relative ineffectiveness of the current BCG vaccine, the development of improved TB vaccines has become a necessity for adequate control and elimination of the disease.

Bovine tuberculosis, caused by *M bovis*, is a zoonotic disease and was the cause of many human deaths in the 1930s and 1940s through consumption of contaminated milk and dairy or meat products. Compulsory eradication programs were introduced in many countries based on the slaughter of infected cattle detected by the intradermal tuberculin skin test. However, probably due to a wildlife reservoir, the incidence of TB in cattle has exponentially increased over the last two decades in certain countries, especially Great Britain, constituting a potential public health problem and calling for the development of more specific diagnostic reagents [354].

2.5.3. Bacteriology

Mycobacterium tuberculosis (M tb), the agent of human TB, was discovered in 1882 by Robert Koch and for a long time called after his name (the ‘Koch bacillus’). All members of the Mycobacterium genus share the property of acid-fastness (Ziehl-Neelsen staining), due to their mycolic acid-rich cell wall structure. They include *M. tuberculosis*, *M. africanum*, and *M. ulcerans*, which are primary human pathogens, *M. bovis*, the agent of TB in cattle and other animals, which also can cause disease in humans, and a great many nontuberculous or environmental species, some of which can be pathogenic in humans such as those belonging to the *M. avium-intracellulare* complex.

TB bacilli usually multiply first in the lung alveoli and alveolar ducts and in draining lymph nodes, where they are engulfed by dendritic cells (DCs). They also multiply in the macrophages that were attracted from the bloodstream and are armed to kill the bacteria upon uptake in their phagosomes. However, Mycobacteria can evade the phagosome-lysosome fusion pathway, multiply in the host macrophages and kill them, progressively creating a primary tubercle. The outcome of infection is controlled by CD4⁺ and CD8⁺ T cells, both of which are necessary for the maintenance of a latent state of infection [355] [356]. Delayed cutaneous hypersensitivity develops and together with other cellular immune reactions, leads to the caseous necrosis of the primary complex. Bacilli eventually spread to many parts of the body such as liver, spleen, meninges, bones, kidneys and lymph nodes, where they can either be a source of overtly disseminated TB or, more commonly, remain dormant. Occasional decline in cell-mediated immunity leads to reactivation TB, most frequently seen in adults as a pulmonary disease with infiltration or cavity in the apex of the lung. This is the most common and most infectious form of TB.

CD4⁺ T-cells play a major role in containment of TB infection; progressive TB is usually associated with a Th2 T-cell response, whereas a pure Th1 response, including production of IL-2 and IFN- γ , mediates protection [357]. CD8⁺ T cells and macrophages are involved in the control of mycobacterial infection [358] [359]. The production of Th1 associated cytokines such as IFN γ , TNF α and IL-12 appears to be an essential component of resistance to Mtb infection. The most effective vaccination strategies in animal models have been those that stimulate T cell responses, both CD4⁺ and CD8⁺, to produce these cytokines.

The tuberculin skin test has long been used as evidence of TB infection or as a sign of adequate response to BCG vaccination, although no clear relationship between delayed-type hypersensitivity and protective immunity could be established [346]. A number of antigens found in *M. tuberculosis*, including Ag85, MPT64, ESAT-6 and CFP10, have been identified which may play a major role in cellular immunity and the induction of a protective IFN- γ response.

2.5.4. Vaccines

2.5.4.1. BCG

By culturing a *M. bovis* isolate from a cow for a period of 13 years and a total of 231 passages, Calmette, a physician, and Guérin, a veterinarian, created an attenuated variant of *M. bovis*, Bacille Calmette–Guérin (BCG). BCG was first tested in infants as an oral vaccine in 1921. New methods of administration were later introduced, such as intradermal, multiple puncture, and scarification [360]. BCG vaccination was included in the WHO Expanded Programme on Immunization (EPI) as of 1974: since then, approximately 100 million children received a BCG vaccine each year, and, to date, well over 4 billion people have been vaccinated with BCG since the historical immunization in 1921.

However, WHO stopped recommending BCG vaccination at birth as of 2007, at least for infants at risk for HIV infection, as data showed that about 417 per 100 000 infants developed disseminated BCG disease due to antenatal infection with HIV [361]. Disseminated BCG disease typically presents with the same symptoms as severe TB and shows a CFR of 75%-86% [362] [363].

As shown by sequencing, the original BCG strain lost the RD1 region of the *M. bovis* genome in the course of the selection process. This region encodes major TB antigens including culture filtrate protein 10 (CFP-10) and early secretory antigen target 6 (ESAT-6) [364] [365] [366] [367]. Major BCG vaccine strains in use today differ even further from the original BCG strain and from each other, with “stronger” strains (Pasteur 1173 P2, Danish 1331) being more reactogenic and, presumably, more immunogenic, than “weaker” strains (Glaxo 1077, Tokyo 172) [346] [368].

No other widely used vaccine is as controversial as BCG. Its effects in large randomized, controlled, and case–control studies, have been widely disparate, from excellent protection against TB to no protection. Most studies have demonstrated that BCG vaccines afford a higher degree of protection against severe forms of TB, such as meningitis and disseminated TB, than against moderate forms of the disease. The efficacy of neonatal BCG vaccination also wanes with age [369]. Studies that evaluated meningitis or miliary TB demonstrated that BCG can provide good protection against these serious forms of TB in young children, with reported efficacy ranges from 46–100%. In contrast, efficacy against pulmonary TB, which is more prevalent in adolescents and adults, has ranged from 0–80%. Overall, BCG is only at best credited with a 50% efficacy [370] [371].

Efficacy of BCG vaccination also appears to vary with geographic latitude – the farther from the equator, the more efficacious the vaccine. Presumably, exposure to nonpathogenic mycobacteria, which is more intense in warm climates, induces a degree of protective immunity in exposed populations, masking potential protection from BCG.

Vaccination with BCG nevertheless remains the standard for TB prevention because of its efficacy in preventing life-threatening forms of TB in infants and young children, and also because it is the only vaccine available, is inexpensive, and requires only one encounter with the baby. The fact that BCG does not protect against pulmonary TB in adults has however prompted the search for new, improved TB vaccines. Dozens of TB vaccine candidates have been tested in recent years in animal models, including subunit protein and peptide vaccines, DNA vaccines, rationally attenuated *Mtb* strains, recombinant BCG, and live vectors expressing immunodominant *Mtb* antigens (for reviews, see [372] [373] [374] [375]).

2.5.4.2. Recombinant BCGs.

Among the many innovative new approaches that have been tested, one has been to reengineer BCG strains to endow them with the capacity of expressing immunodominant *Mtb* antigens. This approach has yielded a series of vaccine candidates, such as:

- A recombinant BCG vaccine (BCG30) that was engineered at University of California at Los Angeles (USA) to express the 30 kD major secretory protein Ag85B [376] [377]. The vaccine was tested in Phase I trial in the USA and elicited markedly enhanced central and effector memory Ag85B-specific CD4⁺ Th1 and CD8⁺ T cell immunity compared with the parental BCG Tice strain and induced a

significant number of Ag85B-specific T cells capable of inhibiting intracellular mycobacteria [378]. The development of this vaccine is currently on hold for regulatory concerns.

- A BCG::RD1 recombinant, in which the RD1 segment of the *M. tuberculosis* genome has been reintroduced, resulting in the expression of ESAT-6 and Ag85A proteins. This new BCG strain, developed at the Pasteur Institute, Paris, showed increased persistence and improved protection against challenge with virulent Mtb in animal models [379] but meets with safety concerns for its use in humans.

- Another recombinant BCG, VPM 1002 (previously known as rBCG:[delta] ureC-Hly), was engineered at the Max Planck Institute for Infection Biology in Berlin (Germany) to express listeriolysin O, which increases MHC class I presentation [380], and its urease gene was deleted in order to prevent neutralization of the acidic pH in phagosomes. This recombinant BCG was found to be devoid of pathogenicity for SCID mice and provided greatly improved protection against aerosol TB in the mouse model. This vaccine is currently phase I clinical trials.

- AERAS-422, developed by the Aeras Global TB Vaccine Foundation, which expresses Mtb antigens 85A, 85B, and Rv3407, together with perfringolysin, a pore-forming protein similar to listeriolysin, is currently in late preclinical development and planned to enter into Phase I trials by the end of 2010 [381].

The VPM 1002 and AERAS-422 vaccines have been found to be more efficacious than classical BCG against Mtb challenge in animal models, including against challenge with the Beijing strain of Mtb which is relatively insusceptible to BCG [382].

2.5.4.3. Live attenuated Mtb mutants

Another approach has relied on the engineering of live attenuated mycobacterial strains, either auxotrophic mutants of Mtb [383] [384], or regulatory mutants such as the PhoP knock-out mutant [385]. PhoP is a gene which controls the expression of several virulence genes of Mtb. The PhoP/PhoR Mtb mutant has shown very good protection in mice and was one of the rare new TB candidate vaccines to show significant improvement over BCG in the guinea pig aerosol model [386]. Concern that auxotrophic mutants could revert to full virulence *in vivo* has led to the engineering of mutants bearing two independent unlinked deletions [387].

2.5.4.4. Subunit and DNA vaccines

Due to safety concerns, in particular in immunocompromised persons, as well as to technical challenges regarding manufacture and reproducibility, live mycobacteria vaccines are not the product of choice of most vaccine manufacturers. Many new TB vaccines approaches are therefore focused on recombinant subunit vaccines, DNA vaccines [388], or live-vector recombinant vaccines that express a variety of Mtb antigens.

An analysis of the Mtb proteome led to the identification of about 45 potential vaccine antigens. Among those, purified mycobacterial antigens such as Ag85A, Ag85B, HSP65, the R8307 protein, a 36kD proline-rich mycobacterial antigen, or the 19kD and 45kD proteins have been found to induce protection levels in mice similar to that obtained with BCG when presented in combination with Th1-inducing adjuvants [389] [390]. Subunit vaccines based on the same proteins in adjuvants used in combination with BCG have resulted in better protection against experimental challenge of cattle with *M bovis* than BCG vaccine on its own [391].

Most promising among these were Ag85 (A and B), and the Mtb9.8, Mtb9.9, Mtb11, Mtb32, Mtb39, Mtb41 and ESAT-6 proteins [392] [393] [394] [395], which were prioritized for vaccine development based on their ability to stimulate PBMC responses from more than 50% of healthy PPD+ donors who presumably have contained their infection due to protective CD4⁺ T cell responses [342] [396].

Some of these proteins have been fused together, such as Mtb32 and Mtb39, yielding M72, which was found to be highly immunogenic and protective in monkeys when formulated in the AS02A adjuvant from GSK, or antigens 85B and ESAT-6, whose fusion product also induced strong protective immune responses in monkeys [397] [398]. ESAT-6 could advantageously be replaced by TB10.4 [399]. M72 is being developed by Corixa Corp and GSK and was found to be safe and immunogenic in Phase I clinical trial [400].

Antigen 85A, a mycolyl-transferase that is involved in cell wall biosynthesis, is also considered a leading candidate for inclusion in a TB vaccine. In addition, a multi-epitope polypeptide, as well as nonproteinic antigens such as mycolic acids and carbohydrate moieties, are being developed as candidate antigens.

2.5.4.5. *Live recombinant TB vaccines*

Modified vaccinia virus Ankara (MVA) was engineered to express Mtb Ag85A (MVA85A) and tested successfully in a BCG prime-recombinant MVA boost strategy in the mouse challenge model, the guinea pig aerosol challenge model and the cynomolgous macaque challenge model [386], before undergoing Phase I clinical studies first in the UK, then in a high TB endemicity setting in The Gambia [343] [401]. The MVA recombinant was found to boost BCG-primed and naturally acquired anti-mycobacterial immunity. Interestingly, the MVA85A vaccine induced up to 30-fold greater cellular immune responses in BCG-primed than in BCG-naive individuals, even if BCG had been administered as long as 38 years before the MVA boost. Another Phase I clinical trial recently took place in South Africa [402]. The BCG/MVA85A prime-boost strategy was also tested in cattle, resulting in significantly wider Ag85A-specific T cell responses and higher frequencies of Ag85-specific IFN- γ secreting cells [403]. Currently, the prime-boost strategy is being tested in a Phase IIa trial in South Africa, and a Phase IIb trial is planned to begin in early 2009 on 4 months-old infants who had a BCG vaccination at birth. The MVA85A vaccine is currently the most advanced candidate TB vaccine [404].

Live, nonreplicative adenoviruses expressing Mtb antigens have been developed by the Aeras Global TB Vaccine Foundation (USA) and Crucell (Netherlands). Advanced TB vaccine candidates in this category include AERAS 407, a replication-defective adenovirus 35 construct that expresses Mtb Ag85A, Ag85B and TB10.4, and went through Phase I trial in South Africa and South America; and AERAS X05, a *Shigella*-delivered recombinant double-stranded RNA encoding Ag85A, Ag85B and Rv3407, which is expected to enter clinical trials shortly. The immunogenicity of a BCG-AdAg85A prime-boost regimen in cattle was found to be significantly greater than that of either vaccine separately [405].

It is clear that, from now on, human clinical studies will act as the principal driving force for the development of new TB vaccines. Testing of such a wide variety of vaccine types using different immunization strategies directed against a sole pathogen is unique in the history of vaccine development. It will make the valid comparison of clinical data most challenging [406]. Therefore, it will be all the more important that this effort be tightly coordinated to provide maximal comparability and transparency. WHO is working with all stake holders in the field to standardize key parameters such as trial entry criteria, endpoints, immunoassays, etc. The main players in this area include the US National Institute for Allergy and Infectious Diseases (NIAID), the Aeras Global TB Vaccine Foundation, supported by the Bill and Melinda Gates Foundation, the Wellcome Trust, a network of European researchers supported by the European Commission, the European and Developing Countries Clinical Trial Partnership, and pharmaceutical manufacturers including GlaxoSmithKline (GSK), IDRI-Corixa, Crucell, SanofiPasteur and Emergent Biosolutions. Discussions are ongoing between these major players to develop a plan for the design and funding of Phase III efficacy trials in appropriate field trial sites in Africa and Asia [375].

2.6. [The A/2009 H1N1 influenza virus pandemic](#)

2.6.1. *Introduction*

Since the global H1N1 influenza virus pandemic of 1918, influenza virus gene reassortment has been well documented and observed to occur frequently between human virus subtypes, between human and avian and among avian influenza viruses. Such reassortments led to the global pandemics of 1957 (H2N2) and 1968 (H3N2) [407] [408]. Although A/H1N1 viruses continued to circulate among humans, seasonal epidemics of influenza A virus from 1968 to 2009 were dominated by A/H3N2 virus variants generated by antigenic drift [409] [410], until, In early April 2009, a new influenza A (H1N1) virus brutally emerged among humans in California and in Mexico, quickly spreading worldwide through human-to-human transmission, and generating the first influenza pandemic of the twenty-first century [411] [412]. The virus was found to be antigenically unrelated to human seasonal influenza viruses but genetically related to viruses known to circulate in pigs. In view of its likely swine origin, it is often referred to as 'swine-origin influenza virus' (S-OIV) A/H1N1, or 2009 A (H1N1) influenza virus.

Swine H1N1 influenza viruses had been circulating in pig populations for at least 80 years but too often lacked surveillance and molecular characterization. In 1998, a new triple-reassortant H3N2 virus emerged in the North American pig population, comprising genes from classical swine H1N1, North American avian and human H3N2 influenza viruses. Co-circulation and mixing of this North American triple-reassortant with viruses of swine lineage generated further H1N1 and H1N2 reassortant swine viruses. In Europe, an avian H1N1 virus ('avian-like' swine H1N1) was first isolated from pigs in Belgium in 1979 [413], and gradually replaced classical swine H1N1 viruses [414]. The 'avian-like' virus lineage spread all over Europe and Asia while also reassorting with other influenza virus strains. In Asian pig populations, the classical swine H1N1 virus lineage still circulated together with the 'avian-like' swine H1N1, H1N2 reassortants and the North American H3N2 triple-reassortant [415]. Multiple lineages of influenza A viruses have been found to co-circulate during any single season and to undergo frequent reassortment which may in turn have a major impact on antigenic evolution [416].

Molecular studies of the new A/H1N1 pandemic virus genome showed that it was derived from several viruses which had been circulating in pigs for a long time, namely the North American H3N2 triple-reassortant, the classical swine H1N1 lineage, and the Eurasian 'avian-like' swine H1N1 virus (see details under Virology). Initial transmission to humans is believed to have taken place at least several months before recognition of the first outbreak and phylogenetic data even suggest that the reassortment of swine lineages may have occurred years before emergence in humans [417] [418] [419] [420]. Surprisingly however, there has been no evidence so far that swine are playing any role in the epidemiology or in the worldwide spread of the virus in human populations [421].

On June 11, 2009, the World Health Organization raised the pandemic alert to level 6, in view of the number of regions which officially reported A/H1N1 influenza cases in their communities. In view of the rapid spread of the H1N1 virus, its propensity to primarily affect children and young adults, as well as those with an underlying lung or cardiac disease condition [422], and the risk of a possible increase in pathogenicity through further reassortment with avian or human virus strains, the development of a specific vaccine was promptly engaged in collaboration between the World Health Organization, Health Ministers and National Health Agencies and the vaccine industry.

2.6.2. Epidemiology and Disease Burden

2.6.2.1. Epidemiology

The emergence of the pandemic H1N1 influenza virus in humans in early April 2009 in Mexico and California came as a total surprise. The virus first emerged in a little village in Vera Cruz, Mexico, but went unnoticed as no case of illness required hospitalization. The first two cases in California occurred in a 10 year-old boy and a 9 year-old girl who both necessitated hospitalization. The H1N1 strain then quickly spread worldwide through human-to-human transmission. The number of countries, overseas territories or communities that reported laboratory-confirmed A/H1N1 cases in humans was more than 207 on November 22nd, 2009. Most countries in the southern hemisphere reported more pandemic H1N1 in 2009 than any of the seasonal subtypes. In the temperate areas of the northern hemisphere, the spread of the pandemic was more gradual, initially spreading widely in the USA, Spain, Great Britain, Japan and Germany before invading other countries as well in the Fall. In the tropics, rates appeared to be quickly increasing in countries in both Central and South America and Asia, especially in Thailand, but very few data exist regarding the African continent. It is not possible at this time to guess what the future look like. The most pessimistic estimates call for 1 billion to 3 billion people (15% to 45% of the world's population) getting infected.

On the basis of recorded clusters in the USA, the household secondary attack rate was estimated to be 27.3%. In school outbreaks, a typical schoolchild infected on average 2.4 (range 1.8-3.2) other children within the school. The basic reproductive number, R_0 , thus ranged from 1.3 to 1.7 [423]. This is consistent with further pandemic spread causing illness in 25% to 39% of the world's population over a 1-year period, similar to the spread of the 1957-1958 Asian influenza A (H3N2) pandemic.

The actual number of influenza A (H1N1) cases worldwide remains unknown, as most cases are diagnosed clinically and are not laboratory-confirmed

[424] but it most probably is in the order of several millions cases. In most countries, the capacity for laboratory diagnosis has been so severely stressed that virological surveillance had to be restricted to patients attending hospitals [425]. The number of influenza-like illnesses during the 2009 spring outbreak in New York City has been estimated at 750 000- 1 million cases.

2.6.2.2. Age distribution

A characteristic feature of the H1N1 pandemic is that it disproportionately affected so far children and young adults [426]. One of the early American studies showed that, although the age of H1N1 patients in the study ranged from 3 months to 81 years, 60% of patients were 18 years of age or younger [427]. In most countries, the majority of H1N1 cases have been occurring in young people, with the median age estimated to be 12 to 17 years in Canada, the USA, Chile, Japan and the UK. Of the 272 patients with 2009 H1N1 influenza who were hospitalized in the USA from April to mid-June 2009, 45% were under the age of 18 years, whereas 5% only were 65 years of age or older [428]. This age distribution speaks in favor of at least partial immunity to the virus in the older population.

Of note, however, is the fact that if the highest rate of severe disease leading to hospitalization has been in the less than 5 years of age, the highest case fatality rate was recorded in the 50-60 years-old population. Subsequent studies have shown that 33% of humans over 60 years of age had cross-reacting

antibodies to S-OIV A(H1N1) by hemagglutination inhibition test and neutralization tests, but antibody titers to A/H1N1 did not substantially increase after vaccination with a seasonal vaccine, even when formulated with water-in-oil adjuvants [417] [429]. In another study, no neutralizing antibodies against the pandemic A/H1N1 (2009) virus could be found in sera from people born after 1920 [430]. However, a strong conservation of more than 50% of T cell epitopes (whether T-helper or CTL epitopes) was described between the 2009 A(H1N1) S-OIV and the seasonal influenza virus strains used to prepare the 2007 and 2008 influenza vaccines, which would provide a definite level of cross-reactive cellular immunity to the pandemic virus in the vaccinated human population [431]. In addition, the possible role of the NA antigen in cross-protective immunity, which remains poorly explored, should not be forgotten [432].

2.6.2.3. Clinical presentation and severity of the disease

H1N1 is most of the times a rather mild, self-limiting upper respiratory tract illness with (or at times without) fever, cough and sore throat, body aches, fatigue, chills, rhinorrhea, conjunctivitis, headache and shortness of breath. Up to 50% of patients present with gastrointestinal symptoms including diarrhea and vomiting. The spectrum of clinical presentation varies from asymptomatic cases to primary viral pneumonia resulting in respiratory failure, acute respiratory distress, multi-organ failure and death [433]. The H1N1 virus can bind alpha2-3 sialic acid receptors found on the surface of cells located deep in the lungs that seasonal influenza virus cannot bind, suggesting why people with the pandemic flu can experience more severe pulmonary symptoms [434].

Thus, 2% to 5% of confirmed cases in the USA and Canada and 6% of cases in Mexico had to be hospitalized, a fifth of them requiring management in intensive care unit (ICU). The rate of hospitalization could actually be as high as 10% in some cities. Most, but not all of the hospitalized patients had underlying conditions such as cardiovascular disease, respiratory disease including asthma, auto-immune disorders, obesity, diabetes or cancer [428].

Pregnant women, especially in their second and third trimester, also are at a high risk for severe disease [422] [435]. Thus, more than one-third of the pregnant women with confirmed H1N1 infection had to be hospitalized during the current pandemic in the USA due to acute respiratory distress syndrome [436]. Similar findings were reported from Australia and New Zealand, where the number of ICU admissions due to influenza A in 2009 was 15 times the number due to viral pneumonia in recent years: infants from 0 to 1 year of age and adults 25 to 64 years of age were at particular risk, as well as pregnant women, adults with a body mass index greater than 35, and indigenous Australian and New Zealand populations. In-hospital mortality was 16% [437].

The overall H1N1 case fatality rate in Mexico was estimated to be around 0.4% [438]. The average case fatality rate that can be deduced from the laboratory-confirmed cases officially reported to WHO as of 06 August 2009 was much lower (0.08%). Current estimates put the average case fatality rate at 0.15% to 0.25%. H1N1 deaths mostly occur in middle-aged adults (median age around 40-50 years), contrary to seasonal Influenza where fatal disease occurs most often in the elderly (>65 years old). Most of the deaths occurred in patients with an underlying medical condition, but close to one third of the

hospitalized patients who died had no known underlying medical condition. In South Africa, a majority of fatal cases occurred in HIV-infected people, including pregnant women. In a recent study of the first 16 weeks of the pandemic in California, which saw 1088 cases of hospitalization or death, the median age of hospitalized patients was 27 years of age, but the case fatality rate (11%) was definitely the highest in persons 50 years of age and older [439].

From data on medically attended and hospitalized H1N1 patients in Milwaukee and information from New-York City hospitals on numbers of hospitalizations, use of intensive care units (ICU) and deaths, it was estimated that about 1 in 2000 (between 1 in 4000 and 1 in 1000) people in the USA who presented with symptoms of pandemic H1N1 influenza infection died; about 1 in 400 symptomatic cases required treatment in ICU; and 1 in 70 required hospital admission [440]. Among the medically attended cases in Milwaukee, 60% were in the 5-17 years age group, but severity of the cases was by far higher in the 18-50 years age group. Quite higher figures have been reported elsewhere for the H1N1 case fatality rate [441] [442], most likely reflecting a large uncertainty on the true numbers.

The official number of deaths from H1N1 infection worldwide that had been reported to WHO on November 22, 2009 was 7820.

During the pandemic outbreak in Mexico, an estimated 150 000 cases of influenza-like illness with 3312 hospitalizations occurred in metropolitan Mexico City. The economic impact of the pandemic was estimated as >\$3.2 billion (0.3% of gross national product) [443].

2.6.2.4. Transmission

The modes of transmission of the pandemic A (H1N1) 2009 virus appear to be similar to those of seasonal influenza viruses and involve primarily close unprotected contact with large respiratory droplets. The contribution of close range exposure to small-particle droplets expelled when an infected person coughs is unknown but could be more prominent under special conditions such as aerosol-generating procedures. The virus is also likely transmitted through contacts with fomites that are contaminated with respiratory or possibly gastrointestinal fluids [444]. Many S-OIV-infected patients experienced diarrhea, and viral RNA can readily be detected in the feces of these patients, making the potential for fecal-oral transmission a risk to take seriously into account [427].

The incubation period for S-OIV infection appears to range from 2 to 7 days, but most patients probably shed virus from day 1 before the onset of symptoms through 5 to 7 days after. Studies of transmission in animal models show that the H1N1 virus transmits just as efficiently as seasonal flu [445], contrary to earlier findings at the start of the pandemic [446].

2.6.3. Virology

The novel S-OIV A/H1N1 2009 can be grown in canine kidney (MDCK) cell cultures or primary human airway epithelial cell cultures, or in embryonated chicken eggs. Scanning electron microscopy revealed virions of remarkably filamentous shape [430].

Sequence analyses showed the absence of markers associated with high pathogenicity in avian or mammalian species, such as a multibasic hemagglutinin cleavage site [447] or a lysine residue at position 627 in the PB2 protein [448]. The occurrence of a mutation at position 222 in the HA gene segment of H1N1 isolates from post mortem specimens has been recently reported in various countries including Norway, the USA, China, Japan, Brazil and France. Although suspected to be associated with increased pathogenicity, this mutation did not change the antigenicity of the virus nor its susceptibility to anti-viral drugs, nor did it appear to provide the virus with increased transmissibility.

2.6.3.1. Molecular and antigenic characterization

Phylogenetic analyses of A (H1N1) virus isolates reveal a great homogeneity of genomic sequences. The virus is antigenically distant from human seasonal influenza viruses but genetically related to three viruses that circulate in pigs [418] [449], with the HA (H1), NP and NS gene segments coming from the classical swine H1N1 lineage. The H1 sequence could actually be traced back to the 1918 H1N1 pandemic virus (the "Spanish flu"), which has remained endemic in swine and continued to circulate among pigs in Asia, the America's and until the 1980s also in Europe [422] [450] [451].

The NA (N1) and M genes of the 2009 H1N1 pandemic virus come from the 'avian-like' Eurasian swine H1N1 lineage, which emerged in Europe in 1979 after reassortment between a classical swine and an avian H1N1 virus, then spread through Europe and Asia [452], displacing the classical swine H1N1 virus from Europe and generating new reassortants in swine with different human origin influenza A viruses [453].

Finally, the PA, PB1 and PB2 genes of the 2009 pandemic H1N1 virus are from the North American H3N2 'triple-reassortant' lineage, which was first isolated from American pigs in 1998 in which it showed unusual pathogenicity [454] [455] [456]. The name 'triple-reassortant' relates to the fact that the virus has genes of human influenza virus origin, of classical swine influenza virus origin and of North American avian virus origin.

The 2009 S-OIV H1N1 therefore has inherited virus gene segments of all three swine, human and avian origin: its HA, NP and NS gene segments have been inherited from swine classical virus, its NA and M segment from the avian-like Eurasian reassortant lineage, its PB1 segment from human H3N2 virus, and its PA and PB2 segments from North American avian lineage. Indeed, all gene segments of the pandemic A (H1N1) S-OIV were already established in the North-American 'triple-reassortant' (H3N2) swine virus and in the 'avian-like' Eurasian (H1N1) swine virus but no data are available to help evaluate when, where, nor between which parent viruses did the initial reassortment actually occur [417] [419] [420].

Antigenically, all S-OIV isolates look similar to classical swine viruses and to reassortant H1N1 viruses that have been circulating among pigs in the USA over the last decade, showing no antigenic cross-

reactivity with contemporary human seasonal H1N1 viruses. Surprisingly, there is no evidence that pigs play any role in the epidemiology or in the worldwide spread of the pandemic A (H1N1) virus in the human population [421].

2.6.3.2. Pathogenicity in animals

Experimental pathogenicity of the 2009 A/H1N1 S-OIV was tested in mice, ferrets and nonhuman primates [430]. S-OIV replicated more efficiently in the lungs of infected mice, generating earlier bronchitis and alveolitis, and eliciting more markedly increased production of interleukin-10 (IL-10), interferon gamma (IFN- γ), IL-4 and IL-5 than infection with a recent human H1N1 virus (A/Kawasaki/UTK-4). Similarly, it induced in nonhuman primates more elevated fever, more severe lung lesions with oedematous exudate and inflammatory infiltrates and higher antigenic loads in pneumocytes, similar to what was reported for highly pathogenic avian H5N1 influenza viruses [457]. This may have to do with affinity of the virus for alpha2-3 sialic acid receptors in the lower respiratory tract [434]. The pandemic virus also was more pathogenic in ferrets, replicating to higher titers in trachea and lungs than human seasonal H1N1 virus and caused more severe bronchopneumonia with prominent viral antigen expression in the peribronchial glands and alveolar cells. In contrast, it was devoid of overt pathogenicity for pathogen-free miniature pigs, although the virus did replicate efficiently in the respiratory tract of the animals.

The 2009 A(H1N1) S-OIV was also found to be more pathogenic than a seasonal 2007 A (H1N1) virus in ferrets and mice, with extensive virus replication occurring in the trachea, bronchi and bronchioles of the animals, while replication of the seasonal virus was limited to the upper respiratory tract [458]. The 2009 A(H1N1) influenza virus also replicated in the intestinal tract of inoculated ferrets, consistent with gastrointestinal involvement in many human A(H1N1) cases [446]. Transmission of the virus via aerosol or respiratory droplets was also tested in ferrets, and found to be either as efficient as [458] or less efficient than [446] highly transmissible seasonal A(H1N1) virus. The latter observation is in agreement with the observation that the virus may not be that easily transmissible among humans as only 10% of patients' household contacts become infected [459].

2.6.3.3. Sensitivity to antiviral drugs

Genetic and phenotypic analyses indicate that S-OIV is susceptible to neuraminidase inhibitors oseltamivir and zanamivir, but resistant to the adamantanes [460]. Treatment with oseltamivir is efficacious if initiated within the first 36 hrs after infection. The FDA issued an emergency use authorization approving the use of oseltamivir to treat influenza illness in infants under the age of 1 year and for chemoprophylaxis in infants older than 3 months of age.

Close to one hundred A(H1N1) S-OIV isolates have been described that were resistant to oseltamivir. These cases have been sporadic and there was no evidence of further transmission of the resistance marker into the virus population.

2.6.4. Vaccines

Vaccines are considered to be one of the most effective tools, not only to prevent the spread of the influenza virus but also to mitigate the severity of illness and the impact of the disease [461]. Today, the implementation of a pandemic A(H1N1) influenza vaccine in the fastest time is a global priority. This stems both from the rapid spread of the pandemic worldwide, from the fear that the A(H1N1) virus might accidentally gain added virulence through mutations and/or reassortment with other human or avian influenza virus, and from the total lack of cross-immune reactivity observed between the pandemic and seasonal influenza virus strains, making the 2009 seasonal vaccine useless in the fight against the A(H1N1) pandemic.

The development of a pandemic influenza vaccine however raises complex challenges, such as ensuring that sufficient seasonal influenza vaccine will still be available in due time, estimating with accuracy short- and medium-term production capacity of the different producers, reserving part of the foreseen production capacity for under-resourced countries with no or little access to the vaccine, etc [462] [463].

As of June 2009, the total global annual capacity for trivalent seasonal influenza vaccine production stood at 876 million doses, with seven manufacturers responsible for 560 million doses (i.e. 64% of the capacity). In spite of the WHO global pandemic influenza action plan to increase the potential supply of pandemic influenza vaccine [464], the supply of enough pandemic vaccine to immunize the world's population -should this be needed- would therefore take at least four years! Added to which, it was not clear at that early time whether one or two doses of pandemic vaccine would be required to induce full protection, nor whether the use of water-in-oil adjuvants would have the same antigen dose-sparing effect as in the case of the H5N1 vaccines [465] [566]. Finally, the yields of virus production in eggs or cell cultures, which is an important determinant of the amount of vaccine doses that can be manufactured, were not quite up to expectation.

A total of 26 vaccine manufacturers from America, Europe, Russia, Australia and Asia have now developed or are presently developing pandemic A(H1N1) vaccines, whether inactivated whole-virus vaccines, split inactivated vaccines, subunit vaccines, live-attenuated vaccines or other formulations. Of note is the participation of new vaccine manufacturers in China, India, Thailand and South America. All H1N1 vaccines were tested in clinical trials for safety and immunogenicity. Clinical trials still are in progress in certain at-risk subpopulations.

Preliminary reports indicated that a single 15- μ g dose of an inactivated split influenza A (H1N1) 2009 vaccine induced a hemagglutination-inhibition assay titer of 1:40 or more in 96.7% of 18-64 years-old subjects [467]. The robust immune response observed in the 18-49 years-old volunteers cohort was unanticipated, suggesting that there is more similarity between the influenza A (H1N1) 2009 virus and recent seasonal virus strains than had been recognized so far [468]. The NIAID Office of

Communications also reported that among healthy volunteers who received a single 15- μ g dose of either the Sanofi-Pasteur or the CSL Limited inactivated split A (H1N1) 2009 vaccine, a robust immune response was measured in 96% and 80%, respectively, of adults aged 18 to 64, and in 56% and 60%, respectively, of adults aged 65 and older [469].

In a recent Phase II trial on 410 children and 724 adults who received a single - dose (15 μ g HA) of inactivated A(H1N1) vaccine in the USA, protective serological titers of >1:40 were detected at 21 days after vaccination in 45%-50% of 6-35 month-old infants, 69%-75% of 3-9 year-old children, 95-100% of 18-64 year-old adults, and 93%-95% of elderly adults [470]. No vaccine-related severe adverse event occurred, but about 50% of every age and vaccine group reported injection-site and systemic reactions. Similarly, a multi-center, double-blind, randomized trial on 12691 3 years of age or older persons receiving a single-dose (7.5 μ g HA) of a split virion A/H1N1 vaccine in China showed that protective serological titers were detected on day 21 in 76.7% of 3-12 year-old children, 96.8% of 12-18 year-old adolescents, 89.5% of 18-60 year-old adults, and 80.3% of adults older than 60 years. In children, the administration of a second dose of the 7.5 μ g formulation increased the seroprotection rate to 97.7% [471].

The fact that it is possible to induce in adults protective antibody levels against A (H1N1) infection within two weeks of administration of a single dose of vaccine has now been confirmed with every pandemic H1N1 vaccine tested [472], whether split inactivated vaccines containing 15 μ g HA (SanofiPasteur, CSL, Sinovac and others), split inactivated vaccines with a water-in-oil adjuvant containing either 7.5 μ g HA and MF59 (Novartis) [473] or 3.8 μ g HA and AS03 (GSK), or whole-virion vaccines containing 10 μ g HA (Baxter) or 6 μ g HA (Omnivest, Hungary) [474]. The same one-dose schedule applies to intranasal live attenuated influenza virus (LAIV) vaccines (MedImmune in the USA and Microgen in Russia). National authorities have recommended that young children should receive a two-dose schedule, as is the case for seasonal vaccines, but immunogenicity data from clinical trials indicate that with many vaccines a single dose induces appropriate levels of immune responses.

Vaccination against pandemic H1N1 influenza was first implemented in China [475], followed by a large number of countries. The problem still remains, however, of vaccinating the populations living in under-resourced countries, which cannot afford to buy the vaccine, and which depend on donations from governments of industrialized countries and from the pharmaceutical industry. The WHO is efficiently coordinating this effort.

Among the high priority groups for vaccination [476] are health care workers and pregnant women, whose vaccination is a highly cost-effective strategy with substantial benefits to both the infants and the expectant mothers [477] [478]. Other priority groups are individuals with an underlying cardiovascular or respiratory medical condition including asthma, auto-immune disorders and diabetes, as well as young children.

The safety of the pandemic H1N1 vaccines has been thoroughly monitored during the various clinical trials. Current data show that the vaccines are well tolerated and behave as the corresponding seasonal vaccines in terms of safety and lack of severe adverse events. A small number of Guillain Barré

syndromes have been reported after H1N1 vaccine administration in large-scale vaccination campaigns, but they all reverted quickly. Although oil-in-water adjuvanted vaccines have been approved for use in all populations by the European Association EMEA, including pregnant women, their use in the USA raises regulatory problems, as no adjuvanted flu vaccine has ever been licensed in the country and as no fast-track system is in place for their registration [479].

The emergence of the 2009 H1N1 pandemic and its global impact on Public Health have revived the dream of a 'universal' influenza vaccine that could provide solid, broad subtypic protection against influenza viruses and skip the need for yearly seasonal vaccinations. The recent finding that the human immune system can recognize a conserved neutralization epitope on the HA molecule that is shared across several influenza virus subtypes [480] [481], combined with the fact that the well-conserved NP viral nucleoprotein could generate cross-protective cellular immunity [482] are strong arguments in favor of the possibility of developing such a vaccine [483]. It also has been shown that the conserved external region of the ion channel M2 viral protein (M2e) can elicit cross-protection through antibody-dependent cellular cytotoxicity (ADCC) [484] [485] [486] [487]. However, pigs which were vaccinated with a human influenza M2e vaccine [487] showed more severe clinical signs than non-vaccinated control animals when challenged with a swine H1N1 influenza virus. Three out of six vaccinated pigs died after challenge, suggesting that antibodies to human M2e, especially in combination with cell-mediated immune responses, exacerbated swine influenza disease [488]. These results cast doubt on the feasibility of using safely M2e as an immunogen in humans.