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Oral vaccination against plague using *Yersinia pseudotuberculosis*

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Short title: Live oral vaccination against pneumonic and bubonic plague

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Abstract

Yersinia pestis, the agent of plague, is among the deadliest bacterial pathogens affecting humans, and is a potential biological weapon. Because antibiotic resistant strains of *Y. pestis* have been observed or could be engineered for evil use, vaccination against plague might become the only means to reduce mortality. Although plague is re-emerging in many countries, a vaccine with worldwide license is currently lacking. The vaccine strategy described here is based on an oral vaccination with an attenuated strain of *Yersinia pseudotuberculosis*. Indeed, this species is genetically almost identical to *Y. pestis*, but has a much lower pathogenicity and a higher genomic stability. Gradual modifications of the wild-type *Y. pseudotuberculosis* strain IP32953 were performed to generate a safe and immunogenic vaccine. Genes coding for three essential virulence factors were deleted from this strain. To increase cross-species immunogenicity, an F1-encapsulated *Y. pseudotuberculosis* strain was then generated. For this, the *Y. pestis* *caf* operon, which encodes F1, was inserted first on a plasmid, and subsequently into the chromosome. The successive steps achieved to reach maximal vaccine potential are described, and how each step affected bacterial virulence and the development of a protective immune response is discussed. The final version of the vaccine, named VTnF1, provides a highly efficient and long-lasting protection against both bubonic and pneumonic plague after a single oral vaccine dose. Since a *Y. pestis* strain deprived of F1 exist or could be engineered, we also analyzed the protection conferred by the vaccine against such strain and found that it also confers full protection against the two forms of plague. Thus, the properties of VTnF1 makes it one of the most efficient candidate vaccine for mass vaccination in tropical endemic areas as well as for populations exposed to bioterrorism.

Highlights

- VTnF1 is an irreversibly attenuated *Yersinia pseudotuberculosis* vaccine strain
- VTnF1 produces the *Yersinia pestis* F1 surface pseudocapsule
- A single oral dose induces a strong and long lasting protective immunity
- VTnF1 protects efficiently against both bubonic & pneumonic plague
- Vaccination also protects against an F1-mutant of *Y. pestis*

1. Introduction

Plague killed millions of people during three major historical pandemics. The second pandemic, known as the "Black Death", killed around 50% of the population in the Middle Ages and disorganized the European economy. In addition to ancient plague foci in China, the third pandemic seeded new foci into previously virgin territories such as Madagascar, Southern Africa and the Americas. Although currently absent from Europe, the plague persists in large areas in the world, in the rodent reservoir from which it may spread after extended periods of silence. During the last twenty years, plague caused human cases in areas where it had disappeared for decades, like India (1994), and Zambia (1997). More frightening, plague reemergence was also observed in countries surrounding the Mediterranean such as Jordan (1997), Algeria (2003), and Libya (2009). Therefore, since the beginning of the nineties, plague is categorized as a re-emerging disease [1].

Yersinia pestis, the causing agent of bubonic and pneumonic plague, is among the deadliest bacterial pathogens affecting humans. Transmitted from mammals-to-mammals by infected fleas, it infects and destroys the draining lymph node (forming the bubo), before disseminating throughout the body and ultimately causing death. This bubonic form of the disease is the most frequent [2]. Sometimes, contamination of the lungs leads to the emission of highly bacteria-laden aerosols and to human-to-human transmission with direct airways contamination. The primary pneumonic plague ensuing is very acute and almost systematically fatal within as few as 3 days.

The threat of bioterrorism has also recently emphasized the need for improved means to control *Y. pestis*, which is one of the Tier 1 select biological agents that pose a risk to national security [3]. WHO estimated that a bioterrorist attack using aerosols (50 kg) over a large city would cause 150,000 pneumonic plague cases and 36,000 deaths [4]. Large-scale exercises simulating an attack involving plague, revealed that both healthcare facilities and local and national public health agencies would be poorly prepared for such attacks [5].

Antibiotic treatment is the main therapy against plague [6]. However, antibiotic resistant *Y. pestis* strains have been identified. One strain, had gained resistance to 8 antibiotics, including those commonly used for plague treatment and prophylaxis [6, 7] the acquisition of transmissible plasmids, suggesting that other resistant strains are likely to appear [8]. These *Y. pestis* resistant strains may represent a major public health concern if they expand or if they are intentionally spread. In front of such a risk, vaccination might be an efficient way to control plague in humans. However, a vaccine licensed worldwide is currently lacking.

The purpose of this review is to present the vaccine strategy against plague that we used, and to summarize the recent results obtained with VTnF1, the most recent version of our vaccine.

2. Vaccination against plague: the context

The first widely used plague vaccine was the live attenuated *Y. pestis* EV76 developed and formerly used in Madagascar. EV76 was obtained by repeated subcultures in vitro of an initially virulent strain that led to the loss of a genetic element named High Pathogenicity Island (HPI), which is important for the systemic dissemination of *Y. pestis*. EV76 induces a strong humoral response and protects

1 against bubonic plague and pneumonic plague but immunity was not long-lasting [9].
2 However, this vaccine sometimes causes severe side reactions, especially in
3 patients with iron overload. Furthermore, the genome of *Y. pestis* being highly
4 unstable [10, 11], a decline in efficiency of the EV76 vaccine preparations used in
5 different countries was observed because of a genetic drift caused by accumulation
6 of mutations and loss of DNA sequences [9-12]. A derivative of the EV76 strain has
7 been developed in Russia [13]. Showing less secondary effects and renamed NIIEG,
8 it is used in the Russian federation and China.
9

10 A plague vaccine composed of formalin-killed whole-cell *Y. pestis* was licensed in
11 the USA and UK during the 20th century. It was discontinued because it was
12 reactogenic in humans and conferred only short-term protection [9], requiring annual
13 booster immunizations [9]. The USP killed vaccine was recently discontinued in the
14 USA. No new killed plague vaccine has replaced that one.
15

16 The development of new candidate vaccines has received much efforts during
17 the recent years. Some strategies were based on an attenuated strain of *Y. pestis*
18 [14-20] obtained by deletion of virulence genes [21, 22], or by addition of genes, such
19 the *lpxL* gene modifying the LPS [23]. Introduction of *Y. pestis* antigens into
20 *Salmonella* [14] and virus vectors [15, 16], as well as DNA [17-20] were also used to
21 produce plague vaccines. At present, none of these was brought to clinical evaluation
22 phases.
23

24 The US and UK governments developed molecular plague vaccines, called F1-V
25 and RypVax® respectively, which are patented but not yet licensed. Both are
26 composed of recombinant *Y. pestis* F1 and V (LcrV) antigens. F1 is the main protein
27 component of the pseudocapsule, and is a dispensable virulence factor. The V
28 antigen is part of the Type Three Secretion System (TTSS), an essential virulence
29 factor. Two subcutaneous doses of these F1/V vaccines provided 100% protection to
30 mice against bubonic and pneumonic plague [15-17, 24]. However, when F1-V was
31 tested in non-human primates, *Cynomolgus* macaques could be protected against
32 aerosolized *Y. pestis*, but not African green monkeys [25]. Therefore, it is not yet
33 clear whether F1-V could reliably provide protection in humans. F1-V and RYpVax
34 have completed phases I and II trials, but the results of these trials are not yet
35 available. RYpVax development has been halted, whereas F1-V marketing is
36 announced for 2020. Another molecular vaccine has been patented by the Chicago
37 University and is called V10. This vaccine, composed of a shorter form of the V
38 antigen, provided protection against bubonic and pneumonic plague to mice and
39 *Cynomolgus* macaques [26]. No phase I evaluation has yet been announced.
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46 **3. Use of *Yersinia pseudotuberculosis* to vaccinate against *Yersinia pestis***

47 **3.1. The vaccine strategy: a live *Y. pseudotuberculosis* administered orally**

48 The rationale for choosing this approach was to combine the immunogenicity and
49 antigenic complexity of live vaccines with the genetic stability and possible oral
50 administration of *Y. pseudotuberculosis*.
51

- 52 • A live vaccine

53 Live vaccines offer a series of advantages over recombinant vaccines.
54 Preparedness plans against bioterrorist attacks imply stockpiling millions of vaccine
55 doses. However, stockpiles have a finite lifespan and thus demand regular
56 production of new doses, an expensive strategy [27]. Instead, live vaccines can be
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1 rapidly and easily produced in large amounts in an emergency situation, thus
2 requiring more limited permanent stocks. For this reason, they are now viewed as a
3 valuable alternative to molecular vaccines.

4 Subunit vaccines composed of a limited set of antigenic targets may not be
5 effective in case of bacterial gene deletion and antigenic variation, as observed for
6 the F1 and V antigens [10, 28]. It is not the case for live vaccines, because their high
7 antigenic complexity guarantees a response against a broad range of targets. In
8 addition, live vaccines contain antigens in their native and naturally glycosylated
9 molecular forms. Antigens are produced *de novo* as long as the bacteria persist, thus
10 providing a prolonged stimulation of the immune system. Also, live vaccines do not
11 require adjuvants since bacterial antigens (LPS and other pathogen-associated
12 signatures) naturally stimulate the innate immune system. They induce both antibody
13 and cell-mediated responses, and cooperation of these responses is more efficient
14 for the elimination of pathogens [25].

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18 Once developed and validated, live vaccines allow mass production at limited
19 costs, a crucial condition for their use in endemic countries.

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22 • *Y. pseudotuberculosis* instead of *Y. pestis*

23 One of the reasons that limit or prevent the use of *Y. pestis* as live vaccine is its
24 genetic instability. Its genome is prone to frequent rearrangements and loss of
25 genetic material, due to the presence of numerous insertion sequences [10, 12, 29],
26 as described for the initial *Y. pestis* EV76 vaccine strain [30]. An alternative, first
27 proposed by Jenner with his *vaccinia* vaccine against smallpox, was to use a
28 microorganism different from the targeted pathogen (so it does not have its
29 drawbacks), but closely related to it, to trigger cross-species protective immunity.
30 Following this strategy, we decided to use *Y. pseudotuberculosis*, because its
31 genome contains only few insertion sequences and is therefore much more stable
32 than that of *Y. pestis*. Furthermore, *Y. pseudotuberculosis* [31] is much less virulent
33 than the plague bacillus, and the oral route is its natural mode of transmission.
34 Finally, the two species are genetically almost identical [31], with more than 95%
35 chromosomal identity [29], and therefore share a large variety of antigenic targets.

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40 • A single dose vaccination

41 Most molecular vaccines require repeated injections and regular boosts to confer
42 protection over long periods of time. Difficult to perform in the field, repeated
43 injections are considered by public health authorities as a limitation for mass
44 vaccination. Therefore, single dose vaccination represents a key advantage.

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47 • An oral vaccination

48 Oral vaccination is also attractive because it avoids the use of syringes, which
49 are a major source of disease transmission during mass vaccination campaigns, a
50 problem frequently mentioned by WHO in its vaccination guidelines [32].

51 52 53 54 **3.2. Proof of concept: the naturally avirulent *Y. pseudotuberculosis* IP32680**

55 The IP32680 was first tested because it was a naturally attenuated strain
56 showing no virulence in mice [33]. One oral IP32680 inoculation protected 75% of
57 mice against bubonic plague. This validated the strategy that cross-protection
58 provided by *Y. pseudotuberculosis* could protect against a fully virulent *Y. pestis*
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1 strain. However, two inoculations were required to induce high antibody titers and to
2 protect 88% of mice against bubonic plague, and the IP32680 strain poorly protected
3 against pneumonic plague. In addition, the causes of its attenuation were not known
4 and the possibility of its reversion to full pathogenicity could not be excluded. These
5 results prompted us to pursue in this direction and to develop an enhanced version of
6 the vaccine.

7 8 **3.3. Attenuation of *Y. pseudotuberculosis* IP32953**

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10 IP32953, the first sequenced *Y. pseudotuberculosis* strain [29], was used as a
11 genetically defined strain to construct an irreversibly attenuated derivative. This was
12 performed by allelic exchanges of virulence determinants with antibiotic resistance
13 cassettes. The choice of virulence factors to delete was determined by the need to
14 induce a strong loss of virulence, without losing important antigenic targets. Three
15 virulence factors were chosen: the HPI, the YopK toxin, and the PsaA/pH6 antigen
16 [34].
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19 The HPI encodes the siderophore yersiniabactin that allow bacteria to acquire
20 the necessary iron molecules from the iron-deprived environment of the host [35].
21 Loss of the HPI in the *Y. pestis* EV76 vaccine strain used by Girard and Robic in
22 Madagascar was the cause of its attenuation [36]. The entire HPI was deleted using
23 the homologous recombination technique [37].
24

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26 The YopK toxin is part of the set of *Yersinia* outer membrane proteins (Yops) that
27 are injected into the cell cytosol via the TTSS. Yops, which are encoded by the
28 pYV/pCD1 virulence plasmid, target multiple intracellular signaling pathways,
29 impairing both cytokines production and cell survival. YopK [38] is a regulator of Yop
30 translocation and contributes to cell apoptosis [39]. YopK is also an inhibitor of
31 inflammasome activation in targeted host cells [40], preventing the activation of
32 caspase-1 required to produce the IL-1 β and IL-18 cytokines that are important for
33 the host inflammatory response. As a consequence, absence of YopK facilitates the
34 inflammatory and adaptive immune responses.
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37 The pH6 antigen (PsaA) is a fibrillar antigen which, like F1, homopolymerizes to
38 form a fimbrial structure at the surface of the bacteria. In *Y. pestis*, it is part of the
39 pseudocapsule and has adhesive properties to epithelial cells [41]. Its role in
40 *Y. pestis* virulence depends on the route of infection [42-44].
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43 Successive deletions of these virulence factors led to a gradual decrease of
44 virulence of the V674 *Y. pseudotuberculosis* vaccine strain (Figure 1).
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46 **3.4. Production of the *Y. pestis* F1 pseudocapsule by the *Y. pseudotuberculosis*** 47 **vaccine strain**

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49 The pseudocapsule produced by *Y. pestis* at 37°C is a polymer of the F1 subunit.
50 F1 synthesis and assembly at the surface is encoded by the *caf* operon, which is
51 located on the *Y. pestis*-specific pFra plasmid [9]. Although the F1 antigen confers
52 anti-phagocytic properties to the bacteria in vitro [45], it has little or no impact on
53 virulence [46-49]. Immunization with F1 confers protection against plague [50], and
54 *Y. pestis*-infected mice can be treated with monoclonal antibodies against F1 [51]. F1
55 is therefore an excellent target for protective immunity against *Y. pestis*.
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58 F1-encapsulated *Y. pseudotuberculosis* strains were generated by introduction of
59 the *caf* operon into V674. This was first obtained by inserting the *caf* locus into the
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1 pGEN expression plasmid, yielding the V674pF1 strain [34]. However, despite the
2 presence of stabilization elements in the plasmid, spontaneous loss of the pGENcaf
3 plasmid was observed, resulting in a progressive reversion to un-encapsulated V674
4 bacilli. A sustained antibiotic pressure could be applied in vitro to ensure plasmid
5 persistence, but this pressure could not be maintained in vivo. To overcome this
6 difficulty, a VTnF1 version of V674 was generated by inserting the *caf* operon into the
7 chromosome of V674 with a mini-transposon [52]. F1 was found to be
8 homogeneously produced by VTnF1 individual colonies, in amounts comparable to
9 those of *Y. pestis* [53].
10

11 **3.5. Virulence and persistence of the V674 vaccine strains**

12 While wild type *Y. pseudotuberculosis* strain IP32953 has a 50% lethal dose
13 (LD₅₀) for mice of 10⁸ CFU by the oral route, the LD₅₀s of its derivatives were
14 systematically >10¹⁰ CFU. *Y. pseudotuberculosis* colonizes the gut and Peyer's
15 patches, and reaches the spleen and liver where it multiplies. All V674 derivatives
16 were also able to colonize and persist for more than a week in the gut and the
17 Peyer's patches [33, 34, 53]. They also retained the ability to breach the barriers of
18 the Peyer's patches and to reach the spleen and liver [33, 53].
19

20 However, major differences in terms of histopathological lesions were seen
21 between wild type and vaccine strains. IP32953 causes massive necrosis on day 6 in
22 the Peyer's patches [34], with numerous abscesses and abundant inflammatory
23 polymorphonuclear infiltration of the spleen and liver [33]. On the opposite, these
24 organs were little affected by the attenuated strain V674.
25

26 Another key difference was that tissue bacterial loads of the V674 strains were at
27 least 10³ lower than those of IP32953 (Figure 2 and [33]). After two weeks, none of
28 the strains were detected in the spleen, indicating a robust response of the immune
29 system that efficiently controlled bacteria. *Y. pseudotuberculosis* has a tropism for
30 lymphatic tissues, including the spleen. The fact that attenuated strains penetrated
31 but were subsequently eliminated from the spleen may explain their potent
32 immunogenicity.
33

34 Whereas V674 and V674pF1 were still present in the feces and Peyer's patches
35 of immunized mice after two weeks, VTnF1 almost completely disappeared from
36 these sites, and no bacteria were detected in the feces during the six following
37 months [53]. Because the only difference between V674pF1 and VTnF1 is the
38 stability of the encapsulation, the most likely reason for VTnF1 faster clearance is
39 that the host eliminated encapsulated bacteria more easily.
40

41 **3.6. The immune response raised after oral administration of the** 42 ***Y. pseudotuberculosis* vaccines**

43 The three genetically attenuated strains V674, V674pF1 and VTnF1 induced high
44 and comparable IgG titers against antigens common to the three strains [34, 53]. The
45 anti-F1 IgG response (for encapsulated strains) comes in addition to this "core"
46 response. Actually, this anti-F1 antibody response is the major component of the
47 humoral response to the vaccine because F1 is very immunogenic. Furthermore, the
48 F1 antigen is shed and thus can be captured and processed by antigen-presenting
49 cells distant from the site of infection. [34]. On the opposite, an F1 pseudocapsule
50 surrounding bacterial cells may have masked other bacterial antigens. This was not
51 the case since the IgG response induced by VtnF1 was also directed against non-F1
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1 antigens. An immunoblot analysis of IgG target antigens revealed that least ten major
2 proteins were recognized [53]. All IgG isotypes (IgG1, IgG2a, IgG2b, IgG3) were
3 produced [33, 53]. This isotype profile indicates that the cellular immune response
4 included (but was not limited to) Th1 lymphocytes [54], which produce the IFN γ
5 responsible for IgG1 to IgG2 commutation. IgA were also found in the serum and
6 intestinal lavages [34], and probably contributed to the elimination of the vaccine from
7 the intestine.

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9 Sub-unit vaccines are better inducers of a humoral than a cellular response due
10 to the use of Aluminum salts as adjuvant [55, 56]. However, the cellular wing of the
11 adaptive immune response plays an important role against plague [57, 58] and
12 collaborates with the humoral defense to protect against pneumonic plague [59]. Live
13 vaccines are good inducers of a cellular response. Memory cells induced by V674,
14 V674pF1 and VTnF1 produced high levels of IFN γ IL-17 and IL-10. IFN γ is the
15 central cytokine of the type 1 response (Th1), and is essential for both innate and
16 adaptive immunity [60]. IL-17 attracts polymorphonuclear leukocytes and induces
17 them to produce antimicrobial peptides. IL-17-producing T lymphocytes (Th17) are
18 essential to survive pneumonic plague [61, 62]. On the opposite, IL-10 is the
19 prototypical anti-inflammatory cytokine, which limits the adverse effects of
20 inflammation induced by IFN γ and IL-17 [63].

21
22 A clear recall response specific for the F1 antigen could be detected after
23 vaccination with encapsulated strains (V674pF1 & VTnF1) [34, 53]. However, the
24 cellular response against non-F1 antigens was much stronger, with IFN γ and IL-17
25 levels similar to those induced by the non-specific T cell stimulator Concanavalin A.
26 Of note, the un-encapsulated V674 strain was already able to induce this memory,
27 indicating that the presence of F1 was not essential for the recruitment of
28 *Y. pseudotuberculosis*-specific T lymphocytes. Despite the abundance of F1, the
29 strong response to other antigens may thus indicate that numerous antigens
30 triggered their own specific cells, shaping a broad repertoire memory population.
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37 **3.7. Protection against bubonic and pneumonic plague**

38 A one-dose vaccination with the naturally avirulent IP32680 strain or the
39 genetically attenuated V674 protected mice against a subcutaneous (sc) infection,
40 but did not confer full protection (<80%; Figure 3 and [33, 34]). Because our goal was
41 to achieve efficient protection against plague with a single-dose vaccination, we
42 decided to add the highly immunogenic *Y. pestis*-specific F1 antigen to our vaccine
43 strain.
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46 V674pF1 and then VTnF1 used in a single intragastric (ig) dose (10^8 CFU)
47 provided 100% protection against pneumonic plague (challenge of 10^5 *Y. pestis*
48 CFU, i.e. 30 LD₅₀). However, only 80% of the mice vaccinated with V674pF1 survived
49 a high-dose (3,300 LD₅₀) pneumonic plague challenge, whereas 100% of the
50 animals vaccinated with VTnF1 did survive (Figure 3 and [34, 53]. The difference of
51 efficiency between V674pF1 and VTnF1 was even more obvious against bubonic
52 plague: only 80% of the mice vaccinated with V674pF1 survived a moderate (10^3
53 CFU; i.e. 100 LD₅₀) bubonic plague challenge, whereas, VTnF1 provided full
54 protection. VTnF1 also protected 93% of the vaccinated mice against a high (10,000
55 LD₅₀) bubonic plague challenge. The most likely explanation for this difference of
56 protection is the more homogenous and sustained production of the F1
57 pseudocapsule by VTnF1. The F1 antigen can activate macrophages [64], an
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adjuvant favorable for an adaptive immune response. Therefore, the efficiency of VTnF1 may result from a stronger stimulation of macrophages, and possibly also of dendritic cells, thus fostering immunity more efficiently.

The persistence of protection provided by VTnF1 was evaluated after six months (one third of an OF1 mouse lifespan). High anti-F1 IgG titers persisted for six months after vaccination, and the cell capacity to produce IFN γ and IL-17 in response to non-F1 antigens remained high as well. Upon challenge with *Y. pestis* 6 months after vaccination, 93% of the animals were still protected against bubonic plague (100 *Y. pestis* LD₅₀) and 50% of the mice survived pneumonic plague (33 *Y. pestis* LD₅₀), indicating that the protection provided by VTnF1 was long-lasting [53].

3.8. Protection against an F1-negative *Y. pestis* strain

A major challenge of vaccination is antigenic variation. The large majority of *Y. pestis* strains produce F1 [10], but F1-negative *Y. pestis* exist in nature and such a strain has been isolated from a fatal human plague case [28]. F1 is not an essential virulence factor in mammals [26, 65, 66], although F1 contributes to *Y. pestis* pathogenicity in some mouse strains [49]. Although natural F1-negative mutants are scarce, a strain lacking F1 can be easily constructed for evil use using standard and widely available laboratory methods. Such a “weaponization” of *Y. pestis* has been achieved during the cold war [67].

Most subunit vaccines under test comprise the F1 antigen. Girard, who developed EV76, reported that the vaccine efficiency was directly related to the amount of F1 [36], possibly explaining why EV76 failed to protect against F1-negative *Y. pestis* [26, 48, 65]. An immune response focused on F1 also favors the selection of F1-negative mutants [68].

We thus evaluated the immunity provided by VTnF1 against an F1-negative *Y. pestis* strain. We observed that vaccination conferred full protection to mice exposed to an F1-negative CO92, even at high-dose challenge of bubonic (10,000 LD₅₀) and pneumonic plague (3,000 LD₅₀) [53]. Because VTnF1 is a whole-cell vaccine, this efficiency can be ascribed to its antigenic diversity. Antibodies recognizing multiple antigens other than F1 were evidenced [53]. VTnF1 also induced a strong cell-mediated immune response directed toward non-F1 antigens. Thus, protection against the F1-negative strain resulted from the cross-species immunity elicited by *Y. pseudotuberculosis* antigens shared with *Y. pestis*. Why attenuated *Y. pestis* induce an F1-focussed immune response, but not the attenuated F1-encapsulated *Y. pseudotuberculosis* VTnF1 remains an open question.

3.9. Perspectives

The next step is to remove the antibiotic-resistance cassettes that have been introduced into VTnF1 to delete important virulence genes and to insert the *caf* operon into the chromosome. Clinical-grade vaccine doses will then be produced on a large scale according to good manufacturing procedures to perform pre-clinical tests and ultimately start clinical tests.

4. Conclusions

Plague is a serious health problem for several countries in the world and a potential weapon of the bioterrorist threat. The VTnF1 live vaccine provides high-level

1 protection against both bubonic and pneumonic plague after a single-dose
2 immunization, and thus represents an efficient solution to prevent plague mortality.
3 As VTnF1 is easy-to-produce, genetically stable, and irreversibly attenuated, it could
4 be used both for vaccination campaigns in plague endemic countries, and as a fast
5 response tool against a bioterrorist plague attack.
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7 **5. Acknowledgements**

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Legends to figures

Figure 1: Contribution of individual gene deletion to IP32953 attenuation

The median lethal dose (LD₅₀) of the indicated strains by the oral route was measured by inoculating groups of mice with graded doses of bacteria and following survival for 21 days. The LD₅₀ was calculated using the Spearman & Karber method. The dotted line indicates the upper limit of the test.

Figure 2: Comparison of in vivo persistence for the successive vaccine strains

Groups of mice were inoculated with a single oral dose of V674, V674pF1 or VTnF1 (10⁸ CFU) and were sacrificed at the indicated times to evaluate the bacterial loads in feces, Peyer's patches and spleen. Samples were minced and dilutions were plated on selective agar plates containing kanamycin to count colonies, with a detection limit of 10 CFU/sample. Shown are individual values per pellet, per patch or in the whole spleen, respectively. The horizontal line indicates the median. The unpaired Mann-Whitney test was used for statistical analysis: Strains were compared by time and organ and only statistically significant comparisons are shown. *: p ≤ 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

Figure 3: Protective efficacy against bubonic and pneumonic plague achieved with the different V674 vaccine versions

Mice having received a single oral dose of V674, V674pF1 or VTnF1 (10⁸ CFU) were challenged intranasally or subcutaneously to cause pneumonic or bubonic plague, respectively, with usual or high doses (severe challenge) of *Y. pestis* CO92, as indicated. Mouse survival was recorded daily for 21 days. The number of surviving mice/number of animals tested is indicated above the corresponding bar for each condition. The high dose challenge was only performed when 100% protection was obtained against the usual challenge. The Fisher Exact test was used for statistical analysis: *: p ≤ 0.05; ***: p < 0.001.





