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A *Yersinia pestis lpxM*-mutant live vaccine induces enhanced immunity against bubonic plague in mice and guinea pigs

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Abstract

The *lpxM* mutant of the live vaccine *Yersinia pestis* EV NIIEG strain synthesising a less toxic penta-acylated lipopolysaccharide was found to be avirulent in mice and guinea pigs, notably showing no measurable virulence in Balb/c mice which do retain some susceptibility to the parental strain itself. Twenty-one days after a single injection of the *lpxM*-mutant, 85–100% protection was achieved in outbred mice and guinea pigs, whereas a 43% protection rate was achieved in Balb/c mice given single low doses (10^3 to 2.5×10^4 CFU) of this vaccine. A subcutaneous challenge with 2000 median lethal doses (equal to 20,000 CFU) of fully virulent *Y. pestis* 231 strain, is a 6–10-fold higher dose than that which the EV NIIEG itself can protect against.
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1. Introduction

The *Yersinia pestis* EV strain has been used as a human plague vaccine for more than 70 years. The virulent parental strain was initially isolated in 1926 by Girard and Robic from a human case of bubonic plague in Madagascar and then attenuated by continuous *in vitro* passages on nutrient media at room temperature (18–25 °C) for 6 years [1–5]. Different lines or subcultures of the EV strain were employed as live plague vaccines worldwide, resulting in considerable decreases of cases of fatal human plague [1–8]. After the first human vaccination trials in 1932 [3], different *Y. pestis* EV lines were inoculated into more than 10 million people, and there has been no evidence of its reversion to virulence in humans or in development of any detectable persistence of strains within inoculated individuals [2,3,5]. However, there were reports that the *Y. pestis* EV76 strain might possess residual virulence in mice and other animal species [3,4,6–11], raising concerns that this strain could, on occasion, cause disease following vaccination of humans.

One of the most frequently used derivatives of the EV strain is the *Y. pestis* EV76 live vaccine strain. From 1936 to 1991, the EV76 strain was used as a live plague vaccine in

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the USSR and has continued to be used in the countries of the Former Soviet Union (FSU). The most common derivative strain for human vaccination in these countries is the *Y. pestis* EV line NIIEG (designation based on the Russian abbreviation of the Scientific-Research Institute for Epidemiology and Hygiene, Kirov, Russian Federation). The vaccine strain was maintained in this Institute for a prolong period of time after obtaining it from the Pasteur Institute, Tananarive, in 1936 from Girard [2,5]. Also, in both NIIEG and the Russian State Anti-plague Research Institute 'Microbe' (Saratov, Russia) the major protective and immunobiological properties of this strain were first studied and basic manufacturing procedures were optimised [2,5,12,13]. After initial vaccination of more than seventy thousands volunteers, it was found that the *Y. pestis* EV NIIEG showed a relatively low frequency of adverse effects for humans [2,5]. The strain demonstrated a high protective efficacy during its use for the containment of a plague outbreak in Manchuria in 1945–1946 [2,14]. The vaccine was found to be effective after administration by different routes, including subcutaneous (s.c.), cutaneous, inhalation, and intramuscular routes of injection. However, the highest level of specific immunity against plague was achieved either by scarifying the dermis followed by injection or after vaccination by inhalation [2,4,5]. In fact, a single dose of the *Y. pestis* EV NIIEG live vaccine conferred a prompt (day 7 post-vaccination) and pronounced immunity lasting for 10–12 months [2,15]. In contrast to the *Y. pestis* EV76 derivative described by Titball's group that contained genetic lesions in the LCR operon resulting in the inability of the bacteria to provide a low calcium response [6,7], the *Y. pestis* EV NIIEG strain possesses all known virulence markers, except the pigmentation (Pgm) locus. The Pgm-negative phenotype in *Y. pestis* EV76 derivatives was mediated by the spontaneous deletion of a 102-kb chromosomal *pgm* locus encoding hemin-binding and iron transport functions [16]. Three typical plasmids, pFra, pLcr and pPst are present in this vaccine strain and the *Y. pestis* EV NIIEG cells produce V antigen and Yops when grown under low calcium conditions [5,17–19]. The strain *Y. pestis* EV NIIEG whether cultured at 28 or 37 °C prior to inoculation, elicits similar levels of immunity against plague in experimental animal models [5–7,20]. Since the latter part of the 20th century, the *Y. pestis* EV NIIEG strain is the only human anti-plague vaccine licensed in the FSU countries.

Despite a number of desirable characteristics resulting in induction of immunity to plague, the *Y. pestis* EV76 strain, like other live *Y. pestis* Pgm-negative variants, can produce a variety of adverse local and systemic reactions and occasionally can be detected in the liver or spleen, but without detectable bacteremia, following inoculation of different mammalian species [6,9–11]. The *Y. pestis* EV NIIEG strain was reported to be relatively avirulent among the majority of mammalian species injected, including baboons (*Papio hamadryas*) [2,5,12,13,15,17,20]. However, other *Y. pestis* EV76 derivatives, similarly to typical *Y. pestis* Pgm^{-} strains, after being administered to other species of non-human primates, including vervet monkeys (*Cercopithecus aethips pygerythrus*) and African green monkeys (*Chlorocebus aethiops*), produced a fatal bubonic–septicaemic plague [4,5,9–11,21]. Most of these EV76 derivatives can yield severe local and systemic reactions and gross tissue changes independent of the route of inoculation. These reactions are morphologically similar to those induced by a wild-type *Y. pestis* strain but are not accompanied by progressive systemic bacteremia and septicaemia. Nevertheless, these reactions make it impossible to license these *Y. pestis* EV76 live vaccine strain derivatives for human use outside of the FSU [8].

The majority of the adverse reactions are thought to be mediated by the endotoxic activity of the *Y. pestis* lipopolysaccharide (LPS) [5,15,22–27]. To significantly decrease the toxicity of LPS, a set of mutants with a deletion–insertion in the *lpxM* gene in both virulent and attenuated *Y. pestis* strains, including the *Y. pestis* EV NIIEG, were constructed recently [22]. These *lpxM* mutants synthesised a less toxic penta-acylated LPS while the parental strains produced more toxic hexa-acylated LPS under the same growth conditions. A marked increase in protective characteristics of one of the *lpxM* mutants of the *Y. pestis* EV NIIEG in a mouse model was also noted [22].

In this study, we examined in detail the protective properties of the *lpxM* mutant of the *Y. pestis* EV NIIEG with altered sensitivity to the plague diagnostic bacteriophage L-413C [17,28,29] using guinea pigs as well as outbred mice and the inbred Balb/c mouse strain. We also investigated whether this *lpxM* derivative of the *Y. pestis* EV NIIEG spread within the body of experimental animals and also evaluated other aspects of residual virulence [21,30] of this mutant. Finally, we observed a clear increase in protective efficacy of the mutant in comparison with the parental *Y. pestis* EV NIIEG vaccine strain in all animal models, making this *lpxM* derivative a potential vaccine candidate with improved characteristics.

2. Materials and methods

2.1. Bacterial strains, plasmids, and media

The *Y. pestis* strains used in this study are listed in Table 1. The bacteria were stored in lyophilised stocks. All molecular biological procedures, and cultivation of the *Y. pestis* EV strain and its derivatives were performed as previously described [22].

2.2. Construction of lpxM mutants

The *lpxM* mutants of the *Y. pestis* EV NIIEG were constructed by the allelic exchange procedure as described previously [22]. A suicide recombinant plasmid, pMSB3K, containing ∼1.5 kb of the 5′ and 3′ flanking regions of the *lpxM* gene with a 0.59 kb deletion substituted with the marker of resistance to kanamycin (Km^{R}) was introduced

Table 1
The *Y. pestis* strains used in this study

Strain or plasmid	Characteristics	Source or reference
Y. pestis EV line NIIEG	Russian vaccine strain, the derivative of the vaccine *Y. pestis* EV76 strain; $pFra^+$; $pLcr^+$; $pPst^+$ Δpgm; bv. orientalis, ssp. *pestis*; attenuated	[2,5,13,18,19,22,39], TSISCBP[a]
Y. pestis EVΔ*lpxM*	$pFra^+$; $pLcr^+$; $pPst^+$ Δpgm $\Delta lpxM$::*kan*; bv. orientalis, ssp. *pestis*; attenuated	[22], This study
pMSB3K	Suicide vector containing a mutant allele of *lpxM* gene for *Yersinia* mutagenesis, $Ap^R Km^R$	[22]
Y. pestis 231	Virulent wild-type strain isolated from *Marmota baibacina* in 1947 in the Tien Shan highland natural focus, $pFra^+$; $pLcr^+$; $pPst^+$ bv. antiqua, ssp. *pestis*; the s.c. LD_{50} for mice and guinea pigs is $\leq$10 CFU[b]	[19,31,39]

[a] Tarasevich State Institute for Standardisation and Control of Biomedical Preparations, Moscow, Russia.
[b] The s.c. LD_{50} value for the *Y. pestis* 231 strain wt was reported previously by Feodorova et al. [19].

to *Y. pestis* cells by conjugation. Selection of *Y. pestis* merodiploid strains and subsequent screening for the allelic exchange was achieved following sucrose counter-selection. The resulting Km^R colonies grown on plates containing 5% sucrose were tested for the loss of resistance to ampicillin (indicating loss of the suicide vector), followed by probing with the *Y. pestis*-diagnostic bacteriophages Pokrovskaya and L-413C [17,18,28,29,31]. Subsequent PCR analysis was performed to evaluate the success of inactivation of the original copy of the *lpxM* gene in the chromosome of the identified clones by using previously described primers msb5S1.5 and msb3S1.5 [22] which flanked the *lpxM* locus, consisting of the *lpxM* gene together with the 5′ and 3′ flanking regions involved in the allelic recombination. In addition, two new pairs of primers were designed matching a sequence within the *lpxM* gene itself. The primer pair *lpxM*-flnk-F (5′-TGACGCAACTGGTTTTATTCC) and *lpxM*-flnk-R (5′-TATTGCTCTGGATTCGGCTTA) flanked the area of the expected internal deletion, and the primers *lpxM*-del-F (5′-GCATGGACAGGAAATACTGGA) and *lpxM*-del-R (5′-TATTGCTCTGGATTCGGCTTA) were located inside this deletion.

2.3. Bacteriophage susceptibility test

A routine procedure testing the Km^R Ap^S colonies obtained after sucrose counter-selection was performed with the diagnostic plague bacteriophages, L-413C and Pokrovskaya [17,18,28,29,31]. The mutant bacterial cells, as well as cells of the parental strain *Y. pestis* EV NIIEG, used as a positive control (*ca.* 7×10^8 cells), were plated on Hottinger agar. Then, 10 μl portions of 10-fold serial dilutions from 10^{-1} to 10^{-7} of either the phage L-413C or the Pokrovskaya phage stocks were spotted on the plates. The plates were left for 10 min at room temperature (20–22 °C) and then incubated for 12–18 h at 28 °C. The phage activity was determined for each of the strains tested by the presence or absence of a clear spot of lysis on the lawn of *Y. pestis* cells in all dilutions.

2.4. Animals

Animals used in this study to measure residual virulence and protective efficacy of the *Y. pestis* EVΔ*lpxM* mutant in comparative experiments with the parental *Y. pestis* EV strain line NIIEG included: (1) female 6–8-week-old outbred mice weighing 18–20 g (State Research Anti-Plague Institute, Volgograd, Russia); (2) female 6–8-week-old inbred Balb/c mice weighing 18–20 g (Russian State Research Anti-Plague Institute ‘Microbe’, Saratov, Russia), and (3) female adult guinea pigs weighing 250–350 g (State Animal Nursery, Marks, Saratov Region, Russia).

2.5. Determination of residual virulence (innocuity tests)

Groups of outbred mice (10 animals in each group) received s.c. injections (0.2 ml) of a 0.9% saline solution containing either 1×10^5 or 1×10^7, 1×10^8 or 1×10^9 colony forming units (CFU) of either the *Y. pestis* EV NIIEG or the *Y. pestis* EVΔ*lpxM*. Groups of inbred Balb/c mice (seven animals in each group) were injected with either 2×10^2, 1×10^3, 5×10^3 or 2.5×10^4 CFU of each of these *Y. pestis* strains inoculated by the same administration route. Groups of guinea pigs (three animals in each group) were injected s.c. with either 1×10^7, 2×10^9 or 1.5×10^{10} CFU of the *Y. pestis* strains as described above for mice. Control animals (four animals in each group) received a 0.2-ml of 0.9% saline solution alone. For a minimum of 21 days after administration of the vaccines, all animals were monitored daily for the development of signs of adverse reactions, and where appropriate, the time to death was recorded. Homogenates of livers, spleens, lungs, regional and distal lymph nodes, as well as blood samples from all dead animals were inoculated onto Hottinger agar and incubated at 28 °C for 48 h to confirm the spread of the *Y. pestis* bacteria into these tissues. On day 21, surviving animals were bled and euthanised and their livers, spleens, lungs, lymph nodes, and blood examined for the presence of viable *Y. pestis* cells by plating homogenised tissue samples as well as a portion of the blood on Hottinger agar. Additionally, on days 3, 7 and 13 the regional lymph nodes close to the inoculation site in the guinea pigs were palpated and live animals from each group with palpable lymph nodes were euthanised and the organs (see above) and blood were tested for the presence of *Y. pestis* vaccine strains as described above. Smears of the blood of dead animals were made on microscope slides and Gram-stained for evaluation of bacteria within the blood.

2.6. Protective efficacy

Groups of outbred mice (seven animals in each group) and groups of Balb/c mice (from four to seven animals in a group) were immunised by s.c. injection either with the *Y. pestis* EVΔ*lpxM* or the *Y. pestis* EV line NIIEG using the same injection protocol as described in the previous section. Both types of mice received various doses of either the *Y. pestis* EVΔ*lpxM* or the *Y. pestis* EV cells in 0.2-ml of a 0.9% saline solution. Guinea pigs (from three to five animals in each group) were immunised with doses ranging from 4×10^1 to 1.5×10^{10} CFU of the two *Y. pestis* strains as described above. On day 21 after immunisation, all animals were challenged by a s.c. route with the virulent *Y. pestis* 231 strain (lethal dose$_{50}$ (LD_{50}) = 10 CFU) grown on Hottinger agar at 28 °C for 48 h. Challenge doses were 2000 LD_{50} (2×10^4 CFU) for outbred mice or 1200 LD_{50} doses (1.2×10^4 CFU) for Balb/c mice and guinea pigs, given in 0.2 ml saline solution. The efficacy of vaccination was estimated by the number of surviving animals. All infected animals were closely observed over a 21-day period for the development of signs of plague infection. The time to death of the animals was also recorded. The livers, spleens, lungs, lymph nodes, and blood samples from all the animals that succumbed to the challenge were plated on Hottinger agar and incubated at 28 °C for 48 h to verify plague infection. On day 43, the surviving animals were euthanised and bled, and their spleens, lymph nodes, and blood samples were examined by culture to determine if viable *Y. pestis* bacteria were present. All experiments were performed at the relevant biosafety level and in accordance with Russian National Animal Care and Use guidelines.

2.7. Statistical analysis

The Student's *t*-test was used to determine the mean time of death of the treated animals. Fisher's exact test was used to compare survival in animals vaccinated with the Δ*lpxM* strain compared to the animals vaccinated with the corresponding dose of the parental strain.

3. Results

3.1. Selection of lpxM mutants of the Y. pestis EV NIIEG

Following the conjugative transfer into the *Y. pestis* EV NIIEG of the suicide plasmid pMSB3K, we found that all of Km^R Ap^S colonies that originated after sucrose counter-selection were lysed by the *Y. pestis*-specific Pokrovskaya phage. However, L-413C phage did not lyse all of the transconjugates, separating these derivatives into two groups, one of which had a phage-sensitive phenotype (Ph^S) and another that had a phage-resistant (Ph^R) phenotype. Analysis by PCR using three pairs of primers specific to *lpxM* gene and surrounding regions revealed that the Ph^R clones corresponded to a successful inactivation of the chromosomal *lpxM* copy by the mutant allele containing the internal deletion and Km^R marker. In contrast, the Ph^S variants were merodiploids possessing both the intact and mutant alleles, which suggested that the second step of the allelic exchange occurred without elimination of the original copy of the *lpxM* gene. Since Ph^R isolates represented strains with a truly disrupted *lpxM* gene, only clones with this phenotype were used in subsequent studies.

3.2. Residual virulence of the Y. pestis EV NIIEG and the Y. pestis EVΔlpxM in animal models

Mice and guinea pigs are used for routine studies to evaluate residual virulence of live *Y. pestis* vaccine candidates [2,6,17,20,22,23,32]. Thus, we first tested the *lpxM* mutant for its virulence potential in both of these animal species. No systemic spread or deaths were noted among outbred mice inoculated with the *Y. pestis* EVΔ*lpxM* mutant. None of the guinea pigs used to evaluate residual virulence died after inoculation with either the parental vaccine strain or the *lpxM* mutant regardless of the dose administered (1×10^7, 2×10^9 or 1.5×10^{10} CFU per animal). The results of examination by palpation revealed a marked difference in the size of the lymph nodes only at the site of inoculation, but not for lymph nodes obtained from other parts of the body. All the changes in lymph node size strongly correlated with the vaccination dose. Among the vaccines tested, the greatest changes, i.e. a two- to threefold swelling of the lymph nodes, were recorded in those animals inoculated with the *Y. pestis* EVΔ*lpxM* at all immunisation doses given. Notable, but less swelling of the lymph nodes was found in animals challenged with the *Y. pestis* EV NIIEG, which was considered a typical result for this live vaccine strain [15,17]. As was expected from the experiments with the *Y. pestis* EV NIIEG [15,17], on the third day post-inoculation of guinea pigs, no evidence for bacterial spread was found in any specimen at any of the doses used. However, on day 3 post-inoculation, cells of the *Y. pestis* EVΔ*lpxM* mutant were recovered but only from the site of vaccination after administration of the highest dose (1.5×10^{10} CFU). Additional cultures taken 7 and 13 days post-injection did not yield bacteria of either strain in any of the guinea pigs.

When Balb/c mice were used as an indicator of residual virulence, a marked difference was observed in this property when comparing the parental strain, *Y. pestis* EV NIIEG, with the *Y. pestis* EVΔ*lpxM* derivative. Also of note, the Balb/c mice were inoculated with significantly lower doses than those given to the outbred mice. Among the Balb/c mice challenged with the *Y. pestis* EV NIIEG, there was at least one vaccine-related death in each of the groups given different doses and overall 8 of 28 mice tested died from infection with this strain (Table 2). The *Y. pestis* EV NIIEG could be cultivated from all of the tissue specimens tested from the dead mice, including the samples obtained from mice, which died 20 days post-vaccination. The results with the blood smears confirmed the presence of the *Y. pestis* EV NIIEG

Table 2
The results of testing of residual virulence of the *Y. pestis* strains EV NIIEG and EVΔ*lpxM* in Balb/c mice

Vaccine	Inoculation dose (CFU)	No. of survivors/total	% of survivors	Mean (S.D.) time to death of dead mice (days, mean ± S.E.M.)	Specimen culturing results[a]					
					Liver	Spleen	Lung	Region lymph nodes	Distant lymph nodes	Blood
Y. pestis EV NIIEG	2×10^2	6/7	85.7	10.0 ± 0	+	+	+	−	+	+
	1×10^3	4/7	57.1	9.0 ± 1.0	+	+	+	+	+	+
	5×10^3	4/7	57.1	12.3 ± 9.7	+	+	+	+	+	+
	2.5×10^4	6/7	85.7	13.0 ± 0	+	+	+	+	+	+
Y. pestis EVΔ*lpxM*	2×10^2	7/7	100	NA[b]	−	−	−	−	−	−
	1×10^3	7/7	100	NA	−	−	−	−	−	−
	5×10^3	7/7	100	NA	−	−	−	−	−	−
	2.5×10^4	7/7	100	NA	−	−	−	−	−	−
Negative control[c]	0	4/4	100	NA	−	−	−	−	−	−

[a] Positive (+) or negative (−) for growth of *Y. pestis* from culturing of a specimen.
[b] Not applicable, all mice in the group survived.
[c] Mice treated with saline solution only.

bacilli in the blood of all Balb/c mice dead, i.e. completely correlated with the specimen culturing results. In contrast, all of the Balb/c mice inoculated with the *Y. pestis* EVΔ*lpxM* mutant survived and no positive tissue specimens for *Y. pestis* were recovered from these animals after sacrifice at day 21 (Table 2).

3.3. Comparative protective efficacy of the Y. pestis EV NIIEG and the Y. pestis EVΔlpxM in three animal models

In all three animal models tested, immunisation with the *Y. pestis* EVΔ*lpxM* provided a significant level of protection against challenge doses of 12,000–20,000 CFU (1200–2000 MLD) of the wild-type *Y. pestis* 231 strain. In contrast, immunisation of animals with the parental strain of *Y. pestis* EV NIIEG did not provide protection against the wild-type *Y. pestis* strain. In outbred mice vaccination with 1×10^7 to 1×10^9 CFU of the *Y. pestis* EVΔ*lpxM* protected 57–86% of the animals against experimental plague (Table 3). The *lpxM* mutant elicited a reasonable dose-dependent protective efficacy. Mice immunised with the *Y. pestis* EV NIIEG were not protected from death and had only negligible increases in the time to death. A control group of four mice given only 10 CFU of the virulent *Y. pestis* 231 all died from infection, indicating the highly virulent nature of the challenge strain in outbred mice.

In Balb/c mice, the *Y. pestis* EVΔ*lpxM* elicited modest protection (no more than 40% of survivors) after immunisation with relatively small doses of 1×10^3 to 2.5×10^4 CFU (Table 4). No protection was achieved with the smallest immunising dose (2×10^2 CFU). Higher doses were not used as the live vaccine strain EV line NIIEG, is too toxic in Balb/c mice at higher doses and even at the lower doses used, some of the animals immunised with this strain died from the *Y. pestis* infection prior to challenge 21 days after immunisation. None of the Balb/c mice immunised with the live vaccine strain EV NIIEG that lived for 21 days survived infection following challenge with the wild-type *Y. pestis* strain 231 (Table 4).

Among guinea pigs, a marked protective efficacy against challenge with 12,000 CFU of wild-type the *Y. pestis* 231 was achieved following vaccination with single doses of the Δ*lpxM* strain between 10^7 and 1.5×10^{10} CFU/animal (Table 5). No protection of guinea pigs was seen following immunisation with the *Y. pestis* EV NIIEG, even after doses of 1.5×10^{10} CFU were used for immunisation. Results with the guinea pigs clearly validate a superior efficacy of the Δ*lpxM* strain in eliciting protective immunity.

4. Discussion

The current interest in developing vaccines to prevent plague, stemming from the potential use of *Y. pestis* as a bioterrorist threat, has focused on subunit vaccines mainly containing the LcrV component of the type III secretion system and the F1-capsule protein. While several types of

Table 3
Comparative protective efficacy of the *Y. pestis* EV NIIEG and the *Y. pestis* EVΔ*lpxM* in outbred mice after challenge with 20,000 of CFU (2000 MLD) of the wild-type strain *Y. pestis* 231

Vaccine	Immunisation dose (CFU)	No. of survivors/total	% of survivors	Mean (S.D.) time to death of dead mice (days, mean ± S.E.M.)
Y. pestis EV NIIEG	10^5	0/7	0	4.4 ± 0.58
	10^7	0/7	0	5.28 ± 0.01
	10^8	0/7	0	5.14 ± 0.12
	10^9	0/7	0	3.7 ± 0.5
Y. pestis EVΔ*lpxM*	10^5	1/7	14.28	4.8 ± 0.6
	10^7	5/7[a]	71.43	5.5 ± 0.5
	10^8	4/7[b]	57.10	7.3 ± 2.6
	10^9	6/7[c]	85.71	8.0 ± 0
Negative control-1[d]	0	0/4	0	4.5 ± 0.5
Negative control-2[e]	0	0/4	0	6.0 ± 0

[a] $P = 0.02$, Fisher's exact test.
[b] $P = 0.07$, Fisher's exact test.
[c] $P = 0.005$, Fisher's exact test.
[d] Unimmunised mice challenged with 20,000 CFU of the *Y. pestis* 231 strain.
[e] Unimmunised mice challenged with 10 of CFU of the *Y. pestis* 231 strain.

vaccines based on these subunits have shown efficacy in animal protection studies [32–35], the only useful pre-clinical means to evaluate potential plague vaccines, none have yet shown efficacy in humans. Indeed, it may not even be feasible to evaluate the efficacy of plague vaccines in humans due to the infrequent occurrence of significant *Y. pestis* infections in man. However, during the past 75 years, live, attenuated plague vaccines have been used in the FSU and other parts of Asia, demonstrating protective efficacy in the setting of human plague as well as in laboratory animals, which, are, in fact, natural hosts for the plague bacillus [1–8,12–14,20,21]. Within the FSU, a specific derivative of the attenuated EV strain, designated the *Y. pestis* EV line NIIEG, has provided a high level of immunity against both bubonic and pneumonic plague following a single injection [2,5,13–15,17,20]. While there is always concern about toxicity and potential reversion to virulence associated with any live vaccine, to date, the toxicity associated with the EV line NIIEG has been acceptable and no recovery of significant virulence has been observed in vaccinated humans. Thus, given the low cost and ease of preparation of live attenuated vaccines, and the large experience with the *Y. pestis* EV-line vaccines in humans, it is worth exploring improvements to this method of vaccination as a potential means to immunise large populations quickly and inexpensively if the need arises.

In spite of the use of the live, attenuated plague vaccines in many humans, these vaccines still produce a number of side-effects in immunised individuals that limits licensing this vaccine for use in humans in many parts of the world. Thus, we sought to develop the *Y. pestis* EV NIIEG derivative with less toxicity, and an enhanced safety profile, as defined by an inability to detect bacterial spread into the circulation of laboratory animals, that also possessed a high level of protective efficacy in these animals [30]. For this purpose, the *Y.*

Table 4
Comparative protective efficacy of the *Y. pestis* EV NIIEG and the *Y. pestis* EVΔ*lpxM* in Balb/c mice after challenge with 12,000 of CFU (1200 MLD) of the wild-type strain *Y. pestis* 231

Vaccine	Immunisation dose (CFU)	No. of survivors/total	% of survivors	Mean (S.D.) time to death of dead mice (days, mean ± S.E.M.)
Y. pestis EV NIIEG	2×10^2	0/6	0	7.8 ± 0.61
	1×10^3	0/4	0	8.0 ± 0.57
	5×10^3	0/4	0	5.75 ± 1.31
	2.5×10^4	0/6	0	7.6 ± 1.05
Y. pestis EVΔ*lpxM*	2×10^2	0/7	0	6.42 ± 0.48
	1×10^3	3/7	42.8	16.5 ± 2.5
	5×10^3	3/7	42.8	10.25 ± 0.62
	2.5×10^4	3/7	42.8	7.5 ± 1.19
Negative control-1[a]	0	0/3	0	9.3 ± 1.76
Negative control-2[b]	0	0/3	0	11.3 ± 1.32

[a] Untreated mice were challenge with 12,000 of CFU of the *Y. pestis* 231 strain.
[b] Untreated mice were challenge with 10 of CFU of the *Y. pestis* 231 strain.

Table 5
Comparative immunogenicity of the *Y. pestis* EV NIIEG and the *Y. pestis* EVΔ*lpxM* in guinea pigs after challenge with 12,000 of CFU (1200 MLD) of the wild-type strain *Y. pestis* 231

Vaccine	Immunisation dose (CFU)	No. of survivors/total	Percent of survivors	Mean (S.D.) time to death of dead guinea pigs (days, mean ± S.E.M.)
Y. pestis EV NIIEG	40	0/3	0	7.6 ± 1.2
	200	0/4	0	8.75 ± 1.24
	1000	0/3	0	9.6 ± 0.88
	1×10^7	0/7	0	11.57 ± 0.79
	2×10^9	0/6	0	12.0 ± 0.63
	1.5×10^{10}	0/7	0	13.29 ± 1.38
Y. pestis EVΔ*lpxM*	40	0/5	0	8.4 ± 0.68
	200	2/4	50	6.5 ± 0.5
	1000	1/4	25	9.3 ± 0.64
	1×10^7	6/7[a]	85.72	12.0 ± 0
	2×10^9	7/7[b]	100	NA[c]
	1.5×10^{10}	6/6[b]	100	NA[c]
Negative control-1[d]	0	0/1	0	6.0 ± 0
Negative control-2[e]	0	0/1	0	7.0 ± 0

[a] $P = 0.005$, Fisher's exact test.
[b] $P = 0.0006$, Fisher's exact test.
[c] Not applicable, all guinea pigs in the group survived.
[d] Untreated guinea pigs were challenge with 12,000 of CFU of the *Y. pestis* 231 strain.
[e] Untreated guinea pigs were challenge with 10 of CFU of the *Y. pestis* 231 strain.

pestis EV NIIEG derivatives with a deletion–insertion in the *lpxM* gene were constructed [22]. This strain cannot produce a fully hexa-acylated lipid A and it is thus expected that the penta-acylated lipid A made by the *lpxM*-mutant will be less toxic [22]. *lpxM* mutants of some other Gram-negative bacteria have also been reported to be significantly less reactogenic [24,36]. To fully validate the potential of *lpxM*-mutants as live vaccines for plague, we measured the residual virulence as well as the protective activity of the *Y. pestis* EVΔ*lpxM* mutant and compared these outcomes to those elicited by the parental *Y. pestis* EV NIIEG strain.

The *Y. pestis* EVΔ*lpxM* was found to be harmless following subcutaneous inoculation of outbred mice, Balb/c mice and guinea pigs, whereas the *Y. pestis* EV NIIEG vaccine strain showed noticeable toxicity in Balb/c mice, disseminated and caused death in one outbred mouse, but was as attenuated in guinea pigs as was the *lpxM* mutant. Inoculation with the *Y. pestis* Δ*lpxM* did not lead to any vaccine-related death in the animals. Thus, the mutant used in this study, like other *lpxM* mutants of Gram-negative bacteria [24,36], was more attenuated, in comparison with the parental the *Y. pestis* EV NIIEG strain.

Of note, the residual virulence in the *Y. pestis* EV NIIEG vaccine strain is considered to be necessary for the development of adequate immunity against plague [2,5,13,15,17]. However, we found that the more highly attenuated *Y. pestis* EVΔ*lpxM* strain provided a better level of immunity against high doses of the *Y. pestis* 231 compared with the parental *Y. pestis* EV NIIEG, which failed to be effective against the high doses of the wild-type strain evaluated here. In both outbred mice and guinea pigs, doses of the *Y. pestis* Δ*lpxM* > 10^5 (mice) or 10^7 (guinea pigs) demonstrated a high level of protection. In Balb/c mice, which clearly are more susceptible to infection with the current live vaccine strain, modest protective efficacy was achieved when low vaccine doses were used (10^3 to 2.5×10^4 CFU given once). Higher doses of the EVΔ*lpxM* strain were not used in Balb/c mice, as we were comparing this construct's protective efficacy against the attenuated EV NIIEG line, which cannot be administered to Balb/c mice at higher doses without most of the animals succumbing to infection from this strain. However, given the protection elicited in Balb/c mice given low doses of the *lpxM* mutant, it might be expected that higher doses of the *lpxM* mutant might provide even higher levels of protection.

The Russian national criteria for evaluating the potential efficacy of a plague vaccine strain requires that at least 50% of outbred mice and guinea pigs should be protected against a subcutaneous challenge with 200 MLD (2000 CFU) of a virulent *Y. pestis* strain [17]. Under these conditions, the protective efficacy induced by the *Y. pestis* EV NIIEG against experimental plague is in the range of 20–60% depending on the immunising dose [17]. Therefore, to document any potential superiority of the *lpxM* mutant over the current vaccine strain, the challenge dose with virulent *Y. pestis* was increased 6–10-fold, representing 1200–2000 MLD (12,000–20,000 CFU). As expected, the parental vaccine strain did not protect against these elevated doses of the *Y. pestis* 231, while the *lpxM* mutant demonstrated clearly measurable protection. Although this mutant protected Balb/c mice challenged with 1200 MLD of the virulent *Y. pestis* strain at a modest level, given the low vaccine doses used and the high susceptibility of this mouse strain to *Y pestis* infection (1 CFU of strain GB is lethal to 100% of Balb/c mice [6]), the findings in the Balb/c mice are still supportive of the increased vaccine efficacy of the *lpxM* mutant in combination with reduced residual toxicity.

The molecular basis for the reduced residual toxicity but enhanced immunogenicity of the *Y. pestis* EVΔ*lpxM* strain is likely related to the type of lipid A produced by this mutant [22]. During growth in flea vectors at temperatures of 22–28 °C, the major glycoform of *Y. pestis* LPS contains a hexa-acylated lipid A [25–27]. However, at mammalian temperatures (37 °C), *Y. pestis* synthesises primarily a tetra-acylated LPS [25–27] that minimally stimulates the major LPS-responsive pathway involving toll-like receptor (tlr)-4. Mammalian hosts therefore do not readily respond to *Y. pestis* infection, allowing the organism to escape immune recognition, grow locally and spread systemically. An elegant demonstration of the role of the lipid A glycoform in plague virulence was shown by Montminy et al. [23] who engineered a plague strain to only produce a potent tlr-4 stimulating LPS glycoform and found this strain had dramatically reduced virulence in mice. The penta-acylated lipid A produced by the *lpxM* mutant in the already attenuated EV line NIIEG appears to have further reduced residual virulence, particularly as measured in Balb/c mice, while enhancing immunogenicity, likely due to the ability of tlr-4 to react to this lipid A glycoform and initiate an effective acquired immune response. Of note, penta-acylated LPS from Δ*lpxM* mutants of other Gram-negative bacteria have decreased endotoxic activity and were also reported to have a potent adjuvant effect on acquired immune responses, similar to those induced by hexa-acylated lipid A from the parental strains [37,38]. Given that live plague vaccines have been successfully used in humans for more than 70 years in the FSU and other parts of Asia, with reported efficacy against both bubonic and pneumonic plague [2,5,12,13], the *Y. pestis* EVΔ*lpxM* could be an improved candidate vaccine leading to the development of a new generation of more effective and less toxic live plague vaccines.

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