RESEARCH ARTICLE

Yersinia pestis Caf1 Protein: Effect of Sequence Polymorphism on Intrinsic Disorder Propensity, Serological Cross-Reactivity and Cross-Protection of Isoforms

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Abstract

Yersinia pestis Caf1 is a multifunctional protein responsible for antiphagocytic activity and is a key protective antigen. It is generally conserved between globally distributed Y. pestis strains, but Y. pestis subsp. microtus biovar caucasica strains circulating within populations of common voles in Georgia and Armenia were reported to carry a single substitution of alanine to serine. We investigated polymorphism of the Caf1 sequences among other Y. pestis subsp. microtus strains, which have a limited virulence in guinea pigs and in humans. Sequencing of caf1 genes from 119 Y. pestis strains belonging to different biovars within subsp. microtus showed that the Caf1 proteins exist in three isoforms, the global type Caf1NT1 (Ala48 Phe117), type Caf1NT2 (Ser48 Phe117) found in Transcaucasian-highland and Pre-Araks natural plague foci #4–7, and a novel Caf1NT3 type (Ala48 Val117) endemic in Dagestan-highland natural plague focus #39. Both minor types are the progenies of the global isoform. In this report, Caf1 polymorphism was analyzed by comparing predicted intrinsic disorder propensities and potential protein-protein interactivities of the three Caf1 isoforms. The analysis revealed that these properties of Caf1 protein are minimally affected by its polymorphism. All protein isoforms could be equally detected by an immunochromatography test for plague at the lowest protein concentration tested (1.0 ng/mL), which is the detection limit. When compared to the classic Caf1NT1 isoform, the endemic Caf1NT2 or Caf1NT3 had lower immunoreactivity in ELISA and lower indices of self- and cross-protection. Despite a visible reduction in cross-protection between all Caf1 isoforms, our data suggest that polymorphism in the caf1 gene may not allow the carriers of Caf1NT2 or Caf1NT3 variants escaping from the Caf1NT1-mediated immunity to plague in the case of a low-dose flea-borne infection.
Introduction

The outbreaks, epidemics and pandemics of human plague are caused by *Yersinia pestis* subsp. *pestis* strains that possess universal hypervirulence for a wide range of mammals and are ubiquitously distributed [1–4]. The representatives of a more ancestral subsp. *microtus* are endemic within populations of some voles (*Microtus* spp.) and cause only rare sporadic diseases [1, 5] with no human-to-human transmission [5]. Strains of both subspecies can make a proteinaceous capsule first described by Alexandre Yersin [6]. This antiphagocytic capsule [7] is the main component of plague vaccines [8–11] and is the most important target for laboratory diagnosis of plague [12].

Capsule biogenesis is implemented by a conserved chaperone/usher pathway [13]. Caf1 structural subunit is encoded by a 510-nucleotide *caf1* gene. The precursor protein contains 170 amino acids. The typical cleavage site is located between Ala21 and Ala22 residues [14].

Recently it was shown that a single nucleotide substitution found in the bv. caucasica strains Pestoides F [15] and G8786 [16] resulted in Ala48 → Ser48 substitution, while mutations in *Y. pestis* E1979001 (bv. antiqua) and F1991016 (bv. orientalis) resulted only in truncation down to 147 and 130 amino-acid residues giving, most likely, non-functional peptides. More recently, sequencing of *caf1* gene from 41 subsp. *microtus* strains isolated from voles and their fleas in Georgia and Armenia indicated that all of them had the same Ala48 → Ser48 substitution (*caf1* NT2, accession no. EF165977), while the strains isolated from gerbils and susliks of the same region carried the gene with a canonic sequence (*caf1* NT1, accession no. EF165976) [17].

In this study, we provide the first evidence that the allele type NT2 (Ser48 Phe117) is unique to the Transcaucasian-highland and Pre-Araks natural plague foci, while a novel NT3 type (Ala48 Val117) is endemic in Dagestan-highland natural plague focus. This is supported by sequencing data on the *caf1* genes from 119 strains of *Y. pestis* belonging to seven out of eight biovars of subsp. *microtus* [4]. Our computational analysis revealed that the Caf1 isoforms found in *Y. pestis* endemic strains should not make them significantly different in terms of pathogenicity. To test this hypothesis, we evaluated serologic cross-reactivity and cross-protection of the Caf1 isoforms. All the three isoforms could be equally detected by immunochromatography test for plague. When compared to the classic Caf1NT1, endemic Caf1NT2 and Caf1NT3 had lower immunoreactivity in ELISA and lower indices of self- and cross-immunity. However, although a notable reduction in the cross-protection was observed between all isoforms, the polymorphisms in the *caf1* gene may not provide for Caf1NT2 or Caf1NT3 *Y. pestis* strains the possibility to escape from the Caf1NT1-mediated plague immunity in the case of a low-dose flea-borne infection.

Materials and Methods

Bacterial strains and culture conditions

*Y. pestis* intraspecies classification used in this study corresponds to the International Codex of Bacterial Nomenclature [4, 18, 19]. In this study, we used a total of 119 strains of *Y. pestis* representing seven out of eight belonging to subsp. *microtus* biovars, such as caucasica (78), altaica (17), qinghaiensis (2), xilingolensis (3), hissarica (4), talassica (4), and ulegeica (11) [4], as well as belonging to the main subsp. *pestis* a vaccine strain EV line NIIEG (bv. orientalis) and a wild type strain 231 (bv. antique). Characteristics of the strains used for testing of serologic cross-reactivity and cross-protection are shown in Table 1. In addition, avirulent bacteria, Pgm− vaccine strain EV, as well as strains C-376pCD1− and C-824pCD1−, depleted
of the low-calcium-response virulence plasmid were used for all Y. pestis-derived Caf1 preparations.

Bacteria were grown at 28°C for 48 h on brain heart infusion (BHI; HiMedia Laboratories) supplemented with 2% agar at pH 7.2. For Caf1 isolation and purification, bacteria were grown at 37°C in New Brunswick Scientific fermenters with working volumes up to 10 L of liquid aerated media. Growth medium was BHI supplemented with 0.5% yeast extract (Difco). Acidity and oxygen levels were controlled with a specified pO$_2$ value $>10%$. Biomasses were harvested by centrifugation after 48 h.

All handling of samples containing live wild-type Y. pestis isolates was performed in a select agent authorized BSL3 facility under protocols approved by the State Research Center for Applied Microbiology and Biotechnology Institutional Biosafety Committee.

### Sequencing of caf1 genes

The nucleotide sequence of each caf1 gene was determined by direct sequencing of the PCR fragment obtained after amplification of the part of caf1 operon of the corresponding strain. The primers caf1-F (5’-GAATTTGTTCGTGGATTGGA-3’) and caf1-R (5’-TTAAGGAGGGCATATAGC-3’), both flanking the caf1 gene, were located within the caf1A and YPMT1.85 (similar to a fragment of integrase) genes, respectively, and were used for both fragment amplification and direct sequencing. Determined sequences of the caf1 genes were deposited to the GenBank (accession numbers KP641181.1-KP641299.1) and compared to reported sequences of this gene in other Y. pestis strains.)
Intrinsic disorder analysis

Amino acid sequences of three Caf1 isoforms were analyzed for the effect of the polymorphism on the intrinsic disorder propensities of related proteins. The intrinsic disorder of these Caf1 isoforms were evaluated by three disorder predictors, PONDR® VSL2 [21], which is one of the more accurate stand-alone disorder predictors [21–23], PONDR® VLXT [24], which is not the most accurate disorder predictor but has a high sensitivity to local sequence peculiarities which are often associated with the disorder-based interaction sites [25, 26], and a metapredictor PONDR® FIT [27], which is more accurate than each of its component predictors, PONDR® VLXT [24], PONDR® VSL2 [21], PONDR® VL3 [28], FoldIndex [29], and IUPred [30].

Since intrinsically disordered proteins or proteins with intrinsically disordered regions are frequently involved in protein-protein interactions and molecular recognitions [25, 31–43] and undergo at least partial disorder-to-order transitions upon binding [25, 33, 43–50], these potential disorder-based binding sites can be identified by various computational means, such as the ANCHOR algorithm [51, 52].

Isolation and purification of Caf1 isoforms

Avirulent bacteria, Pgm− vaccine strain EV, as well as strains C-376pCD1− and C-824pCD1−, depleted of the low-calcium response virulence plasmid were used for all Y. pestis-derived Caf1 preparations. Cell-free Caf1 was extracted directly from the supernatants of Y. pestis broth cultures and purified by chromatography. Clarified Caf1 supernatant was slowly mixed with 4 M ammonia sulfate (AS) solution to achieve a 1 M final concentration, and precipitate was collected by centrifugation at 15000 × g for 30 min at 4°C. A HiPrep Phenyl FF (High Sub) 16/10 column (GE Healthcare) was used for the initial chromatography step. Prior to sample loading, the column was equilibrated with 5 column volumes of a 20 mM Tris buffer, supplemented with 1 M AS (pH 8.0). Clarified supernatant was loaded and subjected to the following steps: 4 column volumes 1 M AS + 20 mM Tris, pH 8.0; 100%– 0% AS + 20 mM Tris, pH 8.0 over ten column volumes; hold with 0% AS buffer for 4 column volumes. Ten mL fractions were collected for each elution step. All fractions were analyzed using 12.5% SDS-PAGE. The fractions containing protein Caf1 were combined and concentrated by a second passage through a hydrophobic interaction chromatography (HIC) column. Protein was concentrated by a single step elution by 20 mM Tris, pH 8.0. The fractions were desalted on a XK 26/30 column (GE Healthcare) packed with Toyopearl HW-40F chromatographic media (Tosoh Bioscience) and pre-equilibrated with 20 mM Tris, pH 8.0. Ten mL fractions were collected, and purified Caf1 protein was concentrated using Millipore YM-10 membrane for a subsequent storage at -70°C until used.

Ethics Statement

All protocols for animal experiments were approved by the State Research Center for Applied Microbiology and Biotechnology Bioethics Committee ( Permit No: VP-2015/2) and were performed in compliance with the NIH Animal Welfare Insurance #A5476-01 issued on 02/07/2007, and the European Union guidelines and regulations on handling, care and protection of Laboratory Animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm).

Mice

Seven week old female BALB/c mice were purchased from Laboratory Animals Breeding Center (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russia), housed in polycarbonate cages, and maintained in light-controlled (lights on from 7:00 to 19:00) BSL3 room...
at the State Research Center for Applied Microbiology and Biotechnology. The temperature and the humidity of the animal room were maintained at 22°C ± 2°C and 50% ± 10%, respectively. Mice were given tap water and mouse mixed fodder PK-120 (Laboratorkorm, Russia) ad libitum throughout the study. The number of mice for experiments used the minimum number of the necessity. The mice were divided into all groups randomly. In this study, we have used humane endpoints for the infected animals. According to the animal protocol the mice should be euthanized in the animal survival studies, when they became either of the following: lethargic, dehydrated, moribund, unable to rise, non-responsive to touch, or lost more than 10% body mass. Humane euthanasia, CO₂ exposure (anesthesia) using compressed CO₂ gas followed by cervical dislocation has been used by well-trained individuals. We have monitored the health condition of the animals at least twice a day. There was no unexpected death during the entire set of experiments.

**Animal immunization**

Mice were randomly divided into four groups (n = 96 per group) and vaccinated subcutaneously (s.c.) with 10 μg of each Caf1 isoform in 0.1 mL PBS (pH 7.2) adsorbed (1:10, w/w) to the vehicle, aluminum hydroxide gel colloidal suspension (Sigma, USA), or only with the vehicle in PBS as a negative control (placebo). After 30 days, the animals were boosted with an identical dose of the same antigen into the same inoculation site.

**Serologic cross-reactivity**

Immunochromatographic rapid diagnostic test for plague that utilizes the anti- _Y. pestis_ Caf1NT3 monoclonal antibody F19 (State Research Center for Applied Microbiology and Biotechnology, Russia).

Blood samples were obtained under anesthesia with CO₂ gas by retro-orbital route by well-trained individuals. Antibody titers were determined by indirect ELISA a day before and 43 days after the second Caf1 immunization individually in five randomly selected animals from each group of 96 mice immunized with one of the Caf1 isoforms, and the mean titer was calculated. Microtiter plates (Greiner Bio-One, Austria) were coated with 100 ng/well of Caf1 in 0.1 M sodium bicarbonate buffer (pH 9.6) overnight at 4°C. Non-specific binding was blocked with 3% gelatin from cold water fish skin (Sigma) in 0.01 M PBS, pH 7.2. Test sera were added using 2.5-fold serial dilutions in 0.01 M PBS buffer containing 0.05% tween-20 (PBST) and incubated for 2 h at 37°C. After four washes with 0.01 M PBST, 100 μl of sheep anti-mouse IgG conjugated to horseradish peroxidase (GE Healthcare) at a dilution of 1:4000 was added for 1.5 h at 37°C. The plates were washed with PBST and 100 μl of 0.01% o-phenylenediamine-H₂O₂ was added to each well. The reaction was stopped by the addition of 100 μl of 1 M H₂SO₄ per well, and OD was read at 450 nm using EVOLIS Twin Plus System (BIO-RAD, USA). The titer of antibodies was estimated as the maximum dilution of serum giving an OD reading that exceeded the background by 0.1. Background values were obtained from serum samples collected from the animals injected with the vehicle alone.

**Cross-protection**

The ability of an antigen isoform to protect an animal from death after administration of a high dose of a virulent strain producing a different isoform of the same antigen, designated Immunity Index (II) was calculated as the ratio:

\[
II = \frac{LD_{50\text{imm}}}{LD_{50\text{veh}}} \tag{1}
\]
where LD$_{50_{imm}}$ is LD$_{50}$ for animals immunized with an antigen under the study; LD$_{50_{veh}}$ is LD$_{50}$ for vehicle-treated animals.

To estimate LD$_{50}$, 45 days after the booster dose, four groups of mice were infected with virulent strains producing either of the Caf1 isoforms (See Table 1). Each group was subdivided into three subgroups of 32 mice that were challenged with 10-fold dilutions of virulent strains of *Y. pestis* (231 (NT1), C-376 (NT2), and C-824 (NT3)), (a. $2 \times 10^3$ to 2 LD$_{50}$; eight mice for a dose) subcutaneously (in the interior thigh). Animals that succumbed to infection were sacrificed and examined bacteriologically to verify that infection was the cause of death. The remaining animals were observed for three weeks. The animals that survived were humanely euthanized.

### Statistical methods

Data on ELISA were expressed as means ±SEM (standard error of the mean). The LD$_{50}$ and a 95% confidence intervals of the virulent strains for immunized and naïve animals were calculated using the Kärber method [53]. Mortality timeframes were recorded, and the mean life to death time span was calculated for each treatment group. Comparison of the survival curves was performed using Log-rank (Mantel-Cox) test. A *P* value below 0.05 was considered to be significant.

### Results

#### Comparison of the Caf1 antigen sequence heterogeneity

Sequencing of *cafl* genes from 119 *Y. pestis* strains belonging to different biovars within subsp. *microtus* showed that the Caf1 proteins possess three isoforms, the global allele type NT1 (Ala48 Phe117), NT2 type (Ser48 Phe117) peculiar to Transcaucasian highland and Pre-Araks natural plague foci, and a novel NT3 type (Ala48 Val117) endemic for Dagestan-highland natural plague focus. Fig 1 represents the results of multiple sequence alignment of Caf1 isoforms analyzed in this study and shows that the Caf1$_{NT2}$ found in Transcaucasian highland and Pre-Araks natural plague foci is different from the major allele isoform Caf1$_{NT1}$ by having an Ala48→Ser48 substitution, whereas Caf1$_{NT3}$ protein isolated from the Dagestan-highland natural plague focus has a Phe117→Val117 substitution.

![Fig 1](https://example.com/fig1.png)

Fig 1. Multiple sequence alignment of the isoforms of Caf1 protein found in different *Y. pestis* strains.

doi:10.1371/journal.pone.0162308.g001
Intrinsic disorder

To understand if found Caf1 polymorphism has an effect on structural and functional properties of this protein, we evaluated the disorder propensities of Caf1<sub>NT1</sub>, Caf1<sub>NT2</sub>, and Caf1<sub>NT3</sub> isoforms and analyzed the effect of corresponding amino acid substitutions on potential disorder-based binding sites. Results of these analyses are summarized in Fig 2 that compares the disorder profiles obtained for the Caf1 isoforms by PONDR<sup>VSL2</sup> (Fig 2A), PONDR-FIT (Fig 2A) and PONDR<sup>VLXT</sup> algorithms (Fig 2B). This analysis revealed that, although Caf1 is predicted to be mostly ordered protein, it has several disordered regions. Curiously, both substitutions found in Caf1 (the Ala48→Ser48 in Caf1<sub>NT2</sub> and the Phe117→Val117 in Caf1<sub>NT3</sub>) cause noticeable increase in the local intrinsic disorder propensity of the short regions surrounding the corresponding substitutions. Importantly, Fig 2 clearly shows that although the effects of these substitutions on the intrinsic disorder propensities of the Caf1 isoforms are not very strong, there is a reasonable agreement between the results obtained by the three computational tools.

Next, we evaluated the presence of potential disorder-based binding regions in various Caf1 isoforms using the ANCHOR algorithm [51, 52] that utilizes the following criteria: (i) residues of potential disorder-based region belong to a long disordered segment and are not a part of a globular domain; (ii) residues of such a region are not able to form enough favorable contacts with its own local sequential neighbors to fold; (iii) these potential binding residues can form enough favorable interactions with globular proteins upon binding [51, 52]. This algorithm also filters out potential disorder-based regions shorter than six residues. Fig 2B represents the results of this analysis and shows that the Caf1<sub>NT3</sub> and Caf1<sub>NT2</sub> have a very short potential binding site (region 140–142) which was filtered out by the algorithm because of its small size. On the other hand, the Phe117→Val117 substitution found in Caf1<sub>NT3</sub> causes an extension of this site to 4 residues (140–144). Since this length is below the length threshold utilized by ANCHOR algorithm, this potential binding site was also filtered out. Fig 2B also shows that there is some delocalization in the effects of the Phe117→Val117 substitution on the disorder propensity and on the disorder-based binding potential.

Isolation and purification of Caf1 isoforms

Hydrophobic chromatography allowed for isolation of highly purified Caf1<sub>NT1</sub>, Caf1<sub>NT2</sub> isoforms as peaks at 350 mM of the ammonium sulphate gradient, while the Caf1<sub>NT3</sub> elution was spread between 600 and 50 mM under the same elution conditions.

Serologic cross-reactivity

Serologic cross-reactivity has been tested by both immunochromatography and ELISA techniques. The detection limit of immunochromatography was 1.0 ng/mL with the range of 1.0 ng-1.0 μg/mL. Results obtained were identical for all the three isoforms, indicating that all of them could be readily detected by the antibody used in this method.

Fig 3 shows that, after both steps of immunization, the antibody titers estimated by ELISA were 4–7 times higher in the animals immunized by the Caf1<sub>NT1</sub> isof orm, regardless of the antigen adsorbed on the plates. The antibody response to immunization with Caf1<sub>NT3</sub> isof orm was the lowest. All the three isoforms absorbed to the plastic were recognized by the sera of immunized animals.

Interestingly, the booster immunizations resulted in a somewhat lower antibody response.
Yersinia pestis Caf1 Protein Sequence Polymorphism

**A**

![Graph A](image1)

**B**

![Graph B](image2)

**C**

| COLY_YERPE | FGTE_SALTY | OMPT_ECO57 |
|------------|------------|------------|
| ASSQLINISPSFVASTGMLSGLSKHMLDAETG-RKISQDLWIKNVAILKGDISW | --------------- | --------------- |
| --------------- | --------------- | --------------- |
| DPLLQTLNARGMTTSLSASGSGNYVDNENQSE-WTDHSSHPATVNHANEYDNLKV | DPYSFLTLNARGMTTSLSASGSGNYVDNENQSE-WTDHSSHPATVNHANEYDNLKV | DPYSFLTLNARGMTTSLSASGSGNYVDNENQSE-WTDHSSHPATVNHANEYDNLKV |
| --------------- | --------------- | --------------- |
| WLLQDENTRAIAGTAGYQETRFSWATAGGSYSUNNGA-YYYGNFFIFGRVYGRYQRFEM | WLLQDENTRAIAGTAGYQETRFSWATAGGSYSUNNGA-YYYGNFFIFGRVYGRYQRFEM | WLLQDENTRAIAGTAGYQETRFSWATAGGSYSUNNGA-YYYGNFFIFGRVYGRYQRFEM |
| --------------- | --------------- | --------------- |
| PGIPEYVYRLMGAGYQERSYTRARGSYTYSSHEGFRND1GSFPGERA1GQVFQFEFM | PGIPEYVYRLMGAGYQERSYTRARGSYTYSSHEGFRND1GSFPGERA1GQVFQFEFM | PGIPEYVYRLMGAGYQERSYTRARGSYTYSSHEGFRND1GSFPGERA1GQVFQFEFM |
| --------------- | --------------- | --------------- |
| PYIGLACQYQINDFELNALFKEFSWVRAHNDENEH-MBDLTFREKTSGRYTYQINAG | PYIGLACQYQINDFELNALFKEFSWVRAHNDENEH-MBDLTFREKTSGRYTYQINAG | PYIGLACQYQINDFELNALFKEFSWVRAHNDENEH-MBDLTFREKTSGRYTYQINAG |
| --------------- | --------------- | --------------- |
| YTVTNAKVFYAEFTYXDEKGGQTIDNSGDSYS1GCGDAG1SNKNTTYPAGLQYRF | YTVTNAKVFYAEFTYXDEKGGQTIDNSGDSYS1GCGDAG1SNKNTTYPAGLQYRF | YTVTNAKVFYAEFTYXDEKGGQTIDNSGDSYS1GCGDAG1SNKNTTYPAGLQYRF |
| --------------- | --------------- | --------------- |

PLOS ONE | DOI:10.1371/journal.pone.0162308 September 8, 2016 8 / 16
Cross-protection

The results of cross-protection testing are shown in Fig 4 and Table 2. Since the strain 231 was significantly more virulent than the strains C-376 and C-824, the animals were given equal
The NT1 isoform that has been traditionally used in commercial and experimental vaccines proved to be the most protective one. This isoform was 100% protective in mice challenged with 2000 LD$_{50}$ of the strain 231 (100%), 85% when C-376 strain was used, and 40% mice were protected when infected with the C-824 strain (Fig 4). Thus, immunization with NT1 isoform partly protected against infection induced by NT1 and NT2 carriers even at a high dose. Vaccination with NT2 protected from infection induced by NT2 carriers even at a high dose. Vaccination with NT2 protected from infection induced by NT2 carriers even at a high dose.

\[ \text{LD}_{50} \]

numbers of corresponding LD$_{50}$. The NT1 isoform that has been traditionally used in commercial and experimental vaccines proved to be the most protective one. This isoform was 100% protective in mice challenged with 2000 LD$_{50}$ of the strain 231 (100%), 85% when C-376 strain was used, and 40% mice were protected when infected with the C-824 strain (Fig 4). Thus, immunization with NT1 isoform partly protected against infection induced by NT1 and NT2 carriers even at a high dose. Vaccination with NT2 protected from infection induced by NT2 carriers even at a high dose.

| Challenge with Y. pestis strain | Virulence in vehicle-treated mice | Virulence and indices of immunity in mice vaccinated with Caf1 from Y. pestis strain |
|-------------------------------|---------------------------------|--------------------------------------------------------------------------------|
|                               | EV (Caf1$_{NT1}$)              | C-376 (Caf1$_{NT2}$)          | C-824 (Caf1$_{NT3}$) |
| LD$_{50}$ (c.f. u.)*           | Mean time to death              | LD$_{50}$ (c.f. u.)          | Mean time to death  | LD$_{50}$ (c.f. u.) | Mean time to death |
|                               | (days)*                         | (days)                        | (days)             | (days)             | (days)             |
| 231                           | 6                               | 4.6±0.6                       | >3.2×10$^4$        | NA                 | >5334              | 562                | 9.2±1.0            | 94                 | 3.2×10$^5$         | 7.7±1.4            | 527                |
| (Caf1$_{NT1}$)                | 1÷22                            |                               |                    |                    |                    |                    |                    |                    |                    |                    |
| C-376                         | 178                             | 7.8±1.3                       | 7.1×10$^5$         | 6                  | 3996               | 2.2×10$^6$          | 9.3±1.4            | 1264              | 4.0×10$^6$         | 7.8±1.3            | 225                |
| (Caf1$_{NT2}$)                | 45÷708                          | 2.2×10$^5$÷5.6×10$^6$         | 120×2.7            | 1125              | 1.1×10$^6$         | 9.3±1.0            | 1125              | 2.0×10$^6$         | 13                | 2000               |
| C-824                         | 10$^3$                          | 6.3±1.0                       | 1.1×10$^6$         | 12.0×2.7          | 1125              | 1.1×10$^6$         | 9.3±1.0            | 1125              | 2.0×10$^6$         | 13                | 2000               |
| (Caf1$_{NT3}$)                | 251÷3981                        | 2.8×10$^5$÷5.6×10$^7$         | 2.8×10$^5$÷5.6×10$^7$ | 5.0×10$^5$÷1.3×10$^7$ |

*Values are given as means ±95% confidence intervals.

Table 2. Indices of immunity (II) induced by the three Caf1 isoforms.

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doi:10.1371/journal.pone.0162308.g004

doi:10.1371/journal.pone.0162308.t002

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and NT3, but was less effective against NT1 carriers. At the dose equal to 20 LD$_{50}$ of any of the three strains tested, all animals vaccinated with Caf1$_{NT1}$ survived. This data suggests that vaccination with the NT1 isoform of Caf1 provides a better protection against all the three $Y$. $pestis$ variants. All the vehicle-treated mice died within a week after infection regardless of the bacterial stain (data not shown).

Vaccination with the Caf1$_{NT1}$ isoform produced a level of protection that exceeded 5334 times that of control (vehicle-treated) mice. The other two isoforms were less potent resulting in only a 1264- to 2000-fold increase in self-resistance. Thus, calculated indices of immunity are in agreement with the data obtained from the survival curves, suggesting that Caf1$_{NT1}$ vaccination is superior compared to the other two isoforms.

**Discussion**

Recently, it has been demonstrated that some of $Y$. $pestis$ strains isolated from Georgian and Armenian natural plague foci had the NT2 allele of caf1 gene differing from the typical NT1 allele by a single nucleotide replacement causing a substitution of alanine by serine (Ala48 $\rightarrow$ Ser48) [15–17]. Our investigations have proven that this replacement is characteristic of all bv. caucasica strains from Transcaucasian-highland plague foci # 4–6 and bv. caucasica isolates from Pre-Araks natural plague focus # 7. It is possible that similar strains are circulating in neighboring territories of Turkey and Iran. We also found a novel NT3 allele (Ala48 Val117) endemic for Dagestan-highland natural plague focus # 39. But the classic Caf1$_{NT1}$ isoform (Ala48 Phe117) remains the major type within the rest of $Y$. $pestis$.

*In silico* analysis of amino-acid sequence of Caf1$_{NT1}$ performed in several laboratories by different methods aimed at location of B- and T-cell epitopes generated conflicting data [54–57]. Information from the laboratory of Dr. D.N. Rao seems to be the most reliable, as it was confirmed by immunoassays and *in vivo* experiments [54].

Our computational analysis revealed that the Ala48 $\rightarrow$ Ser48 and Phe117 $\rightarrow$ Val117 substitutions found in the Caf1$_{NT2}$ and Caf1$_{NT3}$ isoforms, respectively, have some effects on the local intrinsic disorder propensities of these proteins. Both substitutions cause noticeable increase in the intrinsic disorder propensity, but only latter is expected to have some effect on the disorder-based interactivity of this protein. Even in this case, mutation-induced increase in interactivity is minimal, although behavior of the isoform during hydrophobic chromatography is different: the Caf1$_{NT3}$ elution is spread between 600 and 50 mM of the ammonium sulfate gradient, while Caf1$_{NT1}$ and Caf1$_{NT2}$ isoforms are peaking at 350 mM.

It has been shown that Caf1$_{NT1}$ isoform of capsular protein protects bacteria from phagocytosis [58] and exhausts the complement system by selective activation of C`2 and C`4 components thus preventing complement-mediated opsonization of bacteria [59]. On the other hand, it is one of immunodominant antigens responsible for protective immunity against plague [8, 60, 61]. Accordingly, it has been used as the major molecular target for immunodiagnostics, and as a principal component of the vaccines [8, 60–63]. Important questions of whether Caf1$_{NT1}$-mediated protective immunity can be circumvented by the strains that carry NT2 and NT3 alleles of caf1 gene, and whether current immunoassays can detect Caf1$_{NT2}$- and Caf1$_{NT3}$-producing bacteria have not been addressed before.

In our cross-protection studies, the level of immune response achieved was described in terms of the immunity index. This index represents the difference in challenge dose required to cause death in immunized versus naïve animals (Table 2). Similar data were obtained from the analysis of survival curves (Fig 4). Taking into account that infected fleas can contain up to 5000 $Y$. $pestis$ CFU, but the median number of transmitted bacteria is 82 CFU [2], we can predict that the immunity induced by Caf1$_{NT1}$ isoform is sufficient for protection from a low-dose
flea-borne infection caused not only by a strain with the same isoform, but also by isolates producing Caf1\textsubscript{NT2} and Caf1\textsubscript{NT3} variants. However, this low dose cross-protection may be insufficient in case of infection with high doses of strains producing Caf1\textsubscript{NT2} or Caf1\textsubscript{NT3}.

The antibody titers positively correlated to the immunity index (Fig 5). For instance, the index of immunity in the Caf1\textsubscript{NT1} immunized group was higher than that in the other groups, and these mice had higher antibody titers. In contrast, mice immunized with Caf1\textsubscript{NT2} or Caf1\textsubscript{NT3} had lower immunity index and lower immunoreactivity in ELISA.

All the \textit{Y. pestis} Caf1 protein isoforms tested demonstrated a strong serological cross-reactivity as judged by immunochromatography, suggesting that the monoclonal antibody used in this assay is specific to a shared epitope. It may be reasonable to suggest including the strains with Caf1 endemic isoforms for validation of newly developed immune tests targeting this protein.

The antibody response to the immunizations for all three Caf1 isoforms seems to be lower after the second immunization. We can speculate that this phenomenon may be due to the
different terms of antibody-titer measurement after the first and second immunizations, i.e. post-immunization-exposure increase from the four to six weeks was sufficient for onset of depletion of humoral immune response. Another supposition is that the antibody titers measured by ELISA went down after the booster immunizations might be due to the action of regulatory cells. Noteworthy, they were higher after the first vaccination with the NT1 isoform, suggesting that this standard vaccine protein has a superior immunogenicity compared to the minor isoforms. However, after the boost, this difference substantially disappeared, indicating that NT2 and NT3 proteins can also be used for a vaccine, given that immunization is performed in two subsequent injections. These two seemingly less immunogenic isoforms may in fact prove to be better vaccines and induce a longer lasting immunity to *Y. pestis* due to a lower capacity to induce regulatory cells [64]. To test these possibilities, more experiments have to be performed.

**Conclusions**

The main question addressed in this study was whether specific immunity induced by *Y. pestis* Caf1<sub>NT1</sub> vaccine can be protective against *Y. pestis* strains harboring NT2 or NT3 allele types. Our data clearly demonstrate that animals vaccinated with Caf1<sub>NT1</sub> acquire immunity to all the three bacterial strains tested. Therefore, polymorphism in the caf1 cannot be considered a sufficient instrument that would allow *Y. pestis* an escape from Caf1<sub>NT1</sub>-mediated anti-plague immunity in the case of a low-dose flea-borne infection.

**Acknowledgments**

This research was supported by the Russian Science Foundation (grant 14-15-00599). The authors express their acknowledgment to Dr. Rima Z. Shaikhutdinova for her help and care during the animal experiments.

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