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Stability of variable number of tandem repeat (VNTR) markers in *Francisella tularensis*

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Abstract

The intracellular bacterium *Francisella tularensis* causes the zoonosis tularemia. It is of medical as well as epidemiological interest that a bacterial isolate can be identified on strain level. *F. tularensis* strains can be discriminated through variable number of tandem repeat (VNTR) analysis. The aim of this study was to investigate the stability of a selection of VNTRs in *F. tularensis* live vaccine strain (LVS). Stress may affect the expression of virulence factors and bacteria were therefore subjected to; low pH, heat stress, nutrient limitation or growth in amoeba. The VNTR analysis on DNA from these bacteria showed no changes in repeat copy numbers of the selected markers. Variation was not triggered by these stress conditions. Thus, the identification of strains based on VNTRs seems to be a reliable method.

Keywords

VNTR, *Francisella tularensis*, genotyping, slipped-strand mispairing, amoeba

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Stability of variable number of tandem repeat (VNTR) markers in *Francisella tularensis*

Eva Arvidsson

Sammanfattning

Det är viktigt att kunna identifiera farliga mikroorganismer som kan orsaka sjukdom, detta för att veta vilken skada de kan orsaka och därmed hur man ska agera. Man vill också kartlägga hur mikroorganismen förändras och hur den ser ut på olika platser i världen. I den finaste indelningen av mikroorganismer grupperas de i olika stammar. För noggrann identifiering kan man gå ner på DNA-nivå och därigenom få möjlighet att åtskilja till synes lika organismer som hittats vid olika tillfällen (olika ”isolat”).

Detta examensarbete behandlar stabiliteten hos en viss DNA-struktur, med vars hjälp man kan åtskilja olika stammar av till exempel bakterien *Francisella tularensis* som orsakar harpest. Dessa platser i bakteriens DNA ser olika ut hos olika stammar, de utgör ett slags fingeravtryck. Vad som får DNA-strukturen att förändras vet man inte. I det här arbetet användes olika stressfaktorer för att se om ändringar uppstod medan bakterierna odlades. De utsattes för ändrade pH-värden, frånvaro av näringsämnet järn eller ändrade temperaturer. En del bakterier fick växa inuti amöbaceller (experter på nedbrytning av mikro-organismer) en tid. Resultatet blev att dessa stressbehandlingar inte orsakade ändringar i DNA-strukturen. Det betyder att identifieringsmetoden troligtvis är pålitlig och kan användas i framtiden för snabb och säker identifiering.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet september 2002

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1. Introduction

More than 600 people in Northern Sweden were infected when moving hay in 1966-67. The symptoms were general, such as high temperature, fatigue, malaise and severe headache. Many people showed signs of pneumonia or other respiratory symptoms. Rather soon it was shown to be tularemia. Transmission of this largest recorded outbreak of airborne tularemia was through inhalation of dust from hay contaminated with vole faeces [1, 2].

1.1 *Francisella tularensis* and tularemia

The etiological agent of tularemia, or rabbit fever, *Francisella tularensis*, is a gram-negative, facultative intracellular bacterium. Of its four subspecies only *Francisella tularensis* subsp. *tularensis*, present in North America and also in Slovakia, causes a life-threatening disease in humans. Subspecies *holarctica* has been isolated in Europe, North America and Japan whereas *mediaasiatica* is found only in the Central Asian republics of the USSR [3]. The fourth subspecies *novicida* was isolated from a water sample in Utah 1950. The geographical spread of tularemia is shown in Figure 1.

As a zoonosis, the disease is transmitted from animals to man but not from one human to another. The bacterium occurs naturally in rabbits, hares and rodents; especially microtine such as voles, vole rats and muskrats, as well as beavers. In addition a wide variety of other mammals have been reported to be infected. Transmission to humans occurs through direct contact with infected animals, contaminated water or food, or from vectors such as biting insects or ticks. Airborne transmission is also possible, especially during handling of agricultural products.

Clinical manifestations of tularemia are related to the route of infection. Thus, cutaneous inoculation results in ulceroglandular tularemia, entry through inhalation in an oculoglandular or a pneumonic form and ingestion of bacteria induces oropharyngeal tularemia or the intestinal form. Often though, more than one set of symptoms may be present in the same patient. The disease often begins abruptly with non-specific symptoms such as fever, chills, headache and generalized aches. One form of tularemia lacks early signs and symptoms, making it difficult to diagnose. The typhoidal form is less common but likely to be rapidly fatal [4, 5]. The ulceroglandular form accounts for 80% of clinical illness while the pneumonic form is seen most commonly among laboratory workers obtaining the infection through aerosolized bacteria [6].

There is currently no vaccine available, although laboratory workers have been immunised [2] with an attenuated, live vaccin strain (LVS), which is poorly characterized and not fully protective [7]. Before the invention of antibiotics the mortality rate from infection with the more virulent subspecies *tularensis* was up to 30% [8]. Treatment of tularemia is most successful using the antibiotics streptomycin or gentamicin [6]. Owing to the use of antibiotics the overall mortality is now as low as 2-4% [6]. In addition, it has been reported from the U.S. that incidence has dropped significally since the 1940s, mainly thanks to a decrease in animal trapping and hunting [4].

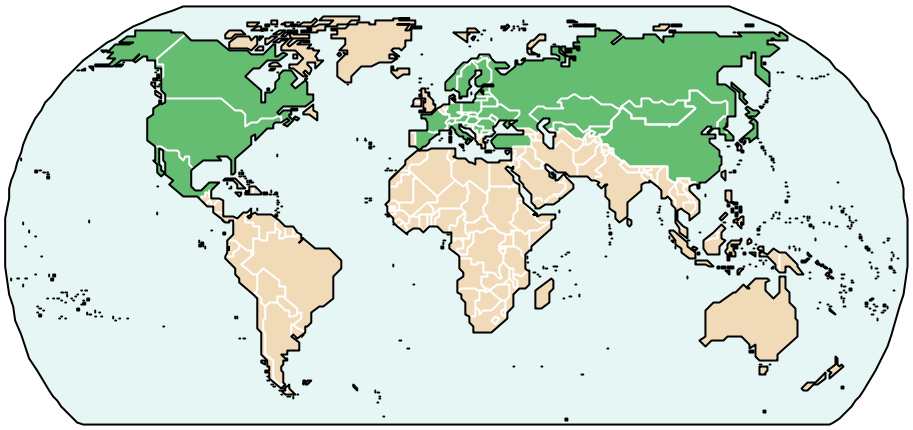

Figure 1. Geographical distribution of tularemia. Only the northern hemisphere is subjected to the disease. (With permission by A. Johansson (2002).)

When bioterrorism is mentioned, after 11th of September 2001 most people think of anthrax and the causing agent *Bacillus anthracis*. However, several more bacteria and viruses group into this category of potential biological weapons. One of them is *Francisella tularensis* and in fact it is more infectious than *B. anthracis* since inhalation of as little as 10-50 organisms [6] of subspecies *tularensis* is sufficient to cause disease in human. This dose is compared to 2 500 – 55 000 particles to cause anthrax [9]. Other microorganisms that are possible to be utilized as biological weapons are *Brucella* species (brucellosis), *Clostridium botulinum* (botulism), *Yersinia pestis* (plague) and variola major (smallpox) [10]. Several reasons make them potent weapons: they can be dispersed through aerosols, the microorganisms and their by-products are stable in the environment, large quantities can be easily manufactured, the civilian population is highly susceptible and the diseases are difficult to diagnose as they occur only rarely [10], e.g. during the last decade about 100 cases of tularemia per year were reported in the United States [11]. Knowledge about *F. tularensis* is limited and in particular concerning its virulence [6]. Therefore, being a potential biological weapon it is important to have more knowledge about this organism.

1.2 Microorganism typing

The identity of a pathogen is wanted of medical as well as epidemiological reasons. While in medicine species identification is important for prevention, diagnosis and treatment of the disease, discrimination among isolates will aid in the establishment of phylogenetic relationships [12]. Typing of microorganisms can give insight into the geographical spread of the disease, track outbreaks and advice in what preventive measures can be taken.

Diagnosing an infectious disease traditionally means depending on measuring metabolic changes induced as a result of microbial growth [13]. *F. tularensis* is difficult to grow, as it requires nutrients that may not be present in the routine media used by microbiology laboratories [6] and it needs a minimum of 48 hours to form colonies. In addition,

cultivation in order to identify *F. tularensis* on subspecies level impose a high risk of infection to the microbiologist and is generally avoided [14]. Clinical diagnosis rely on measuring antibody titers in serum. However, interpretation of *F. tularensis* serology is complicated by cross-reactions with *Brucella* species, *Proteus vulgaris* OX19 and *Yersinia* subspecies. [4, 5]. Moreover, antibodies do not appear until two weeks after infection. In addition, there are tularemia cases that give negative serology tests [14]. Thus, apart from the fact that detection processes are complicated and time-consuming, tularemia patients may be misdiagnosed. Fortunately, there are newly developed methods for more rapid and sensitive diagnosing of tularemia, making their way into routine diagnosis [14, 15].

While phenotypic methods make use of growth characteristics and usually reach to identification at species level, genotypic procedures aim to detect polymorphisms at the level of nucleic acids or to detect allelic variation at the level of enzymes [12]. These methods provide better resolution, are much safer for the microbiologist, are much faster and may be easier to interpret. Changes in the genome may occur through different mechanisms. DNA recombination can give rise to duplications, deletions or inversions and repetitive DNA sequences may cause mobile DNA fragments and polymorphisms through replication slippage [12]. By the incorporation of wrong bases DNA polymerases cause point mutations. Consequently there are interstrain differences that may be detected within various sequential structures. A genotypic method is chosen depending on the degree of conservation of the genome and what resolution is needed (as shown in Figure 2). Thus, adequate complexity of these DNA fingerprints is important.

To detect the genetic variations and different motifs there are methods utilizing e. g. the polymerase chain reaction (PCR) technique, restriction enzymes and/or hybridization techniques based on specific probes. The most detailed method is direct nucleotide sequence analysis. To be useful the methods have to meet several criteria. They must have a high differentiation power to be able to differentiate unrelated strains and to group isolates derived from the same source together. A major concern, especially for the construction of reliable databases where unknown organisms may be compared to known strains, is reproducibility. The same results must be obtained from repeated tests and different laboratories. Other considerations are ease of interpretation, ease of use, cost, and time to get the result. Below is a short outline of some methods in DNA typing, which are also compared in terms of resolution capacities in Figure 2.

1.2.1 Methods for genotyping

The most common molecular typing method is pulsed-field gel electrophoresis (PFGE), in which bacterial isolates are combined with molten agarose and poured into molds. After lysis and digestion with a restriction enzyme the plugs are inserted into an agarose gel and subjected to electrophoresis with changing current. The pulsed field allows separation of very large (10-800 kb) fragments. A fluorescent dye such as ethidium bromide is used for visualization of the banding pattern. This method is highly discriminatory although it takes 2-3 days to complete the analysis [16].

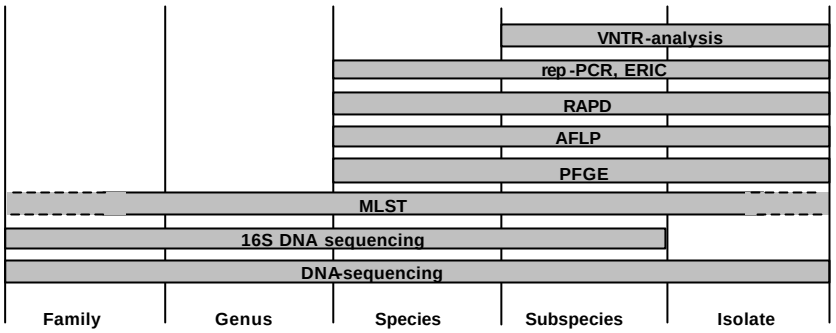

Figure 2. Resolution of some genetic typing methods.

Restriction enzymes in combination with Southern blotting or PCR is used in restriction fragment length polymorphism (RFLP). Either the fragments are transferred from the separating agarose gel to a membrane and a specific probe homologous to the gene to be examined is allowed to hybridize, or the gene of interest is amplified by PCR followed by digestion and separation on agarose gel to make out a characteristic banding pattern. The more laborious blotting techniques have largely been replaced by PCR-based locus-specific RFLP. Because of the limited region of the genome that can be examined, locus-specific RFLP does not give as good level of discrimination as other methods [16], even though it to some extent comes down to the choice of gene to be examined.

More rapid than PFGE, though still having good discriminatory power at strain level, is amplified fragment length polymorphism (AFLP). The most common procedure is allowing two enzymes to digest purified bacterial DNA and then ligating these fragments to linkers containing each restriction site and a sequence homologous to a PCR primer binding site. By designing the primers with two selective nucleotides at the 3' end it is possible to amplify a subset of the genomic restriction fragments. Patterns are visualized through primer labeling or by staining of the agarose gel. AFLP has good reproducibility, but is more laborious than REP-PCR [16].

In random amplified polymorphic DNA (RAPD) a single, random sequence primer of an ideal length of 8 to 10 bases is used with a low annealing temperature in PCR, to give DNA fragments that on an agarose gel give a characteristic banding pattern of a particular strain. Primers that anneal within a few kilobases of each other, in the proper direction, will give rise to the amplification of this DNA region. The method is useful in that it is more discriminatory than e. g. RFLP and can be used for differentiation on strain level, however reproducibility is poor [16].

Another method that is useful for strain typing is repetitive extragenic palindromic (REP)–PCR. There are two sets of repetitive elements used in bacterial typing. REP elements are highly conserved 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem. Enterobacterial repetitive intergenic consensus (ERIC) sequences are 126-bp elements containing a highly conserved central inverted repeat and are located in extragenic regions of the

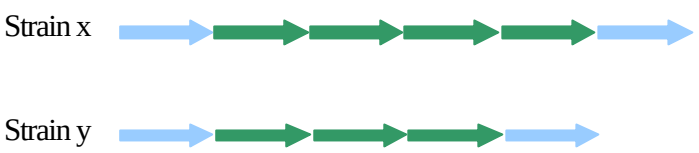

Figure 3. The principle of Variable Number of Tandem Repeats (VNTRs). Blue arrows represent flanking sequences and green arrows the DNA sequence repeated. A different number of repeated sequences is found at the same locus in different strains of the same subspecies.

bacterial genome. Patterns from either element give good strain discrimination, although not quite as good as PFGE [16].

A typing target that shows rapid evolution and is promising for discrimination of individual strains [8] is VNTR, also called simple or short sequence repeat (SSR). These are short nucleotide sequences that are repeated multiple times and they often vary in copy number (as illustrated in Figure 3), creating length polymorphisms that can easily be detected by PCR using flanking primers. Ideally, for discrimination of strains a multiple-locus VNTR analysis (MLVA) system making out a genomic fingerprint specific to each strain, is used. The high mutability of repeat copy number in tandem arrays is the cause for the rapid evolution of these DNA markers and makes them particularly useful in epidemiological analysis of the more genetically homologous microorganisms.

Multiple locus sequence tags (MLST) is another PCR-based typing method, where genetic variation from multiple chromosomal locations is utilized. To identify the variations the nucleotide sequence is determined. Generally, seven to ten genes with house-keeping functions are selected and a stretch of ~500 bp in each is sufficient for detecting minor sequence differences. MLST identifies variation that accumulates slowly within a population and is particularly suited for long-term and global epidemiology [17]. The resolution of this method is thus dependent on the choice of chromosomal loci and the conservation of the bacterial genome. There are hopes for the future in the development of efficient pathogen typing by analysing single-nucleotide polymorphisms (SNPs) on hybridization arrays [18].

Another way of utilizing the sequence technology for bacterial identification is by examining sequence polymorphisms within the rRNA genes (16S and 23S). Ribosomal RNA sequence information has been successfully used in the phylogenetic grouping and discrimination of bacterial species [19] and very rarely also in the discrimination of subspecies.

The ultimate approach in epidemiological research ought to be the sequencing on entire genomes for comparison. Polymorphisms that may be over-seen by other methods would not escape a comparative genomic sequencing. However, to date the process of whole-genome sequencing is both time-consuming and costly and sequencing of small regions only, as in MLST, is more practical.

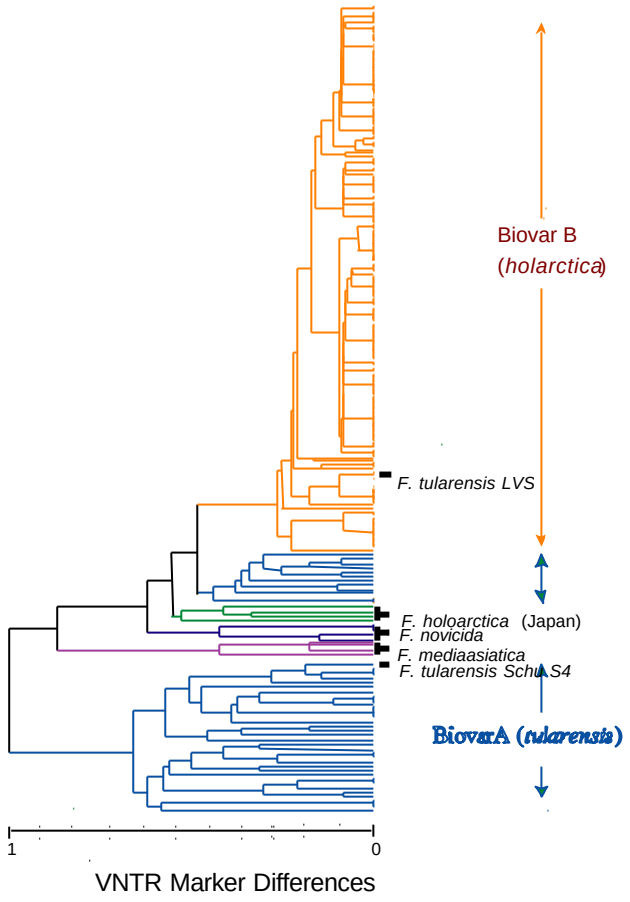

Figure 4. *Francisella tularensis* dendrogram based on VNTR information from 20 loci. *F. tularensis* LVS is the avirulent strain used for research in laboratories. *F. tularensis* Schu S4 is the model genome presently being sequenced. Biovar A is the more virulent subspecies and present predominantly in North America. Each of the four subspecies are represented and strains were derived from different outbreaks and geographical locations. (With permission by A. Johansson (2002).)

1.2.2 Typing of Francisella tularensis

Much effort has been put into the phylogenetical investigation of *F. tularensis* strains. As *Bacillus anthracis* [18], *F. tularensis* is a bacterium of high genetical homogenity [8] and therefore, it is challenging to find a suitable typing method. There are several reasons for typing on strain level: it is important to recognize outbreaks of infections, to discover cross-transmission of pathogens, to identify the source of infection and to reveal particularly virulent strains. Thus, molecular epidemiology is a base for understanding tularemia. The work on both of these bacteria has gained much from the sequencing of their genomes as this allows for a thorough search for genetic polymorphisms. Previous work on *F. tularensis* strains has resulted in the discrimination of subspecies *holarctica* from *tularensis*. However, individual strains could not be resolved [19]. REP- and ERIC-PCR analysis gave the same results [3]. Some other methods, such as RAPD, PFGE,

sequencing of the gyrB gene and RFLP (with probes for insertion sequence elements) were applied but did not show more discrimination [20]. Currently, direct repeats, MLST and VNTR typing systems on *F. tularensis* are being developed and evaluated.

Preliminary results from VNTR analysis on *F. tularensis* strains by Johansson *et al* [21] can be viewed as a phylogenetic tree (Figure 4). More branching within type A (subspecies *tularensis*) indicates a higher genetic diversity. After a dendrogram has been constructed, conclusions could be drawn about the genetic relatedness of different strains and possibly help in understanding the mechanisms of virulence. However, VNTR markers should be used most carefully in evolutionary studies [22].

1.2.3 Variable Number of Tandem Repeats

The fact that some VNTRs have functions implicates that these markers may not all be neutral. Tandem repeat regions located within genes may affect the coding potential of a transcript by causing a frame-shift, blocking of replication, transcription termination or by affecting the efficiency of transcription [22]. However, most prokaryotic repeats are found in extragenic regions [23], of which some have also been assigned different functions. One example is when a repeat region is located between the –35 and –10 sequences of a procaryotic promoter, the efficiency of the promoter is drastically altered by a change in spacing between the two elements [22]. Thus, some VNTRs have functions in genetic regulation and it is speculated that these are shorter (one to six nucleotides) repeats while variability in longer (units of more than 15 nucleotides) repeats has been found in close relationship with alterations in pathogenicity and antigenic variation [23]. This can be seen in cell wall or membrane associated proteins, which in response to repeat variation change the degree of cell surface exposure of the active protein domain [23].

Repeat sequence regions are often important in adaptive behaviour in response to environmental changes [23] and consequently genetic variations at repeat DNA loci may be forced through environmental pressure [22]. For example, the minimal genome hypothesis states that bacteria are under pressure to maintain a minimal functional genome to be able to replicate faster [24, 25] and thus should repeat deletions be favoured over insertions. Also, the capacity for epidemic spread of a microorganism may be affected by the involvement of some tandem repeat loci in gene regulation [22]. Thus, one reason to take caution in using these markers for studies of species evolution is that some VNTRs may evolve under the influence of selective pressure. Also, evolution of the whole genome may not be reflected by the speed of alteration at repeat DNA loci since these vary in repeat number with some frequency [22]. However, while taking into account a possible functional role of a VNTR locus studying the evolution of the tandem repeat region may be useful for monitoring short-term variability within a genome [23].

The positive aspect of choosing VNTRs with a functional role in the genome is that it may be more stable compared to repeat markers of no function.

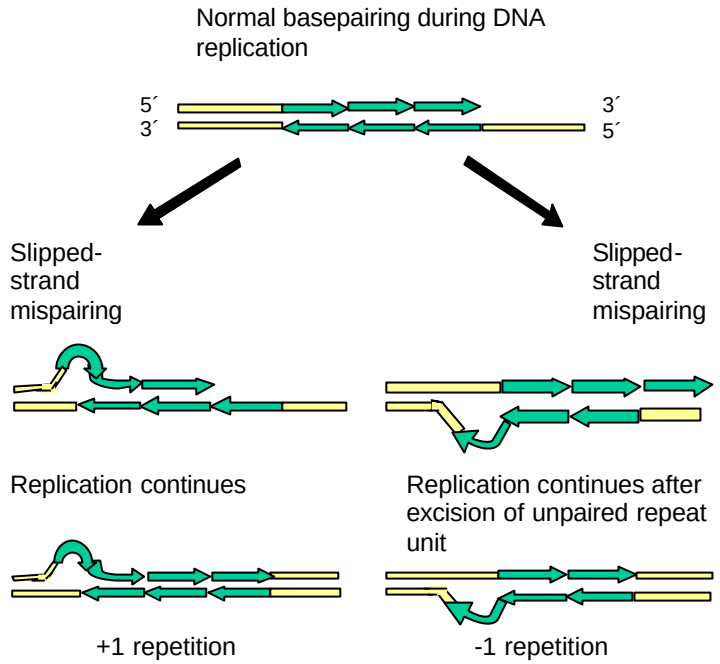

Figure 5. Slipped-Strand Mispairing (SSM), one proposed mechanism explaining the variability in number of tandem repeats. Slippage in the 3´-> 5´ direction (left) results in insertion of a repeat unit. Slippage in the other direction (right) results in deletion of a repeat unit. The deletion is the result of the excision of an unpaired repeat, presumably by the 3´-> 5´ exonuclease activity of DNA polymerase [25].

1.3 Investigating VNTR stability

To adapt the VNTR method as standard procedure for identifying isolates on strain level these markers need to be reliable. The first step on the way has been taken, by finding that this method can discriminate among *F. tularensis* isolates since the genomes of strains differ on these loci. But as these changes in repeat copy numbers occurs with some frequency, it is likely to happen again. The question is how. What is the mechanism of the changes? Is it a spontaneous event or can it be triggered?

The answers to these questions are not known. However, a few mechanisms are likely to contribute and several authors hold it possible that targeted variation within VNTR loci occurs [23].

1.3.1 Mechanisms for VNTR variability

The most accepted explanation for variation in number of repeats at VNTR loci is slipped-strand mispairing (SSM), which may act in combination with inadequate DNA mismatch repair pathways [22, 23]. Figure 5 illustrates how SSM, resulting from the slippage of DNA polymerase during replication, can cause insertions or deletions of repeat units at a VNTR locus. A local denaturation of the double strand and displacement of the strands is followed by the mispairing of complement bases [25]. Then, in this

simplest form of SSM, this results in a bulge in one of the strands, and depending on which strand, there will be either an insertion or a deletion of repeat units. A contributing factor for SSM to occur is thought to be the tertiary structure of repetitive DNA that allows mismatching of neighboring repeats [23]. In addition, one study has correlated neighboring hairpins with the frequency of SSM [23]. Also, the base composition is important, as a higher content of adenosine and thymidine will cause the helix to be more unstable [23]. Although this has not been studied thouroughly, theoretically, a lower melting profile should increase the likeliness of SSM [23]. As DNA elements such as insertion sequences (ISs) and transposons mediate genome rearrangements, they are major evolutionary players [24] that must not be overseen. An alternative explanation of repeat copy number variation is recombination between multiple loci containing homologous repeats [23]. Studies have been conducted in which variation in the size of repeated regions have been observed independently of *recA* and thus very likely to be caused by SSM [23].

In general, variation in number of repeats is supposed to happen by chance. However, seeing the repeat DNA as a possibility of an increased genetic flexibility of the organism [23] is more supportive of a triggered event. If the change in number of repeats can be induced, this may occur more frequently in some bacteria than in others, due to different environmental conditions. No factors that specifically drive the copy number variation of repeat DNA have been identified [23]. However, unknown regulatory mechanisms or factors may exist. Repeat DNA does not seem to change from routine laboratory handling [22]. An estimation of how repeat regions evolve suggests one insertion or deletion event per 1000 replications [26], giving an idea of the speed of these molecular clocks.

1.3.2 Natural reservoir

The organism survives for weeks at low temperatures in water, moist soil, hay, straw and decaying animal carcasses [2]. *F. tularensis* does not form spores and the natural reservoir is mainly unknown. Resent research [27] suggest that such a reservoir in nature might be the amoebae, in which the bacterium survives and replicates. Several other intracellular pathogens, such as Rickettsia-like, *Chlamydia*, *Legionella* and *Mycobacterium* bacteria [28], have been described in amoebae. Figure 6 shows intracellular *F. tularensis* in *Acanthamoeba castellanii*. The organism is pleomorph inside the phagocytic cell.

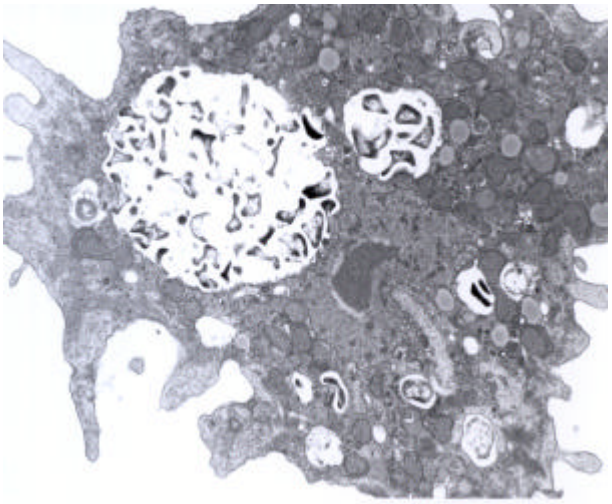

Figure 6. Transmission electron microscopy picture of amoebae with *Francisella tularensis*-containing vacuole at 5000 × magnification, taken at day 9 of infection. Picture was adapted, with permission, from Abd *et al* [27].

Organisms that live intracellularly are able to hide from the immune system and get access to nutrients, co-factors and ATP through their host cell [29]. *F. tularensis* can multiply within a number of different cells, such as macrophage [30] and amoeba [27]. Bacteria are taken into the host cell by phagocytosis. Generally, in phagocytic cells, lysosomes fuse with the phagosome, the pH drops to as low as pH 3.5-4 [31] and this alone has some antimicrobial effect. Intracellular microbes have different ways to cope with the harsh intracellular environment; some prevent lysosome fusion while others escape to the cytoplasm [32]. The defense of *F. tularensis* has not been fully investigated, although in amoebas bacteria keep within vacuoles [27] and lysosomes do not seem to fuse with the cellular compartments containing bacteria. Although *F. tularensis* is able to survive and replicate within *A. castellanii*, the intracellular conditions of amoebae may have some effect on the bacterium.

1.4 Aims

As variation in repeat DNA regions have been associated with alterations in virulence in other bacteria [23] and some VNTRs may not be neutrally evolving, the impact of stress treatment on stability of tandem repeat markers needs to be investigated. This project aimed to monitor any variation in VNTR marker sizes in *F. tularensis* LVS subjected to stress, and from those results deduce whether repeat copy number changes are spontaneous or can be triggered.

2. Materials and methods

2.1 Microorganisms

The amoebae, *Acanthamoeba castellanii* (ATCC 30234), was purchased from the American Type Culture Collection (ATCC), Rockville, MD, United States. *Francisella*

tularensis live vaccine strain (LVS, type B, ATCC 29684) was obtained from US Army Medical Research Institute of Infectious Diseases, Fort Detrik, Frederick, MD, United States. LVS containing green fluorescent protein (GFP) was constructed according to Abd *et al* [27].

2.2 Culture conditions

F. tularensis LVS/GFP was grown for at least 2 days (37ºC, 5% CO_2) on modified Thayer-Martin agar plates containing GC medium base (36 g/l, Difco Laboratories, Detroit, Michigan, United States), haemoglobin (10 g/l, Difco), VitaleX Enrichment (10 mg/l, BBL Microbiology Systems, Becton, Dickinson & Company, MD, United States) and tetracycline (10 mg/l). Bacteria subjected to starvation were put in phosphate buffered saline (PBS) at a concentration of 2×10^7 colony forming units (cfu)/ml and left for 84 days. After that time they were no longer culturable on plates. Chamberlain's medium [44], prepared with and without iron was used, for stress experiments (Table 1) with *F. tularensis* LVS. Optical density was measured at 600 nm by a UV/visible spectrophotometer (Ultrospec® 2000, Pharmacia Biotech, Sweden).

A. castellanii was grown at 30ºC to a concentration of 10^6 cells/ml in ATCC medium 712 containing protease peptone (20.0 g/l), yeast extract (1.00 g/l), distilled H_2O (900 ml/l), 0.40 M $MgSO_4$ (10.0 ml/l), 0.05 M $CaCl_2$ (8.00 ml/l), 0.005 M $Fe(NH_4)_2(SO_4)_2 \times 6\ H_2O$ (10.0 ml/l), 0.25 M $Na_2HPO_4 \times 7\ H_2O$ (10.0 ml/l), 0.25 M KH_2PO_4 (10.0 ml/l) and 2 M glucose solution (50 ml/l).

2.3 Infection of amoebae with bacteria

A. castellanii cells were counted using a Bürker chamber. For infection 4-8 × 10^5 cells/ml were required. Bacterial concentration was estimated by a CO8000 Cell Density Meter (WPA, Cambridge, United Kingdom). Absorbance = 1 corresponds to 2×10^9 cfu/ml. It was estimated that 2×10^6 bacteria/ml were ideally used to infect the amoebae. Tetracycline (10 µg/ml) was added before incubation of the co-culture for 12 days at 30ºC.

2.4 Analysis of viable *Francisella tularensis* within *A. castellanii*

Three ml suspension was taken from co-culture flask and centrifuged at 300 × g for 10 min. After the pellet was washed with FacsWash or PBS it was centrifuged once more. The amoebae pellet was resuspended in 500 µl FacsWash or PBS and then incubated with 100 µg gentamicin for one hour (kept in dark), in order to kill extracellular bacteria. Another 2.5 ml of FacsWash or PBS was added and the suspension was centrifuged as previously. The pellet was dissolved in 100 µl sodium deoxycholate (0.5 %) and incubated for 5 minutes at room temperature to lyse the amoebae. Dilutions 10^{-1}, 10^{-2} and 10^{-3} were made and 100 µl from each dilution was spread on modified Thayer-Martin agar plates. Bacteria were grown at 37ºC with 5% CO_2 for a minimum of two days before examined. Thereafter, DNA was prepared from the bacteria.

Experiment No	Iron	pH	Temp [ºC]
1	+	4	4
2	+	4	37
3	+	4	42
4	+	5.6	4
5	+	5.6	37
6	+	5.6	42
7	+	7	4
8	+	7	37
9	+	7	42
10	-	4	4
11	-	4	37
12	-	4	42
13	-	5.6	4
14	-	5.6	37
15	-	5.6	42
16	-	7	4
17	-	7	37
18	-	7	42
19	+	7	37
20	+	7	37

Table 1. Stress experiment set-up combining different values of pH with temperatures and presence of iron in the medium. The chemically defined, liquid Chamberlain's medium was used. Three samples (No's 8, 19 and 20) were identical in order to indicate the variance of the results.

2.5 Stress experiments

F. tularensis LVS/GFP$^+$ was grown to log-phase in Chamberlain´s [33] complete medium. Bacteria were then centrifuged at 3 000 × g for 10 min in 5 ml Falcon® tubes (Becton Dickinson Labware, NJ, USA) and the supernatant was removed. New medium (with or without iron and with a defined pH) was added and the tubes were placed on a shaking table at 4ºC, 37ºC or 42ºC according to Table 1. After 20 hours, absorbance was measured and culture samples were diluted and plated for viable count. DNA was then prepared from these bacteria.

2.6 Preparation of genomic DNA

Chromosomal DNA was obtained by adding 50-100 µl sample to 900 µl buffer L6 (120 g GuSCN in 100 ml 0.1 M Tris-HCl pH 6.4, 22 ml 0.2 M EDTA pH 8.0 and

2.6 g Triton-X 100) and 5 µl glassmilk (Qbiogene, Canada) and thoroughly mixed. Glassmilk is a positively charged silica particle that binds DNA at high salt concentrations and releases it at low. After 10 minutes incubation in room temperature the solution was mixed and then pelleted by centrifugation at 13 000 × g for 15 seconds. The silica pellet was then washed twice with buffer L2 (120 g GuSCN in 100 ml 0.1 M Tris-HCl pH 6.4) by adding 1 ml of L2, followed by mixing and centrifuging as above. Thereafter, the pellet was washed twice with 70% ethanol and once with acetone and then allowed to dry at 56ºC. After re-suspension of the pellet in 100 µl H_2O it was incubated for 10 minutes at 56ºC and cell debris was pelleted by centrifugation for 2 minutes. The DNA-containing supernatant was transferred to a new tube for spectrophotometric estimation of DNA concentration.

2.7 DNA markers

The highly virulent *F. tularensis* subsp. *tularensis* strain Schu S4, is presently being sequenced at FOI and is > 98% complete. The genomic sequence can be downloaded from the official web-page [34]. By using the REPuter Program developed at the University of Bielefeld, Germany, Johansson *et al* [8] were able to screen the genome for regions with short-sequence tandem repeats (SSTR) of a total length of more than 15 bp. Primers were designed for the amplification of loci with tandem repeats and used to evaluate strains representing the four subspecies. Discriminatory power, represented by Simpson´s index [35], was determined statistically as the average probability of the typing system to assign a different type to two randomly selected unrelated strains. SSTRs with the highest discriminatory power were further analyzed for allelic variability. Amplicon sizes of the 16 SSTRs used in this study with LVS, were determined by sequencing and ranged from 175 to 451 bp [21].

2.8 Dendrogram construction

Johansson *et al* [21] are currently grouping isolates into a phylogenetic tree based on 20 variable loci. The copy included here is not completed, since 5% of the data are still missing. Each isolate was assigned a 20 letter combination dependent on the allele demonstrated at each locus. The isolates were pairwise compared and clustered by using the unweighted pair group method with arithmetic mean (UPGMA) of the software package PAUP4a (D. Swofford, Sinauer Associates, Inc., Sunderland, Mass., USA).

2.9 PCR amplification and analysis of VNTR loci

VNTR [8, 36] primer pairs (Table 2) were purchased with one primer in each pair fluorescently labelled with IRDye™ 800 (MWG-Biotech AG, Germany), in the 5´ end. PCR reagents were obtained from Finnzymes OY, Finland. The reaction mixture contained per 25 µl: about 100 ng of DNA template, 0.3 U DyNAzyme II, 100 µM each of deoxynucleotide triphosphates and 0.8 µM of (each) forward and reverse primers. An initial 2 min of 95ºC denaturation was followed by 35 cycles of denaturation at 95ºC for 45 s, annealing at 55ºC for 45 s and extension at 72ºC for 1 min, followed by a final extension at 72ºC for 7 min.

Primer name	Forward sequence	Reverse sequence
SSTR9a	gttttcacgcttgtctcctatca	caaaagcaacagcaaaattcacaaa
SSTR6b	tttatgataaggatgatttaaaacaaaata	gcttaaatctcgcaataccatgtaat
SSTR3a	gagctggtcaagtttatttaagta	caggattgcttgaacatgata
SSTR16a	gttggcgaacctaaaataatagc	cagctcgaactccgtcatac
SSTR10a	aaacctacaatcaacatctgacaat	ttgttatattaacctcatcagttcaattta
SSTR10b	cgctagatggtgctgatactatctt	cctgctagaaaacccatatttacat
SSTR12	cagaaaatatcactagccaaagttatgta	ttgagtccaccatttagctttatatt
SSTR21	ttttgggttttctctaaacatttcta	caattcagcgaaaccctatctta
SSTR16e	taggcatgacaaaccctgctat	cagctcgaactccgtcatac
SSTR16b	attgggtgatttggatggttg	cagctcgaactccgtcatac
SSTR6a	cgcgccatttggtcctaaa	cttaatttggcttcatcattggtatgt
C1-C6	tccggttggataggtgttggatt	aggcggagatctaggaaccttt
SSTR27	tcagccataccatggattaaaa	gcattgataaccttaagtctttcaaaa
SSTR6c	gctatagcagtaaatgtaggctcaa	acatatcggtggatcactatcaa
SSTR7	ccacagctagccagaccaaat	agtttggcgcgagctaat
SSTR9b	cttatagatgagacacatctgatatttga	ctttctaaatcctgtagcttcacatt

Table 2. Variable Number of Tandem Repeat (VNTR) PCR primer sequences.
SSTR = Short Sequence Tandem Repeats. Numbers indicate repeat unit lengths.

PCR products were diluted 1:15 with loading buffer and 1.5 µl was loaded on a 6% polyacrylamide denaturating gel (BioWhittaker Molecular Applications, Rockland, ME USA). The 50-700 bp sizing standard (LI-COR Biosciences, Lincoln, NE USA) was used for analysis on a LI-COR dna sequencer model 4000L (LI-COR Biosciences GmbH, Bad Homburg, Germany). Observed amplicon sizes were compared to sizes expected in untreated LVS.

3. Results

3.1 Bacterial growth in stress experiments

The *F. tularensis* LVS used here carries a gene expressing tetracycline resistance. The growth in liquid medium in the presence of different concentrations of tetracycline was monitored and showed that the growth was greatly affected by the antibioticum (data not shown). Therefore in the following studies tetracycline was added as a selective supplement only to the agar plates as this did not affect the number of *F. tularensis* colonies. Results from a pre-study in order to estimate the impact of stress treatments on bacterial growth over time while varying one stress parameter at a time, is shown in Figure 7. Bacteria grew very well in pH 4, equally well as under optimal conditions.

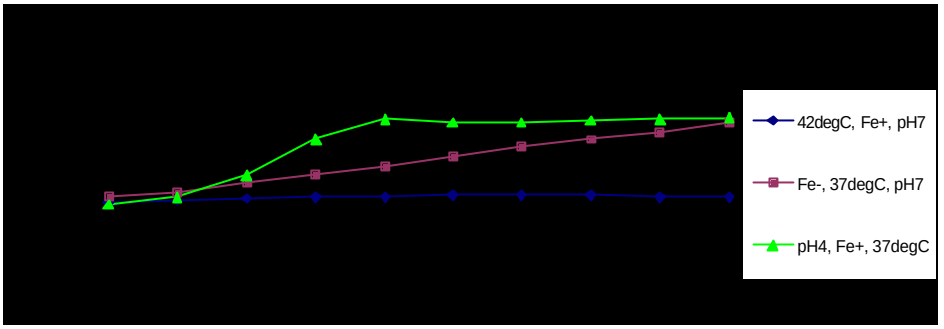

Figure 7. Growth curves in pre-studies for stress experiments. One single stress parameter was varied in each case. Absorbance was measured at 600 nm. The green curve reflects *F. tularensis* LVS growth in pH 4, with an iron source and at 37ºC. A growth curve of pH 7 would look the same. The red curve was derived from growth without iron, while the blue line shows the bacterial growth at 42ºC.

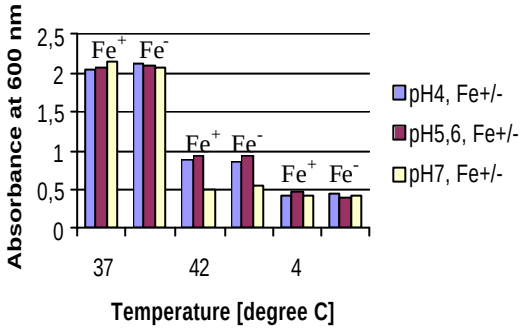

Figure 8. Growth of stress treated *F. tularensis* LVS bacteria. Absorbance was measured after 20 hrs of growth in different media (pH and presence of iron) and temperatures. The left set of columns for each temperature shows growth with iron while the right set derives from *F. tularensis* LVS growth without iron. The different colours indicate different pH.

In medium without any source of iron *F. tularensis* LVS grew slower but eventually reached the same cell density as bacteria grown in pH 4. There was no growth of bacteria at 42ºC. The growth results from the stress experiments, which proceeded for 20 hours, are shown in Figure 8. Absorbance measurements showed that neither availability of iron nor pH seemed to have a significant effect on bacterial growth whereas temperature was critical to growth.

3.2 VNTR analysis

None of the 18 stress treatments (Table 1) resulted in any changes of the 16 VNTRs analyzed. Sizes of DNA fragments from the VNTR markers were determined from a polyacrylamide gel as shown in Figure 9. This gel displays a typical result of a VNTR analysis of DNA from bacteria passed through amoebae, as well as from bacteria subjected to stress. Some VNTR copies did not show on the gel. Table 3 shows the overall VNTR analysis results. It was notable that several markers were not visible in the analysis of DNA from starved bacteria in particular.

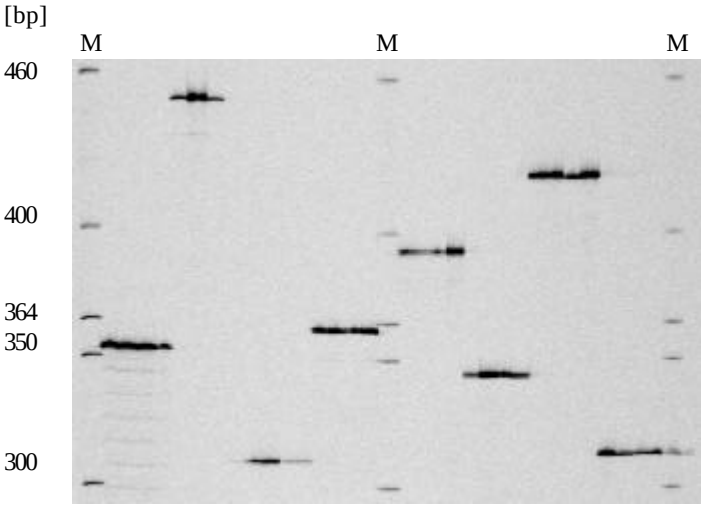

Figure 9. Typical polyacrylamide gel showing VNTR analysis of eight different VNTR markers. The analysis of DNA samples from four stress treatments are shown. M = DNA size marker.

DNA Marker	Size [bp]	LVS	A	B	C
SSTR9	351	+	+	+	+
SSTR6b	451	+	-	-	+
SSTR3	310	+	+	+	+
SSTR16a	361	+	+	+	+
SSTR10a	392	+	+	-	+
SSTR10b	345	+	+	+	+
SSTR12	422	+	+	+	+
SSTR21	311	+	+	+	+
SSTR16e	192	+	+	+	+
SSTR16b	175	+	+	+	+
SSTR6a	390	+	+	+	+
C1+C6	285	+	+	-	+
SSTR27	419	-	-	-	+
SSTR6c	346	+	+	+	+
SSTR7	396	+	+	+	+
SSTR9b	339	+	+	+	+

Table 3. Results from VNTR analysis. DNA from bacteria subjected to different stress treatments were analyzed. Column 'LVS' is the result from analysis on untreated bacteria, LVS cultured on solid medium; column A, LVS after four passages through *A. castellanii*; B, LVS passed once through *A. castellanii*, then starved until they reached the viable but not culturable (VBNC) state; C, summarized results from bacteria subjected to changes in pH, temperature and/or access to iron (as presented in Table 1). A '+' indicates that the expected amplicon size was encountered while a '-' means that nothing was visible on the polyacrylamide gel. For the summarized results (column C) a '+' is shown where all samples had visible amplicons of the expected size.

Where DNA bands could be observed, they were in all cases of the expected size. Thus, no changes in VNTR length were obtained in bacteria neither from the amoebae passages nor from growing under stressing conditions.

4. Discussion

Finding a suitable typing method for discrimination on strain level for a genetically conserved bacterium such as *Francisella tularensis* is highly valuable. Johansson and co-workers (data not published) have identified several tandem repeat markers with the potential to be used for reliable strain identification of bacterial isolates. VNTR markers hold promise in epidemiology, although the mechanisms behind the variability in tandem repeat length and any regulation of this are still unrevealed. However for genetic typing of isolates it is relevant to identify any factors actively affecting the diversity in repeat copy numbers among different isolates. In this study *F. tularensis* live vaccine strain (LVS) was subjected to the harsh intracellular environment of amoebae, starvation or stress; by means of heat chock, different pH and regulated access of iron to determine whether any of these parameters could cause variation in the number of tandem repeats. The rational for selecting these stress parameters were the facts that environmental factors such as iron concentration, pH, oxygen availability and temperature affect virulence in bacteria [37], where for example the transcription of some toxins is controlled in an iron-dependent way, and stress induced by starvation, acidic pH and heat shock may regulate the expression of others [37]. Bacteria need iron for cellular respiration [38] and it has been shown that *F. tularensis* LVS requires iron for growth in macrophages [32]. Growth under limited nutritional conditions is stressful and LVS depleted of an iron source increases its virulence [39]. Sudden changes in nutrition availability and other kinds of stress might have different outcomes in the bacterium.

No VNTR variation was observed in the performed analysis. Thus, change in VNTR length is likely to be spontaneous and not inducible. In the VNTR method marker lengths were analysed. Other mutations that might arise due to these experiments (e. g. point mutations) were not investigated. Possibly, mutational changes can be further propagated by SSM [25] and thus, eventually, cause a change in the VNTR marker region.

Some measure of the impact of the stress treatment was obtained by monitoring bacterial growth. Although both changes in pH and absence of iron are known stress parameters, the only factor that significantly altered growth of *F. tularensis* LVS was temperature.

It is difficult to explain why absence of iron did not affect growth. Since no selective antibioticum was added to the liquid medium for the stress experiments it could not be ruled out that some growth could be assigned to other microorganisms. Although the viable counts on selective solid media were reasonable, contamination, especially at 37ºC, was possible. Since *F. tularensis* LVS that had had no source of iron, could grow on selective agar plates, growth of other microorganisms alone, however, was not sufficient to explain the fact that bacterial growth was high in iron-depleted medium. For *in vivo* growth of *F. tularensis* LVS in macrophages bacteria require access to intracellular iron [32]. One has to remember though that the *in vivo* environment often is

very different from *in vitro* conditions. One likely explanation to *in vitro* growth without iron could be that *F. tularensis* LVS *in vitro* makes use of other metal ions to support growth. However, this observation needs to be further investigated to confirm the growth data obtained during the stress experiments performed here.

The intracellular bacterium seems to have adapted well to different pH, as it grows well in both pH 4 and pH 7. Chamberlain noted that LVS growth in medium with an initial pH of 6.2-6.4 was better than with a pH of 7.0 and that pH was increased to 7.0-7.2 during a 24-hr period [33]. This indicates that the bacteria themselves may be able to regulate pH and may have done so during the stress experiments with varying pH. While growth to some extent indicates how comfortable or stressed bacteria are, events at DNA level is another issue. However, no changes in VNTR copy numbers were observed as a result of intracellular growth in amoebae or during stress treatments.

In the VNTR analysis, some markers were not visible on the polyacrylamide gel. In general this might be overcome by changing the PCR conditions. For viable but not culturable (VBNC) bacteria the DNA bands were often weak and several VNTRs were not amplified at all (Table 3). The problem of amplification of DNA from VBNC cells by PCR has been encountered previously [40, 41] and was explained as either DNA binding proteins or chromosomal supercoiling making DNA inaccessible to PCR primers [40]. Perhaps by using another method for the DNA preparation of bacteria in the VBNC state it is possible to solve this problem.

The results of this study were positive in the sense that stability of the VNTR markers could be shown. However, further studies with amoebae and other stress treatment could be performed. Several parallel or several, e.g. hundreds, successive amoebae infections could result in a change in marker length. Future studies may involve e.g. other stress parameters, such as hydrogen peroxide [42], or radiation [43], which are well recognized mutagens.

In conclusion, stress treatment or growing bacteria within amoebae did not result in changes in VNTR copy numbers, and these observations suggest that length variability is spontaneous or at least not affected by stress. Judging from this study the use of the VNTR method to discriminate among *F. tularensis* strains on DNA level is reliable.

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