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A novel broth medium for enhanced growth of *Francisella tularensis*

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Abstract

Francisella tularensis is a fastidious organism that requires a lengthy incubation time in liquid growth media for detection. The objective of this study was to develop a medium formulation using readily available supplements that enhanced early growth of *F. tularensis*. *F. tularensis* Live Vaccine Strain (LVS) was used to evaluate the growth responses for each of the media formulations tested. Growth in Brain Heart Infusion (BHI) broth supplemented with 2% Vitox, 10% Fildes, and 1% histidine (BVFH) resulted in a significant increase in growth after 8 h incubation compared to other media formulations tested ($P < 0.001$). Virulent strains of *F. tularensis* grown in BVFH medium demonstrated similar enhanced early growth. Cell densities of $3.9 - 5.2 \log_{10} \text{CFU mL}^{-1}$ were obtained after 24 h of growth in BVFH from a $1-2 \text{ cell mL}^{-1}$ starting inoculum of the virulent Type A Schu4 strain, indicating the suitability of this medium in rapidly amplifying low starting titers of *F. tularensis*. Collectively, these results indicate that the novel formulation of the BVFH medium was capable of producing enhanced growth response for *F. tularensis*.

Keywords

rapid methods; virulence; antibiotics; detection; environmental

Introduction

Francisella tularensis, the etiological agent of tularemia, is a category A select agent with a low infectious dose. *F. tularensis* is a fastidious organism requiring cultivation in supplemented growth media (McCoy and Chapin 1912; Rhamy 1933; Payne and Morton 1992). Traditionally, *F. tularensis* is grown in defined media such as Chamberlains or in non-selective media such as Mueller-Hinton broth (Chamberlain 1965; Brown et al. 2004) requiring 3 to 7 days of incubation with shaking and a minimum of 10 days under static conditions to produce visible growth (Ellis et al. 2002). Several studies have used a modified

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Conflict of Interest

Authors have no conflict of interest to disclose.

Brain Heart Infusion (BHI) broth to enhance the growth of *F. tularensis* (Hazlett et al. 2008; Mc Gann et al. 2010) with BHI-grown *Francisella* exhibiting a similar cellular phenotype as mammalian macrophage-passaged *Francisella* cells (Hazlett et al. 2008). A similar BHI formulation was also shown to enhance growth of three commonly studied *Francisella* strains (virulent Type A strain Schu4, attenuated Type B Live Vaccine Strain (LVS), and *F. novicida* strain U112) when compared to Chamberlain's, cation-ion adjusted Mueller-Hinton broth (MHB), and Tryptic Soy broth (TSB) with cysteine (Mc Gann et al. 2010). Similarly, BHI with 5% Fildes medium supported significantly higher cell densities of another fastidious organism, *Haemophilus influenza*, when compared to MHB, BHI, and TSB medium containing various supplements (Bergeron et al. 1987).

The objective of this study was to develop an easily prepared medium capable of supporting significant *F. tularensis* growth after a short incubation time. After development and selection, medium performance was validated with virulent strains of *F. tularensis* subspecies *tularensis* (Type A) and *holarctica* (Type B) as well as *F. novicida* and *F. philomiragia*. A low titer inoculum growth response was also investigated in order to rapidly detect the lowest possible number of viable *F. tularensis* cells.

Results and Discussion

Francisella tularensis LVS was used for the initial testing of various media combinations (Table 1) with results shown in Figure 1. The BVFH medium demonstrated significant growth ($P < 0.001$) at the 8 h time point and had the highest mean $\log_{10}$ increase of 3.89 at 24 h. All other formulations were significantly lower ($P < 0.0001$), with the exception of the BIHg, which was statistically comparable, but still underperformed reaching a $\log_{10}$ increase of 3.60 at 24 h. Notably, the hemoglobin hindered resuspension of bacterial cell pellets during sample preparation for DNA isolation, decreasing its ease of use.

F. tularensis requires cysteine for cultivation; thus BHI alone cannot support its growth (Eigelsbach and McGann 1984). The BI and the BV serve as sources of cysteine, but the BV performed consistently better overall (Figure 1). The manufacturer provides both supplements in lyophilized forms with a separate vial of rehydration fluid. Both have a reconstituted concentration of 100 g L^{-1} ; however, the IsoVitale X contains dextrose in the lyophilized portion and the Vitox contains glucose in the rehydration fluid. Further testing would be required to determine if lyophilized components were responsible for the growth differences observed. At 8 h, there was no significant difference between growth in BI or BV; however, by 24 h, LVS growth was significantly higher in BV than BI ($P < 0.001$) indicating that the Vitox supplement is more supportive of rapid growth of *F. tularensis*. By 24 h, for all media formulations, except for those with antibiotics, LVS growth was significantly higher with additional supplements than for BI or BV alone (Figure 1, $P < 0.001$).

Traditional media for the use of isolating *F. tularensis* incorporated blood in the formulation (Fildes 1920; Rhamy 1933; Gibby et al. 1948; Payne and Morton 1992; Ellis et al. 2002). The Fildes enrichment supplement contains 22.4% sheep blood and 4g of pepsin which results in the generation of hemin (X factor) and nicotinamide dinucleotide (NAD, V factor)

by the enzymatic action of the pepsin on the defibrinated sheep blood. The concentration of Fildes used in the BVFH formulation was 10% which is twice what is recommended. The hemoglobin was received as a 2% solution and was used at a 1% concentration. Although the BIHg formulation was statistically comparable in regards to *F. tularensis* growth at 24 h, the negative impact on sample processing for DNA extraction disqualified this supplement from further testing.

Carnosine, a dipeptide of amino acids beta-alanine and histidine, has been suggested to enhance the growth rate of *F. tularensis* (Lamont et al. 2014). Histidine alone has been shown to decrease the required incubation time by several hours (Hood 1961; Lamont et al. 2014). The results from the current study confirmed that the presence of histidine in BVFH medium increased the growth of *F. tularensis* significantly at 8 h when compared to the BVF and the BVFC formulations ($P < 0.05$) and with BV alone (Figure 1, $P < 0.001$). However, in contrast to Lamont et al. (2014), the enhanced growth of *F. tularensis* in the presence of carnosine was not observed, with no significant differences when compared to BVF formulation. The presence of antibiotics resulted in a lag in growth at 8 h and decreased cell densities after 24 h as compared to formulations without antibiotics (Figure 1). Although antibiotic supplementation to the growth medium may reduce non-*Francisella* background amplification (Petersen et al. 2009), the negative impacts on *F. tularensis* LVS growth by 24 hours excluded their use. Recently, another growth medium for *F. tularensis* ssp. and other pathogenic bacteria has shown encouraging results; however, the medium is neither commercially available nor easily prepared (Becker et al. 2016).

The growth of the virulent strains in BVFH medium performed well but demonstrated significant differences between strain types with the average mean $\log_{10}$ CFU mL^{-1} increase of Type A and B strains being 3.66 and 2.42, respectively, at 24 h (Table 2, $P < 0.0001$). By 24 h, the mean $\log_{10}$ CFU mL^{-1} density of Type B LVS was 3.83 with densities between 2.35-2.48 for the virulent Type B strains (NY98, IN99, and KY99) and 3.07-4.34 for the virulent Type A strains (Schu4, H3565, and F2246) indicating that the cell density of LVS was more comparable to those observed for virulent Type A strains (Table 2). Strains KC1482 and LVS were significantly different ($P < 0.0001$) than their associated type strains with the cell density of KC1482 being significantly lower than those of all the Type A and B strains used in this study (Table 2, $P < 0.05$). Results also demonstrated that *F. novicida* and *F. philomiragia* may not be suitable surrogates for virulent *F. tularensis* of either type if early growth responses are being investigated (Table 2), especially given the genomic and phenotypic differences reported between *F. novicida* and *F. tularensis* Type A Schu4 (Kingry and Petersen 2014). In general, all the strains performed well in the BVFH medium with differences observed between the attenuated (LVS and KC1482) and the surrogate (*F. novicida* and *F. philomiragia*) strains tested. Future research should be aimed at determining the growth requirements and media optimization for each subspecies (Type A and B).

A low titer inoculum test was performed with the Type A Schu4 isolate. Mc Gann *et al.* performed a growth test with a BHI based medium (containing cysteine, nicotinamide adenine dinucleotide, heme-histidine, and glucose) with an 80 cell mL^{-1} starting inoculum for Schu4 which failed to reach a 0.1 A_{600} reading ($6.6 \log_{10}$ CFU mL^{-1}) in 160 h (Mc Gann et al. 2010). However, in the current study, the starting inoculum for Schu4 was 1-2

cells mL⁻¹ which reached a concentration between 3.9 – 5.2 log₁₀ CFU mL⁻¹ in 24 h; indicating that addition of the extra essential growth factors contained in the Vitox and Fildes supplements may be responsible for these differences in Schu4 cell densities. Similarly, growth of the Type B strain, IN99, reached 4 log₁₀ CFU mL⁻¹ in 24 h from a 2–4 cells mL⁻¹ starting inoculum. Generating the highest titer from the fewest cells in the least amount of time is important for a procedure that is intended for use as a detection method for viable cells of *F. tularensis*. The results indicate that the BVFH medium may be a useful tool to help achieve this purpose.

At 0 h, TaqMan qPCR data revealed significant differences between the CFU and the cell equivalence (CE) for both the Type A (*pdpD*) and B (TB_IS*Ftu2*) specific assay as well as a universal *F. tularensis* (23*kDa*) assay (Figure 2, *P* < 0.0001). By 48 h, CFU and CE values were not significantly different for strains Schu4 and IN99, but were for strains H3563 and NY98 (Figure 2A and B, *P* < 0.05). Comparing the performance between primer sets, there was no significant difference between each assay at 48 h. Moreover, there was no observed PCR inhibition for the 23*kDa*, TypeB_IS*Ftu2*, and *pdpD* qPCR assays using the BVFH selected medium, nor was inhibition observed with other commonly used *Francisella* spp. PCR targets, such as *fopA*, *tul4*, *yfdH*, and IS*Ftu2* (data not shown). Thus, these results demonstrate that plate counts and qPCR provide comparable results for quantification when the enriched medium was used.

The need for achieving detectable viable concentrations of *F. tularensis* cells in a timely manner is of great importance for development of rapid analytical methods needed during bioterror or natural outbreak responses and for general study of this fastidious bacterium. Recently, use of culture-based and polymerase chain reaction (PCR) methods have been combined as a way to detect low concentrations of viable pathogens in complex, environmental samples (Létant et al. 2011). This rapid-viability (RV)-PCR method utilizes the change in the cycle threshold (Ct) before and after microbial growth to determine the presence of viable cells; thus a short incubation period is required to obtain significant changes in Ct values. Use of the BVFH medium in the current study resulted in enhanced early growth (by 8–24 h) of *F. tularensis*; thus the BVFH medium could be used to develop a RV-PCR assay for *F. tularensis*.

The objectives in this study were to develop a growth medium combination that (1) enhances the cultivability of *F. tularensis* to detectable and quantifiable levels after a short incubation time of up to 24 h and, (2) is easily prepared with commercially available components and, (3) does not interfere with DNA extraction or inhibit downstream molecular analyses. These results demonstrate that the BVFH medium meets these objectives and can be incorporated into assays requiring the rapid growth of this fastidious organism.

Materials and Methods

Bacteria and growth conditions

The isolates used in this study are listed in Table 3. Work with virulent strains was conducted under BSL-3 conditions and approved by the Institutional Biosafety Committee at the University of Cincinnati. Experiments were performed in triplicate for each strain which

were statically incubated. Media combinations, as recommended for growth of *Francisella* (WHO 2007), were prepared using Brain Heart Infusion (BHI) broth (Becton Dickinson (BD), BBL) as the base along with the following supplements: Hemoglobin and IsoVitale X[™] (BD, BBL); Supplement B (BD, Difco); L-Histidine and Penicillin (Fisher Scientific); Campylobacter Supplement, LCAT Supplement, Polymixin B and Vitox[™] (Oxoid); Fildes Enrichment (Remel); and L-Carnosine (Sigma-Aldrich). The concentrations and combinations evaluated are detailed in Table 1.

Isolates were stored at -80°C in BHI broth with 15% (v/v) glycerol. An aliquot of 0.5 mL thawed suspension of individual frozen stock cultures was transferred to 25 mL of BHI broth containing 2% (v/v) IsoVitale X and statically incubated at 37°C for 48 h for each experiment. Stationary phase cultures were concentrated by centrifugation ($3,000 \times g$ for 10 min at 4°C) and washed three times and resuspended in sterile Butterfields buffer (BB, Hardy Diagnostics). Culture suspensions were acclimated to low nutrient conditions by storage in BB at 4°C for 24 hours prior to use. These resting cells were used as the inocula in the experiments to more closely mimic the physiological state of planktonic cells in the natural aquatic environment (Rice et al. 1990).

For each isolate, the resting cell suspension was diluted and inoculated into 25 mL of BHI + supplements to obtain starting concentrations of 10^2 and 10^3 CFU mL^{-1} as well as a low titer inoculum test of 1-2 cells for isolate Schu4. Each medium formulation was assayed at 0, 8, 24, 48 and 72 h. Bacterial suspensions were diluted in BB and plated onto Chocolate II agar plates (BD, Biosciences). For the spread plate method, 0.1 mL aliquots of the bacterial suspensions were plated in duplicate for each dilution except for the undiluted (10^0) counts, where 0.2 mL of suspension was aliquoted and spread on each of 5 plates. The sum of the counts on the 5 plates represented the number of CFU mL^{-1} . For the spot plate method, bacterial suspensions were serially diluted either 5- or 10-fold in BB and 10 μL aliquots spotted on agar plates (Miles et al. 1938; Gaudy et al. 1963). The limit of detection (LOD) was 1 and $1.95 \log_{10}$ CFU mL^{-1} , for the spread and spot plate method, respectively. Plates were incubated for 48 h (for spot plate method) or 4-7 d (for spread plate method) at 37°C . At each time point, 1 mL was processed for DNA extraction as described below: cells were pelleted by centrifugation for 10 min at $15,000 g$ at 4°C , resuspended in 0.2 mL BVFH and stored at -20°C .

For the Schu4 low titer inoculum experiments, 1.4 mL of BVFH was added to the wells of 96-deep well plates and inoculated with 0.1 mL of the Schu4 suspension. Plates were incubated at 37°C with shaking at 180 rpm. The different time points tested were performed in separate 96-well plates.

DNA extraction and PCR procedure

DNA was extracted using the MasterPure Complete DNA Purification Kit[™] (Epicentre Biotechnologies, USA) according to the manufacturer's protocol except that DNA pellets were resuspended in 50 μL of molecular grade water. TaqMAN qPCR assay for *Francisella* detection was performed using the universal *23kDa* assay (Versage et al. 2003), the Type A-specific *pdpD* assay (Emanuel et al. 2003), and the Type B-specific *ISFtu2* assay (Kugeler et al. 2006). A total reaction volume of 20 μL for each reaction mix contained 10 μL 2×

TaqMan Universal Master Mix (Applied Biosystems, USA), 200nM primer concentration, 6.4nM probe concentration, and 2 µL template DNA. The thermal cycling conditions, performed using the QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific, USA) included a pre-denaturation step at 50 °C for 2 min, 95 °C for 10 min; 45 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s; and a final hold at 45 °C for 5 min.

Statistical analyses

One-way analysis of variance (ANOVA) was performed to determine the effect of culture media on the degree of bacterial growth at 24 hours. Fisher's Least Significant Difference (LSD) multiple comparison test was used to determine the significance of media effect on growth performance. The XLSTAT (version 2014.2.27) program was used to carry out statistical analyses. Prior to performing statistical analyses, CFU data were log₁₀ transformed.

Statistical significance was determined for the virulent strain experiments using two-way Analysis of variance (ANOVA) with a Tukey post-hoc adjustment of *P*-values to account for multiple comparisons.

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Significance and Impact of Study

The need for rapid cultivation of *Francisella tularensis* is essential for detection and monitoring during natural outbreak events or intentional bioterrorism attacks. The addition of selected supplements into the base medium BHI (BVFH) developed in this study enhanced growth of *F. tularensis* Type A1, A2, and B strains compared to BHI alone. Growth of these organisms in BVFH will allow for improved response time should a natural or intentional contamination event occur.

Figure 1.

Change in *F. tularensis* LVS growth in BHI media with various supplements.

F. tularensis strain LVS was grown in various formulations as described in Materials and Methods. Starting concentrations of bacteria at 0 h (T₀) were subtracted from growth (G) levels at 8 h (G_{T8-T0}) and 24 h (G_{T24-T0}). Error bars represent standard error mean. Statistical comparisons were performed between the base medium, BI or BV, to each formulation at the respective time point. * $P < 0.05$; ‡ $P < 0.001$.

Figure 2.

Colony Forming Units (CFU) mL⁻¹ compared to the cell equivalence (CE) generated from the Type A specific primer (*pdpD*), the Type B specific primer (*TB_ISFtu2*), and the universal *F. tularensis* primer (*23kDa*) over time (h) for strain H3563, Schu4 (A), IN99, and NY98 (B). Growth curves were generated using the BVFH medium. * $P < 0.05$ (CFU vs CE)

Table 1.

Media formulations tested in this study

				Abbreviation
Brain Heart Infusion broth				B
2% IsoVitale X TM				BI
2% IsoVitale X TM	2% Supplement B			BIS
2% IsoVitale X TM	5% Fildes Enrichment			BIF ⁵
2% IsoVitale X TM	10% Fildes Enrichment			BIF
2% IsoVitale X TM	10% Fildes Enrichment	0.3% L-histidine		BIFH
2% IsoVitale X TM	1% hemoglobin			BIHg
2% Vitox TM				BV
2% Vitox TM	10% Fildes Enrichment			BVF
2% Vitox TM	10% Fildes Enrichment	0.1% L-histidine		BVFH
2% Vitox TM	10% Fildes Enrichment	0.625 g L ⁻¹ L-carnosine		BVFC
2% Vitox TM	1% hemoglobin			BVHg
2% Vitox TM	10% Fildes Enrichment	10 ⁵ iu L ⁻¹ penicillin	10 ⁵ iu L ⁻¹ polymyxin B	BVFNX
2% Vitox TM	1% hemoglobin	10 ⁵ iu L ⁻¹ penicillin	10 ⁵ iu L ⁻¹ polymyxin B	BVHgNX
2% Vitox TM	10% Fildes Enrichment	0.4 % Campylobacter supplement ¹		BVFCs
2% Vitox TM	10% Fildes Enrichment	1% LCAT Supplement ²		BVFL

Brain Heart Infusion (B) broth is the base medium used in all the formulations tested.

Percentages of L-histidine are a w/v basis, all other percentages are a v/v basis.

Penicillin and Polymyxin B concentrations are given in international units (iu) L⁻¹

¹ contains 2 mg amphotericin B, 15 mg cephalothin, 2500 IU polymyxin B, 5 mg trimethoprim, and 10 mg vancomycin L⁻¹

² contains 1 mg amphotericin B, 6 mg colistin sulphate, 1 mg lincomycin, and 6.5 mg trimethoprim L⁻¹

Table 2.Mean log₁₀ CFU mL⁻¹ increase in BVFH medium

Isolate (type)	Mean log ₁₀ CFU/mL Increase at time (h)			
	8	24	48	72
Schu4 (A)	1.22 (0.06)	4.34 (0.06)	5.89 (0.23)	6.37 (0.04)
F2246 (A)	0.62 (0.03)	3.07 (0.10)	6.12 (0.10)	6.50 (0.13)
H3563 (A)	0.87 (0.07)	3.58 (0.18)	6.12 (0.09)	6.50 (0.20)
Mean	0.90	3.66 [*]	6.04	6.46
SD	(0.05)	(0.11)	(0.14)	(0.13)
NY98 (B)	0.63 (0.06)	2.35 (0.02)	5.45 (0.05)	6.17 (0.13)
IN99 (B)	0.39 (0.08)	2.48 (0.19)	5.85 (0.04)	6.50 (0.07)
KY99-3884 (B)	0.49 (0.06)	2.44 (0.09)	5.61 (0.06)	6.09 (0.08)
Mean	0.50	2.42 [*]	5.64	6.25
SD	(0.07)	(0.10)	(0.05)	(0.09)
<i>F. tularensis</i> KC1482 (A)	0.30 (0.05)	2.07 ^{†‡} (0.08)	4.88 (0.07)	5.48 (0.08)
<i>F. tularensis</i> LVS (B)	1.12 (0.02)	3.83 [†] (0.03)	5.56 (0.17)	5.86 (0.08)
<i>F. novicida</i>	3.73 [#] (0.06)	6.39 [#] (0.14)	7.21 (0.06)	7.63 (0.11)
<i>F. philomiragia</i> 25015	3.72 [#] (0.01)	5.22 [#] (0.21)	5.26 (0.15)	5.46 (0.12)

Average log₁₀ CFU mL⁻¹ starting titer was 2.23 (0.18).

Standard Deviation (SD) is shown in parentheses.

^{*} Average Type A and B strains were significantly different at 24 h ($P < 0.0001$)[†] The attenuated strains were significantly different than their associated type strain ($P < 0.0001$)[‡] KC1482 was significantly lower at 24 h than all the isolates tested ($P > 0.05$)[#] Significantly different than the type A and B strains ($P < 0.0001$)

Table 3.*F. tularensis* strains used in this study

Subspecies (type designation)	Isolate/origin
<i>F. tularensis</i> ssp. <i>tularensis</i> (A1).....	F2246/Maryland
<i>F. tularensis</i> ssp. <i>tularensis</i> (A1).....	H3563/Oklahoma
<i>F. tularensis</i> ssp. <i>tularensis</i> (A1).....	SchuS4/Ohio
<i>F. tularensis</i> ssp. <i>tularensis</i> (A2, attenuated).....	KC1482 ^a /Utah
<i>F. tularensis</i> ssp. <i>holarctica</i> (B).....	IN99/Indiana
<i>F. tularensis</i> ssp. <i>holarctica</i> (B).....	NY98/New York
<i>F. tularensis</i> ssp. <i>holarctica</i> (B).....	KY99-3884/Kentucky
<i>F. tularensis</i> ssp. <i>holarctica</i> (B, attenuated).....	LVS
<i>F. novicida</i>	NR-13/Utah
<i>F. philomiragia</i>	25015/Utah

LVS, live vaccine strain.

^aKC1482 is denoted as ATCC 6223 (Broekhuijsen et al. 2003).