Novel Colorimetric Method for the Detection of Pathogenic *Vibrio vulnificus*

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Abstract: *Vibrio vulnificus* infections are expected to continue to increase with climate change yet current detection methods are either too expensive for wide spread use or unable to distinguish between strains likely to cause disease and those that will not. This study investigates the use of serum and mannitol to develop a culture-based detection method for pathogenic strains. Two genes correlated with virulence were used as indicators of pathogenicity, *vcg* and *pilF*. The medium developed contains 30% serum and 70% mannitol broth, and has accuracies in detecting pathogenic strains of V. vulnificus as high as 74% when *pilF* is used as the indicator of pathogenicity.

1. Introduction

1.1 *Vibrio spp*. and *Vibrio Vulnificus*

Vibrio spp. are Gram negative bacteria that are found in coastal and estuarine waters. There are over 50 species of *Vibrio* that can survive in a wide range of salinities and temperatures. Of these, there are 12 species that are known to cause human infections, most notably *Vibrio cholera* that has long been considered a pathogen of medical importance. While *Vibrio cholera* has been of medical concern previously in the United States and continues to be of concern in much of the developing world, other *Vibrio* species including *Vibrio vulnificus* are considered emerging pathogens (Tantillo 2004). The Centers for Disease Control and Prevention (CDC) reports have shown that while most foodborne diseases have decreased by about 22% between 1996 and 2012, *Vibrio* infections have increased by 116% (CDC). The emergence of a pathogen can be contributed to many factors including ecological and environmental changes, changes in human demographics and behavior, and an increase in the size of the susceptible population (Tantillo 2004). Temperature however is the largest contributing factor to distribution and abundance of pathogenic V. vulnificus. The highest concentrations are seen between 20°C and 30°C and the bacteria can only be found in sediments or in a viable but not culturable (VBNC) state when the water column temperature is below 10°C (Tantillo 2004). The increase in disease incidence has been related to climate change, and as warming continues, the likelihood of disease from *V. vulnificus* may continue to increase (Paz 2007). Climate change leading to warmer waters will not only allow *V. vulnificus* to exist in higher concentrations in places currently at the lower end of its range, but it could also expand the geographic locations in which V. vulnificus is found and lengthen the season in which *V. vulnificus* in shellfish is common.

Vibrio vulnificus infections are most commonly caused by the ingestion of raw or undercooked shellfish as it can be concentrated in oysters and other shellfish that inhabit estuarine waters (Oliver 2005). Concentrations as high as $6 * 10^4$ CFU/g in oyster tissues have been seen in water with

concentration at 7 CFU/ml. Some strains of *V. vulnificus* have been known to cause diseases such as wound infections, gastroenteritis, and primary septicemia. Primary septicemia is a blood infection that also results in lesions and necrotic areas (Horre 1996). Primary septicemia, as well as gastroenteritis, can result from eating raw or undercooked shellfish. Shellfish such as oysters are frequently consumed raw and thus 93% of primary septicemia caused by V. vulnificus are from the consumption of raw oysters (Oliver 2012). 95% of all seafood related deaths are attributed to V. vulnificus (Oliver 2005,2012). Septicemia almost always requires hospitalization and results in death for approximately 50% of patients (Mead 1999). Infections almost always occur in patients with pre-existing conditions such as liver disease or those with compromised immune systems (94% of cases), since these diseases cause elevated iron concentrations in the blood which has also been linked to the bacteria's ability to cause infection (Linkous 1999, Wright 1981, Oliver 2012).

Not all strains of *V. vulnificus* pose the same risk of infection. The species has a large range of genetic variability, and only certain genes are associated with pathogenicity. While there are many genes correlated with virulence, the virulence correlated gene (*vcg*) is commonly used to distinguish strains of possible virulence. The gene has two genotypes, *vcgC* and *vcgE* which often correlated with clinical and environmental strains, respectively (Rosche 2005). Since 90% of vcgC strains have been isolated from clinical samples, they are of the greatest concern (Oliver 2012). However the risk of V. vulnificus infection is relatively low in comparison to total Vibrio vulnificus concentrations. This is partially contributed to low percentage of vcgC strains found in shellfish. About 50-85% of total V. vulnificus found in oysters are the lower virulence vcgE strains (Froelich 2013). One survey of 85 oysters taken from waters in North Carolina and Florida found that 84% of the nearly 900 isolates collected were vcgE strains and that only two of the 85 oysters had higher concentrations of vcgC strains.

Other indicators of pathogenicity include variations of the *pilF* gene. Certain variations of the *pilF* gene have been shown to indicate survivability in human serum and thus contributes to virulence (González 2012). There are other mutations of the *pilF* gene that may also reduce the bacteria's ability to attach to host cells. There is likely not one gene that controls virulence and while *pilF* may contribute to virulence it is not indicative of virulence (Jones 2009). The *pilF* virulent variations may be more inclusive than *vcg* since E-type strains with the possibility of causing infection (primarily wound infections) are also included.

Current FDA regulations do not have set limits on *V. vulnificus* concentrations, but do require action plans when harvesting shellfish under certain conditions. This generally includes refrigeration directly after harvest to prevent further growth of the bacteria (FDA). There may however still be high enough concentrations of the bacteria in the oysters to cause illness, especially in those with other pre-existing conditions. Regular and direct testing of *V. vulnificus* in oysters by the scientific community could further decrease the risk of illness.

Current technologies for detection of *V. vulnificus* include both culture-based and molecular techniques. Culture-based methods such as Chromagar Vibrio™ only detect total vulnificus, and thereby cannot distinguish between stains that are likely pathogenic and those that are not. Medias such as Chromagar Vibrio™ also have low accuracies: approximately 40% (Williams 2013). Molecular techniques, while accurate and effective, require expensive equipment and extensive training. This makes the likelihood of molecular techniques being implemented by small shellfish harvesters or fishermen extremely low. A medium for the detection of pathogenic strains of *V. vulnificus* that could be easily used by non-technical professionals could reduce the risk of illness from shellfish and seafood related deaths.

1.2 Mannitol Fermentation and ABC transport

ATP binding cassette transport or ABC transport is a type of secondary active transport. Protein pores provide selective passage for molecules across cell membranes. These pumps use ATP as the source of energy input and only transport specific molecules. Transporters are specific to the particular substrate and can transport a wide range of substrates including sugars such as mannitol. ATP binding and hydrolysis drive conformational changes that open a passage way for the substrate (Pollard 2008). Since transporters are selective, not all cells have the ability to transport particular substances including mannitol. Only some strains of *V. vulnificus* possess the mannitol fermentation operon that is required to ferment mannitol. The mannitol fermentation operon has been associated with the more virulent C-genotype strains of *V. vulnificus* and in a study conducted by Froelich and Oliver found that all 38 *vcgC* strains tested had the mannitol operon genes while only 8 of 20 *vcgE* strains had the operon (Froelich 2011). This means that the ability to ferment mannitol is primarily seen in *vcgC* strains and while there are some *vcgE* strains that can ferment mannitol, strains that cannot ferment mannitol are of low virulence.

1.3 Role of Serum in Immune Response and Pathogenicity

Serum has been thought to distinguish between pathogenic and non-pathogenic strains of bacteria since it plays an important role in early immune system response. Innate immune responses are the earliest defenses against microbes including bacteria such as *Vibrio vulnificus* (Abbas 2015). Complement is one of the blood proteins that is vital to the innate immune system. Complement responds to any foreign bacteria in the body and is not a learned response. It acts directly to lyse the bacteria (Abbas 2015). In Gram negative bacteria such as *Vibrio vulnificus*, lipopolysaccharides on the outer membrane activate the alternative complement pathway (Abbas 2015). There is always a background concentration on the complement protein C_3 in serum that can undergo spontaneous hydrolysis. Once C_3 is hydrolyzed it undergoes a conformational change that allows it to then interact with Factor B. Factor B and C_3 are then cleaved by Factor D into Bb and Ba. C_3bBb is the C_3 converting enzyme that cleaves additional C_3 into C_3a andC_3b. The process is stabilized by the properdin. The process of cleaving additional C_3 can only take place on activator surfaces such as cell membranes of bacteria. Non-activation surfaces such as the host's cells displace Factor B from C_3b via Factor H (Sullivan).

Once additional C_3 proteins are cleaved, it becomes part of the next enzymatic complex that leads to the terminal components of the membrane attack complex. The membrane attack complex is the process that causes complement-mediated lysis in bacteria. Gram negative bacteria such as *V. vulnificus* are particularly susceptible to complement mediated lysis. The enzymatic complex C_3b_2Bb, or C_5 convertase, causes the cleavage of C_5. The terminal components C_5, C_6, C_7, and C_8 bind on the lipid membrane of the bacteria and begin to cause the membrane to leak. The addition of the final terminal component C_9 also binds, a pore forms on the membrane and unless the cell is able to repair the membrane its contents will leak out (Sullivan). Use of serum in a media can help distinguish pathogenic strains of bacteria since many pathogenic bacteria have evolved to resist innate immune responses including complement mediated lysis (Abbas 2015).

As rates of V. vulnificus infections continue to rise, the need for a fast and inexpensive test method that could be used in by both the shellfish industry and medical professionals is needed. The goal of this study was to use known distinguishing features of pathogenic V. vulnificus to develop a culture based media that can detect which strains are pathogenic. Focus on optimization of the components was to reduce cost of the media to make it widely affordable. The speed and user friendliness will allow wide use even among those with limited experience in microbiological methods.

2. Methods

2.1 Mannitol Fermentation

Initial stages of development for this medium began with testing and optimization of the components. Nine initial strains of *V. vulnificus* were tested in Mannitol/ Phenol Red broth. The broth was made by mixing 15 g of phenol red broth base with 10 grams of mannitol. Approximately 4 mL aliquots of broth were placed into test tubes, capped, and autoclaved at 121°C for 15 minutes. All of the stains used in these experiments are listed in Table 1. with their codes. Phenol Red was used as an indicator of mannitol membrane transport since the fermentation of mannitol sugar by the bacteria produces acid, turning the broth yellow. Mannitol sugar was used in this media since the ability to ferment it is often correlated with virulent stains of V. vulnificus (Drake 2010).Strains known to be *vcg*E and known to ferment mannitol were used. Tubes of mannitol/ phenol red broth were inoculated with 100 uL of broth cultures of *V. vulnificus* and monitored for color change overnight. Positive samples were considered those that turned yellow. Negative samples were orange to red in color.

2.2 Serum resistance

The concentration of serum that the *vcg*C strains could survive in was also optimized. A serum concentration that allowed for growth of only *vcg*C and limited the need for a high amount of serum was used. The goal of optimizing serum concentration was to limit the cost of the medium while maintaining the highest possible accuracy. Serum is a key component in distinguishing between pathogenic and non-pathogenic strains, but it is also the mostly costly component. Minimizing the use of serum will reduce the cost of the medium. Three different experiments were conducted to optimize the serum concentration used. The first tested 10% and 50% serum concentrations with and without heart infusion broth (HI). HI broth is a non-selective medium that promotes the growth of microorganisms (Difco™ & BBL™ Manual, 2nd Edition). This experiment was conducted using strains VV1 (*vcg*C) and VV5 (*vcg*E). Strains were grown up from freezer stock on HI plates at 37° overnight. The relative concentrations were measured using the spectrophotometer with the lowest concentration assumed to be ~10^9 cfu/ml. Broth cultures were diluted to approximately 10^6 cfu/ml with phosphate-buffered saline (PBS) solution. 10 µL of diluted broth cultures were added to the mixtures shown in Table 2. The samples were incubated in the human serum mixes overnight at 37°C and then diluted 10-fold. 100 uL of each diluted broth culture was plated on Thiosulfate-citrate-bile salts-sucrose agar (TCBS) plates and again grown overnight at 37°C.

In the second serum experiment, serum concentrations of 20, 30, 40, and 50% were tested in mannitol-phenol red broth with of two clinical strains (VV1,VV2) and two environmental strains (VV4, VV5). Strains were again grown up from freezer stock on HI plates incubated at 37°C. Colonies were then picked from the HI plates and grown over night at 37°C in HI broth. Again the relative concentrations were measured using the nanodrop with the lowest concentration assumed to be 10^9. Broth cultures were diluted to approximately 10^6 with PBS solution. 10 uL of diluted broth cultures were added to the mixtures shown in Table 3. After overnight incubation at 37°C, relative concentrations were measured using the nanodrop.

Experiment three used similar methods for growing initial broth cultures, but only tested survivability in serum of one environmental (VV8) and one clinical (VV1) strain. 10 uL of broth culture was added to each combination of mannitol and serum in Table 3. Both VV1 and VV8 have the ability to ferment mannitol. The tubes of culture were incubated at 37°C and monitored for color change every hour until all samples were positive.

2.3 Optimal Incubation Time Testing

After the concentration of serum was optimized, a time point was developed. 10 uL of broth culture were added to the serum/mannitol broth mix for the first 9 strains. This was done by inoculating

the mix with broth cultures grown in HI broth at 37°C for 3-4 hours and observing color change every 30 minutes for 5 hours. The time-point was developed from a minimum of six replicates from each strain.

2.4 Final Formula Testing with Additional Strains

Once a time point was developed using the first nine stains, the time point was tested with an additional 10 strains. Strains were grown in HI broth at 37°C for 4 hours and 10 uL of broth culture was inoculated into 1 mL of the standard medium mix. At 2.25 hours, color change was observed.

3. Results

3.1 Mannitol Fermentation and Serum Resistance

Table 4 shows the strains positive for color change in mannitol broth. All strains known to be *vcg*C were positive whereas 3 *vcg*E stains were positive and 3 were negative. The use of mannitol did eliminate some non-virulent strains, but not all. Serum concentrations were also manipulated. The first serum experiment results are shown in Table 5. Both the environmental and clinical strain survived in 10% serum, but neither grew in 50% serum. The second experiment showed that at 30% serum there was still some growth occurring in both *vcg*C and *vcg*E strains and while there may have been growth at 50% the cell concentrations were lower. Table 6 shows the average absorbance of light at 600 nm wavelength (A600 value), a measure of cell density, in 30% and 50% serum after three hours for each strain tested. Experiment 3 results in Table 7 show the time of color change for two strains in serum and mannitol broth at different concentrations of serum. Both strains changed colors at the same time at all concentrations of serum despite VV8 being a *vcg*E whereas VV1 is a vgC.

3.2 Time Point Development and Final Formula Testing

A time point of 2.25 hours was developed to distinguish between *vcg*C and *vcg*E strains in the medium. Figure 1. shows this time point for color change in the detection of *vcg*C. At 2.25 hours, all *vcg*C stains are positive (yellow) while there is only a single positive *vcg*E strain (VV8). When all 19 strains were tested at the 2.25 time point, 16 of 19 (68%) strains were correctly identified as either *vcg*C or *vcg*E. There were no false negatives. The results for each strain are shown in Table 8.

A time point was also developed to distinguish between strains with *pilF* genes correlated with virulence and those that do not. The time point was increased from 2.25 hours to 4 hours to include all *pilF* positive strains shown in Figure 2. This new time point had an accuracy of 78% with the initial nine strains of identifying those with genes correlated with pathogenicity and still contained no false negatives. Additional strains were not tested at the extended time point due to time constraints. However including those strains that exhibited a positive color change within 2.25 hours still had an accuracy rate of 74% with the possibility of 84% accuracy with an extended time point. This test does have a single false negative when all 19 strains are considered, but the extension of the time point may eliminate it. The results for individual strains are shown in Table 9.

Chi-squared tests were performed to confirm that there were statistical differences in media results for pathogenic and non-pathogenic strains. For the *vcg* time point, clinical and environmental strains were significantly different in whether or not they tested positive ($p= 0.0263$). There was not a statistical difference in *pilF* positive and negative strains at the original 2.25 hr time point ($p= 0.0935$). At

an extended time of 4 hrs however, there is a difference between positive and negative *pilF* strains and whether or not they produce a positive result.

4. Cost and Accuracy Comparisons

The cost of the novel colorimetric media is primarily driven by the cost of pooled human serum and thus the optimization of the percentage of serum used for the media was vital to creating an affordable method for detection. The total cost per sample for the new method is $0.65 assuming that only 1 mL of media is needed. The cost for six 100 mL bottles of pooled human serum from Fisher Science is $1,307.06 or $0.65 per sample. Mannitol purchased from VWR International costs $473.85 for 5 kg. When purchased at this large quantity, the cost of mannitol for the medium is about $0.03 per 100 samples. Phenol Red Broth Base from Thermo Scientific™ costs $130.53 when purchased from Fisher Science, or $0.18 per 100 samples. The primary startup cost for the novel medium would be the purchase of a microbiological incubator. Fisher Scientific™ Isotemp™ Microbiological Incubators can be purchased for $2,600. Similar startup costs would be required for all culture-based methods including Chromagar Vibrio. By comparison, traditional PCR costs $1.17 per sample and takes at least 5 hours compared to the 2.5 hours required for the new method (Williams 2013).

5. Discussion

In the United States there has only been national surveillance of Vibrio vulnificus infections since 2007. Previously, infections were only reported the CDC from Gulf States. On average, there are 95 cases of Vibriosis caused by *V. vulnificus* reported every year with 85 cases leading to hospitalization and 35 cases leading to death. The number of cases is likely underreported (CDC). Although the number of infections are low, the cost of a hospitalization and death is high. The total cost associated with the average *V. vulnificus* infection is $2,792,171 per case and an average of 268 million dollars per year is spent on all cases (Scharff 2011). Improved methods of detection and increased surveillance have the potential to greatly decrease these costs and decrease loss of life.

With the rates of *Vibrio* infections expected to continue to increase with climate change and a higher number of susceptible people, the need for a rapid, easy and affordable method of detection becomes increasingly critical. The ability to distinguish *V. vulnificus* strains of the greatest concern will also help to make more informed public health decisions. The combination of human serum and mannitol with phenol red as an indicator has been shown in these experiments to detect pathogenic strains of V. vulnificus with high accuracy. The test can also be adjusted to meet the needs of the specific application. The medium could potentially be modified to detect pathogenic strains of V. vulnificus in shellfish such as oysters, water, and clinical samples.

Serum is an important aspect of the medium since it is known to be an important aspect of the human immune system. Complement is the bactericidal component of serum that has been shown to lower survivability of V. vulnificus in serum, especially *vcg*E type strains (Williams 2014). *vcg*C strains are also able to survive in blood with much lower iron concentrations than *vcg*E type strains (Bogard 2007). Keeping the concentration of serum as low as possible while still distinguishing between strains was vital to keeping the cost of the medium low.

Since the medium can test for two separate genes by altering the time point, the medium has a wide range of applications. The specific kinds of *Vibrio* disease being monitored for should be taken into account. For the testing of oyster tissue, the *vcg* method would likely be the more accurate measurement. The greatest public health concern from the consumption of raw and undercooked oysters is primary

septicemia and almost all cases of septicemia are caused by *vcg*C strains. The time point for *pilF* could also be used if the goal was to be as protective of public health as possible. There are *vcg*E strains that may have the ability to cause primary septicemia although their main route of infection is through wounds. For the inclusion of risk of wound infections especially, *pilF* time point would be the more accurate than *vcg*.

Polymorphisms in *pilF* have been associated with serum resistance and has also been shown that it may be a better measurement of pathogenicity than *vcg* (González 2012). It is a broader measurement since it will also include *vcg*E strains that have possibility to cause infection. While the *vcg* test may be sufficiently protective for oyster tissue testing, *pilF* should be used for water samples if wound infections are also of concern. It would be a better predictor of Vibriosis than *vcg* since any *vcg*E strain that may be causing illness would not be detected.

While there are many genes correlated with virulent strains of V. vulnificus, those that are known to be correlated with resistance to serum such as *pilF* as commonly used. These experiments have shown that there is also potential to develop a medium with serum to detect pathogenic strains. In comparison to other mediums that detect total V. vulnificus the accuracy of this medium is quite high. The further development of such a medium that could be used for both environmental and clinical samples could be used on a wider scale and would be protective of public health.

Tables and Figures

Table 1 Strain Identifications

Strain abbreviation	Strain	*vcg*E or *vcg*C	*pilF*
VV1	VV MO6	C	+
VV2	VV C7184	C	+
VV3	VV YJO16	C	+
VV4	VV JY1701	E	+
VV5	VV JY1305	E	-
VV6	VV E64MW	E	+
VV7	VV ENV1	E	?
VV8	VV SREL 106	E	-
VV9	VV SREL 89	E	-
VV10	VV SREL 46	E	-
VV11	VV AB6-307	?	-
VV12	VV SREL 190	C	+
VV13	VV SREL 119	E	-
VV14	VV SREL 118	E	-
VV15	VV LSU 1014	E	+
VV16	VV LSU 1866	C	+

VV17	VV SREL 28	E	+
VV18	VV SREL 54	E	+
VV19	VV SPRC 10113	E	+
VV20	VV LSU 2098	E	+

Table 2 Serum Experiment 1 Broth Mixtures

	10 % w/	10 % w/o	50 % w/	50% w/o	+ control	- control
PBS (uL)	800	900	400	500	900	1000
HI broth (uL)	100	0	100	0	100	0
Human Serum (uL)	100	100	500	500	0	0

Table 3 Serum Experiment 2 and 3 Broth Mixtures

Serum (uL)	Mannitol (uL)
200	800
300	700
400	600
500	500

Table 4. Color change in Mannitol Broth

Strain	Positive in Mannitol
VV1	Yes
VV2	Yes
VV3	Yes
VV4	No
VV5	No
VV6	No
VV8	Yes
VV9	Yes
VV10	Yes

Table 5 Serum Experiment 1

	VV1	VV5
Control -	NG	NG
Control +	++	++
10% serum + HI	+	+
10% serum	NG	NG
50% serum +HI	NG	NG

50% serum	NG	NG

NG= no growth ++=lawn += some growth

Table 6 Serum Experiment 2, Cell growth after 3 hours

Strain	A600 (30% serum)	A600 (50% serum)
VV1	0.3005	0.0055
VV2	0.0804	0.0215
VV4	0.051	0.0135
VV5	0.2475	0.188

Table 7 Serum Experiment 3, color change time at varying serum concentrations

Percent Serum	VV1 (hrs)	VV8 (hrs)
20%	3.5	3.5
30%	4.5	4.5
40%	5.5	5.5
50%	5.5	5.5

Figure 1 vcg Time Point

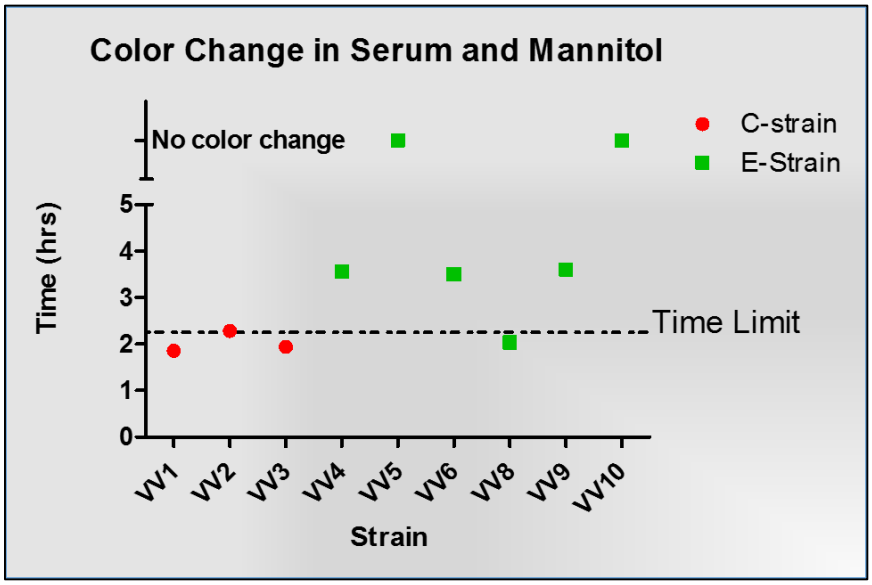

Table 8 Medium test for vcgC strains

Strain	C or E	Positive in Media (2.25 hrs)
VV1	C	+
VV2	C	+
VV3	C	+
VV4	E	-

VV5	E	-
VV6	E (clinical)	-
VV8	E	+
VV9	E	-
VV10	E	-
VV11	?	-
VV12	C	+
VV13	E	+
VV14	E	-
VV15	E	+
VV16	C	+
VV17	E	-
VV18	E	+
VV19	E	+
VV20	E (clinical)	+

Figure 2 pilF Time Point

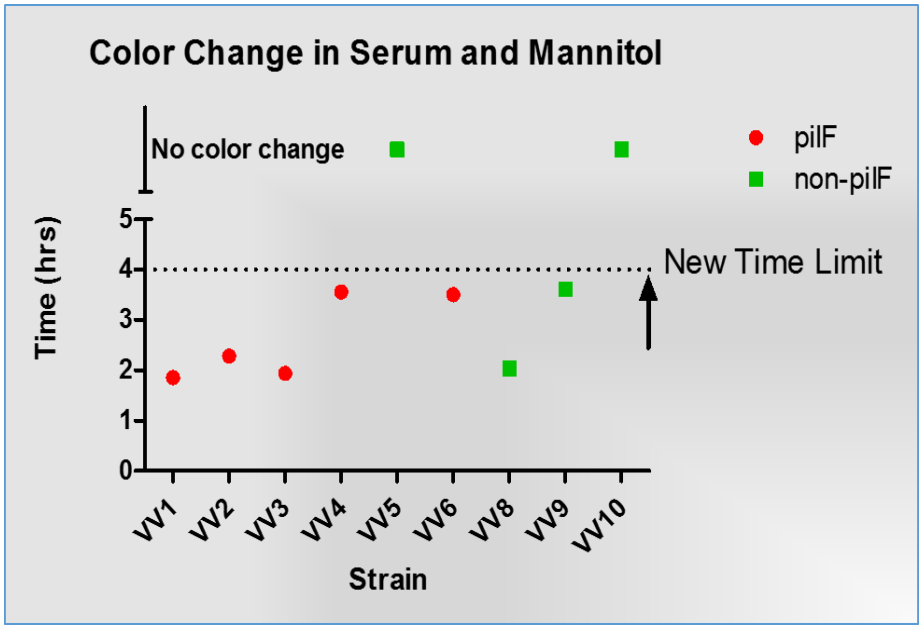

Table 9 pilF Medium Results

Strain	pilF?	Positive in Media (2.25 hrs)	Changes at 4hr time point
VV1	Yes	+	
VV2	Yes	+	
VV3	Yes	+	
VV4	Yes	-	Would become +
VV5	No	-	
VV6	Yes	-	Would become +
VV8	No	+	
VV9	No	-	Would become +
VV10	No	-	
VV11	No	-	
VV12	Yes	+	
VV13	No	+	
VV14	No	-	
VV15	Yes	+	
VV16	Yes	+	
VV17	Yes	-	May become +
VV18	Yes	+	
VV19	Yes	+	
VV20	Yes	+	

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