




## RESEARCH ARTICLE

# REVISED Increased growth temperature and vitamin B12 supplementation reduces the lag time for rapid pathogen identification in BHI agar and blood cultures [version 2; peer review: 2 approved]

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## Abstract







**Background:** Rapid diagnostics of pathogens is essential to prescribe appropriate antibiotic therapy. The current methods for pathogen detection require the bacteria to grow in a culture medium, which is time-consuming. This increases the mortality rate and global burden of antimicrobial resistance. Culture-free detection methods are still under development and are not common in the clinical routine. Therefore, decreasing the culture time for accurately detecting infection and resistance is vital for diagnosis.



**Methods:** This study investigated easy-to-implement factors (in a minimal laboratory set-up), including inoculum size, incubation temperature, and additional supplementation (e.g., vitamin B12 and trace metals), that can significantly reduce the bacterial lag time ( $t_{lag}$ ). These factors were arranged in simple two-level factorial designs using Gram-positive cocci (*Staphylococcus aureus*), Gram-positive bacilli (*Bacillus subtilis*), and Gram-negative bacilli (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, including clinical isolates with known antimicrobial resistance profiles. Blood samples spiked with a clinical isolate of *E. coli* CCUG 17620 (Culture Collection University of Gothenburg) were also tested to see the effect of elevated incubation temperature on bacterial growth in blood cultures.

**Results:** We observed that increased incubation temperature (42°C) along with vitamin B12 supplementation significantly reduced the  $t_{lag}$  (10 – 115 minutes or 4% - 49%) in pure clinical isolates and blood samples spiked with *E. coli* CCUG17620. In the case of the blood

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2. **Rashid Nazir** , COMSATS University Islamabad, Abbottabad, Pakistan

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sample, PCR results also detected bacterial DNA after only 3h of incubation and at three times the CFU/mL.

**Conclusion:** Enrichment of bacterial culture media with growth supplements such as vitamin B12 and increased incubation temperature can be a cheap and rapid method for the early detection of pathogens. This proof-of-concept study is restricted to a few bacterial strains and growth conditions. In the future, the effect of other growth conditions and difficult-to-culture bacteria should be explored to shorten the lag phase.

### Keywords

Lag time, Growth temperature, Vitamin B12, Brain heart infusion, Antimicrobial resistance, Pathogen detection, Blood culture



This article is included in the **Pathogens** gateway.



This article is included in the **Antimicrobial Resistance** collection.

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**REVISED Amendments from Version 1**

1. Gram-positive and gram-negative classification error has been updated in the abstract and introduction.
2. The introduction has been updated, and paragraphs linked together and shortened to include relevant information suggested by the reviewers.
3. The gram-staining column in Table 1 has been updated. A new column on Bacterial doubling time in table 1 has been added.
4. Data related to DNA quantification has been added to the *PCR results* section.
5. The calculation regarding doubling time has been included in the methodology section, and the results are included under the heading '*Bacterial doubling time.*'
6. A new supplement figure on bacterial doubling time results from *E. coli* and *S. aureus* has been added.
7. Based on the reviewer's feedback, we updated the discussion, and relevant references have been added.

**Any further responses from the reviewers can be found at the end of the article**

**Introduction**

The discovery of antibiotics was a breakthrough in therapeutic medicines, which has enabled us to treat critical bacterial infections (Ali *et al.*, 2020). It has been more than 35 years since a new class of antibiotics was discovered, and bacteria are resistant to almost all the presently used antibiotics (Hoffman, 2020). The unavailability of new antibiotics further increases the need for rapid diagnostics of pathogens and their AMR. Early and appropriate antibiotic therapy is crucial in cases of severe bacterial infections and can significantly reduce mortality. One such example is sepsis, a major health problem that accounts for an estimated 31.5 million cases with a mortality rate of 5.3 million deaths globally per year (Fleischmann *et al.*, 2016; Taxis *et al.*, 2020). In the case of sepsis, early treatment with appropriate antibiotics is vital for the patient's survival (Bloos *et al.*, 2017; Ferrer *et al.*, 2014). In septic patients, delaying each hour in prescribing appropriate antibiotics can have lethal consequences (Avershina *et al.*, 2022). Due to the lack of adequate and early diagnostics, clinicians are pushed to prescribe empirical antibiotics to the patients, which in most cases are not very effective and further disseminate antibiotic resistance (Khaledi *et al.*, 2020).

Optimizing the existing diagnostic methods and making them faster and more cost-effective is crucial to decrease the use of empirical and broad-spectrum antibiotics. The current diagnostic procedures rely on culture-based techniques where bacteria are cultured and then identified using different biochemical tests. After that, antibiotic susceptibility testing (AST) is performed, which gives an idea about the resistance or sensitivity of bacteria to different antibiotics. The culture-based methods have a turnaround time of two to four days in most samples. However, in the case of bloodstream infections where the bacterial load is lower, the identification and AST can take up to five days (Briggs *et al.*, 2021; Metzgar *et al.*, 2016). There are many emerging micro- and nanotechnologies for pathogen identification and AST, including both phenotypic (microfluidic-based bacterial culture) and molecular methods (PCR, hybridization probes, nanoparticles, synthetic biology, and mass spectrometry) (Li *et al.*, 2017). Whole genome sequencing (WGS) using the Oxford Nanopore Technology's (ONT) MinION has the potential to detect pathogens and their associated antibiotic resistance genes (ARGs) rapidly, but this technique is still in development, is costly, and not used in clinical settings (Avershina *et al.*, 2022; Taxis *et al.*, 2020). All these techniques are expensive, need dedicated infrastructure, or are time-consuming. Therefore, there is an urgent need to develop cost- and resource-effective methods that can speed up pathogen diagnostics and reduce the mortality from these infections.

We need to understand the bacterial growth requirements to evaluate the possible solutions for the rapid diagnosis of pathogens. Mesophilic bacteria are typically grown at 30 to 37°C in lab procedures. In rich growth media (*i.e.*, Luria-Bertani, Terrific Broth), dense cultures are raised in a reasonably short time of two to six hours using inoculums 1-2% of overnight cultures (ca. 12-14 h). When growing bacteria from clinical samples in blood cultures, however, a far lower inoculum is experienced, *i.e.*, <100 CFU/mL and growth must be pursued for a minimum of 24 h up to several days to detect potential pathogens (Doualeh *et al.*, 2022; Hardy *et al.*, 1992; Waldeisen *et al.*, 2011). The lengthy growth of fastidious bacteria in blood cultures to reach detectable titers is a significant bottleneck in the workflow of testing pathogens for AMR (Menchinelli *et al.*, 2019; Somily *et al.*, 2018; Verroken *et al.*, 2015). Brain heart infusion (BHI) is a nutrient growth medium suitable for blood culture and to culture various fastidious organisms. BHI broth is often used in several application areas, including clinical microbiology testing, life science, biopharmaceuticals, food safety, water safety, and antibiotic sensitivity tests.

**Table 1.** An overview of the different combinations of experiments performed, including information regarding different bacterial strains, their antibiotic susceptibility phenotype, doubling time ( $t_d$ ), and growth variables (i.e., Temperature, Inoculum size, and vitamin B12 supplementation) symbolized  $X_1$ ,  $X_2$ , and  $X_3$ .

Experiment No.	Bacterial strain	Antibiotic susceptibility phenotype	Gram staining	Temperature ( $^{\circ}\text{C}$ ) $X_1$	Inoculum size (v/v %) $X_2$	Vitamin B12 ( $\mu\text{M}$ ) $X_3$	Doubling time ( $t_d$ ) (minutes)
1	<i>E. coli</i> HB101 K2	wild type	Gram negative bacilli	37	0.05	0	28.2
2				42	0.05	0	24.6
3				37	0.05	50	21
4				42	0.05	50	12
5				37	0.5	0	18.6
6				42	0.5	0	13.2
7				37	0.5	50	32.4
8				42	0.5	50	23.4
9	<i>S. aureus</i> NCTC 8325*	wild type	Gram positive cocci	37	0.05	0	33
10				42	0.05	0	29.4
11				37	0.05	50	28.2
12				42	0.05	50	30.6
13				37	0.5	0	25.8
14				42	0.5	0	31.2
15				37	0.5	50	28.8
16				42	0.5	50	21.6
17	<i>P. aeruginosa</i> INN	wild type	Gram negative bacilli	37	0.5	0	47.4
18				37	0.5	50	49.2
19				42	0.5	0	55.2
20				42	0.5	50	53.4
21	<i>B. subtilis</i> INN	wild type	Gram positive bacilli	37	0.5	0	33.6
22				37	0.5	50	36
23				42	0.5	0	30.6
24				42	0.5	50	30

**Table 1.** Continued

Experiment No.	Bacterial strain	Antibiotic susceptibility phenotype	Gram staining	Temperature (°C) X <sub>1</sub>	Inoculum size (v/v %) X <sub>2</sub>	Vitamin B12 (µM) X <sub>3</sub>	Doubling time (t <sub>d</sub> ) (minutes)
25	<i>E. coli</i> CCUG 17620*	wild type	Gram negative bacilli	37	0.5	0	38.4
27				42	0.5	0	30
29	<i>E. coli</i> NCTC 13441*	resistant (ampicillin, extended-spectrum cephalosporins, ciprofloxacin, trimethoprim)	Gram negative bacilli	37	0.5	0	33.6
31				42	0.5	0	23.4
33	<i>S. aureus</i> CCUG 35600*	resistant (methicillin, tetracycline, clindamycin, erythromycin)	Gram positive cocci	42	0.5	0	28.2
35				42	0.5	50	33

\*Clinical strains.

The bacterial growth curve shows four phases: lag phase, log (exponential) phase, stationary, and death phase. Clearly, the time for a culture to reach detectable density, *i.e.*, the length of lag phase growth, is a bottleneck in the diagnostic workflow. In this study, we wanted to investigate easy-to-implement factors (in a minimal laboratory set-up), including inoculum size, incubation temperature, and additional supplementation (*e.g.*, vitamin B12 and trace metals), that can significantly reduce the lag time ( $t_{lag}$ ). These factors were arranged in simple two-level factorial designs using different Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria, including clinical isolates with known AMR profiles (encoding methicillin resistance or extended-spectrum  $\beta$ -lactamases). BHI was used as the growth medium as it is suitable for use as a blood culture medium. Based on the results from experiments with pure bacterial isolates, blood samples spiked with a clinical isolate of *E. coli* CCUG17620 were also tested to see the effect of elevated incubation temperature on bacterial growth in blood cultures.

## Methods

### Bacterial strains

The study focused on seven different bacterial strains covering four different bacterial species. Clinical bacterial strains *E. coli* NCTC13441 and *S. aureus* NCTC8325 were obtained from the National Collection of Type Cultures (NCTC), while *S. aureus* CCUG35600 and *E. coli* CCUG17620 were obtained from the Culture Collection University of Gothenburg (CCUG). Three non-clinical wild-type strains, including *E. coli* HB101 K2, *Pseudomonas aeruginosa* INN, and *Bacillus subtilis* INN, were taken from the internal bacterial collection of Inland Norway University (INN). Additional details are available in [Table 1](#).

### Chemicals

All reagents were obtained from standard lab suppliers. Bacteriological agar and Brain Heart Infusion (BHI) broth for the media preparation were the products of HiMedia Laboratories (Mumbai, India; lot number: BCBH4004V), with BHI consisting of (g/L) calf brain infusion from 200 g (12.5), beef heart infusion from 250 g (5), peptone (10), sodium chloride (5), disodium chloride D (+) glucose (2), disodium hydrogen phosphate (2.5) at pH 7.4. Vitamin B12 or cyanocobalamin was from ThermoFisher, (Massachusetts, USA; number A14894). In addition, we also tested three clinical isolates using a trace metal solution (details in Supplementary Table 1, *Extended data* [[Ahmad 2023](#)]).

### Pre-culture and growth procedures

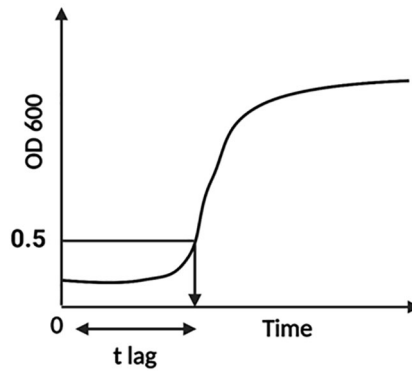
BHI agar and liquid culture media were used to maintain cultures and to examine growth curves. Bacterial samples stored at  $-80^{\circ}\text{C}$  were thawed gently at room temperature, inoculated to BHI broth, and incubated overnight at  $37^{\circ}\text{C}$ . BHI agar plates were prepared by adding 1.5% bacteriological agar powder to BHI broth. An overnight inoculum was streaked out, and plates were incubated at  $37^{\circ}\text{C}$  for 24 h. Single colonies were selected and inoculated into fresh BHI broth. The inoculated BHI broth was incubated at  $37^{\circ}\text{C}$  overnight and served as inoculum for culture experiments (See Supplementary figure S1 for an overview of the complete process, *Extended data*). Experimental liquid cultures were cultivated in shake flasks (500 mL broth in 2L Erlenmeyer bottles). Cultures within the same experimental design were inoculated from the same bacterial preculture suspension to ensure identical starting conditions.

All cultures in BHI broth were incubated at  $37^{\circ}\text{C}$  or  $42^{\circ}\text{C}$  under shaking conditions for up to 6 h, whereas blood cultures were cultivated for 12 h. During incubation, the optical density (OD) was measured at 600 nm every 20-30 minutes, starting from the time of inoculation (0 min) until the bacterial growth reached the stationary phase (approximately 4-5 h). Culture aliquots were immediately cooled and kept on ice until measurement. The OD 600 was plotted *versus* time (min) to obtain the growth curves.

As a practical response value to estimate the effectiveness of treatments, an endpoint of the lag time ( $t_{lag}$ ) was defined, the  $t_{lag}$  being the time (min) from inoculation until the OD 600 value reached a net increase of 0.5. The net OD increase was obtained by subtracting the OD at  $t=0$  from all OD values. The  $t_{lag}$  was obtained by extrapolating at OD 0.5 from the zero-adjusted growth curve. Although this provisional end point is beyond the actual end of lag ([Bertrand, 2019](#)), an extended OD cut-off value was selected to reduce the estimation error when growth curves go from an undefined shallow rise to a steady exponential growth ([Figure 1](#)).

The doubling time ( $t_d$ ) for bacteria was calculated based on the steepest rise in the exponential phase of the growth curve. The OD (log) and time interval in the exponential phase were selected at two points, which showed a specific growth rate ( $\mu$ ). The following formula was used to calculate the bacterial doubling time.

$$t_d = 0.693/\mu$$



**Figure 1. Graphic description of the determination of  $t_{lag}$  from the growth curves used in this study.** The 0.5-intercept represents the net increase in OD 600, *i.e.*, the value subtracted from the optical density (OD) at time zero.

In the formula above, 0.693 is constant, and “ $\mu$ ” is the specific growth rate, which is calculated by using the following equation:

$$\mu = (\text{Ln}(\text{OD}_2) - \text{Ln}(\text{OD}_1)) / (t_2 - t_1)$$

where;

Ln is the natural log

$\text{OD}_2$  is the OD at the endpoint of the exponential phase

$\text{OD}_1$  is the OD at the starting point of the exponential phase

$t_2$  is the time corresponding to  $\text{OD}_2$

$t_1$  is the time corresponding to  $\text{OD}_1$

### Design of experiments for screening of growth variables

A set of growth screening experiments examined the ability of selected variables to cause a reduced lag time. A structured two-level factorial design (Design of Experiments, DOE) was used to observe the effect of incubation temperatures (37°C and 42°C), low (0.05%), and high (0.5%) inoculum, vitamin B12 ( $\pm 50 \mu\text{M}$ ), and trace metal (present y/n) supplements. The design was tested on the wild-type strains (3) and the clinical isolates (4) but with a selection of two or three variables or factors. Table 2 shows the coded and decoded values of the factors tested ( $X_1, X_2, X_3$ ) at a low (-1) and high (+1) level. Thus, either four ( $2^2$ ) or eight ( $2^3$ ) growth experiments were performed for each strain. For a detailed overview of conditions for the individual strains, Table 1 is provided as well as in Supplementary Tables 2-4 (*Extended data*).

The estimated  $t_{lag}$  values from DOE were presented in interaction effect plots, illustrating the impact of a variable (*e.g.*, temperature) with connected experiments on another variable (*e.g.*, vitamin B12 supplement). The main effects of tested variables are shown numerically in descending order in bar charts. The bars are the absolute effect values computed as twice the regression coefficients from fitted DOE models. They show the change in  $t_{lag}$  when a variable goes from a low to a high level when other factors are kept at their average.

**Table 2. The coded and uncoded variables for screening the growth behavior in bacterial cultures.**

Variable	Factor	Levels	
		-1	+1
Temperature (°C)	$X_1$	37	42
Inoculum size (v/v %)	$X_2$	0.05	0.5
Vitamin B12 ( $\mu\text{M}$ )	$X_3$	0	50

## Blood culturing

Clinical strain *E. coli* CCUG17620 was spiked with blood to mimic the sepsis infection. The blood was spiked with 40 CFU/mL from an overnight plate culture of the clinical strain. The lower CFU was used as an inoculum because, in the case of sepsis, the CFU/mL ranges typically from 10-100 (Doualeh *et al.*, 2022). *E. coli* was grown overnight on a BHI agar plate and then suspended in saline to measure the OD. After measuring OD, the suspensions were plated on agar plates to calculate the CFU at different OD values. After optimizing the relation of OD with CFU counts, the desired concentration in the range of 10-100 CFU/mL was used for spiking with human blood. Fresh human blood obtained from healthy donors at Inland Norway University was used for this experiment. Human blood spiked with *E. coli* was mixed with BHI broth medium and incubated at the two study temperatures, *i.e.*, 37 and 42°C. CFU/mL was calculated every 3 h for 12 h by plating out the samples on BHI agar plates. The CFU/mL was calculated using the formula: number of colonies  $\times$  dilution factor/volume of culture plated.

## DNA extraction and PCR from blood cultures

Bacterial DNA from blood culture samples was extracted using QIAamp BiOstic Bacteremia DNA Kit from Qiagen (Germany). The concentration and purity of isolated DNA were determined using the Qubit system with a dsDNA HS assay kit (Thermo Fisher Scientific, USA) and the NanoDrop spectrophotometer. PCR was performed to confirm the presence of *E. coli* CCUG17620 DNA and human DNA in samples taken at 3 h, 6 h, 9 h, and 12 h of incubation at 37°C and 42°C. The primers used for identifying *E. coli* were uspA-F CCGATACGCTGCCAATCAGT and uspA-R ACGCAGACCGTAGGCCAGAT, and human DNA was  $\beta$ -actin (F-CGGCCTTGGAGTGTGTATTAAGTA and R-TGCAAAGAACACGGCTAAGTGT) (Hasan *et al.*, 2016). The PCR reaction was set up in a total volume of 20  $\mu$ L per sample containing 4  $\mu$ L HOT FIREPol MultiPlex Mix Ready to Load master mix from Solis Biodyne (Estonia), 0.5  $\mu$ L of 10  $\mu$ M forward and reverse primers, 8 ng/ $\mu$ L of template DNA and nuclease-free water. The thermal cycling conditions for PCR were initial denaturation at 95°C for 12 min followed by 35 cycles of 95°C for 25 s, 61°C for 50 s and 72°C for 1 min, and a final extension of 72°C for 7 min. The PCR product was run on 1% agarose gel for 45 min at 100V and then visualized on the Gel doc system to determine the presence or absence of our desired bands.

## Statistical analysis

The MODDE 13.0 Pro (Umetrics/Sartorius Stedim Data Analytics AB, Sweden) was used to generate the design matrix, regression coefficients, and statistical analysis. The significance testing ( $p < 0.05$ ) of the DOE screening was performed with ANOVA. All the growth curve graphs are generated using GraphPad Prism 9.4.1.

## Results

### Higher incubation temperature, along with vitamin B12 supplementation, reduces the lag time for bacterial growth

#### *Escherichia coli*

#### *E. coli* HB101 K2

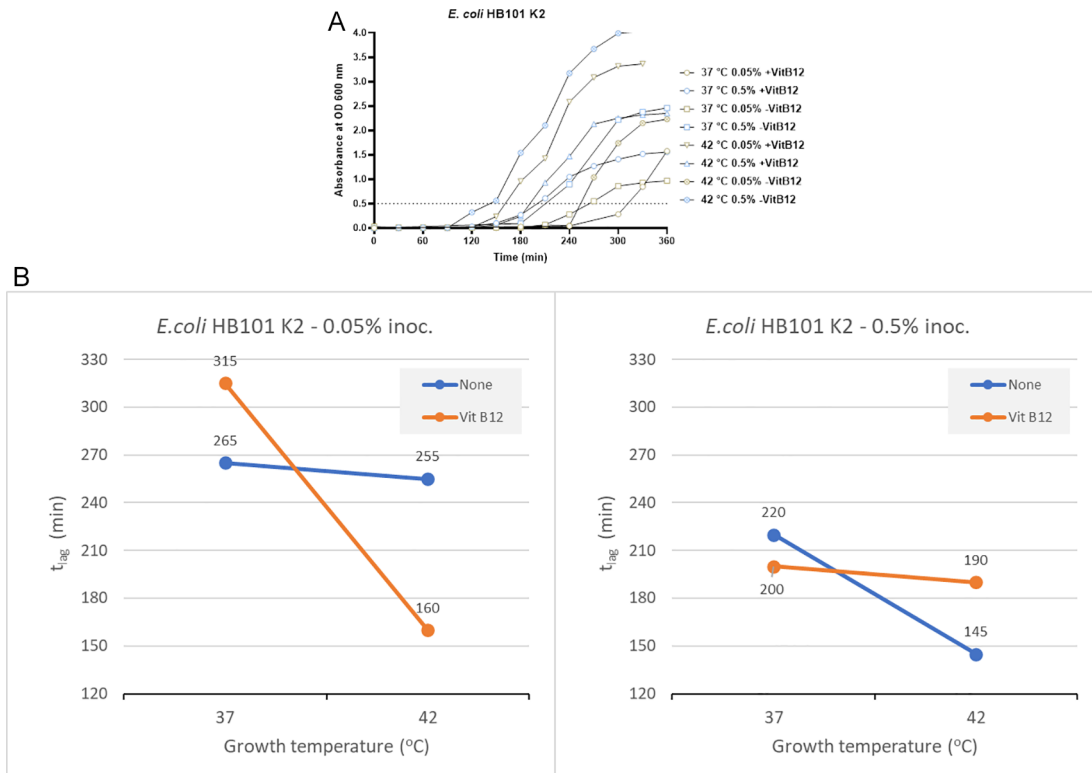
In the absence of vitamin B12 supplementation, the  $t_{lag}$  for bacterial growth was 265 minutes and 255 minutes at 37°C and 42°C, respectively, when an inoculum size of 0.05% was used for culturing (Figure 2A). When using a higher inoculum of 0.5% in the absence of vitamin B12 supplement, the  $t_{lag}$  recorded was 220 minutes and 145 minutes at 37°C and 42°C, respectively (Figure 2A). In the presence of vitamin B12 supplement and using an inoculum size of 0.05%, the bacterial  $t_{lag}$  was 315 minutes (37°C) and 160 minutes (42°C). Similarly, when the inoculum size was increased to 0.5%, with vitamin B12 supplementation, the  $t_{lag}$  was 200 minutes (37°C) and 190 minutes (42°C). The shortest  $t_{lag}$  was recorded to be 145 minutes, which was observed using an inoculum size of 0.5% in the absence of vitamin B12 supplement and incubation at 42°C (Figure 2 and Supplementary Table 2 [Extended data]).

Using an inoculum size of 0.05% and a vitamin B12 supplement, we observed 95 minutes of reduction in the lag time at 42°C compared to 37°C (Figure 2B). In contrast, when the inoculum size was increased to 0.5% along with vitamin B12 supplementation, the  $t_{lag}$  was reduced by 10 minutes at 42°C. Based on the effect plot, it is reported that the sample with no vitamin B12 supplement and 0.5% inoculum size showed the shortest  $t_{lag}$ . But when the inoculum size was decreased to 0.05%, we observed that the sample incubated at 42°C in the presence of vitamin B12 supplement showed the shortest  $t_{lag}$  (Figure 2B). The reduction in  $t_{lag}$  was between 4% and 49% for the above mentioned eight tested conditions.

#### *E. coli* CCUG17620 and *E. coli* NCTC13441

Based on the results from the laboratory strain, we tested the growth of two *E. coli* clinical isolates at 37 and 42°C. *E. coli* CCUG17620 took 170 minutes when incubated at 42°C and 193 minutes at 37°C to reach OD 0.5 without adding vitamin





**Figure 2. Results from *E. coli* HB101 K2 under different growth conditions, including inoculum size, temperature, and vitamin B12 supplementation.** (A) Growth curves of *E. coli* HB101 K2 were obtained in three-factorial designs; at 37°C and 42°C, inoculum size 0.05% and 0.5 %, with and without vitamin B12. The dotted line indicates OD 0.5 for estimating the t<sub>lag</sub> from curves. (B) Effect plot of *E. coli* HB101 K2 in minutes (t<sub>lag</sub>) to reach optical density 0.5.

B12. Similarly, *E. coli* NCTC13441 required 126 and 167 minutes to reach OD 0.5 at 42°C and 37°C, respectively (Figure 3A and Supplementary Table 4 [Extended data]). For the two clinical isolates, *E. coli* CCUG17620 and *E. coli* NCTC13441, the t<sub>lag</sub> was less at 42°C compared to 37°C.

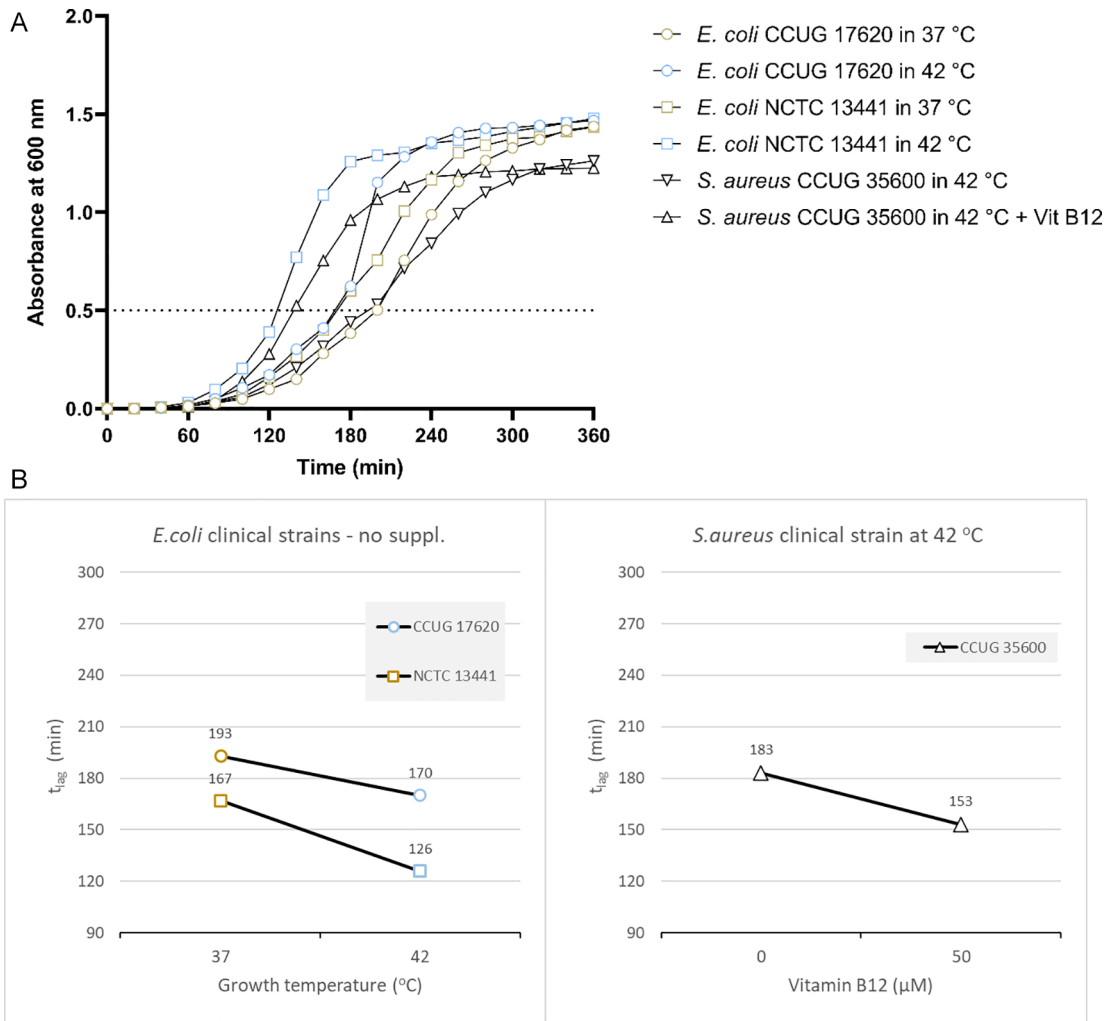
Overall, the effect plot of *E. coli* CCUG17620 and *E. coli* NCTC13441 at inoculum size 0.5% indicated that when the incubation temperature increased (42°C instead of 37°C), the lag time decreased by 15-25% in the tested strains (Figure 3B).

### *Staphylococcus aureus*

#### *S. aureus* NCTC8325

In the case of the clinical isolate *S. aureus* NCTC8325 with no vitamin B12 supplements and 0.05% inoculum size, the time to reach OD 0.5 was 285 minutes and 210 minutes at 37°C and 42°C, respectively (Figure 4A and Supplementary Table 2). But in the presence of vitamin B12 supplementation and lower inoculum size of 0.05%, the t<sub>lag</sub> was 190 minutes (37°C) and 165 minutes (42°C). When the inoculum size was increased to 0.05%, the t<sub>lag</sub> decreased significantly at 42°C (135 minutes) in comparison to 37°C (215 minutes) in the presence of vitamin B12 supplementation (Figure 4A and Supplementary Table 2). We can conclude that incubation at 42°C, along with vitamin B12 supplement, reduces the t<sub>lag</sub> in *S. aureus* NCTC8325.

The effect plot of *S. aureus* NCTC8325 at 0.5% inoculum shows a reduction of 121 minutes in t<sub>lag</sub> at 42°C incubation in the presence of vitamin B12 supplement. At the same time, a reduction of 45 minutes in t<sub>lag</sub> was observed at 0.05% inoculum and 42°C incubation in the presence of vitamin B12 supplement (Figure 4B). The reduction in t<sub>lag</sub> was between 13% and 37% for the eight tested conditions.



**Figure 3. Results for the clinical strains *E. coli* CCUG17620, *E. coli* NCTC13441, and *S. aureus* CCUG35600 under different growth conditions, including temperature, and vitamin B12 supplementation.** (A) Growth curves of clinical bacterial isolates at 37°C and 42°C (*E. coli* CCUG17620, *E. coli* NCTC13441), or with and without vitamin B12 supplement (*S. aureus* CCUG35600 at 42°C). The dotted line indicates OD 0.5 for estimating the  $t_{lag}$  from curves. (B) Effect plot of *clinical samples* in minutes ( $t_{lag}$ ) to reach optical density 0.5.

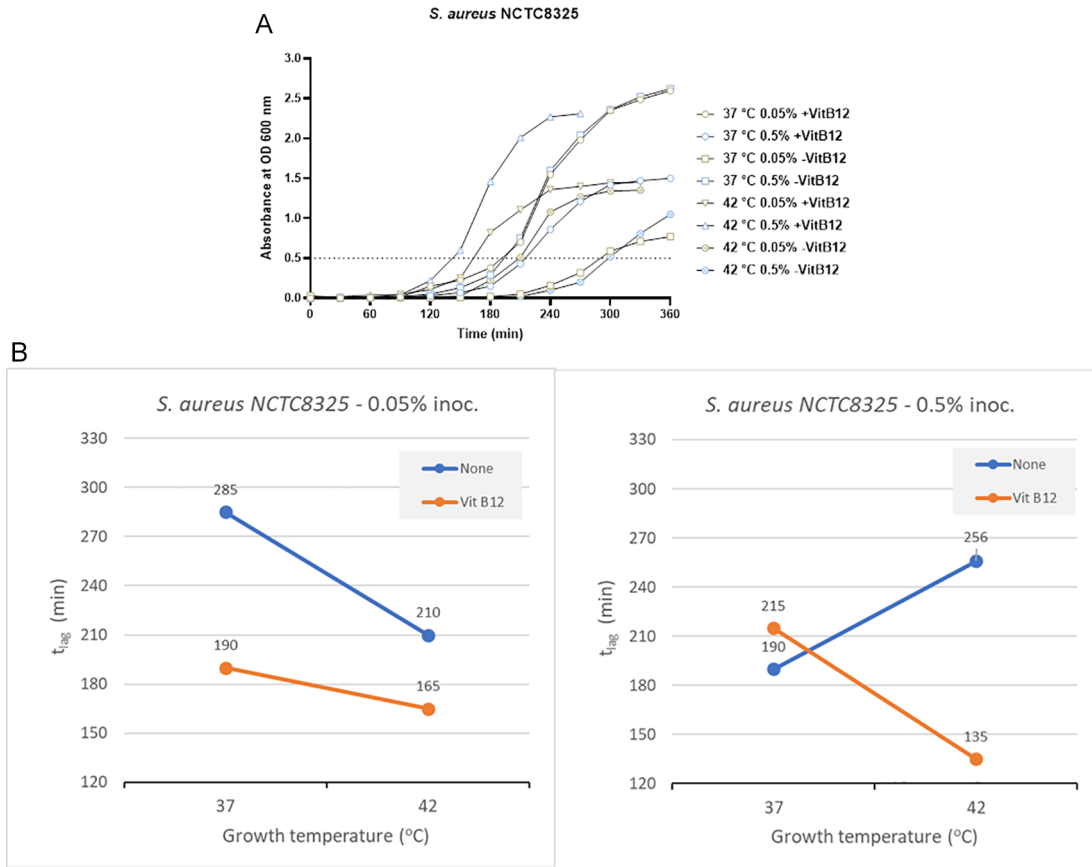
*S. aureus* CCUG35600

Based on the above results, we tested the growth of another clinical strain of *S. aureus*. The effect plot of the clinical isolate *S. aureus* CCUG35600 at inoculum size 0.5% at 42°C co-cultured with vitamin B12 supplement showed a decline in lag time compared to without supplementation. Overall, the shortest  $t_{lag}$  time was observed following the co-culture of bacteria with vitamin B12 supplement (Figure 3A and B). So, the reduction in  $t_{lag}$  was 20%.

*Pseudomonas aeruginosa*

*P. aeruginosa* showed a significant reduction in the  $t_{lag}$  when vitamin B12 supplements were added along with 42°C incubation. It reached OD 0.5 in 185 minutes when vitamin B12 supplement was added along with 42°C incubation. On the other hand, 235 minutes was required with no vitamin B12 supplement and 37°C incubation (Figure 5A).

The effect plot showed a reduction of 30 minutes in  $t_{lag}$  when bacteria were cultured at 42°C in the presence of vitamin B12 supplementation (Figure 5B). The decline in  $t_{lag}$  was between 9% and 20% for the four tested conditions.



**Figure 4. Results for *S. aureus* NCTC8325 under different growth conditions, including inoculum size, temperature, and vitamin B12 supplementation.** (A) Growth curves of *S. aureus* NCTC8325 were obtained in three-factorial designs; at 37°C and 42°C inoculum sizes 0.05% and 0.5 %, with and without vitamin B12. The dotted line indicates OD 0.5 for estimating the  $t_{lag}$  from curves. (B) Effect plot of *S. aureus* NCTC8325 in minutes ( $t_{lag}$ ) to reach optical density 0.5.

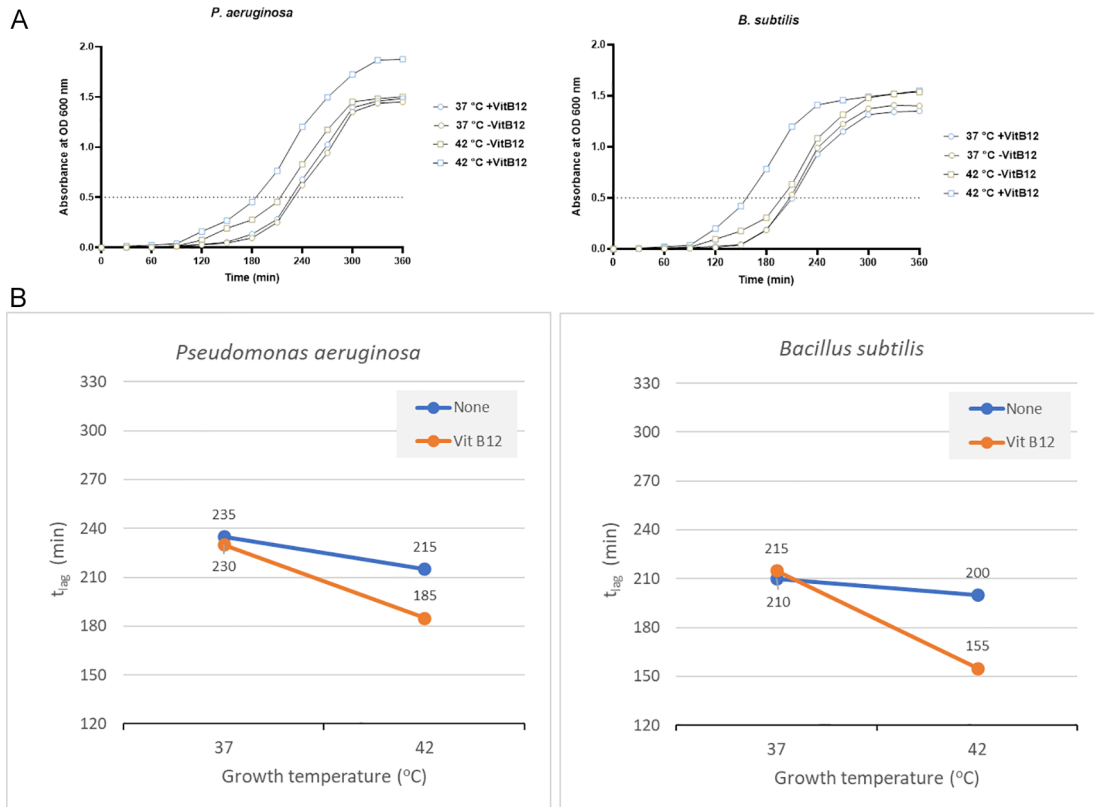
### *Bacillus subtilis*

*B. subtilis* also showed the same pattern as other bacteria, with a  $t_{lag}$  of 155 minutes with vitamin B12 supplement and 42°C incubation. On the other hand, the  $t_{lag}$  was 210 minutes and 215 minutes to reach OD 0.5 when incubated at 37°C with and without vitamin B12 supplement, respectively (Figure 5A and Supplementary Table 3 [Extended data]). The effect plot showed a reduction of 45 minutes in  $t_{lag}$  when bacteria were co-cultured at 42°C in the presence of vitamin B12 (Figure 5B). The reduction in  $t_{lag}$  was between 5% and 28% for the four tested conditions.

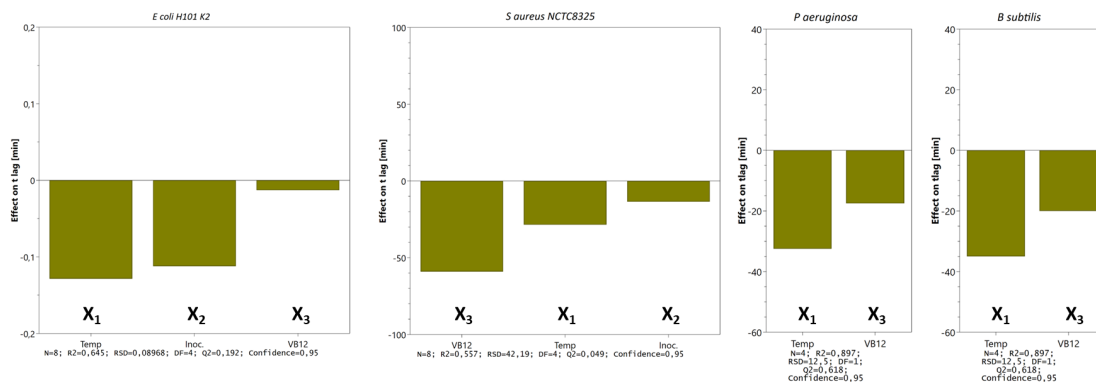
The main effects on  $t_{lag}$  from the variables tested in three- (*E. coli* and *S. aureus*) and two-factor (*P. aeruginosa* and *B. subtilis*) experimental designs are shown in Figure 6. We tested the time to reach OD 0.5 for *S. aureus*, *E. coli*, *P. aeruginosa*, and *B. subtilis* and noticed that incubating the bacteria at 42°C reduces the lag time. However, the clinical isolates (*E. coli* CCUG17620, *E. coli* NCTC13441, and *S. aureus* CCUG35600), which were tested for trace metal solution complement, adversely affected bacterial growth by prolonging the lag time (details in Extended data).

### Bacterial doubling time

The doubling time of all the tested isolates falls between 12 – 55 minutes. The median of the calculated  $t_d$  is 30.6 minutes (n=36), meaning the value closest to the midpoint of the population, i.e., the most 'normal' value. Although the difference in  $t_d$  was insignificant, we still noticed that at 42°C,  $t_d$  is lower (bacteria grow faster) than at 37°C. The difference in  $t_d$  at both temperatures was more pronounced in *E. coli* isolates (*E. coli* HB101 K2, *E. coli* NCTC13441, and *E. coli* CCUG17620) compared to *S. aureus* isolates. We did not see any effect of temperature on  $t_d$  in *P. aeruginosa* and *B. subtilis* isolates (Table 1 and Supplementary figure 3 [Extended data]).



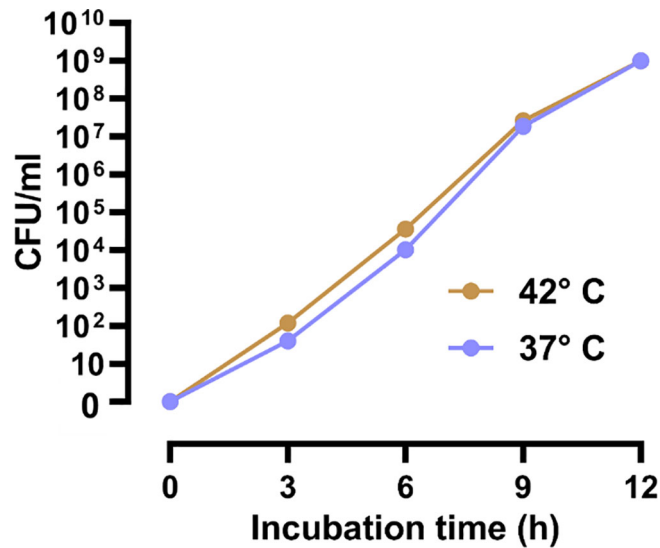
**Figure 5. Results for *Pseudomonas aeruginosa* and *Bacillus subtilis* under different growth conditions, including temperature and vitamin B12 supplementation.** (A) Growth curves of *P. aeruginosa* and *B. subtilis* were obtained in two-factorial designs, at 37°C and 42°C, with and without vitamin B12. The dotted line indicates OD 0.5 for estimating the  $t_{lag}$  from curves. (B) Effect plot of *P. aeruginosa* and *B. subtilis* in minutes ( $t_{lag}$ ) to reach optical density 0.5.



**Figure 6. Main effects on  $t_{lag}$  from the variables tested in three- (*E. coli* and *S. aureus*) and two-factor (*P. aeruginosa* and *B. subtilis*) experimental designs.** Bars represent the change in  $t_{lag}$  (min) when a factor varies from its low to a high level (cfr. Table 1) when all other factors are kept at their averages. Calculated effects are given with 95% confidence. Other statistical features are given at the bottom.

### Higher incubation temperature increases bacterial growth in blood cultures

We also observed that higher incubation temperature could increase bacterial growth in blood cultures as in pure bacterial isolates. The CFU of the blood samples, which were spiked with *E. coli* CCUG17620 and incubated at the two study temperatures, 37°C and 42°C, showed that bacteria could grow much faster in the early stages of incubation at 42°C compared to 37°C. When we calculated the CFU after 3 h and 6 h of incubation, we could see that the CFU/mL of bacteria were three times higher at 42°C than at 37°C. This higher growth of bacteria in the initial hours of incubation is critical for



**Figure 7.** Increased viable counts (CFU/mL) of *E. coli* CCUG17620 spiked in blood cultures cultivated at 37 and 42°C.

identifying the causative pathogen. This information is valuable for infection diagnostic purposes. Further incubation beyond 6 h, narrowed the gap between CFU/mL at 37°C and 42°C. At 12 h of incubation, we got the same CFU at both temperatures (Figure 7).

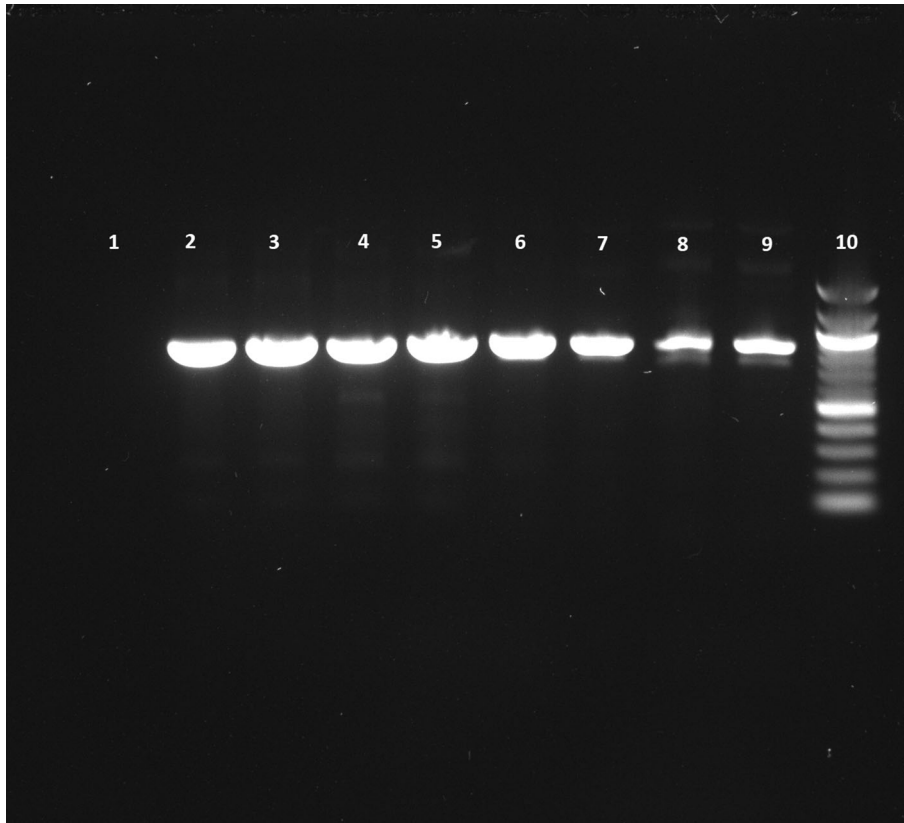
#### PCR results show higher purity of DNA at an increased growth temperature

We performed PCR to confirm the presence of bacterial DNA *versus* human DNA at different incubation time points, as described in Methods. The PCR product run on 1% agarose gel was visualized for the presence of DNA and compared with a 100 bp DNA ladder, which was also run on the gel. We could see the DNA bands for the *E. coli* CCUG 17620 sample even after 3 h incubation. Although the CFU/mL after 3 h of incubation was low, *i.e.*, 40 at 37°C and 120 at 42°C, it was still enough bacterial DNA to be amplified through PCR (Figure 8). For the samples which were incubated for a longer time and had greater CFU, the band signals for *E. coli* DNA appeared stronger in comparison to the human DNA (Figures 8 and 9). We can see from the PCR results that we obtained amplification at both the incubation temperatures (37°C and 42°C), but the DNA band strength and purity were higher at 42°C. The DNA concentration was also higher at increased incubation temperature (42°C) compared to 37°C. The DNA concentration of the samples after 3 h of incubation was 52.4 and 34.3 µg/mL at 42°C and 37°C, respectively. Similarly, we also observed an increased DNA concentration at 6 h (110 µg/mL at 42°C and 82 µg/mL at 37°C) and 9 h (134.4 µg/mL at 42°C and 119.2 µg/mL at 37°C) of incubation in samples which were incubated at a higher temperature. This higher concentration of DNA is beneficial for nanopore sequencing, which can be used to identify the bacteria in real time.

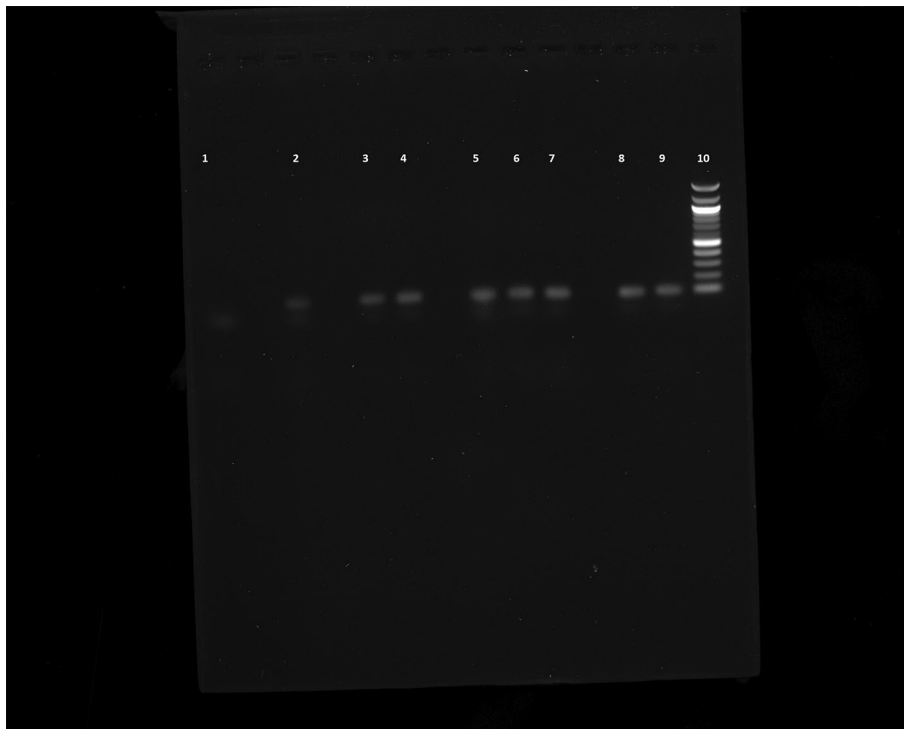
#### Discussion

Early and appropriate detection of pathogens is crucial for treating bacterial infections. However, since the bacterial load is usually very low for infection detection, it is difficult to identify the pathogen and recommend appropriate antibiotic therapy. In the clinical routine, bacteria are grown overnight, requiring a longer time to grow in fresh culture media. Therefore, any method which increases bacterial growth in the initial hours of culturing can be extremely useful in treating patients with different bacterial infections. In the case of sepsis, early and appropriate antibiotic therapy in the first hour of infection can significantly reduce the mortality rate (Avershina *et al.*, 2022; Bloos *et al.*, 2017; Ferrer *et al.*, 2014).

The most common method currently used for identifying bacterial pathogens in clinical samples is based on culture. Although these culturing methods, in most cases, provide information on the causative agent, they are slow and time-consuming. The routine culture-based identification takes around 48-72 hours, and an additional 24-48 hours for antibiotic susceptibility testing. Still, this method is most widely used in clinical diagnosis because it is cost-effective and does not require advanced lab infrastructure. Many other methods, such as whole genome sequencing (WGS), fluorescent in-situ hybridization (FISH), PCR, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS), have the potential for rapid identification of pathogens. However, most of these techniques still require bacterial culturing as the initial step and currently need to be well-developed to be used in clinical settings.



**Figure 8. Blood cultures PCR (*E. coli* CCUG17620).** Lane 1: Negative control (No DNA), Lane 2: 12 h sample (42°C), Lane 3: 12 h sample (37°C), Lane 4: 9 h sample (42°C), Lane 5: 9 h sample (37°C), Lane 6: 6 h sample (42°C), Lane 7: 6 h sample (37°C), Lane 8: 3 h sample (42°C), Lane 9: 3 h sample (37°C), Lane 10: 100 bp DNA ladder.



**Figure 9. Blood cultures PCR (Human).** Lane 1: Negative control (No DNA), Lane 2: 12 h sample (42°C), Lane 3: 12 h sample (37°C), Lane 4: 9 h sample (42°C), Lane 5: 9 h sample (37°C), Lane 6: 6 h sample (42°C), Lane 7: 6 h sample (37°C), Lane 8: 3 h sample (42°C), Lane 9: 3 h sample (37°C), Lane 10: 100 bp DNA ladder.

In this study, we have used different inoculation sizes (0.5% and 0.05%), incubation temperatures (37°C and 42°C), and bacterial growth supplements (vitamin B12 and trace metal solution) to study their effect on the growth of bacteria. We used BHI culture media for bacterial growth because it can support the growth of a wide variety of bacteria. BHI is also suitable for using as a blood culture medium and is similar to the BACTEC blood culture medium, which is extensively used in the clinical microbiology routine. Also, it has been previously used for short-term incubation of positive blood cultures to accelerate the identification of bacteria by MALDI-TOF MS (Torres *et al.*, 2017). We used an incubation temperature of 37°C and 42°C because it is the most commonly used temperature in microbiology for incubating bacteria. Also, it can easily be implemented in a standard laboratory setting (Gutierrez *et al.*, 2018). We have shown that incubating the bacteria at a higher temperature of 42°C instead of 37°C and adding vitamin B12 supplementation in the culture media can significantly reduce the  $t_{lag}$  of bacterial growth. The effect of higher incubation temperature and vitamin B12 supplementation was insignificant on the bacterial doubling time. We did observe a shorter doubling time for *E. coli* isolates at 42°C (Table 1). We hypothesize that it is relatively more important for the bacteria to reduce the lag time than the doubling time. When the exponential growth phase is reached, the bacteria multiply at different rates as the growth machinery has been activated.

We observed reduced  $t_{lag}$  in the pure bacterial isolates and the *E. coli* blood culture using 42°C incubation. The current methods require the blood cultures to be incubated for 24 h or more before identifying pathogens using biochemical tests and then AST. Using this higher incubation temperature (42°C), we can see that after 3 h of incubation, the bacterial CFU/mL was 120, which is three times higher than the standard incubation temperature (37°C). Although the PCR results show bacterial DNA's presence at both temperatures, we can see the better quality of DNA at 42°C by looking at the intensity of bands (Figure 8). This high-quality DNA can be beneficial for performing downstream analyses like PCR or real-time metagenomic sequencing for identifying pathogens and ARGs.

The duration of the lag phase is influenced by several factors such as pH, temperature, nutrients (carbon, nitrogen, phosphorus), oxygen availability, physiological history of the cells, and size of the inoculum (Bertrand, 2019; Mellefont *et al.*, 2003; Rolfe *et al.*, 2012). Many studies have investigated the effect of environmental factors such as pH, temperature, and nutrients on the duration of the bacterial lag phase (Francois *et al.*, 2006, 2007). However, so far, no studies have been done to investigate the effect of vitamin B12 supplements on the bacterial lag phase at different conditions, such as temperature (37°C and 42°C) in connection with inoculum size (0.05% and 0.5%). Other studies have also reported that bacterial lag time depends on the nutrient concentration, such as potassium bicarbonate (KHCO<sub>3</sub>), amino acids, ferrous sulfate, sodium pyruvate, and mucin (Berthelet and MacLeod, 1989). Jiang *et al.* have shown that adding 0.3% mucin to brain heart infusion horse serum (BHI-HS) medium reduced the lag time of *Helicobacter pylori* and enhanced its growth (Jiang & Doyle, 2000). These results are similar to our study, where we have also shown that vitamin B12 supplement in the BHI medium reduces the lag time of bacteria. Vitamin B12 can affect the bacterial lag phase, possibly because it can contribute to different metabolic activities in the bacterial cell, such as the synthesis of nucleic acid and amino acids. Some bacteria can synthesize vitamin B12 on their own, while others require it to be preset in the growth medium (LeBlanc *et al.*, 2011). If vitamin B12 is unavailable in the medium, bacteria need additional time to adapt to the environment and synthesize the necessary enzymes required for vitamin B12 production, which can increase their  $t_{lag}$  (Sultana *et al.*, 2023). Malani *et al.* reported that higher concentrations of sucrose, biotin and pantothenate and lower concentrations of cyanocobalamin (*i.e.*, vitamin B12), CaSO<sub>4</sub>, and glutathione in a growth medium result in larger bacterial inclusion bodies and product expression (Malani *et al.*, 2020). The microbial cell is composed mainly of carbon, hydrogen, nitrogen, and oxygen elements. Other elements such as phosphorus, sulfur, calcium, magnesium, potassium, iron, and sodium are equally crucial for the vital activities of bacterial metabolism (dos Santos *et al.*, 2022). These micronutrients are essential for the enzymatic activities of the bacterial cell and act as prosthetic groups or cofactors for necessary metabolic enzymes (Hood & Skaar, 2012).

It has also been shown that an extended lag phase is advantageous for bacterial survival and helps in the regrowth of bacteria upon the removal of antibiotics (Bertrand, 2019). Li *et al.* evaluated the effect of the bacterial lag phase on the resistance and sensitivity to 12 different antibiotics. They observed that tetracycline and quinolone were the most common antibiotics to which the bacteria showed resistance due to their extended lag phase (Li *et al.*, 2017). Therefore, reducing bacterial lag time is even more critical as it can contribute to drug resistance against the clinically relevant antibiotics used to treat bacterial infections. For all four tested bacteria (*S. aureus*, *E. coli*, *P. aeruginosa*, and *B. subtilis* at OD 0.5), we reported that incubating the bacteria at 42°C reduces the lag time ( $t_{lag}$ ). Studies have reported that bacteria have evolved to regulate their virulence factors due to changes in temperature when they enter from the external environment to the host. Bacteria recognize the host (Konkel & Tilly, 2000; Lam *et al.*, 2014). We hypothesize that faster bacterial growth could lead to the rapid spread of the pathogen and, therefore, a more severe infection that can be fatal.



With an increasing volume of cells in culture media, it takes less time for the bacteria to adapt to the new environment and therefore has a shorter lag time (Robinson *et al.*, 2001; Aamir *et al.*, 2015). In our study, we observed a short lag time for some of the bacteria by using a larger inoculum size of 0.5%, but in *E. coli* HB101 K2 and *S. aureus* NCTC8325, we did not observe the same effect. We did not see any effect of the inoculum concentration on the lag phase in some bacteria because of several factors, such as inherent bacterial characteristics, culture growth phase, and environmental adaptation. Also, some bacteria may have limited ability to utilize the available nutrients even when the inoculum size is larger. Therefore, the inoculum concentration in some of these bacteria might not significantly affect their lag phase (Bidlas *et al.*, 2008).

In this study, we have shown that using vitamin B12 supplements in culture media and incubation at a higher temperature of 42°C can significantly reduce the  $t_{lag}$ . This reduction in  $t_{lag}$  is significant for rapidly detecting pathogens and early and appropriate antibiotic therapy. We have also shown that incubating *E. coli* blood culture at a higher temperature of 42°C reduces the  $t_{lag}$  and enhances the growth of bacteria. It will be beneficial in the future to enrich the culture media with different bacterial growth supplements, which can reduce bacterial lag time and thus allow rapid identification of pathogens. However, to increase the method's reliability and for any potential future use in clinical microbiology, we need to increase the number of bacterial strains analyzed and focus on additional AMR profiles and clinically relevant pathogens. Moreover, the effect of other elements, such as phosphorus, magnesium, calcium, sodium, and iron, as a bacterial growth supplement will be interesting to explore in future research. These results demonstrate the potential that, unlike other molecular-based detection methods, these measures can be cost-effective and do not need any specialized instruments or expertise.

### Data availability

#### Underlying data

Open Science Framework: Ali *et al.*, 2023 F1000 Research, <https://doi.org/10.17605/OSF.IO/HYNTQ> (Ahmad, 2023).

This project contains the following underlying data:

- Ali et al - Raw Data Blood culture Ecoli CFU.xlsx
- Ali et al - Raw data\_ growth curve OD and tlag.xlsx
- Ali et al Raw\_Gel\_image\_Blood\_Culture\_Ecoli\_primers.tif
- Ali et al Raw\_Gel\_image\_Blood\_Culture\_human\_primers.tif

#### Extended data

Open Science Framework: Ali *et al.* 2023 F1000 Research, <https://doi.org/10.17605/OSF.IO/HYNTQ> (Ahmad, 2023).

This project contains the following extended data:

- Supplementary\_Material.docx

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

### Acknowledgments

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## Version 2

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I have seen the changes in the revised version and now the manuscript is approved from my side.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Antimicrobial resistance

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 06 April 2023

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The authors have satisfactorily revised the manuscript as per the comments raised in version-1.

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Microbial interactions, Antimicrobial resistance

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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**Version 1**

Reviewer Report 06 March 2023

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? **Rashid Nazir** 

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The manuscript entitled as “Increased growth temperature and vitamin B12 supplementation reduces the lag time for rapid pathogen identification in BHI agar and blood cultures” published on F1000 Research by Ali *et al.* is an interesting work of applied microbiology. The ms is a proof-of-concept study for future diagnostic improvements. Before its indexing, the following are some comments and observations which are recommended to authors for appropriate response during further revision of their manuscript:

- Please explain the rationale for incubation temperature setting (37 to 42°C) and also provide potential reasons for lag phase changes with/without vitamin B12 in different bacterial stains.
- The bacterial inoculum concentration showed different trends for the lag phase on different temperatures and vitamin B12 supplementation, please discuss different reasons to explain these shifts. Are there any other factors (except the ones studied here) to affect the bacterial lag phase?
- Due to the unique ways in which various bacteria behave and function, the responsiveness and functional capabilities fluctuate as the environment changes. When various environmental factors are activated, not all bacteria or pathogens behave the same way. Some act in opposition to the change, while others react positively. So, how likely is it to expand the observed modification for tens of bacterial pathogens? More precisely, the environmental changes may affect the bacterial physiological functions; in this context, do the tested incubation temperature and vitamin supplementation affect the microbes' pathogenicity or efficiency as well?
- The nutrients are necessary for the bacteria to grow; some are present in significant amounts, while others are scarce. What is the rationale, for authors, to test B12 out of several microbial nutrients?

- As per the study-observations, increased incubation temperature (42°C) reduced the  $T_{lag}$ , may it do the same in real life body-temperature situations? Reduced lag time may enhance the pathogens' survival in compromised immunity scenarios with raised body temperature. Please discuss this practical aspect as well.
- As per the ms title and abstract, the 1<sup>st</sup> impression of the study is more like diagnostic improvements while the introduction start with AMR does not fit in appropriately. 2<sup>nd</sup> paragraph or even the 2<sup>nd</sup> sentence of 2<sup>nd</sup> paragraph might be something to start the introduction and whole AMR scenario may follow in later phase.
- Almost half of the 2<sup>nd</sup> paragraph is on 'Sepsis', I think it narrates the examples of different fatal diseases, so please say it so.
- Page-1, 2 - abstract and last paragraph of introduction: Switch the Gram-positive (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-negative (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria in correct order. Table represented them correctly, though.
- Establish a connecting link of 4<sup>th</sup> paragraph (start with Mesophilic bacteria...) with previous one.
- The 1<sup>st</sup> sentence of the paragraph (starting with 'The bacterial growth curve...') is sufficient, as the rest is quite basic for the microbiology readers.
- As per the ms, bacterial growth reached the stationary phase (approximately 4-5 h), it would be interesting to see the doubling time of the tested bacteria. I am sure the authors would have calculated.
- Figure 8-9: In English, normal reading/writing is from left to write; please correct it 'currently from right to left'.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

I cannot comment. A qualified statistician is required.

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Microbial interactions, Antimicrobial resistance

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 21 Mar 2023

**Rafi Ahmad**

We thank the esteemed reviewer for taking the time to provide us with feedback. The reviewer's comments have helped improve the manuscript. We hope that we have addressed the reviewer's comments sufficiently.

- Please explain the rationale for incubation temperature setting (37 to 42°C) and also provide potential reasons for lag phase changes with/without vitamin B12 in different bacterial strains.

**Response:** The incubation temperature (37 & 42°C) was used in this study because these temperatures can easily be maintained in standard laboratory settings and the most commonly used temperature range (37 to 42°C) for the majority of bacterial strains in microbiology. We used the higher (42°C) and lower (37°C) temperatures in this range to compare their effect on the bacterial growth (Gutierrez et al., 2018).

The relevant text is added in the discussion section of the manuscript (Paragraph 3). The lag time for bacterial growth is dependent on many factors, such as temperature, bacterial strain, and growth medium (availability of nutrients). According to our study, vitamin B12 can affect the lag phase of bacterial growth, possibly because it can contribute to different metabolic activities in bacteria, such as the synthesis of nucleic acid and amino acids. Some bacteria can synthesize vitamin B12, while others require it to be present in the growth medium (LeBlanc et al., 2011). The text has been added in paragraph 4 of the discussion section). If the bacteria do not have vitamin B12 available in the growth medium, they require time to synthesize different enzymes, which is required for the production of vitamin B12 (Sultana et al., 2023). Therefore, the lag time of the bacteria, in that case, is longer.

The relevant text is added in the discussion section of the manuscript (Paragraph 4).

- The bacterial inoculum concentration showed different trends for the lag phase on different temperatures and vitamin B12 supplementation, please discuss different reasons to explain these shifts. Are there any other factors (except the ones studied here) to affect the bacterial lag phase?

**Response:** The reason we did not see any effect of the inoculum concentration on the lag phase in some of the bacteria may be due to several factors, such as inherent bacterial characteristics, culture growth phase, and adaptation to the environment. Some bacteria may have limited ability to utilize the available nutrients even when the inoculum size is larger. Therefore, the inoculum concentration in some of these bacteria may not have any significant effect on their lag phase (Bidlas et al., 2008).

The relevant text is added in the discussion section of the manuscript (Paragraph 6).

Many other factors besides the ones tested here can affect the bacterial lag phase. Some of them are the availability of different nutrients such as carbon, nitrogen, and phosphorus. The pH of the growth medium can also affect the bacterial growth rate and metabolism. Oxygen availability is also important for the optimal growth of bacteria as some bacteria require oxygen for their growth, and others are anaerobic (Bertrand, 2019; Mellefont et al., 2003; Rolfe et al., 2012).

The relevant text is added in the discussion section of the manuscript (Paragraph 4).

- Due to the unique ways in which various bacteria behave and function, the responsiveness and functional capabilities fluctuate as the environment changes. When various environmental factors are activated, not all bacteria or pathogens behave the same way. Some act in opposition to the change, while others react positively. So, how likely is it to expand the observed modification for tens of bacterial pathogens? More precisely, the environmental changes may affect the bacterial physiological functions; in this context, do the tested incubation temperature and vitamin supplementation affect the microbes' pathogenicity or efficiency as well?

**Response:** The effect of higher incubation temperature and vitamin B12 supplementation on tens of bacteria is difficult to predict unless we have specific experimental data for each species. But in this study, we have shown that tested bacterial strains show reduced lag time in the presence of vitamin B12 and higher incubation temperature. This method can potentially be tested for other clinically important bacteria as well. The effect of higher incubation temperature and vitamin B12 supplementation on bacterial pathogenicity and efficiency is difficult to generalize because microbial pathogenicity and efficiency are complex phenomena involving many other factors, such as bacterial virulence factors, host-pathogen interactions, and immune responses. In some cases, higher incubation temperature and/or vitamin B12 can enhance bacterial pathogenicity by increasing its growth rate or virulence factor expression, while in other cases, it may have the opposite or no effect at all (Konkel & Tilly, 2000).

We can hypothesize that faster growth will lead to the rapid spread of the pathogen and, therefore, a more serious infection. As the reviewer has stated, reduced lag time may enhance the pathogens' survival in compromised immunity scenarios with raised body temperature, which will be more pathogenic.

The text has been incorporated into the revised manuscript (Paragraph 5 - Discussion).

- The nutrients are necessary for the bacteria to grow; some are present in significant amounts, while others are scarce. What is the rationale, for authors, to test B12 out of several microbial nutrients?

**Response:** Bacteria require several nutrients to grow, such as carbon, nitrogen, phosphorus, sulfur, etc. Out of all these nutrients, we selected vitamin B12 for testing because it is cheap and easily available (Fang et al., 2017). In our study, we have also used trace elements to see their effect on bacterial growth (Supplementary data). Also, in future studies, the effect of different nutrients on bacterial growth will be interesting to explore, as we have discussed in the manuscript.

The text has been incorporated into the revised manuscript (Concluding paragraph -

Discussion).

- As per the study-observations, increased incubation temperature (42°C) reduced the Tlag, may it do the same in real life body-temperature situations? Reduced lag time may enhance the pathogens' survival in compromised immunity scenarios with raised body temperature. Please discuss this practical aspect as well.

**Response:** An increase in the body temperature is usually called fever which is the body's response to fight any pathogen or infection. In this study, we have shown that higher incubation temperature can reduce the  $t_{lag}$ , which will enhance bacterial growth. However, it is difficult to replicate the identical effect as observed in the human body in the lab. According to some studies, bacteria have evolved to regulate their virulence factors due to changes in temperature when they enter from the external environment to the host. Bacteria recognize the signal provided by the temperature and enhance the production of their virulence factors accordingly within the host (Lam et al., 2014). The growth of bacteria at higher temperatures in the human body can be fatal. We can hypothesize that faster growth will lead to the rapid spread of the pathogen and, therefore, a more serious infection.

The relevant text is added in the discussion (paragraph 5) to highlight this aspect.

- As per the ms title and abstract, the 1st impression of the study is more like diagnostic improvements while the introduction start with AMR does not fit in appropriately. 2nd paragraph or even the 2nd sentence of 2nd paragraph might be something to start the introduction and whole AMR scenario may follow in later phase

**Response:** The text in the manuscript has been updated as per the reviewer's suggestion

- Almost half of the 2nd paragraph is on 'Sepsis', I think it narrates the examples of different fatal diseases, so please say it so.

**Response:** Updated in the revised manuscript.

- Page-1, 2 - abstract and last paragraph of introduction: Switch the Gram-positive (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-negative (*Staphylococcus aureus* and *Bacillus subtilis*) bacteria in correct order. Table represented them correctly, though.

**Response:** Updated in the revised manuscript.

- Establish a connecting link of 4th paragraph (start with Mesophilic bacteria...) with previous one.

**Response:** Updated in the revised manuscript.

- The 1st sentence of the paragraph (starting with 'The bacterial growth curve...') is sufficient, as the rest is quite basic for the microbiology readers.

**Response:** Updated in the revised manuscript.

- As per the ms, bacterial growth reached the stationary phase (approximately 4-5 h), it would be interesting to see the doubling time of the tested bacteria. I am sure the authors would have calculated.

**Response:** The doubling time ( $t_d$ ) for bacteria was calculated based on the steepest rise in the exponential phase of the growth curve. The OD (log) and time interval in the exponential phase were selected at two points, which showed a specific growth rate ( $\mu$ ). The following formula was used to calculate the bacterial doubling time.

$$t_d = 0.693 / \mu$$



In the formula above, 0.693 is constant, and “ $\mu$ ” is the specific growth rate, which is calculated by using the following equation:

$$\mu = (\ln(OD_2) - \ln(OD_1)) / (t_2 - t_1)$$

where;

Ln is the natural log

OD<sub>2</sub> is the OD at the endpoint of the exponential phase

OD<sub>1</sub> is the OD at the starting point of the exponential phase

t<sub>2</sub> is the time corresponding to OD<sub>2</sub>

t<sub>1</sub> is the time corresponding to OD<sub>1</sub>

Relevant text has been added to the methodology (*Pre-culture and growth procedures*), table 1, and results (*Bacterial doubling time*).

- Figure 8-9: In English, normal reading/writing is from left to write; please correct it 'currently from right to left'.

**Response:** Figures are updated in the revised manuscript.

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 02 March 2023

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**Sarita Mohapatra**

Department of Microbiology, All India Institute of Medical Sciences, New Delhi, Delhi, India

The manuscript compares the effect of growth temperature and VitB12 supplementation in reduction of lag time in pathogen growth. I do have the some comments which needs to be addressed:

1. The experiment has been conducted in only one isolate of four different genus. The difference of the bacterial count has been noticed in the initial period of incubation however, most of the experiments showing similar growth pattern over a longer incubatory period. Hence, more number of isolates need to be tested.
2. *Pseudomonas aeruginosa* is known pathogen and its growth at 42°C is taken as one of the identification characters for *P. aeruginosa*. It would be helpful if other Gram-negative bacilli, such as the ESKAPE group of pathogens, were included in the experiment.
3. The Introduction is too long, it should be reduced highlighting the limitations of conventional blood culture technique.
4. How was the CFU measured after 3hr and 6hr incubation?



5. Was DNA quantified? Based on gel picture it is difficult to comment on quantity

Minor comments

1. Correct the gram-positive and gram-negative distribution of bacteria in abstract and other places of manuscript e.g. gram-negative bacilli: *E. coli* & *P. aeruginosa* and gram-positive cocci: *Staphylococcus aureus*, gram-positive bacilli: *Bacillus subtilis*).
2. Full form of CCUG1762 must be written where the term is used first
3. In Table 1 the Gram-stain interpretation should be correctly made as Gram-negative bacilli, or gram-positive bacilli, or Gram-positive cocci, etc.
4. Degree symbol is missing in many places.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Antimicrobial resistance

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 21 Mar 2023

**Rafi Ahmad**

We thank the esteemed reviewer for taking the time to provide us with feedback. The reviewer's comments have helped improve the manuscript. We hope that we have addressed the reviewer's comments sufficiently.

- The experiment has been conducted in only one isolate of four different genus. The

difference of the bacterial count has been noticed in the initial period of incubation however, most of the experiments showing similar growth pattern over a longer incubatory period. Hence, more number of isolates need to be tested.

**Response:** In this study, we tested three *E. coli*, two *S. aureus*, and one isolate of *P. aeruginosa* and *B. subtilis*. We agree that bacterial counts fluctuate more during the initial incubation period. However, this difference in the bacterial count during the initial incubation time can be advantageous for quickly detecting pathogens. In our unpublished work, we have observed that in blood cultures, pathogens with higher bacterial counts during the early stages of incubation allow for faster detection using nanopore sequencing. Sequencing at a timely stage enables the identification of pathogens and their antimicrobial resistance, allowing for swift therapeutic intervention. As this was a proof-of-concept study, we could only test available bacterial strains which we already possess – most of them known pathogens. Future studies should focus on testing more pathogens and using different nutrients to see their effect on bacterial growth.

- *Pseudomonas aeruginosa* is known pathogen and its growth at 42°C is taken as one of the identification characters for *P. aeruginosa*. It would be helpful if other Gram-negative bacilli, such as the ESKAPE group of pathogens, were included in the experiment.

**Response:** In this study, we have included 3 *E. coli* isolates (Gram-negative bacilli), *P. aeruginosa*, and 2 *S. aureus* isolates from the ESKAPE group of pathogens, but we agree with the reviewer's suggestion that other pathogens from this group would be interesting to investigate. As this was a proof-of-concept study, we were not able to include all the bacterial strains and were restricted to those which we have in our lab, and some of them are clinically relevant antimicrobial resistant pathogens. In future studies, the focus should be on testing more pathogens with different conditions to have a broader knowledge of the bacterial growth response to different conditions.

- The Introduction is too long, it should be reduced highlighting the limitations of conventional blood culture technique.

**Response:** The suggested changes have been incorporated into the revised version of the manuscript.

- How was the CFU measured after 3hr and 6hr incubation?

**Response:** The samples were taken out from the incubator after 3 and 6 hours, and a small volume was cultured on BHI agar plates, which were then incubated overnight for the calculation of CFU using the formula: number of colonies × dilution factor/volume of culture plated.

Text has been written in the last sentence of *Blood culturing* in the methods section.

- Was DNA quantified? Based on gel picture it is difficult to comment on quantity

**Response:** Yes, the DNA was quantified, and the details are incorporated into the revised manuscript (Results section: *PCR results*).

#### Minor comments

- Correct the gram-positive and gram-negative distribution of bacteria in abstract and other places of manuscript e.g. gram-negative bacilli: *E. coli* & *P. aeruginosa* and gram-positive cocci: *Staphylococcus aureus*, gram-positive bacilli: *Bacillus subtilis*).

**Response:** The correction has been made in the abstract and introduction section of the

revised manuscript.

- Full form of CCUG1762 must be written where the term is used first

**Response:** The suggested change has been incorporated in the revised manuscript.

- In Table 1 the Gram-stain interpretation should be correctly made as Gram-negative bacilli, or gram-positive bacilli, or Gram-positive cocci, etc.

**Response:** The table has been updated with the suggested changes included.

- Degree symbol is missing in many places.

**Response:** Updated in the revised manuscript.

**Competing Interests:** No competing interests were disclosed.

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