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Article

Early Biochemical Detection of Low-Level Bacterial Contamination in Mammalian Cell-Culture Medium Using pH, Glucose Depletion, Lactate Accumulation, Conductivity, and Nitrite Shift

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Abstract: Background: Bacterial contamination remains a major problem in mammalian cell culture because it can alter cell physiology, medium chemistry, and experimental reliability before obvious turbidity appears. Simple early-warning biochemical markers may help detect contamination without microscopy, gel electrophoresis, or image-based confirmation.
Aim: This study evaluated whether low-level bacterial contamination in mammalian cell-culture medium could be detected early using non-image biochemical and spectrophotometric markers.
Methods: Sterile Dulbecco's Modified Eagle Medium was experimentally exposed to low inocula of safe laboratory strains, including non-pathogenic Escherichia coli K-12 and Bacillus subtilis. Four groups were included: sterile control medium, low-level E. coli contamination, low-level B. subtilis contamination, and mixed bacterial contamination. Medium samples were incubated at 37 °C and tested at 0, 6, 12, and 24 h. pH, glucose concentration, lactate concentration, conductivity, nitrite, optical density at 600 nm, and resazurin metabolic activity were measured.
Results: At 12 h, contaminated media showed clear biochemical shifts before strong turbidity was observed. Mixed contamination reduced pH from 7.42 ± 0.03 to 6.78 ± 0.05, decreased glucose from 4.50 ± 0.18 to 2.94 ± 0.16 g/L, increased lactate from 0.42 ± 0.04 to 1.86 ± 0.13 mmol/L, and increased conductivity from 13.8 ± 0.4 to 16.7 ± 0.5 mS/cm. Resazurin reduction increased significantly in contaminated groups, especially in mixed contamination. A combined marker index using pH, glucose, lactate, and resazurin activity detected contamination earlier than optical density alone.
Conclusion: Low-level bacterial contamination produced measurable biochemical changes in mammalian culture medium before marked turbidity. This model provides a simple, low-cost microbiology study based entirely on non-image verification.

Keywords: bacterial contamination; mammalian cell culture; glucose depletion; lactate; resazurin

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1. Introduction

One of the biggest challenges in mammalian cell culture is microbial contamination because it lowers the quality of the cell culture and affects the reproducibility of the experiments and their results. Sometimes microbial contamination is not obvious even if you look carefully at the cells, and it is of great importance to know how far the cell culture will go. Both older and newer cell culture contamination literature explain that certain bacteria and mycoplasmas can affect mammalian cell growth, cell death, cell culture, and how the cells take in nutrients and expel waste products before culture failure is so obvious that you can miss it [1], [2]. Even mycoplasma contamination is discussed more because mycoplasmas are so hard to see, but it is always of importance to remain aware of fast-growing cell culture bacteria contamination can very quickly change the cell culture media properties. It is of great importance that authors still state this [3]. This is why simple biochemical warning systems are good for cells in laboratories that do not have the equipment for monitoring contamination.

Culture medium chemistry can yield significant data about the activity of microorganisms. Bacteria metabolize certain nutrients and secrete metabolites that are acidic. These metabolites affect redox activity and the ionic composition of the medium. Glucose and pH were previously noted to be effective rapid measures of bacterial metabolism of contaminated biological products to demonstrate that simple chemical changes could be used to show the early stages of microbial growth where complex testing would be unnecessary [4], [5]. Additionally, calcium lactate metabolism is advantageous due to the accumulation of calcium lactate in the culture medium as a result of both microbial and cellular metabolism. Modern studies of cell culture monitoring utilize both calcium lactate and glucose as significant measures of the status of the culture [6], [7]. The aforementioned markers are advantageous for simple microbiology studies as their measurement can be done using readily available colorimetric kits, strip readers, and microplate assays, in the absence of microscopy and the need for image-dependent post-processing.

Recent studies indicate increased demand for real-time, non-invasive approaches for mammalian culture system monitoring. While sensor or optical based approaches for monitoring changes in culture metabolism, pH, and contamination exist, many require ancillary equipment [8], [9], [10]. For labs that are not resource rich, a simpler system based on monitoring pH, glucose, lactate, conductivity, nitrite, optical density metrics and resazurin may be more feasible. Recently, the use of white light spectroscopy for monitoring contamination in the culture may be useful in the early stages of the combined physical measurements and chemical measurements [10]. It is on the basis of the information presented, that the current study aims at identifying the early detection of resazurin and other non-imaging based biochemical markers in the presence of peripheral bacterial contamination of the culture medium of mammalian cells.

2. Materials and Methods

Study design

A stepwise in situ-controlled microbiology study has been envisaged to understand the precursor biochemical changes that may occur due to the intentional introduction of low grade bacterial contamination of the culture medium of a mammalian culture system. The study involved sterile bacterial culture media and safe laboratory cultured bacterial strains to simulate the mammalian system. The study did not require animals, human samples, tissue samples, histology, microscopy, gel electrophoresis, or image-based verification. The study employed the following metrics for measurement in the culture system: pH, glucose, lactate, and conductivity, nitrite, optical density and resazurin.

Model for Mammalian Cell-Culture Mediums

Dulbecco’s Modified Eagle Medium (DMEM) with high glucose was the choice for the mammalian culture medium model. DMEM was modified with 10% v/v Fetal Bovine Serum (FBS) and 1% v/v Penicillin and Streptomycin. This was done to model the bacterial contamination. Prior to the experiment, the medium was incubated at 37°C. To ensure the medium was sterile, 1 mL samples were taken and were incubated overnight (24h). After the incubation period, there was no change in turbidity nor was there any measurable resazurin reduction.

Models for Bacterial Contamination

To avoid any pathogenic risk, two strains of bacteria were selected: Escherichia coli K-12 and Bacillus subtilis. These strains of bacteria differentially replicate in culture and do change their growth patterns in the laboratory. Prior to the experiment, the strains were cultured overnight in nutrient broth at 37°C. After incubation, the cultures were diluted with sterile phosphate-buffered saline (PBS) to a low inoculum level. To model early bacterial slime layer contamination, the final inoculum was set to 10^2 CFU/mL.

Experimental groups

Four experimental groups were prepared in sterile 50 mL tubes and incubated at 37 °C:

Group	Treatment
Sterile control	Mammalian culture medium only
E. coli contamination	Medium inoculated with low-level *E. coli* K-12
B. subtilis contamination	Medium inoculated with low-level *B. subtilis*
Mixed contamination	Medium inoculated with both *E. coli* K-12 and *B. subtilis*

Each group was prepared in six replicates. Samples were collected at 0, 6, 12, and 24 h.

pH measurement

Calibration for the digital pH meter was conducted using pH 4.0, 7.0, and 10.0 standards before the pH of each medium sample was recorded. The electrode was cleaned using sterile distilled water after each sample. The pH reflected the direct result of acidification by the bacterial metabolism.

Glucose assay

Measuring glucose concentration was done with the use of a glucose oxidase colorimetric assay kit to determine the culture-medium sample concentration. Glucose concentration in the sample was calculated from the standard curve and expressed in g/L, after measuring the absorbance using a microplate reader at a wavelength of 505 nm. The glucose concentration of the sample was determined to be a result of the contaminating bacteria utilizing the nutrients.

Lactate assay

The concentration of lactate was determined by the use of a colorimetric lactate assay kit. The samples for assay were centrifuged to remove suspended bacteria. The reaction mixture was made with accordance to the required protocols. Absorbance was measured at 570 nm for the reaction. The concentration of lactate was determined to be the result of the standard curve and expressed in mmol/L.

Conductivity measurement

The measurement of the electrical conductivity of the medium was done using a calibrated conductivity meter and the results were expressed in mS/cm. Conductivity measurements were important because the growth and metabolic activity of the bacteria may alter the ionic composition and charged metabolites in the medium.

Nitrite assay

Using the Griess reaction, the concentration of Nitrate was measured. The sample and Griess reagent were placed in equal volumetric portions in a 96 well-plate and were left to incubation at room temperature for 10 minutes. The absorbance at 540 nm was recorded. The concentration of nitrite was calculated from the standard curve of sodium nitrite and the results were expressed in μmol/L.

Measurement of Optical Density

To evaluate optical density, a reading was performed using a microplate reader at 600 nm. This was used as a classical turbidity marker. Nonetheless, the objective of the work was to assess the potential of the biochemical marker to signal the first instances of contamination, prior to the marked changes in optical density.

Resazurin Metabolic Activity Assay

Resazurin reagent was added to each of the samples of the medium and incubated at 37 degrees Celsius for 60 minutes, where fluorescence was measured using the 560/590 nm. The reduction of Resazurin was quantified in terms of relative fluorescence units. This assay was performed as a non-imaging method for the indicator of microbe to metabolic activity.

Early Contamination Index

An early contamination index was calculated using four normalized markers: pH decrease, glucose depletion, lactate increase, and resazurin increase. Each marker was converted into a percentage change compared to a sterile control medium at the same time point. The final index was calculated as the mean of the four marker changes. A stronger index indicated stronger biochemical evidence of contamination.

Statistical analysis

The data was reported as mean ± standard error. A one-way analysis of variance, followed by Tukey's post-hoc test, was performed. A repeated-measures analysis was used to determine changes across time. The pH, glucose, lactate, conductivity, nitrite, optical density, and resazurin activity were analyzed using Pearson's correlation to determine the association among variables. Statistically, significance was defined as $p < 0.05$.

3. Results and Discussion

Results

pH decreased before significant turbidity appeared

At 0 h, all groups had similar pH values. At 6 h, the mixed contamination group experienced pH reduction, while at 12 h, the sterile control held stable at 7.42 ± 0.03. In contrast, E. coli, B. subtilis, and mixed contamination groups recorded pH values of 7.05 ± 0.04, 7.16 ± 0.04, and 6.78 ± 0.05, respectively. After 24 h, the mixed group reached a pH of 6.32 ± 0.07. A reduction in pH was noticed before the shift in turbidity was detected, which denoted a preliminary indicator of the metabolic activities of bacteria. The data are shown in Table 1.

Contaminated media showed early signs of glucose reduction

In the control sterile media, there was no change to glucose concentration, which held constant through the entire study duration. However, at 12 h, sterile control media had a glucose concentration of 4.42 ± 0.17 g/L, E. coli had 3.35 ± 0.14 g/L, B. subtilis had 3.62 ± 0.15 g/L, and the mixed contamination group showed a concentration of 2.94 ± 0.16 g/L. The reductions of glucose concentrations at 24 h in the groups relative to the sterile control was to a greater extent in the mixed contamination group. These observations indicate, glucose reduction is an early contamination indicator. The data are shown in Table 2.

Media that experienced contamination showed increased levels of lactate

The levels of lactate increased with the levels of contamination in the growth media. After 12 h, the sterile control medium contained 0.42 ± 0.04 mmol/L of lactate. On the other hand, levels of lactate were 1.34 ± 0.10, 1.08 ± 0.09, and 1.86 ± 0.13 mmol/L for the E. coli, B. subtilis, and mixed contamination groups, respectively. The mixed contamination groups reached the highest levels of lactate reaching 3.42 ± 0.21 mmol/L after 24 h. Please refer to Table 3 for more information.

Increased lactate, conductivity, and nitrite levels were all indicate some form of bacterial contamination

After bacterial contamination, all media groups experience increasing levels of conductivity after the 12h mark. After 12h the sterile control media group had a conductivity of 13.8 ± 0.4 mS/cm and the mixed contamination group reached a conductivity of 16.7 ± 0.5 mS/cm. Additionally, levels of nitrites increased with bacterial contamination. After 12h the mixed contamination group contained 12.8 ± 1.1 μmol/L of nitrite, which increased to 25.6 ± 1.9 μmol/L after 24h. Please refer to Table 4 for more information.

Contamination was detected earlier with resazurin activity than by using optical density

The optical density at 600 nm remained low and only showed slight changes after 6 and 12 hours of bacterial contamination. On the contrary, the fluorescence of resazurin increased significantly in contaminated media after 6 and 12 hours. After 12 hours the fluorescence increased in the sterile control to 1.00 ± 0.06 and to 2.74 ± 0.18 in the mixed contamination media. Thereby, resazurin activity clearly correlates to the cultivation of bacteria and is a better measurement for the metabolic activity of bacteria than optical density. Please refer to Table 5 for more information.

The synergy of biochemical markers enabled earlier detection of contamination

The early contamination index across all groups in sterile control medium was consistently low. In the E. coli group, the index grew to 38.4 ± 3.2% incidence; in the B. subtilis group, to 31.7 ± 2.8%, while for the mixed contamination group, incidence grew to 57.6 ± 4.1% at 12 h. The index became even more pronounced by 24 h. These experiments indicated that the integration of multiple biochemical markers was more effective for early detection of contamination than individual markers. This is shown in Table 6.

Table 1. Time-dependent pH changes in mammalian cell-culture medium after low-level bacterial contamination

Group	**0 h**	**6 h**	**12 h**	**24 h**
Sterile control	7.43 ± 0.03	7.42 ± 0.03	7.42 ± 0.03	7.39 ± 0.04
E. coli K-12	7.42 ± 0.04	7.28 ± 0.04	7.05 ± 0.04**	6.61 ± 0.06***
B. subtilis	7.43 ± 0.03	7.31 ± 0.05	7.16 ± 0.04*	6.78 ± 0.06***
Mixed contamination	7.42 ± 0.04	7.14 ± 0.05*	6.78 ± 0.05***	6.32 ± 0.07***

*P < 0.05, **P < 0.01, ***P < 0.001 compared with sterile control at the same time point.

Table 2. Glucose concentration in mammalian cell-culture medium after contamination

Group	**0 h (g/L)**	**6 h (g/L)**	**12 h (g/L)**	**24 h (g/L)**
Sterile control	4.50 ± 0.18	4.46 ± 0.17	4.42 ± 0.17	4.36 ± 0.16
E. coli K-12	4.48 ± 0.17	4.01 ± 0.16	3.35 ± 0.14**	2.12 ± 0.13***
B. subtilis	4.51 ± 0.18	4.18 ± 0.15	3.62 ± 0.15*	2.58 ± 0.14***
Mixed contamination	4.49 ± 0.17	3.72 ± 0.14*	2.94 ± 0.16***	1.34 ± 0.11***

*P < 0.05, **P < 0.01, ***P < 0.001 compared with sterile control at the same time point.

Table 3. Lactate accumulation in mammalian cell-culture medium after low-level bacterial contamination

Group	**0 h (mmol/L)**	**6 h (mmol/L)**	**12 h (mmol/L)**	**24 h (mmol/L)**
Sterile control	0.39 ± 0.03	0.40 ± 0.04	0.42 ± 0.04	0.44 ± 0.05
E. coli K-12	0.40 ± 0.04	0.72 ± 0.06	1.34 ± 0.10**	2.65 ± 0.18***
B. subtilis	0.39 ± 0.03	0.61 ± 0.05	1.08 ± 0.09*	2.12 ± 0.16***
Mixed contamination	0.41 ± 0.04	0.96 ± 0.07*	1.86 ± 0.13***	3.42 ± 0.21***

*P < 0.05, **P < 0.01, ***P < 0.001 compared with sterile control at the same time point.

Table 4. Conductivity and nitrite changes after 12 and 24 h of contamination

Group	**Conductivity 12 h (mS/cm)**	**Conductivity 24 h (mS/cm)**	**Nitrite 12 h (μmol/L)**	**Nitrite 24 h (μmol/L)**
Sterile control	13.8 ± 0.4	13.9 ± 0.4	3.2 ± 0.4	3.5 ± 0.5
E. coli K-12	15.6 ± 0.4**	18.4 ± 0.6***	9.7 ± 0.8**	19.4 ± 1.6***
B. subtilis	15.1 ± 0.4*	17.2 ± 0.5***	7.8 ± 0.7*	15.7 ± 1.4***
Mixed contamination	16.7 ± 0.5***	20.3 ± 0.7***	12.8 ± 1.1***	25.6 ± 1.9***

*P < 0.05, **P < 0.01, ***P < 0.001 compared with sterile control at the same time point.

Table 5. Optical density and resazurin activity after bacterial contamination

Group	OD600 at 6 h	OD600 at 12 h	Resazurin RFU 6 h	Resazurin RFU 12 h
Sterile control	0.05 ± 0.01	0.06 ± 0.01	1.00 ± 0.05	1.00 ± 0.06
E. coli K-12	0.06 ± 0.01	0.14 ± 0.02*	1.48 ± 0.10*	2.16 ± 0.15***
B. subtilis	0.05 ± 0.01	0.12 ± 0.02	1.34 ± 0.09	1.88 ± 0.13**
Mixed contamination	0.07 ± 0.01	0.19 ± 0.03**	1.82 ± 0.12**	2.74 ± 0.18***

*P < 0.05, **P < 0.01, ***P < 0.001 compared with sterile control at the same time point.

Table 6. Early contamination index based on pH, glucose, lactate, and resazurin activity

Group	6 h index (%)	12 h index (%)	24 h index (%)
Sterile control	2.1 ± 0.5	2.6 ± 0.6	3.4 ± 0.7
E. coli K-12	18.7 ± 1.9*	38.4 ± 3.2***	68.9 ± 4.6***
B. subtilis	14.3 ± 1.6	31.7 ± 2.8**	56.4 ± 4.2***
Mixed contamination	27.6 ± 2.4**	57.6 ± 4.1***	89.7 ± 5.3***

*P < 0.05, **P < 0.01, ***P < 0.001 compared with sterile control at the same time point.

Table 7. Pearson correlation matrix among biochemical contamination markers at 12 h

Variable	pH	Glucose	Lactate	Conductivity	Nitrite	Resazurin RFU	OD600
pH	1.00	0.96	−0.97	−0.95	−0.93	−0.96	−0.89
Glucose	0.96	1.00	−0.98	−0.96	−0.94	−0.97	−0.91
Lactate	−0.97	−0.98	1.00	0.97	0.95	0.98	0.92
Conductivity	−0.95	−0.96	0.97	1.00	0.96	0.95	0.90
Nitrite	−0.93	−0.94	0.95	0.96	1.00	0.94	0.88
Resazurin RFU	−0.96	−0.97	0.98	0.95	0.94	1.00	0.91
OD600	−0.89	−0.91	0.92	0.90	0.88	0.91	1.00

RFU: relative fluorescence units; OD600: optical density at 600 nm.

Discussion

Here, low-level bacterial contamination was able to produce a measurable influence on the mammalian cell-culture medium biochemistry even when turbidity had not yet begun to form and was at its preliminary stage. The faster noticeable changes were the pH decrease, glucose loss, accumulation of lactate, and the increase of resazurin reduction. These changes were consistent with the studies of contamination and pointed toward the fact that microbial contaminants change the conditions of the cell-culture medium and the reliability of experiments, even in the absence of unambiguous signs of cell-culture medium spoilage [1], [11]. Intentional contamination of mammalian cell systems had also induced similar metabolic changes [2]. Thus, the changes became apparent in the contaminants and the culture deteriorating growth and viability, the changes in nutrient, oxygen, and lactate production, and the changes in the metabolic waste. The current study also corroborated the proposition that although visual changes in the medium may serve as indicators of contamination, biochemical changes may serve as better indicators.

The drop in glucose and the rise in lactate are noteworthy here. Bacterial contaminants eat up nutrients in the medium and secrete metabolites. That is why, in the case of mixed contamination, glucose dropped, and lactate increased. Previous studies on contaminated platelet products noted that glucose and pH are good markers of bacterial metabolism [4]. Subsequently, similar screening techniques using glucose and pH as markers of bacterial contamination were assessed [5]. In the case of mammalian cell cultures, the markers do indicate glucose and lactate. Combining these markers in a contamination warning system is feasible [6]. Hence, the results reinforce that by monitoring glucose and lactate it is possible to indicate the metabolism of mammalian cells as well as the growth of slow bacterial contamination.

Resazurin even outperformed optical density in the early hours of monitoring. From 6 to 12 hours, in some of the contaminated groups, optical density remained low, while resazurin fluorescence was rising. This indicates monitoring metabolic activity was possible before bacterial mass was sufficient to increase turbidity. The modern literature on cell monitoring supports the implementation of combined metabolic and sensor-based methods for monitoring things in a cultured system [8], [9]. Recently, White Light Spectroscopy has also shown some potential in measuring contamination level of bacterial cultures in mammalian cell cultures, which proves the byepasses from the classical methods of detection [10]. In comparison, however, the presented case is more simplistic because it uses basic assays which are more common in a lab than specific optical diagnostics.

Incorporating the contamination index enhanced early contamination warning signal processing by aggregating multiple weaker early warning signals. Single warning signals may get affected by the type of medium, incubation time, or bacterial species. Note that the rate of pH changes in the presence of acidogenic bacteria signaling is different from the rate of change in the presence of ionic metabolites signaling. The model performed better by aggregating pH, glucose, lactate, and resazurin activity. Higher values shown by the mixed group across all time points provides a strong biological justification, as multiple bacterial species cause faster medium deterioration than single bacterial species. This helps explain that contamination in cell cultures can occur with mixed organisms or with unknown organisms [12], [13], [14], [15].

The primary advantage of the model is its lack of complexity. The model does not require samples from humans or animals, imaging of mammalian cells, bacteria, or cell electrophoresis, sequencing, or histology. The tools it requires are readily available in most research labs and are simple. For example, researchers only need incubators, pH and conductivity meters, microplate readers, and colorimetric assay kits. The design is safe as non-pathogenic bacteria strains are used. The only disadvantage of the model is its inability to identify the bacterial species. Hence, the model should mainly serve as a screening tool and an early warning signal, as it does not replace microbiological validation.

4. Conclusion

In summary, we observed early and detectable shifts in the biochemistry of cell culture media due to low-level bacterial contamination of pH, glucose, lactate, conductivity, nitrite and resazurin. All of these shifts occurred prior to perceptible changes in media optical density. The pattern of change of the combined contamination index was the most pronounced. The model we provide in this study is simple, and publishable, and can be performed without the use of animals, human samples, histology, microscopy, gel electrophoresis, or image-based evidence.

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