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Original Article

Development of Low-Serum Culture Media for the in Vitro Cultivation of *Theileria annulata* S15 Cell Line

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Abstract

Background: The production of bovine theileriosis vaccine involves in vitro cultivation of *Theileria annulata* schizont-infected cell lines. Fetal bovine serum (FBS) is commonly used in animal cell culture, including the *Theileria* cell line. However, we aimed to reduce the amount of serum needed for cell culture by modifying the Stoker culture medium with supplements such as excretion factor and serum substitutes.

Methods: To evaluate the effectiveness of these modifications, techniques such as cell counting, cell viability assays, and genomic analysis were employed in the Parasitic Vaccines Production Department of Razi institute of Iran, from 2020 to 2022. Statistical analysis was used to compare the results of different experimental conditions.

Results: The three experimental media were as effective as the commonly used 10% Stoker medium in supporting the growth and viability of cells.

Conclusion: The significant reduction in the required amount of serum and the remarkable cell growth achieved by using defined serum replacements for the production of cell culture media is a significant step towards the preparation of a proper cell culture medium for the production of bovine Theileriosis vaccine.

Introduction

Complete cell culture media are supplemented with various additives, including undefined components such as fetal bovine serum (FBS) and other serums derived from young and newborn calves, adult cows, horses, and even humans, at a rate of 10-20% (1). Serum may contain contaminants such as mycoplasma, viruses, and prion agents that cause BSE in humans and animals (2). Furthermore, the chemical composition of serum is variable across different batches (3).

To achieve stability and consistency in cell cultures, it is essential to use media with distinct and uniform components in their composition. Therefore, it is recommended to use minimal serum or preferably serum-free media (4). Reducing the amount of serum or partially replacing it with other substances while improving cell and tissue culture methods without serum is a modern scientific approach to finding suitable alternatives for fetal bovine serum (FBS) in cell culture (5). Peptones are natural sources rich in amino acids, peptides, proteins, and growth factors (5).

Animal cell culture typically uses fetal bovine serum (FBS), particularly in cell lines containing *Theileria* schizont, at a rate of 10% to 20% in RPMI cell culture medium. However, due to the unaffordability of FBS for bovine Theileriosis vaccine production, *Theileria* cell lines have been cultured and optimized based on a Stoker medium containing 10% adult bovine serum.

Previous studies have reported on the expression of various genes in *T. annulata* cell lines, including cytokines like interleukin 1 alpha, interleukin 6 and 10, and tumor necrosis factor-alpha (TNF-alpha), as well as genes involved in the transformation of *Theileria* cell lines, such as *T. annulata Peptidyl prolyl isomerase I* gene (TaPin1). These approaches can be considered suitable for investigating the behavior of *Theileria* in experimental cell culture media (6, 7).

We aimed to optimize the Stoker medium by reducing the amount of bovine serum while investigating the characteristics of the cultured cells in terms of morphology, growth rate, cell viability, gene expression level, genomic characteristics measured by PCR method, and examining some metabolites during cultivation. Reducing the required serum for *Theileria* cell culture and using uncomplicated and defined serum substitutes may be appropriate alternatives for preparing the cell culture medium to produce a vaccine.

Materials and Methods

Cell line and cell culture media. *T. annulata* cell line vaccine strain S15, which contained attenuated schizonts was utilized in the Stoker medium, a modified version of the "Eagle cell culture medium" (8, 9). This study was carried out in the Parasitic Vaccines Production Department of Razi Institute of Iran, from 2020 to 2022.

It was consulted with the Research Ethics Committee who determined that our study did not need ethical approval.

Cells were cultured and passaged as described by Hashemi-Fesharki (9). Briefly, the S15 *T. annulata* vaccine cell line was maintained in complete Stoker medium (10% bovine serum, 100 IU/ml Penicillin, 100 µg/ml Streptomycin) in 25-Cm^2 flasks at 37 °C until confluent. Passaging involved transferring cells to fresh flasks with new medium every 3–4 days (typical growth period). For the experiments, cells were passaged at least 30 times in Pyrex Roux bottles in experimental culture media, along with two control media: Stoker containing 10% serum and a basic medium-modified Stoker containing 1% serum.

The minimum level of serum required for cell cultivation was determined and used for further modifications. Briefly, different amounts of bovine serum in the Stoker medium, ranging from 1% to 10%, were prepared

and compared for cell culture analysis to identify the optimal serum concentration. The A2 culture medium, also known as the modified Stoker medium, is a basal culture medium with low serum content and a modified amino acid composition. It is typically supplemented with 1% adult bovine serum to provide essential nutrients for cell growth and proliferation. This culture medium can serve as a starting point for customizing experimental media to meet specific cell culture requirements, such as those for *T. annulata* cells.

In this study, the excretion factor, or the culture supernatant of *Theileria* cells, was used as one of the serum substitutes. The concentration of the culture supernatant in the experimental medium was determined through preliminary tests using different concentrations (2%, 4%, 6%, and 10%) in the basic medium and compared to the positive control of the 10% Stoker medium.

This study employed five peptones for medium supplementation: Tryptone D Type VI (RM 193, Himedia), Casein Hydrolysate Broth (M002A, Himedia), Meat Peptone (RM10933, Himedia), Meat Extract Powder (RM003, Himedia), and Proteose Peptone (RM10932, Himedia). These were individually prepared as 20% (w/v) mother stock solutions sterilized by 0.2 µm filtration and stored at 4°C until use. Each peptone was then added to the culture media at a final concentration of 2 g/L (10).

High costs, poor reproducibility, and batch-to-batch variability of serum in cell culture prompted us to explore cost-effective, accessible, and safe additives as substitutes (11). We evaluated various candidates, including yeast extract, Lactalbumin, dextran sulfate, Diethanolamine, beta-mercaptoethanol, and dextrin white. Initial screening identified promising options, but further testing revealed limitations with some. Notably, beta-mercaptoethanol exhibited detrimental effects at all concentrations and was excluded. Conversely, dextrin white and diethanolamine significantly enhanced cell growth in Stoker media, reducing serum requirements compared to controls. Yeast extract and Lactalbumin have been shown to be effective growth supplements in Stoker medium.

The *T. annulata* schizont-infected vaccine cell line was cultured in a basic 1% serum Stoker A2 medium, which was developed as a modification of the original Stoker 10% serum (S10%) positive control medium. To improve the medium formulation, two amino acids (serine and glycine) and two additives (yeast extract and lactalbumin) were added. In addition, six experimental media (Mixes #1and 2 "M1, M2", Dextrin white "DW", Diethanolamine "DEA", Excretion factor "EF", Meat peptone "MP") were formulated by adding additives and serum substitutes and compared to the 10% Stoker and 1% Stoker A2 media (Table 1).

The growth rate or proliferation was estimated by calculating the doubling time of the cell population (14). The population doubling time (PDT) or growth rate has been calculated using the following formula:

$$PDT\ (hours) = CT / (\log (N_1/N_0) \times 3.31)$$

Where: PDT: Population doubling time (hours), CT: Culture time (hours), N_1: Cell number at the end of the cultivation period, N_0: Cell number at the beginning of the experiment.

The cells containing *Theileria* schizonts were stained using the Giemsa method to facilitate visualization of both the host cell and intracellular schizonts (15).

Measuring Glucose and Urea in the Culture Medium: The measurement of glucose metabolites was performed using an enzymatic colorimetric method with a glucose oxidase kit (Pars Azmoun Company, Iran). The concentration of ammonium was determined using specialized urea (ammonia) testing kits (Pars Azmoun Company, Iran).

Table 1: The experimental low-serum culture media used for cultivating of *T. annulata* S15 Vaccine cell line

Media Name	*Serum %*	*Amino acids composition*	*Vitamins and Minerals*	*Supplements*	*Serum substitute*
Stoker 10%*	10	Normal*	Normal**	Yeast extract 0.2g/L and Lactalbumin 1g/L	-
Basal medium A2	1	Serine and Glycine*	Normal	Yeast extract 0.2g/L and Lactalbumin 1g/L	-
MP medium	1	Serine and Glycine*	Normal	Yeast extract 0.2g/L and Lactalbumin 1g/L	Meat Peptone (MP) 2g/L
EF medium	1	Serine and Glycine*	Normal	Yeast extract 0.2g/L and Lactalbumin 1g/L	Excretion Factor (EF) 2%
DEA medium	1	Serine and Glycine*	Normal	Yeast extract 0.2g/L and Lactalbumin 1g/L	Diethanolamine (Dea) 10uM
DW medium	1	Serine and Glycine*	Normal	Yeast extract 0.2g/L and Lactalbumin 1g/L	Dextrin White 250 mg/L
M1 medium	1	Serine and Glycine*	Normal	Yeast extract 0.2g/L and Lactalbumin 1g/L	Dea 10uM + DW 250 mg/L + EF 2%
M2 medium	1	Serine and Glycine*	Normal	Yeast extract 0.2g/L and Lactalbumin 1g/L	Dea 10uM + DW 250 mg/L + MP 2g/L

Normal *: Standard amounts of amino acids in Stoker medium.
Normal **: Standard amounts of vitamins and minerals in Stoker medium.
Stoker 10%*: Stoker medium, the standard growth medium for vaccine production at Razi institute.
Serine and Glycine* were added to the normal amino acid composition at concentrations of 42 mg/L and 30 mg/L, respectively

The total protein of the culture media was measured using the protein measurement kit from a biochemistry company (Ziest Chem Co., Iran), while necessary controls were taken into account in this study, and the experiments were performed in duplicate.

The optimal osmolality for most cell lines typically ranges between 260 mOsm/kg and 320 mOsm/kg Laboratory osmometers are commonly used to measure the osmotic pressure of solutions, colloids, and compounds,

and can be used to determine the osmolality of liquids (16).

Determining cytokine gene expression and related genes to cell proliferation. The expression levels of *IL-1*, *TNF-a*, and *TaPin1* genes were investigated using the semi-quantitative RT-PCR method, with the housekeeping gene *GAPDH* serving as an internal control (17, 18).

Agarose gel with different concentrations ranging from 1.5% to 2.5% was used based on the size of the PCR product. The results of electrophoresis were imaged using UVIdoc and recorded to compare the patterns.

Gel image densitometry. To compare the results obtained from different samples, all cDNAs produced were first normalized based on the expression level of the *GAPDH* internal standard gene (cDNA normalization). The PCR product of each gene was then photographed and subjected to densitometry after agarose gel electrophoresis. In this process, the density of the PCR product bands of each sequence was determined using PhotoCapture software. The ratio of the density of the target band (cytokines) to the density of the internal standard gene band (GAPDH) represents the relative amount of target gene expression.

Genomic analysis of *T. annulata* cell line. The genomic analysis of *Theileria* cells was conducted using PCR reactions on the *Tams-1* and *Cytochrome b* genes (19, 20).

Statistical Analysis

The data from the samples were recorded in Microsoft Excel analyzed using appropriate statistical tests, including a paired *t*-test to compare cell culture groups in experimental and control media. Differences were considered statistically significant at $P < 0.05$.

Results

The *T. annulata* schizont-infected vaccine cell line demonstrated robust growth in a basic 1% serum Stoker A2 medium, alongside the S10% positive control and six diverse experimental media (Mixes #1 & 2, Dextrin white, Diethanolamine, Excretion factor, and Meat peptone).

The Giemsa-stained cell cultures were examined microscopically for the presence of intracellular *T. annulata* schizonts. Macroschizonts were observed in all samples (Fig. 1).

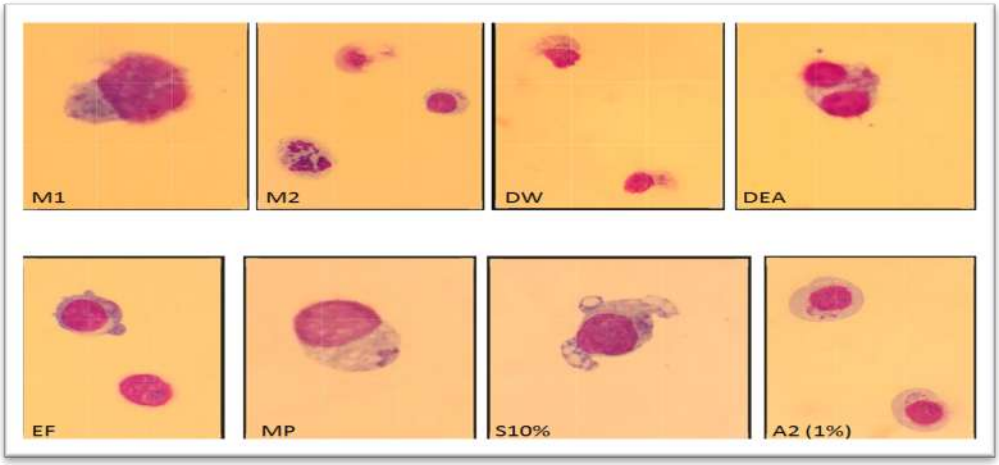

Fig. 1: Giemsa-stained *T. annulata* schizonts infected vaccine strain cell line in experimental cell culture media (M1, M2, DW, DEA, EF, MP) along with two control media. The schizonts can be observed in the cytoplasm of the cells in all examined media (1000X magnification)

In terms of cell counting and pH measurement, the results showed that the highest average cell count was observed in 10% Stoker medium, with a count of $6.62x10^5$ cells per ml, while Dea medium had the lowest cell count, with a count of $5.52x10^5$ cells per ml (Fig. 2). The pH values of both experimental and control media decreased over time, with an average pH level decreasing from 7.4 ± 0.18 to 6.38 ± 0.1.

The statistical analysis of cell counting was conducted by comparing the average number of 30 cell passaging for each experimental medium, as shown in Fig. 2. The results indicated that the highest cell growth was observed in the positive control 10% Stoker medium (6.62 x 10^5 cells/ml), while a reduction of 4.3% was noted in the M2 medium (6.34 x 10^5 cells/ml). The average cell growth for the other six experimental media ranged between 5.52 x 10^5 and 6.34 x 10^5 cells/ml (Fig. 2). Further analysis of specific experimental media revealed that the cell counts in three experimental culture media (MP, DW, Dea) showed a significant decrease compared to the 10% Stoker medium ($P < 0.05$). Conversely, the comparison of cell count in four experimental media (M1, M2, EF, and A2) with a 10% Stoker control medium showed no significant differences ($P > 0.05$) (Fig. 2).

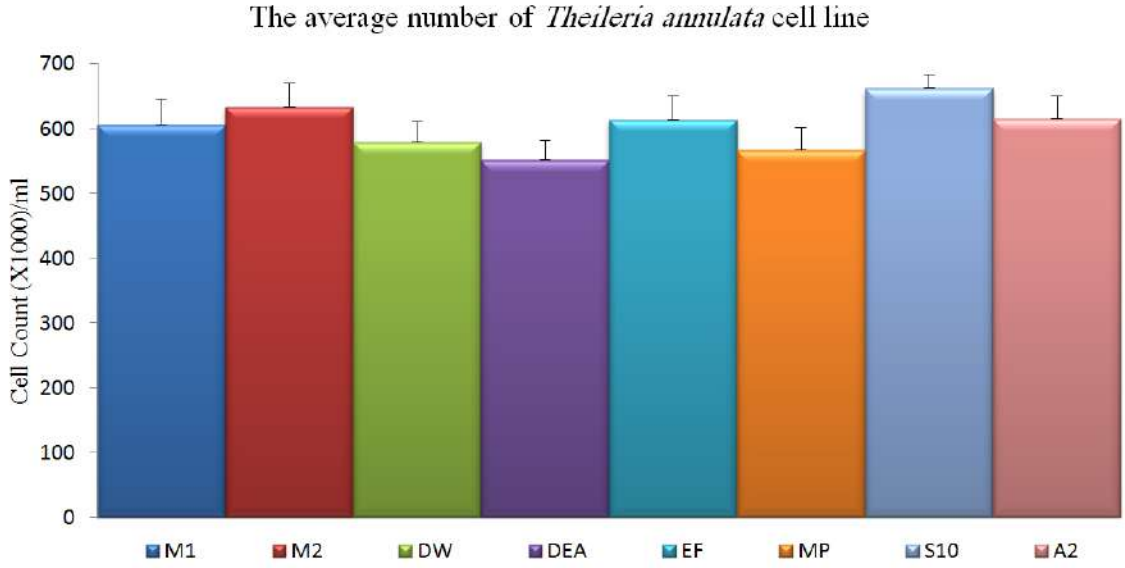

Fig. 2: Comparison of the average number of *T. annulata* vaccine cell line in six experimental (M1, M2, DW, Dea, EF, MP) and two control media (10% Stoker and 1% basic A2 media). The average number of cells in each cell culture medium was determined by performing 30 cell passages. The standard error values for each medium are displayed in the columns (The average number of cells grown in different media was counted as follows: $6.06x10^5$ for M1, $6.34x10^5$ for M2, $5.79x10^5$ for DW, $5.52x10^5$ for Dea, $6.14x10^5$ for EF, $5.67x10^5$ for MP, $6.62x10^5$ for S10% and $6.15x10^5$ for A2)

The highest percentage of cell viability (90%) was observed in the EF and Dea media, while the lowest percentage (82.24%) was observed for the M1 and M2 media. The two control media (10% Stoker and 1% basic A2 medium) showed 87.44% and 88.44% viable cells, respectively. Furthermore, there were no statistically significant differences observed between the examined cell culture media.

The experimental media showed a greater proliferation rate of *Theileria* cells compared to the control media. The highest cell doubling time or growth rate was observed in the positive control 10% Stoker, with a duration of

33.48 hours. The lowest cell doubling time was noted for the M2 medium with a duration of 25.53 hours, indicating a faster proliferation rate of *Theileria* cells in the experimental media.

Glucose was measured at the beginning and end of the growth period. On average, glucose decreased by 44% during cell growth across all eight media. In the positive control medium (Stoker), glucose decreased by 45.7%, while in the experimental media, glucose decreased by 43.8% (Fig. 3). Urea was also measured at the beginning and end of the growth period. On average, urea increased by 294.6% during growth across all eight media. In the positive control medium (Stoker), urea increased by 283%, while in the experimental media, urea increased by 300% (Fig. 3).

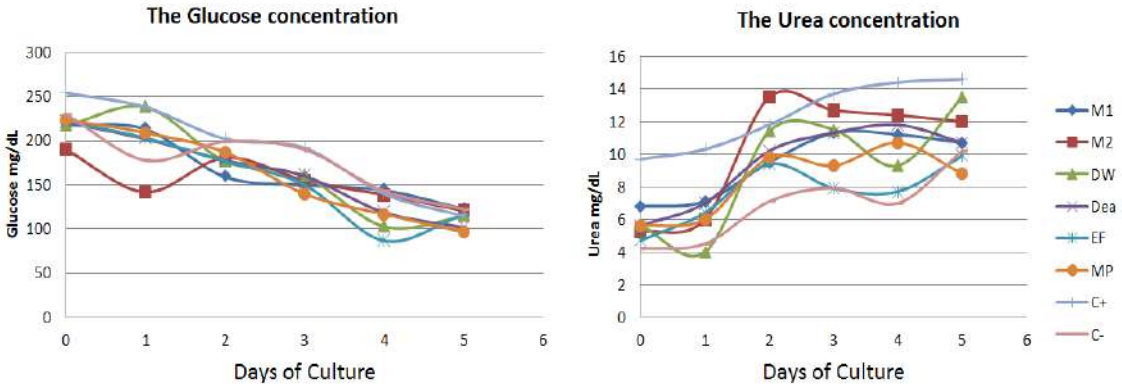

Fig. 3: Glucose and urea levels in examined cell culture media. The graph on the left illustrates the measured amounts of glucose in the supernatant of the eight cell culture media. There was a decreasing trend in glucose levels from day 0 to 5 in all examined media. The graph on the right shows the measured amounts of urea in the supernatant of the cell cultures, which demonstrates an increasing trend in urea levels from day 0 to 5 in all examined cell culture media

The osmolality levels of the fresh cell culture media were measured. The highest value of osmolality was recorded for the 1% basic A2 medium with 326 (mOsm/kg), while the lowest value was recorded for the 10% Stoker medium with 245 (mOsm/kg). The average value of osmolality for the six experimental media was determined to be 307 (mOsm/kg).

The total protein amounts of the experimental media, along with the control media, were measured. The highest amounts of protein content were measured in two cell culture media, Mix #1 and Mix #2 (M1 and M2), with 69 and 71 mg/dL, respectively. However, the protein amounts for the six experimental media ranged between 43 and 46 mg/dL.

The relative expression of target genes was estimated using RT-PCR. The density of internal control cytokines as well as *TaPin-1* PCR bands were documented to calculate the relative expression of target genes (Fig. 4). The results indicate a very similar gene expression pattern for all three studied genes in both examined control media. These findings suggest that the experimental media (1% basic A2) may support the gene expression of the target genes as well as the positive control medium (10% Stoker). Following the PCR assay, amplification of both genes was observed in all grown cells across all examined cell culture media (Fig. 5).

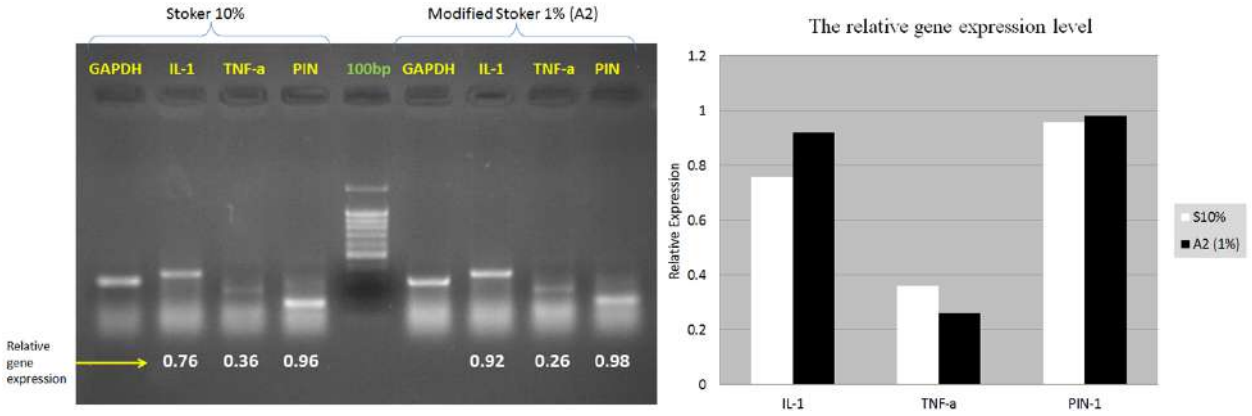

Fig. 4: Gene expression analysis of grown cells in 10% Stoker and 1% basic A2 media. The gel agarose electrophoresis of amplified RT-PCR products of GAPDH, IL-1, TNF-alpha, and TaPin-1 genes is illustrated in the left figure. The four lanes on the left correspond to cells grown in 10% Stoker medium, and the four lanes on the right represent cells grown in 1% basic A2 medium. The right figure displays the level of gene expression of the target genes (IL-1, TNF-alpha, and TaPin-1) in cultured cells in both media, 10% Stoker and 1% basic A2

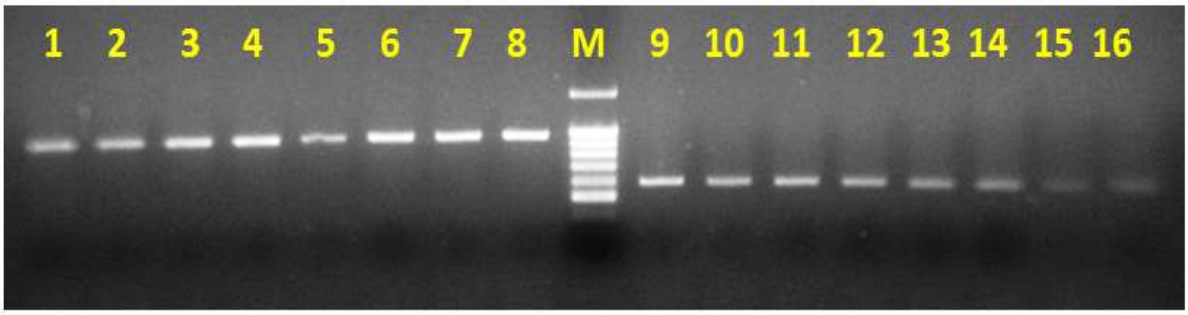

Fig. 5: The PCR products of *Cytochrome b* and *Tams1* genes were analyzed by gel agarose electrophoresis. Both genes were successfully amplified with specific primers in all eight examined cell culture media. The *Cytochrome b* fragments are displayed in lanes 1-8, while the *Tams1* amplicons are shown in lanes 9-16. Lane M represents a 100 bp DNA size marker

Discussion

In this study, among the seven experimental media, three (M1, M2, and A2) exhibited promising results, displaying a notable increase in the growth of vaccine cell lines for mass production. The doubling time of the cultivated cells decreased in comparison to the 10% Stoker control medium, indicating an improvement in growth rate. The osmolality levels for all examined media were within the isotonic range. The measurement of protein content in both experimental and control media showed a range of 43 to 71 mg/dL, which may be attributed to different formulations, especially for serum and peptones used in experimental media. Despite these differences, osmolality values were within an acceptable range, suggesting that these changes did not have an adverse effect on cell growth.

The average percentage of viable cells was calculated for each examined medium, with the EF and Dea media displaying the highest percentage of living cells at 92%, followed by the 1% Stoker (basic A2) medium with a viability of 88.44%. The viability percentage for

the remaining examined media ranged between 82% and 87.92%. These findings suggest that the cultivated cells in the experimental media exhibit high levels of viability.

The measurement of glucose and urea levels in the supernatant of eight examined cell cultures indicated that the cell metabolism was comparable to the positive control. Glucose consumption was observed as the main source of energy, while urea was a natural product of protein metabolism (21).

The results of genomic analysis using PCR with two genes, *Cytochrome b* and *T. annulata merozoite antigen* (*Tams1*), suggested that the growth of the *Theileria* cell line was successful in all the tested media and that the cells maintained their genomic integrity during the growth period (Fig. 5).

Additionally, gene expression assay of *IL-1*, *TNF-alpha*, and *TaPin-1* genes showed that the pattern of *T. annulata* vaccine cell growth in experimental media was similar to the growth and proliferation pattern of vaccine strain cells cultured in 10% Stoker medium (Fig. 5).

The rate of cell growth for the M2 medium was 95.7% compared to the positive control (10% Stoker), but the viability percent for M2-grown cells was higher than for those grown in the 10% Stoker medium.

As a result, the three experimental media (M1, M2, and basic A2) prepared with 1% bovine serum showed positive and satisfactory outcomes in comparison with the control media, indicating their potential for further investigations. The cell growth percentages in M1, M2, and 1% A2 media were 91.5%, 95.7%, and 93%, respectively, in comparison to the 10% Stoker medium.

In this study, yeast extract and peptone were used as substitutes for serum, and previous research has demonstrated their effectiveness in cultivating the protozoan parasite *Leishmania* (22).

In addition to being a substitute for serum, peptone has been shown to stimulate and promote cell growth in various studies. For instance, several commercial peptones were utilized for optimizing the culture of CHO cells, and they resulted in encouraging effects on biomass production (10). Peptone's ability to promote cell growth has also been reported in other studies (23, 24). Although recombinant supplements and additives, such as growth factors, can be used in serum-free media, their utilization is not always cost-effective (25).

Conclusion

This study successfully established a culture system for *Theileria* cell lines in serum-reduced Stoker media supplemented with meat peptone. Importantly, we demonstrate the potential of replacing significant amounts of serum with defined serum substitutes, thereby marking a key step towards developing a cost-effective and well-defined cell culture medium for bovine theileriosis vaccine production.

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Conflict of interest

The authors declare no conflict of interest.

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