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LAB-SCALE PRODUCTION OF ANTI-SCHISTOSOMA MONOCLONAL ANTIBODIES BY HYBRIDOMA CULTURING IN SERUM FREE MEDIUM: ENHANCEMENT OF GROWTH, CRYOPRESERVATION SURVIVAL RATE, AND DIAGNOSTIC ACCURACY.

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Abstract

This study was performed to evaluate the effect of two commercially available serum independent culture media; serum free medium (SFM) and chemically defined medium (CDM), on the growth rate, antibody productivity and post adaptation

cryopreservation and revival reactivity of hybridoma cells compared to the conventional serum based medium (SBM). In addition, the diagnostic efficacy of MAbs secreted in each culture medium was evaluated by testing their performance in sandwich ELISA for antigen detection. Anti- *Shistosoma mansoni* soluble egg antigen hybridoma cell line (7A/8F) secreting previously characterized IgG Kappa mAbs, recognizing repetitive epitopes on *Shistosoma* mansoni soluble egg antigen, were retrieved and propagated in each of the three forementioned media, growth rate and viability were assessed post culturing in each media. The data collected from this study indicated that MAbs secreted from hybridoma cells cultured in SFM were the most abundant, easiest to purify, and the most effective in antigen detection by sandwich ELISA, than those produced in the other two media. Moreover, SFM provided a high quality medium for lab-scale production of MAbs. Moreover, combination of fresh and conditioned medium with DMSO 7.5% was the most promising formulation for the cryopreservation of hybridoma cells cultivated in serum independent media (SFM or CDM).

Keywords: *lab-scale production, monoclonal antibodies, hybridoma, serum*

Introduction

Monoclonal antibodies are greatly acknowledged for their outstanding performance as both diagnostic and therapeutic tools for various diseases. Production of small quantities of less than 0.1 g of purified MAbs is often adequate to meet the needs of many research projects **(Liew *et al* ., 2021).** Lab- scale production of MAbs can be conducted either in vivo or in vitro. In vivo MAbs production is induced by injecting the hybridoma cells into mice peritoneal cavity where they would grow quickly as abdominal tumor, secreting MAbs in ascitic fluid. However, there is likely to be a problem of batch-to-batch variations between animals as well as contamination of the products by non-specific murine immunoglobulin and murine viruses from the host **(Van der Valk *et al*., 2018).** In vitro culture of hybridoma cells in a closed system, is the alternative approach for scaling up of MAbs production, where MAbs secreted in culture medium are harvested and purified . The advantages of in vitro method are its easy performance, relatively low costs, and short production time **(Carvalho *et al*., 2017).**

The cultivation of hybridomas secreting monoclonal antibodies involves the use of media supplemented with fetal bovine serum (FBS). However, the use of FBS is increasingly criticized for scientific, technical, as well as ethical reasons **(Giammarioli *et al*., 2015, Vojgani *et al*., 2018).** The cultivation of the hybridoma cells in media not enriched by serum has the advantage of providing MAbs with a high degree of purity with consequent reduction of non-specific reactions in diagnostic tests **(Manna *et al.*, 2015, Chabaud *et al*., 2016).**

An anti *S. mansoni* hybridoma cell line (7A 8F), produced in monoclonal antibodies production unit at Theodor Bilharz Research Institute, Egypt, was chosen for this study. The 7A 8F MAb is of IgG1 k type, recognizing repetitive epitope on soluble egg antigen (SEA). This MAb was employed as both antigen capturing and detecting in sandwich ELISA for circulating *Schistosoma* antigen (CSA) detection. This study was designed to assess the effect of two serum independent media; serum free medium (SFM), and chemically defined medium (CDM), versus serum based medium (SBM), on the growth rate, monoclonal antibody productivity, and diagnostic efficiency of the 7A 8F hybridoma cells. The culture medium showing exemplary effect on hybridoma cells would be adopted for in vitro lab-scale production of MAbs.

Materials & Methods

1- Ethical statement

All methods were carried out in accordance with relevant guidelines and regulations. This study was reviewed and approved by the ethics committee of Theodor Bilharz Research Institute, and Cairo University.

2- Revival of Hybridoma cells

Hybridoma cell line (7A 8F), secreting MAbs against *S. mansoni* soluble egg antigen (SEA), was kindly provided by the Monoclonal Antibodies Production Unit of TBRI. Revival of cells was carried out according to Goding (1986). The cryopreserved hybridoma cells were thawed rapidly by immersing cryotubes in water bath at 37°C. Thawed cells were transferred carefully into 15 ml sterile centrifuge tube, and 10 ml of 80% fetal bovine serum in RPMI media were added, without disturbing the cell suspension. The cells were centrifuged at 1200 rpm for 10 minutes. The medium was discarded, and the cell pellet was resuspended in SBM at a seeding density of 4 x 10^5 /ml. Revived hybridoma cells were cultured in 25cm^2 tissue culture flasks, and incubated at 37°C in 5% CO_2 .

3- Adaptation of hybridoma cells to their culture in serum independent media:

3.1 Preparation of Culture Media:

i. Serum Based medium (SBM) **:**

RPMI -1640 pH 7.4 supplemented with 20 ml/L Hepes (Gibco. UK), 3 ml/ L Na bicarbonate 7.5%, penicillin (10,000units/ml)/ streptomycin (10mg/ml) (Sigma) in 0.9% sodium chloride and 10 ml L-glutamine (Biochrom, Germany), and 20% fetal calf serum (FCS)((Hyclone, South America) heat inactivated at 56 °C for 30 min, to deactivate the complement-system cytolytic proteins.

ii. Chemically defined medium *(CDM, Gibco, USA)*

CDM supplemented with 8 mM L-glutamine .

iii. ***Serum free medium*** (SFM, Gibco, USA)

iv. Conditioned medium (ConM):

Conditioned medium (ConM) is the hybridoma cells supernatant collected after their culturing for one passage (around 2 to 3 days), when the cells are semiconfluent.

3.2. Adaptation Method:

Adaptation of serum dependent hybridoma cells to their culture in serum independent media (SFM or CDM) started when the cells reached their midlogarithmic phase of growth, before the cells reach full confluency, with hybridoma cells viability > 85%. The cells were seeded at a density of 4 x 10^5 /ml (twice the conventional 2 x 10^5 cells/ ml seeding density). Adaptation was carried out by subculturing of hybridoma cells in increasing concentration of either SFM or CDM in exchange of SBM. This was performed by passaging of hybridoma cells for several times in equal mixture (1:1) of fresh SFM or CDM and conditioned medium (ConM) collected from the previous passage. During adaptation, the cells count & viability were checked on daily basis, using trypan blue dye (Invitrogen, USA) exclusion method according to Patterson, (1979). After full adaption of hybridoma cells to serum independent media, they were propagated to increase their number, by gradual increase of their culture vessels, surface area, starting with 24-well plate, then 6-well plates, then 25 cm2 culture flasks, 75 cm2 flasks and finally 175 cm2 flasks.

4. Lab – scale production process of MAbs :

In vitro raising of monoclonal antibodies at a Lab-scale level was performed by culturing hybridoma cells showing 85%-90% viability in 175 cm^2 culture flasks. Each flask received 100 ml of one of the 3 media, either SBM or SFM or CDM. Each medium was inoculated with hybridoma cells at a density of 4 x 10^5 cells/ml. The cultures were maintained in 5% Co2 at 37^0C, without changing media, to allow for the accumulation of secreted MAbs in the culture supernatant. The flasks were agitated occasionally, and incubated for about 10 days or until most of the cells are dead. To harvest the supernatant, the media was divided into 50 ml falcon tubes, and centrifuged for 10 min at 2800 rpm. The cleared supernatant was filtered through 0.22 µm filter and transferred to sterile 50 ml falcon tubes. The aliquots were stored at -80. This procedure was performed, with some modifications, according to the method of Winzeler and Wang (2013).

5. Assessment of growth rate of hybridoma cells in different culture media

Growth rate was determined as number of viable cells obtained after culture of 4x10^5 /ml hybridoma cells in different media till reaching full confluency. Cell number was counted with a hemocytometer, and their viability was determined using the trypan blue dye exclusion method.

6. Assessment of MAbs level in different culture media:

Monoclonal antibodies, secreted in hybridoma supernatant, were tested for their reactivity to Schistosoma antigen by indirect-ELISA. The ELISA test was performed according to the method of Demerdash et al., 1995.

7. Purification of MAbs from culture supernatant

MAbs secreted in either of three culture media, previously determined to be IgG1, were purified by antibodies purification kit (Abcam UK). The protein content of collected supernatant, before and after purification of antibodies, was estimated by Bio-Rad protein assay (Bradford, 1976), and their reactivity was assessed by indirect ELISA against *S. mansoni* SEA according to Demerdash et al., 1995.

8- Assessment of the effect of different cryopreservation methods on hybridoma cells revival after cryopreservation:

Cultures growing in FBS were cryopreserved in standard cryopreservation medium containing 10% Dimethyl sulfoxide (DMSO, Sigma, USA) and 90% FBS. The hybridoma cells growing in the serum independent media (SFM or CDM) were cryopreserved with standard cryopreservation media, Bambanker™ direct freezing medium (Nippon genetics, Germany) and serum free freezing formula. Parts of propagated adapted cells with more than 90% viability were preserved in liquid nitrogen for future use. For cells growing in CDM, 92.5% CD Hybridoma Medium (50:50 ratio of fresh to conditioned media) and 7.5% DMSO. For cells growing in hybridoma SFM: 92.5% hybridoma SFM (50:50 ratio of fresh to conditioned media) and 7.5% DMSO were used. Furthermore, to evaluate the cryopreservation freezing media, the cryopreserved cells were revived every month for six months.

***9-* Assessment of MAbs performance for detection of in circulating Schistosoma antigen (CSA) by Sandwich ELISA**

9.1. Sample collection

Samples were collected from endemic hot spots in the Nile Delta (Elkhamseeny and Sandala villages in Kafr Elsheikh governorate). Informed consent and full medical history were taken from the patients. Stool samples from patients were collected and examined by Kato-Katz technique to identify *S.mansoni* eggs. Blood samples were collected from thirty two patients diagnosed by detection of *S. mansoni* eggs in their stool (active infection group). Blood samples from 25 age and sex matched healthy endemic individuals were collected (negative control group). The sera were separated and stored at -80°C until used.

9.2. CSA detection by sandwich ELISA

Three sets of sandwich ELISA were performed, each set made use of monoclonal antibodies purified from hybridoma cells cultured in either SBM, or SFM, or CDM. Purified monoclonal antibodies were employed as both antigen capturing and peroxidase-labelled antigen detecting, in sandwich ELISA according to Salah et al.

2002. ELISA microtiter plates (96 wells) were coated with 100ul/well of purified MAbs at a dilution of 1:20 in 0.1 M carbonate buffer pH 9.6. Plates were covered with plate sealers and left overnight at RT. Plates were washed 2 times with 0.01 M PBS, pH 7.4 The remaining sites in the well surface were blocked by incubation for 2h at 37°C with 200µl/well of the washing buffer containing 2.5% FBS. Plates were washed as described before, then 100ul of the diluted serum samples (1:3) were pipetted into the wells of the blocked plate and incubated overnight at 37°C. 100µl of horse raddish peroxidase conjugated MAbs (conjugated according to Tijssen and Kurstak 1984) at dilution 1/50 of in diluent buffer (PBS 0.1M, pH 7.4) were added to each well and the plate was incubated for 1 hour. Wells were then washed 5 times, 3 min each. 100µl / well of substrate solution (OPD in urea hydrogen peroxide) was added to each well and incubated at RT in the dark for 20 min. The reaction was stopped by addition of 50µl / well 8 N H2SO4. The plates were read at 492 nm wave length using microplate ELISA reader.

Statistical Analysis:

Statistical analysis was performed using SPSS 24.0. The values were reported as mean± standard error (SE). Comparison between different hours of samples was performed using analysis of variance (ANOVA). The data were considered significant with p value ≤ 0.05. Receiver Operating Characteristics (ROC) curves was used to determine the accuracy, sensitivity and specificity of sandwich ELISA using MAbs purified from different media.

Results:

1. **Adaptation of hybridoma cells to serum free media:**

Hybridoma cells were adapted to the SFM and CDM by the use of conditioned medium, which is the hybridoma cells supernatent collected after their culturing for one passage. Adaptation was performed by subculturing of hybridoma cells in increasing concentration of either SFM or CDM in exchange with SBM, until the complete serum free condition was reached. This means gradual reduction of FBS content from 20% to 0% (Figure 1).

Hybridoma cells were gradually weaned to grow in serum independent media (SFM and CDM) over 6-8 passages. Their adaptation to CDM needed 6 passages, while their adaptation to SFM took 8 passages. Full adaptation was reached after their passaging for three subsequent cultures in 100% serum independent media. The viability % was adequately maintained at 90% during adaptation to CDM, while a decrease in cell viability to 75% was seen during adaptation to SFM upon reaching 2.5% serum concentration. Therefore, adaptation to SFM needed extra passage in 5% serum concentration to allow an increase in cell viability to 90% before reaching complete adaptation.

2. Assessment of growth rate of hybridoma cells, and MAbs secretion levels in different culture media

2.1. Growth rate Assessment

Hybridoma Cells were cultured for 10 days in the three media, SBM, SFM, and CDM, and their growth rate was evaluated as number of viable cells per culture. For all cultures, the cell concentrations increased in a logarithmic pattern and hit the plateau phase then the cell number starts to decrease in the decline phase. The starting cell density was $4x10^5$ for all 3 cultures. Maximum density reached was 14.2 x 105, 16.1 x 105, and 16.3 x 105, for SBM, SFM, and CDM cultures respectively. This increase in cell density was significantly higher ($p<0.05$) in serum independent cultures (SFM and CDM) than Serum based cultures (Figure 2)

2.2. Antibody Level

The level of secreted monoclonal antibodies in different culture media, determined as OD values at 492nm increased gradually as time elapsed, even during the decline phase of growth, with decrease in cell viability. The highest antibody reactivity was reached in day 10 for all 3 media. The antibody level was significantly higher ($p< 0.05$) in SFM cultures than CDM or SBM cultures (Figure 3).

3. Purification of the monoclonal antibodies secreted in culture supernatant

Supernatants of different media were collected and purified. The protein content of MAbs secreted by hybridoma cell line cultured in SBM medium was higher than those cultured in SFM and the CDM. Antibody reactivity of cells growing in either SBM or CDM showed no significant difference before and after purification. However, antibody reactivity of cells growing in SFM was significantly higher after purification than before purification ($p < 0.01$).

4- Assessment of the effect of different cryopreservation methods on hybridoma cells revival after cryopreservation:

Revival of the cells growing in the SGM using FBS 80% showed high survival rate over 80%. However, revival of the SFM & CDM adapted cells using 80% FBS showed less than 20% survival rate after thawing and died after few passages. Recovery of the adapted cells using 30ml of pre-warmed fresh serum independent media (SFM or CDM) showed higher survival rate (over 80%) ($p < 0.01$) as shown in Figure 4.

Adapted cells preserved in standard cryopreservation medium showed better survival rate when cultured in SFM than CDM ($p <0.05$). While adapted cells preserved in Bambanker media showed no significant difference between SFM and CDM. After revival of the hybridoma cells, the cells were propagated for several passages and the secreted mAbs were assessed for their reactivity against *S. mansoni* SEA by indirect ELISA. There was no significant difference in the reactivity of the mAbs secreted in

either of the three culture media (SGM, SFM, CDM) over 6 month storage period ($p > 0.05$)

5- Detection of CSA in serum samples of patients by sandwich ELISA:

MAbs were employed as both antigen capturing and conjugated peroxidase detecting antibodies for circulating Schistosoma antigen assay by sandwich ELISA. The results of negative control subjects (OD value at 492 nm) were used to determine the cut off value of CSA assay as the mean of their results+ 2 SD of the mean. OD readings above the cut off value were considered positive for CSA (Figure 5).

The CSA level in 57 subjects' sera (25 negative subjects and 32 *S. mansoni* infected patients) was measured using *S. mansoni* MAbs purified from hybridoma cells cultured in any of the three media (SBM, SFM and CDM) by sandwich ELISA. The CSA assay was able to discriminate *S. mansoni* infected patients and healthy controls with variable ranges of sensitivity and specificity according to the medium used for hybridoma cells culture.

Sensitivity of CSA assay was 81.3%, 90 %, and 84.4 % when MAbs were purified from supernatant of hybridoma cells cultured in SBM, SFM, and CDM respectively. While specificity of the assay was found to be 88%, 92 %, and 92 % for the three culture media respectively (Table 1).

Discussion

Disadvantages of using FBS are diverse, including technical and ethical factors **(Radosevic *et al*., 2016).** In monoclonal antibodies production unit (MAPU) at TBRI, panels of mAbs were and still produced against various parasitic antigens. These produced mAbs were employed successfully for effective &sensitive diagnostic approaches. Recently, there is an objective works toward the replacement of the conventional methods of mAbs production and different trails are subjected for this purpose in our labs **(Salah *et al*., 2020).** Testing and adaptation of our preserved hybrid cells with serum free media batch tissue-culture production approach is one of our main near future goals. Therefore, we strive for complete replacement and full adaptation of preserved hybridoma cell lines for successful culturing, with maintained mAbs productivity & reactivity as well as cryopreservation, in SFM. In this study, we explored the performance of the two types of serum independent media (SFM and CDM) on cell growth, cell viability, and antibody production rate of Anti- *S. mansoni* hybridoma cells, compared to SBM.

After revival of hybridoma cells and their culture in SBM, they were adapted to grow in SFM and CDM with gradual reduction in FBS concentration from 20% to 0%. This gradual weaning of hybridoma cells was found to increase their chances of successful adaptation for serum independent media **(Biaggio *et al*., 2015).** . Adaptation was carried out using conditioned medium for sub-culturing of hybridoma cells in increasing concentrations of SFM or CDM, in exchange of SBM. The addition of conditioned

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media, being enriched with cytokines and growth factors originating from culturing of cells for one full passage, was found to improve growth rate and viability of hybridoma cells **(González *et al*., 2007).**

In our study, the cell density was kept above $4x10^5$ cells/ml (double the standard cell density) during the adaptation process. This is because the serum-free cultures contain less proteins than serum-containing medium, so the adapted cells are more sensitive to pH, temperature, osmolality, mechanical forces, and enzyme treatments. Therefore, it is recommended to use higher cell density during the adaptation process **(Caron *et al*, 2018).** Cell viability was monitored throughout the adaptation process. The viability % was maintained at 90% during adaptation process, except for adaptation to SFM at serum concentration 2.5%, when viability decreased to 70%. Therefore, adaptation to SFM needed extra passage in 5% serum concentration to allow an increase in cell viability before reaching complete SFM culture adaptation. According to Caron et al, (2018), if the cell growth declines during any point through the adaptation, the conversion to the previous serum concentration could allow cell growth to stabilize before moving forward with the adaptation. This could be because most serum-free media contain much less protein than serum supplemented media, so they are less able to protect cells from stress in culture. Therefore, several passages may be needed at a given step in the process, before it is possible to proceed to the next **(Johnson, 2006).** After full adaptation, the cell viability of both media reached its maximum (> 90%).

Hybridoma cell culture for Lab- scale level of mAb production was performed in T Flasks 175cm2 with a medium containing all the nutrients needed for cellular growth, and then, cells are inoculated. There is no feeding system with fresh medium or withdrawal of spent medium. As the process runs, nutrient concentration decreases and waste metabolites are produced, lowering cell viability. The hybridoma cells continue to secrete MAb into the culture fluid until cell death occurs. Because the MAb is not metabolized, it accumulates in the culture supernatant. Generally, this type of cultivation reaches a maximum density of $1–2 \times 10^6$ cells/ml, and then the cell viability drops rapidly.

Antibody production may be dependent on the culture medium used for hybridoma cells growth. Some studies suggested a better cell and antibody yield when using serum free media **(Gorfien *et al*., 2000; Even *et al*., 2006).** However, other studies showed that MAbs production is higher in media containing serum, as serum appears to have an enhancing effect on the antibody productivity, or could act as a cell protector from stress **(Manna *et al*, 2015).** This finding is probably related to the high protein content of FBS included in SBM. The bovine IgG contamination due to the use of FBS can be problematic for proper MAbs purification **(Manna *et al*., 2015).**

MAbs extracted from each of SBM, SFM, and CDM cultures were employed for circulating *Schistosoma* antigen (CSA) detection by sandwich ELISA in sera of 32 Schistosomiasis patients and 25 endemic negative controls. These purified MAbs were

successfully discriminated schistosomiasis patients from healthy controls. However, the diagnostic accuracy of MAbs extracted from SFM culture was the highest reaching 91.2% compared to MAbs extracted from SBM, and CDM that showed 84.2% & 87.7% diagnostic accuracy respectively

A relatively lower diagnostic accuracy of sandwich ELISA when using MAb of SBM culture could be attributed to contamination of purified MAbs with some IgG subclasses present in FBS resulting in non-specific binding with antigens or with blocking agents used in immunoassays, resulting in increased background signals and decrease in sensitivity and specificity **(Manna *et al*., 2015).**

Furthermore, we concluded that the serum free freezing formula in which combination of fresh and conditioned medium with 7.5% DMSO, was the most promising formulations for the cryopreservation of hybridoma cells cultivated in SFM and CDM. Hybridoma cells can be cryopreserved at least 6 months without any change in their viability and reactivity. Such good result on cell viability after a cryopreservation is valuable in terms of cell line sustainability and definitely rounds up the whole adaptation process **(Radosevic *et al*., 2016).**

Taken together; compared to SBM, hybridoma cells fully adapted to serum independent media, especially SFM, showed better growth rate, and higher MAbs' production level. In addition, culturing in SFM facilitated recovery and purification of MAbs, allowing for better performance in antigen detection sandwich ELISA. We ended up that culturing of hybridoma cells in SFM using closed culturing vessels, such as large T flasks, could be a reliable and easy process for Lab-scale production of MAbs.

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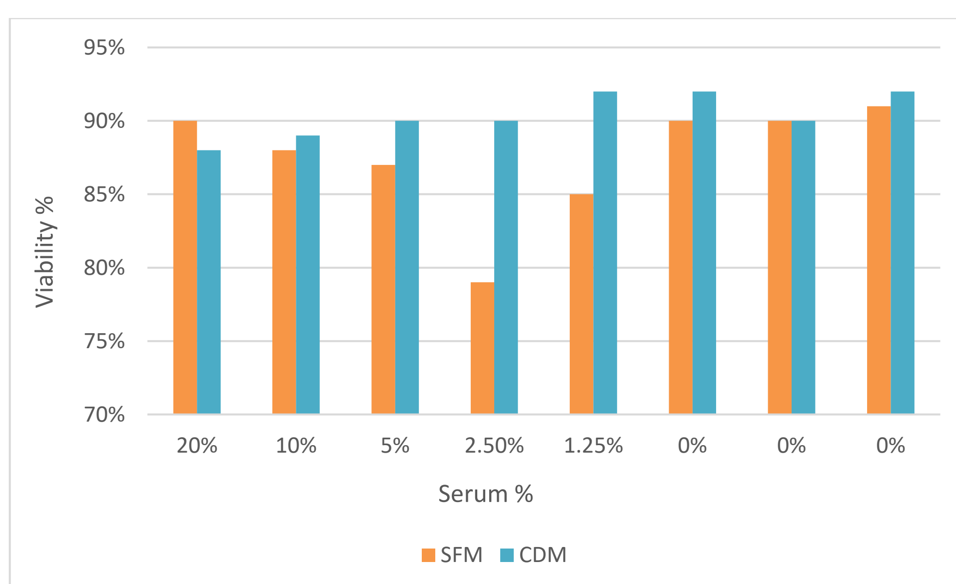

Figure 1: Adaptation of the hybridoma cells to serum independent media (SFM and CDM) by gradual decrease in FBS concentration of their culture medium SGM: serum growth medium, SFM: serum free medium, CDM: chemically defined medium

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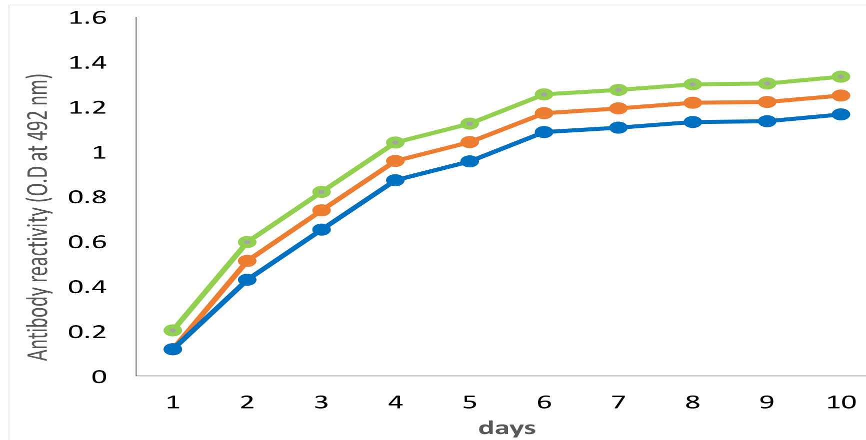

Figure 2: Effect of culture media (SGM, SFM, CDM) on the hybridoma growth rate for cells growing (5x10^5 cells/ml)

Culture media: ( ) SGM, ( ) SFM , ( ) CDM

SGM: serum growth medium, SFM: serum free medium, CDM: chemically defined medium

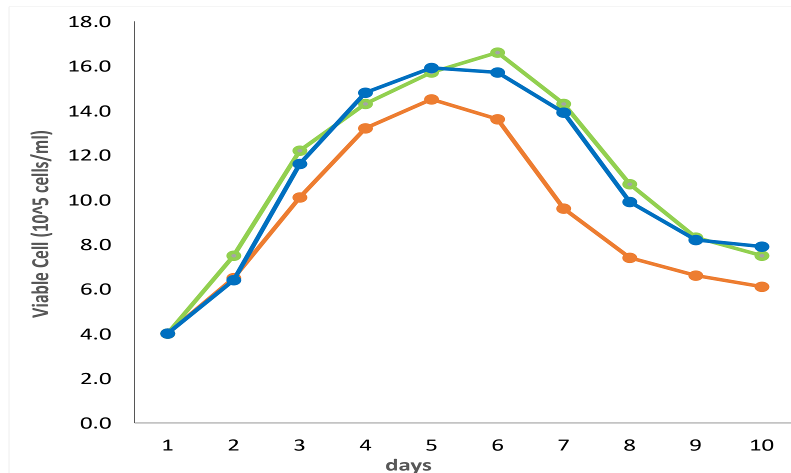

Figure 3: Effect of culture media (SGM, SFM, CDM) on the antibody reactivity of culture supernatant of hybridoma cells growing at different seeding densities. (D) 5x10^5 cells/ml, (F) 6x10^5 cells/ml.

Culture media: ( ) GSM, ( ) SFM and ( ) CDM

SGM: serum growth medium, SFM: serum free medium, CDM: chemically defined medium

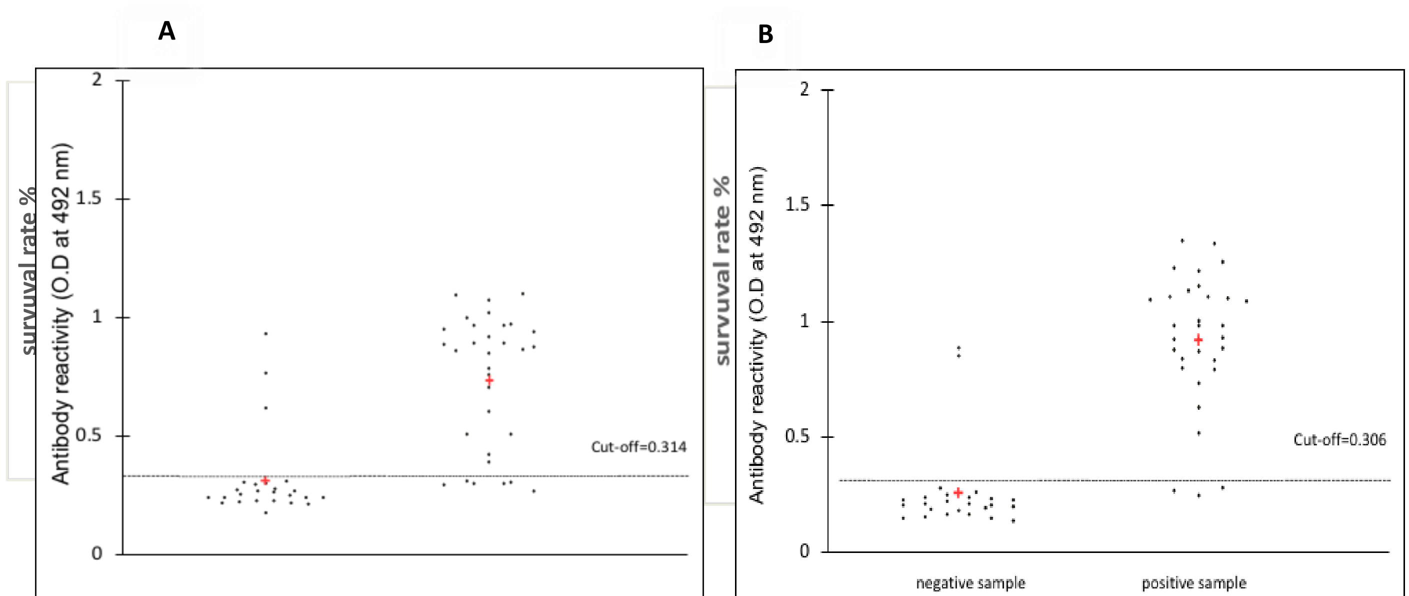

Figure 4: Survival rate of the hybridoma cells after cultured & cryopreserved and thawed in SGM, SFM and CDM 3 (A) and 6 (B) months follow up.

Freezing media: Standard cryopreservation media (SCM), Serum free freezing formula (SFFF) & Bambanker TM Direct freezing media (BFM)

Culture media: ( ) SGM, ( ) SFM and ( ) CDM

A

B

C

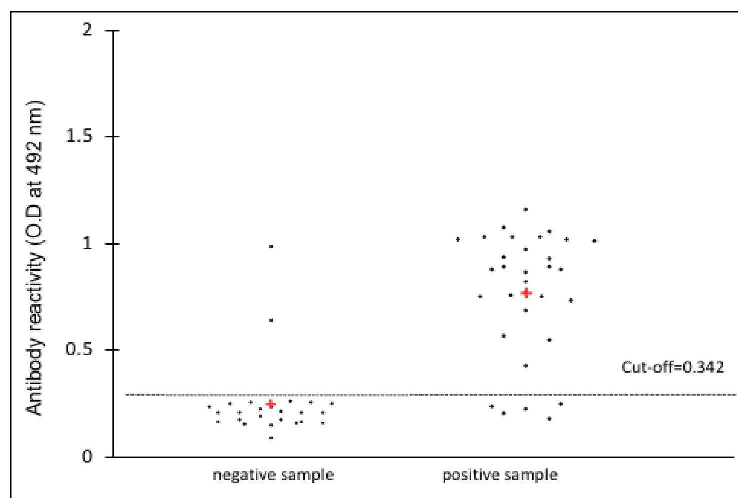

Figure 5: Scattered diagram of CSA level measured as OD readings at 492 nm by sandwich ELISA in a group of Schistosomiasis positive and negative sera, using anti-*S. mansoni* MAbs secreted by hybridoma cells cultured in (A) SDM, (B) SFM and (C) CDM.

Table 1: Sensitivity, specificity and diagnostic accuracy of CSA results performed by purified MAbs from SGM, SFM and CDM by sandwich ELISA.

Media	**Sensitivity %**	**Specificity %**	**Diagnostic Accuracy**
SGM	81.3%	88.0%	84.2%
SFM	90%	92.0%	91.2 %
CDM	84.4%	92.0%	87. 7%

SGM: serum growth medium, SFM: Serum free medium, CDM: chemically defined medium