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Determination of Optimum Conditions for Human Epithelial Type-2 Cell Culture and Fixation on Slides

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ABSTRACT

Objective: To determine optimum conditions for Human Epithelial Type-2 (HEp-2) cell culture and fixation on slides.
Study Design: Cross-sectional study.
Place and Duration of Study: Department of Immunology, Armed Forces Institute of Pathology Rawalpindi, Pakistan from Apr to Sep 2022.
Methodology: Study involved procuring viable HEp-2 cell suspension having different counts and sub-culturing on Teflon coated glass and synthetic plastic slides. Few slides were kept in CO2 incubator and other in normal incubator at varied temperatures. Half of slides were enriched with 10 % Hepes Minimum Essential Medium (HMEM) at timely intervals. Subsequently, slides from each group were fixed using 4 different fixation protocols for different durations and temperatures. After fixation, slides showing better cell growth on microscopy were stored at different temperature wrapped in aluminium foil. Following optimization, staining of slides with 305 known control samples for antinuclear antibodies (ANA) was done. Periodic microscopy was performed by two experienced observers working independently, and observations were noted.
Results: It is better to subculture HEp-2 cells on synthetic plastic slides using viable suspension of four hundred thousand cells per ml and subsequently enrich by 10% HMEM at 90 minutes and 12 hours. Growth is optimal on slides kept at 5% CO2 incubation. For ANA detection fixation of cells at 24 hours with ice-cold methanol for 15 minutes is necessary and then slides can be stained or stored at -60 ^{o}C upto 60 days.
Conclusion: HEp-2 cells can be cultured on synthetic plastic slides and subsequently fixed using methanol.

Keywords: Antinuclear Antibodies (ANA), Cell Culture, Human Epithelial Type-2 (HEp-2), Viral Cytopathic Effects.

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INTRODUCTION

Cell lines are clonal population of immortalized cells propagated in vitro from primary explants which can be kept in laboratory for prolong period of time, with intact phenotypic and functional properties.[1] Their popularity can be estimated by the extensive published literature and American Type Culture Collection (ATCC) which comprise of over 3,600 cell lines from more than 150 species.[2,3] Cell lines have transformed research and are now being used in vaccine preparation, drug testing, antibody production, gene function analysis, artificial tissue generation and production of biological compounds.[4-6]

The Human Epithelial Type-2 (HEp-2) cell line was initially studied on atissue specimen from a patient presenting with a laryngeal carcinoma.[7,8] However, it was later found that the HEp-2 were in fact comprised of cervical adenocarcinoma cells and derived via HeLa cell line contamination. This fact was further supported by isoenzyme analysis, HeLa marker chromosomes, and DNA fingerprinting. HEp-2 cell lines are continuous, cancerous, immortalized, haploid cell lines and in addition to observe viral cytopathic effects, they are gold standard substrate for antinuclear antibody (ANA) detection by indirect immunofluorescence because of presence of over 100 antigens in the nucleus and cytoplasm that may have association with autoimmune diseases. American College of Rheumatology recommends HEp-2 cell for indirect immunofluorescence because of large number of antigens they express.[9,10]

The aim of this study was to culture HEp-2 cell on slides which can be further used to check viral cytopathic effects on unfixed cells or for indirect immunofluorescence after fixation with an appropriate fixative.

METHODOLOGY

This cross-sectional study was conducted at the Department of Immunology Armed Forces Institute of

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Pathology Rawalpindi, Pakistan over a period of 6 months from April to September 2022, after formal approval by Institutional Ethical Review Board (IRB/22/958).

Inclusion Criteria: Patients of either gender, of any age group, presenting for ANA testing, with viable HEp-2 cell suspension having known counts maintained at appropriate temperature and at authorised setup, were included.

Exclusion Criteria: Patients with hemolysed, lipaemic or icteric serum samples, and initial HEp-2 cell suspension having high dead cell count (>10%) were excluded.

Sample size was calculated using the Sensitivity and Specificity calculator by keeping prevalence of ANA 4%, sensitivity 87.7%, specificity 67.8%, and precision 0.10, our sample size came out to be 305.[11] Study involved microscopy of HEp-2 cell lines maintained in T-75 cell culture flasks at National Institute of Health (NIH) Islamabad, Pakistan and subsequently procuring viable HEp-2 cell suspension of different counts after trypsinization of maintained and fully confluent cell lines. Then different quantity of each suspension having different known counts were sub-cultured on wells of Teflon coated glass and synthetic plastic slides. After this, half of the slides from each group were kept in 5% CO2 incubator and other half at different temperatures. Fifty percent of these slides were enriched with Hepes Minimum Essential Medium (HMEM) at 90 minutes, 12, 24, 36 and 42 hours. Microscopy was also performed on all slides at the same time using Olympus CKX53. Few of the slides were rinsed thrice with phosphate buffered saline (PBS) and fixed using 4 different fixation protocols (methanol and acetone, methanol, formaldehyde, acetic acid and ethanol) separately for different time durations and kept at different temperatures which can be seen in Figure-1. After removing from fixatives microscopy was performed and each slide was rinsed with PBS and kept in PBS till it was used, or stored at different temperatures for different time durations wrapped in aluminium foil. After optimization of steps, staining of slides with 305 known control samples for ANA was done and fluorescence was observed using fluorescent microscope BA-310. Microscopy was done by two experienced observers working independently and if two experts disagreed, a consensus was reached by discussion. All microscopic and macroscopic observations were noted on predesigned proforma and dealing with HEp-2 cells was done in biological safety cabinet class II A2 using personnel protective equipment.

RESULTS

As per our results only synthetic plastic slides can be used for HEp-2 cell culture rather than Teflon coated glass slides. Sub-culturing wells of synthetic plastic slides using 20ul HEp-2 cell suspension of 4 hundred thousand cells per ml yielded better results than suspension of 3 and 5 hundred thousand cells per ml (Figure-1A and 1C). Enrichment with 10 % HMEM at 90 minutes and 12 hours is necessary for better growth of cells on plastic slides (Figure-1B, 2 and 3). At 24 hours wells of slide seeded with 4 hundred thousand cells per ml suspension showed maximum confluency and relatively minimum cell death so it is better time for further fixation (Figure-1C, 2 and 3). Methanol is better nuclear fixative however formalin is good for cytoplasmic fixation. For further ANA detection it is best to fix HEp-2 cells slides in ice cold pure methanol for 15 minutes and then subsequently stain or store after wrapping in aluminum foil at -60 ^{o}C for up to 60 days for later use (Figure-1D, 1E, 2 and 3).

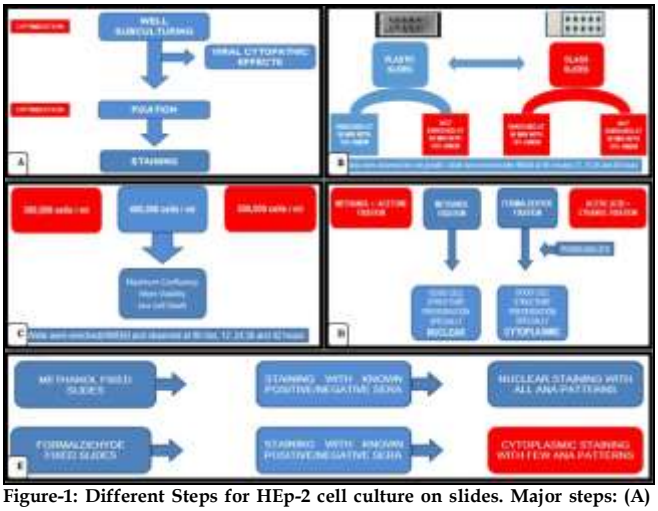

Figure-1: Different Steps for HEp-2 cell culture on slides. Major steps: (A) Comparison of synthetic plastic and Teflon coated glass slides. (B) HEp-2 cell sub-culturing on synthetic slides with different viable suspensions. (C) Comparison of 4 fixation protocols. (D) Staining slides with ANA positive sera and (E) Fluorescent isothiocyanate (FITC) labelled conjugate

DISCUSSION

Certain environmental conditions are required for optimal growth and proliferation of HEp-2 cells including temperature control, suitable pH and osmolality, a surface for cellular attachment, nutritional medium and incubator to maintain stable conditions.[12,13] As per the present study, HEp-2 cells can be cultured on synthetic plastic slides and show

maximum confluency in well at 24 hours if cell suspension having 4 hundred thousand cell per ml count is used and, wells are periodically enriched with 10% HMEM (pH 7.2-7.4) at 90 minutes and 12 hours. Moreover, in our study HEp-2 cells showed better results when placed in 5% CO2 at 35±2°C.

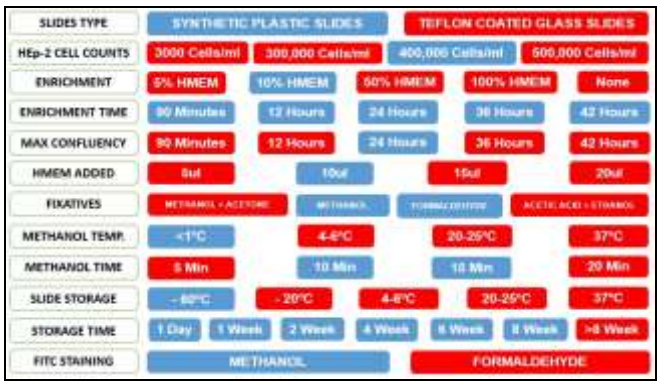

Figure-2: Different steps done during optimization of HEp-2 cell culture on slides and their results (Note blue boxes represent successful/recommended steps whereas red represent unsuccessful/non recommended steps for HEp-2 cell culture)

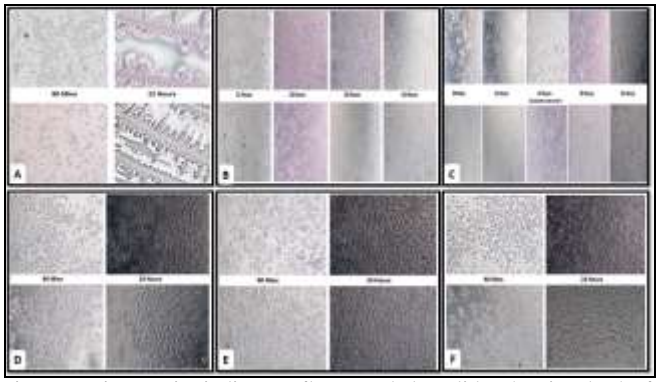

Figure-3: Microscopic Findings. Teflon coated glass slides showing death of cells (A). Synthetic plastic slides not enriched with HMEM (B). Synthetic plastic slides enriched with HMEM(C), Synthetic slides seeded with suspension of 3 hundred thousand cells/ml (D). Synthetic slides seeded with suspension of 4 hundred thousand cells/ml showing maximum confluency(E). Synthetic slides seeded with suspension of 5 hundred thousand cells/ml (F)

One study used a short pre-fixation step with a combination of paraformaldehyde and sucrose followed by a brief permeabilization/fixation step with a mixture of methanol and acetone at -80 °C for immunofluorescence using epithelial cells.[14-16]

Tebo *et al.*, demonstrated that it takes more than one hour for cytosolic proteins in cultured cells to be fixed by formaldehyde. Similar was case other aldehydes, acrolein and glyoxal. They also found a distinct displacement of proteins and lipids, including their loss from cells. Glutaraldehyde resulted in quick fixation in less than four minutes and most cyto-plasmic proteins were also retained. Supplementary addition of formaldehyde surprisingly vanished autofluorescence produced by glutaraldehyde, without compromising fixation speed.[17]

Brito *et al.*, in their study, found that HEp-2 cells fixation with a pure acetone resulted in a 97.5% sensitivity for antinuclear antibody using known positive samples, while only 81.3% sensitivity using alcohol/acetone fixed HEp-2 cells. Fluorescence was more prominent on acetone fixed slides making it easy to read than the alcohol/acetone fixed slides.[18]

In the present study, 4 different fixation protocols including methanol and acetone, methanol, formaldehyde, acetic acid and ethanol were tried on HEp-2 cells cultured on synthetic plastic slides. Among them methanol + acetone and acetic acid + ethanol reacted with synthetic slide material and only methanol and formaldehyde were able to fix HEp-2 cells. Methanol was better nuclear fixative however formalin was good for cytoplasmic fixation. Furthermore HEp-2 cell fixation is best for ANA detection when ice cold pure methanol is used for 15 minutes. Methanol fixed HEp-2 slides can be stored at -60 °C for up to 60 days.

LIMITATIONS OF STUDY

The main limitation of the present study was a limited scope to culture and fixation of HEp-2 cells because of time and financial constraints, however further multicenter studies are needed for extensive validation of HEp-2 slides and fluorescent staining.

CONCLUSION

It is better to subculture HEp-2 cells on wells of synthetic plastic slides using viable suspension of 4 hundred thousand cells per ml, subsequently enrichment by 10% HMEM at 90 minutes and 12 hours. For ANA detection fixation of sub-cultured cells at 24 hours with ice cold methanol is necessary and afterwards fixation slides can be stored at -60 °C for upto 60 days.

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Authors' Contribution

Following authors have made substantial contributions to the manuscript as under:

MZA & DA: Data acquisition, data analysis, critical review, approval of the final version to be published.

AT & RA: Study design, data interpretation, drafting the manuscript, critical review, approval of the final version to be published.

YR & MI: Conception, data acquisition, drafting the manuscript, approval of the final version to be published.

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

REFERENCES

1. Verma A, Verma M, Singh A. Animal tissue culture principles and applications: Anim Biotechnol 2020: 269-8293. https;//doi.org/10.1016/B978-0-12-811710-1.00012-4
2. Kaur G, Dufour JM. Cell lines: Valuable tools or useless artifacts. Spermatogenesis 2012; 2(1): 1-5. https://doi.org/10.4161/spmg.19885
3. Richter M, Piwocka O, Musielak M, Piotrowski I, Suchorska WM, Trzeciak T. From Donor to the Lab: A Fascinating Journey of Primary Cell Lines. Front Cell Dev Biol 2021; 9: 711381. https://doi.org/10.3389/fcell.2021.711381
4. Fujii M, Sato T. Somatic cell-derived organoids as prototypes of human epithelial tissues and diseases. Nat Mater 2021; 20(2): 156-169. https://doi.org/10.1038/s41563-020-0754-0
5. Souren NY, Fusenig NE, Heck S, Dirks WG, Capes-Davis A, Bianchini F, et al. Cell line authentication: a necessity for reproducible biomedical research. EMBO J 2022; 41(14): e111307. https://doi.org/10.15252/embj.2022111307
6. Masters J, Capes-Davis A. Cell-line authentication: End the scandal of false cell lines. Nature 2012; 492(7428): 186. https://doi.org/10.1038/492186a
7. Segeritz CP, Vallier L. Cell Culture: Growing Cells as Model Systems In Vitro: Basic Science Methods for Clinical Researchers Chapter 9. Academic Press 2017: 151-172. https://doi.org/10.1016/B978-0-12-803077-6.00009-6
8. Bahmad HF, Cheaito K, Chalhoub RM, Hadadeh O, Monzer A, Ballout F, et al. Sphere-Formation Assay: Three-Dimensional in vitro Culturing of Prostate Cancer Stem/Progenitor Sphere-Forming Cells. Front Oncol 2018; 8: 347. https://doi.org/10.3389/fonc.2018.00347
9. Gorphe P. A comprehensive review of Hep-2 cell line in translational research for laryngeal cancer. Am J Cancer Res 2019; 9(4): 644-649.
10. Fusi M, Dotti S. Adaptation of the HEp-2 cell line to totally animal-free culture systems and real-time analysis of cell growth. BioTechniques 2021; 70(6): 319-326. https://doi.org/10.2144/btn-2020-0162
11. Dilnessa T ZH. Cell Culture, Cytopathic Effect and Immunofluorescence Diagnosis of Viral Infection. J Microbiol Modern Tech 2017; 2(1): 102.
12. Sherman KE, Rouster SD, Kong LX, Aliota MT, Blackard JT, Dean GE. Zika virus replication and cytopathic effects in liver cells. PLOS One 2019; 14(3): e0214016. https://doi.org/10.1371/journal.pone.0214016
13. Arshad M. Development and Validation of In-house HEp-2 Cell Slides for Detection of Antinuclear Antibodies (ANA) by Indirect Immunofluorescence. J Coll Physicians Surg Pak 2023; 33(12): 292-296. https://doi.org/10.29271/jcpsp.2023.03.292
14. Nagy G, Földesi R, Csípő I, Tarr T, Szűcs G, Szántó A, et al. A novel way to evaluate autoantibody interference in samples with mixed antinuclear antibody patterns in the HEp-2 cell based indirect immunofluorescence assay and comparison of conventional microscopic and computer-aided pattern recognition. Clin Chim Acta 2024; 553: 117747. https://doi.org/10.1016/j.cca.2023.117747
15. Damoiseaux J, Andrade LEC, Carballo OG, Conrad K, Francescantonio PLC, Fritzler MJ, et al. Clinical relevance of HEp-2 indirect immunofluorescent patterns: the International Consensus on ANA patterns (ICAP) perspective. Ann Rheum Dis 2019; 78(7): 879-889. https://doi.org/10.1136/annrheumdis-2018-214436
16. Pullen Jr RL. Antinuclear antibodies. Nurs Made Incredibly Easy 2022; 20(6): 47-48. https://doi.org/10.1097/01.NME.0000884112.83689.31
17. Tebo AE. Recent Approaches To Optimize Laboratory Assessment of Antinuclear Antibodies. Clin Vaccine Immunol 2017; 24(12): e00270-317. https://doi.org/10.1128/CVI.00270-17
18. Brito FA, Santos SM, Ferreira GA, Pedrosa W, Gradisse J, Costa LC, et al. Detection of anti-nuclear antibodies by indirect immunofluorescence on HEp-2 cells: setting the appropriate screening dilution for the diagnosis of autoimmune rheumatic diseases. Rev Bras Reumatol 2014; 54(1): 13-20. https://doi.org/10.1016/j.rbre.2014.02.001