DOI: https://doi.org/10.53350/pjmhs221610804

ORIGINAL ARTICLE

Enzyme Immunoassay for the Detection of Human Chorionic Gonadotropin (HCG) and Placental MRNA Marker a Practical Technique for Forensic Pregnancy Identification in Bloodstains

SHEEBA SHABBIR[1], NUSRAT ALI[2], IFTIKHAR AHMAD[3], AINA KHURSHID[4], MUHAMMAD FARHAN SIDDIQ RAO[5], SABIKA HUSSAIN[6], MUHAMMAD RIZWAN[7]

[1]*Department of Forensic Medicine and Toxicology, HBS Medical and Dental College Islamabad*
[2]*Department of Biochemistry, HBS Medical and Dental College Islamabad*
[3]*Department of Forensic Medicine and Toxicology, Khyber Girls Medical College Peshawar*
[4]*Department of Biochemistry, Multan Medical and Dental College, Multan*
[5]*Institute of Molecular Biology and Biotechnology (IMBB) Bahauddin Zakariya University Multan*
[6]*Assistant Professor, Forensic Medicine, Rawal Institute of Health and Sciences*
[7]*Department of Life Sciences, School of Science, University of Management and Technology (UMT), Lahore, Pakistan*
Correspondence to: Iftikhar Ahmad, Email: iftikhar0912@gmail.com

ABSTRACT

Introduction: The diagnosis of pregnancy from forensic bloodstains can be useful in cases of infanticide, criminal abortions and missing person identification.

Objective: This research illustrated the use of a rapid, precise, and tremendously responsive enzyme immunoassay kit designed for medical usage, which is put to good use in our lab for qualitative HCG detection in blood stains and has proven to be a useful tool in forensic pregnancy identification.

Methods: HCG concentrations had previously been generally known, and total eighty whole blood samples were taken: forty expectant females (every single one between months one to six of pregnancy), twenty healthy young males, and twenty postmenopausal females with good health ration has been cleared in all data.

Results: Enzyme immunoassay is a useful forensic technique for detecting human chorionic gonadotropin hormone in pregnant women**.** In the forty sample batch that was dated for six months, 37 samples (92.5%) yielded positive results, 38 of which (95%) yielded good outcomes in the qualitative analysis, most of them within the sensitivities boundary. The samples that were diluted to 1/100 and 1/200 and kept at room temperature for one week and six months period, respectively, produced just two (5.0%) and five (12.5%) successful results and the samples showed negative results when diluted to 1/200 and stored for six months.

Practical implication This paper reports on human chorionic gonadotropin (HCG) detection in bloodstains based on enzyme immunoassay.

Conclusion: Enzyme immunoassay has proven to be a suitable method intended for detecting an hCG hormone in blood stains, allowing for the qualitative assessment of hCG, and making it particularly interesting for use in forensic science applications.

Keywords: Enzyme immunoassay, human chorionic gonadotropin (hCG), pregnancy, bloodstain

INTRODUCTION

The diagnosis of pregnancy from forensic bloodstains can be useful in cases of infanticide, criminal abortions and missing person identification [1]. However, this task is challenged by the usually small amounts of potentially degraded biological material found at crime scenes that often is of unknown age [2]. Pregnancy diagnostics from bloodstains have been demonstrated via immune detection of pregnancy-specific proteins [3], but the sensitivities achievable by this approach limit its application in a forensic context where often minute dried bloodstains have to be investigated [4]. Consequently, there is a need for more sensitive techniques to detect pregnancy-specific biomarkers from small bloodstains found at crime scenes [5].

A commercial fast enzyme immunoassay was employed to identify the hCG -subunit with good sensitivity and specificity [6]. This is highly relevant for forensic applications for pregnancy identification. When various bloodstains are seen in a forensic investigation it is possible to distinguish those from an expectant female from others [7, 8]. Total estriol, progesterone, and/or human placental lactogen (hPL), as well as human chorionic gonadotropin (hCG), may be present in these stains and evaluated using immunoassay techniques [9].

In addition, Reverse transcription polymerase chain reaction (RT-PCR) diagnostic can be used to detect hCG mRNA transcripts from minuscule bloodstains, involving whole blood, ethidium bromide staining, and agarose gel electrophoresis [10,11]. Forensic pregnancy identification utilizing bloodstains may be employed in situations of criminal abortions and infanticide [12]. Although it is impossible to assess hCG mRNA during the entirety of a healthy pregnancy, blood stains in a forensic environment can be used to determine a woman's gestational age by combining the study of pregnancy-specific human placental lactogen and hCG mRNA biomarkers [13].

This research illustrated the use of a rapid, precise, and tremendously responsive enzyme immunoassay kit designed for medical usage, which is put to good use in our lab for qualitative HCG detection in blood stains and has proven to be a useful tool in forensic pregnancy identification.

MATERIAL AND METHODS

Eighty samples of whole blood were drawn from the femoral vein of subjects whose hCG concentrations were previously disclosed to the general public and forty expectant females (every single one between months one to six of pregnancy), twenty healthy young males, and twenty postmenopausal females with good health.

Bloodstain HCG Determination Using a Qualitative Enzyme Immunoassay: On a commercial test pack HCG serum kit, the eighty bloodstain samples were examined (Abbott Laboratories). An enzyme sandwich immunoassay is used in the kit. For 30 to 60 seconds, 300 µL extracts from the eighty bloodstains were mixed with goat anti-human alpha-hCG alkaline phosphatase conjugation at room temperature and sodium chloride solution was used to wash the mixture. After a 2-min reaction period, colour development began following the addition of three drops of the conjugated enzyme's substrate, chromogenic reagent (registered trademark, Abbott Laboratories).

Using a Quantitative Enzyme Immunoassay to Determine HCG in Bloodstains: For every one of the eighty whole human blood samples used to create the bloodstains, thirty bloodstains and eighty plasmas underwent quantitative analysis. A commercial Abbott Laboratories βHCG 15/15 kit, or a firm segment sandwich immunoassay, was employed for measurement. Observantly

following the directions in the kit's manual, quantitative plasma determination was carried out. To determine the amount of hCG in the forty extracts from pregnancy blood splatters and controls had been added to 100 µL of the extract. Additionally, horseradish peroxidase conjugate with goat anti-human βHCG and goat anti-human βHCG immobilized on polystyrene beads had also been added. Washing the beads helped to get rid of any unbound elements. The o-phenylenediamine (OPD) substrate solution, comprising hydrogen peroxide, was then incubated with the beads. The intensity of colour was assessed using a spectrophotometer calibrated at 490 nm.

RNA isolation, cDNA synthesis and quantitative RT-PCR: Total RNA isolation was performed among participants with the RNeasy plus Mini kit according to the manufacturer's instructions with some modifications. These included the incubation of the cotton swabs and the pieces of filter paper in RLT buffer for 1 h at 4°C followed by acid phenol/chloroform extraction before the loading of whole blood lysate onto the RNeasy spin columns. This purification step significantly increased RNA yield and purity. RNA extracts were treated with DNase using the Turbo DNA-free kit by Ambion (Applied Biosystems) to remove genomic DNA contamination. The quality and quantity of RNA were assessed with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). For the RNA samples designated for the pregnancy specificity determination, cDNA synthesis was carried out with the Transcriptor First Strand cDNA Synthesis kit. RNA extracts designated for the gestation time applicability and sensitivity studies were reverse transcribed with the Revertaid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) with a combination of random hexamer and oligo(dT)18 primers.

Real-time PCR reactions were performed in 15 µl reaction volumes, in duplicate, on an ABI 7300 PCR machine (Applied Biosystems): initial denaturation at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 s, and final annealing and elongation at 60°C for 60 s. After that, the SYBR Green PCR kit was used for the testing of pregnancy specificity and sensitivity detection.

RESULTS

Enzyme immunoassay is a useful forensic technique for detecting human chorionic gonadotropin hormone in pregnant women. The findings from the enzyme immunoassay which is a useful method used to quantify HCG in eighty plasma samples and bloodstain isolate from forty expectant females are displayed in Table 1. At one week and six months, these latter ones' ageing stages were assessed. The range of hCG activity of forty blood stains and eighty plasma samples from the pregnant group is also displayed in Table 1.

The outcomes of the qualitative hCG analysis of the eighty human plasma samples are shown in figure 1, and they were significant for the entire pregnant category while being inconclusive including all control, which included the twenty older males and twenty postmenopausal female specimens**.** To ascertain the impact of these variables on hormone stability, the stability of the HCG marker in blood samples from pregnant women was examined using a qualitative immunoassay at various temperatures and for varying amounts of time (up to six months).

The qualitative findings are listed in Table 2. The forty samples in each of the 4 sets of stains under investigation exhibited positive results of comparable strength in the bloodstain isolate held (1 week and 3 months) at 56^{0}C. The final two samples that had been retained at room temperature for six months had an encouraging outcome in favour of the forty samples that had been kept there. Of these, 36 (90%) had HCG activity levels that were over the specificity threshold (35 mIU/mL hCG, per the test pack manual), which was met by the final two samples. In the forty sample batch that was dated for six months, 37 samples (92.5%) yielded positive results, 38 of which (95%) yielded good outcomes in the qualitative analysis, most of them within the sensitivities boundary.

Table 1**:** Outcomes from the measurement of hCG in the plasma of forty expectant women, twenty male, twenty postmenopausal women, and isolates of stains of forty expecting women.

Range of Activity in mIU/mL										
	0-1000	1000-2000	2000-3000	3000-6000	6000-12000	12000-20000	20000-30000	30000-50000	50000-100000	Above 100000
40 Plasma pregnancy		2		4			1	19	2	2
20 Plasma postmenopausal females	15									
20 Plasma males	15									
40 Extracts bloodstains (1-Week Period)	2	2	2			13	8	2	1	
40 Extracts –bloodstains (6 months old)	27	2			1					

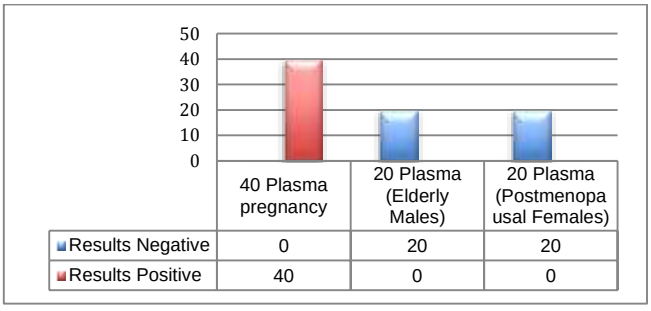

Figure 1: Forty blood samples' responses to an enzyme-immunoassay for a qualitative HCG assessment.

Table 2: Analysis of old bloodstains from forty Expectant Female's reactions

Time	Room Temperature	56^{0}C
1 week period	40	40
3-month period	40	40
6-months periods	40[a]	37[b]

The samples, which were diluted to 1/100 and held for a week, three months, and 56^{0}C, respectively, produced 34 positives out of the 40 specimens in each batch (85.0%), as shown in Tables 3 and 4. Negative results were obtained with bloodstain extracts that had hCG concentration ≤ 15,000 mIU/mL. In contrast, among isolates at 1/100 diluted using bloodstains accumulate for 6 months period at room temperature, a blood sample with only an hCG concentration of 130-145 mIU/mL, which also exceeds the maximum recognition rate of the assay, yielded only two successful outcomes (5.0%). In the qualitative HCG determination, all of the samples from the group that had been aged for 6 months periods at 56^{0}C and 1/100 dilution produced negative results.

The findings of the laboratory tests at a dilution of 1/200 were as follows: five samples (12.5%) were screened positive at equally room temperature and 56^{0}C lots using a one-week storage phase. For the lots that matured for three months at both temperatures, there were four favourable results.

Table 3: Analysis of old bloodstains from forty expectant women's reactions

Extract Dilutions = 1/100	Room Temperature	56°C
1 week period	34	34
3-month period	34	34
6-months periods	2[a]	-

Table 4: Reaction involving aged bloodstains of forty expecting women.

Extract Dilutions = 1/200	Room Temperature	56°C
1 week period	5	5
3-month period	4	4
6-months periods	-	-

DISCUSSION

Significant advancements in hCG identification techniques with improved accuracy and specificity in blood cultures have been made over the past decade [14]. According to previous studies, the qualitative enzyme immunoassay is legitimately regarded as being adequately sufficiently precise for the diagnosis of hCG action in bloodstains of expectant females, in addition to the reality that it has a high level of accuracy [15,16].In the Abbott manual, 2-ng/mL hCG is specified as the sensitivity threshold for the HCG serum test pack. The actual sensitivity limit, however, is closer to 0.8 ng/ml HCG, according to our laboratory measurements. As a result, this test can identify HCG activity. Whole blood is needed in incredibly small volumes. Taking into account that after 40 days of pregnancy, pregnant women's typical plasma levels of HCG range between 80 ng/mL. For this test, a viable bloodstain would only need to be created using 20 µL of whole blood. The previous research, the results of the qualitative hCG analysis of the 93 human plasma samples were significant for the entire pregnant category but inconclusive for all control, which included the 30 older males and 45 postmenopausal female specimens [17,18]. The study was consistent with our study's findings which showed the qualitative hCG analysis of the eighty human plasma samples.

Similar to Vallejo's (1990) work, the samples that were diluted to 1/100 and 1/200 and kept at room temperature for six months and one week, respectively, produced just two (5.0%) and five (12.5%) successful results and the samples showed negative results when diluted to 1/200 and stored for six months [19,20].Hormone breakdown does not appear to be taking place in the stains that have been aged for up to three months because HCG activity does not seem to be altered. Contrarily, a significant decline in hormone activity is seen after six months of ageing. Within the experimental range, the storage temperature had essentially little impact on the stability of HCG in cotton cloth bloodstains.

Our data demonstrate that RT-PCR allows the detection of pregnancy-specific hPL and βhCG mRNA transcripts from small blood stains. The hPL-specific RT-PCR assay applied was sensitive enough to reliably detect hPL transcripts throughout the pregnancy and from as little as 0.25 cm2 of the dried bloodstain. Furthermore, the hPL transcript was shown to be stable enough for successful amplification from the dried blood of up to 2 months of age, expectedly older. Therefore, we propose hPL-specific RTPCR as a new molecular tool for pregnancy diagnostics from blood stains found at crime scenes. Moreover, our results indicate that a combined analysis of hPL and βhCG mRNA markers may allow estimating the gestational age of a woman from her blood stain left behind, potentially adding to the value of forensic pregnancy diagnosis for crime scene investigations.

CONCLUSIONS

Enzyme immunoassay has proven to be a suitable method intended for detecting hCG hormone in blood stains, allowing for the qualitative assessment of hCG. It is a quick, sensitive, and highly specific method that is notably useful for bloodstain pregnancy diagnosis, making it particularly interesting for use in forensic science applications.

REFERENCES

1. Kayser, M. (2009). Forensic pregnancy testing: a special case in molecular diagnostics. *Expert Review of Molecular Diagnostics*, *9*(2), 105-107.
2. Kayser, M., & De Knijff, P. (2011). Improving human forensics through advances in genetics, genomics and molecular biology. *Nature Reviews Genetics*, *12*(3), 179-192.
3. Vergote G, Heyndrickx B, Paredes M, Daels J (1991) Forensic determination of pregnancy hormones in human bloodstains. J Forensic Sci Soc 31:409–419.
4. Vallejo G (1990) Human chorionic gonadotropin detection by means of enzyme immunoassay: a useful method in forensic pregnancy diagnosis in bloodstains. J Forensic Sci 35:293–300.
5. Ng EK, Tsui NBY, Lau TK, Leung TN et al (2003) From the Cover: mRNA of placental origin is readily detectable in maternal plasma. Proc Natl Acad Sci U S A 100:4748–4753.
6. Lin, Z., Wang, X., Li, Z. J., Ren, S. Q., Chen, G. N., Ying, X. T., & Lin, J. M. (2008). Development of a sensitive, rapid, biotin–streptavidin based chemiluminescent enzyme immunoassay for human thyroid stimulating hormone. *Talanta*, *75*(4), 965-972.
7. Silva, S. S., Lopes, C., Teixeira, A. L., De Sousa, M. C., & Medeiros, R. J. F. S. I. G. (2015). Forensic miRNA: potential biomarker for body fluids?. *Forensic Science International: Genetics*, *14*, 1-10.
8. Sijen, T. (2015). Molecular approaches for forensic cell type identification: on mRNA, miRNA, DNA methylation and microbial markers. *Forensic Science International: Genetics*, *18*, 21-32.
9. Khan, S., Katabuchi, H., Araki, M., Nishimura, R., & Okamura, H. (2000). Human villous macrophage-conditioned media enhance human trophoblast growth and differentiation in vitro. *Biology of reproduction*, *62*(4), 1075-1083.
10. Hoon, D. S., Sarantou, T., Doi, F., Chi, D. D., Kuo, C., Conrad, A. J., ... & Guiliano, A. (1996). Detection of metastatic breast cancer by β-hCG polymerase chain reaction. *International journal of cancer*, *69*(5), 369-374.
11. Tsui NBY, Chim SSC, Chiu RWK et al (2004) Systematic microarray based identification of placental mRNA in maternal plasma: towards non-invasive prenatal gene expression profiling. J Med Genet 41:461–467
12. Gauvin, J., Zubakov, D., van Rhee-Binkhorst, J., Kloosterman, A., Steegers, E., & Kayser, M. (2010). Forensic pregnancy diagnostics with placental mRNA markers. *International journal of legal medicine*, *124*(1), 13-17.
13. Kayser, M. (2009). Forensic pregnancy testing: a special case in molecular diagnostics. *Expert Review of Molecular Diagnostics*, *9*(2), 105-107.
14. Sturgeon, C. M., Berger, P., Bidart, J. M., Birken, S., Burns, C., Norman, R. J., ... & IFCC Working Group on hCG. (2009). Differences in recognition of the 1st WHO international reference reagents for hCG-related isoforms by diagnostic immunoassays for human chorionic gonadotropin. *Clinical chemistry*, *55*(8), 1484-1491.
15. Liu, J., Mosavati, B., Oleinikov, A. V., & Du, E. (2019). Biosensors for detection of human placental pathologies: a review of emerging technologies and current trends. *Translational Research*, *213*, 23-49.
16. Burd, E. M. (2010). Validation of laboratory-developed molecular assays for infectious diseases. *Clinical microbiology reviews*, *23*(3), 550-576.
17. González-Martínez, M. Á., Puchades, R., & Maquieira, Á. (2018). Immunoanalytical technique: enzyme-linked immunosorbent assay (ELISA). In *Modern Techniques for Food Authentication* (pp. 617-657). Academic Press.
18. Butler, S. A., Khanlian, S. A., & Cole, L. A. (2001). Detection of early pregnancy forms of human chorionic gonadotropin by home pregnancy test devices. *Clinical chemistry*, *47*(12), 2131-2136.
19. Vallejo, G. (1990). Human chorionic gonadotropin detection by means of enzyme immunoassay: a useful method in forensic pregnancy diagnosis in bloodstains. *Journal of Forensic Science*, *35*(2), 293-300.
20. Moyes, A. J., Khambata, R. S., Villar, I., Bubb, K. J., Baliga, R. S., Lumsden, N. G., ... & Hobbs, A. J. (2014). Endothelial C-type natriuretic peptide maintains vascular homeostasis. *The Journal of clinical investigation*, *124*(9), 4039-4051.