EXPERIMENTAL STUDY FOR HUMAN SKIN IDENTIFICATION USING A SPECIFIC GENE MARKER AT DIFFERENT STORAGE DURATIONS

Heba A. Abd El Razik *;Nadia A. Kotb*; Abla A. Ali*; Amany A. Bayoumi*, and Laila A. Rashed.**

*Forensic Medicine & Clinical Toxicology, Faculty of Medicine, Cairo University.

**Biochemistry department, Faculty of medicine, Cairo University.

ABSTRACT

The detection of human skin through mRNA-based profiling is very useful for forensic investigations. The aim of this study was definitive identification of human skin at different time intervals using an mRNA marker late cornified envelope gene 1C. Ten middle aged healthy volunteers of both sexes were recruited for this study. Skin samples controlled with blood samples were taken from the candidates to test for the presence of our targeted mRNA marker. Samples were kept at dry dark conditions to be tested at different time intervals (24 hours, one week, three weeks and four weeks) for detection and relative quantification of the targeted marker by RT PCR. This study verified the high specificity and sensitivity of mRNA marker in the skin at different storage times up to three weeks under the study conditions.

INTRODUCTION

At present, there is a strong trend in forensic genetics for the development of alternative approaches for identifying the cellular origin of biological stains from crime scene samples. Many laboratories are focused on the identification of tissue-specific messenger RNA (mRNA) markers for the development of an assay to detect forensically relevant human body fluids **(Visser et al., 2011).**

The structure of RNA is similar to DNA, its function is time limited resulting in relatively short half-lives. One central assignment of RNA is the conversion of genetic information into proteins and the regulation of this process. The two strands of DNA are separated and RNA is synthesized by RNA polymerases complementary to the coding strand **(Vennemann and Koppelkamm, 2010).**

Within forensic genetics, messenger RNAs (mRNAs) had increasingly gained popularity regarding their potential to distinguish between human body fluids and other forensically relevant tissues. Alternative methods for cell-typing include tissue-specific miRNAs, DNA methylation and microbial markers **(Goray et al., 2010).** miRNAs are small (20–24 nucleotides) regulatory RNAs which are strongly associated with members of a class of proteins called Argonautes , which makes them very stable and advantageous when dealing with degraded forensic samples . Also epigenetic DNA methylation markers had been described that can differentiate between some tissue types. Both miRNA and DNA methylation markers seem promising, but still in its infancy as for instance more markers are needed to discriminate the forensic range of body fluids. **(Frumkin et al., 2010).**

Ribonucleic acid (RNA) is known

for its instability because of the present ribonucleases. However, recent studies had reported that RNA isolated from some forensic stains showed unexpectedly high stability. Using gene expression on aged blood and saliva stains, scientists identified blood and saliva specific messenger RNA (mRNA) markers that showed stable expression patterns in stains up to 180 days of storage, and some of these markers showed successful and reliable amplification in much older stains, such as 16 years old blood stains **(Zubakov et al., 2008).**

Diffrent studies report the development of molecular methodologies for the specific identification of skin in forensic samples. They utilized immunological staining and quantitative PCR mRNA profiling assays to evaluate the expression of various **cytokeratins** in order to distinguish the presence of mucosal or epidermal epithelial cell, demonstrating the ability to identify mucosal epithelial cells using Ck4 (KRT4) expression and epidermal epithelial cells using Ck10 (KRT10) expression. However the tissue specificity of these markers is not absolute, since Ck10 expression was observed in some vaginal samples **(Schulz et al., 2010).**

Another skin-specific markers were identified including late cornified envelope genes 1C, interleukin 1 family member 7 (IL1F7) and chemokine (c-c motif) ligand 27.There were recently detected **(Hanson et al., 2012).**

PARTICIPANTS & METHODS

I. Participants selection:

- **Inclusion criteria:** 10 middle aged healthy volunteers from both sexes (health staff at Kasr Al-Aini hospital) between 25 & 40 years**.**
- **Exclusion criteria:** people with skin diseases.

II. Design of work:

This study was divided into two parts:

A- pilot study: aim of pilot study was
 – Choice of method of sampling. Skin swab or touch.

B- Prediction of specificity of marker for skin. Fresh blood samples were taken as control samples and examined for detection of skin marker.

C- Detection of degradation of targeted marker in swab samples after different storage durations in dark dry environment**:**

Swab samples taken after informed consent from each participant were divided into 4 parts:

1) **The first was** examined after 24 hours for detection of the targeted mRNA marker.
2) **The second was** examined after one week.
3) **The third was** examined after three weeks.
4) **The fourth** was examined after four weeks**.**

D- Prediction of chosen skin marker in both sexes after different storage periods in dark dry environment.

Materials & Reagents:

1. Sterile cotton swabs.
2. Cartoon box with a cover to preserve the samples.
3. **Reagents for RNA extraction and transcription:**
   a) SV total RNA extraction kit.
   b) Di ethyl pilocarpine -treated water.
   c) Superscript II reverse transcriptase and buffer, oligodeoxythymidine (dT) 12-18 or random hexamer primer; dNTP.
   d) RNase inhibitor.

4. **Reagents for qPCR: (Quiagen)**

(A) SYBR Green I qPCR mixture (this mixture includes dNTP, Taq DNA polymerase, reaction buffer and the fluorescent dyes gene specific primers).

(B) Plastic white strip qPCR tube (200 µl volume) with optical clear strip caps or white 96-well qPCR plates with optical clear seal sheets and press applicator.

(C) Housekeeping gene.

5. Human Placental Ribonuclease Inhibitor for inhibition of RNase activity.
6. First strand buffer
7. Deoxynucleotide triphosphates (dNTPs) dATP, dTTP, dGTP, dCTP are used for extension of primers.
8. Random hexamers: primers for reverse transcription of RNA (Stratagene).
9. DEPC.

Procedure

The current work was carried out according to: (promega, Madison, USA)(2013)

Collection of samples.

Blood samples: 5 fresh blood samples (200 micro litres) were collected by thumb prick method to be used as control samples.

Skin samples: was collected using sterile dry cotton swabs.

Isolation of RNA from the skin:

The isolation of intact RNA requires four essential steps:

1- Effective disruption of cells

2-Denaturation of nucleoprotein complexes.

3-Inactivation of endogenous ribonuclease (RNase) activity.

4-Removal of contaminating DNA and proteins.

The reverse transcription of extracted RNA was done

Three µl of random primers were added to the 10 µl of RNA which was denatured for 5 minutes at 65°C in the thermal cycler.

Quantitative PCR.

The gene-specific forward and reverse primer pair was normalized. Each primer (forward and reverse) concentration in the mixture was 5 pmol/ µl.

Data analysis.

RESULTS

Pilot study results:

Both skin swab and touch have the same results as a method of sampling.

late cornified envelope gene 1C results

1-The targeted marker could not be detected in blood samples taken as control.

2-The presentation of late cornified envelope gene 1C at different time intervals (24 hours, one week, three weeks and four weeks) was shown in table (1) where the highest mean value was after 24 hours (11.90±2.42) and the lowest mean value was after three weeks (7.56±2.56) as illustrated in **Figure (1).** No marker could be detected at four weeks. There are significant differences between gene expressions at different time intervals (P value =0,003).

3- Gender difference: there was no significant difference regarding the marker values at different time intervals when comparing between males and females skin samples as shown in table(2).

Table (1): Description of late cornified envelope gene 1C values in all swab samples at different time intervals

Duration	Mean	Standard Deviation
24 hours	11.90	2.42±
One week	8.91	2.61±
Three weeks	7.56	2.56±
Four weeks	0	0
P value	0,003	

P value ‹0.05 is significant

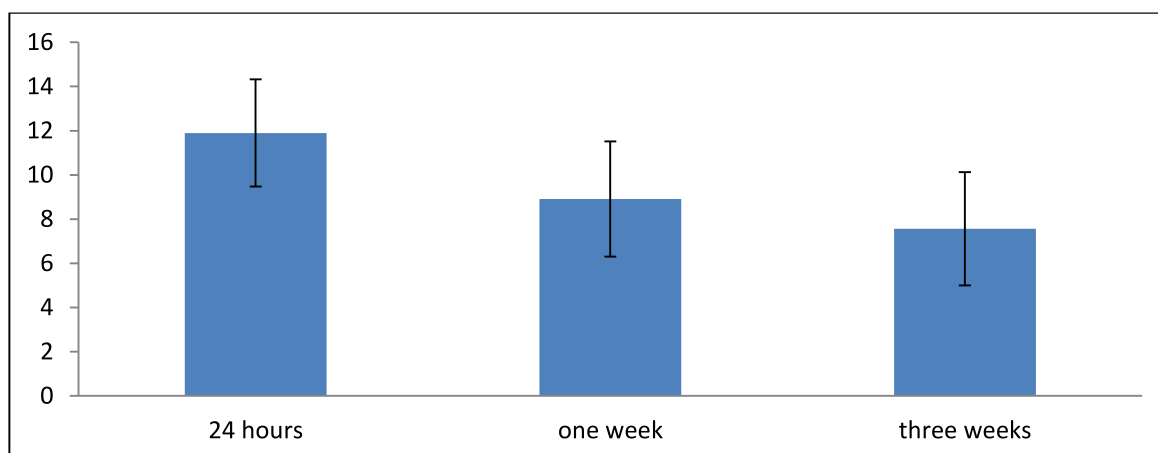

Figure (1) Description of late cornified envelope gene 1C values in all swab samples at different time intervals

Table (2): Comparison between males and females regarding late cornified envelope gene 1C values at different times

Duration	Sex				P value
	Male(no.5)		Female(no.5)		
	Mean	Standard Deviation	Mean	Standard Deviation	
24 hours	12.10	2.06±	11.69	2.84±	0.713
One week	9.00	2.69±	8.81	2.67±	0.874
Three weeks	6.98	2.61±	8.14	2.50±	0.322
Four weeks	0	0	0	0	0

P value ‹0.05 is significant.

DISCUSSION

Dedicated whole-genome expression array analysis in samples from forensically relevant body fluids that were stored for various time intervals has previously shown to deliver stable mRNA markers useful for forensic tissue identification (**Zubakov et al., 2009**).

In the current study, control blood samples were used for assessment of the relative gene expression in blood in comparison to that in skin swabs as regard the targeted marker. The results showed that they were significantly found in skin swabs only, while not even traces were detected in blood samples. These results prove that blood, as an example of biological samples is free of the targeted skin markers. Therefore it may be concluded that the used skin markers are

specific to skin tissues.

This result coincides with those of **Hanson et al., (2011)** who stated that the expression of potential skin candidates identified through literature searches and whole transcriptome profiling were evaluated in all forensically relevant biological fluids and tissues (skin, blood, semen, saliva, vaginal secretions and menstrual blood). True RNA species were identified and demonstrated by processing the same samples without reverse transcriptase as well as DNA samples. From an evaluation of over 100 candidates, late cornified envelope gene 1C demonstrated a high degree of specificity for skin.

Hanson et al., (2012) stated that additional specificity testing was performed for each of the skin markers using a more optimal and realistic input amount. The majority of all skin samples were detected at this input whereas no detection was observed in any of the other body fluid samples. This study confirmed the high degree of skin specificity of each of the candidates. In addition to body fluid samples, the expression of each candidate was evaluated in a panel of twenty human tissues. Significantly, no cross-reactivity was observed for these tissues.

The work of **Gomes et al., (2013)** also supported the high specificity of both the saliva- and skin-specific mRNA markers and suggested the possibility to easily discriminate between skin and saliva in one reaction using the developed mRNA pentaplex system. The high sensitivity shown, in particular of the skin gene markers, also supports the usefulness of these mRNA markers in the identification of contact trace evidence.

In the present study, the mean values of targeted marker at the studied time intervals showed that the highest mean value was after 24 hours (11.90±2.42) while the lowest mean value was after three weeks (7.56±2.56). **Our current study showed that the expression of the targeted gene** disappeared at the fourth week from time of the collection**.**

This is in accordance with **Visser et al., (2011)** who showed that, some skin markers tend towards decreasing detection levels with increasing storage time.

Regarding gender influence in the current study, the obtained results showed no significant difference between both sexes as regard the expression and quantification of the marker in the skin swab samples.

Similar results were reported by **Visser et al., (2011)** who collected palmer skin (n=15) samples from healthy volunteers (eight men, seven women, ranging from 22 to 42 years of age) under informed consent and there was no significant difference detected regarding sex.

CONCLUSION

The reverse transcription polymerase chain reaction (RT -PCR) is a useful technique to analyze mRNA expression derived from the skin. It is highly sensitive and allows a quantification of rare transcripts and small changes in gene expression. Besides, it is easy to perform, provide the necessary exactness and produce reliable as well as rapid quantification results.

The present work verified that the late cornified envelope gene 1C**, the selected gene for skin using quantitative RT-PCR,** are highly specific and sensitive to skin tissues as

it was not found in the control blood samples.

late cornified envelope gene 1C can be detected up to three weeks of storage.

There is significant decrease of gene expressions by increasing time.

There is no significant difference between both sexes as regards the targeted marker values at the studied **different time intervals** (24 hours, one week, three weeks and four weeks).

RECOMMENDATIONS

Although it is evident that qRT-PCR assay has become a useful and important technology in the clinical diagnostic laboratory, it must be used appropriately and it is essential to be aware of its limitations if it is to fulfil its potential.

Further work is required to seek new candidates and include more markers for the identification of skin. Any additional markers that may be discovered in the future could be added to the analysis where additional and more sensitive markers would contribute to the more reliable identification of skin.

Further studies of skin markers are needed at different sites of skin (scalp, limbs, trunk.....) which may help to better reconstruction of crime events.

REFERENCES

Frumkin,D.; Wasserstrom,A.; Budowle,B. and Davidson,A.(2010): DNA methylation-based forensic tissue identification, Forensic Sci. Int. Genet. volume 7(1):136-142.

Gomes, I.; Strohbucker, B.; Rothschild, M.A. and Schneider, P.M. (2013): Evaluation of mRNA specific markers using a pentaplex system for the identification of skin and saliva from contact trace evidence. Forensic Science International: Genetics Supplement Series ,4 : e180–e181.

Goray, M.; Eken, E.; Mitchell, R.J. and van Oorschot, R.A. (2010) : Secondary DNA transfer of biological substances under varying test conditions. Forensic Sci. Int. Genet, 4: 62–67.

Hanson, E.; Haas, C.; Jucker, R. and Ballantyne, J. (2011): Identification of skin in touch/contact forensic samples by messenger RNA profiling. Forensic Science International: Genetics Supplement Series, 3 : e305–e306.

Hanson, E.; Haas, C.; Jucker, R. and Ballantyne, J.(2012): Specific and sensitive mRNA biomarkers for the identification of skin in 'touch DNA' evidence. Forensic Science International: Genetics, 6 : 548–558.

Schulz, M.M. ; Buschner, M.G.; Leidig, R.; Wehner, H.D. ; Fritz, P. ; Habig, K.; Bonin, M.; Schutz, M.; Shiozawa, T. and Wehner, F. (2010) : A new approach to the investigation of sexual offenses-cytoskeleton analysis reveals the origin of cells found on forensic swabs. J. Forensic Sci, 55: 492–498.

Vennemann, M. and Koppelkamm, A. (2010): mRNA profiling in forensic genetics I: Possibilities and limitations. Forensic Science International, 203: 71–75.

Visser, M.; Zubakov, D.; Ballantyne, K.N. and Kayser, M. (2011) : mRNA-based skin identification for forensic applications. Int. J. Legal Med., 125: 253–263.

Zubakov, D.; Hanekamp, E.; Kokshoorn, M.; van Ijcken, W. and Kayser, M. (2008): Stable RNA markers for identification of blood and saliva stains revealed from whole genome expression analysis of time-wise degraded samples.Int. J. Legal Med. 122 :135–142.

Zubakov, D.; Kokshoorn, M.; Kloosterman, A. et al. (2009): New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains. Int. J. Legal Med.,123: 71

دراسة تجريبيه للاستعراف على الجلد الادمي باستخدام الدلالة الجينية الخاصة في فترات تخزينيه مختلفة

هبه عبده عبد الرازق* ، أد. ناديه عبد المنعم قطب* ، أد. عبلة عبد الرحمن علي* ، د. اماني عبد الفتاح بيومي* ، أد. ليلى أحمد راشد.**

* قسم الطب الشرعي والسموم الإكلينيكية، كلية طب قصر العيني، جامعة القاهرة، ** قسم الكيمياء الحيوية، كلية طب قصر العيني، جامعة القاهرة.

الملخص العربي

لقد أتاحت التقنيات الحديثة الكشف عن الحمض النووي الريبوزي الرسول للاستدلال على مختلف أنواع الخلايا البشرية المتواجدة بكميات صغيرة في الأنسجة أو في سوائل الجسم المختلفة، مثل الدم، السائل المنوي، اللعاب، إفراز الحيض والإفرازات المهبلية. كما أمكن أيضا الكشف عن خلايا الجلد الآدمي وغيرها باستخدام هذه التقنيات الحديثة.

ويبقي تفاعل البوليمراز المتسلسل أكثر التقنيات حساسية للكشف عن مستهدفات الحمض النووي الريبوزي الرسول وأصبح استخدامه من أكثر الطرق المفضلة للتقدير الكمي الثابت للحمض النووي الريبوزي الرسول.

وقد اجري هذا البحث على عشره من المتطوعين من الجنسين بعمل مسحات من الجلد وحفظها في مكان جاف مظلم لفترات زمنيه متزايدة. ولإثبات حساسيه ودقه الكشف عن الحمض النووي الريبوزي الرسول في الجلد تم استخدام عينات من دم المتطوعين. **وقد ثبت** خلوها جميعا من الحمض النووي الريبوزي الرسول المميز للجلد.

كما تبين انه يمكن الكشف عنه حتى بعد مرور ثلاث اسابيع من التخزين واختفاؤه في الاسبوع الرابع ووجد ان هناك فروق ذات دلاله إحصائية بزيادة وقت التخزين. وبدراسة تأثير الجنس على الحمض الريبوزي الرسول للجلد **تبين انه** لا توجد فروق ذات دلاله إحصائية بين الذكور والاناث في العينات التي شملتها الدراسة.