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Forensic molecular study using ITS region as a clue and possible fungal outbreak pre-warning indicator

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Abstract : The PCR primers ITS1 and ITS4 are commonly used to gain wide acceptance for work with fungal Internal Transcribe Sequences (ITS) that amplify the highly variable ITS1 and ITS2 regions. ITS region was evaluated for mixed human blood sample differentiation. Some of these fungal pathogens can affect both human and animal and can contaminate the environment, or may establish new enzootic foci. Total of 70 blood samples were collected in the EDTA tube that include (20 healthy (non-symptomatic) human blood samples, 20 sheep blood samples, 20 chicken blood samples and 10 human blood of leukemia patients. Blood culture has showed no results even when Blood agar plates were incubated for a week. PCR result showed that mycosis in human leukemia patient 70% in comparison with 30% in non-symptomatic samples. Positive human blood can be differentiated in mixed samples using this primer pair. Chicken and sheep were found infected by 40% and 20% respectively. Fungal infection detection using ITS region can facilitate determination of geographical area and human involvement in some forensic cases where mixed blood samples are present. Determination of fungal strain aid in putting a cut edge in zoonotic infection outbreak and provide information in possible bio-terrorism attack origin.
Key words: Forensic microbiology, ITS region, Fungal infection.

Introduction:

Microbial forensics is a new branch of the forensics field focuses on the person or people who perpetrated a crime, and on the victims and their related microbiology. Microbial forensic analysis is became very important in the detection of reliable molecular variations among microbial strains and their use to infer the origin, relationships, or transmission route of a particular isolate[1]. One of the most important microbes are Fungi that are widely spread in the environment and cause many diseases for both human and animals. Some of them are zoonotic. Systemic infections occur when fungi get into the bloodstream and generally cause diseases that are more serious. Systemic fungal infections usually attack a person with a suppressed immune system, or by invasive fungus that is common in a certain geographic area. Genomics is increasing the power of microbial forensics through the design of gene-based diagnostic tests and directing analysis. The genomes of some of the important zoonotic pathogens (e.g. *Salmonella typhimurium* and *Escherichia coli* O157:H7) have been sequenced as what have been done with many important strains that might be used as biological agents threat (e.g. *B. anthracis*) [2,3].

Sequencing give an indication of the geographical area and source of infection in order to determine either the pathogen in a disease outbreak or poisoning bio-crime where a poisonous fungus is involved [4].

Fungal infection investigation is necessary in forensic cases that involve drugs and toxins analysis for both pre and postmortem cases since fungal metabolites can alter the drug chemical characteristics and make it undetectable or at least with false dose analysis results [5].

Very few studies manipulated fungal infection and toxins as a non-human DNA in forensic studies. Therefore, the aim of the present study was to evaluate a routine, high throughput, fast and sensitive molecular analysis in investigating fungal systemic infection using the PCR primers ITS1 and ITS4 that aid in human blood differentiation in some forensic cases. Add to that, to evaluate fungal infection load in animals that might act as source of an outbreak where a swift action is urgent to limit the scale of any outbreak and reassure the public when events are under control.

Materials and Methods:

Sample collection

Total of 70 blood samples were collected in the EDTA tube that include (20 healthy (non-symptomatic) human blood samples, 20 sheep blood samples, 20 chicken blood samples and 10 human blood of leukemia patients). Pathogenic *Aspergillus niger* donated from the DNA research center, University of Babylon, was used as positive control.

Blood culture

Blood culture was done in triplicates and as following. About 0.5ml of blood were inoculated in 10ml Brain heart infusion broth (HIMEDIA) tubes then incubated at 37ºC for 24 hours. Thereafter, 0.5ml of culture broth was spread on Blood agar (HIMEDIA) plates and incubated at 37ºC for a week.

DNA extraction from blood

Genomic DNA was extracted from the whole blood (for individual and mixed blood samples) using Reagent genomic DNA Kit (Geneaid, Twain) according to the manufacturer instruction with modification by further protein cleanup using phenol/Chloroform (1:1).

DNA Extraction from fungi

Genomic DNA was extracted from *Aspergillus niger* isolated from phlegm of a patient, using Fungal/bacterial DNA Extraction Kit (ZYMO research, USA) and according to the manufacturer instructions.

Polymerase Chain Reaction (PCR)

PCR reactions were conducted using Thermal Cycler (Cleaver scientific, UK). The PCR (recipe and cycle) conditions were optimized in order to get the best possible yield utilizing about 40ng of the extracted DNA.

PCR Amplification of the ITS region

About 500-800bp of the internal transcribed spacer (ITS) region [6], of fungal rRNA genes was amplified using the fungal universal primers ITS1 as a forward primer and ITS4 as a reverse primer (Table 1) and as described by . The optimized recipe in Table (2) was followed and the PCR reaction was conducted using thermocycler (cleaver scientific, UK). The cycle conditions were as following, pre-denaturation at 95ºC for 1 min.; 35 cycles of (denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1 min); final extension at 72ºC for 10 min followed by holding at 4ºC. A 5µl of the amplicon was electrophoresed through 1% agarose gel then examined and photographed using Bio-documentation/ Quantum ST5 (VILBER, France).

Table (1): Primers used in this study [7].

Name	Sequence	Tm(ºC)
ITS1	5` TCCGTAGGTGAACCTGCGG 3`	61
ITS4	5` TCCTCCGCTTATTGATATGC 3`	55.3

Table (2): The optimized recipe components of a single 20µl PCR reaction.

Reagent	Volume (µl)	Final concentration
Master mix (*i*-Taq, Intron)		
Water (sterile, nuclease free)	11	
Primer ITS4 (10 mM)	2	1 mM
Primer ITS1 (10 mM)	2	1 mM
DNA template (20-40 ng/ml)	5	1-2 ng/ml
Total	20	

Results and Discussion:

Blood Culture

Blood culture has showed no results even when Blood agar plates were incubated for a week. This is expected since many fungal strains need weeks to appear in culture. Add to that, positive blood culture results were obtained in less than 50% of clinical cases [8,9].

PCR

PCR result shows that mycosis in human leukemia patient 70% in comparison with 30% in non-symptomatic samples which give an indication for high susceptibility of leukemic people for being infected due to immune suppression. Detection of fungus in 30% of apparently healthy people give an indication of a possible disease outbreak either from contact animals like chicken and sheep were 40% and 20% found infected respectively or other sources, especially with a high percentage of human positive results in comparison with animal results (Table 3) and (Figure 1).

With the PCR amplicon gel electrophoresis results (Figure 2), we have found that a (1:1) mixture of positive (H1) and negative (H2) human blood sample show a positive band (lane 4). This result indicates the presence of H1 blood in the mix and can be used as a supportive clue with involvement of H1 in the forensic case.

Table (3): PCR results of the tasted blood sample

Sample	Positive	Negative	Positive results (%)
Human	6	14	30
Human Leukaemia Patient	7	3	70
Chicken	8	12	40
Sheep	4	16	20

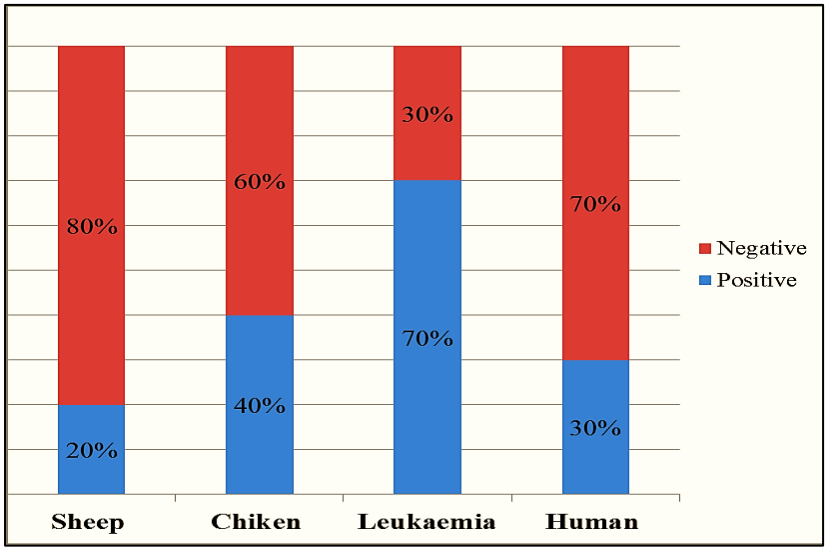

Figure (1): PCR results of the tested blood samples showing the mycosis percentage.

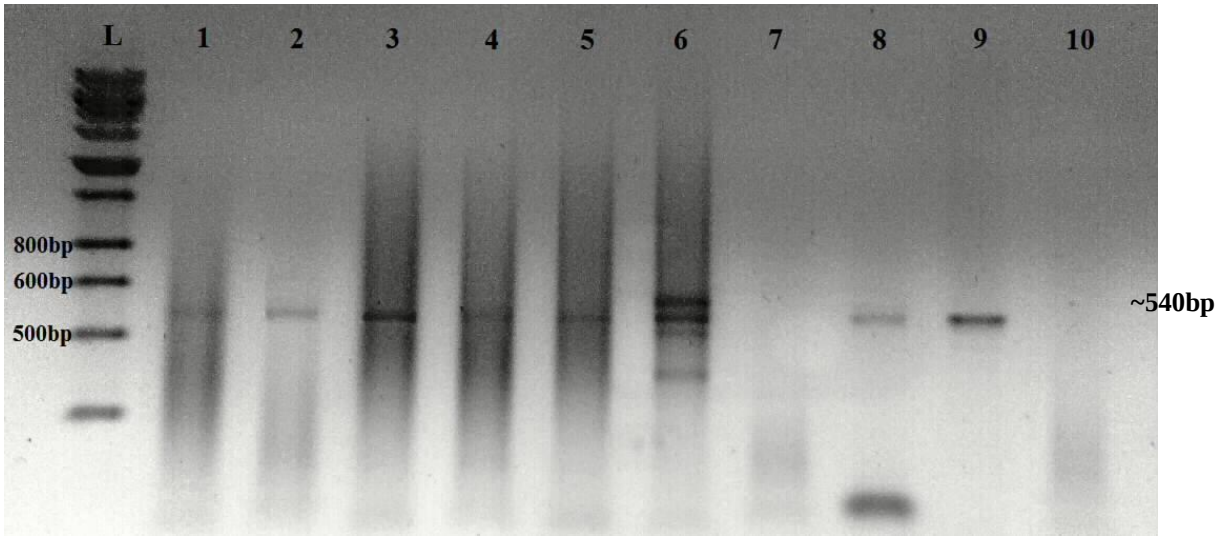

Figure (2): Agarose gel electrophoresis of PCR amplicon. L: DNA size marker. 1: YF (Positive Control), 2: HF3, 3: SH2, 4: H1+H2, 5: CH2, 6: CH3, 7: H2, 8: H3, 9: H1, 10 (negative control) Electrophoresis conditions: Agarose concentration 1 %, power applied: 100 V (7mA / cm), time of run: 45 min. staining method; precast red fast.

Conclusion

PCR analysis for fungal infection using ITS primer pair appeared valuable in detecting fungi in human blood rapidly with high sensitivity in comparison with routine laboratory culture procedures. PCR can act as a key clue in court after fungus strain determination by sequencing that give an indication of the geographical area, source of infection and toxigenic strains determination. Add to that, determination of a zoonotic infection and/or a possible bio-terror action in a disease outbreak or poisoning bio-crime where a poisonous fungus is involved.

Fungal infection investigation is necessary in forensic cases that involve drugs and toxins analysis for both pre and postmortem cases since fungal metabolites can alter the drug chemical characteristics and make it undetectable or at least with false dose analysis [5,10].

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