CHAPTER.5
POLYMERASE CHAIN REACTION FOR EARLY DETECTION OF FUNGI

Introduction:
Our earlier studies have demonstrated the burden of fungal infections in deep tissue of diabetic foot wounds were very high. The current conventional culture methods to identify fungi in deep tissue specimens are based on the biochemical reactions and morphological features of the colonies. This technique not only requires an expert mycologist but also is time consuming, non-specific, would mislead species identification. Though it is known that DNA based fungal identification is the gold standard, such techniques are not routinely applied for fungal detection in diagnostic tool now-a-days.

Aims:
Standardize PCR technique for early detection of fungi in deep tissue of diabetic foot wounds

Methods and materials:
DNA isolation: Homogenized the tissue in 100ul of 1XTNE. To it 25ul of 20%SDS and 12.5ul of Proteinase K added and incubated at 55°C for 3 hours. After that 250ul Phenol and 250ul Chloroform: Isoamyl alcohol was added and incubated at room temperature for 30mnts and then centrifuged at 14000rpm for 15mnts. To the upper aqueous phase 500ul Chloroform: Isoamyl alcohol was added and centrifuged at 14000rpm for 15mnts. Added 25ul of 3M Sodium Acetate and 1ml absolute Ethanol to the upper aqueous phase and centrifuged at 14000rpm for 10mnts. Washed the DNA pellets with 1ml of 70% Ethanol and centrifuged 10000rpm, dried and dissolved in 50ul of 1X TE and stored over night at 4°C. The same method was done to isolate DNA from fungal colony.

PCR Mix: Mix volume containing 5ul DNA, 0.5ul dNTP, 0.5ul forward primer, 0.5ul reverse primer, 10X buffer 2.5ul, distilled 3.5ul and Taq DNA Polymerase.
**Pan fungal primer:** ITS 1 (5' -TCC GTA GGT GAA CCT GCG G) and ITS 2 (5' -GCTGCG TTC TTC ATC GAT GC).

**PCR reaction program:** Initiation of denaturation and activation of Taq DNA is done at 95°C for 10mnts, denaturation is done at 94°C for 15sec. 45 cycles of primer annealing at 60°C at 30sec, primer extension at 72°C at 30sec, final extension done at 72°C at 30sec and held at 4°C.

**Agarose Gel Electrophoresis:** 5ul of PCR product mixed with 5ul of loading dye was run in Agarose gel for 20mnts and visualized in UV light transillumination.

**Results**
We isolated DNA from 17 types of fungal species and did the PCR using panfungal primer. The figure 6 shows the PCR products in AGE.

![Agarose Gel Electrophoresis](image)

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**Fig.5.1. Agarose gel showing PCR products of various fungi isolated from DFWs**

We isolated DNA from 150 deep tissue specimen (100 specimen infected with fungi and 50 specimen without fungal infections) and PCR was performed. The table 5.1 shows the sensitivity, specificity and positive predictive rates of the test applied. The sensitivity of the test was 30%, specificity was 100% and positive predictive rate was 100%.
Table. 5.1: Sensitivity and Specificity of the PCR technique for identifying in tissue of DFWs

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<th>Diseased (n)</th>
<th>Non-Diseased (n)</th>
<th>Total (n)</th>
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<tr>
<td><strong>Test Positive</strong></td>
<td>30 (TP)</td>
<td>0 (FP)</td>
<td>30</td>
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<tr>
<td><strong>Test Negative</strong></td>
<td>70 (FN)</td>
<td>50 (TN)</td>
<td>120</td>
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<td><strong>Total (n)</strong></td>
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<td>50</td>
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**Conclusion:** We developed a molecular based technique for early detection of fungi from deep tissue of diabetic foot wounds, which would help the clinicians to start antifungal therapy the very next day after surgical interventions for DFWs.