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Molecular Detection of Fumonisin Producing *Fusarium* Species of Freshly Harvested Maize Kernels Using Polymerase Chain Reaction (PCR)

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ABSTRACT: Fumonisins are a new class of toxic secondary metabolites produced by *Fusarium* species namely *Fusarium moniliforme* and *Fusarium proliferatum* that have recently come under intense scientific investigations. Thirty-two *Fusarium* isolates namely, *Fusarium moniliforme* (12 isolates), *Fusarium proliferatum* (5 isolates), *Fusarium anthophilum* (4 isolates), *Fusarium oxysporum* (3 isolates), *Fusarium sporotrichioides* (3 isolates), *Fusarium pallidoroseum* (3 isolates) and *Fusarium solani* (2 isolates), other four isolates of *Aspergillus flavus* (2 isolates) and *Alternaria alternata* (2 isolates), isolated from freshly harvested maize kernels were subjected to PCR analysis. The objective of this study was to develop a PCR-based detection kit for detection of fumonisin-producing *Fusarium* species. One set of primers based on the conserved ITS region of the genus *Fusarium* was used to differentiate the genus *Fusarium* from other fungal genera such as *Aspergillus* and *Alternaria.* The other set of primers specific to fumonisin producing 'FUM 1gene' region of *Fusarium* was used to differentiate the fumonisin producing *Fusarium* species from non-fumonisin producers. Results of the study revealed that PCR-based technique could be used not only to differentiate the *Fusarium species* from other genera of fungi but also to identify fumonisin-producing *Fusarium moniliforme, F. proliferatum* and *F. anthophilum.*

KEY WORDS: ***Fusarium* species, Maize, Fumonisin-producers, Molecular detection technique, Polymerase Chain Reaction.**

INTRODUCTION

The genus *Fusarium* represents one of the major fungal genera, which are ubiquitous in distribution, and are found frequently on freshly harvested and stored agricultural commodities such as cereals (Hussein et al., 1991; Wilson et al., 1995; Marasas, 1996). Among cereals, maize is the major food commodity in which the natural occurrence of *Fusarium* and fumonisins have been reported from different parts of the world including USA (Ross et al., 1991) Brazil (Sydenham et al., 1992), Asia (Ueno et al., 1993), Italy (Ritieni et al., 1997), Costa Rica (Viquez et al., 1996) and Hungary (Fazekas et al., 1998). In India, high levels of fumonisins have been reported in maize kernels infected with *F. moniliforme* (Chhatterjee and Mukherjee, 1994), as well as in poultry feeds (Shetty and Bhat, 1997).

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Gelderblom et al. (1988) first reported the occurrence of fumonisins in the cultures of *Fusarium moniliforme.* This finding came as a breakthrough into nearly a century of investigation on the animal and human diseases associated with the consumption of maize contaminated with *F. moniliforme*. The different *Fusarium* species that have been reported to produce fumonisins include *Fusarium moniliforme* Sheldon; *Fusarium proliferatum* (Matsushima) Nirenberg; *Fusarium nygamai* Burgess and Trimboli; *Fusarium anthophilum* (A. Braun) Wollenweber; *Fusarium dlamini* Marasas, Nelson and Toussoun; *Fusarium napiforme* Marasas, Nelson and Rabie; *Fusarium thapsinum* Klittich, Leslie, Marasas, Nelson and *Fusarium globosum* Rheeder, Marasas and Nelson. The reports of International Agency for Research on Cancer (IARC) in 1995 revealed that fumonisin contamination in various agricultural commodities and their consumption by humans and animals is responsible for several health problems. Fumonisins have been shown to have carcinogenic properties in mice. These toxins have also been reported to be hepatotoxic and cancer initiating in rats (Gelderblom et al., 1988; Voss et al., 1990), to cause

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pulmonary edema in pigs (Harrison et al., 1990), and equine leukoencephalomalacia in horses (Kellerman, et al., 1990; Ross et al., 1991).

Maize (*Zea mays* L.) is one of the major cereal crops in India. The crop is grown in both Rabi and Kharif (monsoon and winter) seasons. *Fusarium* contamination is easily detected in the field, because of fungal colonization that may cause visible disease symptoms when toxins are produced inside the maize kernels (Janardhana et al., 1999). The quality of grains, particularly after wet weather in the early or late monsoon season is often lowered, following colonization by fungi, with the consequent risk of mycotoxin contamination (Wilson et al., 1995).

Detection of fumonisin-producing fungal species by conventional methods is a labor- and time-consuming task that requires expertise in chemical analysis and fungal taxonomy. This is particularly complex in case of the genus *Fusarium* because of the existence of several and often-conflicting taxonomic treatments and a large number of closely related species of this genus, which has been entirely classified on micro-morphological characteristics. Molecular detection tools have been used to detect *Fusarium* species contamination in cereals (Bluhm et al., 2004; Patino et al., 2004; Abd-Elsalam et al., 2003). In the present investigation polymerase chain reaction (PCR) technique was used to detect fumonisin-producing *Fusarium* species.

MATERIALS AND METHODS

Collection of maize samples

Maize samples were collected from different districts of Karnataka State, during April 2004 through April 2005. Samples were brought to the laboratory using sterilized polythene bags and representative samples were obtained after sampling by cup method.

Sample plating and isolation of *Fusarium* species

Maize samples were subjected to both standard blotter method and agar plating method (Mathur and Kongsdal, 2003). Maize kernels (200) from each sample were plated on moist blotters as well as on agar medium (10 kernels per plate). For selective isolation of different *Fusarium* species Dichloran Chloramphenicol Peptone Agar (DCPA) (Andrews and Pitt, 1986) and Malachite Green Agar 2.5 (MGA 2.5) medium (Bragulat et al., 2004) were used. The plates were then incubated in alternating periods of 12h darkness and 12h of light at 25 ± 2°C for 7 days. After incubation, plates were visualized for *Fusarium* colonies by macroscopic observation. Suspected *Fusarium* colonies were transferred onto Potato Dextrose Agar (PDA) medium to identify them up to the species level using fungal Keys and Manuals (Booth, 1977; Keith, 1996; Singh et al., 1999). Fungal isolates were maintained on Czapek Dox Agar slants at 4°C for further studies.

Fungal cultures and DNA isolation

All fungal isolates were freshly inoculated in 500 µl Potato Dextrose broth in 2 ml eppendorf tubes and incubated at room temperature for 4 days. The resulting mycelium was used for the DNA extraction using the modified procedure of Zhang et al. (1998). The mycelial mat was pelleted by centrifuging at 5000 rpm (REMI C24 cooling centrifuge) for 5 min. The pellet was ground in microfuge tubes with blunt ends of disposable pipette tips in 500 µl of extraction buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl, pH 8.0, pre-heated at 65°C) and incubated at 65°C for 15 minutes. During incubation, the mixture was briefly vortexed 2-3 times. The samples were then treated with 500 µl of phenol: chloroform (1:1) and vortexed for one min and the supernatant was taken after centrifugation at 3000 rpm for 5 min at 4°C. DNA was precipitated with an equal volume of ice-cold isopropanol, and incubated at -20°C for 60 min and again centrifuged at 8000 rpm for 8 min at 4°C. The pellet obtained was rinsed with 70% ethanol, air-dried, resuspended in 50 µl of nucleic acid free water and used directly for PCR.

Primers for PCR amplification

Two sets of primer were used as described by Bluhm et al. (2004). One set of primer was used from the conserved ITS DNA region specific to *Fusarium* genus (ITS Forward – AACTCCCAAAC CCCTGTGAACATA, ITS Reverse – TTTAACGG CGTGGCCGC) and the expected size of amplicon was 431 bp. Another set of primer specific for fumonisin production was used from 'FUM 1 gene' of *Fusarium* species (FUM1 Forward – CCATCAC AGTGGGACACAGT, FUM1 Reverse – CGTATC GTCAGCATGATGTAGC) and expected amplicon size was 183 bp. Primers and reagents for PCR analysis were obtained from Genei, Bangalore.

PCR mixture and conditions

PCR was performed using Advanced Thermus 25 Thermocycler (Peqlab, Germany). PCR mixture (25 µl) contained 2 µl of DNA sample, 10X PCR buffer, 25 mM $MgCl_2$, 2 mM dNTPs, 20 pmol of each forward and reverse primer and 0.5 µl (3U/µl) of Taq DNA polymerase. The PCR conditions for ITS

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and FUM 1 regions include 94°C for 4 min for initial denaturation, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, primer extension at 72°C for 1 min. The final extension was set at 72°C for 10 min. Ten µl of the PCR product was electrophoresed on 1.5% agarose gel, stained with ethidium bromide, illuminated and documented using Biorad UV Transilluminator.

RESULTS AND DISCUSSION

Fungal species

The following fungal species viz., *Fusarium moniliforme* (12 isolates), *Fusarium proliferatum* (5 isolates), *Fusarium anthophilum* (4 isolates), *Fusarium oxysporum* (3 isolates), *Fusarium sporotrichioides* (3 isolates), *Fusarium pallidoroseum* (3 isolates), *Fusarium solani* (2 isolates), *Aspergillus flavus* (2 isolates) and *Alternaria alternata* (2 isolates) were identified. Among these *F. moniliforme*, *F. proliferatum* and *F. anthophilum* were suspected fumonisin-producers.

Fusarium moniliforme was characterized by mostly zero-septate, clavate microconidia with a flat base produced on monophialides in chains in the aerial mycelium (Fig. 1A). The colonies on PDA were creamish to peach to vinaceous on the obverse and pale cream to salmon to violet or blue on the reverse. The macroconidia were observed rarely which were mostly 3-septate in some isolates. Since *F. moniliforme* resembles *F. proliferatum* in most of the characters except for the formation of polyphialides, extra caution was exercised in order to avoid confusion with *F. proliferatum* that are morphologically related (Fig. 1B). *F. anthophilum* was characterized by the formation of whitish peach to creamish mycelia on PDA medium. Micro-morphological characters include the formation of microconidia, which are circular to ovoid or ellipsoidal and 0-1 septate and macroconidia, which are 3-5 septate. Microconidia are found in false heads from simple phialides as well as polyphialides and never in chains. Secondary phialides are highly branched (Fig. 1C).

All 36 fungal isolates, which included 32 isolates of 7 species of *Fusarium*, 2 isolates of each of *Aspergillus flavus* and *Alternaria alternata* were subjected to PCR analysis using 'ITS' genus specific and 'FUM 1 gene' specific primers. The results are presented in Table 1.

All 32 isolates of *Fusarium* species were positive for the ITS region. The expected 431 bp amplified ITS DNA product was detected in all 32 *Fusarium* isolates except *Aspergillus flavus* and *Alternaria alternata*, which were used as control (Fig. 2A).

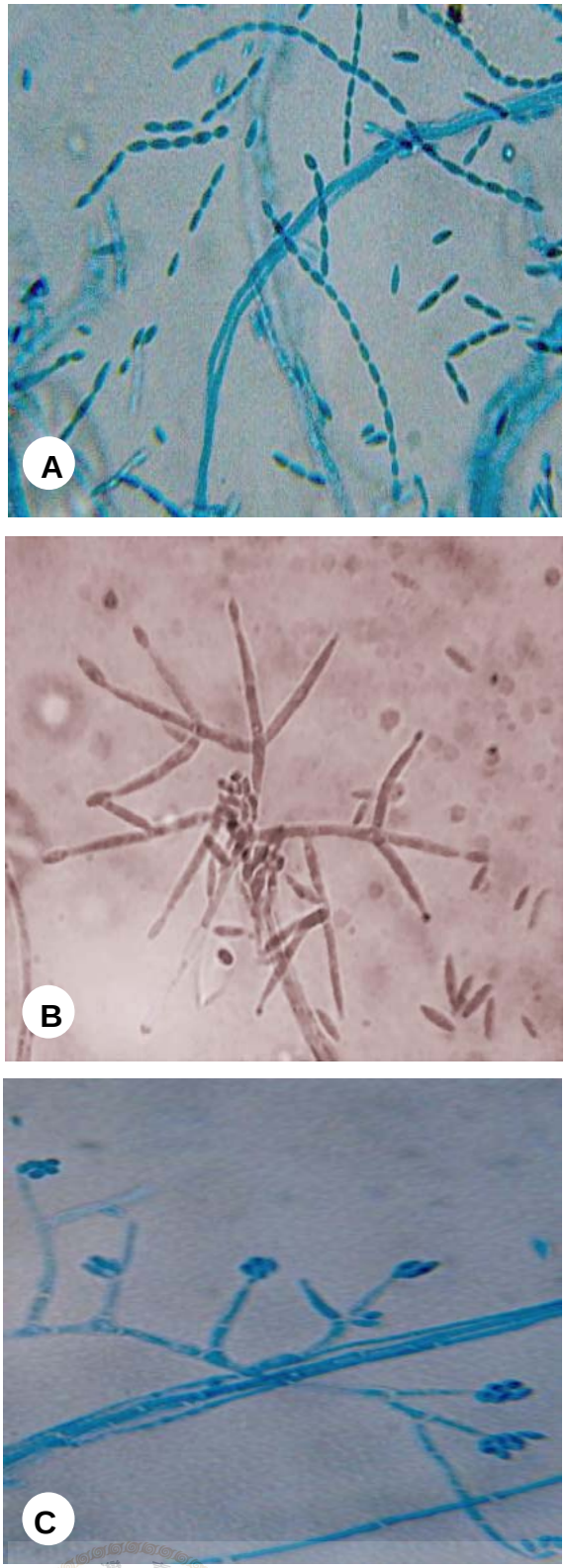


Fig. 1. A: *Fusarium moniliforme* – chains of microconidia borne on monophialides. B: *Fusarium proliferatum* microconidia borne on polyphialides. C: *Fusarium anthophilum* – microconidia in false heads.

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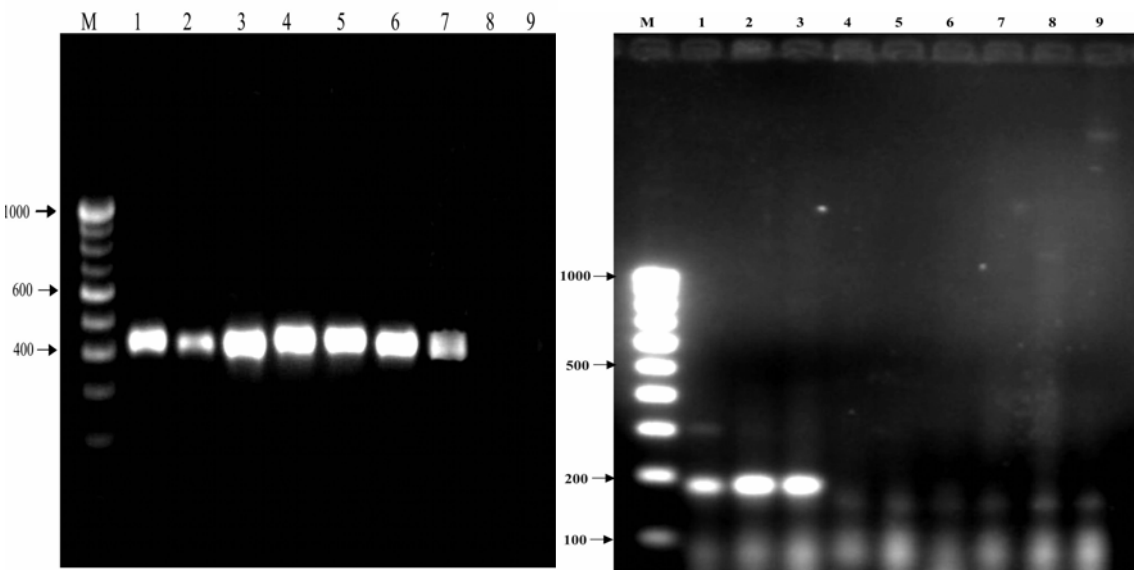


Fig. 2. A: Agarose gel showing amplified products using primers for ITS region. Lane M: 100bp DNA ladder. Lane 1: *Fusarium moniliforme.* Lane 2: *Fusarium proliferatum*. Lane 3: *Fusarium anthophilum*. Lane 4: *Fusarium oxysporum*. Lane 5: *Fusarium sporotrichioides*. Lane 6: *Fusarium pallidoroseum*. Lane 7: *Fusarium solani*. Lane 8: *Aspergillus flavus*. Lane 9: *Alternaria alternata*. B: Agarose gel showing amplified products using primers for FUMI region. Lane M: 100bp DNA ladder. Lane 1: *Fusarium moniliforme*. Lane 2: *Fusarium proliferatum*. Lane 3: *Fusarium anthophilum*. No bands were observed in Lane 4 *Fusarium oxysporum*. Lane 5: *Fusarium sporotrichioides*. Lane 6: *Fusarium pallidoroseum*. Lane 7: *Fusarium solani.* Lane 8: *Aspergillus flavus*. Lane 9: *Alternaria alternata*.

Table 1. Table showing the number of fungi tested and number of fungi showing positive for ITS and FUM 1 gene as analyzed by PCR.

Fungi	Number of isolates	Positive for ITS region	Positive for FUM 1gene
Fusarium moniliforme	12	12	2
Fusarium proliferatum	5	5	3
Fusarium anthophilum	4	4	3
Fusarium oxysporum	3	3	0
Fusarium sporotrichioides	3	3	0
Fusarium pallidoroseum	3	3	0
Fusarium solani	2	2	0
Aspergillus flavus	2	0	0
Alternaria alternata	2	0	0

On the other hand when all 32 isolates of *Fusarium* species were analyzed by PCR for fumonisin producing ability using FUM 1 gene based primers, the expected DNA fragment of 183 bp was amplified only in 2 out of 12 isolates of *Fusarium moniliforme*, 3 out of 5 isolates of *Fusarium proliferatum*, and 3 out of 5 isolates of *Fusarium anthophilum*. Totally 8 isolates of *Fusarium* species showed a positive result with FUM1 gene set of primers. No bands were seen in all other isolates of *Fusarium* and genera of *Aspergillus* and *Alternaria* (Fig. 2B). The results were contrary as *F. oxysporum* has been reported to produce fumonisin (Abbas and Ocamb, 1995). However, this necessarily does not imply that all strains of *Fusarium moniliforme*, *Fusarium proliferatum*, *Fusarium anthophilum* and *Fusarium oxysporum* are fumonisin-producers as is evident from our investigation.

The genus *Fusarium* is one of the most economically important groups of fungi causing diseases in maize. It is a non-obligate pathogen and has been detected on maize, sorghum, wheat, cotton, beans, tomatoes, peanuts, bananas, soybean, green peppers and several forages. Most, if not all, *Fusarium* species are capable of producing mycotoxins like fumonisins, which are important in terms of human and animal health. Fumonisins produced by few strains of *Fusarium moniliforme*, *F. proliferatum* and *F. anthophilum* are heat-stable and known to cause Leukoencephalomalacia in horses (Wilson and Marsonport, 1971); Pulmonary edema in swine (Harrison et al., 1990); Hepatotoxicity and Hepatocarcinogenecity in rats (Gelderblom et al., 1988).

The routine analysis of maize and other cereals for toxigenic *Fusarium* species is hindered by difficulties associated with standard methods for isolating and identifying *Fusarium* species. Traditionally *Fusarium* species have been differentiated by morphological characteristics such as presence or absence of micro conidia, shape and size of macroconidia, colony morphology, pigmentation and growth rates. Often morphological analysis is time consuming and requires considerable expertise and skill (Nelson et al., 1983).

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PCR-based detection techniques have provided an alternative to microbiological identification of *Fusarium* species. Random Amplified Polymorphic DNA PCR techniques have been used for species-specific PCR assays. Another approach for group-specific or species-specific detection involves the design of PCR primers for the polymorphic regions of rDNA. Several researchers have employed polymerase chain reaction (PCR) primers from genes directly involved in fumonisin-biosynthesis, like, FUM1 to identify groups of fumonisin-producing *Fusarium* species (Bluhm et al., 2004; Patino et al., 2004; Abd-Elsalam et al., 2003).

India, being an agriculture-based country, produces a variety of food crops throughout the year. Non-scientific methods of agricultural practices, poor storage facilities and unfavourable environmental conditions during pre- and post-harvest handling of cereal grains are responsible for the contamination, infection and colonization by fumonisin-producing fungi (Shetty and Bhat, 1997; Janardhana et al., 1999; Bhat et al., 1997). Systematic investigations have not been made in India with special reference to *Fusarium* biodiversity, occurrence, toxicity, and extent of fumonisin contamination on various corn-based animal and poultry feeds and agricultural commodities particularly cereals such as maize, paddy and sorghum, which are grown and consumed widely.

A report from India described an acute but self-limiting, food-borne disease outbreak in villages consuming moldy corn containing up to 64.7 mg fumonisins/kg (Shetty and Bhat, 1997; Bhat et al., 1997). The survey was carried out in 50 villages belonging to 6 districts of Andhra Pradesh in India. Nearly 1429 people were affected, and the disease was observed only in adults who consumed the moldy grains of sorghum and not in pregnant women and children who did not consume sorghum.

Maize and sorghum are the two important staple food crops cultivated as major rain fed crops in most of the semi-arid areas in the Deccan plateau of South India. Due to intermittent rain at the time of harvest, a number of *Fusarium* species contaminate grains and render the grains unsuitable for human and animal consumption. Realizing the importance of the genus *Fusarium*, International Society of Plant Pathologists Committee on *Fusarium* has been established and two International Conferences were held exclusively on the genus *Fusarium*. The work in India by Bhat et al. (1997) is witness to the extent of problem faced by people of India who are living below the poverty line. Accurate field survey and epidemiological studies on occurrence of *Fusarium* and extent of fumonisin contamination need to be initiated and the study of agroclimatic conditions that favour the growth of these moulds and toxin production would generate sufficient data to know the exact problem and losses caused by *Fusarium* species. By such means it is also possible to relate the health hazards associated with the consumption of such mouldy grains by animals and humans. Sensitive techniques for detection of toxigenic fungi are the urgent need of the hour to prevent the entry of these toxic substances into the food chains.

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利用聚合酶連鎖反應 (PCR) 偵測從新鮮收成玉米粒分離出來的會產生 Fumonisin 的鐮胞菌株

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摘　　要

Fumonisins 是新種類的有毒二次代謝物，由鐮胞菌 (*Fusarium* species) *Fusarium moniliforme* 和 *Fusarium proliferatum* 產生，這兩種鐮胞菌近來被科學界廣泛研究。從新鮮收成玉米粒分離出來 32 株 *Fusarium*，分別是 *Fusarium moniliforme* (12 株)，*Fusarium proliferatum* (5 株)，*Fusarium anthophilum* (4 株)，*Fusarium oxysporum* (3 株)，*Fusarium sporotrichioides* (3 株)，*Fusarium pallidoroseum* (3 株) 和 *Fusarium solani* (2 株)，以及 *Aspergillus flavus* (2 株) 和 *Alternaria alternate* (2 株) 都用來進行 PCR 分析。本研究目標為發展出一套聚合酶連鎖反應 (PCR) 偵測系統，用以偵測產生 Fumonisin 的鐮胞菌株。一組參照鐮胞菌屬具保留性的 ITS 區域設計的核酸引子 (primer) 用來區分鐮胞菌屬和其他真菌菌屬，例如 *Aspergillus* 和 *Alternaria*。另一組參照鐮胞菌產生 fumonisin 的″FUM 1 基因″區所設計的核酸引子用來區分產生 fumonisin 的鐮胞菌種和不產生 fumonisin 的鐮胞菌種。研究結果顯示，利用 PCR 技術不僅可以用來區分鐮胞菌屬和其他真菌菌屬，也可以用來鑑定產生 fumonisin 的 *Fusarium moniliforme*，*Fusarium proliferatum* 和 *Fusarium anthophilum*。

關鍵詞：鐮胞菌、玉米、Fumonisin 生產者、分子偵測技術、聚合酶連鎖反應。

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