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THE APPLICATION OF PCR IN THE DETECTION OF AFLATOXGENIC ISOLATES OF *Aspergillus flavus* IN PEANUT

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ABSTRACT

Aflatoxin contamination of peanut seeds , results from growth in kernels by toxigenic strains of the fungus *Aspergillus flavus* Link. This can occur in the field (Pre-harvest) when severe late - season drought stress occur and during storage (post - harvest) when improper conditions of moisture and temperature exists. Current investigations aims the detecting *A. flavus* in commercial peanuts production area, moreover, some isolated mycoflora were found to be associated with seeds during (2006-2007) growing seasons. Analyses of 198 seed samples recorded (13.1%) aflatoxin contaminated kernels belonging to 26 samples with aflatoxin detectable levels ranked from (1.6 – 40) ppb. *A. flavus* total DNA from both toxigenic and non-toxgenic isolates obtained from seeds or soil at five geographic locations was subjected to RAPD technique (random amplified polymorphic DNA). Phonetic and caldistic analyses of the data, based on bootstrap analyses, indicated that the RAPD system was unable to distinguish between aflatoxigenic and non aflatoxigenic *A.flavus* strains. Therefore, the present study supports the application of that technique for strain characterization and preliminary evolution.
Keywords: Peanut – aflatoxin – *Aspergillus flavus* – RAPD technique.

INTRODUCTION

Peanut is one of the most important agricultural commodities in the new reclaimed soils in the Northern part of Egypt, from the East and West of the Nile Delta to the North of Cairo . The total cultivated area of peanut was 145.000 feddans in the report presented by the European commission . In west Delta, 38.092 feddans was the total harvested peanut area in 2005 season and classified as follows: 21.991 feddans, belongs to Al-Entelak area, 9.028 to Tiba, 5.361 to Al-Bostan, 1.616 to west, Al-Nubaria and just 96 feddan to Al-Hamam.

Aspergillus is a cosmopolitant mold, therefore peanut seeds have already contaminated since they are cultivated . *A. flavus* and *A. parasiticus* can invade peanut seeds in the field and post harvest . Poor storage of peanuts can lead to an infection by the mould fungus releasing the toxic aflatoxin.

Aflatoxins are polyketide highly toxic secondary metabolites produced by important food contaminating spices *A. flavus* and *A. parasticus* and sometimes by *A. calavtus* (Wilson, and Stansell 1983). The four main aflatoxins produced,are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2) and aflatoxin G_2 (AFG_2) which are furanocoumarine derivatives and potent liver carcinogens for a wide variety of animals and humans (Beasley,. 1990 ,Smith *et al*., 1999, Cary *et al*, 2000).

It has been established that the occurrence of aflatoxins is influenced by favorable conditions which include high moisture content and high

temperature. Hence, the extent of contamination will vary with geographic location, agricultural and agronomic practices and the susceptibility of the peanuts to fungus before they are harvested and during storage and processing periods. Mycotoxin frequently contaminates peanuts, corn and also, rice wheat and other grains stored in unfavorable conditions (Lee *et al*., 2004) , it is not easy to identify the contaminated and non-contaminated nuts (Beasley, 1990).

Aflatoxin could be formed while the nuts are formed. Mold are frequently form spores to protect themselves from unfavorable environmental condition, such as low relative humidity and low water activity because the spore is resistant to very dry condition. When the environmental condition is altering , the mold grows. Based on the aforementioned information, post harvest handling has to be conducted rapidly. Harvest is frequently followed by drying until the moisture content of peanuts is unfavorable for the bats. The combination of moisture content in materials, high temperature and high relative humidity is an appropriate condition for the formation of aflatoxin. Therefore, extra treatment is needed .

Aflatoxins are a potential cause of liver damage, cirrhoses and liver cancer. It has been recognized as a substance that retains to high temperature. However, it is produced when the mold being stress by severe change of temperature and humidity and aflatoxin B_1 is the most dangerous toxin for both animal and human health (Commission Decision ,2004).

Mycotoxins are compounds produced from fungi that contaminate food stuff and have determinate effects on humans and animals. They are referred to as secondary metabolites which are low molecular weight compounds that do not contribute to the bioenergetics of the producing fungi. The metabolites required energy to be produced. Production is restricted to certain taxa or strains within taxa. They often exert their effects external to the organisms but not exclusively. On the other hand, primary metabolites are the well-known almost-universal compounds involved in life such as (a) DNA, (b) protein, and (c) the small compounds involved in glycolysis and the hexose monophosphate shunt mechanisms.

The use of PCR to identify mycotoxin fungi is attracting considerable attention ,these methods are based on genes separate from mycotoxins biosynthesis. However, there are only a few mycotoxins about which the biochemistry has been determined sufficiently to enable the development of gene probes of the pathway.

Conventional plating out methods will indicate specific viable fungi from a sample. Analysis of the pure culture (e.g., by chromatography) will inform if it can produce particular mycotoxins. PCR analysis will detect relevant genes in a sample (and it is possible to determine whether, or not such genes are expressed). Chromatography, for example, will determine if a sample actually contains particular mycotoxins and by implication that a producing fungus was present at some stage. This is the field of metabolites. The levels of detection for mycotoxins are extremely sensitive and now concentrations of as low as 10^{-5} (10^{-5} = $mg.kg^{-1}$) can be conceived with HPLC.

Furthermore, it is possible to determine of all the genes of a pathway can be expressed by incubating a sample and assessing whether concentrations of the mycotoxin increase.

MATERIALS AND METHODS

Tested samples were obtained from peanut crops and soil collected from five controlled experiment conducted distinct sites, namely El Hamam, West-Noubaria, El Bostan, Tiba, al-Entelak belonging to (West-Delta, Egypt). The cultivated area was approximately forty thousands feddan.

Isolation and identification of mycoflora:

Seed samples:

One sub-sample of 30 g was taken from each peanut sample, and then disinfected by immersion in 2% sodium hypochloride solution for 3 min, followed by 3 rinsing with sterile distilled water. From the sub sample, 30 seeds were randomly taken and cultured on Petri dishes containing dichloran raised bengal agar with chloramphenicol (DRBC) 10 kernels/dishes. Incubation was carried out at 25ºC for 5 days.

Soil samples were analyzed according to the method described by Swanson *et al*. (1992). For soil mycoflora isolation, Martin medium was used. The plates was incubated at 25ºC for 10 days and observed daily.

Mycelial growth were obtained from peanut, derived from the same set of kernels (according to the methodology proposed by Delp *et al*.,1986), and from superficial soil samples (0-2 cm depth) around each peanut plant at all locations. Colonies whose morphology grossly resembled that of *A. flavus* (colour of the top surface, and reverse, appearance, topography, and contours) were isolated on potato dextrose agar (PDA) and identified according to micromorphological criteria by the microculture technique (Riddel ,1950), making use of the shape, colour and size of the phialides, vesicles, conidias and conidiophores (Pitt & Hocking, 1997).

Culture media:

Dichloran raised bengal agar with chloramphenicol (Cotty & Cardwell, 1999 - DRBCOXOID) pH 5.6, supplemented with chloramphenicol, was used for the isolation and quantification of fungi from peanut kernels.

Martin's medium (Martin ,1950) was used for the isolation of soil fungi.

Sabouraud dextrose agar (OXOID) pH5.6, supplemented with chloramphenicol, was used for the identification and maintenance of fungal isolates. Czapeck medium was used for culturing *A. flavus* prior to DNA extraction. Coconut-agar (Lin & Dianese ,1976) was used to test the fungal isolates for aflatoxin production (where as, this specific media differentiated between aflatoxigenic and non-aflatoxigenic *A. flavus* isolates using (NUV) examination test).

Evaluation of toxigenic potential and confirmatory test:

The toxigenic potential of *A. flavus* differentiated selected isolates was evaluated according to Lin and Dianese method, (1976). The procedure used involved extraction with methanol/4% KCl solution (v/v) (9:1), followed by clarification of the extract with ammonium sulfate and partitioning to

chloroform. The content was filtered through filter paper (Whatman no. 1) then evaporates to dryness. Suspended extracts were quantified by thin-layer chromatography (TLC) using aflatoxins standards (Schuller *et al*. ,1973). Confirmation of the identified aflatoxins was carried out derivatization using trifluroacetic acid (Przybylsky, 1975). The TLC detection limit was 2 µg/kg.

DNA extraction*:* All fungal isolates were maintained on Sabouraud dextrose agar and further inoculated into flasks containing Czapeck liquid medium prior to total-DNA extraction. After 2-3 days of incubation, the contents of the flasks were aseptically filtered and the mycelial mass was ground with a mortar and pestle in liquid nitrogen. DNA was extracted as described by Innis and White ,(1990). The amount of DNA (obtained from single extraction) for each sample was estimated by electrophoresis in 1.5% agarose gels stained with ethidium bromide; DNA concentrations were normalized to about 2.5 ng μl^{-1} by visual comparison with known standards (Gibco).

RAPD PCR*:* Total DNA (10 ng) was submitted to PCR with 5 well established random primers already used with *A. flavus* (Bayman & Cotty, 1993 Chao *et.al*.,2006): P160: 5' CATGGCCACC 3'; P117: 5' TGGCGT CTCCA 3'; P54: 5' GGCGATTTTTGCCG 3'; P10: 5' GCGGCCATAGCAAC 3'; PM1: 5' GCGTAAGCGGGGCATATG 3'. Smaller concentrations of DNA were tested in a pilot experiment, but very low concentrations did not result in a good reproductive technique. The choice of primers currently in use was motivated by our concern to reproduce standard experimental conditions that favor more meaningful comparisons between different works (Welsh & McClelland, 1990). Reaction mixtures were subjected to an incubation step at 94ºC for 5 min followed by 45 cycles of 1 min at 94ºC, 2 min at 35ºC, and 3 min at 72ºC. In the end, samples were kept at 4ºC until use. The amplification conditions followed a rigorous standardization, including the use of the same *Taq*-Polimerase (Gibco) and thermocycler (Perkin, model 2.400) sources for all experiments.

In order to confirm that all bands in RAPD reactions were products of the target DNA and not technical artifacts, negative controls with sterile distilled water (Willians *et al*., 1990, Diaz-Guerra *et al*., 2000) were introduced instead of DNA samples during extraction with liquid nitrogen. Whenever bands appeared in the negative control (on two occasions very faint bands were detected) the whole run was discarded. Amplifications were carried out in triplicate, which were preferentially run on different days. The amplification products were separated by electrophoresis in 1.5% agarose (Gibco) gels stained with ethidium bromide. For each sample, the result selected was the repetition with greatest consistency: clear view of bands and conformity between the repetitions. Very tenuous bands were not considered due to the low stringencies used.The results of the band patterns of each gel were noted down in matrices for each primer and after collected together in a single matrix, used for cladistic and phonetic analysis, each one with their own presumptions for the characters in question.

Phenetic analysis*:* The phenetic analysis was based on the un-weighted pair group method using arithmetic averages (UPGMA - Swofford & Olsen, 1990), which groups by similarities sometimes used in conjunction with RAPD

(Bayman & Cotty, 1991) that is part of the PAUP phylogenetic analysis package (Swofford, 1998). The adopted assumption for inclusion of characters into the program was: absence of band = 0; presence of band = 1.
Cladistic analyses*:* The cladistic analyses were carried out according two methods based on PARCIMONY by the PAUP Phylogeny program (Berbee *et al*., 1995).

Consensus phylogenetic trees (cladograms) were constructed between the different strains taking into account the characters of the whole set of primers. The basic assumptions (approaches) were: irreversibility of characters (assumption: absence of band = 0; presence of band = 1) and Dollo (assumption: predominant character = 0; rare character = 1).

The topology of the trees from these two analyses was compared in order to evaluate the degree of concordance between them. Reweighting characters was performed until two identical trees were obtained for two consecutive finds (Swofford, 1998). This procedure allows getting closer to the best tree, especially for RAPD data.

RESULTS AND DISCUSSION

Aspergillus flavus has received greater attention than any other mycoflora as it is a very important toxigenic fungus who produces aflatoxins, which have a potent carcinogenic effect and their acute poisonous act for animals and humans. Although, not all members of that species is toxigenic.

Data Table 1 show that El-Bostan region has a highly contamination percentage with *A. flavus* (19%) followed by Tiba, Al-Entelak, West – Nubaria, then El-Hamam region with lowered levels (17, 15, 8 and 7%).

The total mycoflora detection levels were also varied in peanut seeds samples related to their commercial production location,i.e., 49, 47, 44, 28 and 23 % infection for Tiba, El-Bostan, Al-Entlak, West-Nubaria then El-Hamam region. The isolated mycoflora were indentified as : *A. flavus, A. niger, Fusarium sp., Rhizoctonia solani, Rhizopus stolonifer, Sclerotium sp.* and *Foma sp*. (Fig 1).

Table (1) :Mycoflora detectable levels (%) from naturally infected peanut seeds.

Isolated Fungi	West -Delta collected regions				
	El Hamam	West-Noubaria	El Bostan	Tiba	Al Entlak
Aspergillus flavus	7	8	19	17	15
Aspergillus niger	2	3	9	12	9
Fusarium sp.	7	5	4	4	7
Rhizoctonia solani	3	4	2	5	6
Rhizopus stolonifer	3	4	5	7	3
Sclerotium sp	1	3	6	4	3
Foma sp	0	1	2	0	1
Total	23	28	47	49	44

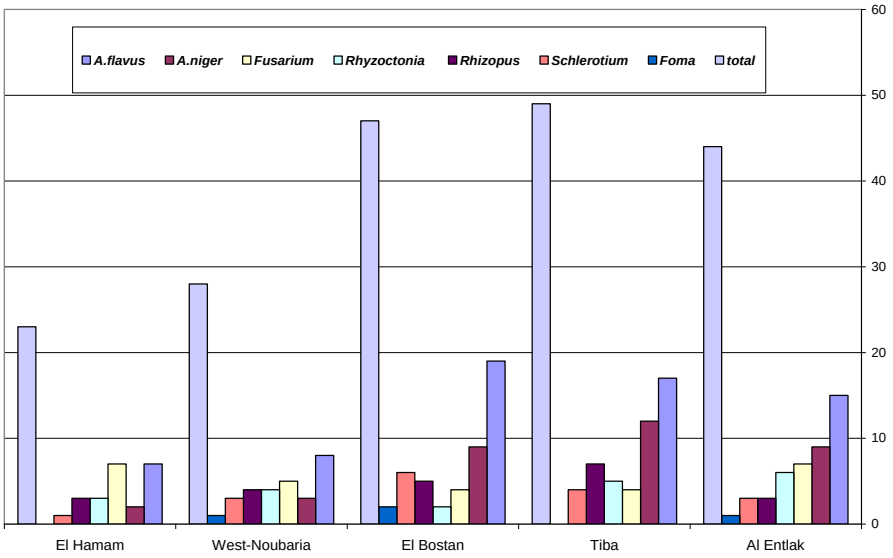

Fig. (1): Mycoflora detectable levels (%) from naturally infected peanutseeds

Aflatoxin detection in seeds:

Examination of 198 peanut seed samples obtained from the different five locations belonged to West-Delta region during 2006- growing season, showed that 13.1% of the seeds found to be contaminated with aflatoxins (Table 2). This coincides with the understanding that the aflatoxin producing fungus is particularly associated to peanut seeds. Whereas, three samples within 35 examined samples, contaminated with aflatoxin as represented about (8.6%) of El-Hamam tested samples, with aflatoxin detectable levels ranged between (8.0 – 21) ppb.

Table (2): Aflatoxin detectable levels (ppb) in west – Delta peanut seed samples:

Seed sources	Examined samples	No. aflatoxin detectable samples with its percentage (%)	Aflatoxin ranges (ppb)
El-Hamam	35	3 (8.6)	8.0 – 21
West-Nubaria	90	10 (11.1)	2.0 – 40
El-Bostan	33	7 (21.0)	8.0 – 33
Tiba	25	4 (16.0)	5.5 – 20
Al-Entlak	15	2 (13.3)	1.6 – 22
Total samples	198	26 (13.1)	1.6 - 40

Aflatoxin detected levels as (AF) b_1 (µgLKg = ppb).

Other ten samples within 90 samples collected from west-Nubaria having (11.1%) of the tested samples recorded (2.0 – 40) ppb aflatoxin.

While, Al-Bostan 33 samples appeared 7 aflatoxin contaminated samples, containing (8.0 – 33) ppb calculated as (21.0%) of the samples. The same evaluation was carried out with Tiba 25 samples which detected only 4 aflatoxin contaminated samples which detected only 4 aflatoxin contaminated samples and Al-Entlak 15 samples which detected just two aflatoxin contaminated samples with percentages of (16.0%) and (13.3%) of their examined samples and aflatoxin detectable amounts ranged from (5.5 – 20) and (1.6 – 22) ppb respectively.

Therefore, the present work revealed that there was 26 peanut seed samples were contaminated with differed aflatoxin levels ranked from (1.6-40) ppb within 198 subjected to examination process.

Differentiation between toxigenic and non-toxigenic *A. flavus* isolates:

Data presented in (Table 3) showed that isolates of *A. flavus* obtained from either seeds or soil samples related to its respective sites and toxigenic potential although, just ten of the examined isolates symboled as (H-2, N-2, B-2, T-2 and E-2) from seeds and others, as (H-2s, N-2s, B-2s, T-2s and E-2s) from soil samples, proved to be toxigenic and, in this respect, it has been shown that this study may not represent models situation, as it has analyzed whether there was a detectable genomic difference between these obtained isolates and the non toxigenic ones. None of the analyses revealed any differentiating pattern . Similar results have been described in other studies with *Aspergillus* spp., showed no correlation between DNA band profiles and production or non-production of mycotoxyins (Bayman & Cotty, 1993, Croft & Varga ,1994, Jovita & Bainbridge, 1996). Furthermore, Tran-Dinh *et al*., (1999) found no relationship between RAPD-based band profile and toxin production in *A.flavus*. However, on one investigation with isolates of *Aspergillus* sect. Flavi using RAPD, Egel *et al*., (1994) grouped strains with similar toxigenic capacities, in a more subtle differentiation than the simple classification of toxin producers and non producers.

Geographic isolation*: t*o wonder what would happen if RAPD was not suitable for characterization, and led to unreliable data, is an interesting speculation.

One would expect to form trees by random grouping of samples and that, only eventually, isolates from the same origin would group together. However, this was not observed.

In fact, the comparison between topologies of the cladistic and phenetic analyses revealed high degree of concordance. The agreement among different methodologies is one of the indicators of the consistency of the method adopted (Bayman & Cotty, 1993, Berbee *et al*., 1995). All in all, it is worth noting that Tran-Dinh *et al*., (1999) have grouped several isolates of *A. flavus* in a clear association with their origins, prompting them to endorse the use of RAPD as a reliable and reproducible methodology with no significant artifacts. In the present work, we have obtained a non-random array, but one cannot assume that this is a fully discriminatory result and, thus, RAPD usefulness is debatable. Same results was mentioned by Geiser *et al*. ,(1998) who were unable to find any geographic distribution pattern to work with RFLP. The fact that samples from one region have segregate with samples

from another region hampers any attempt to categorically state that there is an association between their origin and their genotyping**.**

Table (3)*: Aspergillus flavus* isolates: abbreviations with respective sites,substrates of origin and toxigenic potential

Isolates Abbreviation*	Source	Region	Toxigenic potential
H-1	Peanut	El Hamam	Absent
N-1	Peanut	West-Noubaria	Absent
B-1	Peanut	El Bostan	Absent
T-1	Peanut	Tiba	Absent
E-1	Peanut	Al Entlak	Absent
H-1s	Soil	El Hamam	Absent
N-1s	Soil	West-Noubaria	Absent
B-1s	Soil	El Bostan	Absent
T-1s	Soil	Tiba	Absent
E-1s	Soil	Al Entlak	Absent
H-2	Peanut	El Hamam	Present
N-2	Peanut	West-Noubaria	Present
B-2	Peanut	El Bostan	Present
T-2	Peanut	Tiba	Present
E-2	Peanut	Al Entlak	Present
H-2s	Soil	El Hamam	Present
N-2s	Soil	West-Noubaria	Present
B-2s	Soil	El Bostan	Present
T-2s	Soil	Al Entlak	Present
E-2s	Soil	Al Entlak	Present

*** as (H-2, N-2, B-2, T-2 and E-2) from seeds and others, as (H-2s, N-2s, B-2s, T-2s and E-2s) from soil samples.H,N,B,T,E mean El Hamam, West-Noubaria, El-Bostan, Tiba, Al-Entlak regions at (West-Delta, Egypt) .**

This data could be important if we think in terms of a microbiologic control, like what was carried out recently with the malaria mosquito, spreading of the non-toxigenic strain to remove the toxigenic one. Elegant and promising in theory, this approach to controlling micro toxins faces a problem if there or isn't a genetic rearrangement between strains of the Flavi section. This possibility could produce the worst of both worlds, a toxigenic strain even more competitive, removing the others.

Even considering the advantages of being simple, low costing, and applicable to large and genetically unknown collections, these traits, are not enough for electing RAPD as a sole typing methodology. However, the indication of consistence observed show that RAPD-based data could aggregate important information and enrich genotyping studies, especially if they are interpreted in combination with results obtained by other means (Bos & Stuart ,1995).

Of course the importance of RAPD depends on the chosen primers that can vary in an incredibly wide manner. This makes this technique hostage to

a systematic adoption by part of the scientific community, in a “fashion” trend. It is clear that the use of a specific primer is connected to its discriminatory ability, but for the ease of establishing new primers that can eventually be used as standard; we believe that the technique lends itself better to an anarchical diversity than an agreement.

So the study observed that the potential hazard associated with aflatoxin in peanut seeds has been serious. The risks posed to health in Egypt can be further lowered by reduced exposure. For instance those when have pre-existing liver disease may consider avoiding peanut, or through using roasting process, where roasting at 150 ^{o}C for 30 min. led to 70.0% and 79.8% reduction of AFB, and AFG, respectively (Ogunsanwo *et al*., 2004). Finally, it must be discarded any peanut that looked moldy, damped, shriveled and discolored.

Conclusion

There are toxigenic and non-toxigenic *A. flavus* variants, but the necessary conditions for expressing the toxigenic potential are not fully understood. Therefore, we have studied total-DNA polymorphism from toxigenic and non toxigenic A. flavus strains isolated from peanut crops and soil at five geographic locations in the West Delta of Egyptl. Total DNA from each A. flavus isolate was extracted and subjected to polymerase chain reaction amplification with five random primers through the RAPD (random amplified polymorphic DNA) technique. Phenetic and cladistic analyses of the data, based on bootstrap analyses, led us to conclude that RAPD was not suitable to discriminate toxigenic from non toxigenic strains. But the present results support the use of RAPD for strain characterization, especially for preliminary evaluation over extensive collections.

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استخدام تكنيك PCR فى الكشف عن عزلات الفطر اسبرجلس فلافس المنتجة للأفلاتوكسين فى الفول السودانى

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مركز البحوث الزراعية- معهد بحوث إمراض النباتات- الجيزة- مصر.

استهدفت الدراسة الكشف عن عزلات الاسبرجلس فلافس والتي قد تصيب بذورالفول السوداني في مناطق إنتاجها وتمييز العزلات المنتجة للافلاتوكسين عن مثيلتها الغير منتجة وذلك في عينات البذور والتربة أخذت من خمس مناطق منزرعة وتابعة لنطاق غرب الدلتا بمصر. وهى مناطق (الحمام – غرب النوبارية - طيبة – البستان – الانطلاق) بالإضافة إلى عزل وتعريف للفطريات التي قد تصاحب البذور المحصودة حيث تراوحت نسب عزلها ما بين ٢٣-٤٩%في البذور.

كما اهتمت هذة الدراسة بتقدير نسب عينات البذور الملوثة بالسم الفطري الافلاتوكسين وخاصة بأنها الجزء المخصص للاستهلاك الادمى . حيث أظهرت النتائج احتواء ١٣,١% من العينات بواقع ٢٦ عينة للبذور ضمن ١٩٨ عينة مختبرة على مستويات متفاوتة من الافلاتوكسين تراوحت كمياتها ما بين (١,٦- ٤٠) جزء في المليون.

كما استهدف البحث استخدام تكنيك RAPD بغرض التمييز بين عزلات الاسبرجلس فلافس المنتجة وغير المنتجة للافلاتوكسين والمعزولة من البذور أو التربة حيث تبين من الدراسة عدم كفاءة التكنيك في التمييز بين تلك العزلات ويوصى بتطبيقه في عمليات تعريف العزلات بغرض تقييمها.