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MYCOLOGICAL STUDIES ON CHICKEN-VISCERA WITH THE AID OF RAPD-PCR TECHNIQUE AS A TOOL FOR CONFIRMATION

(With 2 Tables and 5 Figurs)

By

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دراسة عن الفطريات المتواجدة في أحشاء الدواجن باستخدام الوراثة الجزيئية

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لقد تم تجميع عدد ١٠٠ عينة عشوائيا من أحشاء الدواجن المذبوحة في مختلف مجازر الدواجن بالنمسا (٢٠ عينة من كل من القونصة ، القلب ، الأمعاء ، الكبد ، الطحال) وذلك لفحصها ميكولوجيا مع استخدام الوراثة الجزيئية كوسيلة تأكيديه .حيث وجد أن الأمعاء والمعدة الطاحنة (القونصة) أكثر الأعضاء تلوثا بالفطريات ٤,٤ × ١٠° ، ٢.٦ × ١٠⁴ /١جم علي التوالي ، ومن ناحية أخري فان عينات القلب والطحال وجدت خالية تماما من التلوث بالفطريات. أيضا تم عزل عدد ٨٥ عترة فطريات من العينات التي فحصت وكان معظمها من مجموعه الأسبرجلس والتريكوديرما بنسبة ٢٠% ، ١٤.١% بالترتيب ، ولهذا فقد أجريت علي هذه المجموعة اختبارات أخري تأكيدية باستخدام الوراثة الجزيئية. وأخيرا تم مناقشة الآثار الضارة علي صحة الإنسان نتيجة تلوث أحشاء الدواجن بالفطريات في المجازر كما تم تناول الإجراءات الصحية التي يجب اتخاذها في مجازر الدواجن لمنع أو تقليل هذا النوع من التلوث.

SUMMARY

A total of 100 samples of chicken-viscera were collected from different poultry-slaughtering plants in Austria; 20 each of gizzard, heart, intestine, liver and spleen. Samples were subjected to mycological examination for isolation and identification of various mould species. It was found that intestine and gizzard were heavy contaminated with moulds than other examined visceral organs of the slaughtered chicken; 4.4 X 10^5 and 2.6 X 10^4 /1g of the samples, respectively. On the other hand, the fungal contamination was not detected in all samples of heart and spleen. Eighty-five mould strains were isolated from examined samples, the majority of which were *Aspergillus glaucus* group (20.0%) and *Trichoderma*

(14.1%). The isolated species of *Aspergillus glaucus* (anamorph: *Eurotium*) group and *Trichoderma* were further identified by using RAPD-PCR (Random Amplified Polymorphic DNA – Polymerase Chain Reaction) technique. Finally, the harmful effect on human health resulting from mould contamination of chicken-viscera and the hygienic measures adopting in poultry-slaughtering plants were fully discussed.

Key words: Mycological - chicken - viscera - PCR - Aspergillus –mould.

INTRODUCTION

The poultry industry has undergone a tremendous change over the past half century. The development of a completely vertically integrated production system which often includes everything from the primary breeder to the handling of by-product and distribution of the further processed product to restaurants and retail outlets is common. This continuous intensive production of poultry meat creates new challenges for food scientists where it is considered as a major contributor to the pollution of the environment, rather than other forms of microbial contamination as wastes, sewage and human activities (Anthony, 1998 and Aletor et al., 2000).

Nursey (1997) indicated that mould contamination of poultry meat may occur at any point along the production chain; in feed raw materials, compound poultry feed, poultry flocks or processing. Species of *Aspergillus, Fusarium* and *Penicillium* could be isolated from chicken's mash, while *Alternaria, Aspergillus*, *Penicillium* and *Mucor* were detected in litter (Skrinjar et al., 1995)*Aspergillus flavus, Fusarium moniliforme* and *Penicillium chrysogenum* were isolated from poultry feed samples accompanied with the production of aflatoxins , T-2 toxin , fumonisin and zearalenon (Hess et al. , 1995). *Trichoderma* and *Phialophora* species could be isolated from decaying matter, foodstuffs, animal tissues and poultry feed (Samson et al, 1996). While Santos et al. (1996) found that *Aspergillus* species occur in 64% of the samples of compound poultry feed and aflatoxin B1 in 5% of the same samples but at values above the legal limits. Rudy (1991) stated that mould contamination in poultry industry begins from broiler incubators and he isolated mainly *Aspergillus* species from incubators and dead chicks affected with *aspergillosis*. Grewal (1988) found that chicken manure harbor harmful fungal flora, thereby acting as a major source of contamination in poultry meat industry.

These filamentous fungi and their mycotoxins may enter the gastrointestinal tract in association with ingested food and can cause serious problems for the organism. Chicken fed on mouldy feeds had low weight gain and showed hepatotoxicosis due to mycotoxic contamination. Aflatoxins, zearalenone and T-2 toxins were mainly detected in such feed (Bocarov-Stancic et al. 1995 and Khan et al, 1998). Many mycotoxins that contaminate poultry feed are potent neurotoxins, carcenogens and may cause degenerative changes in liver, spleen and kidney (Atlas, 1995).

Some species of fungi need more experiences during their identification by classical methods. Right now with this revolution in the molecular techniques by using polymerase chain reaction techniques, those problematic strains did not need so much effort to do identified well. Random PCR approaches are being increasingly used to generate molecular markers which are useful for taxonomy and for characterizing fungal populations. RAPD-PCR assays have been used extensively to define fungal populations at species, intraspecific, race and strain levels. In general, most studies have concentrated on intraspecific grouping, although others have been directed at the species level.

Genus *Eurotium* is an important in this study because some species of this genus well known as mycotoxin producers. So the genus was subjected to be confirmed by using RAPD – PCR techniques. This method has been employed by Yuan et al. (1995) to discriminate between *Aspergillus sojae* and *A. parasiticus*. *Trichoderma* as a genus was subjected for analysis by PCR-fingerprinting techniques by several researchers (Arisan-Atac et al., 1995; Kuhls et al., 1995; Meyer et al., 1992; Turner et al., 1997 and Lieckfeldt et al., 1998).

Therefore, this study was planned to evaluate the mould contamination in chicken- viscera (gizzard, heart, intestine, liver and spleen) in slaughter- houses by conventional methods. Furthermore, species of *Aspergillus glaucus* (anamorph. *Euorotium*) group and *Trichoderma* strains were subjected to further identification by using RAPD-PCR technique.

MATERIALS and METHODS

A large number of different methods has been developed for examination of mycoflora in foods based on media, water activity and temperature. In order to find optimal detection and isolation media for food-borne fungi, lower water activity, lower temperature and higher carbohydrate level should be kept than bacteriological media. The following conventional standardizing methods were carried out according to Samson et al. (1996).

1.Sampling:

A total of 100 samples of chicken-viscera collected from different poultry-slaughtering plants in Austria; 20 each of gizzard, heart intestine, liver and spleen. The samples transported in an insulated ice bag to the laboratory without delay. Ten-fold dilutions up to 10^6 using sterile peptone water (0.1%) were prepared. Malt extract and Czapeck's-Dox agar (pH: 4.5) used for plating. The plates incubated at 25° C for 5 – 7 days and examined daily for detection of mould colonies.

2-Isolation and identification:

Detected colonies in Petri- dishes were inoculated with sterile mycological needles into sterile slope agar (2% Malt extract agar, pH: 7.0) and incubated at 25°C for 5 days. The summation of inoculated Malt extract slopes multiplied by correspondent dilutions were expressing the total mould count per one gram (TMC/g) of the sample. Identification of mould species carried out on pure cultures based on 3-point method and slide-culture technique. These methods of differentiation between mould species mainly depending on their taxonomic information and morphology of the colony, as well as, pigmentation of the reverse surface and fungus structure, according to Rippon (1982) and Samson et al. (1996).

3- Molecular technique:

The strains of *Eurotium* group (anamorph : *Aspergillus glaucus*) and *Trichoderma* were the majority of mould species isolated from chicken-visceral samples, therefore they subjected to further identification with the aid of RAPD-PCR analysis as follows:

a- DNA extraction:

Trichoderma strains were cultured in 100 ml. Erlenmeyer-flasks containing 25 ml (per liter: 10 g pepton (Difco); 2.8 g ammonium sulfate; 4 g KH_2PO_4; 10 g Na_2HPO_4, 10 ml of amplified Czapek conc.: 7 g $MgSO_4$; 0,05 g $CuSO_4$; 0.1 g $FeSO_4$ $7H_2O$; 0.1 g ZnS O_4 $7H_2O$, the final pH was adjusted to 5.0) while *Eurotium* strains were cultures in flasks containing 25 ml (per liter : 1g K_2HPO_4; 10 ml Czapek concentrate, 5 g yeast extract and 200 g sucrose) for one week using a rotator shaker (30°C at 150 rpm). The mycelium was collected by filtration and ground to fine powder in a liquid N2. Fifty mg. of the powder transferred to 1.5 ml. Eppendorf tube and mixed with 700µ / 2 X CTAB buffer .The tubes incubated at 65°C for 30 min., then 700µ of chloroform were added and the mixture vortixed briefly. The resulting mixture centrifuged at a maximum speed of 500 rpm for 30 min. and the cleared supernatant was mixed with 600µ of isopropanol chilled to -20°C. The mixture was centrifuged at the maximum speed of 500 rpm for 5 min. and the resulting pellet washed twice with 1 ml of 70% ethanol the pellet was dried under vacuum and dissolved in 100µ TE (10 mM Tris, 1 mM EDTA,pH 7.5) buffer. The DNA concentrations were evaluated by agarose gel electrophoresis.

b- RAPD-amplification:

PCR conditioned and separation of RAPD-PCR fragments were carried out according to Messner et al. (1994). Using the primers ofV6(5'dTGCAGCGTGG; Lopandic et al., 1996) and M13 (5' dGAGGGTGGCGGTTCT K.O'Donnell et al., 1999).Synthesis of primers performed by (Codon Genetical Systems, Vienna, Austria), using a model 392 DNA synthesiser (Applied Biosystems, Foster city, CA, USA). The temperature profile of primers was subjected for denaturation at 98°C for 15 sec.; annealing at 40°C for 90 sec. and extension at 72°C for 100 sec. to a total of 40 cycles.

Results and Discussion:

1- Total mould count

It was found that mean values of total mould counts per one gram (TMC / g) in examined samples of gizzard, intestine and liver were 2.6 x 10^4, 4.4 x10^5 and 1.5 x 10^2 , respectively ,while moulds not detected in all samples of heart and spleen, as recorded in Table (1) . The obtained counts declared that intestine and gizzard were heavy contaminated with moulds than other visceral organs collected from poultry-plants and this may be attributed to the entry of moulds into the gastrointestinal tract in association with ingested poultry feed (Hess et al., 1995 and Khan et al. , 1998).

2-Isolated mould species:

Results shown in Table (2) revealed that 85 mould strains belonging to 9 genera were isolated from the examined chicken-viscera , the main of which were *Eurotium* 17 (20.0%) and *Trichoderma* 12 (14.1%) species. Moreover, some species of *Absedia*, *Fusarium*, *Rhizopus*, *Thamnidium*, *Mucor*. *Alternaria* and *Phialophora* were detected in lower percentages.

Frequency of the identified strains of *Eurotium* (anamorph: *Aspergillus glaucus*) group in this study were; *E. amstelodami* (5.9 %), *E. chevalieri* (9.4 %), *E. herbariorum* (2.4 %) and *E. rubrum* (2.4 %), as declared in Table (2) and Fig. (1, 2, 3 and 4). These fungi are xerophilic, optimally growing on low water activity, and highly toxic for man and animal (El-Kady et al., 1994 and Samson et al. 1996). It was found that species of *E. amstelodami* , *E. chevalieri* and *E. rubrum* have the ability to produce sterigmatocystine which may cause hepatoma and cholangiocarcinoma in animals (Davis , 1981 and Sabah , 1987). While, Leitao et al. (1987) stated that some strains of *E. chevalieri* , *E. ruber* and *E. repens* isolated from poultry feed could produced aflatoxins B_1, B_2, G_1 and G_2 . Aflatoxins are considered as potent carcinogens and known to cause death in sheep and cattle, as well as, may be involved in some human disease conditions (Atlas et al., 1995).

Trichoderma spp. which isolated in this study included *T. harzianum* (9.4 %) and*T. viride* (4.7%), as revealed in Table (2) and Fig. (5). These species are usually growing rapidly on mycological media with green shades due to conidium production. During their growth, they can produce toxic metabolites mainly trichodermin and gliotoxin which have a deleterious effect on respiratory process and cell membrane function, as well as, they can produce pulmonary oedema, hepatic lesions and renal dysfunction (Mortimer, 1970 and Domsch et al., 1993).

The saprobic fungus *Absedia corymbifera* which also growing rapidly and covering the whole Petri-dish within one weak, was isolated from examined organs in a frequency of 12.9 %, as tabulated in Table (2). The fungus is widely distributed in nature; in soil, stored grains, poultry feed, air, animals and man (Hesseltine and Ellis, 1973, O'Donnell, 1979 and Von Arx, 1981).

Fusarium colonies usually growing fast, pale or bright-colored in yellow, brownish, pink, reddish, violet or lilac shades. The isolated species in this study were *Fusarium poae* (7.1 %) and *F. oxysporium* (3.5%). These strains are highly toxic for human and animals because of their ability in production of several types of mycotoxins as fuminisins and trichothecenes. Fumonisins have been associated with lung oedema in animals and human oesophageal cancer in South Africa (Nelson et al., 1993). Trichothecenes have been implicated in haemorrhagic syndrome in animals and human disease epidemics in Eastern Europe and Japan (Marasas et al., 1984).

Rhizopus fungus belonging to Zygomycetes group which are heterothalic and saprobic, though some species attack plants, animals and humanbeings. Two species were isolated from the visceral examined samples; *R.stolonifer* (2.4 %) and *R.oryzae* (2.4 %).

Thamnidium eligans is belonging to Zygomycetes group and has a minimal available moisture (a_w) of 0.94 (Frizavd and Samson, 1991). The fungus were isolated from the examined samples in frequency of 9.4 % of the total isolated moulds as indicated in Table (2)

Mucor racemosus which detected in frequency of 8.2 % of the isolated strains, is widely distributed in soil, food, poultry feed and animal tissues. It is usually appear as white colony on media, becoming brownish-gray with age (Hanlin, 1973).

Alternaria alternata was identified in a rate of 8.2 %, as tabulated in Table (2). It is a common saprophytic fungus which can grow at a minimal a_w 0.85 and a wide range of temperature –5 to 36° C, thereby it could be isolated from many kinds of foodstuffs, soil and air (Ellis, 1971 and Domsch et al. , 1993).

One species of *Phialophora* (*P. fastigata*) isolated from the examined samples in lower percentage (2.4 %). The fungus could be also detected in foodstuffs, animal tissues and diseased human (Schol-Scwarz, 1970 and Domsch et al., 1993).

3-RAPD-PCR analysis:

Figs. (1,2,3,4 and 5) indicated that all studied *Eurotium* group (*E. amstelodami*, 5 strains; *E. chevalieri*, 8; *E. herbariorum*, 2 and *E. rubrum* 2) and *Trichoderma* strains (*T. harzianum*, 8 strains and *T. viride*, 4) were identical with their corresponding type strains stored in culture collection of the Institute of Applied Microbiology (IAM), Univ. of Agricul. Scien., Vienna. Theses results confirmed the identification with conventional methods, as tabulated in Table (2). The same results were obtained by Lieckfeldt et al. (1999) who distinguished two types of *Trichoderma viride* by using PCR fingerprinting.

The obtained results in this study declared that fungal contamination of the chicken-viscera, particularly intestine and gizzard, frequently occurred in poultry-slaughtering plants. Such contamination may be attributed to the pollution of poultry-feed or poor hygienic measures adopting during slaughtering, evisceration and handling of the carcasses. The direct hazard to human health resulting from consumption of poultry meat contaminated with moulds or their mycotoxins needs to be carefully controlled. Therefore, rigid attention for hygienic design, cleaning of equipment and sanitation procedures and for eliminating sources of contamination in the processing environment are usually sufficient to avoid a significant build-up of fungi during poultry processing

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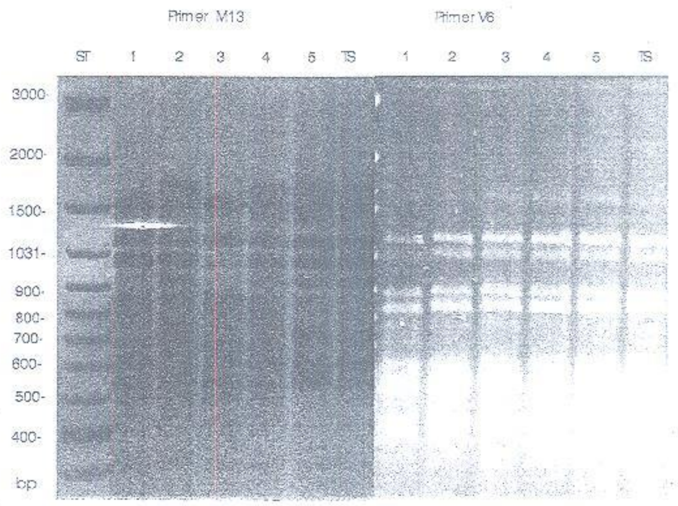
Table 1: Total moulds count/1 g from 100 samples of chicken-viscera from different poultry slaughtered plants.

Sources	Gizzard	Intestine	Liver
Minimum	2.1×10^4	3.1×10^5	1.1×10^2
Maximum	3.1×10^4	5.7×10^5	1.9×10^2
Mean	2.6×10^4	4.4×10^5	1.5×10^2
Stand. error			
Stand. deviation			

Table 2: Frequencies of isolated mould species from 100 samples of chicken-viscera.

Samples / Mould sp.	Gizzard		Intestin		Liver		Total	
	No.	F%	No.	F%	No.	F%	No.	F%
Absidia corymbifera	4.0	4.7	5.0	5.9	2.0	2.4	11.0	12.9
Alternaria alternata	0.0	0.0	4.0	4.7	3.0	3.5	7.0	8.2
Eurotium								
E. amstelodami	0.0	0.0	3	3.5	2.0	2.4	5.0	5.9
E. chevalieri	2.0	2.4	6	7.1	0.0	0.0	8.0	9.4
E. repens	0.0	0.0	2	2.4	0.0	0.0	2.0	2.4
E. rubrum	.1.0	1.2	0.0	0.0	1.0	1.2	2.0	2.4
Fusarium								
F. poae	2.0	2.4	1.0	1.2	3.0	3.5	6.0	7.1
F. oxysporum	3.0	3.5	0.0	0.0	0.0	0.0	3.0	3.5
Mucor racemosus	3.0	3.5	3.0	3.5	1.0	1.2	7.0	8.2
Phialophora fastigiata	2.0	2.4	2.0	2.4	1.0	1.2	5.0	5.9
Rhizopus								
R. oryzae	0.0	0.0	2.0	2.4	0.0	0.0	2.0	2.4
R. stolonifer	3.0	3.5	4.0	4.7	0.0	0.0		
Thamnidium elegans	2.0	2.4	4.0	4.7	2.0	2.4	8.0	
Trichoderma								
T. harzianum	0.0	0.0	8.0	9.4	0.0	0.0		
T. viride	4.0	4.7	0.0	0.0	0.0	0.0		
Total	26.0	30.6	44.0	51.8	15.0	17.6	85.0	100

[a] moulds not detected at all samples of heart and spleen.



Fig(1) RAPD patterns of *Eurotium amstelodami* isolates primed by M13 (5' dGAGGGTGGCGGTTCT K.O'Donnell *et al.*, 1999) and V6 (5'dTGCAGCGTGG: Lopandic *et al.*, 1996) comparing with type strain (VIAM1067).

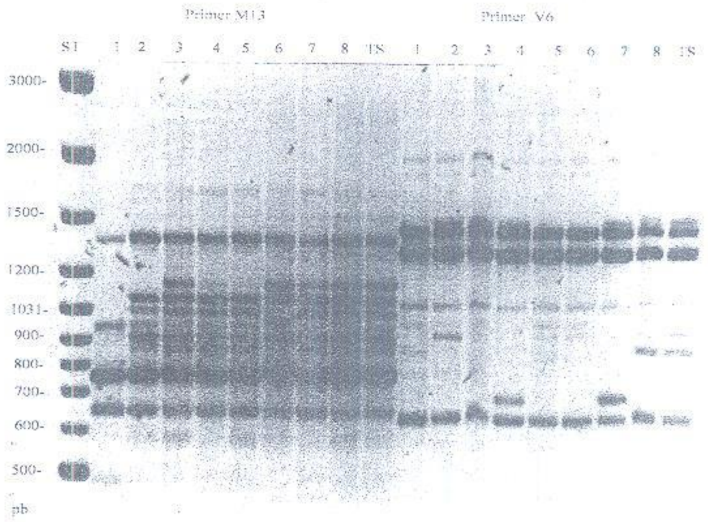


Fig.(2) RAPD fragments of *Eurotium chevalieri* isolates primed by M13 (5' dGAGGGTGGCGGTTCT K.O'Donnell *et al.*, 1999) and V6 (5'dTGCAGCGTGG; Lopandic *et al.*, 1996) comparing with type strain (VIAM541).

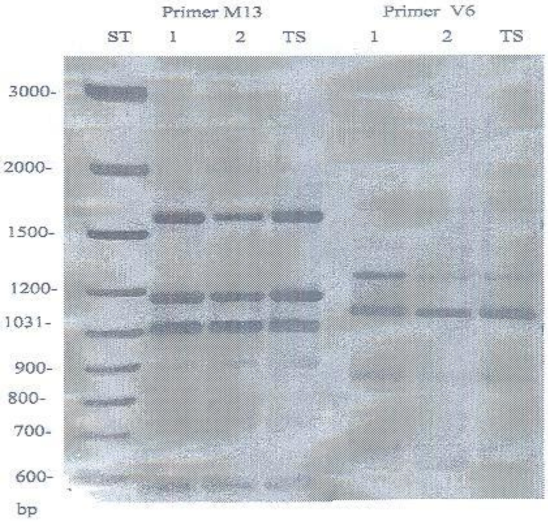


Fig. (3) RAPD patterns of *Eurotium herbariorum* isolates primed by M13 (5' dGAGGGTGGCGGTTCT K.O'Donnell *et al.*, 1999) and V6 (5'dTGCAGCGTGG; Lopandic *et al.*, 1996) comparing with type strain (VIAM 542).

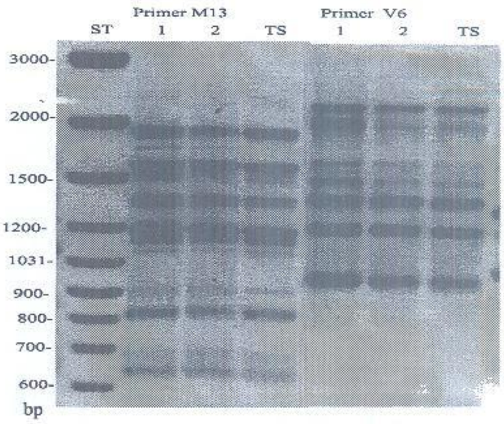


Fig. (4) RAPD fragments of *Eurotium rubrum* isolates primed by M13 (5' dGAGGGTGGCGGTTCT K.O'Donnell *et al.*, 1999) and V6 (5'dTGCAGCGTGG; Lopandic *et al.*, 1996) comparing with type strain (VIAM69).

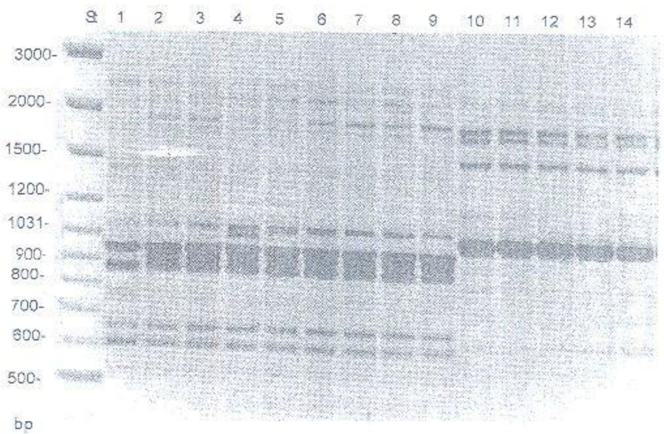


Fig 5. RAPD isolates of strains primed by M13 (5' dGAGGGTGGCGGTTCT K.O'Donnell *et al.*, 1999) and V6 (5'dTGCAGCGTGG; Lopandic *et al.*, 1996). Lanes 1-8 *Trichoderma harzianum* isolates, lane 9 is *T. harzianum* type strain (VIAM 3241), lanes 10-13 *T. viride* isolates and lane 14 is *T. viride* type strain (VIAM 3009).