Trichoderma pseudokoningii Rifai isolation from Egyptian immunocompromised cattle with *Mycobacterium bovis* infection

Helmy Ahmed Torky[1]
[1]*Department of Microbiology, Faculty of Veterinary Medicine, Alexandria University, Egypt.*
&
Eman Khalifa Sedeek khalifa[2]
[2]*Department of Microbiology, Animal Health Research Institute, Alexandria, Egypt. Correspondence should be addressed to Eman, K.; email: dr_eman_vet@yahoo.com*

Cell phone number: 01002546397
Work Number: 03/4950528
Fax number: 03/4950528
Postal address: Egypt, Alexandria, Animal Health Research Institute, Alexandria station of rail way.

Trichoderma pseudokoningii Rifai isolation from Egyptian immunocompromised cattle with *Mycobacterium bovis* infection

Helmy Torky[1] & Eman Khalifa[2]
[1]*Department of Microbiology, Faculty of Veterinary Medicine, Alexandria University, Egypt.* [2]*Department of Microbiology, Animal Health Research Institute, Alexandria, Egypt. Correspondence should be addressed to Eman, K.; email: dr_eman_vet@yahoo.com*

Recently, *Trichoderma* species are emerging as potent fungal pathogens in immunocompromised human. We report first three cases of *Trichoderma pseudokoningii* Rifai pulmonary infection in the Egyptian tuberculous dairy cattle with *Mycobacterium bovis,* from heart of a generalized bovine TB in a cow over 5 years old, mediastinal lymph node of pulmonary TB in a cow of 3 years old, and lung of mixed pulmonary and digestive BTB in a cow of 4 years old. As well as we have developed a pathogenisity test technique for *Trichoderma pseudokoningii* Rifai infection in 3 G. pigs by intraperitoneal injection of 2 G. pigs with mixed infection of *Mycobacterium bovis* and *Trichoderma pseudokoningii* Rifai; death of both animals 14 days, thereafter, and by injection of 1 G. pig with single infection of *Trichoderma pseudokoningii* Rifai; death of animal 21 days, thereafter. We did not report any animal case along review of literature.

Opportunistic fungal infections have increased in recent years in immunocompromised patients[1,2]. *Trichoderma* strains have been detected on the skin, in the lung and as causative agents of peritonitis in peritoneal dialysis patients[3,4]. Six species of the genus *Trichoderma* (*T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. pseudokoningii*, *T. citrinoviride*, and *T. viride*) that have been identified as etiologic agents of infection in immunocompromised hosts. That *T. longibrachiatum* is the main human pathogen species within the genus[6].

TB infection co-exists with an alteration in the immune system[7] and disseminated fungal infections increased in immunocompromised patients[8], significantly when the immune system of infected individual becomes suppressed[9].

For the first time , here we report isolation and identification of 3 strains of *Trichoderma pseudokoningii* Rifai from pulmonary infection in tuberculous dairy cattle with *M. bovis*, and developed a model for

studying the effect of co-infection of *Trichoderma pseudokoningii* Rifai and *M. bovis* in 2 G. pigs by intraperitonial injection of *M. bovis*[10], and *Trichoderma pseudokoningii* Rifai, which revealed acceleration of pulmonary lesions formation and death of both G. pigs throughout 14, and in single infection of *Trichoderma pseudokoningii* Rifai in 1 G. pig, which died 21 days post injection.

RESULTS

BTB prevalence in Egyptian dairy cattle

Out of 1350 cattle examined by single intradermal cervical tuberculin test/bovine purified protein derivatives (SICTT/B-PPD), 32 (2.4%) were positive, of them 22 (68.75%) showed visible lesions (VL), while 10 (31.25 %) showed non visible lesions (NVL).

Mycobacteriological examination of the collected tissue and lymph nodes from slaughtered SICTT positive cattle

Out of 32 slaughtered cattle samples, 10 (31.3%) AFB identified by ZN, 23 (71.9%) positive histopathologically, 17 (53.1%) *Mycobacterium bovis* isolated and identified and confirmed by LCD-array, M-PCR, and genotyping.

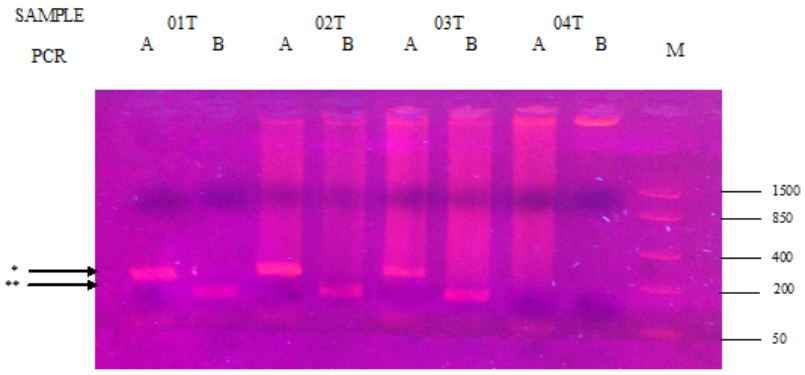

Fig. 2 Agarose gel electrophoresis of PCR product DNA following LCD array. * = PCR amplicon Mix A: Genus mycobacterium (ITS region of rRNA cluster) and ** = PCR amplicon Mix B: *M. tuberculosis* complex (IS6110 Element of M. tuberculosis complex), M: DNA 50bp marker. 01T (Mesentric L. N.), 02T (Lung 1), 03T (Liver 2), and 04T (Lung 2).

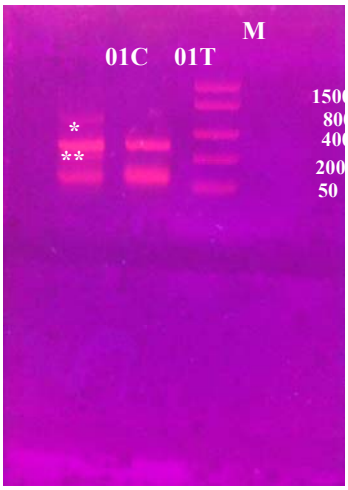

Fig. 3 Identification of AFB by M-PCR. DNA extracted from pooled AFB isolates and intestinal L. N. tissue samples were used as template for m-PCR amplification of the RvD1Rv2031c and the IS6110 sequences. Amplification products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide (10 µg/ml).Lane M: 50 bp DNA ladder (GenLadderTM 50s, inno-train); lane 01C and lane 01T: m-PCR products of AFB isolated from suggestive TB lesions (pooled culture sample and from pulmonary TB tissue sample, respectively), *: Indicated the position of the fragments of 500 bp (diagnostic for *M. bovis*), while **: Indicated the position of the 245 bp fragment (diagnostic for M. tuberculosis complex members). It was noted that there were some differences in the intensity of the bands among different isolates which may be due to difference in the genomic DNA extraction or due to impurities of the extracted DNA.

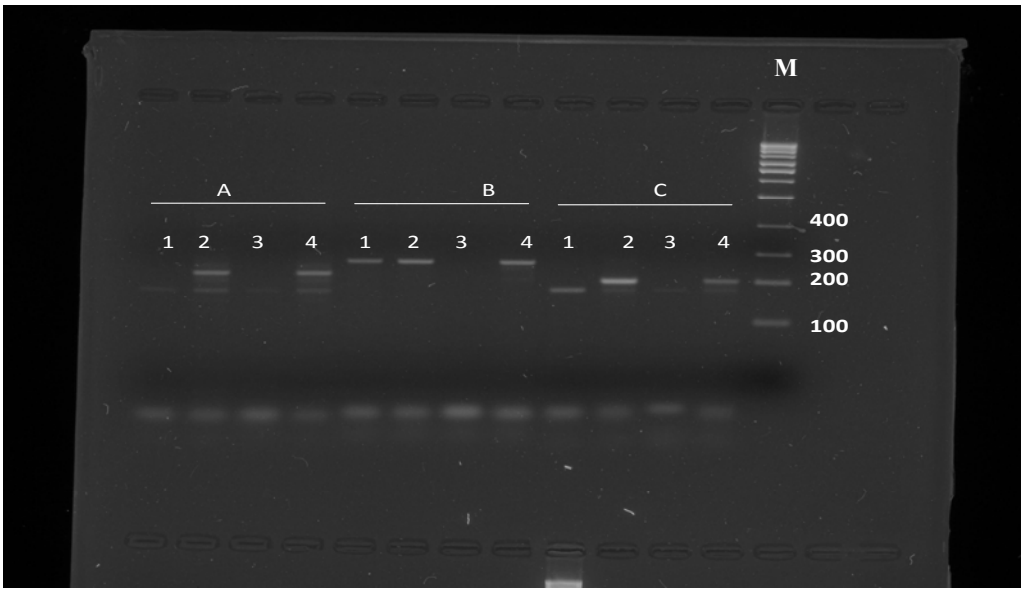

Fig. 4 Confirmation of *M. bovis* by genotyping of random 4 isolates recovered from tuberculin reactor cattle (Miru 26, VNTR 3232, and Miru 24). The number 1- 4 indicates the strain number and the letters A-C indicate the PCR reaction where A`- Miru 26, B- VNTR 3232, and C—Miru24. M (100 bp DNA marker).

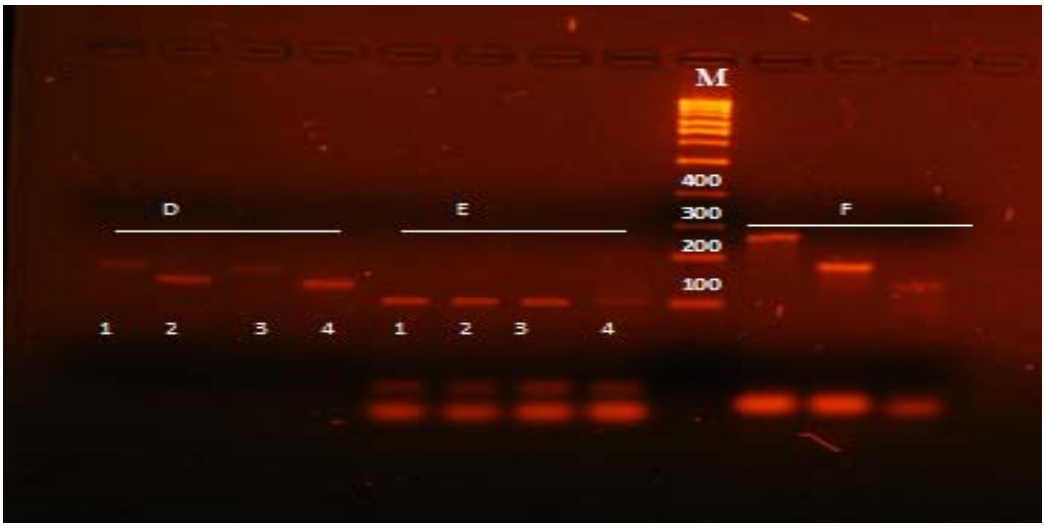

Fig. 5 Confirmation of *M. bovis* by genotyping of random 4 isolates recovered from tuberculin reactor cattle (QUB4156c and QUB 1451). The number 1- 4 indicates the strain number and the letters D-F indicate the PCR reaction where D- QUB 4156c, E- QUB 1451, while F group was done as a random repeat control for the results and to compare the different lengths beside the 100 bp DNA marker.

Mycological examination of the collected tissue and lymph nodes from slaughtered SICTT positive cattle

Out of 32 slaughtered cattle samples, we have recorded the isolation and identification of the following fungi: *Trichoderma pseudokoningii* Rifai 3 isolates (Fig. 6 & 7), *Penicillium aurantiogriseum* Dierckx 1 isolate, *Aspergillus fumigatus* 2 isolates, and *Candida albicans* 9 isolates. The 3 strains of *Trichoderma pseudokoningii* Rifai and 1 strain of *Penicillium aurantiogriseum* Dierckx were sent to Assiut University Mycological Center (AUMC) for confirmation of identification and have been deposited in the culture collection of AUMC Center, under AUMC numbers of 7986, 7988, 7989, and 7987, respectively.

Fig. 6: Macroscopic appearance of *Trichoderma pseudokoningii* Rifai (surface).

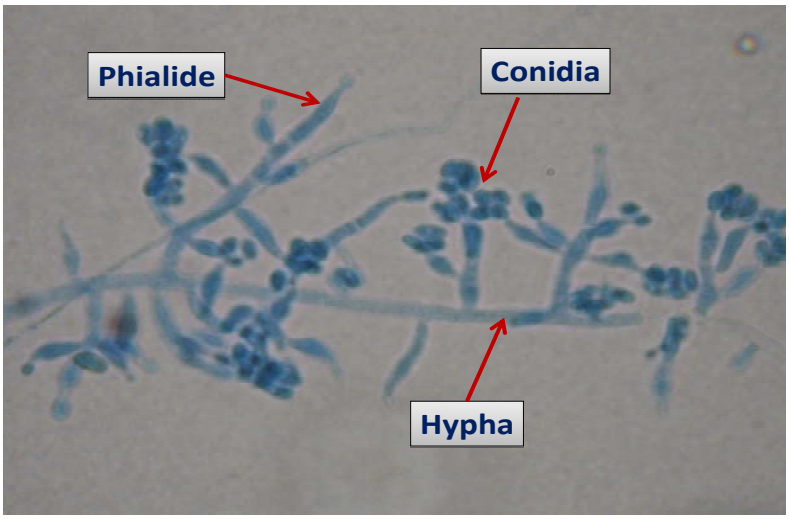

Fig. 7: Microscopic appearance of *Trichoderma pseudokoningii* Rifai (phialides in groups topped by mass of conidia, original magnification X400).

Pathogenisity test:

Although literatures of pathogenisity test of *Trichoderma pseudokoningii Rifai* in experimental animals have not been available yet, we could perform the pathogenisity test in 3 G. pigs by our technique. Where two G. pigs infected with mixed (*M. bovis* and *Trichoderma*), after mixed infection, 2 animals showed no increase in body weight; although they were eat very well; at 18th day from the first dose both of them died, PM examination revealed that the progress of formation of tuberculous granuloma, as well as congestion of lungs, heart, pleura, and liver. *M. bovis* and *Trichoderma* re-isolated and re-identified (retro-culture). **Fig. 8** and **Fig. 9** showed the histopathological result of both infections.

While about the 1 G. pig injected with *Trichoderma* only, after the infection, the animal showed no increase in body weight; although they were eat very well; at 24th day from the first dose it died, PM examination revealed that congestion of lungs, heart, pleura, and liver. *Trichoderma* re-isolated and re-identified (retro-culture).

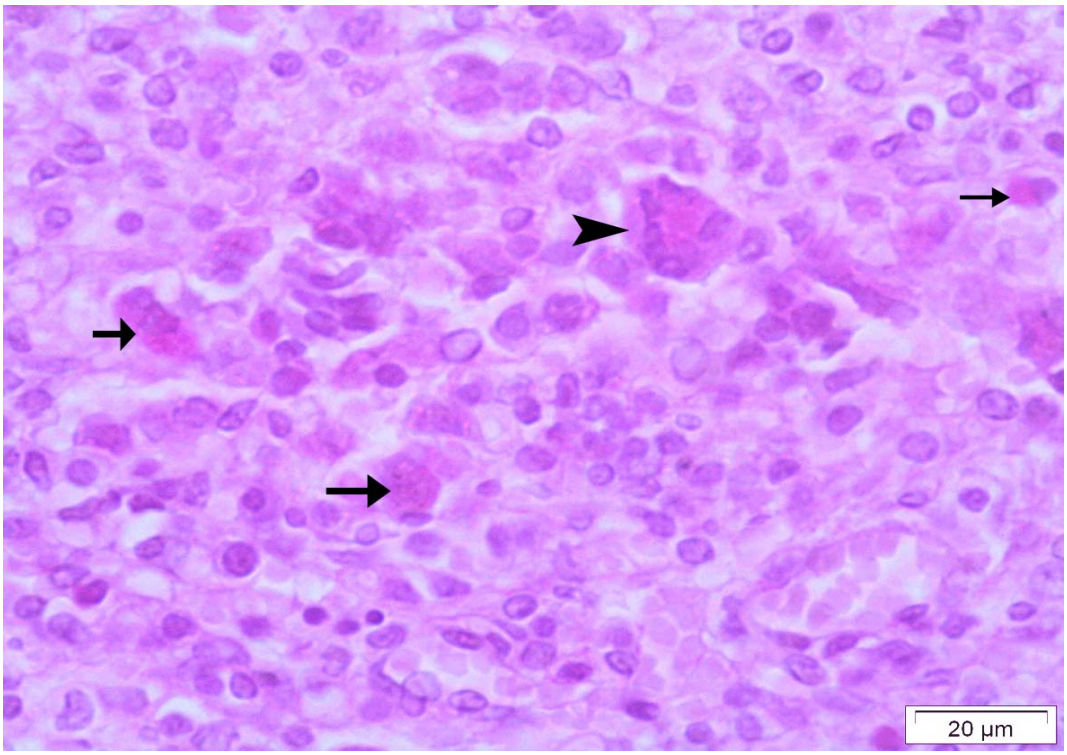

Fig. 8 Light micrograph of lung tissue showing granulomatous reaction containing Langerhan's giant cell (arrowhead) as well as PAS reacting fungal particles (arrow). PAS stain counter by hematoxylene (Mixed infection).

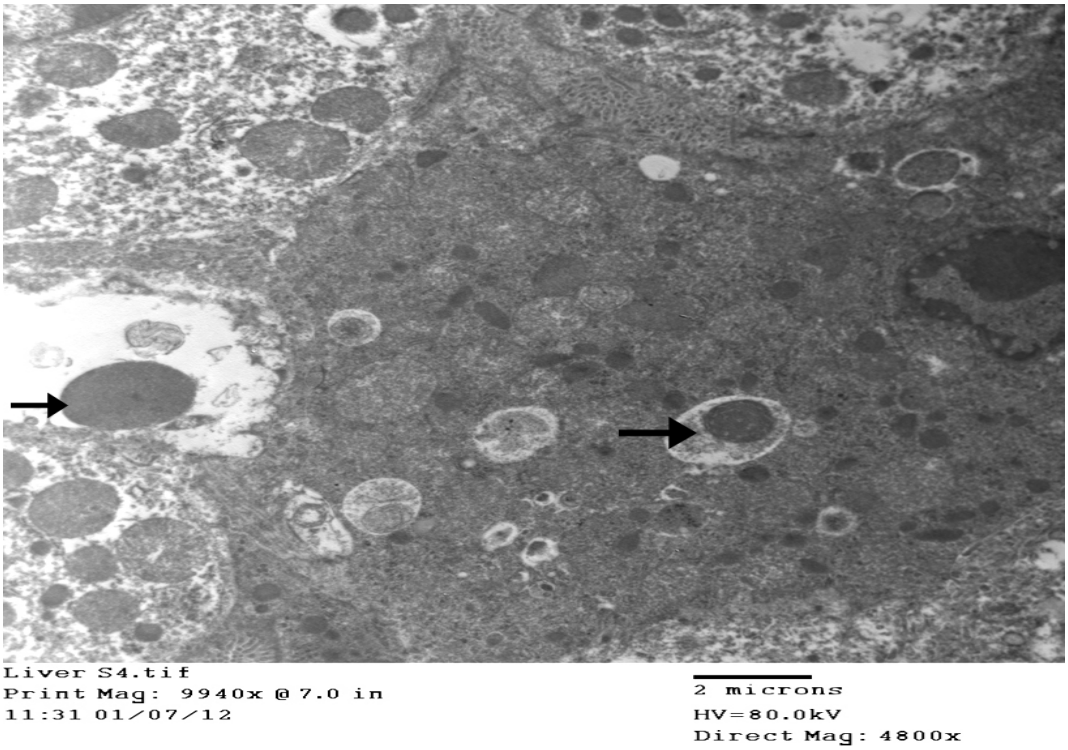

Fig. 9 Transmission electron micrograph of the liver tissue showing presence of spherical electron dense particles in the sinusoidal lumen as well as in the hepatic cell (arrow) seems to be a fungal particle (Mixed infection).

DISCUSSION

Opportunistic fungal infections have been observed with increasing frequency in recent years in immunocompromised patients. Several data were published in the last decade about the clinical importance of the filamentous fungal genus *Trichoderma*, indicating that *Trichoderma* strains besides their agricultural and biotechnological importance may be potential opportunistic pathogens in immunocompromised hosts as well[11].

Trichoderma longibrachiatum, a filamentous fungus, was recently described as an emerging pathogen in immunocompromised patients**.** As recorded in invasive lung infection by *T. longibrachiatum* in a patient with hematologic malignancy. The infection mimicked invasive pulmonary aspergillosis and was successfully treated with a combination of voriconazole and caspofungin[12].

Trichoderma species are filamentous fungi that were previously considered to be culture contaminants. Recently, with the increasing number of risk population, they are described as an emerging pathogen in immunocompromised patients. *Trichoderma longibrachiatum* is the most common species involved in *Trichoderma* infection. *Trichoderma longibrachiatum* skin infection in a renal transplant recipient has been recorded[13].

Tuberculosis in cattle and other domestic animals is above all caused by two members of *Mycobacterium tuberculosis* complex (MTC): *M. bovis* and *M. caprae*[14-16]. However, occasional occurrence of tuberculosis due to *M. tuberculosis* species with concurrent tuberculous lesions has supported by the Ministry of Agriculture of the Czech Republic (Grant No. MZE 0002716201). And the genotypic detection of insertion element IS6110 is a reliable assay to identify species that belong to MTBC and has been applied to studies of MTBC infections in cattle, although the identification of IS6110 by PCR fails to differentiate *M. bovis* from *M. tuberculosis*[17].

The guinea pig is well known to be a susceptible animal model to TB that develops granulomas with prominent central caseation and extensive connective tissue deposition, yet knowledge of the arrangement of these elements over time remains obscure. In addition, the influx of T-cell subsets, only recently described in the mouse model**,** has yet to have been documented in the guinea pig[18].

Our findings on G. pigs indicated that *Trichoderma pseudokoningii* Rifai is a pathogenic mould for cattle and its co-existence with TB accelerated the progress of TB.

With the increased incidence of bovine tuberculosis (BTB), as well as its zoonotic nature, its complexity could be placed on both mycobacteria and associated systemic mycosis as primary or secondary causative agent

of the disease, since a limited number of studies has been carried out to review their association in the tuberculous-like lesions.

The immune response plays a fundamental role in the outcome of *Mycobacterium tuberculosis* complex infection. It is clear that the immune system reacts efficiently in the vast majority of infection. This is particularly evident in the case of TB, where most cases infected by the tubercle bacillus (~ 90%) do not develop the disease throughout their lifetime. Nevertheless, the risk of developing the disease increasing considerably when TB infection co-exists with an alteration in the immune system.

Currently, due to the upsurge of systemic mycotic infection, the epidemiology of tuberculosis has been greatly affected as many systemic mycotic infected cattle are co-infected with tuberculosis, the incidence of the disease may rise in coming years.

Although the correlation between in vivo lab animal histopathological results and that in cattle is poor yet, it may reflect the synergistic action of both microorganisms in co-working together in immunocompromised cattle.

METHODS

1350 Egyptian dairy cattle were examined by SICTT according to laws of General Organization of Veterinary Services (GOVS); positive reactors were slaughtered then tissue samples from tuberculous-like lesions from (lung, trachea, heart, pleural membrane, liver, kidney, spleen, intestinal mucosa, ovaries, and peritoneal membrane and their corresponding lymph nodes were collected and examined mycobacteriologically and mycologically for the evidence of *Mycobacterium* species as well as fungal elements. For *Trichoderma* species isolation; the culture of the speciemen was made on Sabouraud chloramphenicol agar (BioMerieux) at 30^{o}C and after 10 days yielded a filamentous fungus. It was identified as *Trichoderma* spp. on the basis of their macroscopic morphology (flat green colonies) and microscopic characteristics (septate hyphae, branched at right angles, with short conidiophores, phialides in groups of 2-4, flask-shaped, widely splaying out, chlamydospores present) (Fig. 6 &7)[28]. Thereafter, pathogenisity test performed by injection of mixed infection of both *M. bovis* and *Trichoderma pseudokoningii* Rifai in two Guinea pigs, and by single infection with only *Trichoderma pseudokoningii* Rifaiin one Guinea pig.

Mycobacteriological examination

Samples prepared (ref. 19), cultivated onto modified Lowenstein-Jensen medium (ref. 20), the yielded isolates identified morphologically (ref. 21), then isolated mycobacteria stained by ZN stain (ref. 20).

Histopathological examination

Collected samples were examined histopathologically (ref. 22).

PCR examination

Tissue samples as well as yielded *Mycobacterium* isolates processed (ref. 23) and examined by LCD-array (ref. 24), then by M-PCR (ref. 25), and finally by Genotyping (ref. 26).

Mycological examination

Samples prepared and cultivated (ref. 27), then mould and yeast identified (ref. 28, 29, 30, 31& 32), *Trichoderma* was identified by key features based on the types of branching systems of conidiophores, the phialide disposition, and the character of the phialospores (ref. 33 & 34), then yeast confirmed by PCR (ref. 35).

Pathogenisity test

Injection of *M. bovis* (ref. 10), and in this investigation we used our technique in injection of *Trichoderma pseudokoningii* Rifai, singly or mixed with *M. bovis*.

Preparation of spore suspension (CFU: Colony Forming Unit=Spore):

The isolates were subcultured on Sabouraud Dextrose Agar slants, on which the fungus is able to sporulate readily, and incubated at 35°C. Inoculum suspensions prepared from fresh, mature (10 day old) cultures. Cover colonies with approximately 5 ml of sterile water supplemented with 0.1% Tween 20. Then, the conidia were rubbed carefully with a sterile cotton swab (Collection swab; EUROTUBO, Madrid, Spain) and transferred with a pipette to a sterile tube. The resulting suspensions were homogenized for 15 seconds with a gyratory vortex mixer at 2,000 rpm (MS 1 Minishaker; IFA, Cultek, Madrid, Spain). Appropriate dilutions were performed in order to get the right concentration for counting in a haemocytometer chamber (Neubauer chamber; Merck S.A., Madrid, Spain). All inoculum preparations were checked for the presence of hyphae or clumps. If a significant number of hyphae was detected (> 5% of fungal structures), the 5 mL suspension transferred to a sterile syringe attached to a sterile filter with a pore diameter of 11 μm (Millipore, Madrid, Spain), and filtered and collected in a sterile tube. This step removes hyphae and yields a suspension composed of spores. If clumps were detected, the inoculum was shaken again in the gyratory vortex mixer for further 15 seconds. This step was repeated as many times as necessary, until clumps are no longer encountered. The suspension adjusted with sterile distilled water to a concentration of 2-5x10^6cfu/mL by counting the conidia in a haemocytometer chamber. Then the suspension diluted 1:10 with sterile distilled water to obtain a final working inoculum of 2–5 x10^5 cfu/mL (ref. 36, 37 & 38).

MIXED (*M. BOVIS* AND *TRICHODERMA*) INFECTION:

Mixed dose= 0.5 ml of *M. bovis* solution (2g colony diluted in 10 ml saline) plus 0.5 ml of *Trichoderma* spore solution.
Single dose= 0.5 ml of *Trichoderma* spore solution.
Intranasal inhalation with *Trichoderma* spore.
1st day: 2 G. pigs (250g) were injected by mixed dose intraperitonially. As well as intranasal inhalation in each side were applied.
4th day: given single dose (intraperitonially plus intranasal inhalation).

SINGLE (*TRICHODERMA*) INFECTION:
Single dose= 0.5 ml of *Trichoderma* spore solution.
Intranasal inhalation with *Trichoderma* spore.
1st day: 1 G. pig (250g) was injected by the 1st single dose intraperitonially. As well as intranasal inhalation in each side were applied.
4th day: given 2nd single dose (intraperitonially plus intranasal inhalation)
22nd day: given (intranasal inhalation only).
23rd day: given (intranasal inhalation only).

ACKNOWLEDGMENTS
We thank A. Nafady for help with histopathological sections. Professor Doctor of Pathology. Faculty of Veterinary Medicine. Assiut University. Egypt.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

---

1. Rex, J. H., Walsh, T. J. & Anaissie, E. J.: Fungal Infection in iatrogenically compromised hosts. Adv. Int. Med. **(43)**, 321– 371 (1998).
2. Fleming, R. V., Walsh, T. J. & Anaissie, E. J. Emerging and less common fungal pathogens. Infect. Dis. Clin. North. Am. **(16)**, 915–933 (2002).
3. Rota, S., Marchesi, D., Farina, C. & de Bievre, C. *Trichoderma pseudokoningii* peritonitis in automated peritoneal dialysis patient successfully treated by early catheter removal. Perit. Dial. Int. **20**, 91–93 (2000).
4. Myoken, Y. *et al*. Fatal necrotizing stomatitis due to *Trichoderma longibrachiatum* in a neutropenic patient with malignant lymphoma: a case report. Int. J. Oral Maxillofac. Surg. Dec; 31**(6)**: 688-91(2002).
5. Chouaki, T. *et* al. Invasive Infection Due to *Trichoderma* Species: Report of 2 Cases, Findings of In Vitro Susceptibility Testing, and

Review of the Literature. J. Clin. Infect. Dis. **(35)**, 1360-1367 (2002).

6. Christina, K. *et al*. In vitro activity and synergism of amphotericin B, azoles and cationic antimicrobials against the emerging pathogen *Trichoderma* spp. J. Antimicrobial Chemotherapy. **58**, 1058–1061 (2006).
7. Schlesinger, L. S., Bellinger-Kawahara, C. G., Payne, N. R. & Horwitz M. A. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. J. Immunol., 144 **(7)**, 2771-80 (1990).
8. Miguel, D. D. *et al*. Nonfatal pulmonary *Trichoderma viride* infection in an adult patient with acute myeloid leukemia: report of one case and review of the literature. Diag. Microbiol. and Infec. Dis. **(53)**, 33–37 (2005).
9. Dye, C. Global epidemiology of tuberculosis. Lancet. **367**, 938-940 (2006).
10. Shriniwas. Susceptibility of guinea pig to *Mycobacterium tuberculosis*. Indian J. Tuberculosis; 13 **(1)**: 27-33(1965).
11. Kredics, L *et al*. Clinical importance of the genus *Trichoderma*. Acta. Microbiol. Immunol. Hung. **50**, 105-117 (2003).
12. Alanio, A. *et al*. Invasive pulmonary infection due to *Trichoderma longibrachiatum* mimicking invasive Aspergillosis in a neutropenic patient successfully treated with voriconazole combined with caspofungin. Clin. Infect. Dis. 46 **(10)**, 116-8 (2008).
13. Trabelsi, S., Hariga, D. & Khaled, S. First case of *Trichoderma longibrachiatum* infection in a renal transplant recipient in Tunisia and review of the literature. Tunis Med. Jan. 88 **(1)**, 52-7 (2010).
14. Pavlik, I. *et al*. The last outbreak of bovine tuberculosis in cattle in the Czech Republic in 1995 was caused by *Mycobacterium bovis* subspecies caprae. Veterinarni Medicina. **47**, 251–263 (2002).
15. Prodinger, W. M.; Eigentler, A.; Allerberger, F.; Schonbauer, M. and Glawischnig, W. Infection of red deer, cattle, and humans with *Mycobacterium bovis* subsp. caprae in western Aust. J. Clin. Microbiol. 40, 2270–2272 (2002).
16. Erler, W. *Et al*. Molecular fingerprinting of *Mycobacterium bovis* subsp. caprae isolates from Central Europe. J. Clin. Microbiol. **42**, 2234–2238 (2004).
17. Leite, F. C. Q *et al*. Isolation and identification of mycobacterium from livestock specimens and milk obtained in Brazil. Mem. Inst. Oswaldo Cruz. **98**, 319-323 (2003).
18. Gonzalez-Juarrero, M. *et al*. Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection

with *Mycobacterium tuberculosis*. Infect. Immun. **69**, 1722–1728 (2001).
19. Forbes, B. A., Sahm, D. F. & Weissfeld, A. S. Bailey & Scott□s diagnostic microbiology. Text book. 10th Ed. Mosby. Inc. Pp: 724-740 (1998).
20. Collee, J. G. *et al.* Mackie and McCartney Practical Medical Microbiology 14th Ed., Fourteenth Edition. Churchill Livingstone, New York Edinburgh, London. 838-841(1996).
21. Kent, P. T. & Kubica, G. P. Public health mycobacteriology: a guide for the level III laboratory, Atlanta, CDC (1985).
22. Bancroft, J. D. and Stevens, A. Theory and practice of histological techniques. 2nd Ed. Churchill Livingstone. 482-518 (1982).
23. Sambrooke, J., Fritscgh, E. F. & Meniates, T. Molecular cloning**.** A laboratory manual, 2nd Ed. Cold spring Harbor. 141 (1989).
24. Aragón, L. M. *et al*. Rapid detection of specific gene mutations associated with isoniazid or rifampicin resistance in *Mycobacterium tuberculosis* clinical isolates using non-fluorescent low-density DNA microarrays. J. Antimicrobial Chemotherapy, **57**, 825–831(2006).
25. Figueiredo, E. E. S. *et al*. Identification of *Mycobacterium bovis* isolates by a multiplex PCR. Braz. J. Microbiol. **40**, 231-233 (2009).
26. Supply, P. *et al*. Proposal for Standardization of Optimized Mycobacterial Interspersed Repetitive Unit-Variable-Number Tandem Repeat Typing of *Mycobacterium tuberculosis.* J. Clin. Microbiol. 44 **(12)**, 4498–4510 (2006).
27. Cruickshank, R. *Et al.* Medical Microbiology.12th ed., Vol. II. Livingstone. Limited, Edinburg, London and New York (1975).
28. Koneman, E. W. *Et al.* Color atlas and text book of diagnostic Microbiology. 5th Ed. Lippincott-Raven Publishers. Philadelphia (1997).
29. Dierckx, R. P. Fungi, Anamorphic fungi, Penicillium. Annales de la Société Scientifique de Bruxelles 25(1): 1-88 (1901).
30. Pitt, J. I. The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces. Academic Press Inc (London) LTD. 634 pp (1979).
31. Moubasher, A H. Soil Fungi in Qatar and Other Arab Countries, University of Qarar Press, Doha , Qatar, 566 pp (1993).
32. Samson, R. A., Hoekstra, E. S. & Frisvad, J. C. Introduction to food and air borne fungi 7th Edition, Fungal Biodiversity Centre CBS, Utrecht, the Netherlands. 389 pp (2004).
33. Rifai, M. A. A revision of the genus *Trichoderma*. Mycol. Pap. 116: 1-55 (1969).

34. de Hoog, G. *Et al.* Hyphomyces: Trichoderma. Atlas of clinical fungi. Eds, GS de Hoog, and J. Guarro. Utrecht, The Netherlands7 Centraalbureau voor Schimmelcultures, Universitat Rovira I Virgili, 2nd ed, vol (1) pp. 943(2000).
35. Marinho, S. A. *et al*. Identification of Candida spp. by phenotypic tests and PCR. Braz. J. Microbial. 41, 286-294 (2010).
36. Aberkane, A. *et al.* Comparative evaluation of two different methods of inoculum preparation for antifungal susceptibility testing of filamentous fungi. *J. Antimicrob. Chemother.*; **50**: 719-722 (2002).
37. Petrikkou, E. *et al.* Inoculum standardization for antifungal susceptibility testing of filamentous fungi pathogenic for humans. *J Clin Microbiol*.; **39**: 1345-1347 (2001).
38. Rodriguez-Tudela, J. L. *et al.* Interlaboratory evaluation of hematocytometer method of inoculum preparation for testing antifungal susceptibilities of filamentous fungi. *J. Clin. Microbiol*.; **41**: 5236- 5237 (2003).